Learning and Memory Outcomes with Variations in Rodent Models of Menopause: Interactions Between Age, Gynecological Surgery, and Ovarian Hormone Shifts Alter the Course of Healthy Female Aging

by

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ABSTRACT

The experience of menopause, typically occurring in midlife, can be markedly diverse, with surgical or transitional onset that results in alterations in circulating ovarian hormones and reproductive cyclicity. Such diverse experiences can coincide with the onset of symptoms and indications that impact long-term health outcomes. Indeed, prior work has shown that various facets of the menopausal experience can modulate later cardiovascular, immune, cognitive, and affective risks, making menopause a critical timepoint during aging. While clinical research provides great insight into numerous variables of interest, preclinical research can extend insights into these areas more directly by probing putative mechanisms driving long-term health risks as well as systematic assessment of therapeutic interventions. Reproductive senescence in the female rat differs from that of humans, as ovarian hormone decline and follicular depletion are less pronounced at midlife in the rodent. With advances in rodent modeling, preclinical researchers can probe questions pertaining to age and menopause independently, facilitating systematic exploration of factors affecting aging. This dissertation explores the impact of chronological aging, menopause etiology, and reproductive hormone alterations using a multidisciplinary approach, evaluating numerous dimensions of behavioral and physiological changes including immunology, endocrinology, and behavioral neuroscience. Assessments of chronological aging, both in normal aging and neurodegenerative rodent models, showed that patterns of memory decline differ by memory type, with midlife highlighted as a unique timepoint for learning and memory changes. Using several distinct rodent models of menopause in conjunction with a novel preclinical hysterectomy model, differences in cognitive and

physiological profiles were observed. Notably, these effects depended upon age at surgery and ovarian status. Finally, evaluations of hormone therapy which were mapped on to variants of clinically-relevant menopause models provided further context into the patterns of nuanced cognitive effects revealed within the clinical literature. Collectively, these dissertation chapters delineate a myriad of factors to consider when evaluating cognitive and physiological outcomes associated with menopause. Maximizing communication and collaboration across preclinical and clinical realms of menopause research will best leverage and facilitate translational outcomes. Such exchanges will ultimately create a framework to propel the understanding of hormone-brain-cognition relationships, optimizing care for women at midlife and beyond.

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GENERAL INTRODUCTION

Research questions surrounding reproductive functioning as aging ensues are aided by both clinical and preclinical research working in concert to identify variables of interest and theorize mechanisms of action. Indeed, rodent work has vastly expanded our understanding of questions pertaining to reproductive development, maturity, and aging in humans. This dissertation seeks to address the role of rodent models of menopause in expanding our understanding of how the experience of reproductive senescence uniquely interacts with aging to produce distinct cognitive profiles that extend across the spectrum of aging. I will specifically focus on the impacts of various surgical and hormonal alterations in menopause and subsequent impacts on memory, affective or anxiety-like, and immune outcomes, as well as discuss putative neurobiological mechanisms that drive these behavioral changes. Finally, I will expand on how these findings may prompt further areas of research within the clinical realm, and together inform clinical care, with the hopes of optimizing outcomes for those experiencing this life stage.

Similarities between the Female Reproductive Tract and Brain of Rodents and Humans: A Brief Overview of Anatomy & Physiology

Female rodents and humans have similar reproductive anatomy, with the same general structures including the ovaries, fallopian tubes or oviducts, uterus, cervix, and vagina. Notably, in development in humans, the Mullerian ducts in females fuse to form a singular uterine body, where the rodent Mullerian ducts fuse to a lesser degree, resulting in a bifurcated uterus with two lateral horns (Cunha et al., 2019). In both species, these reproductive structures form a complex system with brain and endocrine structures called the hypothalamic-pituitary-gonadal (HPG) axis, which plays a significant role in ovulatory cyclicity commencing at puberty and the eventual shift into reproductive

senescence (Koebele & Bimonte-Nelson, 2016). Indeed, humans and rodents even have comparable hormone changes across the cycle (see Figure 1); however, there are several differences in the staging of such cyclicity. Humans experience a menstrual cycle, wherein ovarian follicles mature and are ovulated while the uterine endometrium grows, which is shed without sufficient hormone stimulation as a result of pregnancy (Jabbour et al., 2006). The menstrual cycle, which lasts an average of 28 days (Chiazze et al., 1968), is characterized by 3 distinct phases (see Figure 1): the follicular phase, where 17beta-estradiol (E2) and progesterone levels gradually rise; the periovulatory phase, where estradiol levels peak and progesterone levels accelerate corresponding with an LH surge that initiates ovulation; and the luteal phase, where progesterone levels are highest before dropping back to moderate levels, and estradiol is maintained at a moderate level to return to the early follicular phase (Reed & Carr, 2018).

Conversely, rodents do not undergo a menstrual cycle, as they do not shed their endometrium, but it is instead resorbed within the uterus (Koebele & Bimonte-Nelson, 2016); instead, they experience what is termed an estrous cycle. In preclinical research, the estrous cycle is tracked by changes in cell population and density within the lining of the rodent vaginal epithelium (Goldman et al., 2007). There are 4 stages within the rodent estrous cycle, which typically occurs across 4-5 days (Koebele & Bimonte-Nelson, 2016): metestrus, diestrus, proestrus, and estrus (Figure 1). Metestrus is characterized by progesterone levels that briefly increase due to the release of progesterone from the corpus luteum following ovulation and estradiol levels that are relatively low; vaginal cytology indicates the mixed presence of cornified cells, leukocytes, needle-like cells, and round epithelial cells. During diestrus, progesterone and estradiol levels remain low,

and vaginal smears reveal a large number of leukocytes, with or without cornified cells and round cells. At proestrus, estradiol and progesterone levels peak, triggering the LH surge that initiates ovulation; vaginal cytology during this phase shows clustered, round epithelial cells and some cornified cells. Finally, during estrus, estradiol and progesterone levels decline, and there is a dense presence of cornified cells when conducting vaginal cytology (Bimonte-Nelson et al., 2021; Koebele & Bimonte-Nelson, 2016). While each individual stage may differ across the two species, reproductive cycling overall reveals a similar pattern of changes in the levels of ovarian hormones coincident with ovulation, making rodents useful in understanding how various exposures across the lifespan might alter reproductive outcomes.

Similar to reproductive anatomy and physiology, rodents and humans share a similarity in brain structures as well, with analogous functions associated with key brain regions. Indeed, not only is the anatomy of the rat brain quite similar to that of humans, but brain development in both species follows a largely similar path to adulthood (Semple et al., 2013). Additionally, many of the same brain structures common amongst the two species are interconnected in similar networks, and overlap in their impact of certain behaviors. Such neurological similarities have allowed for great advances in the study of various brain structures and the neurobiological mechanisms behind subsequent behaviors. For example, hippocampal lesion work in rats has given further insight into the role of the hippocampus in establishing mental schemas, but has identified the maintenance and updating of such schemas as hippocampally-independent processes (Tse et al., 2007). In addition to similar anatomy and physiology, rodent and human brains are similarly sensitive to hormonal stimulation, making the former excellent models for

evaluating the cognitive impacts of ovarian hormone modulation. Just as there are critical periods of brain re-organization in the human as a result of hormonal stimulation, such as those experienced during puberty (Feinberg & Campbell, 2010), so too do rats experience critical windows wherein hormonal exposures can alter long-term brain and behavioral trajectories (Koebele & Bimonte-Nelson, 2015). When considering the similarities in functioning of the reproductive and neurological systems of the female rat and human, it is apparent why rodents are considered suitable models for further evaluating questions pertaining to the organizational and activational effects of hormone modulations across the female lifespan.

Female Reproductive Senescence in Rats and Humans

Unlike that of the reproductive years, the experience of reproductive senescence in both female rats and humans is quite different. Briefly, women experience menopause, which occurs on average at age 52 (N. Santoro et al., 2021). Menopause is the result of ovarian follicular depletion and a dysregulation of the HPG axis, yielding a myriad of symptoms, sometimes lasting a decade or more (Gracia & Freeman, 2018), and can confer risks to long-term health (Harlow & Derby, 2015). The mechanism associated with instigating menopause, whether attributable to HPG axis dysregulation or ovarian follicular depletion, remains unclear. The rodent experience of reproductive senescence, termed estropause, occurs over a period of time that approximately begins at 10-12 months of age (Lu et al., 1979), and results in HPG axis dysregulation not coincident with ovarian follicular depletion (Koebele & Bimonte-Nelson, 2016). Unlike that of humans, significant work has been done in rodents to characterize the mechanism behind such endocrine dysregulation, and has identified the brain playing a critical role; indeed,

modulation of in the pulsatile release of gonadotropin-releasing hormone (GnRH) by the hypothalamus are responsible for a decreased surge in luteinizing hormone (LH) and subsequent alterations in cyclicity in the aging rodent (Wise, 1982a, 1982b). Such findings point to further inspection of reproductive-endocrine-brain relationships to better characterize reproductive senescence and aging in both species. The following sections will break down these differing forms of reproductive senescence and address observed ovarian, endocrine, and immune changes within both species.

Ovarian Profiles

It is well-established that women are born with the entirety of their ovarian follicular reserve, approximately 250,000-500,000 follicles per ovary (Gougeon, 2010); for female rodents, cases of adult neo-oogenesis remain controversial (Johnson et al., 2004). While some natural follicular depletion occurs within adolescence and early adulthood in both species (Gosden et al., 1983; Gougeon, 2010) as a result of apoptotic processes, it is in midlife where the ovarian profiles of the two species begin to diverge. For humans, in addition to the ovulation of a select number of these follicles, apoptotic processes accelerate with aging and reach a climax at midlife, as the ovarian follicular pool is depleted to around 1,000 remaining follicles per ovary by menopause onset (Gougeon, 2010; Hansen et al., 2008). Levels of anti-Mullerian hormone (AMH), which is produced by granulosa cells of small follicles and plays a role in inhibiting further recruitment of follicles, decline across this period, serving as a marker of the ovarian follicular reserve (Freeman et al., 2012). In the rodent, there is no such rapid depletion in the ovarian follicular reserve; while follicle counts continue to decline, ovarian follicles at all stages can be found late into chronological aging (Koebele, 2019). However, there

is some evidence that rodents at very advanced ages may experience near-total follicular depletion and ovarian failure (Meites et al., 1980). Rodents in estropause will experience irregular cyclicity, with some anovulatory cycles, and will eventually result in a persistent state of estrus or an eventual persistent diestrus cycle phase (Finch, 2014; Koebele & Bimonte-Nelson, 2016).

Endocrine Profiles

During the early stages of the menopause transition, ovarian follicular depletion and resultant declines in circulating AMH and inhibin B result in irregular follicular growth and development and cause erratic estrogen levels that can exceed those of the follicular phase during a typical menstrual cycle (N. Santoro et al., 2021). Coincidently, due to reduced follicle quality, the usual increase in progesterone following ovulation can be diminished, resulting in a lack of sufficient opposition to these estrogen increases (Lobo, 2022; Meites et al., 1980) (Figure 1). It is notable that during the menopause transition, the fluctuation in estrogen and progesterone levels is highly variable across women (Grub et al., 2021) and across cycles (O'Connor et al., 2009). After in the later phases of the menopause transition, when ovulation and cycling cease due to lack of follicular development, circulating levels of estrogen and progesterone drop to very low, often undetectable, levels, and the pituitary gonadotropins LH and follicle stimulating hormone (FSH) are elevated (Lobo, 2022; N. Santoro et al., 2021; N. Santoro & Randolph, 2011) (Figure 1).

With aging in the female rodent, the hypothalamus and pituitary are the nexus of reproductive-related changes. As the rhythmic releases of GnRH are disrupted within the hypothalamus at the beginning of estropause, there is a dampened and delayed surge of

LH in proestrus (Downs & Wise, 2009) which precedes irregular estrous cyclicity. Additionally, sensitivity of LH to increases in estradiol decreases across this period, further attenuating these LH surges with ovulation (Downs & Wise, 2009). Such dysregulation ultimately results in estrogen levels that stabilize at moderate to high levels, with moderate progesterone levels (Koebele & Bimonte-Nelson, 2016; Lu et al., 1979; Meites et al., 1980) (Figure 1). Eventually, levels of circulating estrogens and progesterone may further decline at extreme ages (Meites et al., 1980), as animals reach a persistent diestrus state.

Immune Profiles

In both species, reproductive cycling is an immune event; indeed, ovarian follicular maturation and ovulation are assisted by chemokines such as tumor necrosis factor-alpha (TNF-alpha), which is secreted by granulosa cells to promote secretion of FSH (Ye et al., 2016). Further, the apoptotic process that occurs throughout reproductive aging indicates that the ovary gradually develops a pro-inflammatory phenotype. Initiation of such apoptotic processes, associated with the bcl-2 protein family, are thought to be related to level of inflammatory factors TNF-alpha and interleukin-6 (IL-6) (Hsu & W Hsueh, 2000). Within humans, menopause is associated with accelerated atresia around midlife, indicating an increase in immune system activation at the ovarian level. Additionally, there is evidence that the modulation of ovarian hormones that accompanies the menopause transition also plays a role in immune outcomes in midlife. With the decline in circulating estrogens, it is observed that there is an overall increase in circulating proinflammatory cytokines (Gameiro et al., 2010). Additionally, during this time, women begin to transition into a state of immunosenescence, which is characterized

by an overall decrease in functioning of both adaptive and immune responses, including weakening of physical barriers (e.g. skin and mucosa), lower reactivity of B and T lymphocytes, and lower overall antibody response to vaccines (Gameiro et al., 2010; Ghosh et al., 2014). This evidence collectively points to menopause as a critical immune event that can alter long-term immune outcomes in women later in life.

In rats, the different experience of reproductive senescence, with ovarian cyclicity and relatively higher circulating estrogens and progesterone, would suggest a differential immune profile with reproductive aging to that of women. While the role of estropause in immune outcomes in the rodent is not as well-characterized, there is evidence that there are still marked changes in inflammation with aging. Indeed, the immunosenescent profile of the rodent shares many similarities to that of humans (Serre-Miranda et al., 2022), with some suggestions that the immune profile in the rodent rain is similar to that of postmenopausal women (Mishra et al., 2020). Further work is needed in this critical area of research to determine the extent to which female rodents can provide an adequate model for immune aging in women.

Menopause Etiology: Rodent Models that Parallel the Clinic

As noted in the sections above, female rodents and humans have divergent experiences of reproductive senescence. Where the rodent estropause results in moderate estrogen and progesterone levels, and a gradual decline in ovarian follicular reserve, the human experience of menopause involves a reduction in circulating estrogens and progesterone to near-undetectable levels, and a state of ovarian failure. Notably, in women, transitional menopause occurs in conjunction with chronological aging, making it exceptionally difficult to tease apart whether symptomology and risk factors are due to

aging or menopause, or potential interactions thereof. While surgical menopause, due to removal of the ovaries or uterus, is possible in women across the lifespan, the study of subsequent outcomes can be obscured by extraneous variables that are quite complicated to disentangle. These differences between the rodent and human experience of reproductive senescence may initially seem like setbacks, but they actually provide greater opportunity for further preclinical investigation. Indeed, because the rat does not undergo follicular and hormone depletion that ensues with aging, with the appropriate methodology we can experimentally induce follicular depletion or surgical menopause when we so choose. This allows us to dissociate effects of aging from the effects of follicular depletion or ovarian/uterine status, with far fewer confounding variables and a better ability to investigate mechanisms behind subsequent health outcomes. The issue remains as to how to model these diverse experiences of menopause in the rodent so that they best represent clinical experiences of women in as many dimensions as possible. This section will cover the many experiences of menopause in women, as well as the tools and techniques used by preclinical researchers to address clinical research questions surrounding menopause and long-term health outcomes.

The Menopause Transition

Modeling the menopause transition in the rodent is difficult, given the differences in endocrine and ovarian profile with reproductive senescence across the two species (see sections above). However, a model has been developed that allows for an evaluation of transitional menopause within the rodent by using 4-vinylcyclohexene diepoxide (VCD) (see Figure 2). VCD is an ovatoxin that, when administered routinely to the rodent across a series of injections, results in ovarian follicular depletion and hormonal alterations over

the course of a 90-day period (Acosta, Mayer, Talboom, Tsang, et al., 2009). VCD works by targeting the primordial and primary follicular pool within the ovary and accelerating atretic processes within these tissues (Hoyer et al., 2001; Hu et al., 2001). It has been demonstrated that VCD activates proapoptotic machinery specifically within ovarian tissues, including various caspase cascades (Hu et al., 2001). The resulting profile is one of significantly reduced levels of primordial, secondary, and antral follicles, as well as corpora lutea, and reduced serum progesterone levels (Acosta, Mayer, Talboom, Tsang, et al., 2009; Koebele et al., 2017; Koebele, Hiroi, et al., 2021; Koebele, Mennenga, et al., 2020). However, it should be noted that while work in mice has shown estrogen reductions with VCD (Mayer et al., 2004), there is no such consistent estrogen reduction in the VCD model for the rat.

With the development of the VCD rodent model of transitional menopause, we are able to observe changes in critical menopause variables across a period of ovarian follicular depletion, as well as beyond the onset of ovarian failure. Furthermore, given the need for VCD injections to initiate ovarian follicular depletion, we are able to address age-dependent outcomes in a systematic manner by inducing ovarian failure at key timepoints or stages in the reproductive lifespan of the rodent. Initial work with the VCD model in rats found cognitive deficits following ovarian failure, as well as changes in serum progesterone levels (Acosta, Mayer, Talboom, Tsang, et al., 2009). Further work evaluating young and middle-aged rats undergoing a model of transitional menopause found that young rats that underwent follicular depletion demonstrated impaired spatial memory early in the follicular depletion process (Koebele et al., 2017), mirroring clinical work suggesting greater vulnerability for long-term cognitive deficits in those that

experience premature menopause (Ryan et al., 2014). Beyond memory evaluations, research has shown increased anxiety-like behaviors in rats treated with VCD (Reis et al., 2014); research has already begun to pursue a neural mechanism, and reductions in serotonergic expression in the amygdala and hippocampus have been identified as potential drivers of such behaviors (Pestana-Oliveira et al., 2018). Beyond cognitive and affective outcomes, the VCD rodent model of transitional menopause has also been utilized to better understand cardiovascular risks across the menopause transition (Konhilas et al., 2020). While VCD is becoming more popular within the field of preclinical research, many domains relevant to menopause, including vasomotor and inflammatory outcomes, have yet to be addressed. Taken together, while it is not a perfect model of menopause, its dynamic nature and the yielding of comparable ovarian profile provides preclinical menopause researchers with a useful tool to assess critical health outcomes and provide greater insight to clinical work.

Surgical Menopause

While most women experience a transition into postmenopausal life, many others experience surgical reproductive tract alterations that can impact reproductive cyclicity. Oophorectomy, or surgical removal of the ovaries, which can be performed for malignant, benign, or even prophylactic reasons across the reproductive lifespan (Rocca et al., 2007), results in an abrupt cessation in circulating ovarian hormones. More common still is hysterectomy, or surgical removal of the uterus; it is the second most common gynecological surgery, after caesarian section (Merrill, 2008). The sections below will explore these two forms of surgical menopause, as well as their preclinical correlates.

Ovary removal. In women, surgical menopause in the form of a bilateral salpingo-oophorectomy (BSO), or surgical removal of the ovaries and fallopian tubes, appears to be most common in the years just preceding menopause, but can happen at any point in the reproductive lifespan (Rocca et al., 2021). Typically, BSO occurs with concurrent hysterectomy or following a prior hysterectomy; indeed, BSO is performed concomitantly in approximately 47% of all hysterectomy surgeries (Novetsky et al., 2011). However, clinical care has more recently shifted towards a minimalist approach, encouraging reproductive structures be left intact when possible. This shift has resulted in a general decline in BSO over the last few decades, particularly in premenopausal women (Z. Erickson et al., 2022). This trend is in part due to the fact that, when BSO is performed before age at natural menopause, the abrupt loss in circulating ovarian hormones and disruption of HPG axis functioning results in an immediate post-menopausal state. Research has shown that such abrupt hormonal alterations can confer lifelong health risks (Adelman & Sharp, 2018); indeed, BSO before menopause onset is associated with increased risk of coronary heart disease, stroke, dementia, depression, and anxiety (Parker, 2014; Parker et al., 2009; Rivera et al., 2009; Rocca et al., 2014, 2021). Furthermore, stark shifts in immune system functioning become apparent following BSO, with studies showing prolonged increases in CRP and inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-alpha) following ovarian removal (Au et al., 2016; Cantatore et al., 1995; Kalyan et al., 2011; Pacifici et al., 1991). Such pro-inflammatory shifts associated with BSO also appear to impact long-term immune outcomes, as surgical menopause increases risk for autoimmune disorders such as lupus (Costenbader et al., 2007).

Rodent models can provide profound insight into the mechanisms behind such health risks associated with ovarian removal. Indeed, in the domain of menopause modeling, ovariectomy (Ovx), or surgical removal of the ovaries in the rodent, has been traditionally used to analyze effects pertaining to ovarian hormone loss via surgical menopause (see Figure 2). Such work has shown cognitive deficits associated with abrupt loss in circulating ovarian hormones via Ovx (Koebele et al., 2019; Wallace et al., 2006) that can be rescued by administration of estrogens such as E2 (Bimonte & Denenberg, 1999; Prakapenka et al., 2018). The Ovx model is also important in providing a clean hormonal slate for further assessment of various hormone or drug treatments that may interact with circulating ovarian hormones. While Ovx effects on various cognitive and physiological systems have been well-characterized in the preclinical literature (Puga-Olguín et al., 2019; Zeibich et al., 2021), providing insight into one form of surgical menopause, other surgical menopause etiologies are less characterized.

Uterus removal. Hysterectomy is a commonly performed gynecological surgery, with estimates that one in nine women will undergo the procedure in their lifetime in the United States (Wright et al., 2013). Hysterectomy can be performed alone, or in conjunction with ovarian removal; like that of BSO, prevalence of surgery varies with age, but is most common in the years just preceding menopause (Merrill, 2008; Whiteman et al., 2008). Additionally, like BSO, incidence of hysterectomy has generally declined over the last few decades, particularly when performed without concomitant ovarian removal (Merrill, 2008). Indeed, while there was a period of time wherein the non-pregnant uterus was thought to play minimal to no role in broader cognitive and physiological functioning, being “a quiescent and, to a large degree, useless organ”

(Navot & Williams, 1991), newer clinical research has pointed to a role of the uterus in regulating ovarian functioning, as well as later health outcomes. There is evidence that women who undergo hysterectomy with ovarian conservation during reproductive years transition to menopause earlier than women with an intact uterus and ovaries (Chan et al., 2005; Farquhar et al., 2005; Kaiser et al., 1989; Moorman et al., 2011); however, other work points to more temporary changes in ovarian function or no such changes (Beavis et al., 1969; Chalmers et al., 2002). Beyond a differential experience of menopause, hysterectomy appears to have a profound effect on long-term health. Hysterectomy surgery alone has been found to increase early-onset dementia risk by 38% (Phung et al., 2010), where detrimental effects were increased with a younger age at surgery. Likewise, hysterectomy has been shown to be detrimental to mood and affect, with increased risk of de novo anxiety and depression (Laughlin-Tommaso et al., 2020). Such outcomes suggest that the uterus is involved either more directly in brain-related outcomes, or via its relationship with the ovaries, the uterus can impact the brain and behavior; however, this research area remains under-explored.

Rodent work evaluating outcomes associated with hysterectomy have likewise been under-evaluated, but more recent work is shedding a light on the role of the uterus in ovarian and brain outcomes. Evaluations of ovarian changes as a result of hysterectomy in the rat suggest that the ovaries might depend upon the uterus to control ovulation (Özdamar et al., 2005; Tanaka et al., 1994), but further research is needed to track the long-term ovarian outcomes associated with this hysterectomy model. When considering the cognitive outcomes associated with hysterectomy, our laboratory has developed a novel model to assess such effects of this surgical menopause variant, with

or without concomitant ovarian removal (see Figure 2). Preliminary work suggests that the surgical removal of the uterus in the rat results in learning and memory deficits, as well as some indications of ovarian hormone changes (Koebele et al., 2019). However, follow-up work evaluating intervals post-surgery up to one year demonstrated that, while cognitive deficits persist, ovarian and endocrine outcomes do not, suggesting that the cognitive effects of ovarian removal are not secondary to the ovaries (Koebele, 2019). This novel rodent model should be utilized to evaluate other variables of interests identified by the clinic for women following hysterectomy, including affective, cardiovascular, and inflammatory outcomes.

Management of Menopause in the Clinic and Corresponding Preclinical Insights

Menopause is often a dynamic and transitional event, but it can occur as a more abrupt event, secondary to surgery removing some or all of the reproductive tract. Scientific discovery and clinical insights thus far have supported the hypothesis that just as the trajectory of menopause is not uniform across aging women, the optimal therapeutic tactic will not be uniform across aging women either… just as menopause etiology can be diverse, symptoms due to menopause can be diverse. This section will explore the symptoms and indications associated with menopause, the current therapeutics, and how preclinical research can provide greater insight into optimizing health outcomes during the menopause transition.

Common Symptoms & Indications

Clinical researchers have gone to great lengths to try to capture the diverse experiences of menopause, finding patterns in signs and symptoms across different populations to predict health outcomes and mitigate associated risks. Indeed, works such

as the Stages of Reproductive Aging Workshop (STRAW), and its follow-up STRAW+10, have characterized menopause stages by predominant symptoms and hormone profiles to aid in quantification of the menopause experience into discrete phases, while acknowledging that the time of each stage, and time across stages, can vary greatly across individuals (Harlow et al., 2012; Soules et al., 2001). These stages of menopause include key distinctions between the early menopause transition, where menstrual cycles are somewhat irregular, and the late menopause transition, where there are periods of intermittent amenorrhea (Soules et al., 2001). Such nuanced assessments of the menopause transition provide opportunity for greater clarity in the evolution of the diverse experiences of menopause.

During each of these stages, the menopause transition can yield an array of symptoms that impact quality of life and increase the risk for life-altering health outcomes. The Study of Women's Health Across the Nation (SWAN), which was initiated in 1996 in a racially diverse sample of women, has provided researchers with a better understanding of permanent and transient changes that women undergo throughout menopause and across aging (El Khoudary et al., 2019). Menopause can impact genitourinary, cardiovascular, metabolic, and sexual health, resulting in night sweats/hot flashes, sleep disturbances, and central and visceral adiposity as well as altered risk factors for osteoporosis, diabetes, and cardiovascular disease (El Khoudary et al., 2019; el Khoudary et al., 2020). Midlife is a particular life phase in women where chronic pain begins to increase (Pavlović & Derby, 2022), with increases in rheumatoid arthritis and migraines. Researchers have found that the experience of menopause, and the duration and severity of symptoms encountered, has a profound impact on aging outcomes. Work

completed using data from the SWAN evaluating vasomotor symptoms (VMS) has shown that women experience these symptoms on average for over 7 years during this transition, far longer than researchers and clinicians initially thought (Avis et al., 2015). In women that had earlier initiation of VMS, these symptoms lasted longer into postmenopausal life, sometimes for a decade or longer (Avis et al., 2015). Notably, racial or ethnic groups were observed to have differential experiences of VMS, where African-American women had the longest duration of VMS of average of over 10 years (Avis et al., 2015). It is critical that more clinical work focuses on highlighting groups traditionally underrepresented in scientific and medical research for further investigations of the complex variables associated with differential menopause experiences.

Negative impacts on mood, anxiety, and cognition have also been observed across the menopause transition, although there is evidence that these effects are most prominent during the transition to menopause and in early postmenopause, and that they can attenuate later in postmenopause (Bromberger et al., 2011; Drogos et al., 2013; Greendale et al., 2009, 2010; Maki et al., 2018; Weber et al., 2013; Willi et al., 2021). There are links between affective outcomes in menopause and other symptoms; indeed, more severe and lasting VMS are associated with higher depressive symptoms and greater perceived stress (Avis et al., 2015). Like that of VMS, there are likely individual differences in vulnerability and resilience to menopause-related outcomes on psychosocial variables. For example, individual fluctuations of estrogens early in perimenopause have been related to psychosocial menopausal symptoms, as demonstrated in a study assaying a urinary metabolite of estradiol showing that its fluctuations were associated with depressive symptoms, negative affect, and elevated

levels of sadness, rejection, and anger (Gordon et al., 2019). Additional work has demonstrated that psychosocial and health-associated risk factors in women have been related to the trajectory of depressive symptoms in midlife when followed over 15 years (Bromberger et al., 2019). Further work should be done to evaluate the mechanisms behind affective outcomes associated with key stages in the menopause transition, as well as potential relationships between symptoms that may drive the increased risk for depression or anxiety disorders in vulnerable individuals.

Beyond affective changes in menopause, there are negative cognitive outcomes associated with certain experiences of menopause. Initially, some researchers thought that the memory and attentional deficits associated with the menopause transition, described as a "brain fog", may have been secondary to other symptoms such as lack of sleep or VMS, and were temporary (Sullivan Mitchell & Fugate Woods, 2001). However, more recent clinical work has shown that cognitive deficits associated with menopause are more mechanistically linked to the physiology of this transitional state. Indeed, subjective memory complaints have been shown to correspond with objective cognitive evaluations in women in midlife (Drogos et al., 2013). Work evaluating women with severe VMS found that verbal memory deficits were associated with objective VMS, and not subjective experience of VMS, suggesting that the mechanisms behind these two symptoms of menopause are physiologically linked (Maki et al., 2008). Recent work has also shown indications that these memory deficits can persist into postmenopausal life in certain individuals, and confer greater risk for severe decline later in life. In a recent longitudinal study evaluating a sample of low-income women, which included a large proportion of women belonging to racial groups traditionally underrepresented in health

research, clinically significant cognitive impairments in learning and memory were observed from premenopause to early perimenopause, with these impairments persisting into postmenopause (Maki et al., 2021). While much is currently unknown regarding the mechanism behind these memory deficits, researchers think that the brain may undergo a key period of re-organization in menopause, where dysregulation of the HPG axis results in critical neurobiological shifts. During this period, prior or current exposures could render women more susceptible to more persistent cognitive deficits and disorder once the brain and body reach a new equilibrium in postmenopausal life (Koebele & Bimonte-Nelson, 2015, 2017). Research similar to that described here, identifying complex health-related factors that impact long-term health outcomes, is critical to ultimately yield discoveries that translate to the clinic thereby initiating a real impact on the quality of life, health outcomes, and longevity for women during aging.

Hormone Therapy

To alleviate some of the symptoms associated with menopause, either brought about through surgical or transitional means, women can be prescribed menopausal hormone therapy (HT). HT is comprised of an estrogen, with the most common being E2, the most potent endogenous estrogen in women, or conjugated equine estrogens (CEE), derived from pregnant mares (N. Santoro et al., 2021). For any woman with an intact uterus, a progestogen is an essential component of HT as it is necessary to prevent estrogen-induced endometrial hyperplasia (Pinkerton et al., 2017). Progestogens are a class of hormone that include endogenous progesterone, as well as synthetically-derived molecules that act on the progesterone receptor (Sitruk-Ware, 2005). The most common progestogens found in HT include micronized progesterone, medroxyprogesterone

acetate (MPA), norethindrone acetate (NETA), and levonorgestrel (LEVO) (N. Santoro et al., 2021). While certain HT formulations can be taken orally, HT is also available as a transdermal patch or topically applied to the skin, providing cyclic or tonic routes of hormone delivery (N. Santoro et al., 2021).

Notably, this discussion of endogenous or naturally circulating versus synthetic hormones is distinct from the discussion of "bioidentical" hormones. The term "bioidentical" does not have a consistent definition; in a scientific context, this term should be stringently used to mean that the molecular and chemical structure of the compound is identical to that of endogenously circulating E2 produced by the body (Files et al., 2011; The Endocrine Society, 2006). However, the term "bioidentical hormones" is generally considered a marketing term not recognized by the scientific community (Pinkerton, 2014). Indeed, there was a movement toward the unsubstantiated concept of specialized bioidentical hormone therapy, termed compounded bioidentical hormone therapy (CBHT), where "personalized" formulations are created by pharmacists based on evaluations of individual hormone levels. These formulations can be problematic for several reasons. Firstly, ovarian hormone levels fluctuate significantly depending upon menopause stage, cycling patterns, diurnal variations, etc. (el Khoudary et al., 2019; Pinkerton et al., 2017), making it difficult to ascertain patterns from serum evaluations alone. Additionally, the notion that HT to rescue declines in endogenous hormones across menopause is flawed; rather HT is prescribed in the formulation necessary to alleviate menopausal symptoms (Files et al., 2011). In addition to CBHT being potentially misleading given the notion that women need a specific, individualized dose to treat their menopause indications, it is also potentially dangerous, given that these formulations

have not been rigorously evaluated by the FDA. Pharmacies creating CBHT have very little oversight, and have been previously found to work in non-sterile environments (Simon, 2016), with as many as 90% of CBHTs also failing tests of potency (Files et al., 2011). For these reasons, CBHT is *not* recommended by the scientific and medical communities, including leading organizations providing recommendations for menopausal treatment and care (ACOG, 2014; Baber et al., 2016; Files et al., 2011; Neves-E-Castro et al., 2015; Pinkerton et al., 2017; Stuenkel et al., 2015; The Endocrine Society, 2006).

Generally, menopausal VMS is the most common indication for the use of HT, but HT is known to have an impact on several other menopause symptoms, including risk of bone fracture and osteoporosis, genitourinary symptoms, and cognition and affective outcomes (NAMS, 2022). The last 20 years of research, at the clinical and preclinical level, have provided immense advances in our understanding of HT and its impact on the experience of menopause and subsequent aging, exploring variables of interest far beyond its indications of use. This next section will briefly explore the clinical and preclinical work surrounding HT that has given rise to the current theories surrounding its impact on cognitive, affective, and immune outcomes.

Clinical HT research: The WHI and beyond. While smaller-scale work evaluating the cognitive and physiological effects of HT has occurred for decades (Caldwell & Watson, 1952; Duff & Hampson, 2000; Kimura, 1995) and largely indicated a beneficial role of estrogen-containing HT on cognition with aging, the arguably most impactful clinical evaluation of HT was the Women's Health Initiative (WHI). The WHI was a long-term national health study that recruited over 20,000 postmenopausal women

aged 50-79, beginning with recruitment in the early 1990s before abruptly concluding in the early 2000s (Chester et al., 2018). While the primary aim of the WHI was to evaluate the effects of administration of CEE alone or CEE+MPA (for uterus-intact participants) on cardiovascular health, the study also evaluated risk for breast cancer, stroke, endometrial cancer, pulmonary embolism, hip fracture, and overall mortality (Chester et al., 2018). A subset study, termed the WMI Memory Study (WHIMS), evaluated cognitive outcomes and risk of dementia with HT use in women aged 65 or older (Coker et al., 2010). While the study was supposed to last for over a decade, a 2002 report found a persistent adverse event risk for CVD and breast cancer, ending the CEE+MPA trial early; the CEE only trial shortly followed, ending in 2004 (Burger et al., 2012). When evaluating the data from the WHIMS, there was a shocking increase in dementia risk in HT users (Coker et al., 2010; Shumaker et al., 2003), changing the field's perceptions of the beneficial effects of HT on cognition across the menopause transition. However, there were several problems with this report. First, the interruption of the study resulted in a skewed depiction of long-term outcomes such as dementia risk, particularly for younger participants in the CEE or CEE+MPA groups. Additionally, recruitment was skewed towards older, postmenopausal participants that were not symptomatic, resulting in a poor reflection of the population taking HT (Chester et al., 2018).

Since the WHI, clinical researchers have worked carefully to better understand the conflicting findings within the field surrounding HT use. Alternative to the WHI, the KEEPS evaluated administration of either transdermal E2 or oral CEE, with micronized progesterone given to those with an intact uterus, in women that were recently menopausal (Gleason et al., 2015). Participants were evaluated primarily for

cardiovascular outcomes, but additional analyses were performed to assess affective and cognitive health (V. M. Miller et al., 2021). While no cognitive differences were observed across these two estrogen regimens, researchers found that both HT formulations improved sleep and VMS, and oral CEE resulted in improvements in depression and anxiety symptoms not seen in women using transdermal E2 (V. M. Miller et al., 2021). Another such study evaluating the effects of E2 and CEE on cognition found that menopausal women taking E2 performed better on a verbal memory task than women taking CEE in a sample of women at-risk for Alzheimer's disease (Wroolie et al., 2011). Such work suggests that HT type may play a role in subsequent cognitive outcomes. Different experiences of menopause may also result in divergent outcomes regarding cognitive benefits with HT use. However, these questions are difficult to address in clinical research, as hysterectomized individuals require a different HT formulation than those that are uterus-intact, and oophorectomy is frequently performed with hysterectomy (Novetsky et al., 2011). While differing patterns of effects have been shown in HT formulations that only contain an estrogen versus those with an estrogen plus a progestin (Coker et al., 2010; Maki & Sundermann, 2009), more careful research design is needed to directly address this research question as to whether surgical versus natural menopause alter response to HT.

Preclinical HT research: Key insights. As clinical researchers have made great progress in their evaluation of the long-term outcomes associated with menopausal HT, so too have preclinical researchers addressed questions surrounding HT through the use of various menopause models. Early questions surrounding the impact of ovarian hormone on brain and behavior in the rodent have paved the way to more complex

questions surrounding the effects of hormone therapy across various menopause etiologies. Indeed, the Ovx model of surgical menopause has been used as a "blank slate" model to study effects of ovarian hormones for decades (Koebele & Bimonte-Nelson, 2016). Such early preclinical research has shown beneficial effects on learning and memory outcomes with estrogen administration, but these effects are dependent upon estrogen type and menopause model. Our laboratory has evaluated administration of E2 in surgical and transitional rodent models of menopause, where following surgical menopause strong working memory benefits are observed with E2 (Bimonte & Denenberg, 1999) and in the transitional model of menopause, both learning benefits and retention deficits are observed with E2 (Koebele, Mennenga, et al., 2020). These differing cognitive effects depending upon menopause experience are also observed with CEE, where our laboratory has demonstrated that CEE administration is beneficial for spatial working memory performance in a rodent model of surgical menopause, but impairs cognition in the transitional menopausal model (Acosta et al., 2010). When these two estrogens were directly compared to one another in ovariectomized rats, E2 generally afforded greater cognitive benefits concerning spatial working memory and reduced anxiety-like behaviors (Hiroi et al., 2016).

While much work has been done to evaluate the cognitive effects of estrogens, the cognitive and affective outcomes associated with progestin administration are less characterized. Work from our laboratory using the Ovx model has shown that some progestogens, such as progesterone or MPA, evaluated in the WHI, are detrimental to cognition (Bimonte-Nelson et al., 2004; Braden, Andrews, et al., 2017; Braden et al., 2010, 2011), other progestins, such as LEVO or drospirenone, can be beneficial to

learning and memory outcomes (Koebele et al., 2022; Prakapenka et al., 2018). Researchers have begun to investigate the mechanisms behind these differing effects. When considering progesterone, GABAergic modulation has been identified as a large contributor to the cognitive outcomes associated with its administration in Ovx models; our work has shown that administration of bicuculline, a GABAergic antagonist, obviates the learning and memory deficits associated with progesterone (Braden et al., 2015). However, the mechanism behind the differing cognitive effects of progestins is much more difficult to ascertain. Progestins are derived from parent molecules that impart differential affinity for and biological activity on androgenic, estrogenic, and other steroid receptors (Kuhl, 2005); each of these receptors are known to play a role in cognitive outcomes. Further complicating this research is the fact that effects of these progestins appear to differ somewhat by menopause model. Our work in VCD model of transitional menopause has shown neutral outcomes with LEVO administration (Koebele, Hiroi, et al., 2021) and some beneficial learning effects with MPA administration (Peña, 2019). Further work must be done to characterize the effects of these progestins, as well as the neurobiological mechanism behind these effects; this investigation could result in key advances for optimizing HT outcomes for uterus-intact menopausal women.

More recent work has taken a greater translational approach in evaluating combined estrogen plus progestin HT, and emphasizing the use of ovary-intact models of menopause. Given that many women must take an estrogen + progestin HT formulation throughout the menopause transition, and that progestins have been shown preclinically to have their own unique effects on cognition (Braden, Andrews, et al., 2017), it is critical to understand how these combined HT formulations might have different cognitive

outcomes than solely estrogen-containing HT. Early work with progesterone and E2 has shown that they generally seem to play opposing roles in learning and memory outcomes, both in Ovx (Bimonte-Nelson et al., 2006) and VCD (Koebele, Hiroi, et al., 2021) models; this also appears true when discussing their combined effects on anxiety-like behavior (Hiroi & Neumaier, 2006). Other progestogen plus estrogen combinations have revealed interactive effects of learning and memory. In our laboratory, we analyzed a regimen of E2, the progestin Levonorgestrel (LEVO), and the combined E2+LEVO (both hormones in the HT patch Climara Pro) in Ovx rats to understand their independent and interactive effects. We found that, while E2 and LEVO alone both benefit cognition, when administered together these cognitive benefits disappear (Prakapenka et al., 2018). Again, interactive effects were observed in the VCD model, where E2 and LEVO alone did not impact anxiety-like or depressive-like behaviors, but the combined E2+LEVO administration resulted in alterations in both measures (Koebele, Hiroi, et al., 2021). Further preclinical research into the cognitive effects of combined estrogen + progestin HT formulations is critical to providing the best possible care for women entering the menopause transition to maintain cognitive prowess.

Menopause as a "Critical Window" & HT Outcomes

As discussed in the sections above, the WHI made a significant impact on clinical HT research. However, recent research does not emphasize the initial findings of this large clinical trial; rather, further inspection of WHI data revealed a key variable that has altered the course of the field, and become a guiding principle of current research concerning the effects of HT on long-term health outcomes. The "critical window hypothesis", also known as the "critical timing hypothesis", of menopausal hormone

therapy states that there is a critical window within the menopause transition and early postmenopausal life, where the brain is undergoing re-organization as a result of declining ovarian hormone levels and HPG axis dysregulation; during this phase, the psychological and physiological effects of estrogen-containing HT are most optimal (Chester et al., 2018; Koebele & Bimonte-Nelson, 2016; Maki, 2013). As mentioned previously in this chapter, a major flaw of the WHI was that it recruited individuals that had undergone menopause years to decades before study onset; these individuals may have been outside the optimal range for HT use, obscuring outcomes for clinically-relevant individuals closer to menopause. Indeed, further inspection of WHI data has found that these startling statistics concerning risk of stroke or breast cancer only hold for the oldest participants, where women aged 50-59 in the HT groups show reductions in all-cause mortality compared to placebo, and some protective effects against dementia (Manson et al., 2017). Work in the years since the WHI has revealed much to the field regarding the optimization of HT use. Even large institutions like the North American Menopause Society (NAMS) have updated their recommendation according to the critical timing hypothesis, recommending HT use for the treatment of VMS and prevention of osteoporosis in women without contraindications if younger than 60, or within 10 years of menopause (Pinkerton et al., 2017). These findings were certainly groundbreaking for both the clinical and preclinical research communities, but a closer inspection of preclinical work reveals evidence for this critical timing hypothesis long before the release of WHI data. The following sections will explore the clinical and preclinical research that has built and supported the critical timing hypothesis, as well as work that has expanded our understanding of the putative mechanism behind this window.

Impacts on Long-Term Cognitive Aging

Preclinical research done prior to the release of the WHI results and the formulation of the formal "critical window hypothesis" demonstrated that estrogen's beneficial effects on cognition are dependent upon timing in relation to surgical menopause initiation. In an experiment designed by Robert B. Gibbs (2000), ovariectomized rats were given different regimens of estrogen or estrogen and progesterone either immediately after ovariectomy, 3 months following surgery, or 10 months following surgery. When evaluated on a spatial learning and memory task 12 months after surgery, it was found that immediate or early treatment of estrogen and estrogen + progesterone benefitted cognition relative to treatment controls, where treatment at the 10-month postsurgical timepoint resulted in no cognitive benefits (Gibbs, 2000). This paper was key to the discussion of a critical window for the effectiveness of estrogen to relieve the cognitive deficits associated with surgical ovary removal in the preclinical literature, and has repeatedly been analyzed across a variety of tasks and treatment paradigms (Daniel, 2013).

Results from early clinical work also indicated what was eventually known as the critical window theory of menopause. Although smaller than the WHI, work by Duffy and colleagues evaluating HT use in postmenopausal women aged 45-65 revealed verbal and spatial working memory improvements relative to placebo (Duff & Hampson, 2000). In the REMEMBER pilot study, early HT users outperformed late users on tests of general cognition and attention (MacLennan et al., 2006), suggesting that early HT use in menopause affords the greatest benefits. Studies in surgically menopausal women also found benefit with immediate estrogen administration, with improved immediate recall

relative to placebo (Phillips & Sherwin, 1992). Alternatively, administration of estrogen-containing HT outside of this critical window has been shown to result in neutral or even detrimental cognitive outcomes (Resnick et al., 2009; Shumaker et al., 2003). This pioneering work, along with preclinical research, paved the way for further pursuits of age and menopause-stage dependent questions surrounding HT use and duration.

Later clinical work has solidified and built upon this theory of menopause as a critical window to further provide context to these HT effects. When data from the younger cohort of the WHI (WHIMSY) was analyzed following initial results, researchers found that, for women aged 50-54 at initiation of CEE-containing HT with and without MPA, there were no cognitive benefits or deficits associated with HT use; it is possible that even earlier intervention with HT use in the menopause transition would have yielded beneficial cognitive outcomes (Espeland et al., 2017). These findings are corroborated by work that expands on the WHI. The KEEPS trial, mentioned in previous sections, has specifically focused on non-hysterectomized, recently menopausal women, providing greater insight into the health outcomes associated with HT, and have evaluated more common formulations of HT (Gleason et al., 2015). Similarly, evidence from the Cache County study, a study specifically evaluating dementia in elderly residents, suggests a protective effect of HT during menopause, where HT use within 5 years of menopause onset was associated with a 30% decreased risk in AD, where later use of HT was associated with increased AD risk (Shao et al., 2012). While this observational work is necessary for understanding general relationships, a large-scale clinical trial like the WHI, guided by the shortcoming of its predecessor, would provide

significant benefits to the field in understanding this critical window of menopause, and how HT formulation or menopause etiology might shift this window.

Preclinical work has made advances in the identification of a putative mechanism for this window of opportunity with menopausal HT. To assess the cognitively beneficial effects of estrogens in old age, work done by the Foster laboratory has shown that aged rodents need a higher dose of estrogen (Foster et al., 2003), suggesting a potential shift in estrogen receptor (ER) density with aging in key areas for cognitive outcomes, such as the hippocampus. Indeed, Foster has shown that rats demonstrate a decline in ERalpha with aging, suggesting that ERalpha's role in the cognitive outcomes associated with estrogen administration is critical. In line with the timing hypothesis, Foster posits that the ratio of ERalpha to ERbeta is critical in determining the cognitive outcomes associated with estrogens, where with aging, this shift to a lower ratio necessitates a greater level of estrogen to rescue age-related memory decline. Furthermore, the experience of menopause may shift this curve, such that the potential for cognitive benefit with estrogen stimulation is blunted, resulting in a lack of cognitive benefits observed with estrogen administration postmenopause. However, Foster suggests that there is a brief window wherein ERalpha levels can be maintained by estrogen-containing HT, such that ERalpha levels may stabilize with aging, resulting in lasting cognitive benefit (Foster, 2012b). While progress has been made, identification of the neurobiological mechanism for the pattern of cognitive outcomes associated with the critical window of menopause and HT use via preclinical modelling is vital for optimization of HT guidance for the clinic.

Impacts on Lifelong Risk of Anxiety/Depression

The critical window theory of menopause is also supported by findings surrounding anxiety and depression. Risk of depression increases across the menopause transition and into early postmenopause, independent of prior history of depression (Bromberger et al., 2011); in postmenopausal life, this risk actually decreases over time (Freeman et al., 2014). Surrounding surgical menopause, studies have shown an increased risk of de novo anxiety and depression with BSO before menopause onset (Böös et al., 1993; Rocca et al., 2018), with some evidence of estrogen-based HT alleviating this risk (Böös et al., 1993), where other report no effect of HT on affective outcomes (Rocca et al., 2018). Indeed, antidepressant selective serotonin reuptake inhibitors (SSRIs), as well as estrogen-containing HT, have been suggested to treat menopausal depression (Bromberger & Epperson, 2018), with evidence showing that the two might interact to augment anti-depressive effects (Halbreich et al., 1995). While these studies provide a promising lens into the risk of anxiety and depression, such evaluations are quite complex to evaluate clinically. Preclinical work has tried to address the underlying mechanism behind such behaviors, with some findings that estrogens may have a protective effect on cholinergic activity (Dumas et al., 2012; Gibbs, 2010) and increase plasticity (McEwen, 2002) within key brain regions for cognitive, attentive, and affective outcomes, such as the hippocampus and prefrontal cortex. Indeed, work has even been done to begin to address the timing hypothesis with regard to estrogen-induced hippocampal plasticity, suggesting that an extended period of hormone deprivation reduced sensitivity of the hippocampus to increased dendritic spine density observed with estrogen administration (McLaughlin et al., 2008). This work points to a link between memory outcomes and vulnerability to stress with menopause onset; however, more

preclinical research is needed to address these questions. Primarily, there is still much to uncover about the neurobiological mechanisms behind depression and the general increased risk in depression across adulthood in females relative to males (Eid et al., 2019). With this foundation, preclinical work should look to using more translational HT formulations and menopause models to understand whether the "window" for HT to optimally benefit affective outcomes mirrors that which provides optimal learning and memory outcomes.

Impacts on Lifelong Immune Health

The timing hypothesis also seems to be critical when considering the immune outcomes associated with menopausal HT use. For women that have undergone surgical removal of the ovaries and uterus, early estrogen-containing HT administration seems to combat the shift in serum proinflammatory cytokine levels, with surgery-induced increases in IL-1 and TNFalpha reaching baseline levels within 2-4 weeks following HT use (Pacifici et al., 1991). Furthermore, it appears that HT rescues the increases in cytotoxic killer T cells in serum, suggesting shift towards a more anti-inflammatory profile with HT use (Kumru et al., 2004). This inflammatory shift with exogenous estrogens seems not only limited to surgical menopause, but also carries over to HT use in women that have undergone the natural menopause transition, where post-menopausal increases in serum chemokines, such as IL-8, are observed (Abu-Taha et al., 2009); this shift, however, is presented in somewhat of a different context. While there is a pattern for overall decrease in T cells, the main findings associated with HT use following natural menopause include an increase in responding of T cells following stimulation (Porter et al., 2001). While this may initially seem to counter the effects of HT use with

BSO, it is important to view these findings in the context of basal levels vs upon activation. As immunosenescence not only characterized by a low level of inflammation, but is also characterized by a blunted response to injury or infection, it would appear that HT might aid the immune system in increasing the response to infection or injury post-menopause. While this work has advanced the field's understanding of the timing hypothesis in the context of immune function and inflammation, further work is needed to probe additional variables such as HT type or menopause etiology. Additionally, greater focus is needed on longitudinal work that properly evaluates a myriad of variables that relate to immune system functioning, including risk of autoimmune disease.

Notably, preclinical research corroborates these clinical findings, and further indicates that the immunoprotective benefits of estrogen are only present briefly following surgical menopause. Indeed, work performed by Abu-Taha and colleagues has shown that E2 reduces the low-grade systemic inflammation that follows Ovx in female rodents (Abu-Taha et al., 2009). Similar work done in aged Ovx rats suggests that E2 can reduce levels of neuroinflammation in the dentate gyrus (Kireev et al., 2014), suggesting a potential for beneficial hippocampal-dependent outcomes. Finally, work performed by Suzuki and colleagues in 2007 demonstrated that, if administered within days of Ovx, E2 can lower serum cytokines and protect against brain damage and inflammation via stroke (Suzuki et al., 2007); delayed administration of E2 weeks after Ovx showed no protective effects following stroke, suggesting that E2's benefits on cognitive and brain health are dependent upon timing in reference to ovarian hormone cessation. While such work is critical in understanding the broader implication of the critical window hypothesis, evaluations of different HT formulations, particularly combination therapies, as well as

other menopause models, would provide great insight. Behavioral analyses should be performed to assess the extent to which relationships exist between immune outcomes and the learning and memory or affective outcomes described in the sections above; such work would contribute greatly to our understanding of the neurobiological mechanism responsible for this phenomenon.

Summary & Exploration of Dissertation Goals

The experience of midlife is complex in women, with ovarian and endocrine shifts that impact the trajectory of aging, behaviorally and in the brain. While clinical research has made great strides in identifying key variables for further research, the value of rodent models of menopause in independently assessing reproductive and chronological age to identify putative mechanisms that give rise to behavioral phenomena is clear. With knowledge of the most pressing questions surrounding menopause and healthy female cognitive aging, the following chapters seek to address the role of chronological aging, menopause etiology, and ovarian hormone profile in driving brain and behavioral outcomes through the use of various rodent models. Chapter 2 begins this series with an assessment of working and reference memory relationships in young and aged female rats, identifying nuanced shifts in learning and memory performance across chronological aging. Chapter 3 assesses a similar question, but utilizes a rat model of Alzheimer's disease, the TgF-344 AD rat model, to characterize the pattern of learning and memory deficits surrounding the middle-age timepoint in females. Chapter 4 again assesses the role of female chronological aging in modulating learning and memory outcomes, but in the rodent model of hysterectomy, with and without concomitant ovarian removal. Chapters 5 and 6 explore the role of the VCD model of transitional

menopause, either alone (Chapter 5) or in concert with the rodent model of hysterectomy (Chapter 6), in modulating behavioral outcomes in the female rat. These chapters address the role of ovarian and endocrine changes that correspond with the menopause transition in modulating memory and anxiety-like behaviors. Chapter 7 begins to assess questions surrounding HT through the evaluation of the selective progestin segesterone acetate, SGA, in the Ovx model of surgical menopause. Finally, Chapter 8 evaluates the role of estrogen-based HT in altering inflammatory, anxiety-like, and memory outcomes using two surgical menopause models, Ovx and hysterectomy, in middle-aged female rats. The outcomes of this dissertation are manifold, highlighting the critical role that preclinical rodent modeling plays in investigating key questions pertaining to long-term brain and behavioral outcomes associated with diverse experiences of menopause. Such work can provide further areas for clinical research to explore, and can provides insight for clinical care of menopausal women across the trajectory of aging.

CHATPER 2

AGE IMPACTS THE BURDEN THAT REFERENCE MEMORY IMPARTS ON AN INCREASING WORKING MEMORY LOAD AND MODIFIES RELATIONSHIPS WITH CHOLINERGIC ACTIVITY

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ABSTRACT

Rodent aging research often utilizes spatial mazes, such as the water radial-arm-maze (WRAM), to evaluate cognition. The WRAM can simultaneously measure spatial working and reference memory, wherein these two memory types are often represented as orthogonal. There is evidence, however, that these two memory forms yield interference at a high working memory load. The current study systematically evaluated whether the presence of a reference memory component impacts handling of an increasing working memory load. Young and aged female rats were tested to assess whether aging impacts this relationship. Cholinergic projections from the basal forebrain to the hippocampus and cortex can affect cognitive outcomes, and are negatively impacted by aging. To evaluate whether age-related changes in working and reference memory profiles are associated with cholinergic functioning, we assessed choline acetyltransferase activity in these behaviorally-tested rats. Results showed that young rats outperformed aged rats on a task testing solely working memory. The addition of a reference memory component deteriorated the ability to handle an increasing working memory load, such that young rats performed similar to their aged counterparts. Aged rats also had challenges when reference memory was present, but in a different context. Specifically, aged rats had difficulty remembering which reference memory arms they had entered within a session, compared to young rats. Further, aged rats that excelled in reference memory also excelled in working memory when working memory demand was high, a relationship not seen in young rats. Relationships between cholinergic activity and maze performance differed by age in direction and brain region, reflecting the complex role that the cholinergic system plays in memory and attentional processes across the

female lifespan. Overall, the addition of a reference memory requirement detrimentally impacted the ability to handle working memory information across young and aged timepoints, especially when the working memory challenge was high; these age-related deficits manifested differently with the addition of a reference memory component. This interplay between working and reference memory provides insight into the multiple domains necessary to solve complex cognitive tasks, potentially improving the understanding of complexities of age- and disease- related memory failures and optimizing their respective treatments.

Introduction

The field of learning and memory has historically utilized a variety of methods to assess spatial navigation during learning and memory tasks, ranging from Tolman's sunburst maze to test rodents (Tolman, 1948) to the virtual reality techniques used in more recent human neuroimaging studies (Spiers et al., 2001; see review: Burgess et al., 2002). There is a long history of work showing memory decline during normal aging in humans (Cherry et al., 1993; Salthouse et al., 1989; Weaver Cargin et al., 2007; see reviews: Cohen et al., 2019; Glisky, 2007; Harada et al., 2013) and in rodent models (Barnes, 1979; Bimonte et al., 2003; Bizon et al., 2009; Frick et al., 1995; see reviews: Bettio et al., 2017; Foster, 2012; Gallagher & Nicolle, 1993; Rodefer & Baxter, 2007). Mazes requiring rodents to navigate through space to successfully solve a cognitive task have been used for decades (Bimonte-Nelson, 2015a), often focusing on hippocampal-related spatial learning and memory, where performance has consistently been shown to decline with age (Schimanski & Barnes, 2015). There is a rich history of theory and operational definitions conceptualizing the way that maze tasks are learned and solved, which has informed and driven much of the learning and memory literature (Baddeley & Hitch, 1974; Brown et al., 1993; for reviews: Bimonte-Nelson, 2015a; O'Keefe & Nadel, 1978). Spatial learning and memory in rodents can be categorized into multiple domains, most commonly spatial working memory, which is a form of short-term memory that must be constantly updated, and spatial reference memory, which is a form of long-term memory that remains fixed across time (Bimonte-Nelson et al., 2015; Bizon et al., 2009; Foster, 2012; Frick et al., 1995; for origin of definitions see: Olton, 1979; Olton & Papas, 1979; Jarrard et al., 1984). In the preclinical literature, working, and reference memory

are often described as representing two separate memory systems, and are quantified as such (Auger & Floresco, 2014; Bimonte-Nelson, 2015d; Bimonte-Nelson et al., 2003, 2015; Braden et al., 2011; Camp et al., 2012; Dennes & Barnes, 1993; Hiroi et al., 2016; Jarrard et al., 1984, 2012; Olton, 1979).

Prominent in the rodent literature is the use of the radial-arm maze (RAM) (Bimonte-Nelson et al., 2004; Braden et al., 2011; Koebele et al., 2019; Olton, 1979; Olton & Samuelson, 1976; Prakapenka et al., 2018; Ward et al., 1999; see reviews: Olton, 1979; Bimonte-Nelson et al., 2010, 2015), a multi-arm apparatus requiring rodents to locate a reinforcer at the ends of particular arms, often by navigating through space using distinctive extramaze cues for optimal performance. The RAM is a win-shift task, with optimal performance involving the animal receiving a reinforcer in one location and then "shifting" away to a new location for the next reinforcer, rather than returning to the initial site of reinforcement within a testing session; this is accomplished by presenting reinforcement only upon first entry into a given arm (Bimonte-Nelson et al., 2015). In this manner, the RAM evaluates working memory, as an animal must update where it has already visited within a session. As reinforcers are removed and trials progress within a day, more items need to be remembered and the working memory load increases. The RAM can test working memory alone by providing a reinforcement in every arm, or the protocol can be modified to simultaneously test working memory and reference memory (Bimonte-Nelson et al., 2015). When evaluating working and reference memory simultaneously on the RAM, only a specific subset of the arms is associated with reinforcement; in this manner, the arms that contain a reinforcer become working memory arms, and arms that never contain a reinforcer become reference memory arms

(Bimonte-Nelson et al., 2015). The capability of the RAM to efficiently and simultaneously assess working memory and reference memory, particularly when there are concerns for prior cognitive testing effects, generalization, or satiation with an extended behavioral battery (Blokland & Raaijmakers, 1993; Serrano Sponton et al., 2018), makes the RAM especially useful when evaluating variables likely to be impacted by these concerns. On the appetitively-motivated land version of the RAM, performance in aged rats has been shown to be compromised compared to young rats (Bimonte-Nelson et al., 2015; Luine & Hearns, 1990; Luine & Rodriguez, 1994; Ward et al., 1999).

Our laboratory has shown that rodents display age-related deficits in both working and reference memory domains on the water radial-arm maze (WRAM), a water-escape version of the RAM; this has been demonstrated on the 12-arm (Bimonte et al., 2003), and the 8-arm (Bimonte-Nelson et al., 2003, 2004; Koebele et al., 2017) WRAM tasks. While we have shown age-related deficits on WRAM tasks with both working and reference memory components, even when the number of working memory arms and the working memory load differed between the 12-arm and 8-arm mazes, it has not been determined whether the presence of reference memory arms impacted these outcomes. Deciphering whether age impacts the ability to handle an increasing working memory load when reference memory arms are not present is critical to better understand the parameters of age-associated memory changes.

Using the WRAM, we have found that errors made into working and reference memory arms typically occur in concert, indicating that working and reference memory demands impact each other (Braden, Andrews, et al., 2017; Braden et al., 2011; Koebele et al., 2019; Mennenga, Gerson, Koebele, et al., 2015; Mennenga, Koebele, et al., 2015;

Prakapenka et al., 2018). Here, we evaluate relationships between working and reference memory by systematically testing working memory performance with and without the presence of reference memory demands. We test whether the ability to handle an increasing working memory load is impacted by the simultaneous necessity to handle reference memory information, as well as whether successful learning and maintenance of reference memory is affected by an increasing working memory load. Since we aim to better understand these relationships with aging, both young and aged rats are included in the current experiment.

While decades of spatial navigation work has demonstrated that aging impacts both spatial working memory outcomes (Bimonte et al., 2003; Coppola et al., 2014; Uresti-Cabrera et al., 2015) and spatial reference memory outcomes (Frick et al., 1995; Gage et al., 1984), much of this early work was done with males. More recent work testing both males and females during aging underscores the importance of characterizing unique cognitive outcomes in females (Benice et al., 2006; Bimonte et al., 2000; Bowman, 2005; Talboom et al., 2014). This is especially poignant given the recent position statements from the National Institutes of Health to include females in clinical and preclinical research (Clayton & Collins, 2014; L. R. Miller et al., 2017), and with the burgeoning research area of menopause and hormone therapies as aging ensues (Braden et al., 2011; Gibbs, 2003; Koebele et al., 2017; Lewis et al., 2008; Luine & Rodriguez, 1994). Therefore, we aim to characterize how aging in females impacts the relationship between spatial working and reference memory.

Cholinergic projections from the basal forebrain to the hippocampus and cortex play important roles in spatial learning and memory, in part *via* effects on stimulus

detection and attention (Everitt & Robbins, 1997; Gibbs, 2010; Koebele & Bimonte-Nelson, 2017; Parikh & Bangasser, 2020; Venkatesan et al., 2020). These projections are negatively affected by normal aging and with neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Beach et al., 2000; Drachman et al., 1980; Gibbs, 2003; Mesulam et al., 1987; Mufson et al., 2002; Schliebs & Arendt, 2011). Here, in behaviorally tested females, we evaluate choline acetyltransferase (ChAT) activity in the hippocampus and frontal cortex as an indicator of cholinergic function. This experimental design provides the unique opportunity to further explore how age and differential memory demands modulate the relationship between cholinergic activity and cognitive performance. Collectively, this work characterizes relationships between spatial reference and working memory outcomes in young and aged female rats, and explores whether ChAT activity in specific brain regions varies in relationship to memory depending on task demands at these two age timepoints. We hypothesize that the addition of reference memory will detrimentally impact working memory trajectory as the load increases, which will exhibit a greater burden with age. Furthermore, we hypothesize that these outcomes will correlate with cholinergic findings in regions critical to spatial learning and memory.

Methods

Animals

Twenty young (4 months of age) and 19 aged (21 months of age) female Fischer-344 rats were ordered at the same time from the National Institute on Aging colony at Charles River Laboratories in Raleigh, North Carolina. All animals arrived in the same shipment, were pair-housed upon arrival, and were maintained together in the same room,

which was set on a 12-h light/dark cycle (lights on at 7:00a.m.). Rats were given food and water freely throughout the study. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Arizona State University and adhered to the standards of the National Institutes of Health.

Timeline

The experimental timeline is depicted in Figure 3A. Behavioral testing was run on the same timeline for all subjects, beginning 21 days after their arrival. Ten young rats and nine aged rats were assigned to the 8-Arm WRAM (Figure 3B), and separate groups of 10 young rats and 10 aged rats were assigned to the 12-Arm WRAM (Figure 3C). Behavioral testing began each morning at the same time of day, and the 8-Arm WRAM was in a room separate from the 12-Arm WRAM. Animals were removed from their pair-housed cage in the vivarium, and placed individually into their own separate testing cage. Upon completion of testing for that day, each animal was returned to its pair-housed cage in the vivarium. Testing order was counter-balanced by age within each testing room to control for age-related effects due to testing order. After WRAM testing, rats were evaluated for their ability to perform the procedural components of a water-escape task using the 1-day Visible Platform (VP) task. Rats were euthanized and the brains dissected the day following completion of the behavioral battery.

Eight-Arm Water Radial Arm Maze

The 8-Arm WRAM was used to test working memory as working memory load increased, as described previously (Bimonte-Nelson, 2015e). Figure 3B contains a

schematic of the 8-Arm maze configuration. Escape platforms were hidden ~3 cm under the surface of the water in seven of the eight arms, and the water was made opaque with the addition of non-toxic black paint. The temperature of the water was kept at a range of 18–20°C. Salient external cues were placed in the testing room, including two panels on the walls outside the maze, the door, a row of tables with heating lamps, and the experimenter at the starting arm.

The subject was released into the start arm and given 3 min to find a platform. If the rat did not find a platform in the allotted trial time of 3 min it was guided to the nearest platform. Once on the platform, the rat remained for a total for 15 s before returning to the warmed home cage for a 30 s inter-trial interval. During this time, the platform recently found was removed, and the maze was cleaned of debris and water was swept over to eliminate any possible odor cues. The animal was then brought back to the start arm, where it was allowed to find another platform. This continued until all seven platforms were located; in this way, the working memory load was increased as each trial progressed, since rats needed to remember not to return to arms found previously that day. Once all seven platforms were located, the rat was returned to its heated home cage and testing began for the next animal. Testing continued in this fashion for a total of 12 days.

Arm entries were counted when the tip of the rat's snout passed 11 cm into each arm, marked by a line on the outside of the maze arms. Measures of working memory errors were quantified as done for previous research using the WRAM (Bimonte-Nelson, 2015e; Braden et al., 2010; Prakapenka et al., 2018). Working Memory Correct (WMC) errors were the number of entries into an arm that previously contained a platform for

that session. Start Arm (START) errors were the number of entries into the start arm; as expected, animals made few START errors on the WRAM task, and therefore analyses of START errors were not included in subsequent analyses for the 8-Arm WRAM. The sum of all errors made for each trial was defined as Total Memory (Total) errors. Of note, for this 8-Arm maze, Total errors were nearly identical to that of WMC errors across testing days due to the low number of start arm entries, as schematically depicted in Figure 4. Errors were evaluated for each daily session and were analyzed across all days of testing.

Twelve-Arm Water Radial Arm Maze

To determine the impact of the addition of reference memory on working memory performance as working memory load increases, the 12-Arm WRAM was used as described previously (Bimonte et al., 2003). Figure 3C contains a schematic of the 12-Arm maze. Platforms were hidden about 3 cm under the surface of the water, which was made opaque with non-toxic black paint, at seven of the 12 arms, leaving four arms without platforms in addition to the start arm. Thus, like the 8-Arm maze, the 12-Arm maze had 7 arms platformed. This was done to ensure an equal number of working memory items across both mazes, such that the working memory information that needed to be handled increased in equal increments across trials for both the 8-Arm maze and the 12-Arm maze. In the 8-Arm task, there was a start arm and no reference memory arms, whereas in the 12-Arm task there was a start arm plus four reference memory arms. This allowed for a rigorous and systematic evaluation of the addition of reference memory to a working memory task. The temperature of the water was likewise kept at a range of 18–20°C. Each subject had unique platform locations that were semi-randomly determined and remained fixed for each animal across days throughout the experiment. Salient

external cues were placed in the testing room, including two panels on the walls outside the maze, the door, a row of tables with heating lamps, and the experimenter at the start arm.

The testing procedure for the 12-Arm task was identical to the testing procedure for the 8-Arm task. WMC errors were the number of entries into an arm that previously contained a platform for that session. Reference Memory (RM) errors were the number of first entries into an arm that has never contained a platform for that particular animal, referred to as a reference memory arm (this included the start arm). Working Memory Incorrect (WMI) errors were any subsequent entry into an arm that had never contained a platform; in this manner, WMI errors are a working memory failure by repeatedly entering a reference memory arm in the search for an escape platform. Total errors were the sum of all errors made across a trial. Errors were evaluated for each session and were analyzed across all days of testing.

Visible Platform Task

After WRAM testing was completed, rats received 1 day of testing on the VP task as previously described (Bimonte-Nelson, 2015c; Braden et al., 2010; Koebele et al., 2017; Prakapenka et al., 2018), to ensure that all subjects were able to successfully complete the procedural components of a water-escape task. Rats were placed into a rectangular tub (100 × 60 cm) with water kept at 18–20°C, and external maze cues were covered by an opaque curtain that encircled the maze. Subjects were given 90 s to locate a clearly visible black platform protruding 4 cm from the water throughout testing. Once the platform was found, the rats remained there for 15 s before being returned to its warm home cage with an inter-trial interval of 5–8 min. Subjects were given six trials, with the

location of the platform varying in space semi- randomly, and the drop-off location remaining constant across trials. Performance on the VP task was evaluated *via* latency (seconds) to the platform.

Tissue Collection

The day following the conclusion of behavioral testing, rats were deeply anesthetized using isoflurane, decapitated, and brains were rapidly dissected and frozen. The dissected tissues were weighed in pre-weighed microcentrifuge tubes and stored at −70°C until analysis commenced. Dissections were performed using ice cold saline for the following brain regions as described by Paxinos and Watson (1998): frontal cortex (plates 5– 14), perirhinal cortex (plates 39–42), entorhinal cortex (EC; plates 39–42), the dorsal hippocampus (plates 33–35), and the ventral hippocampus (plates 39–42). For each brain, the frontal cortex was first collected from the dorsal portion of the whole brain. Next, the brain was cut in the coronal plane and the dorsal hippocampus was collected. Finally, a secondary coronal cut was made posterior to the first cut, where the ventral hippocampus and the entorhinal and perirhinal cortices were collected.

ChAT Activity Analyses

To prepare brain tissues collected at sacrifice for ChAT activity analyses, cold sonication medium containing 10mM EDTA and 0.5% Triton X-100 was prepared and added in a ratio of 10 µl solution per 1mg tissue, where tissue weight was measured as a wet weight during sacrifices; each sample was then sonicated. After sonication, each sample was aliquoted and assayed to ascertain its total protein concentration (Bradford, 1976). The ChAT activity of each sample was determined using a radioenzymatic method that has previously been published (Gibbs et al., 2011), wherein the total production of

[^{3}H]- acetylcholine was measured under a 37° C reaction for 30 min in a medium with a final acetyl-CoA concentration of 0.25 mM (30,000–40,000 d.p.m./tube; Sigma Inc., St. Louis, MO), with 10 mM EDTA, 10 mM choline chloride, 0.2 mM physostigmine-sulfate, 300 mM NaCl, and 50 mM sodium phosphate buffer (7.4 pH). The determination of background was performed by processing identical tubes without the addition of sample. Reactions were terminated in 4 mL 10 mM cold sodium phosphate buffer, then with the addition of 1.6 mL acetonitrile containing 5 mg/mL tetrephenylboron. Phase separation was obtained by adding 8mL of Econo-Flour scintillation cocktail (Packard Instruments, Meriden, CT) and by counting the total cpm while in the organic phase. The difference between the total cpm and the background cpm was then utilized to estimate the total amount of ACh produced per sample. Aliquots of each diluted sample was used in determining the total protein in each reaction tube, as well as for collecting the total ChAT activity. The average ChAT activity was calculated from three reaction tubes per sample, where ChAT activity was represented as pmol of ACh synthesized/hr/µg protein.

Statistical Analyses

For behavior assessments, data were analyzed using an omnibus repeated measures ANOVA with Maze (two levels: 8-Arm and 12-Arm) and Age (two levels: Young and Aged) as independent variables, Errors as the dependent variable, and Trials within Days as repeated measures. While 12 days of baseline testing were conducted, Day 1 of WRAM testing was excluded from all analyses. On Day 1, animals are water-maze naïve; therefore, the first day of testing is considered to be a training session and is typically excluded from subsequent analyses (Bimonte-Nelson et al., 2015; Hiroi et al., 2016). As an initial assessment, to evaluate overall performance on the WRAM, omnibus

repeated measures ANOVAs were run to examine the main effects of Age and Maze on Total errors across Days 2–12 of testing. Similar analyses were run for WMC errors for both mazes. In addition, for the 12-Arm WRAM only, as it was the only task with a reference memory component, we analyzed RM errors (first entry into a reference memory arm) and WMI errors (repeat entries into these reference memory arms). Based on prior publications from our laboratory concerning blocking (Bimonte-Nelson et al., 2015), particularly those involving the 12-Arm WRAM (Bimonte et al., 2003), days of WRAM testing were blocked into two separate phases to distinguish the learning and memory retention portions of testing: Days 2–7 were the Acquisition Phase of testing, and Days 8–12 were the Asymptotic Phase of testing. Each phase of testing was analyzed separately using repeated measures ANOVA for WMC errors for both the 8-Arm and 12-Arm mazes, as well as for WMI and RM errors for the 12-Arm WRAM. RM errors by definition are capped at 5 total errors within a testing day (the first entry into each of the 5 RM arms), making performance for a given trial heavily dependent on performance during previous trials for this measure. Therefore, main effects of Age were analyzed for this measure, but effects of Trial or interactions with Trial were not probed, as is the common procedure in our laboratory (Bimonte-Nelson et al., 2004; Braden, Andrews, et al., 2017; Braden et al., 2015; Mennenga, Koebele, et al., 2015). In the case of a significant Age × Maze interaction for WMC errors, the variables Age and Maze were analyzed separately as repeated measures ANOVA, with Maze or Age as the independent variable, Errors as the dependent variable, and Trials within Days as the repeated measure. In the case of a significant Trial × Maze, Trial × Age, or Trial × Age × Maze interaction for either testing phase, all trials were analyzed individually as repeated

measures ANOVAs, with Maze and/or Age as the independent variables and Days as the repeated measure. Upon significant Age × Maze interactions for an individual trial, the variables Age and Maze were analyzed separately as repeated measure ANOVAs, with Maze or Age as the independent variable, Errors as the dependent variable, and Days as the repeated measure. For all behavioral analyses, alpha was set at $p < 0.05$, and marginal findings were set at p values between 0.05 and 0.10.

Pearson r correlations were performed, along with Fisher's tests of significance, to determine the relationship between RM errors made in earlier trials (T2) with WMC errors made on the highest working memory load trial (T7) on Day 12 for the 12- Arm WRAM, with young and aged groups undergoing separate analysis. This was performed to determine if the reference memory errors made earlier within a testing day correlated with a greater number of WMC errors made at the end of the testing day; such a relationship would indicate that reference memory ability early within a session impacts working memory ability at the end of a session, when working memory load is highest.

ChAT activity was analyzed for the dorsal and ventral hippocampus, frontal cortex, entorhinal cortex, and perirhinal cortex using a series of ANOVAs with Age and Maze as the independent variables and ChAT activity as the dependent variable (pmol of ACh produced/hr/µg protein).

Pearson r correlations were performed with Fisher's tests of significance to determine the relationship between ChAT activity and WMC errors for the Asymptotic Phase (Days 8– 12), which were averaged across days and summed across trials. These correlations were run for each of the four groups separately (8-Arm Young, 8-Arm Aged, 12-Arm Young, 12-Arm Aged).

Results

Water Radial-Arm Maze

Overall WRAM performance. Maze components included seven spatial working memory arms in both the 8-Arm and 12-Arm mazes, plus the addition of reference memory arms in only the 12-Arm maze. WMC errors (entries into previously platformed arms, referred to as working memory arms) were analyzed for both mazes, and the unique addition of reference memory arms in the 12-Arm WRAM yielded analysis of RM errors (first entries into unplatformed arms, referred to as reference memory arms) and WMI errors (re- entries into unplatformed, or reference memory, arms). To aid the collective interpretation, we visually represent the proportion of WMC errors that contribute to Total errors from the 8-Arm WRAM and the 12-Arm WRAM; we did this for each age group separately (Figure 4). As expected, the majority of Total errors in the 8-Arm WRAM were WMC errors; however, on the 12- Arm WRAM, WMC errors accounted for only a portion of Total errors, likely due to the presence of the reference memory arms which give rise to the potential to commit additional error types.

Analysis of maze performance revealed a main effect of Day for Total errors [$F_{(10, 350)} = 2.28$, $p < 0.05$], with errors decreasing across Days 2–12, indicating that rats did indeed learn the WRAM task (data not shown). For Total errors [$F_{(1, 35)} = 318.11$, $p < 0.0001$], as well as WMC errors [$F_{(1, 35)} = 46.91$, $p < 0.0001$], there was an effect of Maze across Days 2–12, whereby rats in the 12-Arm WRAM made more errors than those in the 8-Arm WRAM. This finding indicates that the addition of a reference memory component in the 12-Arm WRAM resulted in rats making more WMC errors as

compared to rats tested on the 8-Arm WRAM, which does not contain this additional reference memory component (data not shown). There also was a main effect of Age for Total errors across Days 2–12 [$F_{(1, 35)} = 17.35$, $p < 0.001$; data not shown], indicating that aged rats made more errors than young rats. This main effect of Age was also seen in WMC errors [$F_{(1, 35)} = 8.41$, $p < 0.01$; data not shown], for Days 2–12, such that aged rats made more WMC errors than did young rats, regardless of maze. Main effects of Age for RM errors [$F_{(1, 18)} = 5.22$, $p < 0.05$] and WMI errors [$F_{(1, 18)} = 14.69$, $p < 0.01$] for rats tested on the 12-Arm WRAM exclusively were also detected across Days 2–12 (data not shown).

Working memory performance for the Acquisition Phase (Days 2-7). Analysis of performance during the Acquisition Phase revealed a main effect of Maze [$F(1, 35) = 13.70$, $p < 0.001$], whereby rats tested in the 12-Arm WRAM performed worse than those in the 8-Arm WRAM (Figure 5A), as well as a significant Age × Maze interaction [$F(1, 35) = 4.31$, $p < 0.05$]. Further analyses showed that within the 8-Arm WRAM, aged rats performed worse than young rats [$F(1, 17) = 9.68$, $p < 0.01$], but within the 12-Arm WRAM there was no difference in performance with age. For young rats, there was a main effect of Maze for the Acquisition Phase [$F(1, 18) = 30.84$, $p < 0.0001$], with young rats tested on the 8-Arm WRAM performing better than those tested on the 12-Arm WRAM. No significant effect of Maze was seen for aged rats. Significant interaction effects for Trial × Age [$F(5, 175) = 2.88$, $p < 0.05$], Trial × Maze [$F(5, 175) = 3.25$, $p < 0.01$], and Trial × Age × Maze [$F(5, 175) = 5.28$, $p < 0.001$] were also found during the Acquisition Phase, indicating that rats performed differently throughout the series of

trials depending on age and maze (Figure 5B). Subsequent analyses of each trial alone showed that the rats tested on the 12-Arm WRAM performed worse than those tested on the 8-Arm WRAM across the majority of trials, suggesting that the presence of a reference memory component impacted performance regardless of whether the working memory load demand was low or high. Specifically, main effects of Maze were found on Trial 2 [$F(1, 35) = 8.10$, $p < 0.01$], Trial 3 [$F(1, 35) = 5.41$, $p < 0.05$], Trial 4 [$F(1, 35) = 8.23$, $p < 0.01$], and Trial 6 [$F(1, 35) = 17.82$, $p < 0.001$]. For the highest working memory load trial, Trial 7, there was an Age × Maze interaction [$F(1, 35) = 8.21$, $p < 0.01$], with aged rats tested on the 8-Arm WRAM making more errors on Trial 7 than young rats on the 8-Arm WRAM [$F(1, 17) = 13.74$, $p < 0.01$]. In contrast, no age effects on Trial 7 were present for the 12-Arm WRAM. Analysis of the performance of young rats on Trial 7 during the Acquisition Phase revealed an effect of Maze, whereby young rats tested on the 12-Arm WRAM made more errors than those tested on the 8-Arm WRAM [$F(1, 18) = 9.92$, $p < 0.01$]; this effect was not found for aged rats (Figure 5B).

Working memory performance for the Asymptotic Phase (Days 8-12).

Analysis of performance during the Asymptotic Phase revealed a main effect of Age [$F(1, 35) = 4.40$, $p < 0.05$], whereby aged rats made more working memory errors than young rats, and a main effect of Maze [$F(1, 35) = 23.21$, $p < 0.0001$], where rats tested on the 12-Arm WRAM, evaluating both working and reference memory, made more errors than those tested on the 8-Arm WRAM, evaluating solely working memory (Figure 6A). While there was not a significant Age × Maze interaction for the Asymptotic Phase, due to the nature of the relationships between Age and Maze that we noted during the

Acquisition Phase, these same analyses were conducted within the Asymptotic Phase. Within the 8-Arm WRAM there was a main effect of Age [$F(1, 17) = 4.68$, $p < 0.05$], whereby aged rats made more errors than their young counterparts. This was not true of rats tested on the 12-Arm WRAM. Furthermore, there were effects of Maze for both young [$F(1, 18) = 13.64$, $p < 0.01$] and aged [$F(1, 17) = 9.79$, $p < 0.01$] rats, with rats of each age tested on the 12-Arm WRAM making more WMC errors than those tested on the 8- Arm WRAM. There also were interactions for both Trial × Age [$F(5, 175) = 3.49$, $p < 0.01$] and Trial × Maze [$F(5, 175) = 3.25$, $p < 0.01$], indicating that, similar to the Acquisition Phase of testing, performance during the Asymptotic Phase differed across trials depending upon age and maze (Figure 6B). Subsequent analyses of each testing trial revealed a main effect of Maze [Trial 2: $F(1,35) = 11.10$, $p < 0.01$; Trial 3: $F(1, 35) = 6.90$, $p < 0.05$; Trial 4: $F(1, 35) = 14.36$, $p < 0.001$; Trial 5: $F(1, 35) = 20.94$, $p < 0.0001$; Trial 6: $F(1, 35) = 7.35$, $p < 0.05$; Trial 7: $F(1, 35) = 6.75$, $p < 0.05$], with rats tested on the 12-Arm WRAM making more errors than those tested on the 8-Arm WRAM. For the highest working memory load trial (Trial 7), there was a main effect of Age [$F(1, 35) = 4.56$, $p < 0.05$] and a significant effect of Maze [$F(1, 35) = 6.75$, $p < 0.05$, as mentioned previously], where aged rats made more errors than young rats, and rats tested in the 12-Arm WRAM made more errors than those tested on the 8- Arm WRAM. Much like in the Acquisition Phase of testing, there was an effect of Maze for young rats, where young rats tested on the 8-Arm WRAM performed better than those tested on the 12-Arm WRAM [$F(1, 18) = 8.99$, $p < 0.01$], and this Maze effect was not present for aged rats. Regarding an effect of Age within the solely working memory task, aged rats tested on the 8-Arm WRAM performed worse than young rats tested on the 8-Arm WRAM [$F(1,$

17) = 4.92, $p < 0.05$]. The two ages did not differ for WMC errors on the 12-Arm WRAM. The presence of an age effect for WMC errors in the 8-Arm WRAM, but not the 12-Arm WRAM, indicates that the addition of a reference memory component in the 12-Arm WRAM overpowered any age-related differences for this evaluation of working memory (Figure 6B).

Collectively, these data suggest that, regardless of phase of learning, the addition of a reference memory component in the 12-Arm WRAM resulted in both young and aged rats making a larger number of WMC errors compared to the 8- Arm WRAM, where multiple reference memory arms were not present. Thus, for the 12-Arm WRAM, age did not appear to play a significant role for WMC performance, as both young and aged rats displayed poor working memory performance for this measure. However, for the 8-Arm WRAM, age played a significant role, whereby age-related deficits were seen with an increasing working memory load for WMC errors.

Assessment of age-related differences in working memory and reference memory performance when reference memory requirements are added. After addressing the differences in performance between the 8-Arm WRAM and the 12-Arm WRAM for both young and aged rats using WMC errors, the only measure shared by both mazes, the reference memory arm component of the 12-Arm WRAM was analyzed alone to determine age-related outcomes. Thus, within the 12-Arm WRAM, performance was additionally quantified via assessment of first entries into arms that never contained a platform (RM errors) and repeat entries into arms that never contained a platform (WMI errors). In this manner, we were able to evaluate the unique effects that the addition of

reference memory arms had on both reference memory and working memory performance for both aged and young rats.

When evaluating RM errors in the Acquisition Phase, the main effect of Age was not significant, indicating that young and aged rats did not differ in maintaining reference memory items while learning the task. For the Asymptotic Phase, there was a main effect of Age [$F_{(1, 18)} = 5.47$, $p < 0.05$] (data not shown), whereby aged rats made more RM errors than young rats, suggesting that young rats better maintained reference memory information at the end of testing on the maze that evaluated both working and reference memory.

For WMI errors, during the Acquisition Phase, there was a marginal main effect of Age [$F_{(1, 18)} = 3.81$, $p = 0.07$], whereby aged rats tended to make more WMI errors while learning the task than did young rats (Figure 7A). Additionally, during the Acquisition Phase, there was a significant Trial × Age interaction [$F_{(6, 108)} = 4.46$, $p < 0.001$; Figure 7B], indicating that the effects of Age on WMI errors were trial-dependent. Further analyses demonstrated that on Trial 6, with a high working memory load, there was an effect of Age, whereby aged rats made more entries into unplatformed arms than young rats [$F_{(1, 18)} = 14.70$, $p < 0.01$]. Likewise, across the Asymptotic Phase (Days 8–12), there was a main effect of Age [$F_{(1, 18)} = 6.30$, $p < 0.05$], where aged rats made more WMI errors than young rats, displaying that the working memory capabilities of aged rats suffered relative to young rats with the addition of a reference memory component (Figure 7C). The Trial × Age interaction was not significant (Figure 7D).

Correlations between reference memory and working memory in the 12-Arm WRAM. To further investigate the role that the addition of a reference memory component to a working memory task plays in working memory performance in young and aged rats, a correlation was performed for each age separately between RM errors on Day 12 for Trial 2, and WMC errors on Day 12 for Trial 7, in the 12-Arm WRAM. In this manner, we evaluated whether reference memory errors made on the earliest trial were related to working memory errors made on the last trial, when working memory load was most taxed. This allowed for evaluation of the role that reference memory prowess, demonstrated early within a testing day, played in later working memory outcomes. There was a positive correlation for the aged group [$r(8) = 0.73$, $p < 0.05$; Figure 8], indicating that aged rats that made more reference memory errors on an early trial tended to make more working memory errors when working memory was most burdened. There was no correlation for young rats. This further indicates the non- orthogonality of reference memory and working memory in the WRAM task, particularly for aged rats when working memory is sufficiently taxed.

Visible Platform Task

Motor and visual acuity for solving the procedural components of a water-escape task were analyzed using the VP task. Groups did not differ from each other across testing trials in the ability to locate and navigate to the visible platform following the first introductory trial [Trials 2–6; Effect of Age: $F(1, 35) = 0.01$, p = n.s.; Effect of Maze: $F(1, 35) = 0.89$, p = n.s.; Age × Maze Interaction: $F(1, 35) = 0.43$, p = n.s.]. The average escape latency for each group by the final trial was no greater than 10s, indicating that

rats had the visual and motor acuity necessary to complete a water escape task (Figure 9). Given that our laboratory has shown that this task identifies rats with motor issues (Mennenga, Gerson, Dunckley, et al., 2015), the findings here indicate that the young and aged rats in this experiment did not have significant motor issues that would hinder maze performance.

ChAT Activity Assays

ChAT activity was analyzed for the dorsal hippocampus, ventral hippocampus, frontal cortex, perirhinal cortex, and entorhinal cortex. For the ventral hippocampus and the frontal cortex, aged rats exhibited higher levels of ChAT activity than young rats [main effect of Age for ventral hippocampus: $F_{(1, 35)} = 6.30$, $p < 0.05$; main effect of Age for frontal cortex: $F_{(1, 35)} = 9.56$, $p < 0.01$; Figures 10A,B]. For the dorsal hippocampus, entorhinal cortex, and perirhinal cortex, there were no main effects of Age (data not shown). There were no main effects of Maze, nor were there any Age × Maze interactions, for any of the brain regions analyzed.

Correlations between ChAT activity levels in each brain region and WRAM performance (WMC errors made averaged across Days 8–12) were analyzed to further investigate the relationships between these variables, and to assess whether these subsequent relationships vary by age and memory type. For aged rats tested on the 8-Arm WRAM, there was a positive correlation between ChAT activity in the ventral hippocampus and WMC errors [$r(7) = 0.84$, $p < 0.01$; Figure 10C], whereby more working memory errors made specifically on the 8-Arm WRAM correlated with higher levels of ventral hippocampus ChAT activity for aged rats. For young rats tested on the 8-Arm WRAM, the opposite relationship was found for the frontal cortex; indeed, there

was a negative correlation between frontal cortex ChAT activity and WMC errors [$r(8) = -0.78$, $p < 0.01$; Figure 10D], whereby higher ChAT activity in the frontal cortex in young rats was correlated with fewer working memory errors on the 8-Arm WRAM.

Discussion

The current study demonstrated that the addition of a reference memory component impaired working memory performance in both young and aged female rats throughout testing on the WRAM task. However, the type of memory failure that increased after the addition of the reference memory component differed between the ages. Specifically, aged rats made more working memory errors than young rats on the working memory only task, and these comparably higher working memory errors in aged rats persisted when reference memory was added to the task. Aged rats evaluated on the combined working and reference memory task also made more reference memory errors than young rats. These effects were pronounced for the latter portion of testing. Thus, young rats learned to successfully handle the additional reference memory challenge, while aged rats did not. This yielded an age-related failure to maintain reference memory information in the combined working and reference memory task.

Even though the number of working memory arms was constant in both the sole working memory task and the combined working and reference memory task, there were age-related impairments in the ability to handle an increased working memory load only in the task that tested exclusively working memory. Thus, the addition of a reference memory challenge further burdened the ability to handle an increasing working memory load in young rats, while in aged rats, the working memory load was likely already taxed maximally even without the reference memory challenge. These findings correspond to

previous work demonstrating age-related deficits in performance on a multitude of spatial learning and memory tasks (Bimonte et al., 2003; Coppola et al., 2014, 2015; Frick et al., 1995; Koebele et al., 2017). Our current findings also show that the presence of both a reference memory and working memory component combined with a sufficient working memory load results in a working memory impairment in young rats that is comparable to the impairment in aged rats when they are tested solely on a working memory task. Overall, both young and aged rats were able to learn the procedural components of the WRAM, as shown by their improved performance across all days of WRAM testing. Additionally, young and aged rats did not differ in their ability to perform the procedural components of a water-escape task as tested in the VP task, suggesting that differences in WRAM scores were not attributable to differences in visual or motor competencies necessary to complete this battery.

For Total errors, there was an effect of Age across all testing days, whereby aged rats made more errors of each memory type than did young rats. This indicated that, regardless of maze type, aged rats were less able to handle the cognitive complexity of the spatial memory task than their younger counterparts; this age-effect is a replication and extension of prior work showing age-related impairments in spatial learning and memory (Barnes, 1979; Barnes et al., 1980; Bimonte et al., 2003; Frick et al., 1995; Shukitt-Hale et al., 2004). These findings also complement the human literature, wherein aged individuals demonstrate poorer spatial navigation as compared to young individuals, which largely stems from a deficit not in encoding or recognizing landmarks, but in the temporospatial ordering of these landmarks (Wilkniss et al., 1997). Even though both mazes had seven working memory arms to remember, overall maze performance was

poorer when reference memory arms were added on to the demand. Specifically, rats tested on the combined working and reference memory 12-Arm WRAM made more Total errors into those 7 working memory arms than rats tested on the 8-Arm WRAM. Notably this effect occurred regardless of age such that in both young and aged rats, spatial learning and memory was more heavily taxed by the addition of a reference memory component on the WRAM task.

Analyses of performance at the highest working memory load demonstrated unique maze effects between young and aged rats. When comparing WMC errors in both testing phases at the highest working memory load trial, young rats made fewer errors on the working memory only task as compared to the task that evaluated reference memory as well, as noted by the Maze effect in young rats. In contrast, there was no Maze effect for aged rats, indicating that the addition of a reference memory component did not further increase working memory impairments in the aged cohort. These findings support the tenet that the sole spatial working memory task is so challenging for aged rats that the addition of a reference memory component does not further burden working memory. This phenomenon likely contributed to the emergence of age-related effects that differed depending on maze type. Indeed, for both testing phases, at the highest working memory load, aged rats tested in the working memory only task made more errors on the pure working memory measure than young rats. In contrast, at the highest working memory load, young and aged rats did not differ in their ability to maintain working memory information on the combined working and reference memory task, suggesting that adding a reference memory component impacted the observation of age-related effects.

On the working memory only task there were age effects on the pure working memory measure, while on the combined working and reference memory task, the profile of age-related decrements shifted from pure working memory to first and repeat failures of reference memory information, the latter of which can be defined as a working memory process. While there were no age effects on entries into working memory arms (WMC errors) when both working and reference memory were present, there was an age-related deficit on both first and repeat entries into reference memory arms (RM and WMI errors). These results demonstrate that aged rats were not only challenged by the presence of 7 working memory arms, which were found in both mazes, but that the addition of reference memory arms led to additional working memory failures in revisiting these reference memory arms, a challenge that young rats were able to learn across testing. These findings suggest that there are differences in the manner with which young and aged female rats experience or solve this complex reference and working memory task. In rodents and other species, aging can impact strategies used to solve spatial memory tasks (Barnes et al., 1980; Begega et al., 2001; Coppola et al., 2014, 2015; Rodgers et al., 2012); it is possible that the differential outcomes for young and aged rats in the current study are the result of different strategies utilized to solve this complex water-escape task. Such findings have also been observed following manipulations in ovarian hormone levels in female rodents (Korol & Kolo, 2002); this is critical, as gonadal hormone levels change across the lifespan in the female rat, and could result in differential strategy utilization as a function of age (Korol & Pisani, 2015).

Measuring both working and reference memory simultaneously in the 12-Arm WRAM provided the unique opportunity to determine if rats that excelled in reference

memory when there was minimal working memory demand (Trial 2) also excelled in working memory when demand was maximal (Trial 7). Thus, we asked, does higher reference memory ability relate to higher working memory ability at its highest load? Our results showed that this relationship was present in aged rats, but not in young rats. Specifically, for aged rats, there was a significant positive correlation, with aged animals that made more reference memory errors tending to also make more working memory errors at the highest challenge. This positive relationship between the two error types further supports the tenet that the two measures are not orthogonal in aged rats, presenting an exciting opportunity for further investigation into the relationship between working and reference memory, and putative age-related differences in this relationship. For young rats, these two error types did not correlate. One explanation for this is that young animals made very few reference memory errors on the last day of baseline testing (Day 12), yielding little variability within the group. It therefore seems likely that while the measures of reference memory and working memory are logistically scored and quantified independently for the WRAM, they are not functionally orthogonal from a psychophysiological systems approach. Probing and deciphering reciprocal relationships between these memory types will enrich and diversify analytic interpretations of maze data, especially in age-related contexts. That the presence of working memory might impact reference memory outcomes should be carefully considered. For example, systematically examining reference memory when tested in concert with working memory, as done here with the WRAM, as compared to testing on a task where the sole measure is reference memory, as done with the win-stay Morris Maze, can aid interpretation of not only reference memory findings, but relationships amongst working

and reference memory as working memory demand shifts from minimal to maximal difficulty. Indeed, reference memory outcomes on the WRAM (when working memory is also present) often do not parallel reference memory outcomes on the Morris Maze (when working memory is not present), for example, with pharmaceutical or genetic manipulations (Acosta et al., 2010; Braden, Andrews, et al., 2017; Camp et al., 2012; Holter et al., 2019).

The literature linking memory, aging, and the cholinergic system led us to evaluate age-related differences in cholinergic activity after exposure to tasks requiring different memory types and memory demands. ChAT activity assays were performed in the dorsal hippocampus, ventral hippocampus, frontal cortex, perirhinal cortex, and entorhinal cortex, and these values were correlated with cognitive functioning. Aged female rats exhibited greater ChAT activity in the ventral hippocampus and frontal cortex compared to young rats, while age did not impact ChAT activity in the entorhinal cortex, perirhinal cortex, or dorsal hippocampus. These findings contribute to a larger body of literature studying aging in rodents, non-human primates, and humans, showing degradation of cholinergic functioning with aging in brain regions critical to learning and memory (Araujo et al., 1990; Haley et al., 2011; Rinne, 1987; Tayebati et al., 2002). Notably, much of this prior work was conducted in behaviorally naïve subjects. Behavioral testing itself is likely to significantly impact cholinergic outcomes, as this has previously been shown for neurotrophins (Bimonte et al., 2003), and studies have shown that the cholinergic system can be modified with food reward (Aitta-Aho et al., 2017), as well as with testing on a spatial discrimination task (Toumane et al., 1988). Assessing how complex behavioral testing impacts cholinergic function in specific brain areas

amongst the broader system could add new dimensions and interpretations to age-related questions. For example, ChAT activity in the basal forebrain was a strong predictor of age-related spatial learning deficits as tested on the reference memory version of the Morris Maze in rats (Gallagher et al., 1990). Recent research has shown that cholinergic inputs modulate cue detection, cue salience, and top-down regulation of how cues are used to influence behavior (for reviews: Parikh & Bangasser, 2020; Venkatesan et al., 2020). These attentional processes are strongly linked to performance on memory tasks, as there is significant overlap between brain regions involved in both attention and memory (see review: Chun & Turk-Browne, 2007). In solving complex spatial navigation tasks, rodents typically rely heavily upon the presence of extramaze cues to locate the hidden platform(s); cholinergic output in related brain areas is therefore likely affected by this attentional demand. Hence, the observed increases in ChAT activity in both the frontal cortex and ventral hippocampus in aged female rats may be compensatory in nature, reflecting the brain's attempt to compensate for weaknesses elsewhere by ramping up cholinergic activity to ultimately yield improved attentional and memory outcomes.

The current findings suggest that with aging, when navigating a complex spatial maze task, cholinergic activity increases in brain regions important for spatial learning and memory outcomes; however, these increases in activity are insufficient to alter behavioral outcomes, as aged rats still demonstrated repeated learning and memory failures. These findings are in accordance with work from human studies (Dumas & Newhouse, 2011). Indeed, increases in prefrontal and parietal activity have been observed *via* functional MRI during evaluation on working memory and visual attention tasks in aged adults relative to young adults (Cabeza et al., 2004). This increase in brain activity

with aging in regions critical to working memory processing is thought to be in response to a sufficient challenge; in fact, optimal activity within these brain regions to maximize working memory performance is shifted higher in aged adults than young adults evaluated *via* MRI (Rypma & D'Esposito, 2000). Furthermore, postmenopausal women administered the antimuscarinic drug scopolamine, known to impair working memory, showed increased MRI frontal lobe and hippocampal activity relative to placebo (Dumas et al., 2011). These complimentary findings shown in both the clinical and preclinical literature are further supported by the data shown herein. Specifically, for aged rats tested in the solely working memory WRAM, there was a positive correlation between ventral hippocampus ChAT activity and WMC errors, with greater cholinergic activity associated with increased working memory errors. This could be predicted in a compromised system, which ramps up cholinergic activity to compensate for age- related memory deficits. This is consistent with the assertion that "aging acts as an intervening variable" (Sarter & Bruno, 1998) in experiments designed to evaluate the vulnerability and restorative capacity of basal forebrain cholinergic projections, at least in the context of injury and degenerative processes. Conversely, for young rats, the relationship between cholinergic activity and behavioral outcomes is inverted: in a system that is not excessively challenged by the attentional demands of a spatial working memory task, greater cholinergic activity is sufficient to drive improvements in performance. This was reflected in the correlation for young rats whereby greater cholinergic activity in frontal cortex was associated with fewer working memory errors, thereby reflecting that increased cholinergic functioning is associated with better working memory outcomes in young adulthood. These findings demonstrate that, with aging, cholinergic modulation

may be insufficient to reverse deficits in learning and memory processing, as aged rats remained impaired across the task; this fits with the broader understanding in the field that, with aging, the brain undergoes a multitude of changes beyond that of cholinergic dysregulation (Koebele & Bimonte-Nelson, 2017). The current work underscores that aging is a complex biological process, where behavioral outcomes are driven by a wealth of neurobiological changes. Future work should continue to evaluate the role that attentional demand, in addition to working memory and reference memory demand, plays in altering cholinergic outcomes across the female aging trajectory in both the clinical and preclinical domains.

Conclusion

Taken together, the current findings support the precept that young rats are more capable of performing spatial working memory tasks compared to aged rats, and that the addition of a reference memory component yields a decrease in working memory performance with an increasing working memory load, such that young rats perform similarly to their aged counterparts. This interference of reference memory items on the working memory system suggests that the two are not orthogonal as once believed, and should be considered carefully when interpreting data from instruments that are often used to measure both working and reference memory constructs simultaneously, vs. tasks that measure them individually. Analyses of ChAT activity in both the ventral hippocampus and frontal cortex revealed an age effect, whereby aged rats had greater ChAT activity than young rats. The rigorous behavioral testing on a spatial learning and memory task likely impacted this outcome *via* a compensatory mechanism, whereby cholinergic output is increased in aged rats to aid in the attentional and mnemonic

processing necessary to perform this complex spatial navigation task. Correlations demonstrate relationships between working memory and cholinergic activity within distinct brain regions across the two age groups, specific to the purely working memory task. This study highlights the complex relationships between age, cognitive demand, and cholinergic activity in discrete brain regions. Exploring relationships between working and reference memory can provide innovative breadth and depth into study outcomes, supplementing interpretations of better or worse performance including interactions between memory systems and attentional demands. The evaluation of not only working memory and reference memory outcomes in isolation, but of working and reference memory outcomes simultaneously and their relationships to one another, can give us the translatability necessary to better understand the complexities of age and neurodegenerative-related memory failure. This is critical, as in everyday life, people engage in activities that demand multiple cognitive domains simultaneously, including reference memory and working memory. Deciphering varied neurobiological profiles, as related to the ability to handle multidimensional memory demands and challenges, can optimize therapeutics for targeted functional outcomes in populations where cognition is impacted.

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CHAPTER 3

TASK-DEPENDENT LEARNING AND MEMORY DEFICITS IN THE TGF344-AD RAT MODEL OF ALZHEIMER'S DISEASE: THREE KEY TIMEPOINTS THROUGH MIDDLE-AGE IN FEMALES

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ABSTRACT

The TgF344 rat model of Alzheimer's disease (AD) provides a comprehensive neuropathology presentation, with age-dependent development of tau tangles, amyloid-beta (Aβ) plaques, neuronal loss, and increased gliosis. The behavioral trajectory of this model, particularly relating to spatial learning and memory, has yet to be fully characterized. The current experiment evaluated spatial working and reference memory performance, as well as several physiological markers of health, at 3 key age points in female TgF344-AD rats: 6-months, 9-months, and 12-months. At 6 months of age, indications of working and reference memory impairments were observed in transgenic (Tg) rats on the water radial-arm maze, a complex task that requires working and reference memory simultaneously; at 12 months old, Tg impairments were observed for two working memory measures on this task. Notably, no impairments were observed at the 9-month timepoint on this maze. For the Morris maze, a measure of spatial reference memory, Tg rats demonstrated significant impairment relative to wildtype (WT) controls at all 3 age-points. Frontal cortex, entorhinal cortex, and dorsal hippocampus were evaluated for $A\beta_{1\text{-}42}$ expression via western blot in Tg rats only. Analyses of $A\beta_{1\text{-}42}$ expression revealed age-dependent increases in all 3 regions critical to spatial learning and memory. Measures of physiological health, including heart, uterine, and body weights, revealed unique age-specific outcomes for female Tg rats, with the 9-month timepoint identified as critical for further research within the trajectory of AD-like behavior, physiology, and pathology.

Introduction

As of 2021, the Alzheimer's Association estimates that in the United States alone, 6.2 million individuals are living with Alzheimer's disease (AD), and almost two-thirds of these cases are women (Alzheimer's Association, 2021). There are currently no disease-modifying treatments to halt or attenuate the progressive neurodegeneration, memory loss, and cognitive decline that accompany this disease, making AD a tremendous public health crisis. Only after decades of pathological progression does clinical symptomology begin to present in patients with probable AD (Braak et al., 2011; Perl, 2010; Vickers et al., 2016). This lengthy preclinical stage adds to the difficulty of finding efficacious treatments, as the burden of brain changes is extensive and potentially irreparable by the time of clinical diagnosis. As such, it is pertinent that researchers characterize the trajectory of prodromal symptoms of AD, which will aid in identifying reliable measures for early diagnosis and treatment.

Transgenic rodent (Tg) models aim to reproduce pathological and behavioral characteristics of patients with AD, with the hope of probing the lengthy and largely silent prodromal stages of the disease. Cohen and colleagues generated a Tg rat model (TgF344-AD) that expresses the human amyloid precursor protein (APP) with the Swedish mutation and the mutant human presenilin-1 lacking exon 9 (PSEN1dE9) (R. M. Cohen et al., 2013). Several studies have recently demonstrated the promise of the TgF344-AD rat in modeling and enriching our understanding of the pathological and cognitive changes associated with human AD. Specifically, TgF344-AD rats develop soluble amyloid-beta (Aβ) oligomers that later aggregate into Aβ plaques, as well as endogenous hyperphosphorylated tau leading to neurofibrillary tangle-like structures that

develop in an age-dependent manner (R. M. Cohen et al., 2013). Additionally, this model presents with neuroinflammation, cerebrovascular dysfunction, reduced synaptic transmission, and frank neuronal loss in AD-associated regions of the brain (Joo et al., 2017; Smith & McMahon, 2018; Wu et al., 2020).

While the temporal development of pathology in this model seems to recapitulate that seen with clinical AD, the behavioral characterizations conducted thus far using the TgF344-AD rat model have yielded conflicting results. Currently, the age at which impairments in specific memory domains emerge in the TgF344-AD model is unclear. One study demonstrated spatial reference memory deficits measured by the Morris Water Maze (MWM) in TgF344-AD rats compared to wildtype (WT) rats as early as 6 months of age (Rorabaugh et al., 2017), whereas other studies have reported no significant spatial learning and reference memory deficits at this timepoint via the MWM or the Barnes maze (R. M. Cohen et al., 2013; Pentkowski et al., 2018). However, at 6 months of age, Cohen and colleagues detected a tendency toward impairment in TgF344-AD rats compared to WT rats in the reversal phase of the Barnes task, assessing cognitive flexibility, which is thought to depend, in part, on the frontal cortex (R. M. Cohen et al., 2013). The discordance of the studies performed to date, in addition to the early indications of memory impairment, warrant a more nuanced evaluation of spatial learning and memory at this 6-month timepoint, in which the detection of burgeoning cognitive deficits may depend on the specific type of memory being assessed as well as the paradigm used to assess it.

Further evaluations of spatial memory have been conducted for the TgF344-AD rat beyond 6 months of age. In fact, between 7-13 months of age, a similar pattern of

discordant findings has been reported. One study evaluating spatial navigation strategy and spatial reference memory found that as early as 7-8 months of age, TgF344-AD rats showed a decrease in the directness of their swim path trajectory to the escape platform in the MWM, but not a significant impairment when analyzing swim path length, suggesting that TgF344-AD rats utilized a different navigation strategy without having overt spatial reference memory impairments at this 7-8 month timepoint (Berkowitz et al., 2018). In this same study, robust impairments in spatial reference memory were detected in TgF344-AD rats at 10-11 months of age. Notably, other studies have reported no significant differences in spatial reference memory between TgF344-AD and WT rats in the Barnes maze at 12-13 months of age, nor impairments in spatial or non-spatial recognition memory via two variants of the novel object recognition task in 12 month-old TgF344-AD rats (Morrone et al., 2020; Wu et al., 2020). TgF344-AD rats between 9-12 months of age showed impaired spatial memory, but intact non-spatial recognition memory, relative to WT rats, suggesting a dissociation of effects depending on task type (Galloway et al., 2018). Importantly, the initial characterizations of the TgF344-AD rat model by Cohen and colleagues found no observable sex differences on the tasks assessed, and consequently, a majority of the subsequent studies on this model combined male and female rats (R. M. Cohen et al., 2013). This may account for some of the discordant behavioral findings, as previous AD rodent models have displayed sex differences in cognitive function and neuropathology (Pang et al., 2022; Yang et al., 2018), and from a clinical perspective, it is well established that females are at a greater risk of developing AD compared to males (Mielke et al., 2014). Overall, using different paradigms to assess specific memory domains, combining sexes and ages, as well as a

lack of statistical power to detect nuanced memory changes, likely contribute to the lack of accordance in behavioral findings. As such, these studies prompt further investigation into the complex behavioral phenotype of the TgF344-AD rat model, particularly in females, as they are disproportionately affected by AD, and at critical age ranges for this model from young adulthood through middle-age.

The current experiment aimed to assess how spatial learning and memory changes manifest in 6, 9, and 12-month old female TgF344-AD rats, with the goal to elucidate the timeline of nuanced behavioral changes in working and reference memory domains. While many of the aforementioned studies focused on spatial navigation and long-term memory, no studies have systematically characterized spatial working memory performance as a variation of cognitive load in early adulthood. Prior work has indicated that changes in working memory may be one of the earliest cognitive symptoms associated with AD (Bianchini et al., 2014; Jahn, 2013; Zokaei & Husain, 2019), and working memory impairments have been shown to reliably predict the progression from mild cognitive impairment (MCI), a prodromal stage of AD, to AD dementia (Kirova et al., 2015). The present study utilized the water radial-arm maze (WRAM) to test spatial working and reference memory performance as memory load increases (Bimonte-Nelson et al., 2015). These assessments testing an increasing working memory demand provide a more sensitive measurement of cognitive change and a reduced likelihood of ceiling effects for animals in a preclinical stage of development. Physiological markers of health were also evaluated in female TgF344-AD rats and WT controls, and elements of brain pathology were evaluated in the Tg rats, with the goal of constructing a comprehensive picture of how the TgF344-AD rat model could translate to the presentation of AD in

women. These assessments provide opportunities to discover relationships among cognitive, select brain pathology, and health related markers at the earliest stages of functional AD symptom onset.

Methods

Subjects

To establish a transgenic colony at Arizona State University, a male rat carrying the APP/ PSEN1dE9 transgenes and 4 female WT rats on a Fischer 344 NHSD background were obtained from the University of Southern California. These rats were used to breed subsequent generations of experimental rats within our laboratory, of which 30 WT sexually inexperienced female rats and 30 Tg sexually inexperienced female rats were used for the current study. For each genotype, rats were aged to 6, 9, and 12 months at the onset of behavioral testing, making for the following experimental groups: 6-month Tg rats (n=10), 6-month WT rats (n=10), 9-month Tg rats (n=11), 9-month WT rats (n=10), 12-month Tg rats (n=9), and 12-month WT rats (n=10). One subject from the 6-month Tg group was excluded from all analyses due to abnormal behavioral results and protein concentrations as analyzed via western blot. All rats were kept on a 12-hour light/dark cycle (light on at 7:00am) with *ad libitum* access to food and water. All experimental protocols were approved by the Arizona State University (ASU) Institutional Animal Care and Use Committee and were in accordance with guidelines from the National Institutes of Health (NIH). Furthermore, the study was completed in accordance with ARRIVE guidelines. For a brief overview of the experimental timeline, see Figure 11.

Animal Breeding and Housing

Breeding all experimental subjects involved pairing either 2 WT females with a Tg male, or 2 Tg females with a WT male within a cage. Transgenic status was confirmed via Polymerase Chain Reaction (PCR; see Genotyping section below) before selection as a breeder. A systematic breeding schedule was developed for this study to ensure that rats were the specific ages of 6, 9, and 12 months old during simultaneous behavioral evaluation. Breeding pairs were placed together in a cage a month prior to the target birth date of a litter, as the typical rat gestation period is 21 days. Litter size was between 3 to 14 pups. Before the litters were born, the male rat was removed from the cage to avoid disturbance of the dam or the litter.

To achieve the proper distribution of Tg and WT rats for each age cohort in the current study, a minimum of 10 breeding pairs were placed together. In order to breed litters with sufficient numbers of subjects, there was a broader range of ages within each cohort than initially anticipated. The 9-month cohort bred according to our optimal schedule, while the 12-month cohort breeding yielded rats closer to 11 months of age at behavioral testing, and the 6-month cohort yielded an age range of 5-6 months at behavioral testing.

On postnatal day 10, rat pups were identified as male or female by anogenital distance. At this time, a small razor blade was used to snip the tip of the tail ($\leq$ 3 mm) of each pup. Tail snips were stored at -70ºC until later genotyping. To identify each subject, ear punches were performed directly following tail cuts. On postnatal day 21, rat pups were weaned from their dam and same-sex housed into littermate pairs. During breeding, when there was an odd number of males or females, the remaining rat was individually housed. No experimental animals were single-housed in the current study; if an

experimental animal did not have a littermate, it was paired with a non-experimental animal of the same sex and age. Only females were used as experimental subjects in this study. Males were culled or used as future colony breeders.

Genotyping

PCR was used to confirm the presence or absence of the human mutant APP transgene with tail samples collected from each rat, as previously published (Mifflin et al., 2021). First, DNA from tail samples was digested using a solution with a 24:1 ratio of Sodium Chloride-Tris-EDTA buffer and proteinase K, totaling 500ul per sample. Samples were incubated overnight in a water bath heater set to 55°C. DNA was extracted from each sample by centrifuging samples at 15,000 rpm for 10 minutes at 4°C. Subsequent supernatant was added to 500ul of isopropyl alcohol. This solution was vigorously shaken to isolate the DNA before being placed into a Speedvac for 3 minutes to remove all liquid. The DNA strand for each sample was then resuspended in 150 µM TE buffer and placed back into the 55°C water bath to incubate overnight. The following day, DNA samples were added to a solution with a 7:1:12.5 ratio of distilled water, 3 primers (PRP internal control, PRP reverse, and APP), and Taq buffer. Samples were then placed in a thermocycler to amplify DNA, and a gel was prepared by heating 1.8% agarose in 1X Tris-borate EDTA. For fluorescence of DNA bands, 10ul GelRed nucleic acid gel stain was added to the gel preparation. Following use of the thermocycler, 1X green loading dye was added to each sample, and the samples were subsequently pipetted into each gel well, along with an appropriate ladder. Gels were run at 200mV for 30 minutes before being placed under UV light to capture photos of the protein bands using a BioRad Gel Doc XR. A lower band (400bp) corresponded to the presence of the APP

transgene and indicated that the corresponding rat was Tg; a higher band (750bp) indicated a WT rat.

Body Weights and Vaginal Cytology

Weekly body weights were collected for 9 weeks across the duration of the experiment to determine the effects of genotype and age on body weight, as well as to assess overall animal health. One month prior to the initiation of behavioral testing, vaginal smears were completed for all subjects to monitor vaginal cytology with age and across genotype using a protocol previously established in our laboratory (Koebele & Bimonte-Nelson, 2016). Vaginal smears were collected for 8 consecutive days, approximately 2 full estrous cycles, where smears were classified as 1 of 4 phases: estrus, metestrus, diestrus, and proestrus. Estrus was characterized by the presence of mostly cornified cells; metestrus by a mixture of cornified cells, round epithelial cells, needle-like cells, and leukocytes; diestrus by leukocytes with or without cornified cells; and proestrus by epithelial round cells with or without cornified cells. Characterization of each phase was completed in correspondence with previously published work (Goldman et al., 2007; Koebele & Bimonte-Nelson, 2016).

Behavioral Battery

Behavioral testing was carried out at the same time each morning (8:00AM, +/- 30 minutes) in two separate rooms for greater efficiency. Counterbalancing was performed to ensure that there was an even distribution of ages and genotypes (i.e. 50% WT, 50% Tg) across both rooms.

Water radial-arm maze (WRAM). For the current experiment, the WRAM was utilized to assess spatial working and reference memory, as previously done in our

laboratory (Koebele et al., 2019; Koebele, Mennenga, et al., 2020; Prakapenka et al., 2018) using established protocols (Bimonte-Nelson, 2015d). The WRAM involved an 8-arm apparatus filled with 18-20°C water located in a room with robust extra-maze cues to aid in spatial navigation. Such cues included a large black and white striped rectangle (22" by 55") mounted to the wall in front of the maze, as well as a large black rectangle (22" by 55") on the adjacent wall. Other extra-maze cues included a desk, a long table, and the tester (who remained in a fixed location while the rats swam). At the ends of 4 of the 8 maze arms, platforms were hidden 2-3 cm under the water surface, further obscured by non-toxic black paint in the water. Platform locations were semi-randomized across subjects and counterbalanced across experimental groups, but were maintained across all days of testing for a given rat. At the beginning of a testing day, rats were placed into individual testing cages and brought into the testing rooms in groups of 10. At the beginning of a testing session, the rat was placed into the start arm and given 3 minutes to swim to 1 of the 4 hidden platforms. If the rat did not find a platform within the maximum trial time, it was led to the nearest platform, at which point the trial was complete. If the rat found the platform within the 3-minute trial time, the rat was allowed to remain on the platform for a total of 15 seconds before being removed from the maze and placed into its heated testing cage for a 30 second inter-trial interval (ITI). During the ITI, the just-found platform was removed, and the maze was swept for odor cues. After the ITI, the rat was placed back into the start arm of the maze and given 3 minutes to swim and navigate to a remaining platform. Trials continued in this manner until all 4 platforms were located, allowing for an incremental increase in working memory load across testing trials, as rats had to remember not to re-visit arms where platforms were

located earlier within a day. Upon completion of the 4th trial, the rat was returned to its testing cage, and behavioral testing for the next rat was initiated. Testing was completed across 2 testing rooms simultaneously for all experimental subjects. Rats were tested in this manner for a total of 12 baseline days of testing. On the 13th day of WRAM testing, a 6-hour delay was implemented between trials 2 and 3 to evaluate delayed memory retention.

Performance on this task was evaluated via errors, or the number of entries a rat made into an arm that did not contain a platform prior to locating a platform on a given trial. Errors were further categorized into 3 types: Working memory correct (WMC) errors, reference memory (RM) errors, and working memory incorrect (WMI) errors. WMC errors were entries into arms that previously contained a platform, indicating the rat remembered a correct platform location but failed to update their memory by recognizing they had already found that platform during that day of testing. RM errors were first entries into a non-platformed arm for a given testing day, reflecting a failure in long-term memory. WMI errors were each subsequent entry into a non-platformed reference memory arm for a given testing day, demonstrating the rat failed to update they already visited that arm and were not rewarded.

Morris water maze (MWM). Upon completion of the WRAM, rats commenced MWM testing the following day to evaluate spatial reference memory, as done previously in our laboratory (Hiroi et al., 2016; Prakapenka et al., 2018) using published protocols (Bimonte-Nelson, 2015b). The MWM consisted of a large circular pool filled with 18-20°C water made opaque with non-toxic black paint, surrounded by robust extra-maze cues to aid in spatial navigation. These cues included a large black and white striped

rectangle (22” by 42”) mounted to the wall in front of the maze, as well as a large black rectangle (22” by 28”) with a smaller white rectangle inside it (11” by 14”) on the opposing wall. In addition to these cues, a computer desk, a long table, and the tester (who remained in a fixed location while the rats swam) served as additional extra-maze cues. In the northeast quadrant of the maze, a platform was submerged 2-3cm underneath the water, where it remained for the duration of baseline testing. Across all baseline testing trials, rats were dropped off semi-randomly from 4 different locations (labeled N, S, E, W) around the maze and given 1 minute to swim to the platform. If the rat did not find the platform within the 1-minute trial time, it was led to the platform and the trial was terminated. Once on the platform, the rat remained there for 15 seconds before being placed back into its testing cage. The maze was then swept for odor cues and debris before the next rat was placed into the maze. The ITI for this task was approximately 10-15 minutes, as all rats completed one trial before moving on to the next trial. Rats completed 4 trials per day for 5 testing days. On the 5th testing day, a final 5th trial was added as a probe trial, where the platform was removed from the maze. The rat was allowed to swim for 1 minute, permitting evaluation of spatial localization to the platform.

MWM performance was assessed by swim distance and swim latency to the platform. To evaluate these measures, a video camera and computer equipped with Ethovision 6 (Noldus Instruments, Wageningen, The Netherlands) were used to record trials. For the probe trial, percent swim distance in the quadrant that previously contained the platform (Target Quadrant) and percent swim distance in the quadrant opposite the platform (Opposite Quadrant) were recorded for each rat.

Visible platform (VP). Following the MWM task, rats were evaluated on the single day VP task, which assesses visual and motor acuity, as well as ability to perform the procedural components necessary to solve a water-escape task. The VP task followed established protocols (Bimonte-Nelson, 2015c) that have been used in prior work from our laboratory (Koebele et al., 2019). This task consisted of a 100 cm x 60 cm tub filled with clear 18-20°C water surrounded by a large curtain to obscure any extra-maze cues. The back of the tub contained a black platform, placed 2-3cm above the water's surface. The platform was semi-randomly moved to 3 different locations across all 6 testing trials. At the beginning of the testing day, a rat was dropped off at the starting location and given 90 seconds to reach the platform. If the rat did not locate the platform in the given trial time, the rat was led to the platform, and the trial ended. If a rat was unable to locate the platform within this time on multiple trials, we considered this subject for exclusion from the data analysis on all behavioral tasks, as this could indicate an impaired ability to complete a water-escape based task. Upon locating the platform, the rat remained there for 15 seconds before being removed and placed into its heated testing cage. The maze was then swept for odor cues and debris before the next rat began its trial. Testing continued in this fashion, with an average ITI of 5-8 minutes for all 6 testing trials. Swim latency to platform was recorded for VP performance.

Tissue Collection

After conclusion of the behavioral battery, all rats were euthanized to collect tissues for further processing. At euthanasia, rat ages were approximately 7 months (termed the 6-month groups), 10 months (termed the 9-month groups), and 13 months (termed the 12-month groups). Rats were deeply anesthetized with isoflurane before

blood was collected via cardiocentesis. Hearts were then weighed (wet weight). Brains were extracted for rapid dissection, as done in previous work from our laboratory (Braden et al., 2011). Brain dissections were performed using Eagle Medium kept on ice; dissected regions including the right Frontal Cortex (FC; Plates 5-14), right Entorhinal Cortex (EC; Plates 39-42), and right Dorsal Hippocampus (dHPC; Plates 33-35) were collected as previously described (Paxinos & Watson, 1998). Upon collection of each region, tissues were placed in individual pre-weighed microcentrifuge tubes, weighed, and stored at -70°C until further analysis. Left and right ovaries and uterine horns were also collected from each subject, trimmed of any abdominal fat, and weighed (wet weight).

Western Blot Protein Analysis

Samples of FC, EC, and dHPC brain regions from the right hemisphere of each Tg subject (N=30) were processed for relative expression of $A\beta_{1\text{-}42}$ via western blot protein analysis. Tissue samples kept at -70°C were first suspended in a 1:25 weight-to-volume ratio of RIPA buffer solution (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris HCl), protease inhibitor (Millipore-Sigma) and phosphatase inhibitor (Millipore-Sigma), with sample tubes kept thereafter over ice. Tissues were homogenized using probe sonication (Ultrasonic Processor, Cole Parmer, IL, USA), and homogenate was centrifuged at 10,000 rpm for 10 minutes at 4°C. The resulting supernatant was collected and aliquoted to avoid freeze-thaw cycles for the samples. Aliquots were stored at -70°C. The protein concentration of each sample was determined using a bicinchoninic acid protein assay (BCA; Thermo-Fischer Scientific, Pittsburgh, PA, USA), where samples were run in duplicate and their concentration

values averaged, with no higher coefficient of variation between the duplicate samples than 11%. One sample from the dHPC of a subject in the 9-month Tg group had a protein concentration that exceeded the level of detection for this assay, resulting in the exclusion of this sample from western blot analysis.

Samples of differing ages were counterbalanced across each gel. Each gel also contained a positive and negative control from aged subjects in the colony that were confirmed to be Tg and WT, respectively. The NuPAGE PowerEase electrophoresis system was used for tissue processing. Samples for a given region were loaded in a 4-12% NuPAGE Bis-Tris gel in an XCell SureLock Mini-Cell (Invitrogen, Carlsbad, CA, USA), each with 10ug protein and run with MES running buffer. After protein separation, transfer was completed using an Immobilon polyvinylidene difluoride membrane. Following protein transfer, the membrane was incubated in 5% nonfat milk for 1 hour at room temperature before being washed with 1X TBST. The membrane was then incubated at 4°C overnight in the primary antibody for $A\beta_{1\text{-}42}$ (1:1,000; Cell Signaling #12843S) and the loading control beta-actin (1:20,000; Cell Signaling #4970S) in 5% milk. The following day, the membrane was washed with 1X TBST before incubation for 1 hour at room temperature in the secondary antibody anti-rabbit HRP (1:500; Cell Signaling #7074). The membrane was washed with 1X TBST and immunoreactivity was visualized with enhanced chemiluminescence (Lumiglo and peroxide solutions, Cell Signaling #7003S) and developed on a Konica SRX-101A Film Processor (Tokyo, Japan). Densitometry analyses were completed for resulting films using ImageJ software based upon previously established protocols (Gallo-Oller et al., 2018). $A\beta_{1\text{-}42}$ expression

was normalized to the expression of the loading control beta-actin for each gel. A total of 9 gels were run across all 3 brain regions.

Statistical Analyses

Statistical analyses were completed using StatView software. All behavioral data, with the exception of the WRAM delay and MWM probe trial, were analyzed using a series of planned comparisons which investigated potential effects of Genotype for each age (i.e., 6-month Tg vs. WT, 9-month Tg vs. WT, 12-month Tg vs. WT). This allowed us to address whether there were behavioral or neurobiological differences across genotype for each specific age cohort. All analyses were two-tailed, with an alpha level set at 0.05. Results were considered marginal where the *p* value was between 0.05 and 0.10.

For WRAM data, repeated measures ANOVAs were utilized for each set of planned comparisons within an age cohort, with Genotype as the independent variable, Errors as the dependent variable (with separate analyses for WMC, RM, and WMI errors), and Days and Trials as the repeated measures. Based on prior publications from our laboratory that have demonstrated differences in baseline testing days corresponding to performance in task acquisition and maintenance (Braden, Andrews, et al., 2017; Koebele, Mennenga, et al., 2020; Mennenga, Gerson, Koebele, et al., 2015; Prakapenka et al., 2018), WRAM data were analyzed following the set of planned comparisons outlined above in 2 separate blocks: the Acquisition Phase (Days 2-7) and the Asymptotic Phase (Days 8-12). These 2 blocks were also chosen based on the WRAM learning curve across all days of testing for these rats, whereby all rats had a steeper decrease in making errors from Days 2-7 while acquiring the task rules, with performance plateauing from

Days 8-12, maintaining task performance. For data collected during the WRAM delay, each experimental group was analyzed individually with a repeated measures ANOVA, where WMC errors served as the dependent variable, and Trial 3 performance on the last baseline day of testing (Day 12) versus Trial 3 performance directly following the delay (Day 13) served as the repeated measure.

MWM data were analyzed using a repeated measures ANOVA in the same set of planned comparisons as previously described, where Genotype was the independent variable, Swim Distance (cm) or Swim Latency (s) to the Platform was the dependent variable, and Trials within Days was the repeated measure. The probe trial was analyzed for each experimental group using a repeated measures ANOVA, where percent swim distance in the Target (NE) or Opposite (SW) Quadrant served as the within-subjects dependent variable.

For the VP task, repeated measures ANOVA were run for the same set of planned comparisons, with Genotype as the independent variable, Latency (s) to the Platform as the dependent variable, and Trials as the repeated measure.

Weekly body weight data were analyzed using repeated measures ANOVA with the same set of planned comparisons as described above, where Genotype was the independent variable, Body Weight was the dependent variable, and Weeks was the repeated measure. Other organ weights collected at the end of the experiment were analyzed using ANOVA with the same set of planned comparisons, where Genotype served as the independent variable and Wet Weight or the ratio of Wet Weight to Body Weight as the dependent variable.

Data from all Tg subjects for western blot protein analyses were analyzed via ANOVA, with Age as the independent variable and normalized relative expression of $A\beta_{1\text{-}42}$ as the dependent variable, and individual analyses were run for each brain region. With a significant effect of Age, *post hoc* analyses were performed using Fisher protected least significant difference (PLSD). One subject in the 6-month Tg group had levels of $A\beta_{1\text{-}42}$ in several brain regions that far exceeded that of the group; this subject was subsequently removed from all behavioral and brain analyses.

To determine putative relationships between behavioral, physiological, and neurobiological outcomes within Tg rats, a series of Pearson r correlations were completed with Fisher's tests of significance. Specifically, WMC and WMI errors summed across trials and averaged across days for each block of WRAM testing were correlated with $A\beta_{1\text{-}42}$ expression in the FC, dHPC, and EC to determine whether there was a relationship between working memory performance and amyloid-beta expression in these brain regions. MWM Swim Latency and Distance summed across trials and across days were correlated with $A\beta_{1\text{-}42}$ expression in the FC, dHPC, and EC to determine if there was a relationship between reference memory performance and $A\beta_{1\text{-}42}$ expression in these regions. Additionally, body weight at the end of the experiment was correlated with $A\beta_{1\text{-}42}$ expression in the FC, dHPC, and EC to determine whether a relationship existed between physiological health and amyloid-beta protein expression. These correlations were performed separately for each Tg experimental group (i.e., intraclass correlations). Given the exploratory nature of these correlations, corrections for multiple comparisons were not performed; the threshold of significance was set to $p < 0.01$ for all correlations.

Results

Vaginal Cytology

Vaginal smears performed across 8 consecutive days resulted in observations of age-typical estrous cyclicity, with most rats exhibiting normal cycles 4-5 days in length. Generally, the number of rats with irregular estrous cyclicity (e.g., prolonged cycles, persistent estrus/diestrus) increased with age: only 1/10 WT subject in the 6-month cohort was observed to have an irregular cycle, where 10 (5/10=WT, 5/11=Tg) rats in the 9-month cohort and 7 (5/10=WT, 2/9=Tg) rats in the 12-month cohort had irregular estrous cyclicity. These irregular estrous cycles did not appear to differ by genotype. This would indicate that WT and Tg rats had normally functioning ovaries, where there were indications of irregular cycles with increasing age as commonly observed in prior research (Koebele & Bimonte-Nelson, 2016; Talboom et al., 2014).

Behavioral Battery

WRAM - Baseline testing. Analysis of performance during the Acquisition Phase captures learning of the WRAM task, and analysis of performance during the Asymptotic Phase captures task memory maintenance. For RM errors during the Acquisition Phase, there was a marginal main effect of Genotype for the 6-month groups [$F_{(1,17)} = 3.65$, $p = 0.0732$, whereby Tg rats tended to make more RM errors compared to WT rats (Figure 12A). There were no other effects for RM errors for the 9 or 12-month groups for this initial phase of testing (Figure 12B,C), nor were there main effects or interactions with Genotype for WMC or WMI errors for any age-specific planned comparisons.

For RM errors during the Asymptotic Phase, there was a marginal main effect of Genotype for the 6-month planned comparison [$F_{(1,17)} = 4.27$, $p = 0.0544$], such that Tg rats made marginally more RM errors than WT rats (Figure 12D). Although there were no RM effects for the 9-month groups (Figure 12E), for the 12-month planned comparison, there was a main effect of Genotype for RM errors [$F_{(1,17)} = 14.87$, $p = 0.0013$], with Tg rats making more errors than WT rats in this oldest cohort, demonstrating a reference memory impairment among Tg rats (Figure 2f).

During the Asymptotic Phase of WRAM testing, for WMC errors, there was a marginal effect of Genotype for the 6-month cohort [$F_{(1,17)} = 3.09$, $p = 0.0967$], where Tg rats tended to make more errors than their WT counterparts (Figure 13A). While there were no significant Genotype effects for the 9-month cohort (Figure 13B), for the 12-month cohort, there was an effect of Genotype [$F_{(1,17)} = 4.90$, $p = 0.0408$], where Tg rats made more WMC errors than WT rats (Figure 13C). There were no Trial x Genotype interactions for WMC errors across any of the age-specific planned comparisons.

For WMI errors during the Asymptotic Phase, planned comparisons of the 6-month groups yielded a marginal effect of Genotype [$F_{(1,17)} = 3.27$, $p = 0.0883$], whereby Tg rats tended to make more errors than WT rats (Figure 13D). No effects of Genotype were present for the 9-month groups (Figure 13E). Similar to WMC errors, there was an effect of Genotype for WMI errors during the Asymptotic Phase for the 12-month groups [$F_{(1,17)} = 8.50$, $p = 0.0096$], with Tg rats making overall more WMI errors than WT rats (Figure 13F). Furthermore, there was a significant Trial x Genotype interaction for this planned comparison [$F_{(3,51)} = 10.02$, $p < 0.0001$]. When the highest working memory load trial, Trial 4, was probed separately, there was a Genotype effect [$F_{(1,17)} = 10.18$, $p =$

0.0054], where 12-month Tg rats made more errors compared to 12-month WT rats (Figure 13G).

WRAM - Delayed memory retention. To evaluate delayed memory retention, a 6-hour delay was implemented between Trials 2 and 3 on the final day of WRAM testing. Performance was evaluated by comparing WMC errors made on Trial 3 the final day of baseline testing (Day 12) to WMC errors made on Trial 3 directly following the delay (Day 13). For 6-month WT rats [$F_{(1,9)} = 12.24$, $p = 0.0067$] and 6-month Tg rats [$F_{(1,8)} = 12.00$, $p = 0.0085$], there was a main effect of Delay for WMC errors, where rats made more errors following the implementation of a delay (Figures 14A and 14B, respectively). There were no effects of the delay for the 9-month rats (Figures 14C and 14D). There was a main effect of Delay for WMC errors for the 12-month WT group [$F_{(1,9)} = 15.78$, $p = 0.0032$], where WT rats made more errors directly following the delay (Figure 14E); there was no delay effect for 12-month Tg rats (Figure 14F).

MWM. MWM data were analyzed across all 5 days of baseline testing to evaluate reference memory performance. For each set of age-specific planned comparisons, when evaluating swim distance to the platform for Days 1-5, there was a main effect of Genotype [6-month groups: $F_{(1,17)} = 5.63$, $p = 0.0297$; 9-month groups: $F_{(1,19)} = 9.41$, $p = 0.0063$; 12-month groups: $F_{(1,17)} = 11.37$, $p = 0.0036$] (Figure 15A-C). Likewise, when evaluating swim latency to the platform collapsed across all days and trials of the MWM, there was an effect of Genotype for each set of planned comparisons [6-month groups: $F_{(1,17)} = 7.00$, $p = 0.0170$; 9-month groups: $F_{(1,19)} = 11.31$, $p = 0.0033$; 12-month groups: $F_{(1,17)} = 7.76$, $p = 0.0127$] (Figure 16A-C). These collective data indicate a transgenic-

induced reference memory impairment, as Tg rats at each age had greater distances and longer latencies to reach the platform than respective WT rats.

For each experimental group, when evaluating percent total swim distance in the Target (NE) Quadrant versus the Opposite (SW) Quadrant during the probe trial, there was a main effect of Quadrant [6-month Tg: $F_{(1,8)} = 46.98$, $p = 0.0001$; 6-month WT: $F_{(1,9)} = 174.68$, $p < 0.0001$; 9-month Tg: $F_{(1,10)} = 11.91$, $p = 0.0062$; 9-month WT: $F_{(1,9)} = 52.79$, $p < 0.0001$; 12-month Tg: $F_{(1,8)} = 9.53$, $p = 0.0150$; 12-month WT: $F_{(1,9)} = 45.46$, $p < 0.0001$] (Figure 15D), where each group swam a greater percent of the total distance in the NE quadrant, where the platform was previously located, compared to the opposite SW quadrant. This indicates that each group spatially localized to the platform by the end of testing.

VP. To ensure that subjects had the visual and motor acuity necessary to complete the procedural components of a water escape task, VP data were analyzed for all experimental groups according to our set of planned comparisons. Across all 6 trials, when evaluating latency to the platform, there was a main effect of Trial for each set of planned comparisons [6-month groups: $F_{(1,17)} = 5.85$, $p = 0.0001$; 9-month groups: $F_{(1,19)} = 4.98$, $p = 0.0004$; 12-month groups: $F_{(1,17)} = 5.28$, $p = 0.0003$] (data not shown), with latency decreasing across testing trials; there was no effect of Genotype for any of the planned comparisons. For the 12-month groups, there was a significant Trial x Genotype interaction [$F_{(5,85)} = 2.98$, $p = 0.0158$]. This interaction appeared to be dependent upon Trial 1, where on average, Tg rats had longer swim latencies than their WT counterparts; this is likely due to exploratory behavior, and not the result of an inability to complete the

task. Overall, these effects suggest the rats were capable of performing a water-escape task, and that age and genotype did not significantly alter this ability.

Physiological Markers of Health

Body weight. Across the duration of the experiment, for each set of planned comparisons, there was a main effect of Genotype for body weights [6-month groups: $F_{(1,17)} = 10.66$, $p = 0.0046$; 9-month groups: $F_{(1,19)} = 65.20$, $p < 0.0001$; 12-month groups: $F_{(1,17)} = 5.04$, $p = 0.0384$] (Figure 17A-C), where Tg rats weighed more than their WT counterparts across all ages assessed. For each age, there was an effect of Week [6-month: $F_{(8,136)} = 13.06$, $p < 0.0001$; 9-month: $F_{(8,152)} = 37.88$, $p < 0.0001$; 12-month: $F_{(8,136)} = 23.19$, $p < 0.0001$], with weights increasing until the initiation of water maze testing, and thereafter the weights began to decrease (likely due to maze-related physical activity).

Uterine and ovary weights. For the uterine tissue collected at euthanasia, there was no effect of Genotype on wet weight for the 6-month and 9-month timepoint planned comparisons; however, for the 12-month timepoint, there was an effect of Genotype, where WT rats had heavier uteri than their Tg counterparts [$F_{(1,17)} = 5.08$, $p = 0.0377$] (Figure 17D-F). When evaluating ovary weights collected at euthanasia, there were no effects of Genotype across any of the age-specific planned comparisons (Figure 17G-I).

Heart weights. Hearts weights collected at euthanasia revealed a marginal Genotype effect for the 6-month timepoint [$F_{(1,17)} = 3.03$, $p = 0.0998$] and a significant Genotype effect for the 9-month timepoint [$F_{(1,19)} = 8.36$, $p = 0.0094$], with Tg hearts weighing more than WT hearts (Figure 17J-K). At 12-months, there was no effect of Genotype on heart weight (Figure 17L). When evaluating heart weight with respect to

body weight, as has previously been done (Turdi et al., 2009), the pattern of results changed: at the 9-month timepoint, there was a significant effect of Genotype [$F_{(1,19)} = 6.94$, $p = 0.0163$], where the WT heart-to-body weight ratio was greater than that of the Tg rats (data not shown). There were no other significant Genotype effects across the other age-points.

Western Blot Protein Analysis

Western blot protein analysis for the FC revealed a main effect of Age for A$\beta_{1\text{-}42}$ expression, normalized to the loading control beta-actin [$F_{(2,26)} = 48.84$, $p < 0.0001$], where relative A$\beta_{1\text{-}42}$ expression increased with age (Figure 18A). Likewise, there was a main effect of Age for relative A$\beta_{1\text{-}42}$ expression in the dHPC [$F_{(2,25)} = 24.65$, $p < 0.0001$] (Figure 18B) as well as in the EC [$F_{(2,26)} = 29.80$, $p < 0.0001$] (Figure 18C), in which greater expression was observed with increasing age. Cropped images of representative bands from these blots are shown in Figure 18 to best demonstrate target bands. There were no effects of Age for the loading control beta-actin across any brain region. These results indicate that, across these timepoints, Aβ expression is increasing within brain regions critical to learning and memory outcomes for female Tg subjects.

Correlations

Correlations between western blot and behavioral outcomes were performed for each experimental group. Specifically, correlations were run for A$\beta_{1\text{-}42}$ expression in the FC, dHPC, and EC with WMC and WMI errors across the Acquisition and Asymptotic Phases of WRAM testing as well as with Swim Latency and Distance for MWM. No significant correlations were found for these variables. In addition, correlation analyses were run for body weights at the end of the experiment and A$\beta_{1\text{-}42}$ expression in the FC,

dHPC, and EC to evaluate potential relationships between physiological and neurobiological outcomes in Tg rats. For the 9-month Tg group, there was a significant negative correlation between normalized A$\beta_{1\text{-}42}$ expression in the FC and body weight (r = -0.84, p = 0.0006), such that with increasing Aβ expression, there was a corresponding decrease in body weight **(**Figure 18D). There were no other significant correlations across any of the other experimental groups, or in any other brain region.

Discussion

The present study examined the manifestation of spatial learning and memory impairments as well as A$\beta_{1\text{-}42}$ protein expression in female TgF344-AD rats across 3 different age-points. In doing so, we aimed to better characterize the cognitive and neurobiological trajectory of this relatively novel transgenic rat model of AD, while focusing on key timepoints critical to female aging, as clinically, females are more susceptible to developing AD. Our results demonstrated that, while the trajectory of AD-like pathology followed a linear path, the behavioral trajectory of these rats was more complex, with consistent indications of early working memory impairment at the 6-month timepoint before a robust impairment at the 12-month timepoint. Furthermore, physiological characterizations revealed unique transgenic, age-dependent outcomes that are promising in providing the field with avenues for further research into the full characterization of AD.

Behavioral evaluations on the WRAM revealed that, across reference memory and working memory performance, early indications of learning and memory impairments in the TgF344-AD rat were present in the 6-month cohort. These repeated patterns of marginal transgenic impairment across each memory error type, along with

prior work demonstrating marginal impairment of reversal learning in 6-month old TgF344-AD rats on the Barnes maze (R. M. Cohen et al., 2013), form a behavioral profile that would suggest potential significant impairment if the working memory load was increased. Prior work in young, non-transgenic rats has demonstrated a lack of significant treatment- or age- related impairment when the working memory load is low, with more profound effects observed with a higher working memory load (Bernaud et al., 2021; Bimonte et al., 2003; Bimonte & Denenberg, 1999). The early indications of memory impairment within the 6-month cohort observed here are clinically relevant, as researchers have put forth efforts to find tasks that might be able to detect AD-related memory impairments at earlier timepoints (Marra et al., 2016) and that can distinguish AD from other forms of dementia (Zokaei et al., 2020). Prior work has indicated that there are prodromal changes in working memory functioning with AD (Bianchini et al., 2014; Jahn, 2013; Zokaei & Husain, 2019), leading researchers to conclude that spatial navigation tasks, similar to those used in the current experiment, should be included in early clinical evaluations to detect early manifestations in functional decline with AD (Coughlan et al., 2018, 2019; Tu et al., 2015). Future work should better characterize these early indications of working memory impairment, as they may aid in detection of incipient cognitive decline and correspond to key points of pathological progression in the TgF344-AD rat model. Such relationships could hold therapeutic potential, as current treatments for AD are only initiated after the disease has progressed profoundly in its pathological and cognitive trajectory (Cummings et al., 2021; Selkoe, 2011).

Notably, where indications of spatial learning and memory impairment were observed with Tg rats in the 6-month cohort, the 9-month rats had no such genotype

effects on WRAM performance. While previous work has shown spatial navigation deficits on the MWM in 10-11 month old TgF344-AD rats (Berkowitz et al., 2018), it is possible that the 9-month old Tg rats within our study utilized a different spatial strategy to solve this task to compensate for potential hippocampal compromise. In solving tasks like the WRAM, rats rely on a hippocampal-dependent allocentric strategy, where robust extra-maze cues are often utilized to spatially navigate to the goal arm (Olton, 1979). However, rats may also use a striatal-dependent egocentric strategy, which relies on body orientation as the reference frame, following a path of sequential right or left turns (Korol & Wang, 2018; Olton, 1979). Prior work in aged rats and humans has demonstrated impaired allocentric navigation, where egocentric strategies remain largely successful (Begega et al., 2001; Harris et al., 2012). In the case of AD, where hippocampal functioning may be compromised due to severe neuropathology, prior research has found that AD patients show more severe deficits when forced to use an allocentric strategy (Kalova et al., 2005; Nedelska et al., 2012). Therefore, it is possible that Tg rats at the 9-month timepoint shift from an allocentric strategy to an egocentric strategy due to significant neuropathological development. Future research should further investigate this potentially critical timepoint in the TgF344-AD model through examining their ability to solve a spatial navigation task when forced to only use an allocentric strategy, and whether this correlates with some critical timepoint in the trajectory of hippocampal neuropathology.

At the 12-month timepoint, significant working memory impairments were observed in TgF344-AD rats relative to WT controls on the WRAM during the Asymptotic Phase of testing. This finding corroborates prior literature, where deficits in

spatial learning and memory have been observed at 10-11 months of age on the MWM (Berkowitz et al., 2018) and 15 months of age on the Barnes maze (R. M. Cohen et al., 2013). Further, the trial-specific genotype effects for WMI errors at the 12-month timepoint indicate that rats did not differ in spatial working memory performance until the highest working memory load, where TgF344-AD rats had difficulty handling the significant strain on working memory processing as compared to WT rats. These findings indicate that, even at the 12-month timepoint, more nuanced behavioral assessments are necessary to gain insight into genotype-induced behavioral effects. Further work should be done to elucidate specific, fine-tuned cognitive domains to create a comprehensive behavioral profile which could provide further insight into key trajectories earlier in disease progression.

In contrast to the effects observed on the working and reference memory-dependent WRAM, results on the MWM indicated reference memory impairments for TgF344-AD rats relative to WT rats at every age-point assessed, that is at 6, 9, and 12 months old. Our noted impairment on the MWM is consistent with prior work (Berkowitz et al., 2018) showing significant spatial reference memory impairment on the hidden platform version of the MWM by 10-11 months of age in TgF344-AD rats compared to WT counterparts, with Tg-related impairments demonstrated in distance to the platform as well as multiple measures of qualitative swim segmentation outcomes including overall search trajectories and platform-direct swim paths. Markedly, in the 10-11 month-old cohort, female Tg rats demonstrated a lower proportion of direct trajectories to the hidden platform relative to all other groups including male Tg rats, with indications of similar effects in Tg females at the earlier ages as well (Berkowitz et al., 2018). The

current findings highlight the critical need to evaluate sex as a variable when both sexes are tested in preclinical research concerning aging, AD, and AD-related dementias; collapsing across sexes without accounting for potential sex-related effects can lead to misleading interpretations. We also note here that MWM outcomes are different from the pattern of effects observed for RM outcomes on the WRAM. This coincides with several studies from our laboratory indicating that assessing reference memory in conjunction with working memory differs from assessments of one memory type alone, effects seen in both young and aged females (Bernaud et al., 2021; Braden, Andrews, et al., 2017; Mennenga, Koebele, et al., 2015).

The physiological measures further characterize this model by providing information on genotypic differences beyond that of cognition across 3 age-points. Weekly measurements of body weight demonstrated that, at each age, female Tg rats weighed more than their female WT counterparts, suggesting that this TgF344-AD genetic profile may have a significant impact on key physiological factors such as satiety, exercise, and metabolism. This finding within the current AD rat model may seem unexpected, as weight loss is consistently observed in patients with AD (Gillette-Guyonnet et al., 2007). However, in rodent models of AD, body weight outcomes have been shown to be impacted by sex, age, and type of AD model (Garvock-de Montbrun et al., 2019; Knight et al., 2012; Mifflin et al., 2021; Pugh et al., 2007; Robison et al., 2020; Stover et al., 2015). In fact, one study demonstrated that 3xTg mice weighed more than their WT counterparts in young adulthood; however, by 12 months of age the Tg mice weighed less than WT mice even though they consumed more food, supporting the age-related development of a hypermetabolic profile (Knight et al., 2012).

Like body weight, heart weights also indicate a unique physiological profile for the TgF344-AD model. We found that the 9-month timepoint was distinct, with female Tg hearts weighing more than female WT hearts. However, when the ratio between heart weight and body weight was considered, it was revealed that, relative to body weight, Tg hearts weighed less than their WT counterparts distinctly at this 9-month timepoint. Further work assessing the trajectory of metabolic and cardiovascular changes across age in the TgF344-AD model will clarify how other organ systems interact with AD pathology. Finally, reproductive system evaluations collected throughout the study (vaginal cytology) and at sacrifice (uterine weight) suggest that reproductive aging may differ across the Tg and WT rats. This is an exciting area for further research, as relationships between ovarian hormone changes and AD-like behavior and neuropathology with aging have been established preclinically (Carroll et al., 2007) and clinically, with work showing that abrupt loss of circulating ovarian hormones due to oophorectomy increases risk for dementia decades later in life (Rocca et al., 2007).

Western blot results of relative A$\beta_{1\text{-}42}$ expression provided insight into the progression of AD-like pathology, with increasing levels of A$\beta_{1\text{-}42}$ expression at each age in brain areas critical to learning and memory functioning. Our current work fits with the prior literature surrounding the TgF344-AD model, such that amyloid deposition has been detected at 6 months of age in the hippocampus (R. M. Cohen et al., 2013). The fact that pathology is present before robust behavioral deficits in the TgF344-AD rat model is profoundly useful for clinical translation, as this more closely mirrors the clinical trajectory of AD (Braak et al., 2011). Amyloid beta was the pathological feature chosen for the present study in order to corroborate previous work done by Cohen and colleagues

(R. M. Cohen et al., 2013) as well as to focus the resources available for this study on this brain measurement and the primary behavioral outcomes. Future research should characterize the other neuropathological facets of this AD model, such as the presence of tau pathology resulting in neurofibrillary tangles, gliosis, and neuronal loss, expanding upon the initial work of Cohen and colleagues (R. M. Cohen et al., 2013), to determine its effectiveness at each age-point in modeling the progression of AD. Finally, the evaluation of relative A$\beta_{1\text{-}42}$ expression in the brain allowed for us to determine whether relationships existed between pathology and behavioral or physiological measures for each age. There were no significant correlations between behavioral outcomes and A$\beta_{1\text{-}42}$ expression, and this lack of relationship seems to parallel clinical AD research. Indeed, while decades of research have been dedicated to the study of senile plaques as the characteristic hallmark of AD, there is a general lack of correlation between disease manifestation and overall amyloid pathology (Berg et al., 1998; Snowdon, 1997). Recent research indicates that the clinical features of AD more closely align with tauopathy as well as soluble Aβ oligomers, rather than the insoluble fibrillar amyloid within dense-core plaques, as neuronal and synaptic loss commonly parallel these pathological trajectories (Haass & Selkoe, 2007; Lane et al., 2017). By further evaluating the pathology of the TgF344-AD rat model in the future, additional work can determine whether relationships exist between other neuropathological markers of disease progression and behavior. The current investigations did, however, result in the discovery of a significant negative correlation between A$\beta_{1\text{-}42}$ expression in the FC and body weight for female 9-month Tg rats, indicating that increased AD-like pathology was associated with decreased body weight. This finding fits with those of the clinical literature, where

researchers have found that greater weight loss is associated with further progression of AD (White et al., 1998). This provides greater context to the main effect of body weight also mentioned earlier: while Tg rats weighed more than their WT counterparts at this 9-month timepoint, greater body weight was associated with lower $A\beta_{1\text{-}42}$ expression. Future work should further investigate this 9-month age as a putative critical period within this model, with an eye toward its potential for therapeutic intervention and cross-system interdisciplinary interpretations.

In summary, in the herein interdisciplinary experiment assessing select behavioral, physiological, and pathological outcomes of the TgF344-AD rat model across multiple key ages, we chose to focus on females as they are disproportionately affected by AD in the clinic, and they are understudied preclinically with most experimental approaches evaluating males solely or combining across the sexes, with reference to AD-related questions. Specifically, our data provide insights into the behavioral, physiological, and pathological profiles of female TgF344-AD rats across 6-12 months of age. Our battery of spatial learning and memory found unique outcomes indicating impairment as early as 6 months of age, with profound working and reference memory impairment by 12 months. The lack of significant spatial working memory impairment at 9 months of age, in conjunction with a unique correlation between physiological and brain outcomes at this age-point, suggest that this may be a critical time for female TgF344-AD rats. Additionally, this study highlights the importance of considering the broader physiological consequences that coincide with behavioral deficits and pathology across this AD-like trajectory, with unique findings for body weight, heart weights, and uterine weight. Such systems-wide approaches in the clinic with AD diagnosis and

treatment could improve outcomes. Our results highlight the importance of the initiatives driving the field toward a greater focus on cross-systems multidisciplinary research, where an emphasis has been placed on personalized medicine (Forloni, 2020; Vanitallie, 2013) and the identification of biomarkers to assist in detecting early AD progression in vulnerable patients (Reiman, 2017).

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CHAPTER 4

The data presented in this chapter are currently unpublished observations. A subset of data from this experiment were, in part, presented in my master's thesis at Arizona State University (2020).

YOUNG ADULT AND MIDDLE-AGE RATS DISPLAY UNIQUE WORKING MEMORY IMPAIRMENT FOLLOWING HYSTERECTOMY

Contribution: I was the graduate student principal investigator for this experiment. A subset of the data presented in this chapter are from my master's thesis work under Dr. Heather Bimonte-Nelson. Following my thesis work, I then completed the Western blot protein assays to analyze relative levels of expression of the inflammatory marker IL-10, along with analyses of ovarian hormones via blood serum and ovarian morphology.

ABSTRACT

Hysterectomy is the second most common gynecological surgery performed in women. While some of these surgeries involve removal of the uterus alone, others involve concomitant removal of the ovaries. While clinical literature has historically suggested notion that the nonpregnant uterus is dormant, more recent clinical findings suggest that hysterectomy is associated with cognitive detriment. Notably, an earlier age at hysterectomy, with or without concomitant ovarian removal, has been shown to increase dementia risk, implicating age at surgery as a variable of interest. While preclinical work in a rodent model of hysterectomy has demonstrated spatial working memory impairments when performed in young adulthood, the role of age at surgery has yet to be addressed. The current experiment utilized a rodent model of hysterectomy to investigate the importance of age at surgery in post-surgical cognitive outcomes and to evaluate relative expression of neurobiological markers of activity (FosB and ΔFosB) and inflammation (IL-10) in regions critical to spatial learning processes. Young adult and middle-aged female rats underwent sham, hysterectomy, or hysterectomy with ovariectomy surgery, and were tested on a behavioral battery that evaluated spatial working and reference memory performance. Following the behavioral battery, animals were euthanized and brain tissues from the Dorsal Hippocampus, Entorhinal Cortex, and Frontal Cortex were processed via Western Blot for relative FosB, ΔFosB, and IL-10 expression. Behavioral analyses demonstrated that animals receiving hysterectomy, regardless of age or ovarian status, were generally impaired in learning a complex spatial working memory task. However, rats that received hysterectomy in middle-age uniquely demonstrated persistent working memory impairment, particularly with a high working

memory demand. Correspondingly, ovarian follicle counts indicated a differential impact of hysterectomy dependent upon age at surgery. However, there were no effects of surgery at either age for any protein expression analysis across all three brain regions. Taken together, these findings suggest that age at surgery plays an important role in learning and memory outcomes following hysterectomy, and demonstrate the need for further research into the role of the uterus in communications between the reproductive tract and brain.

Introduction

Hysterectomy, or removal of the uterus, is the second most common gynecological surgery, with the first being cesarean section (Carlson et al., 1993). As such, up to one third of women in the United States experience this gynecological surgery by the age of 60 (Carlson et al., 1993; Whiteman et al., 2008). For being so common, hysterectomy is an under-researched area within the broader menopause literature, particularly as compared to oophorectomy, or surgical removal of the ovaries in women. While hysterectomy can be performed alone, there are cases where the uterus is removed in conjunction with the ovaries. This decision to remove the ovaries with the uterus involves many complex factors, but one critical factor is age or reproductive stage at surgery. Because many women who undergo hysterectomy have not yet experienced menopause, the ovaries are conserved in approximately half of all hysterectomy cases to prevent an abrupt surgical menopause and its associated negative health outcomes (Bhattacharya & Jha, 2010; Wright et al., 2013). That is, ovarian conservation allows for women to maintain circulating ovarian hormone levels until the natural transition into menopause in midlife. However, if a woman is at high-risk for certain gynecological cancers or other complications, the ovaries are removed at the time of hysterectomy as a prophylactic measure, regardless of reproductive stage; this results in an abrupt loss of circulating ovarian hormones, sometimes decades before expected menopause onset (Lowder et al., 2010). While hysterectomy itself is understudied, even more under-researched are the decades of like that follow, especially in cases where the ovaries are conserved.

Although the ovaries are generally thought to function normally following hysterectomy (Chalmers et al., 2002), there is also evidence to suggest that hysterectomy surgery impacts ovarian outcomes during aging. Specifically, research has indicated that women receiving hysterectomy with ovarian conservation can experience disrupted ovarian function, potentially due to modulation of ovarian blood flow, leading to an earlier point of ovarian failure than women who do not receive such gynecological surgery (Chan et al., 2005; Farquhar et al., 2005; Moorman et al., 2011). Such research is complicated by the fact that, in a typical clinical setting, menopause is difficult to characterize in women that have undergone hysterectomy. Indeed, as menopause is defined retrospectively by one year of amenorrhea (NAMS, 2014), many women that receive hysterectomy earlier in life would already be considered menopausal by clinical standards. Instead of using these clinical criteria, menopause in hysterectomized women is primarily indicated by the presence of associated symptoms, which include hot flashes, sleep disturbances, and mood and cognitive changes (Koebele & Bimonte-Nelson, 2016; The Practice Committee of the American Society of Reproductive Medicine, 2004). While useful for practitioners, this definition of menopause is problematic from a research perspective, as these symptoms can vary greatly in incidence, severity, and duration between women (Gracia & Freeman, 2018; N. Santoro et al., 2021). As a supplement, measurement of serum hormones such as follicle-stimulating hormone (FSH) levels in blood can be collected to confirm menopause status. FSH is collected due to the fac that, in later reproductive years, FSH levels begin to increase through the menopause transition and remain elevated in postmenopause (Burger, 2006; Burger et al., 1999; Harlow et al., 2012; Koebele & Bimonte-Nelson, 2016; Soules et al., 2001). Levels

of serum FSH exceeding 40 IU/liter have been adopted as a marker of the late menopausal transition, particularly in the presence of other indications, such as vasomotor symptoms (Burger et al., 1999; Randolph et al., 2006). However, much like the use of menopause symptoms, FSH levels can vary across women greatly, making it a problematic marker of menopause without sufficient longitudinal information. Indeed, serum FSH levels as a stand-alone measurement are too unreliable to consistently identify the initiation of reproductive senescence (Su & Freeman, 2009). However, there are indications of small yet significant elevations in FSH following hysterectomy without concomitant oophorectomy (Cooper & Thorp, 1999), suggesting some degree of ovarian response to uterine removal. Like that of FSH, most literature indicates that levels of ovarian hormones, such as 17β-estradiol or progesterone, largely remain stable long-term following hysterectomy (Beavis et al., 1969; Chalmers et al., 2002; Kaiser et al., 1989; Souza et al., 1986), where some evaluations report at least short-term changes in serum estradiol and progesterone levels (Vuorento et al., 1992; Xiangying et al., 2006). Collectively, such reports of changes in gonadotropin and ovarian hormone levels following hysterectomy, at least in the short-term window following surgery, suggest that further research is necessary into the potential role of the uterus in regulation of the hypothalamic-pituitary-ovarian (HPO) axis.

Findings implicating the uterus as a participant in a network of communication between the ovaries, pituitary, and brain are in contrast to the long-standing characterization of the uterus as a reproductive organ that is responsive to stimulation via the ovaries, but otherwise plays a minimal role in HPO axis feedback. Indeed, the uterus has historically been described as a “useless organ” outside of its role in maintaining a

pregnancy in clinical guidelines (Navot & Williams, 1991). Given the aforementioned findings concerning ovarian changes following hysterectomy, along with evidence that the uterus contains both gonadotropin- and steroid hormone- receptors (Lessey et al., 1988; Reshef et al., 1990; Stilley et al., 2014; Toft & Gorski, 1966) and is innervated along the central and peripheral nervous systems (Brauer & Smith, 2015; Gnanamanickam & Llewellyn-Smith, 2011), it is possible that the role of the non-pregnant uterus in HPO axis functioning has largely been underestimated.

In the last 20 years, clinical literature has indicated that the uterus may indeed send and receive neural inputs important for maintaining cognitive health, and that hysterectomy may disrupt healthy cognitive functioning with aging. Hysterectomy, both alone and with concomitant oophorectomy, has been associated with an increased risk of dementia (Phung et al., 2010; Rocca et al., 2007). Interestingly, an earlier age at hysterectomy alone is related to an exacerbation of this increased risk of dementia and cognitive decline, paralleling findings associated with premenopausal oophorectomy (Rocca et al., 2012). In contrast, one study found a small reduction in risk of Alzheimer's disease associated with hysterectomy (Imtiaz et al., 2014); however, the majority of the women who underwent hysterectomy were postmenopausal at the time of surgery (Imtiaz et al., 2014), implicating age at surgery as a key variable in cognitive outcomes following hysterectomy. Indeed, it is possible that hysterectomy leaves the brain vulnerable to cognitive deficits in an age-dependent manner, where following early hysterectomy, the young adult brain has not yet undergone the reorganizational processes that occur across the menopause transition (Koebele & Bimonte-Nelson, 2015), leaving it ill-poised to respond to endocrine or other changes as a result of uterine removal.

Given these indications concerning the role of the uterus as a potential bi-directional participant in feedback between the brain and ovaries, the utilization of a preclinical model is warranted to systematically investigate such relationships. Preclinical rodent models of surgical menopause can provide valuable insight into the behavioral and neurobiological mechanisms behind such phenomena. Indeed, rodents have well-studied reproductive and neurological structures that are comparable in many ways to those of humans. However, one important difference between rodents and humans is the female experience of reproductive senescence. The rodent form, termed estropause, does not result in ovarian follicular depletion or significant decreases in circulating estrogens and progesterone(Koebele & Bimonte-Nelson, 2016). This makes them an excellent tool for studying age-dependent phenomena without having to concede interactions with reproductive status, as is done in the human literature, making them an excellent model for preclinical assessments of surgical menopause (Acosta, Mayer, Talboom, Tsang, et al., 2009; Bimonte-Nelson et al., 2003; Koebele & Bimonte-Nelson, 2016). For example, the preclinical rodent model of surgical removal of the ovaries, known as Ovariectomy (Ovx), has provided significant contributions to the knowledge of the cognitive effects of the abrupt cessation of circulating ovarian hormones (Bimonte & Denenberg, 1999; Bimonte-Nelson et al., 2004; Camp et al., 2012). Ovx has been demonstrated to be detrimental to cognition when animals undergo surgery in adulthood (Bimonte & Denenberg, 1999; Daniel et al., 1999), but can be beneficial when surgery occurs when animals are aged and are evaluated at an extended timepoint post-surgery (Bimonte-Nelson et al., 2003). These preclinical findings correspond with the clinical literature, showing cognitive impairment following surgical menopause as compared to the natural

menopause transition (Nappi et al., 1999) and an increased risk of dementia for those who undergo oophorectomy before menopause onset (Rocca et al., 2007, 2011).

Until recently, there had been no systematic evaluations of reproductive senescence that included the presence or absence of the uterus as a variable of interest. Our laboratory recently sought to fill this gap in the literature by developing a rodent model of hysterectomy to evaluate whether surgical removal of the uterus uniquely impacted cognitive outcomes (Koebele et al., 2019). In this experiment, young adult female rats received either hysterectomy, both alone and with concomitant Ovx, bilateral Ovx, or sham surgery, and after six weeks underwent a cognitive battery to assess spatial working and reference memory (Koebele et al., 2019). This study found spatial working memory impairment with hysterectomy surgery as compared to the other variations in gynecological surgery, as well as compared to sham controls. Upon inspection of circulating ovarian hormone levels and ovarian cytology at euthanasia, there was some evidence of hormonal changes following hysterectomy, but such changes were not present for measures of ovarian follicle morphology (Koebele et al., 2019). The model has provided significant evidence concerning the role of the uterus in cognitive functioning, with indications that this relationship is indeed not secondary to ovarian changes as a result of uterine removal (Koebele et al., 2019). While this work is integral in further understanding the relationship between the uterus and the brain, several questions remain concerning the cognitive outcomes associated with this form of gynecological surgery. Following that of the Ovx literature, the next question seems clear in the case of preclinical hysterectomy when observing clinical trends for dementia risk: the role of age at surgery in hysterectomy-induced cognitive outcomes must be evaluated.

While the role of age at surgery is a critical research factor in understanding the cognitive effects of hysterectomy, it is also important that preclinical work explore potential neurobiological mechanisms that might better characterize this relationship. Currently, the neurobiological underpinnings behind such learning and memory impairment remains elusive. In order to develop effective therapies to postpone or prevent this cognitive impairment in women following gynecological surgery, it is critical to understand these neural mechanisms. One potential mediator of these uterine-brain interactions could be the inflammatory system, which is modulated both by surgery (Alam et al., 2018) and by aging (Mishra & Brinton, 2018). Indeed, menopause has been identified as a unique period of immune shifts, with increased levels of proinflammatory markers (Abu-Taha et al., 2009) and increased risk of complications or onset of immune-related diseases (Fish, 2008). Furthermore, ovarian removal has been shown to induce lasting proinflammatory shifts in both humans and in rodents (Pacifici et al., 1991; Pfeilschifter et al., 2002). Notably, few clinical studies have evaluated inflammatory outcomes following hysterectomy with ovarian conservation, but the uterus has been shown to contain regionally-specific densities of inflammatory factors across the menstrual cycle and in postmenopause (Givan et al., 1997). Such work indicates that the removal of the uterus could have a significant impact on inflammatory outcomes, both peripherally and in brain.

Another potential mediator of such brain changes associated with learning is Delta (Δ) FosB. ΔFosB and its full-length complement, FosB, are transcription factors of the immediate early gene *fosB*. Generally speaking, these immediate early genes are activated following stimulation or activity, triggering the release of these transcription

factors. FosB and ΔFosB can heterodimerize with transcription factor Jun from the immediate early gene *jun* to form activator protein -1 (AP-1) complexes, which in turn may regulate gene expression (Carle et al., 2007; Ruffle, 2014). Whereas FosB is a characteristically unstable transcription factor with a half-life of approximately an hour (Carle et al., 2007; Zerial et al., 1989), ΔFosB is unique in its relative stability with a half-life of days to weeks (Carle et al., 2007; Ruffle, 2014), making the latter a useful tool for assessing long-term change within the brain. ΔFosB has largely been studied in the addiction literature, where ΔFosB expression becomes upregulated in the nucleus accumbens (a brain region important for addiction learning) with repeated exposure to drugs of addiction, such as cocaine, and remains persistently elevated after drug cessation (Nestler et al., 2001). Of note, ΔFosB has also recently been implicated in spatial learning, where the number of FosB/ΔFosB-immunoreactive cells was increased in the CA1 region of the hippocampus following learning of the Morris Water Maze (MWM) task for rats compared to naïve controls, as well as compared to rats that learned the non-spatial version of the MWM, where these cells were demonstrated to increase for all tested animals in the dentate gyrus (Eagle et al., 2015). Given the prolonged nature of ΔFosB, evaluation of the expression of FosB and ΔFosB in brain regions important for learning and memory following hysterectomy surgery might provide insight into the neurobiological mechanisms underlying these observed cognitive changes.

The current experiment sought to characterize the role of age at surgery for hysterectomy, both alone and in conjunction with ovary removal, in altering post-surgical learning and memory outcomes using a rodent model. Young and Middle-aged female rats underwent either Sham, Hysterectomy, or Hysterectomy with concomitant Ovx.

Spatial learning and memory were evaluated via a behavioral battery six weeks post-surgery. At the end of the experiment, relative protein expression of FosB, ΔFosB, and IL-10 were analyzed via Western Blot in the Entorhinal Cortex and Dorsal Hippocampus, as well as the Frontal Cortex (IL-10 only) to evaluate potential brain changes in regions associated with spatial learning and memory as a result of surgery in young adulthood and middle-age.

Methods

Subjects

Thirty-two 5 month-old (Young) and thirty 11 month-old (Middle-Age) virgin, reproductively-intact female Fischer-344 (CDF) rats were obtained from the National Institute on Aging colony at Charles Rivers Laboratories (Raleigh, NC). Rats were pair housed randomly upon arrival, and were provide food and water ab *libitum* while kept on a 12-hour light/12-hour dark cycle daily for the duration of the study. Rats were given one week to acclimate before surgeries and initiation of the experiment. All experimental procedures were performed with approval from the Arizona State University (ASU) Institutional Animal Care and Use Committee, and followed the standards of the National Institutes of Health.

Experimental Design

A detailed overview of the experimental timeline can be found in Figure 19A.

Surgical procedure. Rats within each age group (5 months and 11 months) were randomly assigned to one of three surgical groups: Sham, Hysterectomy, or Hysterectomy with concurrent Ovx (Ovx+Hysterectomy); this resulted in a total of six surgical groups: Young-Sham, Young-Hysterectomy, Young-Ovx+Hysterectomy,

Middle-Age-Sham, Middle-Age-Hysterectomy, Middle-Age-Ovx+Hysterectomy. Surgeries were performed one week after the rats arrived at ASU, and were conducted using a protocol previously established within this laboratory (Koebele et al., 2019). For all surgical procedures, rats were anesthetized via inhaled isoflurane and received 1.0mg/kg meloxicam and 1.2mg/kg buprenorphine for pain management, along with 5.0ml of an isotonic solution to ensure postsurgical hydration. Each surgery involved a ventral midline incision through the skin and peritoneum; groups receiving sham surgery received only this surgical manipulation. Groups receiving hysterectomy surgery had each uterine horn ligated and cut below the ovary, preserving the oviduct, before being separated from attached abdominal fat. This fat was separated down the length of the uterine horn until the utero-cervical junction was reached, wherein it was ligated and cut above the cervix at the base of the uterine body. Rats receiving Ovx+Hysterectomy surgery had ovaries and uterus removed by separating the surrounding abdominal fat until the utero-cervical junction was reached, where it was ligated and cut at the base of the uterine body, preserving the cervix. For all surgical groups, muscle incisions were sutured using dissolvable Vicryl suture, with bupivacaine applied to the muscle surface for further pain management prior to skin closure. The skin incision was closed via surgical staples for all subjects. Rats recovered under a heat lamp and were single-housed for seven days following surgery, before being re-pair housed with their original cage-mate for the duration of the study. Two animals in the Young-Hysterectomy group died following surgery, resulting in a total of 10 rats per treatment group.

Body weights & vaginal cytology. Baseline weights were collected before surgery, and weights were recorded weekly throughout the duration of the study. Three

weeks after surgery, vaginal smears were conducted daily for eight consecutive days to monitor estrous cyclicity following surgery, as well as to confirm successful Ovx surgery for the Ovx+Hysterectomy groups. Vaginal smears were classified according to established protocols (Goldman et al., 2007) as proestrus, estrus, metestrus, or diestrus. Proestrus was classified by the presence of clustered round epithelial cells with some cornified cells. Estrus was classified by the predominant presence of cornified cells. Metestrus was characterized by the presence of cornified cells, round cells, leukocytes, and some needle-like cells. Diestrus was characterized by the predominant presence of leukocytes, sometimes with the presence of cornified cells or round epithelial cells. Vaginal smears were conducted again on the day prior to euthanasia and on the day of euthanasia for all subjects to confirm estrous cyclicity in ovary-intact subjects at this timepoint, as well as to confirm Ovx status for Ovx+Hysterectomy rats for both ages.

Behavioral Battery

Six weeks following surgery, all rats underwent behavioral testing to evaluate spatial working and reference memory performance.

Water radial-arm maze. All rats were first evaluated via the win-shift version of the water radial-arm maze (WRAM), which was used to examine spatial working and reference memory (Bimonte-Nelson et al., 2015). The WRAM is an eight-arm apparatus that is situated in a testing room surrounded by salient visual cues to aid in spatial navigation. The maze is filled with room temperature water (18-20°C), where the surface of the water is made opaque with non-toxic black tempera paint. Four out of the eight arms contain hidden platforms, which animals must locate to successfully complete the

task. Each rat was randomly assigned to a set of platform locations, which were kept fixed for each individual subject across all days of testing.

At the start of each trial, the rat was released from the starting arm and given three minutes to successfully locate a hidden platform; if the rat did not find a platform within the allotted time, it was gently guided to the nearest platform by the experimenter. Once on the platforms, the rats were required to remain there for a total of 15 seconds before being removed by the experimenter and placed into a heated testing cage for a 30 second inter-trial-interval (ITI), wherein the found platform was removed and the maze was swept to distribute any odor cues. After completion of the ITI, each rat was once again dropped off at the starting arm to begin another trial; this continued for a total of four trials per day, such that all platforms were located for each individual animal. In this manner, working memory load increased across each trial for a given subject, as each rat needed to remember not to visit arms where platforms had previously been located for that day. Testing continued in this fashion for a total of 12 baseline days, where on Day 13, a six-hour delay was implemented between Trials 2 and 3 to evaluate delayed memory retention.

Performance on the WRAM was quantified by totaling the number of entries into non-platformed arms, here referred to as errors, for each trial. An arm entry was recorded as an error when the tip of the rat's snout passed 11cm from the opening of the arm, which was clearly marked on the outside of the maze arms. These errors were further split into orthogonal error types that represent spatial working and reference memory performance, as has been done previously in this laboratory (Bimonte et al., 2003; Bimonte-Nelson, 2015d; Braden, Andrews, et al., 2017; Koebele et al., 2019). These

error types were defined by the following criteria: an entry into a previously platformed arm within a testing day was noted as a working memory correct (WMI) error; the first entry into a non-platformed arm for a given day of testing was noted as a reference memory (RM) error; and subsequent entries into non-platformed arms for a given testing day was noted as a working memory incorrect (WMI) error.

Morris water maze. One day following the completion of WRAM testing, all subjects were evaluated on the Morris water maze (MWM), which measures spatial reference memory performance (Bimonte-Nelson, 2015b; Bimonte-Nelson et al., 2015; Morris et al., 1982). The MWM consists of a large circular tub, 188 cm in diameter, filled with cool water (18-20°C) which is made opaque with non-toxic black tempera paint. A platform (10 cm diameter) is placed in the northeast (NE) quadrant of the maze and sits hidden below the surface of the water, where it remains across all testing trials. Robust visual cues are placed around the room to aid in spatial navigation.

In this task, each rat was dropped off at a series of designated starting locations (North, West, South, East) that are semi-randomized across testing days. Each subject was given 60 seconds to locate the hidden platform for each trial; if the platform was not located in the allotted time, the animal was gently guided by the experimenter onto the platform. Once located, the rat remained on the platform for a total of 15 seconds before being removed and placed into its heated testing cage for an approximate 10 minute ITI. Each rat performed four testing trials per day across 5 days; an additional 5th trial, referred to as the probe trial, was added at the end of the 5th day of MWM testing, where the platform was removed and each animal was observed swimming for 60 seconds to evaluate spatial localization to the platform. Performance on the MWM was quantified by

calculating the distance traveled by each subject to reach the platform for all testing trials, as well as swim latency to the platform, using the Ethovision tracking system (Noldus Instruments, Wageningen, The Netherlands).

Visible platform task. Following the conclusion of MWM testing, each rat was evaluated on the visible platform (VP) task to assess ability to perform the procedural components of a water-escape task (Bimonte-Nelson, 2015c). The VP apparatus is a rectangular tub filled with clear cool water (18-20°C) that contains a black platform, sitting 4cm above the surface of the water. A curtain that surrounded the tub was used to attenuate the use of spatial cues while performing the task. Each rat was dropped off at the designated starting location, and was given 90 seconds to locate the platform. Once located, the animal remained there for 15 seconds before being removed and placed into its heated testing cage, with an inter-trial interval of approximately 5-10 minutes. Each animal completed a total of 6 trials, wherein the platform location was moved semi-randomly across trials between three distinct locations. Performance on this task was quantified by latency (in seconds) to locate the platform for each trial.

Euthanasia

Ten days following the completion of the behavioral battery, all animals were euthanized at approximately 7 months of age (Young) or 13 months of age (Middle-Age). Rats were deeply anesthetized with inhaled isoflurane before blood was collected via cardiocentesis to allow for blood serum analysis of various ovarian hormone levels. After decapitation, brains were rapidly removed, where the left hemisphere was post-fixed in 4% paraformaldehyde for 48 hours before being transferred into a 0.1M phosphate buffered solution for subsequent analysis. The right hemisphere was raw dissected to

collect frontal cortex, cingulate cortex, dorsal hippocampus, entorhinal cortex, perirhinal cortex, temporal cortex, and ventral CA1/CA2 brain regions. These dissected tissues were individually weighed and flash frozen at -70°C for further analysis.

Ovaries were collected from the ovary-intact animals (Sham and Hysterectomy groups), trimmed of excess fat, individually weighed, and placed into vials filled with 10% formalin for 48 hours before being transferred into a 70% ethanol solution for further analysis. Uteri from the Sham subjects were collected, trimmed of excess fat, and the wet weight was collected.

Ovarian Follicle Counting

FYXX Foundation (Flagstaff, AZ) received one randomly-selected ovary form each rat that was fixed upon euthanasia to conduct all ovarian tissue processing and follicle quantification. First, the oviduct was separated from the ovary before the Leica TP1020 tissue processor was used to process tissues. Ovaries were then individually embedded in paraffin wax and sectioned using a semi-automatic rotary microtome at a thickness of 5um. Every 10th section was mounted to slides before undergoing staining via Gills 2 hematoxylin and counterstaining via eosin Y-phloxine B. All slides were then cover-slipped, and scanned with the 3D HisTech DESK scanner. Of these scans, every 20th section was analyzed for presence of primordial, primary, secondary, and antral follicles, using established criteria (Haas et al., 2007). If any follicles showed apparent signs of atresia, they were not counted. Primordial cells were classified by a single layer of squamous granulosa cells around the oocyte. The following formula was used to estimate the count of primordial cells: $N_t = (N_0 \times S_t \times t_s)/(S_0 \times d_0)$, where N_t represents the total follicle estimate, N_0 represents the number of follicles observed in the

ovary, S_t represents the total number of sections, t_s represents the thickness of each section in um, S_0 represents the total number of sections observed, and d_0 represents the mean diameter of the cell nucleus (Gougeon & Chainy, 1987). Primary follicles were identified by their dingle layer of cuboidal granulosa cells. Secondary follicles were identified as containing several layers of granulosa cells, where antral follicles were identified as having two or more granulosa cell layers and a fluid-filled antral space inside the follicle. All counts for primary, secondary, and antral follicles were completed by a simple sum. Finally, corpora lutea were counted as they appeared throughout the entire sample of tissue.

Serum Hormone and Gonadotropin Evaluations

Blood collected at euthanasia was stored in on ice for at least 30 minutes before being placed in the centrifuge at 4C for 20 minutes at 2000 rpm. Serum was then aliquoted into two identical tubes, and stored at -20C until shipment. Samples were shipped to the Wisconsin National Primate Research Center (WNPRC; Madison, WI) overnight on dry ice to determine serum concentrations of 17β-estradiol (E2), progesterone, androstenedione, and anti-Mullerian hormone (AMH) for all ovary-intact subjects (i.e. all Sham and Hysterectomy groups), as well as luteinizing hormone (LH) and follicle stimulating hormone (FSH) for all experimental subjects. Concentrations of E2, progesterone, and androstenedione were determined using LC-MS/MS. Concentrations of AMH were determined using EIA (Ansh). Concentrations of LH and FSH were determined using a commercially available multiplex kit (EMD Millipore).

Western Blot Protein Analysis

Dorsal hippocampus and entorhinal cortex, collected from the right hemisphere of each subject at euthanasia, were evaluated for FosB expression and ΔFosB expression via western blot protein analysis. These two regions, as well as right frontal cortex, were evaluated for IL-10 expression via a separate series of western blots. Raw tissue samples that were flash frozen were suspended in a 1:25 weight-to-volume ratio of RIPA buffer solution (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris HCl), protease inhibitor (Millipore-Sigma, CAT#5892791001) and phosphatase inhibitor (Millipore-Sigma, CAT#524625), and thereafter kept on ice. Tissue samples were then homogenized via probe sonication (Ultrasonic Processor, Cole Parmer, IL, USA), before being centrifuged at 10,000 rpm for 10 minutes at 4°C. The resulting supernatant was then collected, aliquoted, and frozen at -70°C for further analysis. Protein concentration for each sample was determined using a bicinchoninic acid protein assay (Thermo-Fisher Scientific, Pittsburgh, PA, USA), with samples run in duplicate; a consistent measure of protein concentration was obtained when the coefficient of variation was less then 10% across duplicates.

Treatment groups were counterbalanced and evenly distributed across each gel. Tissue processing was completed using the NuPAGE PowerEase electrophoresis system. Samples for a given region were loaded at an equal protein concentration and run with MES running buffer on a 4-12% NuPAGE Bis-Tris gel in an XCell SureLock Mini-Cell (Invitrogen, Carlsbad, CA, USA). After protein separation, an Immobilon polyvinylidene difluoride membrane was utilized for protein transfer. The membrane was then blocked in 5% nonfat milk for 1 hour at room temperature before being washed with 1XTBST. The membrane was then incubated overnight in 5% milk with the primary antibody for

anti-FosB (1:1000; Abcam, ab184938) or IL-10 (1:1000; Abcam, ab9969) at 5°C. The following day, the membrane was washed with 1XTBST before incubation in 5% milk with the secondary antibody anti-rabbit HRP (1:2000; Cell Signaling #7074) for 1 hour at room temperature. The membrane was then washed again before being developed using chemiluminescence (Lumiglo and peroxide solutions, Cell Signaling #7003S) with a film developer (Konica SRX-101A Film Processor, Tokyo, Japan). The predicted kDa for FosB, deltaFosB, and IL-10 were approximately 36, 48, and 20kDa, respectively. Resulting films were scanned as JPEG files at 600dpi for subsequent densitometry analyses, which were completed using ImageJ software (Gallo-Oller et al., 2018). FosB and ΔFosB expression were normalized to beta-tubulin expression for each gel. A total of 10 gels were run across the two brain regions for FosB and ΔFosB analyses. For IL-10 analyses, a total of 13 gels were run across all three brain regions.

Statistical Analyses

All statistical analyses were completed using StatView software. For the behavioral and brain data, except those from the WRAM delay, MWM probe, and VP task, all analyses were conducted separately for the Young and Middle-Aged rats, with two-group planned comparisons set to independently compare the three surgical groups. All analyses were two-tailed, wherein the alpha level was set to 0.05. Results were designated as marginal if the p value was between 0.05 and 0.10.

Water radial-arm maze. WRAM data were first analyzed as an omnibus repeated measures ANOVA across all baseline days of testing (Days 2-12), with Age and Surgery as the independent variables and Days and Trials as the repeated measures, to determine the general learning patterns for this task for each of the error types (WMC,

WMI, and RM errors). Based upon prior publications demonstrating behavioral differences across days of WRAM testing corresponding to task acquisition and maintenance (Bimonte-Nelson et al., 2015; Koebele et al., 2019; Mennenga, Koebele, et al., 2015; Prakapenka et al., 2018), the WRAM data were then analyzed for each planned comparison and each error type after being separated into three blocks: Days 2-4, the Early Acquisition Phase; Days 5-9, the Late Acquisition Phase; and Days 10-12, the Asymptotic Phase (see Figure 19B). A repeated measures ANOVA was used to analyze data collected from the WRAM, with Surgery as the independent variable, and Days and Trials as the repeated measures. The dependent variable for these analyses was Errors, whereby separate analyses were conducted for WMC, WMI, and RM errors.

For data collected during the WRAM delay, each treatment group was evaluated separately for WMC and WMI errors using a repeated measures ANOVA, whereby Errors served as the dependent variable, and Trial 3 performance on the last baseline day of testing (Day 12) versus Trial 3 performance post-delay (Day 13) served as the repeated measures.

Morris water maze. One rat in the Middle-Age-Hysterectomy group was unable to complete MWM testing due to a benign skin condition unrelated to surgical assignment, and this animal was excluded from these analyses. MWM data were analyzed using a repeated measures ANOVA, with Surgery as the independent variable and Trials within Days as the repeated measures, as there were four testing trials per day over the course of five days. Swim distance to the platform (cm) served as the dependent variable for each planned comparison. The probe trial was analyzed using an ANOVA, where the percent of swim distance in the Target (NE) vs. Opposite (SW) Quadrant

served as the dependent measure; this probe analysis was performed separately for each treatment group.

Visible platform task. Data collected from the VP task were analyzed using a repeated measures omnibus ANOVA, where Age and Surgery served as the independent variables, Trials as the repeated measure, and Latency to the platform as the dependent measure.

Body weight analysis. Body weight data were analyzed using ANOVA, with the same planned comparisons used for behavioral analyses, where Surgery served as the independent variable and Body Weight at euthanasia served as the dependent variable.

Ovarian follicle counting. Ovarian follicle counts were analyzed as ANOVA, where the same planned comparisons were used for behavioral and other analyses, but without the comparisons involving the Ovx+Hysterectomy groups (i.e. Sham vs. Hysterectomy, for both Young and Middle-Aged rats). For these analyses, Surgery served as the independent variable, and counts of primordial, primary, secondary, and antral follicles, and corpora lutea served as the dependent measure. Two samples of ovaries from the Middle-Age-Hysterectomy group, as well as one ovary from the Middle-Age-Sham group and one ovary from the Young-Sham group could not be analyzed, and are therefore missing from statistical analyses.

Serum hormone and gonadotropin evaluations. Serum hormone concentrations for E2, progesterone, androstenedione, AMH, LH, and FSH were analyzed via ANOVA. Analyses followed the same set of planned comparisons outlined above, but without the Ovx+Hysterectomy groups for E2, progesterone, androstenedione, and ASH analyses. Surgery served as the independent variable for all analyses, and serum concentrations of

each hormone served as the dependent measure. If a sample fell outside of the limit of detection (i.e. too high/low in value), it was set to the value of the lower/upper limit of detection for the assay.

Western blot protein analysis. Western blot analyses were completed using ANOVA, with Surgery as the independent variable, and normalized FosB, ΔFosB, or IL-10 expression as the dependent variable for each planned comparison.

Results

Water Radial-Arm Maze

The WRAM evaluated spatial working and reference memory performance. With the initial omnibus analysis, there was a main effect of Day for each memory measure, with errors decreasing across testing days (Days 2-12) for WMC ($F_{(10,540)} = 9.543$, $p < 0.0001$), WMI ($F_{(10,540)} = 22.268$, $p < 0.0001$), and RM errors ($F_{(10,540)} = 18.283$, $p < 0.0001$), demonstrating learning of the WRAM task (data not shown). For each memory measure, there was no significant Day x Age x Surgery interaction, demonstrating that groups did not differ in rate of WRAM learning across all baseline testing days. All subsequent analyses of WRAM data during baseline testing involved the use of planned comparisons and blocking of testing days to further characterize differences in acquisition and memory retention phases of WRAM testing.

Early acquisition phase. For the Early Acquisition Phase (Days 2-4), planned comparisons for the Young-Sham and Young-Ovx+Hysterectomy groups revealed a significant Trial x Surgery interaction for WMC errors ($F_{(2,36)} = 4.396$, $p < 0.05$; Figure 20A), where post hoc analyses revealed a marginal effect of Surgery for Trial 4 ($F_{(1,18)} = 4.083$, $p < 0.10$) such that rats that received Ovx+Hysterectomy surgery in young

adulthood tended to make fewer WMC errors than those in the Sham group (Figure 20B). However, this effect of Surgery was not present for middle-aged animals. No other planned comparison in either Young or Middle-Aged rats revealed significant effects of Surgery or Trial x Surgery interactions for WMC errors during the Early Acquisition Phase. There were no statistically significant effects for WMI or RM errors for any of the planned comparisons during the Early Acquisition Phase.

Late acquisition phase. For the Late Acquisition Phase (Days 5-9), there were no statistically significant effects across any of the planned comparisons for WMC errors.

Analyses of WMI errors in the Late Acquisition Phase for planned comparisons between Young-Sham and Young-Hysterectomy groups revealed a Trial x Surgery interaction for WMI errors ($F_{(3,54)} = 4.093$, $p < 0.05$; Figure 21A). Subsequent analyses revealed that at the highest working memory load trial, Trial 4, there was a marginal Surgery effect ($F_{(1,18)} = 3.947$, $p < 0.10$), with Hysterectomized rats in young adulthood tending to make more WMI errors than their Sham counterparts (Figure 21B). No other planned comparison in either Young or Middle-Aged rats revealed significant effects of Surgery or Trial x Surgery interactions for WMI errors during this phase of testing.

For the Late Acquisition Phase, there were no significant effects for RM errors across any of the planned comparisons.

Asymptotic phase. During the Asymptotic Phase of WRAM testing (Days 10-12), planned comparisons between Middle-Age-Sham and Middle-Age-Hysterectomy groups revealed a marginal effect of Surgery for WMC errors ($F_{(1,18)} = 3.425$, $p < 0.10$), where animals that received hysterectomy surgery in middle-age tended to make more WMC errors than Sham animals (Figure 22A). Additionally, analyses for rats in the

Middle-Age-Sham and Middle-Age-Hysterectomy groups revealed a Trial x Surgery interaction ($F_{(2,36)} = 4.228$, $p < 0.05$; Figure 22B). Such interactions with Trial were also present for planned comparisons between Middle-Age-Hysterectomy and Middle-Age-Ovx+Hysterectomy groups ($F_{(2,36)} = 4.838$, $p < 0.05$), as well as for Young-Hysterectomy and Young-Ovx+Hysterectomy groups ($F_{(2,36)} = 3.746$, $p < 0.05$), for WMC errors during task maintenance (Figure 22B).

Analyses for these sets of planned comparisons at each individual trial revealed that, at the moderate working memory load trial, Trial 3, there was a main effect of Surgery for the Young-Hysterectomy and Young-Ovx+Hysterectomy planned comparison ($F_{(1,18)} = 4.688$, $p < 0.05$), where rats that had their ovaries and uterus surgically removed in middle-age made more WMC errors than those that only underwent uterine removal in middle-age (Figure 22C). At the highest working memory load trial, Trial 4, there was a main effect of Surgery for the Middle-Age-Hysterectomy and Middle-Age-Ovx+Hysterectomy planned comparison ($F_{(1,18)} = 4.682$, $p < 0.05$), where hysterectomy alone in middle-age resulted in greater WMC errors than removal of both the ovaries and uterus (Figure 22D). Additionally, there was a marginal effect of Surgery for the Middle-Age-Sham and Middle-Age-Hysterectomy planned comparison at this high working memory load trial ($F_{(1,18)} = 4.367$, $p < 0.10$), where rats that receiving hysterectomy surgery in middle-age tended to make more WMC errors than those receiving sham surgery (Figure 22D). No other planned comparison revealed significant effects of Surgery or Trial x Surgery interactions for WMC errors during task maintenance.

When analyzing WMI errors during task maintenance, there was a marginal effect of Surgery across all trials for the Middle-Age-Sham and Middle-Age-Hysterectomy ($F_{(1,18)} = 4.118$, $p < 0.10$) and Middle-Age-Sham and Middle-Age-Ovx+Hysterectomy ($F_{(1,18)} = 4.245$, $p < 0.10$) planned comparisons, where surgical removal of the uterus, with or without removal of the ovaries, in middle-age resulted in a tendency for greater WMI errors as compared to Sham rats (Figure 23A). Similarly, for both of these planned comparisons, there were significant Trial x Surgery interactions (Middle-Age-Sham vs. Middle-Age-Hysterectomy: $F_{(3,54)} = 3.999$, $p < 0.05$; Middle-Age-Sham vs. Middle-Age-Ovx+Hysterectomy: $F_{(3,54)} = 4.245$, $p < 0.01$; Figure 23B). When analyzing each trial, it was revealed that for Trial 4, the high working memory load trial, there were marginal effects of Surgery for the Middle-Age-Sham vs. Middle-Age-Hysterectomy planned comparison ($F_{(1,18)} = 4.281$, $p < 0.10$) and the Middle-Age-Sham vs. Middle-Age-Ovx+Hysterectomy planned comparison ($F_{(1,18)} = 4.245$, $p < 0.10$), where rats that underwent uterine removal in middle-age, regardless of ovarian status, tended to make more WMI errors than Sham rats (Figure 23C). No other planned comparison revealed significant effects of Surgery or Trial x Surgery interactions for WMI errors during this portion of the task.

For the Asymptotic Phase of WRAM testing, there were no effects of Surgery or Trial x Surgery interactions for RM errors across any of the planned comparisons.

Delayed memory retention. To evaluate delayed memory retention on the WRAM with the implementation of a 6-hour delay between Trials 2 and 3, performance was evaluated on Trial 3, the immediate post-delay trial. Specifically, Trial 3 for the last baseline day of testing, Day 12 (Baseline) was compared to performance on Trial 3 on the

day of the delay, Day 13 (Delay). For WMC errors, the Young-Sham ($F_{(1,9)} = 22.500$, $p < 0.01$), Young-Hysterectomy ($F_{(1,9)} = 16.000$, $p < 0.01$), Young-Ovx+Hysterectomy ($F_{(1,9)} = 7.309$, $p < 0.05$), Middle-Age-Sham ($F_{(1,9)} = 31.154$, $p < 0.001$), and Middle-Age-Ovx+Hysterectomy ($F_{(1,9)} = 5.651$, $p < 0.05$) made significantly more errors on the post-delay trial as compared to baseline indicating delay-induced impairment (Figure 24). Notably, no effect was seen for the Middle-Age-Hysterectomy group. There were no delay-induced effects for WMI errors.

Morris Water Maze

For each set of planned comparisons, in evaluating MWM performance across Days 1-5, there was a main effect of Day (Young-Sham vs. Young-Hysterectomy: $F_{(4,72)} = 35.740$, $p < 0.0001$; Young-Sham vs. Young-Ovx+Hysterectomy: $F_{(4,72)} = 24.762$, $p < 0.0001$; Young-Hysterectomy vs. Young-Ovx+Hysterectomy: $F_{(4,72)} = 33.597$, $p < 0.0001$; Middle-Age-Sham vs. Middle-Age-Hysterectomy: $F_{(4,68)} = 21.764$, $p < 0.0001$; Middle-Age-Sham vs. Middle-Age-Ovx+Hysterectomy: $F_{(4,72)} = 27.132$, $p < 0.0001$; Middle-Age-Hysterectomy vs. Middle-Age-Ovx+Hysterectomy: $F_{(4,68)} = 24.132$, $p < 0.0001$) for distance to the platform, with distance decreasing across testing days, indicating learning of the task (Figure 25A). There was no effect of Surgery nor an Age x Surgery interaction for any of these planned comparisons.

Finally, analyses of probe trial performance revealed an effect of Quadrant for each treatment group (Young-Sham: $F_{(1,9)} = 60.851$, $p < 0.0001$; Young-Hysterectomy: $F_{(1,9)} = 73.493$, $p < 0.0001$; Young-Ovx+Hysterectomy: $F_{(1,9)} = 120.132$, $p < 0.0001$; Middle-Age-Sham: $F_{(1,9)} = 18.126$, $p < 0.01$; Middle-Age-Hysterectomy: $F_{(1,8)} = 98.046$, $p < 0.0001$; Middle-Age-Ovx+Hysterectomy: $F_{(1,9)} = 58.383$, $p < 0.0001$; Figure 25B).

For each group, a greater percentage of total swim distance was spent in the Target Quadrant (NE) as opposed to the Opposite Quadrant (SW), indicating spatial localization of the platform.

Visible Platform Task

For the VP task, there was a main effect of Trial ($F_{(5,270)} = 7.246$, $p < 0.0001$), with latency to the platform decreasing across trials (Figure 26). There was no effect of Surgery or Age, or any interactions of Age or Surgery with Trial for this task, indicating groups did not differ in visual or motor acuity necessary to perform this task. For the final testing trial, no animal had greater than 15 seconds latency to platform, indicating all subjects were capable of performing the procedural components necessary to solve a water-escape task.

Vaginal Cytology

Vaginal smears were performed for eight consecutive days, beginning three weeks after surgery. Rats that underwent Ovx+Hysterectomy surgery showed blank or diestrus-like smears, demonstrating successful surgical removal of the ovaries. Rats in the Sham and Hysterectomy groups showed normal estrous cycles of 4-5 days in length, demonstrating continued post-operative ovarian function. Vaginal smears were again collected on the day prior to euthanasia and the day of euthanasia. Ovx+Hysterectomy groups continued to display blank or diestrus-like smears, whereby Sham and Hysterectomy groups displayed normal estrous cycle activity. There were no perceptible differences in estrous cyclicity across Young and Middle-Age groups within a given surgery type.

Body Weights at Euthanasia

At euthanasia, body weights were collected for each subject. Analyses showed that there was a main effect of Surgery for the following planned comparisons: Young-Sham vs. Young-Ovx+Hysterectomy ($F_{(1,18)} = 73.118$, $p < 0.0001$), Young-Hysterectomy vs. Young-Ovx+Hysterectomy ($F_{(1,18)} = 59.684$, $p < 0.0001$), Middle-Age-Sham vs. Middle-Age-Ovx+Hysterectomy ($F_{(1,18)} = 22.836$, $p < 0.001$), and Middle-Age-Hysterectomy vs. Middle-Age-Ovx+Hysterectomy ($F_{(1,18)} = 62.844$, $p < 0.0001$). In each case, surgical removal of the ovaries resulted in an increase in body weight. Interestingly, there was also a marginal effect of body weight for the Middle-Age-Sham vs. Middle-Age-Hysterectomy planned comparison ($F_{(1,18)} = 4.279$, $p < 0.10$), where rats that received Hysterectomy in middle-age tended to have lower body weights than Sham rats (data not shown).

Ovarian Follicle Counts

Counts of primordial follicles revealed no significant effect of Surgery for either set of planned comparisons (Figure 27A). For primary follicles, there was a marginal effect of Surgery for the Young-Sham vs. Young-Hysterectomy planned comparison ($F_{(1,17)} = 4.106$, $p < 0.10$), where rats that received Hysterectomy in young adulthood had elevated primary follicle counts relative to Sham rats (Figure 27B). There were no effects of Surgery for Secondary follicles (Figure 27C). For Antral follicles, there was a Surgery effect for the Middle-Age-Sham and Middle-Age-Hysterectomy groups ($F_{(1,15)} = 8.441$, $p < 0.05$), where Hysterectomy rats showed reduced antral follicle counts relative to Sham rats in middle-age (Figure 27D). There were no Surgery effects for corpora lutea (Figure 27E).

Serum Hormone and Gonadotropin Evaluations

Serum evaluations of E2 concentrations with Sham and Hysterectomy subjects revealed a main effect of Surgery for the Middle-Age-Sham and Middle-Age-Hysterectomy groups ($F_{(1,18)} = 8.116$, $p < 0.05$), where hysterectomized rats had elevated E2 in middle-age as compared to age-matched Sham rats (Figure 28A). There was no effect of Surgery for rats in young adulthood. Additionally, serum concentrations of androstenedione had a main effect of Surgery for the Middle-Age-Sham vs. Middle-Age-Hysterectomy planned comparison ($F_{(1,18)} = 5.249$, $p < 0.05$), with rats that underwent Hysterectomy in middle-age having greater serum androstenedione than rats that underwent Sham surgery at this same age (Figure 28B). Surgery effects were not present in young adulthood for serum androstenedione. There were no Surgery effects for serum progesterone (Figure 28C) or AMH (Figure 28D) across any planned comparisons.

Analyses for serum LH concentrations revealed main effects of Surgery for the Young-Sham vs. Young-Ovx+Hysterectomy ($F_{(1,18)} = 21.089$, $p < 0.001$), Young-Hysterectomy vs. Young-Ovx+Hysterectomy ($F_{(1,18)} = 22.755$, $p < 0.001$), Middle-Age-Sham vs. Middle-Age-Ovx+Hysterectomy ($F_{(1,18)} = 119.226$, $p < 0.0001$), and Middle-Age-Hysterectomy vs. Middle-Age-Ovx+Hysterectomy planned comparisons ($F_{(1,18)} = 36.344$, $p < 0.0001$), with surgical removal of the ovaries at either age resulting in an increase in serum LH levels relative to Sham and Hysterectomy groups (Figure 28E). There were no differences in LH levels at either age between Sham and Hysterectomy groups. Serum FSH concentrations revealed main effects of Surgery for the Young-Sham vs. Young-Ovx+Hysterectomy ($F_{(1,18)} = 5.584$, $p < 0.05$), Young-Hysterectomy vs. Young-Ovx+Hysterectomy ($F_{(1,18)} = 6.045$, $p < 0.05$), Middle-Age-Sham vs. Middle-Age-Ovx+Hysterectomy ($F_{(1,18)} = 26.473$, $p < 0.0001$), and Middle-Age-Hysterectomy

vs. Middle-Age-Ovx+Hysterectomy planned comparisons ($F_{(1,18)} = 25.625$, $p < 0.0001$). Similar to the effects on LH levels with Ovx, FSH levels were elevated by Ovx surgery at both ages relative to the ovary-intact groups (Figure 28F). There were no effects of Surgery for FSH levels between Sham and Hysterectomy groups at either age.

Western Blot Protein Analysis

Across all sets of planned comparisons, there were no effects of Surgery for normalized FosB expression, normalized ΔFosB expression, or normalized IL-10 expression in any brain region (data not shown).

Discussion

The current study used the novel rodent model of hysterectomy to investigate whether age at hysterectomy surgery, with or without concomitant ovarian removal, impacted subsequent cognitive, physiological, and neurobiological outcomes. Indications of hysterectomy-induced working memory deficits were present in young adult female rats during the later stages of learning on the WRAM, but only when the ovaries remained intact. For rats that received hysterectomy in middle-age, regardless of ovarian status, indications of working memory deficits were present during the maintenance phase of the task, particularly at the highest working memory load trial. Additional changes in ovarian follicle counts and hormone levels following hysterectomy in middle-age suggest that this timepoint may be particularly vulnerable to changes following hysterectomy surgery.

The working memory deficits observed in the Young-Hysterectomy rats relative to the Sham control rats during the latter portion of task acquisition replicate the findings of cognitive deficits from prior work with this hysterectomy model (Koebele et al., 2019).

Given clinical findings showing increased risk of dementia risk with earlier age at hysterectomy (Phung et al., 2010; Rocca et al., 2012), it was expected that rats receiving hysterectomy in middle-age would show diminished cognitive deficits. However, the current experiment found more consistent cognitive deficits were present at this age-point, regardless of ovarian status, indicating greater impact of hysterectomy at this unique age point. It is possible that this discrepancy between the clinical literature and the current study is partially attributable to a moderating clinical variable, as individuals who undergo hysterectomy surgery at different ages might have different health conditions that prompt such gynecological surgery, which could be driving this differential dementia risk. Alternatively, the differences between female reproductive senescence in rats and in humans (Bimonte-Nelson et al., 2021; Koebele & Bimonte-Nelson, 2016), which produce different hormonal and neurobiological profiles across species at this age, may be driving these divergent cognitive outcomes with hysterectomy in middle-age.

Overall, both young and middle-aged rats demonstrated an ability to learn and perform spatial navigation tasks, regardless of surgery. Indeed, rats were able to learn the procedural components of the WRAM, as a main effect of Day was found across Days 2-12, where errors made decreased across days of testing for all error types across all subjects. On the MWM, significant effects of Quadrant on the probe trial for each treatment group indicated spatial localization of the platform. During the VP task, animals also demonstrated the ability to successfully complete a water-escape task, as main effects of Trial were found without a significant interaction with Age or Surgery.

Within the Early Acquisition Phase of testing, for WMC errors, animals that received hysterectomy with concomitant Ovx in young adulthood, but not middle-age,

tended to make fewer errors than Sham rats when evaluated at a high working memory load. This early task facilitation in young adult Ovx+Hysterectomy rats was observed previously in our laboratory (Koebele et al., 2019). This facilitation in acquisition of a spatial navigation task could be attributable to the impact that ovarian hormone depletion can have on attentional processes and strategy preferences (Korol & Kolo, 2002; Mcgaughy & Sarter, 1999).

In the Late Acquisition Phase, there is a unique tendency for hysterectomy-induced impairment observed for young animals alone, where animals in the Young-Hysterectomy group tended to make more WMI errors at the highest working memory load as compared to those in the Young-Sham group; such impairment was not observed for those that received surgery in middle-age. This finding is a replication of prior work in the hysterectomy model showing working memory impairment (Koebele et al., 2019). Furthermore, it indicates that animals receiving hysterectomy surgery in young adulthood are at a unique disadvantage in learning a spatial working memory task, particularly when working memory is highly taxed.

When analyzing the Asymptotic Phase, the resulting effects largely contrasted those found during Acquisition on the WRAM. For WMC errors, animals receiving hysterectomy with concurrent Ovx in young adulthood were found to be impaired at the moderate working memory load trial as compared to Sham animals. Where earlier analyses of task acquisition found learning facilitation, rats receiving hysterectomy plus Ovx in young adulthood were uniquely impaired on a spatial working memory task at a moderate cognitive load by the maintenance phase. This would strongly indicate a different strategy being used to perform this spatial task; prior work has shown that with

low estradiol, female rats may be more likely to select a nonspatial "response" strategy that relies on processing outside the hippocampus (Korol et al., 2004). When looking at effects for middle-aged rats, those receiving hysterectomy were found to make marginally more errors than Sham rats, indicating a tendency for impairment at the final portion of the task. Indeed, when the highest working memory load was evaluated, it was found that rats receiving hysterectomy in middle-age tended to make more errors than their Sham counterparts, and made significantly more errors than those that received concomitant Ovx and hysterectomy. This pattern of effects suggests that hysterectomy without ovarian removal at middle-age confers a unique risk of spatial learning and memory detriments. The pattern of effects for WMI errors in the Asymptotic Phase are similar overall to that of WMC errors. Analyses across all 4 trials, but particularly at the highest working memory load trial, found that rats receiving hysterectomy in middle-age, with or without concomitant Ovx, tended to make more errors than their sham counterparts. With WMC and WMI errors demonstrating the same pattern of cognitive impairment with hysterectomy in middle-age at a high working memory load, it seems evident that the effects of hysterectomy on working memory performance, as evaluated on the WRAM, indeed differ depending upon age at surgery. These behavioral findings sit in contrast to the aforementioned experimental hypothesis that younger age at hysterectomy surgery would demonstrate exacerbated cognitive impairment as compared to those receiving surgery in middle-age, based upon findings from the clinical literature (Phung et al., 2010; Rocca et al., 2012). However, there are some differences between such clinical findings and the current experimental paradigm: namely, that the current experiment evaluated cognition at a fairly short interval post-surgery, whereas the

majority of clinical research has evaluated dementia risk associated with hysterectomy decades after surgery. Recent work in the Bimonte-Nelson laboratory using the hysterectomy model has evaluated spatial learning and memory at longer post-surgical timepoints (Koebele, 2019), but only in animals that receive surgery in young adulthood. Indications from this work so far point to a sustained cognitive impairment for young adult animals receiving hysterectomy surgery, even 12 months after initial surgical intervention(Koebele, 2019). Such research should be extended into evaluating longer post-surgical timepoints for animals that receive hysterectomy in middle-age, where impairment could be more severe at short-term evaluations, but could dissipate more quickly with aging than with animals that receive hysterectomy in young adulthood.

Evaluation of delay-induced impairments on the WRAM revealed that all groups except for the Middle-Age-Hysterectomy group were impaired by the implementation of a delay between Trials 2 and 3. Impairment with WRAM delay has previously been demonstrated in such groups receiving gynecological surgery (Koebele et al., 2019). While it may seem unexpected that the group demonstrating greatest impairment during the final baseline days of testing would not be impaired with the implementation of a delay between trials, it is possible that this group was already sufficiently taxed during baseline testing, such that the delay did not appear to present any additional challenge. This laboratory has previously found that animals that are sufficiently burdened during baseline days of testing demonstrate no further impairment following implementation of a delay on a spatial working memory task (Engler-Chiurazzi et al., 2011). Finally, evaluation of MWM did not demonstrate significant differences in spatial reference memory performance due to surgery. These findings concur with those demonstrated in

prior work using this model (Koebele et al., 2019), where no differences in reference memory performance were detected with hysterectomy or Ovx surgery.

Notably, age at surgery also seems to play a role in ovarian outcomes, as follicle counts differed depending upon hysterectomy surgery and age. In young rats, hysterectomy surgery resulted in a marginal increase in primary follicles, potentially indicating a greater number of follicles are exiting the non-growing follicular pool. Such follicular activation is thought to originate within the ovary, rather than as the result of hormonal stimulation (Fortune et al., 2000). Kit ligand, produced by granulosa cells, is thought to be partially responsible for this activation. Experimental inactivation of its receptor c-kit halted production of primary follicles in developing female mice (Yoshida et al., 1997), demonstrating its ability to initiate the exit of follicles from the primordial pool. In the current study, modulation of gene expression of the kit ligand withing the ovary could operate in an age-dependent manner, as no such increase in primary follicles was observed in middle-aged rats following hysterectomy. Future work should explore putative ovarian gene expression changes as a result of hysterectomy, and whether age plays a critical role in these outcomes.

In contrast to the increases observed in primary follicles following hysterectomy in young adulthood, middle aged rats that underwent hysterectomy had reduced antral follicle counts as compared to their age-matched controls. Serum hormone evaluations also revealed some unique effects of hysterectomy surgery in middle-age. While, as predicted, LH and FSH levels rose with surgical removal of the ovaries at both timepoints as compared to ovary-intact rats (Koebele et al., 2019; Wise & Ratner, 1980), hysterectomy uniquely in middle-age resulted in elevations in serum E2 and

androstenedione. This finding sits in contrast to the tendency for declining E2 and androstenedione levels with hysterectomy in young adulthood that have been previously reported (Koebele et al., 2019). It is critical to note here that these two findings have different age-matched Sham groups: while the prior study may have found that hysterectomy results in declining levels on a background milieu of a reproductively healthy adult rat, the current study indicates that the ovaries may be differentially sensitive to hysterectomy-induced changes in middle-age. While female rodents do undergo a form of reproductive senescence, it differs in many ways from that of humans. While young adult rodents have circulating hormone levels that parallel those of women during their reproductive years, with cyclic fluctuations in 17β-estradiol and progesterone (Koebele & Bimonte-Nelson, 2016), rodents in late middle-age undergo a reproductive transition known as estropause, characterized by a persistent estrus cycle phase with moderate and stable circulating levels of 17β-estradiol and moderate gonadotropin levels (Koebele & Bimonte-Nelson, 2016; Lu et al., 1979). Indeed, when looking at the current study, the variability in serum E2 and androstenedione levels seems to drop in middle-age, suggesting that these rats are not experiencing regular estrous cycles. Therefore, it is possible that the ovaries in the middle-age rodent have a unique response to uterine removal that creates a distinct hormone profile.

While questions remain as to why androstenedione and E2 levels become elevated following hysterectomy in the middle-aged rat, these increased levels may explain the ovarian follicle effects found in the current study. Work in rodents has shown that elevated androgens can promote apoptosis in mature antral follicles; notably, estrogens have been shown to counteract these effects (Billig et al., 1993). Such androgenic and

follicular outcomes are common in individuals with poly-cystic ovarian syndrome (PCOS), a disease characterized by infrequent ovulation or anovulation, and excess ovarian androgens (Franks & Hardy, 2018). Future work in this model should characterize not only healthy follicles, but atretic follicles to better understand these relationships between hormonal and ovarian outcomes in middle-age. It is possible that the elevated E2 levels in the current study are also a result of ample androstenedione, as androstenedione can be converted into estrone via aromatase, where estrone is further converted to estradiol (Franks & Hardy, 2018). Such conversions could occur at the level of the ovary, or occur in adipose tissues (Kuhl, 2005); further studies should pursue a better understanding of potential metabolic changes with aging in the female rodent to better characterize these outcomes.

The putative ovarian and hormonal changes as a result of hysterectomy in middle-age are important to understand in the context of this model, yet the differences between reproductive senescence in females between humans and rodents present some challenges here regarding translational interpretation. Future work could assess a clinically-relevant rodent model of the menopause transition, the 4-vinylcyclohexene diepoxide (VCD) model. VCD, when administered gradually over a series of 15 injections, has been demonstrated to selectively deplete the ovarian follicular pool, leading to eventual ovarian failure via accelerated atresia (Hoyer et al., 2001; Hu et al., 2001; Mayer et al., 2002), similar to that of menopausal women. Additionally, this laboratory has demonstrated that some serum alterations in ovarian hormone levels for VCD-treated rats are similar to those seen in menopausal women (Acosta et al., 2010; Acosta, Mayer, Talboom, Tsang, et al., 2009). Given the translational value of the VCD model, future

work could expand the use of the hysterectomy model to better characterize the spatial learning and memory outcomes, as well as ovarian and hormonal outcomes, associated with uterine removal at the start of the menopause transition via VCD administration, with a rapidly depleting ovarian follicular reserve and fluctuating ovarian hormone levels.

In the current experiment, there were no significant main effects for any neurobiological measures across several brain regions of interest. The lack of effect on FosB/ΔFosB expression with hysterectomy suggest that overall activity levels are not impacted by surgery at this six-week timepoint. Prior work with this model found changes in FosB and ΔFosB expression in middle-age following hysterectomy, but these evaluations took place six months after surgery (Koebele, 2019). It is possible that initial brain changes following hysterectomy lead to a cascade that results in modified activity levels, represented by changes in FosB and ΔFosB expression, in the months following surgery. This would follow the clinical literature, which shows increased risk for dementia sometimes decades after hysterectomy surgery (Phung et al., 2010). Future work should evaluate longer intervals post-surgery to determine whether the trajectory of neurobiological changes after hysterectomy depend upon age at surgery. The lack of changes in IL-10 expression with hysterectomy, with or without ovarian removal, demonstrate that this traditionally classified anti-inflammatory cytokine does not shift as a result of reproductive tract manipulations in either young adulthood or middle-age rats. While prior work in rodent models of oophorectomy have demonstrated reductions in IL-10 in regions critical to spatial learning and memory (Eid et al., 2020), indicating an overall shift towards a greater pro-inflammatory profile, it is possible that the additional removal of the uterus in the current study differentially shifted the subsequent

inflammatory profile. Indeed, rather than reducing expression of anti-inflammatory cytokines, it is possible that hysterectomy impacts the immune system by increasing pro-inflammatory cytokine expression (e.g. IL-6, TNF-alpha), or through another means, such as modulation of macrophage levels, etc. Further work should be done to evaluate the potential impact of uterine removal, with or without concomitant ovarian removal, on subsequent immune function.

Conclusion

The current experiment demonstrates that age at hysterectomy surgery, alone and with concomitant ovarian removal, modulates spatial learning and memory outcomes. Hysterectomy in young animals negatively impacted spatial working memory during learning, whereas surgery in middle-age showed impairments in the maintenance phase of WRAM testing. Furthermore, unique ovarian and hormonal outcomes with hysterectomy were observed, particularly at in middle-age, indicating an age-differential response of the ovaries and broader HPG axis with uterine removal. This investigation into the role of age at hysterectomy surgery, both alone and with concomitant ovarian removal, in modulating cognitive outcomes has provided significant insight into the importance of the nonpregnant uterus across the female reproductive lifespan, where the uterus might participate in communications between the reproductive tract and brain. Indeed, this work contributes to the larger body of literature providing evidence opposite the notion that the nonpregnant uterus is a dormant, useless organ, thereby supporting the tenet that the uterus plays a larger role in HPO axis functioning and modulates cognitive outcomes. The current experiment has expanded the questions that future work should now address, particularly concerning whether the unique effects observed in middle-age

with uterine removal are attributable to age, reproductive stage, or putative interactions with other critical variables. Once these effects are more clearly delineated, options for clinical management can be more directly pursued to yield promise toward a healthy trajectory during aging in women, even with variances in menopause etiology.

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CHAPTER 5

The data presented in this chapter are currently unpublished observations.

THE COGNITIVE AND OVARIAN OUTCOMES OF DIFFERING METHODS OF VCD ADMINISTRATION IN THE FEMALE RAT AS A MODEL OF TRANSITIONAL MENOPAUSE

Contribution: I was the graduate student principal investigator for this experiment under the mentorship of Dr. Heather Bimonte-Nelson.

ABSTRACT

Preclinical rodent models of menopause provide critical insight into key health factors that can be further probed in the clinic. Unlike prior models that have relied on surgical manipulation of the reproductive tract, the 4-vinylcyclohexene diepoxide (VCD) model of menopause chemically induces ovarian follicular depletion, making it an apt model of the menopause transition. However, different experimental protocols have be used to create the VCD model within the preclinical menopause literature. These protocol variations can putatively impact overall animal health as well as endocrine and ovarian factors relevant to the scientific question at hand. Notably, there has yet to be a systematic assessment of these methodologies for endocrine, ovarian, and behavioral changes. The current study evaluated the effect of administering VCD using different vehicles (50% DMSO/ 50% Saline vs. Sesame Oil) and different routes of administration (IP vs SubQ) on ovarian and cognitive outcomes. Results demonstrate that, overall, the methods evaluated yield consistent profiles of ovarian follicular depletion and mild cognitive deficits. However, there were methodological challenges with each protocol, highlighting the importance of choosing a protocol that is most suitable given the experimental parameters and outcomes of interest.

Introduction

While menopause is clinically confirmed retrospectively after one year of amenorrhea, on average at the age 52 (NAMS, 2014), ovarian and endocrine systems undergo critical shifts on average 4-6 years before menopause onset (Harlow et al., 2012); this period of time is termed the menopause transition. The menopause transition serves as an important period of time in the female lifespan that can alter the trajectory of healthy aging and increase the risk for some age-related diseases. During these years, dysregulation of the hypothalamic-pituitary-ovarian (HPO) axis results in irregular menstrual cycling, and erratic levels of circulating estrogens and progesterone, which is concurrent with a decline in ovarian follicles (Harlow et al., 2012). Ultimately, menopause results in ovarian follicular depletion, and low or undetectable circulating estrogen and progesterone levels, along with elevated levels of the gonadotropins follicle stimulating hormone (FSH) and lutenizing hormone (LH) (Bimonte-Nelson et al., 2021). Such ovarian and hormone changes across these years coincide with the onset of symptoms that include hot flashes, cognitive and sleep disturbances, and mood alterations (Koebele & Bimonte-Nelson, 2016; NAMS, 2014; Sturdee et al., 2017; Weber et al., 2013). Key parameters relating to this experience, including age at menopause onset, duration of this transition, and symptom severity play a role in long-term health outcomes. Indeed, factors relevant to the experience of menopause can alter risk for autoimmune, cardiovascular, and mood-related disorders, as well as dementia (Biglia et al., 2017; Ryan et al., 2014; Shuster et al., 2010; Zhu et al., 2019). For this reason, a great deal of clinical research has been done to identify such risk factors, as well as potential therapeutics, with the ultimate goal of improve health outcomes for individuals in

menopause (Biglia et al., 2017; el Khoudary et al., 2020; Shuster et al., 2010). However, little is still known surrounding the mechanisms behind certain risk factors, which stifles the ability to identify efficacious therapeutics and prevention strategies for women undergoing this transition.

Where clinical trials can provide information about the menopause transition, preclinical menopause research can also aid in the study of this critical period; through systematic experimental designs and modeling, preclinical rodent work can identify variables of interest and putative interventions, and can begin to investigate questions surrounding mechanisms of conferred risk. Rodents have similar brain and reproductive structures, and can display an array of complex behaviors, allowing for the assessment of cognitive and physiological changes with reproductive senescence and across aging (Bimonte-Nelson et al., 2021; Koebele & Bimonte-Nelson, 2016). It should be noted that there are some complexities in modeling menopause in the rodent: namely, the female rodent does not experience ovarian follicular depletion and declining estrogen across its form of reproductive senescence, known as estropause (Koebele & Bimonte-Nelson, 2016). What may seem like a challenge, however, actually confers an advantage in addressing certain questions surrounding the menopause transition using preclinical models: this distinction between ovarian failure and chronological aging allows for the separation of age-related risks from those of menopause. Indeed, interventions can be performed to initiate the onset of menopause-like reproductive profiles, either chemically or surgically, to observe resulting outcomes in the rodent in an age-dependent manner.

For decades, the established preclinical model of menopause was the ovariectomy (Ovx) model of surgical menopause, which requires surgical removal of the ovaries in the rodent (Koebele & Bimonte-Nelson, 2016). The Ovx model has a hormone profile that resembles that of surgically postmenopausal women, as ovarian removal results in a steep decline in circulating estrogens and progesterone, and step elevation in FSH and LH levels (Koebele & Bimonte-Nelson, 2016). While much progress has been made within the field in characterizing surgical menopause outcomes using the Ovx rodent model, the model is not optimal for investigating questions surrounding the menopause transition. This is because the Ovx model does not allow for a gradual change in ovarian hormone levels, and necessitates removing the ovaries, which are a source of androgens and other hormones, even after follicular depletion occurs (Mayer et al., 2004). To better model the menopause transition, the 4-vinylcyclohexene diepoxide (VCD) rodent model may be used. The VCD rodent model of transitional menopause involves a series of injections that, by targeting primordial and primary follicles, results in ovarian follicular depletion and alteration to the ovarian hormone profile that more closely corresponds to that of menopause (Hoyer et al., 2001; Hu et al., 2001; Mayer et al., 2002). Additionally, VCD-induced follicular depletion results in memory deficits that mirror those reported by individuals in the menopause transition (Acosta, Mayer, Talboom, Tsang, et al., 2009; Schaafsma et al., 2010). Such outcomes allow researchers to evaluate the impact of various factors, including the use of hormone therapies, on various critical dimensions of his transitional stage, and to further probe the mechanisms behind various risks associated with menopause.

VCD is a useful tool in the context of modeling transitional menopause, but its potential for clinical translation is currently stifled by a lack of consistency in treatment protocol. Indeed, with different laboratories developing their own protocols for using the VCD model, there are varied VCD administration methods across research groups, potentially affecting endocrine outcomes as well as overall health of these experimental subjects. In our laboratory, VCD is administered via a series of intraperitoneal injections, given at a concentration of 180 mg/kg in a 50%/50% dimethyl sulfoxide (DMSO) and saline vehicle (Acosta et al., 2010; Acosta, Mayer, Talboom, Tsang, et al., 2009; Koebele et al., 2017; Koebele, Hiroi, et al., 2021; Koebele, Mennenga, et al., 2020). We find consistent spatial learning and memory deficits, along with ovarian follicular depletion and some hormonal changes; however, we have observed that animals lose significant weight across the procedure, and there can be other complications from the series of injections. Other laboratories have published using a variety of methods, doses of VCD, vehicles for administration, and routes of administration (Hu et al., 2001; Mayer et al., 2002; Muhammad et al., 2009; Pestana-Oliveira et al., 2018; Reis et al., 2014), with inconsistent reporting of subsequent ovarian and hormone changes. Vehicle and route of administration are known to affect pharmacokinetics, and can subsequently impact physiology and behavior in the rodent (Turner et al., 2011). Within the context of menopause modeling, prior work has demonstrated that different vehicles for estradiol administration result in distinct behavioral profiles in middle-aged female rats (Prakapenka et al., 2020), suggesting that these different VCD administration methodologies could also differentially modulate cognitive and physiological outcomes. Critically, none of these differing methods have been systematically evaluated to ensure

consistent ovarian, endocrine, and behavioral outcomes; while these works claim to be modeling transitional menopause in the rodent, it is unclear what degree of translational success each method of VCD administration can provide.

The current study seeks to address this discrepancy in the literature by directly comparing different methods for VCD administration within the middle-aged female rat, including both differing vehicles (DMSO/Saline vs Sesame Oil) and routes of administration (intraperitoneal vs subcutaneous) that are commonly observed in the VCD literature, and observing subsequent cognitive and ovarian profiles. Not only will these measures provide greater clarity regarding whether these models aptly reflect these dimensions of the menopause transition, but they will also highlight the methodological challenges of each method, in the hopes of identifying the optimal form of VCD administration in modeling this critical window.

Methods

Subjects

Sexually-inexperienced female Fischer 344-CDF 7–8-month-old rats (N=50), free of cataracts, arrived to the Arizona State University (ASU) Department of Psychology vivarium from the National Institute on Aging (NIA) Colony at Charles River Laboratories (Raleigh, NC). Upon arrival, animals were pair-housed and maintained in a temperature- and humidity- controlled room on a 12-hour light/dark cycle (lights on at 7:00am), and given food and water *ad libitum*. Due to the emergence of the COVID-19 pandemic, animals remained at the ASU facility for approximately 4 months before the start of the current experiment; therefore, the experiment began with rats aged 12-13

months. All animal protocols were approved by the ASU Institutional Animal Care and Use Committee (IACUC), and were conducted in accordance with guidelines from the National Institutes of Health (NIH). Figure 29 demonstrates the full experimental timeline.

VCD Administration

VCD was administered based upon prior work in our laboratory and others (Koebele et al., 2017; Mayer et al., 2004; Reis et al., 2014) at a daily dose of 160 mg/kg (FYXX, Flagstaff, AZ). Daily VCD injections were determined by the animal's body weight. All VCD and Vehicle injections were conducted on a 15-injection schedule, as previously done in our laboratory (Acosta, Mayer, Talboom, Tsang, et al., 2009; Koebele et al., 2017; Koebele, Hiroi, et al., 2021). Injections were performed on a Monday/Tuesday/Thursday/Friday schedule to allow for animal recovery. If an animal's weight fell below 90% of their initial body weight, injections were halted until sufficient weight was gained to continue injections.

Due to concerns that administration of large volumes of Sesame Oil subcutaneously could result in poor absorption, VCD was dissolved at roughly twice the concentration in Sesame Oil (120 mg/ml) than that of DMSO/Saline (63.33 mg/ml), resulting in a smaller volume being delivered to animals receiving Sesame oil injections. Vehicle injection volumes were matched to the comparable volume of VCD injections at the rats' average body weight, resulting in either 0.5ml DMSO/Saline or 0.25ml Sesame oil injections.

Initial experimental design. Initially, this project addressed the experimental question of evaluating different VCD administration paradigms commonly used within the literature. As the literature surrounding this transitional menopause model not only differs regarding the vehicle type for VCD delivery, but also the route of VCD administration (Koebele et al., 2017; Mayer et al., 2002; Pestana-Oliveira et al., 2018), the current experiment evaluated two of the most common vehicles and routes of administration: 50%/50% DMSO/Saline (DMSO/SAL) vs Sesame Oil (Oil) vehicle deliveries of VCD, administered as Intraperitoneal (IP) vs Subcutaneous (SubQ) injections. Finally, in addition to the exploration of these two variables within the VCD literature, the current experiment initially included a general handled control group. The addition of a handled, but not injected, control group allowed us to isolate Vehicle effects in addition to VCD effects. This is critical, as prior work in our laboratory has demonstrated effects of different delivery vehicles that modulate behavioral outcomes (Prakapenka et al., 2020). Thus, the experiment began with the following treatment groups (n=10/group): Handled Control, IP-Vehicle-DMSO/SAL, IP-VCD-DMSO/SAL, SubQ-Vehicle-Oil, and SubQ-VCD-Oil.

During the first week of VCD administration, difficulties in SubQ administration of VCD resulted in the modification of the experimental treatment groups. Specifically, a few days after beginning injections, SubQ-VCD-Oil rats began to develop chemical burns on the skin around the injection site. At that point, it was unclear if these rats could continue to participate in the study; therefore, a new experimental design was developed, such that the questions surrounding differential VCD administration could still be addressed.

Updated experimental design. During this time of uncertainty, we still sought to evaluate VCD injections delivered in sesame oil. To more directly compare vehicle types in light of these complications, the Handled Control treatment group was changed to IP injections of VCD delivered in Sesame Oil (IP-VCD-Oil). To better serve as a Vehicle control, the previous SubQ-Vehicle-Oil group received all remaining injections via the IP route, and was thereafter referred to as the IP-Vehicle-Oil group.

The SubQ-VCD-Oil group, which developed the skin lesions, were evaluated by the clinical veterinarians, and had resolving lesions within 48 hours of halting VCD injections. The veterinarians deemed such lesions not serious enough to halt the study, resulting in rats in the SubQ-VCD-Oil group continuing to be included in the experiment and receive VCD treatment.

Therefore, the updated experimental design included the following groups: IP-Vehicle-DMSO/SAL, IP-VCD-DMSO/SAL, IP-Vehicle-Oil, IP-VCD-Oil, and SubQ-VCD-Oil.

With these treatment groups and the above noted injection paradigm, all Vehicle animals completed the series of 15 injections without a break in the administration schedule. For VCD-treated rats, one rat in the IP-VCD-Oil group and one rat in the SubQ-VCD-Oil group died due to complications surrounding VCD administration. One rat in the IP-VCD-DMSO/SAL group missed the final VCD injection due to severe weight loss. All other VCD animals completed the series of 15 injections, with 8/10 rats in the IP-VCD-DMSO/SAL group, 9/10 rats in the IP-VCD-Oil group, and 5/10 rats in

the SubQ-VCD-Oil group needing at least one break in the VCD injection schedule due to weight loss below 90% of their starting weight.

Body Weights and Vaginal Cytology

In addition to body weights being collected during each injection session, body weights were recorded for each subject on a weekly basis through to the conclusion of the study to assess individual health. Final body weights at euthanasia were collected to determine whether these VCD paradigms result in differing long-term physiological outcomes.

Two weeks prior to behavioral assessment, 88-96 days after the first Vehicle or VCD injection, eight days of vaginal cytology was undertaken for each rat to assess estrous cyclicity, following previous protocols (Koebele, Hiroi, et al., 2021). Cotton-tipped swabs were soaked in sterile saline before being gently inserted into the vaginal opening. A light microscope (Fischer Scientific Micromaster; CAT #12-561-4B) was used to classify estrous cycle phase as either estrus-, metestrus-, diestrus-, and proestrus-like as done previously (Goldman et al., 2007; Koebele et al., 2019; Koebele, Hiroi, et al., 2021).

Behavioral Battery

At 103-111 days following the first VCD injection, all rats were evaluated on a behavioral battery to assess spatial working and reference memory performance. This battery included the Water Radial-Arm Maze (WRAM), evaluating spatial working and reference memory; the Morris Water Maze (MWM), evaluating spatial reference

memory; and the Visible Platform (VP) Task, a control task evaluating the rats' visual and motor acuity necessary to solve a water-escape task.

WRAM. The WRAM is commonly used in our laboratory to evaluate spatial working and reference memory performance (Bernaud et al., 2021; Bimonte-Nelson, 2015d; Koebele et al., 2019; Prakapenka et al., 2018). The maze is an 8-arm apparatus that is filled with water and surrounded by robust extra-maze cues to aid in spatial navigation. The maze water is kept 18-20C and is dyed with black paint to obscure the 4 platforms that are hidden about 2-3cm under the surface at the ends of 4 of the 8 maze arms. Platform locations were semi-randomized across treatment groups and counterbalanced across testing squads and rooms but remained constant across testing days for a given rat. Rats were brought into the room in individual testing cages in squads of 8. One rat was then dropped off at the starting arm of the maze and given 3 minutes to swim and locate a platform. If the rat did not locate the platform in the allotted time, it was led to the nearest platform. Once on the platform, the rat reminded there to spatially localize for 3 minutes before being placed briefly back into its testing cage under a heat lamp during a 30-second inter-trial-interval. During this period, the tester removed the just-found platform and swept the maze for odor cues. After this 30-second interval, the rat was placed back into the maze at the starting arm and was given 3 minutes to find one of the remaining platforms. Trials continued in this fashion until all 4 platforms were found (i.e. 4 testing trials). In this way, working memory demand increased with each trial, as rats needed to remember not to revisit platform locations already visited within a testing day. After all 4 trials were completed, the rat remained in their testing cage, and the next rat began behavioral testing. Testing was continued in this manner across all 12

baseline days. On the 13th day of WRAM testing, trials 2 and 3 were interrupted by a 6-hour delay to assess delayed memory retention.

Working and reference memory performance on the WRAM was assessed in the form of errors, or entries a rat made into an arm that did not contain a platform. These errors were counted under the designation of Total Errors. To better assess working and reference memory items separately, errors were also further broken down into the following orthogonal domains: working memory correct (WMC) errors, or entries into a previously platformed arm, reference memory (RM) errors, or first-time entries into unplatformed arms, and working memory incorrect (WMI) errors, or repeated entries into unplatformed arms.

MWM. After WRAM testing, reference memory performance was assessed on the MWM, as done previously in our laboratory (Braden, Andrews, et al., 2017; Koebele et al., 2019; Prakapenka et al., 2018) with established protocols (Bimonte-Nelson, 2015b). The MWM apparatus is a large circular pool filled with 18-20C water obscured with black non-toxic paint and surrounded by robust extra-maze cues. The maze contains a platform hidden in the NE quadrant 2-3 cm under the water's surface that was kept constant across testing trials. Like that of the WRAM, rats were brought into the room in individual testing cages in squads of 8. Rats were dropped off semi-randomly at one of 4 maze quadrants (N, S, E, W) for each testing trial and given 60 seconds to navigate to the platform. If the rat did not find the platform in the allotted time, it was led to the platform. Rats remained on the platform for 15 seconds before being placed back into their heated testing cage. The maze was swept for odor cues before the next rat was tested

on the maze, as all rats completed the same trial before the next trial began; in this fashion, inter-trial-intervals were about 8-10 minutes. Animals were tested for 4 trials per day for 5 days. On the 5th testing day, an additional 5th trial was added as a probe trial, where the platform was removed, and all rats were given 60 seconds to swim in the maze to assess spatial localization to the platform.

MWM performance was assessed via swim distance (cm) and swim latency (sec) to the platform. These analyses were conducted by recording testing trials on a computer equipped with Ethovision (Noldus Instruments, Wageningen, The Netherlands). Probe trial performance was assessed as the proportion of distance travelled in the target quadrant (NE), which contained the platform during baseline testing, versus that of the opposite quadrant (SW).

VP. To assess the rats' ability to complete the necessary elements of a water-escape task, including visual and motor acuity, rats were evaluated on the VP task using established protocols (Bimonte-Nelson, 2015c) after completion of the MWM task. The VP apparatus consisted of a large rectangular pool filled with 18-20C water free of paint and surrounded by a curtain to obscure potential extra-maze cues. A platform whose base was 2-3cm above the surface of the water, was placed into one of three semi-random locations along the back of the apparatus. Similar to WRAM and MWM, rats were placed into individual testing cages in testing squads of 8. A trial began when a rat was placed into the maze at the starting location and given 90 seconds to locate the visible platform. When the platform was located, the rat was given 15 seconds on the platform before being placed into their heated testing cage. The maze was then swept for odor cues before

the next animal began its trial. There was an inter-trial interval of 8-10 minutes, as all rats in a squad were tested on a trial before moving to the next trial. Performance on the VP task was assessed via latency (sec) to the platform.

Euthanasia and Tissue Collection

Two days after completion of the behavioral battery, all rats were euthanized at approximately 16-17 months of age to collect tissues for further processing. The inhalant isoflurane was used for euthanasia before blood was collected via cardiocentesis. Brains were collected and dissected into various brain regions for further processing. Ovaries (R&L) and uteri were also collected, trimmed of excess fat, and weighed (g) to determine the impact of VCD on reproductive physiology. Ovaries were then placed into vials with 10% buffered formalin; forty-eight hours later, vials were changed over to 70% ethanol until further analysis.

Ovarian Follicle Counting

One ovary from each rat fixed at euthanasia was randomly selected for processing and follicle quantification, which was completed by FYXX Foundation (Flagstaff, AZ, USA). The oviduct was first separated from the ovary before processing using a Leica TP1020 tissue processor. Each ovary was then embedded in paraffin and sectioned using a semi-automatic rotary microtome at 5um thick. Every 10^{th} section was mounted to slides before being stained with Gills 2 hematoxylin and counterstained with eosin Y-phloxine B then manually cover-slipped. Slides were scanned with the 3D HisTech DESK Scanner, where every 20^{th} section was analyzed for the following viable follicle types using established criteria (Haas et al., 2007): primordial, primary, secondary, and

antral follicles. Follicles were counted as viable if they contained no apparent signs of atresia. Primordial cells were classified by a single layer of squamous granulosa cells around an oocyte. These cells were estimated by the formula $N_t = (N_0 \times S_t \times t_s)/(S_0 \times d_0)$, with N_t represents the total follicle estimate, N_0 represents the number of follicles observed in the ovary, S_t represents the total number of sections, t_s represents the thickness of each section in um, S_0 represents the total number of sections observed, and d_0 represents the mean diameter of the cell nucleus (Gougeon & Chainy, 1987). Primary follicles differed in that they included a single layer of cuboidal granulosa cells, where secondary follicles contained several layers of granulosa cells. Antral follicles had two or more granulosa cell layers and a fluid-filled antral space within the follicle. Counts for primary, secondary, and antral follicles were completed by simple sum. Finally, corpora lutea were counted as they appeared across the entire sample.

Statistical Analyses

StatView software was used for all statical analyses. Planned comparisons were carried out for analyses, with the exception of the WRAM delay, the MWM probe, and the VP task analyses, to address direct questions relating to these different VCD administration protocols. Planned comparisons were as follows: IP-Vehicle-DMSO/SAL vs. IP-VCD-DMSO/SAL, IP-Vehicle-Oil vs. IP-VCD-Oil, and IP-Vehicle-Oil vs. SubQ-VCD-Oil. All statistical analyses were two-tailed with the alpha level set to 0.05. Results were considered marginal when the *p* value ranged from 0.05 to 0.10.

WRAM baseline testing. WRAM data were blocked into the Acquisition Phase (Days 2-7) and Asymptotic Phase (Days 8-12) prior to statistical analysis, based upon the

presentation of the learning curves (see Figure 30) and upon prior work in our laboratory demonstrating differing effects across task learning and task maintenance (Braden et al., 2017; Koebele et al., 2020). For these testing blocks, repeated measures ANOVAs were conducted in the set of comparisons mentioned above, where Treatment was the independent variable, Errors (WMC, WMI, or RM) served as the dependent variable, and Days within Trials served as the repeated measures.

WRAM delayed memory retention. WRAM delay data were analyzed as a repeated measures ANOVA for each treatment group individually, where the dependent variable was WMC errors, and performance on Trial 3 of Days 12 (Baseline) and Day 13 (Delay) served as the repeated measures.

MWM. Data from the MWM were analyzed using the planned comparisons above in a series of repeated measures ANOVAs, with Treatment as the independent variable, Distance (cm) to the Platform as the dependent variable, and Days within Trials as the repeated measures. Probe trial data were analyzed for each treatment group individually as an ANOVA, with percent swim distance in the Target (NE) and Opposite (SW) Quadrant served as the dependent variable.

VP. To assess potential differences in ability to solve a water-escape task by treatment group, performance on the VP task was analyzed using an omnibus repeated measures ANOVA, with Treatment as the independent variable, Latency (s) to the Platform as the dependent variable, and Trials as the repeated measures.

Body weights and uterine/ovary weights. Body weights, as well as uterine and ovary weights, collected at sacrifice were assessed using the same set of planned

comparisons in a series of ANOVA, where Treatment served as the independent variable, and Weight (g) served as the dependent variable. For ovary weights, weight of the R and L ovary were summed before statistical analysis.

Ovary follicle counting. Ovarian follicle counts were analyzed in sets of planned comparisons as ANOVA individually for primordial, primary, secondary, and antral follicles as well as corpora lutea, with follicle counts as the dependent measure and Treatment as the independent measure.

Results

Vaginal Cytology and Body Weights

Assessments of vaginal cytology revealed that rats treated with Vehicle displayed typical smears for the middle-aged timepoint, with cycling that was somewhat irregular and lengthened in the estrus and diestrus phases (Goldman et al., 2007). For rats treated with all three VCD protocols, smears oscillated mostly between diestrus and estrus, with little to no emergence of proestrus-like smears, suggesting disrupted estrous cyclicity previously reported in the literature (Koebele, Mennenga, et al., 2020).

Analysis of body weights at sacrifice demonstrated no differences between treatment groups (data not shown), suggesting that VCD, regardless of treatment protocol, did not yield long-term changes in body weight relative to Vehicle controls.

Behavioral Battery

WRAM – baseline testing. Analyses of the Acquisition Phase revealed a Trial by Treatment interaction for the IP-Vehicle-Oil vs. SubQ-VCD-Oil planned comparison for WMI errors [$F_{(3,51)} = 5.23$, $p < 0.01$] (Figure 31A). Analyses of each subsequent trial

found that on Trial 3, the moderate working memory load trial, there was an effect of Treatment [$F_{(1,17)} = 9.77$, $p < 0.01$], where rats treated subcutaneously with VCD made more WMI errors than the sesame oil Vehicle control rats (Figure 31B). There were no other effects of Treatment, or Trial by treatment interactions, for any other error type across any planned comparison during the Acquisition Phase of the WRAM.

For the Asymptotic Phase, analyses of WMC errors demonstrated a marginal effect of Treatment for the IP-Vehicle-DMSO/SAL vs. IP-VCD-DMSO/SAL planned comparison [$F_{(1,18)} = 4.16$, $p < 0.10$], where rats treated with VCD tended to make more working memory errors than the DMSO/Saline Vehicle rats (Figure 32A). For the IP-Vehicle-Oil vs. IP-VCD-Oil planned comparison, there was an effect of Treatment [$F_{(1,17)} = 8.79$, $p < 0.01$], with IP-VCD-Oil-treated rats making more errors than rats in the Vehicle-Oil group (Figure 32A). Likewise, the IP-Vehicle-Oil vs. SubQ-VCD-Oil planned comparison revealed a marginal Treatment effect for WMC errors [$F_{(1,17)} = 3.65$, $p < 0.10$], with subcutaneously VCD-Oil-treated rats making marginally more errors than the sesame oil-vehicle-treated rats (Figure 32A). Additionally, this planned comparison revealed a marginal Trial x Treatment interaction [$F_{(2,34)} = 2.93$, $p < 0.10$] (Figure 32B); analyses of each trial demonstrated that for Trial 2, or the low working memory load trial, there was an effect of Treatment [$F_{(1,17)} = 5.17$, $p < 0.05$], where VCD-induced impairment was robust when administered subcutaneously via sesame oil as compared to the IP-Vehicle-Oil group (Figure 32C). For Trial 4, the high working memory load trial, there was a marginal Treatment effect for this planned comparison [$F_{(1,17)} = 3.56$, $p < 0.10$], where SubQ-VCD-treated rats tended to make more working memory errors than their sesame oil Vehicle counterparts (data not shown).

Analyses of WMI errors during the Asymptotic Phase of the WRAM revealed a Trial x Treatment interaction for the IP-Vehicle-Oil vs. IP-VCD-Oil planned comparison [$F_{(3,51)} = 3.10$, $p < 0.05$] (Figure 33A); analyses of each trial individually found that for Trial 4, the high working memory load trial, there was a marginal effect of Treatment [$F_{(1,17)} = 3.50$, $p < 0.10$], where rats treated via VCD via IP injections of sesame oil tended to make more errors than their sesame oil Vehicle counterparts (Figure 33B). No other Treatment effects were found for any error type across the Asymptotic Phase of the WRAM.

WRAM – delayed memory retention. Analyses following the implementation of a delay between Trials 2 and 3 in the WRAM task did not reveal a Delay effect for the IP-Vehicle-DMSO/SAL, IP-VCD-DMSO/SAL, IP-Vehicle-Oil, or IP-VCD-Oil groups (Figure 34A-D). However, a significant effect of Delay was found for the SubQ-VCD-Oil group [$F_{(1,8)} = 9.66$, $p < 0.05$], with more WMC errors made after a 6-hour delay was implemented on the last WRAM testing day (Figure 34E).

MWM. Analyses of performance on the MWM across all baseline trials found effects of Day [IP-Vehicle-DMSO/SAL vs. IP-VCD-DMSO/SAL: $F_{(4,72)} = 17.91$, $p < 0.0001$, IP-Vehicle-Oil vs. IP-VCD-Oil: $F_{(4,68)} = 23.28$, $p < 0.0001$, IP-Vehicle-Oil vs. SubQ-VCD-Oil: $F_{(4,68)} = 18.27$, $p < 0.0001$] and effects of Trial [IP-Vehicle-DMSO/SAL vs. IP-VCD-DMSO/SAL: $F_{(3,54)} = 11.15$, $p < 0.0001$, IP-Vehicle-Oil vs. IP-VCD-Oil: $F_{(3,51)} = 8.00$, $p < 0.001$, IP-Vehicle-Oil vs. SubQ-VCD-Oil: $F_{(3,51)} = 12.97$, $p < 0.0001$] for each of the planned comparisons (learning curves displayed in Figure 35A), with

swim distance decreasing across testing days and across trials. No Treatment effects were found for analyses of swim distance to the platform across any planned comparison.

Probe trial analyses of each treatment group individually demonstrated significant effects of Quadrant [IP-Vehicle-DMSO/SAL: $F_{(1,9)} = 35.23$, $p < 0.001$, IP-VCD-DMSO/SAL: $F_{(1,9)} = 52.95$, $p < 0.0001$, IP-Vehicle-Oil: $F_{(1,9)} = 7.84$, $p < 0.05$, IP-VCD-Oil: $F_{(1,8)} = 9.25$, $p < 0.05$, SubQ-VCD-Oil: $F_{(1,8)} = 9.06$, $p < 0.05$], where rats in each treatment group swam a greater proportion of swim distance across the probe trial in the Target Quadrant (NE) as opposed to the Opposite Quadrant (SW), demonstrating spatial localization to the platform (Figure 35B).

VP. To assess the ability to perform the elements necessary to complete a swim navigation task, as well as to assess putative treatment group differences in this control measure, VP data were assessed across all groups. Analyses revealed a significant effect of Trial [$F_{(5,215)} = 5.55$, $p < 0.0001$], with latency to the platform generally decreasing from Trial 1 to Trial 6 (Figure 36). By Trial 6, no rat needed longer than 19 seconds to reach the visible platform, with an overall average of 3.37 seconds in latency to the platform by the final trial. No effects of Treatment or Treatment by Trial interactions were present, indicating that groups did not differ in their ability to perform this control task.

Uterine and Ovary Weights

Assessments of uterine weight at sacrifice revealed effects of Treatment across all planned comparisons [IP-Vehicle-DMSO/SAL vs. IP-VCD-DMSO/SAL: $F_{(1,18)} = 25.75$, $p < 0.0001$, IP-Vehicle-Oil vs. IP-VCD-Oil: $F_{(1,17)} = 11.79$, $p < 0.01$, IP-Vehicle-Oil vs.

SubQ-VCD-Oil: $F_{(1,17)} = 4.15$, $p < 0.10$], where VCD treatment resulted in some degree of uterine atrophy as compared to each Vehicle control (Figure 37A). Likewise, there was a significant effect of Treatment across all planned comparisons for summed ovary weights [IP-Vehicle-DMSO/SAL vs. IP-VCD-DMSO/SAL: $F_{(1,18)} = 140.96$, $p < 0.0001$, IP-Vehicle-Oil vs. IP-VCD-Oil: $F_{(1,17)} = 17.92$, $p < 0.001$, IP-Vehicle-Oil vs. SubQ-VCD-Oil: $F_{(1,17)} = 36.77$, $p < 0.0001$], with VCD-treated rats having smaller ovaries than those of their respective Vehicle controls (Figure 37B), a result of follicular depletion that has previously been observed in rodents with VCD treatment (Mayer et al., 2002, 2004).

Ovary Follicle Counting

Analyses of ovarian follicles found that the various VCD-treated groups had distinct profiles from those of Vehicle-treated rats that were fairly consistent across VCD treatment methodology. For primordial follicles, there was a Treatment effect for each set of planned comparisons [IP-Vehicle-DMSO/SAL vs. IP-VCD-DMSO/SAL: $F_{(1,18)} = 111.04$, $p < 0.0001$, IP-Vehicle-Oil vs. IP-VCD-Oil: $F_{(1,17)} = 44.71$, $p < 0.0001$, IP-Vehicle-Oil vs. SubQ-VCD-Oil: $F_{(1,17)} = 46.26$, $p < 0.0001$], with reductions in follicle counts in VCD-treated rats relative to their respective Vehicle controls (Figure 38A). Primary follicles also had a pattern of Treatment effects [IP-Vehicle-DMSO/SAL vs. IP-VCD-DMSO/SAL: $F_{(1,18)} = 19.89$, $p < 0.001$, IP-Vehicle-Oil vs. IP-VCD-Oil: $F_{(1,17)} = 33.60$, $p < 0.0001$, IP-Vehicle-Oil vs. SubQ-VCD-Oil: $F_{(1,17)} = 14.37$, $p < 0.01$], but VCD-treated rats here had elevated counts as compared to their corresponding Vehicle-treated rats (Figure 38B). Secondary follicle counts had significant effects of Treatment [IP-Vehicle-DMSO/SAL vs. IP-VCD-DMSO/SAL: $F_{(1,18)} = 180.75$, $p < 0.0001$, IP-Vehicle-Oil vs. IP-VCD-Oil: $F_{(1,17)} = 49.54$, $p < 0.0001$, IP-Vehicle-Oil vs. SubQ-VCD-

Oil: $F_{(1,17)} = 48.30$, $p < 0.0001$], where VCD groups had reduced counts compared to corresponding Vehicle groups (Figure 38C). Likewise, Treatment effects were found for antral follicle counts [IP-Vehicle-DMSO/SAL vs. IP-VCD-DMSO/SAL: $F_{(1,18)} = 69.44$, $p < 0.0001$, IP-Vehicle-Oil vs. IP-VCD-Oil: $F_{(1,17)} = 10.74$, $p < 0.01$, IP-Vehicle-Oil vs. SubQ-VCD-Oil: $F_{(1,17)} = 8.07$, $p < 0.05$] (Figure 38D) and corpora lutea counts [IP-Vehicle-DMSO/SAL vs. IP-VCD-DMSO/SAL: $F_{(1,18)} = 301.32$, $p < 0.0001$, IP-Vehicle-Oil vs. IP-VCD-Oil: $F_{(1,17)} = 121.56$, $p < 0.0001$, IP-Vehicle-Oil vs. SubQ-VCD-Oil: $F_{(1,17)} = 44.59$, $p < 0.0001$] (Figure 38E), with reduced counts in VCD rats relative to Vehicle rats, regardless of methodology. Overall, results demonstrate ovarian follicular depletion for all VCD groups, regardless of methodology.

Discussion

The current project sought to characterize the efficacy of three different VCD administration paradigms used throughout the literature as a preclinical rodent model of the menopause transition. Whether VCD was administered as a series of intraperitoneal or subcutaneous injections, across two different vehicles, there were consistent patterns of learning and memory deficits, as well as similar ovarian follicular profiles that indicate each method could serve as an efficacious model of the menopause transition. The direct comparison of administration protocols done here has allowed for better understanding of the challenges in using each protocol, where greater transparency is critical within the literature to identify the best method of VCD administration for a given research question.

Overall, all animals treated with either VCD or Vehicle through either route of administration demonstrated overall learning on the WRAM and MWM, indicating that VCD did not completely impair spatial navigation. Additionally, groups showed comparable performance on the VP thereby demonstrating that the various methods of VCD administration did not limit the ability or motivation to perform such water-escape tasks, as has been previously shown in our laboratory (Acosta et al., 2010; Acosta, Mayer, Talboom, Tsang, et al., 2009; Koebele, Mennenga, et al., 2020). Interestingly, all three VCD administration protocols showed indications of working memory deficits on the WRAM, but the patterns of these deficits differed. Specifically, rats in the IP-VCD-DMSO/SAL group, the treatment group most often used in our laboratory, showed only marginal impairments in task maintenance for WMC errors on the WRAM. Prior work in our laboratory has found that, while VCD can augment Age effects in young and middle age treated rats, young rats appear to display more robust working memory deficits with VCD as compared to their Vehicle counterparts (Koebele et al., 2017). Given that age-sensitive behavioral effects of VCD have been reported, it is critical that future work establish behavioral outcomes of VCD treatment in young rats with an Oil vehicle, as this work has been less characterized. In the current experiment, both IP and SubQ injections of VCD in an Oil delivery vehicle resulted in working memory deficits as compared to oil vehicle controls. Where rats in the IP-VCD-Oil group demonstrated significant impairment as evaluated in WMC errors during WRAM task maintenance, rats in the SubQ-VCD-Oil group demonstrated impairments across both task learning and task maintenance, as well as delay-induced impairments, suggesting greater overall impairment with this treatment paradigm. Such nuanced differences in behavioral

outcomes with VCD could be leveraged to ask more nuanced questions concerning learning and memory effects observed across the menopause transition. For example, while subjective reports of "forgetfulness" are common across the menopause transition, some researchers have found objective impairments for those in this transition that reflect memory deficits (Schaafsma et al., 2010), and others report effects concerning learning deficits (Greendale et al., 2011). Preclinical models such as the VCD model may therefore be leveraged to assess these learning or memory deficits separately to maximize translational to the specific clinical population.

Like the behavioral outcomes observed in the current experiment, uterine and ovarian analyses demonstrate overall consistent outcomes for all three methods of VCD administration. In the current experiment, all three VCD administration paradigms resulted in reductions in uterine weight as compared to their respective Vehicle controls. While these findings are in contrast with those from our laboratory, finding no difference in uterine weight (Acosta, Mayer, Talboom, Tsang, et al., 2009; Koebele et al., 2017), other laboratories report mixed findings concerning uterine weight changes as a result of VCD administration (no change: Frye et al., 2012; Thompson et al., 2002; VCD-induced decrease: Muhammad et al., 2009). Additionally, while reductions in primordial, secondary, and antral follicles, as well as corpora lutea, follow prior literature evaluating the effects of VCD on rodent ovarian morphology (Acosta, Mayer, Talboom, Tsang, et al., 2009; Haas et al., 2007; Koebele et al., 2017; Koebele, Mennenga, et al., 2020; Mayer et al., 2004; Reis et al., 2014), the significant increase in primary follicle counts with VCD treatment observed across all three methodologies represents a distinct pattern from that of prior literature, showing decreased primary follicle counts in rats following VCD

treatment in rats (Koebele et al., 2017; Koebele, Mennenga, et al., 2020). Such discrepancies between prior work and the current experiment may be, in part, due to the use of different rat strains in VCD research. Indeed, recent work in our laboratory has reported different ovarian follicular outcomes with VCD across the Fischer 344 NIH and CDF rat strains (Koebele, Hiroi, et al., 2021; Koebele, Mennenga, et al., 2020). In NIH-strain rats, primary follicles decreased with VCD administration, where with CDF-strain rats, VCD induced an increase in primary follicle counts, consistent with findings from the current experiment. The two strains differ by six single-nucleotide polymorphisms (SNPs) (National Institute on Aging, n.d.), but the characterization of putative phenotypic changes as a result of these SNPs is incomplete. It is possible that this strain difference also is responsible for these differences in uterine outcomes with VCD. Future work should further explore why these genetic strain differences might modulate VCD outcomes, and take care when using VCD as a model of transitional menopause to note the potential for strain differences outside of the reproductive tract, including hormone levels and behavioral profiles. However, the overall picture of ovarian outcomes from the current experiment indicates a state of ovarian failure across each of the three VCD protocols, given the significant reductions in primordial, secondary, and antral follicles, as well as corpora lutea.

Beyond the consistencies surrounding the behavioral and physiological profiles of these three VCD administration paradigms, it would be remiss to not acknowledge their differing methodological challenges. While some prior work has listed the risks and challenges associated with different forms of VCD administration in female rats, including peritonitis, skin lesions, and weight loss (Koebele et al., 2017; Muhammad et

al., 2009), most work does not report the specifics of methodological issues surrounding VCD administration (Acosta et al., 2010; Keck et al., 2007; Reis et al., 2014). The current experiment outlines the importance of this clarity, as different routes of VCD administration and delivery vehicles resulted in distinct challenges in protocol implementation and outcomes, even with all three doses of VCD and schedules of administration being identical. Rats in the SubQ-VCD-Oil group developed skin lesions that resolved shortly after injections, but complicated injections with the buildup of scar tissue; while not life-threatening, these injections were clearly caused discomfort for the rats. However, SubQ injections resulted in less weight loss overall, and fewer incidences of missed injections. When comparing the IP administration paradigms used in the current experiment, while neither resulted in skin lesions, both the Oil and DMSO/Saline injections resulted in significant weight loss and missed injection days, prolonging the administration window. It is important that researchers are made aware of these differential challenges to optimize experimental parameters.

Overall, the current experiment demonstrates that use of IP or SubQ administration of VCD, delivered in Oil or DMSO/Saline vehicle, results in a pattern of cognitive deficits and ovarian follicular depletion that are consistent with claims of this preclinical model of the menopause transition. Importantly, the greatest differences observed between these protocols came from the challenges surrounding their implementation. Where IP injections resulted in weight loss that delayed injections, SubQ injections resulted in skin lesions and appeared to cause additional discomfort. The proper reporting of research methods is crucial to performing quality, replicable science. Greater care must be taken within our field to make knowledge of these challenges more

readily available to researchers that are interested in questions surrounding menopause. This is particularly relevant as institutions continue to encourage researchers to include both sexes in preclinical animal work (Clayton & Collins, 2014; L. R. Miller et al., 2017) and engage in interdisciplinary and collaborative research (Bimonte-Nelson et al., 2021). Those looking to use VCD as a translational model must consider the variants of the VCD protocols in the context of the practical strengths and weaknesses, and select a VCD administration protocol that is optimized for their research question and experimental timeline. Finally, new protocols for VCD administration should undergo a similar level of rigorous evaluation to that of the experiment above, including analysis of behavioral and physiological outcomes, to better character the limitations of this preclinical model of transitional menopause.

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CHAPTER 6

The data presented in this chapter are currently unpublished observations.

AN ASSESSMENT OF FOLLICULAR DEPLETION PRIOR TO HYSTERECTOMY IN THE FEMALE RAT: IMPACTS ON LEARNING AND MEMORY OUTCOMES AND ANXIETY-LIKE BEHAVIORS

Contribution: I was the graduate student principal investigator for this experiment under the mentorship of Dr. Heather Bimonte-Nelson.

ABSTRACT

Hysterectomy, or surgical removal of the uterus, is a gynecological surgical procedure that has only recently been acknowledged as a critical variable when understanding the role of menopause etiology in driving long-term health outcomes. Some research has found that hysterectomy increases risk of dementia, with an earlier age at surgery associated with higher risk. It is unclear whether such age-related differences are attributable to chronological age or menopause status. Where this can be difficult to evaluate clinically, use of preclinical rodent models can provide further context by separating these two variables. The current study utilized rodent models of hysterectomy and induced experimental transitional menopause via 4-vinylcyclohexene diepoxide (VCD) to assess whether cognitive outcomes following hysterectomy are altered if preceded by ovarian follicular depletion. Rats arrived to the laboratory at 2-3 months of age before treatment of Vehicle (50%/50% DMSO/Saline) or VCD (160 mg/kg) was initiated. After a 90-day waiting period to achieve follicular depletion, rats received either Sham or Hysterectomy surgery. Six weeks following surgery, rats were tested on a behavioral battery that tested spatial working and reference memory via the water radial-arm maze (WRAM) and Morris water maze, as well as locomotor and anxiety-like behavior via the Open Field Test. Neither VCD nor Hysterectomy manipulations impaired cognition during the initial learning portion of the WRAM task in Vehicle-treated rats. However, when these reproductive experimental manipulations were combined, there were some behavioral impairments. Indeed, rats that underwent Hysterectomy plus VCD showed a marginal learning deficit on the WRAM as well as a

significant delayed memory retention deficit. Since any group with Hysterectomy demonstrated this delayed memory retention impairment, extended temporal parameters of memory may be particularly sensitive to Hysterectomy. During the mid-learning phase of the WRAM, Hysterectomy facilitated working memory performance. Analyses of ovary weights revealed that VCD, regardless of uterine status, resulted in decreased weights at euthanasia. Further work should explore whether unique interactions between Hysterectomy surgery and VCD alter ovarian morphology, as well as the role of ovarian failure subsequent to Hysterectomy surgery in altering cognitive profiles.

Introduction

Hysterectomy, or surgical removal of the uterus, is the second most common gynecological surgical procedure (Carlson et al., 1993). Hysterectomy surgery can occur at any point in a woman's lifespan; while more than half of all surgeries are performed before the average age at menopause, many such surgeries are performed either during the menopausal transition or early in postmenopausal life (Wright et al., 2013). Indeed, the years preceding menopause are the most common to undergo hysterectomy (Merrill, 2008). That hysterectomy surgery is performed across vastly different reproductive life stages can make for complications in studying its impacts on long-term health outcomes in women. However, there are some indications that hysterectomy is associated with an increased risk for diseases such as dementia, cardiovascular disease, and depression (Howard et al., 2005; Laughlin-Tommaso et al., 2020; Phung et al., 2010; Rocca et al., 2012). Notably, some of these risks are dependent upon age; clinical work has shown that younger age at hysterectomy confers a greater risk of dementia (Phung et al., 2010). Rather than age, these effects may be dependent upon menopause stage prior to surgery. It is possible that hysterectomy impacts some degree of ovarian functioning to increase such disease risk, but only while HPG axis functioning is intact. There is some clinical literature indicating that ovarian morphology and hormone secretions may be impacted by hysterectomy surgery (Laughlin et al., 2000; Moorman et al., 2011; Nahás et al., 2003; Souza et al., 1986), while others suggest either temporary changes (Gökgözoglu et al., 2014) or no such change in the ovaries post-hysterectomy (Chalmers et al., 2002; Simpson et al., 2005). Such observations are critical for optimizing health outcomes, but

addressing the putative mechanism behind such risks is challenging when age and reproductive stage cannot easily be dissociated in clinical research.

While clinical research may be limited in its further investigation of such questions surrounding ovarian status and hysterectomy-induced outcomes, preclinical rodent models can be of great use in this domain of research. Indeed, the female rat does not undergo age-dependent ovarian follicular depletion (Koebele & Bimonte-Nelson, 2016). The 4-vinylcyclohexene diepoxide (VCD) model of transitional menopause, which induces ovarian follicular depletion and yields a hormone profile more comparable to that of menopause through a series of injections, may be used to parse out the impacts of age and menopause stage on associated outcomes (Bimonte-Nelson et al., 2021). Preclinical investigations of age versus menopause stage have been carried out for VCD combined with ovariectomy, or surgical removal of the ovaries in the rodent. Such work has demonstrated distinct cognitive outcomes if ovaries are removed with or without prior VCD treatment (Acosta, Mayer, Talboom, Tsang, et al., 2009). To aid in the investigation, a new rodent model of hysterectomy has been recently developed in our laboratory, which demonstrates cognitive deficits that persist at long intervals post-surgery (Koebele, 2019; Koebele et al., 2019). However, these investigations have only been performed for rats in young adulthood, where hormone levels and ovarian functioning at this age are more comparable to that of pre-menopausal women. Until now, there has been no assessment of hysterectomy with follicular depletion to fully address interactions with menopause.

The current study utilized our laboratory's novel rodent model of hysterectomy as well as our VCD model of transitional menopause to assess putative changes in cognitive

profiles following hysterectomy if preceded by ovarian follicular depletion. Young female rats were administered either VCD or Vehicle injections to induce ovarian follicular depletion across a period of 90 days. After this time, rats underwent either Sham of Hysterectomy surgery before being evaluated on a behavioral battery that assessed spatial working and reference memory, as well as anxiety-like and locomotor behavior. Additional analyses were performed at euthanasia to detect gross uterine and ovarian changes. This work provides greater context into the role that ovarian follicular status may play in altering hysterectomy's observed cognitive deficits independently from chronological aging.

Methods

Subjects

Sexually-inexperienced female Fischer 344-CDF rats, aged 3-4 months (N=48) and free of cataracts, arrived at the Arizona State University (ASU) vivarium in the Department of Psychology from the National Institute on Aging (NIA) Colony (Charles River Laboratories; Raleigh, NC). All rats were pair-housed upon arrival and kept in a room with controlled temperature and humidity that kept a 12-hour light cycle (lights on at 7:00am). Food and water were provided *ad libitum*. Rats were allowed to acclimate at the facility for 2-4 weeks before beginning experimental intervention. All experimental interventions followed guidelines provided by the NIA, and were approved by the Institutional Animal Care and Use Committee (IACUC) at ASU. Figure 39 demonstrates the experimental timeline, behavioral battery, and treatment groups.

VCD Administration

VCD administration followed an established protocol from prior work in our laboratory (Acosta, Mayer, Talboom, Tsang, et al., 2009; Koebele et al., 2017). For rats receiving VCD treatment (n=24), 160mg/kg VCD (FYXX, Flagstaff, AZ), dissolved in a 50%/50% solution of dimethyl sulfoxide (DMSO) and saline, was administered via intraperitoneal (IP) injection daily based upon body weight. Injections for all Vehicle-treated rats (n=24) were kept at a dose of 0.5ml DMSO/Saline, regardless of individual body weight. VCD or Vehicle injections were administered daily on a Monday/Tuesday/Thursday/Friday injection schedule across a series of 15 total injections. If a rat fell below 90% of their starting body weight at any point during this period of time, injections were halted until the rat gained sufficient weight to be able to continue administration. Two subjects (both receiving VCD) died during VCD injections, and veterinarian-assisted necropsy indicated they were likely due to complications related to these IP injections.

Body Weights and Vaginal Cytology

Following the completion of VCD or Vehicle injections, weekly body weights were performed to track potential physiological changes with follicular depletion and additional experimental manipulations. These body weight assessments were carried out each week until euthanasia. Additionally, to evaluate follicular depletion and putative modulation with Hysterectomy, vaginal smears were performed using established protocols (Goldman et al., 2007; Koebele et al., 2019) at two different points across the experimental timeline: (1) 88 days after the first VCD injection, but before Sham or Hysterectomy surgeries, and (2) 20 days after Sham/Hysterectomy surgeries (120 days after the first VCD injection). Both sets of smears were performed across 8 consecutive

days, where a small cotton-tipped applicator soaked in sterile saline was inserted into the vaginal opening; subsequent sample was viewed on a slide under a light microscope (Fisher Scientific Micromaster). Vaginal smears were classified as either estrus, metestrus, diestrus, and proestrus. Estrus was characterized by predominantly cornified cells, and metestrus was characterized by the presence of cornified cells, round cells, leukocytes, and potential needle-like cells. Diestrus was characterized by the predominant presence of leukocytes, with or without the presence of cornified cells. Proestrus was characterized by the presence of clustered round epithelial cells, with some occasional cornified cells.

Hysterectomy Surgery

At 100 days after the first VCD or Vehicle injection, rats underwent either Sham or Hysterectomy ("Hyst") surgery following established protocols (Koebele et al., 2019), resulting in a total of 4 experimental groups: Vehicle-Sham, VCD-Sham, Vehicle-Hyst, and VCD-Hyst. Surgical procedure involved anesthetization via inhaled isoflurane, where 1.0mg/kg meloxicam and 1.2 mg/kg buprenorphine SLR were administered for pain management to all rats. Additionally, rats were given 5.0 ml lactated ringers solution to ensure postsurgical hydration. Both Sham and Hysterectomy surgeries involved a ventral midline incision through the skin and peritoneum. Hysterectomy surgeries additionally required the uterine horn to be ligated and cut below the left and right ovary, preserving the oviduct, before being separated from the attached abdominal fat down the length of the horn. When the utero-cervical junction was reached, the uterus was ligated and removed from the body cavity above the cervix. All surgeries had muscle sutured closed using dissolvable Vicryl suture, where bupivacaine was topically applied to the muscle

before skin closure with surgical staples for further pain management. In recovery, rats were monitored in individually housed cages under a heat lamp; all rats remained single-housed for 7 days before being re-pair housed with their original cage mate. Surgical staples were removed beginning 10 days following surgery, when skin looked well healed. Five rats died from surgical complications (1 from the Vehicle-Sham group, 3 from the VCD-Hyst group, and 1 from the VCD-Sham group). The final size of each group before behavior testing was as follows: Vehicle-Sham (n=11), VCD-Sham (n=9), Vehicle-Hyst (n=12), and VCD-Hyst (n=9).

Behavioral Battery

142 days after the first VCD injection, or six weeks following surgeries, all rats were tested on a behavioral battery that evaluated spatial working and memory performance, as well as anxiety-like and locomotor behavior. One rat in the VCD-Sham group was excluded from all analyses except those from the WRAM due to death during behavioral testing that was unrelated to experimental manipulation.

Water radial-arm maze. Rats were first evaluated on the win-shift version of the water radial-arm maze (WRAM), used to assess spatial working and reference memory performance (Bimonte-Nelson, 2015d; Bimonte-Nelson et al., 2015). The WRAM apparatus, which has eight arms and is filled with 18-20°C water dyed black with non-toxic paint, is situated in a room with robust extra-maze cues to aid in spatial navigation. Four out of the eight arms contain platforms that are fully submerged under the water's surface, which the animals must locate to solve this maze task. Platform locations are randomly assigned to individual rats, but are counter-balanced across treatment groups and testing rooms.

To start a trial, a rat is dropped off from the starting arm; the rat is then given three minutes to locate a platform. If a platform is not found within the allotted trial time, the rat is led to the platform. Once on the platform, the rat must remain there for a total of 15 seconds to spatially localize, before being placed by the experimenter into their individual heated testing cage for 30 seconds. This 30-second inter-trial-interval (ITI) provides time for the experimenter to remove the recently-found platform and sweep the maze with a net to distribute any odor cues. After the ITI is complete, the rat is once again dropped off from the starting arm, and is given 3 minutes to find one of the remaining platforms; in this fashion, 4 trials are performed per day for each subject, as they are required to locate each of the submerged platforms. Given that the rats need to remember not to visit arms where platforms had previously been located for that day, the working memory load increases across trials. Testing continued for a total of 12 days before, on the 13th day, a 6-hour delay was implemented between Trials 2 and 3 to assess delayed memory retention.

Performance on the WRAM was quantified as errors, or entries into an arm that did not contain a platform, for each trial. An arm entry was recorded if the rat passed 11cm into the arm, marked on the outside of the maze. Errors were further broken down into 3 subtypes that represent differential working and reference memory demands: a working memory correct (WMC) error, quantified by an entry into a previously platformed arm; a reference memory (RM) error, or the first entry into an unplatformed arm within a testing day; and a working memory incorrect (WMI) error, quantified as a repeat entry into an unplatformed arm.

Morris water maze. Following the completion of WRAM testing, rats were given one day for rest before beginning the Morris Water Maze (MWM) task, evaluating spatial reference memory (Bimonte-Nelson, 2015b; Bimonte-Nelson et al., 2015). The MWM apparatus is a large (188cm in diameter) circular tub, which is filled with 18-20°C water made opaque with black non-toxic tempera paint. In the testing room, there are robust extra-maze cues to aid in spatial navigation. An escape platform is submerged underneath the northeast (NE) quadrant of the maze, and it remains in the NE throughout all testing trials on the task.

During testing, a rat is dropped off at one of a series of starting locations (North, East, South, West) and is given 60 seconds to locate the platform. If the rat does not locate the platform before the 60 second trial time, the experimenter guides the rat to the platform. Once on the platform, the rat remains there for 15 seconds to spatially localize before being placed into its heated testing cage for a 10-minute ITI. In this fashion, each rat receives 4 testing trials per day for 5 days. On the 5^{th} day, a final 5^{th} probe trial is conducted, where the platform is removed, and rats swim in the maze for 60 seconds; this probe trial evaluates spatial localization to the platform. Performance on this task was quantified by measuring the latency (s) and distance swam (cm) to the platform using the Ethovision tracking system (Noldus Instruments, Wageningen, The Netherlands).

Visible platform. The following day that MWM testing was completed, rats were evaluated on the visible platform (VP) task, a control task which evaluates the ability to perform the procedural components of a water-escape task (Bimonte-Nelson, 2015c). The VP apparatus is a large rectangular tub filled with 18-20°C clear water that contains a large black platform that extends 4cm above the water's surface. The platform is moved

to three distinct locations within the maze semi-randomly across trials. To eliminate the use of spatial cues, a large curtain surrounded the maze. To perform the task, a rat is dropped off at a designated starting location and given 90 seconds to locate the platform. Once on the platform, the rat remained there for 15 seconds before being placed back into their testing cage for a 10-minute ITI. All rats completed a total of 6 trials; performance was quantified as latency (s) to the platform for each trial.

Open field test. After the VP task, rats were given two days to acclimate before begin evaluated on the Open Field Test (OFT), a task evaluating anxiety-like behavior and locomotion using protocols established in prior work (Hiroi & Neumaier, 2006; Koebele, Hiroi, et al., 2021). The OFT apparatus was a large 100cm by 100cm square black plexiglass box that was sectioned digitally into 25 smaller, equally-sized squares to represent the corners, center, and small center of the arena. Odormute™ was used to clean the maze on the day before testing to eliminate foreign odors. Testing was conducted in the dark, with an infrared light and camera positioned above the maze; the camera was connected to Ethovision tracking system software (Noldus Instruments, Wageningen, The Netherlands). For OFT, rats were placed in their individually-housed testing cages and placed into the behavioral suite 30 minutes before testing to acclimate to their surroundings. During testing, rats were brought in individually and removed from their testing cages to be placed into the box along the center of the North wall to initiate a trial. The experimenter then left the room, and the rat's behavior in the box was recorded for 10 minutes. After the trial had ended, the experimenter re-entered the room and removed the rat from the box; the maze was then cleaned thoroughly with water to spread any remaining odors before the next rat began testing. OFT performance was evaluated

by calculating the time spent (s) in the corners, center, and small center of the arena, as well as the distance traveled (cm) in each of these zones; total distance (cm) was also calculated.

Euthanasia

One week after evaluation on the OFT, rats were euthanized by deeply anesthetizing them with isoflurane and performing cardiocentesis to collect blood from the heart. Body weights were taken at euthanasia to determine the physiological effects of VCD and Hysterectomy. Various brain regions critical to learning and memory processes were also raw dissected at euthanasia and frozen at -70°C for future research using previously established protocols (Braden et al., 2010, 2011) and according to plate designations (Paxinos & Watson, 1998). Ovaries were removed from all subjects, trimmed of fat, and individually weighed to determine effects of VCD and Hysterectomy on the ovaries. The uterus was also removed from each Sham subject and trimmed of excess fat before being weighed to evaluate effects of VCD on the uterus.

Statistical Analyses

All statistical analyses were performed using StatView software. One rat in the VCD-Sham group was excluded from all analyses except those from the WRAM due to death during behavioral testing that was unrelated to experimental manipulation. For all analyses, results were designated marginal if p-values were between 0.10 and 0.05, and significant if $p < 0.05$.

WRAM. Data were analyzed using a series of repeated measures ANOVAs with a 2x2 design, where Injection (VCD vs. Vehicle) and Surgery (Sham vs. Hysterectomy) served as the independent variables, Days and Trials served as the repeated measures, and

WMC, WMI, and RM errors served as the dependent variables. Based upon prior work in our laboratory demonstrating differing behavioral patterns and distinct effects pertaining to hormonal and surgical manipulations corresponding to task acquisition and maintenance (Bimonte-Nelson et al., 2015; Koebele et al., 2019; Mennenga, Koebele, et al., 2015), WRAM data were analyzed within each error type as 3 separate blocks: Days 2-4, the Early Acquisition Phase; Days 5-9, the Late Acquisition Phase; and Days 10-12, the Asymptotic Phase.

For data collected during the WRAM delay, individual treatment groups were evaluated separately for WMC errors using a repeated measures ANOVA, where Errors served as the dependent variable, and performance on Trial 3 of the last baseline day of testing (Day 12) versus performance for the same trial directly following the delay (Day 13) served as the repeated measures.

MWM. MWM data were analyzed using a repeated measures ANOVA with the same 2x2 design, with Injection and Surgery as the independent variables, Days within Trials as the repeated measures, and swim distance (cm) and latency (s) to the platform as the dependent variables. For the probe trial, each individual treatment group was analyzed via a repeated measures ANOVA, where the percent swim distance served as the dependent variable and the Target Quadrant (NE) versus the Opposite Quadrant (SW) served as the repeated measure.

VP. Data collected from the VP task were evaluated using a repeated measures ANOVA with the same 2x2 design, where Injection and Surgery were the independent variables, Trials were the repeated measures, and latency (s) to the platform was the dependent variable.

OFT. OFT performance was analyzed via a series of ANOVA, using the same 2x2 design with Injection and Surgery as the independent variables, and time spent (s) in the corners, center, and small center served as the dependent variables. Additionally, OFT analyses were performed with total distance (cm), and distance in the corners, center, and small center as the dependent variables.

Body weights at euthanasia. Body weights, collected at euthanasia, were analyzed using an ANOVA, where Injection and Surgery as the independent variables and weight (g) served as the dependent variable.

Ovary & uterine weights. Uterine weights, collected from all Sham rats at euthanasia, were analyzed using an ANOVA, with Injection serving as the independent variable and wet weight (g) serving as the dependent variable. Weights of both ovaries (R+L) for all rats were analyzed using an ANOVA, with Injection and Surgery as the independent variables and weight (g) as the dependent variable.

Results

Vaginal Cytology

Inspection of vaginal smears 88-95 days following VCD administration found that, while Vehicle rats displayed a normal 4-5 day estrous cycle, VCD-treated rats displayed irregular smears, mainly alternating between periods of estrus and diestrus, as previously observed (Acosta, Mayer, Talboom, Tsang, et al., 2009; Koebele, Mennenga, et al., 2020). Following Sham or Hysterectomy surgery, injection with VCD or Vehicle dominated the presence of typical or atypical vaginal smears. Specifically, all Vehicle rats displayed typical 4-5 day estrous cycles, as expected following uterine removal (Koebele et al., 2019). Rats treated with VCD displayed disrupted estrous cyclicity,

where most rats exhibited a persistent or prolonged estrus phase, switching briefly to diestrus, as previously demonstrated (Acosta, Mayer, Talboom, Tsang, et al., 2009).

WRAM

Early acquisition phase. During the Early Acquisition Phase (Days 2-4), analyses revealed a marginal Injection x Surgery interaction for WMC errors [$F_{(1,37)}$ = 3.083, $p < 0.10$], indicating a tendency in differing Surgery effects dependent upon VCD or Vehicle injections (data not shown). However, no subsequent analyses probing this interaction were significant. Additionally, WMC error analyses demonstrated a Trial x Injection x Surgery interaction [$F_{(2,74)} = 3.703$, $p < 0.05$] (Figure 40A); analyses of each individual trial revealed that for Trial 4, the high working memory load trial, there was a significant Injection x Surgery interaction [$F_{(1,37)} = 4.976$, $p < 0.05$] (Figure 40B). In this high working memory load trial, rats in the VCD-Hyst group made marginally more WMC errors than those in the Vehicle-Hyst group [$F_{(1,19)} = 3.954$, $p < 0.10$]; there was no such VCD effect in rats that received Sham surgery (Figure 40B).

When evaluating WMI errors during the Early Acquisition Phase there was a marginal Trial x Surgery x Injection interaction [$F_{(3,111)} = 2.629$, $p < 0.10$], where for Trial 1, the low working memory load trial, there was a marginal effect of Surgery [$F_{(1,37)}$ = 2.963, $p < 0.10$], of Injection [$F_{(1,37)} = 2.963$, $p < 0.10$], and a marginal Injection x Surgery interaction [$F_{(1,37)} = 2.963$, $p < 0.10$] (data not shown). However, subsequent analyses did not reveal any individual group differences (data not shown).

For RM errors, there were no significant effects of Injection or Surgery during the Early Acquisition Phase.

Late acquisition phase. Analyses for WMC errors during the Late Acquisition Phase (Days 5-9) did not reveal any effects of Injection or Surgery, nor any interactions of Trial with Injection and/or Surgery.

For WMI errors, there was a main effect of Surgery [$F_{(1,37)} = 5.529$, $p < 0.05$], where hysterectomized rats, collapsed across injection, made fewer errors than Sham rats (Figure 41A). Additionally, analyses revealed a Trial x Surgery interaction [$F_{(3,111)} = 4.348$, $p < 0.01$] (Figure 41B), where at the highest working memory load trial, Trial 4, there was an effect of Surgery [$F_{(1,39)} = 5.282$, $p < 0.05$], with Hysterectomy rats making fewer working memory errors than Sham rats (Figure 41C). These results indicate that, regardless of VCD or Vehicle injection, Hysterectomy surgery was facilitative to working memory functioning, particularly with a high burden, during the Late Acquisition Phase.

RM error analyses did not result in any effects of Injection or Surgery.

Asymptotic phase. During the Asymptotic Phase (Days 10-12), there were no effects of Injection or Surgery, nor any interactions of Trial with Injection and/or Surgery for any error type.

Delayed memory retention. Analyses of delayed memory retention on the WRAM were performed for WMC errors from Trial 3 on the last baseline day of testing (Day 12) and the first post-delay trial, Trial 3, on Day 13. Effects of delay were not present for Vehicle-Sham (Figure 42A) or VCD-Sham (Figure 42B) groups. Both Hysterectomy groups revealed a significant effect of Delay [Vehicle-Hyst: $F_{(1,11)} = 9.483$, $p < 0.05$ (Figure 42C); VCD-Hyst: $F_{(1,8)} = 19.692$, $p < 0.01$ (Figure 42D)], indicating delay-induced impairments on the WRAM.

MWM

For MWM performance across Days 1-5, there was a main effect of Day for both swim distance to the platform [$F_{(4,144)} = 64.475$, $p < 0.0001$] (Figure 43A) and latency [$F_{(4,144)} = 55.893$, $p < 0.0001$] (data not shown), with both decreasing across testing days, indicating learning of this reference memory task. There were also significant effects of Trial for both measures [Swim Distance: $F_{(3,108)} = 10.044$, $p < 0.0001$; Latency: $F_{(3,108)} = 9.514$, $p < 0.0001$], with both also decreasing across trials within a testing day. However, no significant effects of Injection or Surgery were present for MWM swim distance or latency, and there were no significant Injection x Surgery interactions.

Analyses of the probe trial revealed significant effects of Quadrant for each treatment group [Vehicle-Sham: $F_{(1,10)} = 96.940$, $p < 0.0001$; VCD-Sham: $F_{(1,7)} = 90.571$, $p < 0.0001$; Vehicle-Hyst: $F_{(1,11)} = 132.022$, $p < 0.0001$; VCD-Hyst: $F_{(1,8)} = 37.039$, $p < 0.001$], indicating that all groups were able to spatially localize the platform (Figure 43B).

VP

Analyses of the VP task revealed a main effect of Trial [$F_{(5,180)} = 10.469$, $p < 0.0001$], with latency to the platform decreasing across trials (Figure 44). There were no interactions of Trial with Surgery or Injection, but there was a significant Trial x Injection x Surgery interaction [$F_{(5,180)} = 2.954$, $p < 0.05$] (data not shown); this was likely due to the spike in latency in the VCD-Sham group on Trial 2. In spite of this interaction, all groups generally decreased in latency across trials, with no rat having a swim latency to the platform greater than 20 seconds by Trial 6. These results indicate that, overall, groups did not differ in their ability to complete the procedural components necessary to solve a water-escape task.

OFT

For OFT analyses, there was no effect of Surgery or Injection, or any interaction, across measures of total distance, or distance or time spent in the corners, center, or small center of the box, indicating no differences in locomotor or anxiety-like behavior (data not shown).

Body Weights at Euthanasia

There was an effect of Injection for body weights collected at euthanasia [$F_{(1,36)} = 4.484$, $p < 0.05$], whereby VCD-treated rats, collapsed across surgery, weighed more than those treated with Vehicle (Figure 45A).

Ovary/Uterine Weights

Analyses of uterine weights in Sham rats revealed an Injection effect [$F_{(1,17)} = 8.419$, $p < 0.01$], where rats treated with VCD had smaller uterine weights than those treated with Vehicle (Figure 45B), seen in prior work using VCD (Mayer et al., 2002, 2004). Likewise, analyses of ovary weights for all groups revealed a main effect of Injection [$F_{(1,36)} = 20.341$, $p < 0.0001$], where, collapsed across surgical groups, rats treated with VCD had smaller ovaries than those treated with Vehicle (Figure 45C); these effects have also been seen previously with VCD-induced ovarian follicular depletion (Mayer et al., 2002, 2004).

Discussion

The current experiment evaluated the impact of prior ovarian follicular depletion on hysterectomy-induced cognitive deficits through the use of the VCD and hysterectomy preclinical rodent models. Young female rats were administered VCD or Vehicle across a series of IP injections; 90 days later, following the course of ovarian follicular depletion,

rats received either Sham or Hysterectomy surgery. Six weeks following surgery, evaluations of spatial working and reference memory demonstrated surprising patterns of hysterectomy-induced facilitation, as well as for VCD-induced deficits, where the latter were only found for hysterectomized rats. While the working memory deficits associated with the model of hysterectomy were not observed, unique factors surrounding the VCD paradigm, including the early life stress of such IP injections, may have altered subsequent outcomes. Further work will be needed to address the extent to which ovarian follicular depletion and hysterectomy potentially interact to produce unique cognitive and physiological profiles that better inform clinical insights.

WRAM learning curves, as well as MWM probe trial analyses, suggest that all groups demonstrated spatial working and reference memory ability. The Trial effect for the VP task also demonstrated that all groups were capable of completing such spatial navigation tasks, as has previously been demonstrated with the VCD and hysterectomy models (Koebele et al., 2019; Koebele, Hiroi, et al., 2021). Notably, during early learning on the WRAM task, there were unique interactions between ovarian and uterine status, but not as would be predicted given the clinical literature. While clinical literature suggests that ovarian failure would lead to diminished effects of hysterectomy (Phung et al., 2010), the current study found that VCD treatment yielded marginally greater errors in hysterectomized subjects, with no effects in sham-treated animals. Such findings suggest that hysterectomy exacerbates VCD-induced impairments. While prior work has shown VCD-induced follicular depletion results in cognitive impairment without subsequent surgery (Acosta, Mayer, Talboom, Tsang, et al., 2009), it is possible that VCD effects would be more pronounced if the working memory burden were increased.

Indeed, prior work suggests that young female rats need a greater working memory load to demonstrate sufficient burden (Bernaud et al., 2021).

During the latter portion of the WRAM learning phase, a hysterectomy-induced performance facilitation was observed. While cognitive deficits were expected given the prior literature (Koebele, 2019; Koebele et al., 2019), facilitation during the learning portion of the task has also been observed in our laboratory following hysterectomy (Koebele et al., 2019). It is possible that VCD could obscure the cognitive deficits induced by hysterectomy surgery; however, such facilitation was found regardless of VCD or Vehicle injection. Rather, the experience of such repeated injections at a young age, regardless of either treatment, may have impacted subsequent cognitive outcomes. The injection paradigm used yielded substantial weight loss, and involved repeated restraint and IP injections; such chronic, unpredictable stress early in life can have significant impacts on outcomes later in life. Indeed, such forms of chronic stress have been shown to induce cognitive dysfunction that is lasting (D'Amico et al., 2020), and research suggests that early life stress can re-organize both cognitive and emotional signaling within the brain through modulation of the hypothalamic-pituitary-adrenal (HPA) axis (Y. Chen & Baram, 2016). Research has shown interactions between HPA axis dysfunction and subsequent reproductive dysfunction (Magiakou et al., 1997), where chronic stress early in life can increase risk of precocious puberty and early menopause (Elias et al., 2005; Pesonen et al., 2008). Similar work evaluating early hormone exposures has demonstrated unique cognitive outcomes later in life, positing that there such exposures can alter critical windows across the lifespan, including menopause (Koebele & Bimonte-Nelson, 2015). Were these rats to have experienced sufficient

stress, whether receiving VCD or Vehicle injections, it is possible that their cognitive networks, as well as HPG axis functioning, could have been altered such that impacts of hysterectomy were distinct from that of prior work. Further research evaluating chronic stress in early adulthood in the rat and its impact on subsequent reproductive and cognitive profiles later in life would provide further context into the role of VCD in modulating hysterectomy-induced outcomes in the current experiment. To eliminate the potential interpretative challenge of modulating a stress response early in life with VCD, such injections could be given at a later age, and hysterectomy therefore evaluated at a middle-age timepoint, which more closely mirrors that of women in the clinic.

MWM and OFT results from the current study revealed no effect of VCD or hysterectomy; no reference memory impairment or affective changes were found with either model of menopause in prior work from our laboratory (Acosta, Mayer, Talboom, Tsang, et al., 2009; Koebele, 2019; Koebele et al., 2019). Regarding the physiological outcomes from the current project, it appears that VCD dominated subsequent outcomes, where body weights were elevated, and uterine and ovary weights were much reduced, regardless of surgical status. Such findings follow prior work (Mayer et al., 2002, 2004), and add to the notion that these early life experiences may have obscured later surgical manipulation of the reproductive tract. Further analyses of ovarian morphology and serum hormone levels will assist in charactering the putative interactions between VCD and hysterectomy surgery within the current study.

Assessments of the effects of hysterectomy with or without prior ovarian follicular depletion in the female rat, as evaluated in the current study, provided surprising cognitive outcomes. The hysterectomy-induced facilitation observed here

could indicate that early experience of the VCD injection paradigm obscured the interactions we sought to investigate. Future work could further pursue a similar, clinically-relevant question by performing hysterectomy surgery, then following with the VCD model ovarian follicular depletion. Such work would be able to address whether hysterectomy surgery early in adulthood modulates the experience of menopause and ovarian failure. Given that rats would be experiencing VCD administration in adulthood, as evaluated previously in our laboratory (Acosta, Mayer, Talboom, Tsang, et al., 2009; Koebele, Hiroi, et al., 2021; Koebele, Mennenga, et al., 2020), there would be no such concerns of early life stress. While VCD and hysterectomy models are useful to the preclinical literature, they provide the greatest strength to the clinic through a recognition of their strengths and limitations (Bimonte-Nelson et al., 2021).

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CHAPTER 7

The data presented in this chapter are currently undergoing peer review for publication in a scientific journal. Preliminary data from this experiment were, in part, presented at the Society for Neuroscience conference in 2019.

EVALUATIONS OF MEMORY, ANXIETY, AND THE GROWTH FACTOR IGF-1R AFTER POST-SURGICAL MENOPAUSE TREATMENT WITH A HIGHLY SELECTIVE PROGESTIN

Contribution: I was the graduate student principal investigator for this experiment under the mentorship of Dr. Heather Bimonte-Nelson.

ABSTRACT

Progestogens are a key component of menopausal hormone therapies. While some progestogens can be detrimental to cognition, there is preclinical evidence that progestogens with a strong progesterone-receptor affinity benefit some molecular mechanisms believed to underlie cognitive function. Thus, a progestin that maximizes progesterone-receptor affinity and minimizes affinities to other receptors may be cognitively beneficial. We evaluated segesterone-acetate (SGA), a 19-norprogesterone derivative with a strong progesterone-receptor affinity and no androgenic or estrogenic-receptor affinity, hypothesizing that it would enhance cognition. Middle-aged rats underwent Sham or Ovariectomy (Ovx) surgery followed by administration of medroxyprogesterone-acetate (MPA; used as a positive control as we have previously shown MPA-induced cognitive deficits), SGA (low or high dose), or vehicle (one Sham and one Ovx group). Spatial working and reference memory, delayed retention, and anxiety-like behavior were assessed, as were memory- and hormone- related protein assays within the frontal cortex, dorsal hippocampus, and entorhinal cortex. Low-dose SGA impaired spatial working memory, while high-dose SGA had a more extensive detrimental impact, negatively affecting spatial reference memory and delayed retention. Replicating previous findings, MPA impaired spatial reference memory and delayed retention. SGA, but not MPA, alleviated Ovx-induced anxiety-like behaviors. On two working memory measures, IGF-1R expression correlated with better working memory only in rats without hormone manipulation; any hormone manipulation or combination of hormone manipulations used herein altered this relationship. These findings suggest that SGA does not benefit spatial cognition after surgical menopause, and that surgical

menopause with or without SGA disrupts relationships between a growth factor critical to neuroplasticity.

Introduction

Menopause, which is defined retrospectively by one year of amenorrhea, occurs naturally at an average age of 52 (Pinkerton et al., 2017). During the transition through menopause, estrogen and progesterone levels fluctuate and eventually decline, while FSH and LH levels rise (El Khoudary et al., 2019). Coincident with these changes in hormone profile is the onset of symptoms such as hot flashes, mood alterations, sexual dysfunction, increased osteoporosis risk, and memory disturbances (El Khoudary et al., 2019; Pinkerton et al., 2017). To combat some of these symptoms, hormone therapy (HT) may be prescribed. HT contains an estrogen component, such as conjugated equine estrogens (CEE) or 17β-estradiol (E2), and can include a progestogen for those with a uterus to prevent estrogen-induced endometrial hyperplasia and cancers (Shifren et al., 2019). Progestogens are a class of hormones that include progesterone, its metabolites, and the synthetic progestins, which mimic the biological activity of endogenous progesterone. It is notable that, while the uterus is often the primary target of progestin-based therapies, exogenous hormone administration can lead to a cascade of effects throughout the body, including changes in mood and increased risk for cardiovascular disease or stroke after menopause (Gleason et al., 2015; Pinkerton et al., 2017).

While a variety of different progestins are clinically available in HT formulations, the cognitive effects of progestins are still poorly understood. Some studies in women suggest that estrogens in HT can have beneficial cognitive effects during the menopause transition and into postmenopausal life (Braden, Dassel, et al., 2017; Duff & Hampson, 2000; Maki et al., 2011), where other studies report that combination HT can result in cognitively neutral outcomes, or even impairments to cognitive performance (Coker et

al., 2010; Gleason et al., 2015; Resnick et al., 2006). Notably, the cognitive effects of progestogens when given alone have not been well characterized in clinical research. Some work suggests that progesterone administration does not significantly affect cognitive outcomes in menopausal women (Henderson, 2018), but the same level of clinical research has not been done with regard to the variety of clinically available progestins. Preclinically, a number of studies evaluating progestogens in rodent models of menopause suggest that while some progestins are detrimental to cognition, a select few have beneficial or neutral cognitive effects (Braden, Andrews, et al., 2017; Braden et al., 2011; C. A. Frye et al., 2013; Koebele, Hiroi, et al., 2021; Prakapenka et al., 2018).

The distinct progestin effects observed within the clinical and preclinical literature are likely a result of their complex pharmacology and bioactivity. Progestins vary in their chemical structure and composition largely due to the variety of parent molecules from which these synthetic hormones are derived. These varied parent molecules form several different classes of progestins, including 19-nortestosterone derivatives (estranes and gonanes), 17α-hydroxy-derivatives (pregnanes), 19-norprogesterone derivatives (norpregnanes), and spirolactone derivatives (Davtyan, 2012; Schindler et al., 2003). Novel progestins have continued to be synthesized frequently throughout the last few decades, and these differences in origin of each progestin lead to a unique pharmacological bioactivity of estrogenic, androgenic, and glucocorticoid receptor affinities beyond their affinity for the progesterone receptor, which in turn can impact cognitive outcomes. For example, administration of medroxyprogesterone acetate (MPA), a 17α-hydroxyprogesterone derivative that has moderate progesterone receptor and mild androgen receptor affinity, has been demonstrated to result in impaired spatial

memory functioning in middle-age female ovariectomized (Ovx) rats relative to vehicle controls (Braden, Andrews, et al., 2017; Braden et al., 2010). Of note, progesterone itself, with strong progesterone receptor and mild glucocorticoid receptor affinity, has also been found to be detrimental to spatial learning and memory in some rodent models (Bimonte-Nelson et al., 2004; Braden et al., 2015). Not all progestins result in deleterious effects on learning and memory. Levonorgestrel, a 19-norestosterone-derived gonane with strong androgen receptor and strong progesterone receptor affinity, has been demonstrated by our laboratory as beneficial to spatial learning and memory compared to vehicle control treatment in the Ovx rat (Braden, Andrews, et al., 2017; Prakapenka et al., 2018). However, these beneficial memory effects were obviated when levonorgestrel was paired with an estrogen (Prakapenka et al., 2018). Given the prevalence and wide range of progestins prescribed clinically, it is crucial to define which progestins are cognitively detrimental, neutral, or even beneficial to optimize HT formulations for the clinic.

Distinct from the cognitive outcomes outlined above, some preclinical research has demonstrated neurogenic and neuroprotective effects of progestogens with strong progesterone receptor affinities in response to traumatic brain injury or stroke (Fréchou et al., 2015; Kumar et al., 2017; L. Liu et al., 2010). Indeed, administration of progesterone, as well as the progestin segesterone acetate (SGA; trade name, Nestorone®), resulted in increased bromodeoxyuridine+ cell ratios within the hippocampus of female rats, suggesting increased hippocampal neurogenesis following progestin treatment (L. Liu et al., 2010). Progestogens including progesterone and its metabolite, allopregnanolone, are implicated in neuroprotection in the context of stroke recovery (A. Liu et al., 2012). Both neuroprotective and neurogenic effects of progestogens show strong links to their degree

of progesterone receptor activity (Hussain et al., 2011; A. Liu et al., 2012). Therefore, it is possible that the cognitive effects of various progestogens could be optimized in the context of menopausal HT through the selection of a progestogen with a high affinity for the progesterone receptor, particularly in the context of hippocampal-dependent learning and memory. A promising candidate is the progestin SGA, a 19-norprogesterone derivative, which has a pharmacological profile that displays robust progestational activity, at 100 times that of progesterone, without any androgenic or estrogenic activity (Kumar et al., 2000). SGA has been examined *in vivo* for its pharmacokinetic properties and bio-distribution profile (Prasad et al., 2010), and behavioral outcomes have been evaluated with SGA treatment in rodent models of stroke (Tanaka et al., 2019). However, the effects of SGA on learning and memory have not been fully explored within the context of menopausal HT.

The current study evaluated daily SGA treatment at two clinically-relevant doses in a rat model of surgical menopause to examine learning and memory effects of this novel progestin, hypothesizing SGA-induced cognitive enhancement. In addition to evaluations of SGA administration, we evaluated the cognitive effects of treatment with MPA, a progestin found in HT and evaluated clinically in the Women's Health Initiative (Resnick et al., 2006; Shumaker et al., 2003). MPA treatment served here as a positive control, with predictions for cognitive deficits, as we have previously found working and reference memory deficits when compared to Ovx rats treated with the vehicle (Braden, Andrews, et al., 2017; Braden et al., 2010, 2011). Finally, we sought to better characterize putative relationships between the brain and behavior with SGA treatment by evaluating neurobiological changes after progestin administration and investigating

potential correlations between these two domains. Specifically, we evaluated relative expression of glutamate decarboxylase (GAD) 65 and GAD 67, proteins that have been shown by our laboratory to be modulated by administration of both MPA and progesterone in the Ovx rat (Braden et al., 2010) as well as to correlate with working memory performance in MPA-treated subjects (Braden et al., 2011). We additionally evaluated relative expression of insulin-like growth factor 1 receptor (IGF-1R), based on studies demonstrating that IGF-1 and its receptor regulate neurogenesis (Nieto-Estévez et al., 2016) and promote neuroplasticity (Dyer et al., 2016), are responsive to changes in ovarian hormone signaling (Rettberg et al., 2014; Zhao et al., 2012), and can impact hippocampal memory outcomes (B. S. Nelson et al., 2014). In addition, SGA increased expression of IGF-1 and IGF-1R in the frontal cortex of young, ovary-intact rodents in prior research (S. Chen et al., 2018). We predict that there will be memory benefits of SGA in the Ovx rat, and that this will be related to the upregulation of IGF signaling in associated brain regions. Collectively, this experiment provides insight into whether SGA, with high progestogenic activity and lack of estrogenic or androgenic activity, could be a potential cognitively-beneficial, and therefore advantageous, progestin for menopausal HT formulations.

Methods

Subjects

Fifty twelve-month old Fischer-344-CDF virgin female rats were received from the National Institute on Aging colony at Charles Rivers Laboratories (Raleigh, NC, USA). The rats were pair-housed on a 12-hour dark/light cycle, with food and water given *ad libitum*. They were given one week to acclimate to the new environment before

the commencement of the study. All procedures were conducted with approval from the Arizona State University IACUC and followed the standards set by the National Institutes of Health, and complied with ARRIVE guidelines. See Figure 46 for the experimental timeline, including surgeries, treatment administration, and the behavioral battery.

Surgery

Forty of the fifty rats underwent Ovx surgery from the dorsolateral aspect under acute isoflurane inhalation anesthesia, as done in previous hormone administration studies in our laboratory (Braden, Andrews, et al., 2017; Braden et al., 2010, 2011; Prakapenka et al., 2018). After dorsolateral incisions were made in the skin and peritoneum, a Vicryl ligature was applied to each uterine horn, and each ovary and uterine horn tip were removed. The remaining ten rats received sham surgery, which involved dorsolateral incisions into the skin and peritoneum, but not removal of the ovaries or ligation of the uterine horns. For all rats, dissolvable Vicryl sutures were applied to the muscle, topical 0.25% bupivacaine (Marcaine®, Pfizer Pharmaceutical, Hospira Inc., Lake Forest, IL) was applied for local analgesia, and surgical staples were used to close the skin incision. Rats were subcutaneously administered 2 mL saline to prevent dehydration and 5mg/mL/kg carprofen (Rimadyl®, Pfizer Pharmaceutical, Hospira Inc., Lake Forest, IL) for pain management. Subjects were single-housed upon the completion of surgery, and re-pair-housed 48 hours after surgery. Vaginal smears were carried out 23 days after Ovx surgery for 8 consecutive days, using previously established protocols from our laboratory (Koebele et al., 2019). Cells were collected from each rat at the vaginal opening using a cotton-tipped applicator and 0.9% sterile saline. Samples were placed onto a slide, where they were classified as metestrus,

diestrus, proestrus, or estrus according to well-established protocols (Acosta, Mayer, Talboom, Zay, et al., 2009; Goldman et al., 2007). This was done to confirm that all Ovx rats were acyclic, displaying few to no cells within the vaginal epithelium, as well as to confirm that all Sham rats were normally cycling, as displayed by the diversity of cells within the vaginal epithelium (Engler-Chiurazzi et al., 2011; Koebele et al., 2019; Koebele & Bimonte-Nelson, 2016).

Treatment Administration

Two days after surgery, Ovx rats were randomly assigned to receive daily 0.3 mL subcutaneous injections of either vehicle (Ovx-Vehicle, n=10), a 0.35 μg dose of SGA (Ovx-SGA Low, n=10), a 0.70 μg dose of SGA (Ovx-SGA High, n=10), or a 0.70 mg dose of medroxyprogesterone acetate, or MPA (Ovx-MPA, n=10), which were continued throughout the duration of the study. The rats that underwent sham surgery received daily 0.3 mL subcutaneous vehicle injections (Sham-Vehicle, n=10) throughout the duration of the study. The daily 0.35 μg and 0.70 μg doses of SGA were chosen based on the effective daily doses of SGA administered to women in clinical trials for the transdermal implant, which contained 100 μg of SGA, and the vaginal ring, which contained 200 μg of SGA (Sitruk-Ware, 2006; Sitruk-Ware et al., 2003), modified for the rat based on body weight. The daily 0.70 mg dose of MPA was chosen based on previously published data from our laboratory showing impairments for spatial working memory with MPA administration in rodent models of menopause (Braden, Andrews, et al., 2017; Braden et al., 2010, 2011).

Behavioral Battery

After thirty-two days of daily subcutaneous injections, rats were evaluated on a behavioral battery to assess learning and memory performance, the ability to perform the procedural components of a water-escape task, as well as anxiety-like and locomotor behaviors. Tasks included the Water Radial-Arm Maze (WRAM), the Morris Water Maze (MWM), the Visible Platform Task (VP), and the Open Field Test (OFT).

Water radial-arm maze (WRAM). Rats were first evaluated on the win-shift WRAM, which was used to assess spatial working and reference memory performance (Bimonte & Denenberg, 1999; Bimonte-Nelson, 2015c; Braden, Andrews, et al., 2017; Koebele et al., 2019; Prakapenka et al., 2018). The maze apparatus was comprised of a circular arena with eight arms that branched out from the center, and was filled with 18-20ºC water, which was made opaque through the use of non-toxic black paint. Salient external cues were placed around the testing room to aid in spatial navigation. Of the eight arms, four arms contained platforms hidden under the surface of the water. These platform locations were semi-randomized across subjects, but remained the same for each individual subject across all days of testing.

At the start of each trial, the subject was released at the start arm and given three minutes to locate a platform. If the animal did not find a platform in the allotted time, it was guided to the nearest platform. Once on the platform, the animal remained there for a total for 15 seconds before the experimenter returned the animal to the heated testing cage for a 30 second inter-trial interval (ITI). During this time, the recently-found platform was removed, and the maze was cleaned with a net to eliminate any potential odor cues. After the ITI was completed, the animal was once again released at the start arm in search of one of the remaining platforms. This continued until all of the platforms

were located; in this way, the working memory load was increased with each trial, as rats needed to remember not to return to arms explored previously within a given day. After all four platforms were found, the animal was finished testing for the day and was brought back to its heated testing cage. The next animal in the testing squad then began its first trial, and so continued until all rats completed their testing trials. WRAM testing continued in this fashion for a total of twelve consecutive days. On Day 13, a 4-hour delay was implemented between trials 2 and 3 to examine delayed memory retention.

Performance on the WRAM was evaluated for each rat by summing errors, defined as the number of entries into arms that did not contain a platform, for each trial. An arm entry was recorded when the tip of the rat's snout passed 11 cm into the arm, marked by a line on the outside of the maze arms. These errors were further separated into three measures of working and reference memory, as previously done in our laboratory (Bimonte-Nelson, 2015c; Bimonte-Nelson et al., 2003; Braden et al., 2011; Mennenga, Koebele, et al., 2015), using the following definitions: the first entry into a non-platformed arm within a day was defined as a reference memory (RM) error; a re-entry into a non-platformed arm within a day was defined as a working memory incorrect (WMI) error; and an entry into a previously platformed arm within a day was defined as a working memory correct (WMC) error.

Morris water maze (MWM). Following completion of the WRAM, rats began MWM testing the next day to evaluate spatial reference memory (Bimonte-Nelson, 2015a). The MWM apparatus was comprised of a circular tub, 188 cm in diameter, filled with 18-20ºC water made opaque with black non-toxic paint. A platform, 10 cm in diameter, was submerged in the northeast (NE) quadrant of the maze. The platform

remained in the same location across all testing trials and all days of testing. Robust spatial cues were placed around the room to aid in spatial navigation. At the beginning of each trial, a rat was dropped off at one of four designated starting locations (N, S, E, W), which were semi-randomized across trials and across days. For each trial, subjects were given 60 seconds to locate the hidden platform. Once found, the rat remained on the platform for 15 seconds before being placed back into its heated testing cage for an approximately 10 minute ITI. Each rat received 4 trials per day for each of the 5 days of testing. An additional 5th trial, the probe trial, was implemented on the last day of MWM testing, where the platform was removed from the maze. Rats then swam for 60 seconds during this probe trial to evaluate spatial localization to the platform. Each rat's swim path was recorded using the Ethovision tracking system (Noldus Instruments, Wageningen, The Netherlands), and total swim distance (cm) to the platform was analyzed along with latency (s) to the platform.

Visible platform task (VP). One day after the completion of MWM testing, performance on the VP task was evaluated to confirm the visual and motor capability of each animal to perform the procedural components of a water-escape task (Bimonte-Nelson, 2015b; Braden et al., 2011; Prakapenka et al., 2018). The apparatus was comprised of a rectangular 100 cm x 60 cm tub filled with clear water kept at 18-20ºC. A curtain that surrounded the tub was used to attenuate the use of spatial cues while rats completed this task. A black platform, 10 cm in diameter, was situated in the tub such that it remained 4 cm above the surface of the water throughout testing. The location of the platform was semi-randomly distributed among three different locations across all six testing trials. A trial began once the rat was dropped off at a designated location along the

edge of the tub furthest from the platform. Each rat was given 90 seconds to locate the visible platform. Once the rat found the platform, it remained there for 15 seconds before being placed back into its heated testing cage, with an ITI of approximately 5-10 minutes. Testing continued in this fashion until all six testing trials were completed, where latency (s) to the platform was analyzed to evaluate ability to perform the procedural components necessary to complete a water-escape task.

Open field test (OFT). Following a one-day break in behavior testing after the completion of VP, the OFT was completed to evaluate anxiety-like and locomotor behavior, using previously established protocols for testing and interpretation that have been utilized with exogenous ovarian hormone administration paradigms (Hiroi & Neumaier, 2006). The apparatus was comprised of a large 100 x 100 cm black square box made of plexiglass, which was digitally sectioned into 25 smaller squares, equal in size, representing the corners, center, and small center of the arena. Odormute™ was used to thoroughly clean the apparatus 24-hours prior to testing to eliminate foreign odors. The apparatus was positioned in a room with dim red lights to simulate darkness under a camera with tracking capabilities (Noldus Instruments, Wageningen, The Netherlands). The rats were placed in their testing cages at the start of the day, and were given 30 minutes to acclimate to their surroundings in the behavioral suite before testing. Rats were then brought individually in their testing cages into the testing room. Next, they were removed from their testing cage and placed into the box along the center of the north wall to initiate a trial, with the drop-off location kept consistent across rats. The experimenter then left the room and the animal's behavior in the box was recorded for ten minutes. After completion of the trial, the experimenter re-entered the room to remove

the rat from the box and place it back into its testing cage, where it was subsequently brought into the lit behavioral suite with the other testing cages. The apparatus was then cleaned thoroughly with water to spread any odors evenly before the next animal was brought in. In addition to total distance traveled (cm), time spent (s) and distance traveled (cm) were recorded for each animal in the center, in the small center, and in the corners of the apparatus.

Euthanasia and Tissue Collection

Between 24 and 48 hours after completion of the behavioral battery, rats were euthanized. Rats were deeply anesthetized with isoflurane and brains were extracted. The right hemisphere was rapidly dissected using previously established laboratory protocols (Braden et al., 2010, 2011; Prakapenka et al., 2018). Brain dissections were performed according to plate designations (Paxinos & Watson, 1998) to collect frontal cortex, the CA1/CA2 region of the dorsal hippocampus, and entorhinal cortex from the right hemisphere. For each brain, the right frontal cortex was first taken from the dorsal aspect of the brain, before cuts across the coronal plane were made to gain access to the right CA1/CA2 region of the dorsal hippocampus and right entorhinal cortex. These raw dissected brain regions were individually weighed, and then immediately frozen and stored at -70℃ until further analysis. The uterus was also removed from each subject, trimmed of excess fat, and weighed to examine the potentially stimulating effects of progestin treatment on the uterus, as well as the effect of ovarian hormone deprivation on the uterus following Ovx, which has been well characterized (Braden et al., 2010; Koebele et al., 2019).

Western Blot Protein Analysis

Right frontal cortex, right dorsal hippocampus, and right entorhinal cortex were evaluated via Western blot protein analysis for relative expression of GAD 65, GAD 67, and IGF-1R. Raw tissue samples were suspended in a 1:25 weight-to-volume ratio of RIPA buffer solution, which contained 150mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 50mM Tris HCl, protease inhibitor (Millipore-Sigma, CAT#5892791001), and phosphatase inhibitor (Millipore-Sigma, CAT#524625). Following suspension in RIPA buffer, all samples were subsequently kept on ice before samples were homogenized via probe sonication (Ultrasonic Processor, Cole Parmer, IL, USA). After all samples were homogenized, they were centrifuged at 10,000 rpm at 4ºC for 10 minutes, where the resulting cleared supernatant was collected, aliquoted, and kept at -70ºC for future analysis. The total protein concentration was determined for each sample via a bicinchoninic acid protein assay (Thermo-Fisher Scientific, Pittsburgh, PA, USA).

Samples were counterbalanced across treatment groups and evenly distributed across each gel. The NuPAGE PowerEase electrophoresis system was used to complete tissue processing, where samples for a given region were loaded at an equal protein concentration into a 4-12% NuPAGE Bis-Tris gel matrix in an XCell SureLock Mini-Cell (Invitrogen, Carlsbad, CA, USA) and run with MOPS running buffer. Following protein separation, membrane transfer was performed using an Immobilon polyvinylidene difluoride membrane. The membrane was then blocked in 5% milk for one hour at room temperature before being washed with 1X TBST. The membrane was then incubated at 5ºC overnight in 5% milk that contained primary antibody for anti-GAD65 (1:5000; Abcam, ab26113), anti-GAD67 (1:10000; Abcam, ab26116), anti-IGF-1R (1:1000; Cell

Signaling, 9750S), and anti-beta-actin, the loading control (1:20000; Cell Signaling, 4970S). The next day, the membrane was washed with 1X TBST before being incubated at room temperature for one hour in 5% milk that contained the secondary antibodies anti-rabbit horse radish peroxidase (HRP) (1:2000; Cell Signaling, 7074S) and anti-mouse HRP (1:2000; Cell Signaling, 7076S). The membrane was then washed with 1X TBST and developed using chemiluminescence (Lumiglo and peroxide solutions, Cell Signaling, 7003S) and a film developer (Konica SRX-101A Film Processor, Tokyo, Japan). The resulting films were scanned and saved as JPG files at 600dpi for densitometry analysis using ImageJ software, following previously established protocols (Gallo-Oller et al., 2018). For each gel, GAD65, GAD67, and IGF-1R expression were normalized to beta-actin expression.

Statistical Analyses

All statistical analyses were completed using StatView software. One animal in the Sham-Vehicle group was excluded from all analyses due to premature death unrelated to experimental manipulations. Additionally, one animal in the Ovx-Vehicle group was excluded from all analyses due to a technical error.

For neurobiological and behavioral assessments, data were analyzed using a series of planned comparisons to examine the effects of ovarian hormone loss (Sham-Vehicle vs. Ovx-Vehicle groups), as well as the individual effects of exogenous hormone administration (Ovx-Vehicle vs. Ovx-MPA, Ovx-Vehicle vs. Ovx-SGA Low, and Ovx-Vehicle vs. Ovx-SGA High), as has been done in previous publications (Acosta, Mayer, Talboom, Zay, et al., 2009; Braden, Andrews, et al., 2017; Braden et al., 2011; Koebele,

Hiroi, et al., 2021). For all analyses, results were designated marginal if *p*-values were between 0.05 and 0.10, and significant if $p < 0.05$.

Water radial-arm maze. Data were analyzed using repeated measures ANOVAs with Treatment as the independent variable, and Trials within Days as the repeated measures. Errors served as the dependent variable for these analyses, with separate analyses conducted for each error type. Data were initially analyzed in an omnibus repeated measures ANOVA for all baseline days of testing (Days 1-12) for WMC, WMI, and RM errors to determine whether rats learned the task across testing days by decreasing in errors, and subsequent analyses were performed in the series of planned comparisons as described above. Based on prior publications from our laboratory (Bimonte et al., 2003; Bimonte-Nelson et al., 2003, 2004, 2015; Braden, Andrews, et al., 2017; Engler-Chiurazzi et al., 2011; Mennenga, Gerson, Koebele, et al., 2015), data from WRAM testing was blocked into two groups to distinguish performance during task acquisition and maintenance: Days 2-7, referred to as the Acquisition Phase, and Days 8-12, referred to as the Asymptotic Phase. Each phase was analyzed separately using repeated measures ANOVA for WMC, WMI, and RM errors. For all statistical analyses, alpha was set at $p < 0.05$.

To analyze delayed memory retention on the WRAM, a repeated measures ANOVA was conducted for each treatment group separately, whereby WMC and WMI errors served as the dependent variables, and performance on Trial 3 for Day 12, the last day of baseline testing, and performance on Trial 3 for Day 13, the immediate post-delay trial, served as the repeated measures.

Morris water maze. Repeated measures ANOVA were utilized to analyze MWM data. Treatment was the independent variable, Swim Distance to Platform (cm) was the dependent variable, and Trials within Days were the repeated measures. For all baseline MWM trials, the same set of planned comparisons were utilized as were used for the WRAM data. The probe trial was analyzed using an ANOVA, with each treatment group analyzed separately; Percent Swim Distance in the Target Quadrant (NE) vs. that of the Opposite Quadrant (SW) served as the dependent measure.

Visible platform task. To determine whether rats differed in their ability to complete the procedural components necessary to solve a water-escape task, data collected from the final trial (Trial 6) of the VP task were analyzed using ANOVA, with the same set of planned comparisons used for MWM and WRAM data. Treatment was the independent variable and Latency to Platform (s) was the dependent variable.

Open field test. OFT results were analyzed using ANOVA, with the same set of planned comparisons as used for other behavioral analyses, where Treatment served as the independent variable, and Total Distance Travelled (cm), Time Spent in the Corners (s), Time Spent in the Center (s), and Time Spent in the Small Center (s) served as the dependent variables, along with Distance in the Corners (cm), Distance in the Center (cm), and Distance in the Small Center (cm). Two subjects (both in the Ovx-MPA group) were excluded from OFT analyses due to a technical error.

Body and uterine horn weights. Body weight and uterine horn weight data were analyzed using ANOVAs, with the same set of planned comparisons as stated above. Treatment served as the independent variable, and body/uterine weight at euthanasia (g) served as the dependent variables for these analyses.

Western blot protein analysis. Western blot protein expression data were analyzed using ANOVA. Treatment was the independent variable, and normalized relative GAD65 expression, normalized relative GAD67 expression, normalized relative IGF-1R expression, or relative beta-actin expression served as the dependent variable for each planned comparison. Outlying values were excluded from analyses of a given neurobiological marker within a given brain region if they: (1) did not pass the Grubbs test for outlier detection (Grubbs, 1969), and (2) were more than 2 standard deviations above or below the corresponding treatment group mean. There were two outliers for GAD67 expression in the Dorsal Hippocampus (1 in the Sham-Vehicle group and 1 in the Ovx-Vehicle group), one outlier for GAD67 expression in the Entorhinal Cortex (from the Ovx-Vehicle group), and one outlier for GAD65 in the Frontal Cortex (from the Ovx-SGA Low group) based on these criteria.

Correlations between WRAM performance & normalized GAD65, GAD67, and IGF-1R expression. Pearson r correlations were conducted with Fisher's r-to-z transformations to determine statistical significance of relationships between densitometry data and WRAM performance data for each treatment group. Relative normalized relative GAD65, GAD67, and IGF-1R expression in the right frontal cortex, right dorsal hippocampus, and right entorhinal cortex were correlated with WMI and WMC errors, averaged across trials, for the Acquisition and Asymptotic Phases of WRAM testing. These correlations were performed within each treatment group (i.e., intraclass correlations). Corrections for multiple comparisons were not performed for these correlations due to their exploratory nature, but correlations were only deemed significant if they had values of $p < 0.01$.

Results

Water Radial-Arm Maze

Baseline testing. Across all baseline testing days, there was a main effect of Day collapsed across all groups for WMC errors [$F_{(11,473)} = 3.37$, $p < 0.001$], WMI errors [$F_{(11,473)} = 13.44$, $p < 0.0001$], and RM errors [$F_{(11,473)} = 7.13$, $p < 0.0001$], with errors made on the WRAM decreasing across days, indicating that rats learned this complex spatial memory task (data not shown).

For the planned comparison between the Ovx-Vehicle and Ovx-SGA Low treatment groups, there was an effect of Treatment for WMC errors during the Acquisition Phase [$F_{(1,17)} = 8.03$, $p < 0.05$] (Figure 47A), whereby the Ovx rats treated with the low dose of SGA made more WMC errors than Ovx rats treated with the vehicle. Additionally, there was a Trial x Treatment interaction for the Ovx-Vehicle vs. Ovx-SGA Low planned comparison [$F_{(2,34)} = 4.52$, $p < 0.05$] (Figure 47B); further analyses revealed that for the highest working memory load trial, Trial 4, Ovx rats administered the low dose of SGA made more WMC errors than Ovx rats administered vehicle [$F_{(1,17)} = 6.81$, $p < 0.05$]. For the Ovx-Vehicle vs. Ovx-SGA High planned comparison, there was a marginal Trial x Treatment interaction [$F_{(2,34)} = 2.53$, $p < 0.10$]; subsequent analyses did not find a significant effect of Treatment for any individual testing trial (data not shown). In analyzing WMC errors during the Acquisition Phase, there were no other significant effects of Treatment, or Trial x Treatment interactions, for any other set of planned comparisons.

Similar to WMC errors during the Acquisition Phase, there was a main effect of Treatment for the Ovx-Vehicle vs. Ovx-SGA Low planned comparison for WMI errors

during the Acquisition Phase [$F_{(1,17)} = 7.41$, $p < 0.05$] (Figure 48A), where Ovx rats treated with the low dose of SGA made more errors than rats in the Ovx-Vehicle group. Additionally, for WMI errors in the Acquisition Phase, there was a Trial x Treatment interaction for the Ovx-Vehicle vs. Ovx-SGA Low planned comparison [$F_{(3,51)} = 7.94$, $p < 0.001$] (Figure 48B); upon analyzing the highest working memory load trial, Trial 4, the Ovx-SGA Low group made more errors than the Ovx-Vehicle [$F_{(1,17)} = 14.23$, $p < 0.01$].

Likewise, for the planned comparison between the Ovx-Vehicle and the Ovx-SGA High groups, there was a marginal Trial x Treatment interaction for WMI errors during the Acquisition Phase [$F_{(3,51)} = 2.44$, $p < 0.10$]; upon subsequent analysis of each individual trial, there was no significant effect of Treatment (data not shown).

There were no other significant effects of Treatment, or Trial x Treatment interactions, for WMI errors during the Acquisition Phase for any other set of planned comparisons, nor were there effects of Treatment for RM errors during task acquisition. Additionally, there were no significant effects of Treatment, or Trial x Treatment interactions, across the sets of planned comparison for the Asymptotic Phase for any error type.

Delayed memory retention. A 4-hour delay was implemented between Trials 2 and 3 on the final day of WRAM testing to evaluate delayed memory retention (Figures 49A-E). For the Ovx-Vehicle group, there was a marginal effect of Day for WMC errors, [$F_{(1,8)} = 4.39$, $p < 0.10$] (Figure 49B), where Ovx rats administered the vehicle tended to make more WMC errors following implementation of the delay. Likewise, Ovx rats treated with MPA or the low dose of SGA exhibited delayed memory retention

impairments, making more WMC errors following the delay relative to baseline [Day effect for WMC errors for the Ovx-MPA and the Ovx-SGA High groups: $F_{(1,9)} = 7.36$, $p < 0.05$ and $F_{(1,9)} = 9.99$, $p < 0.05$, respectively] (Figure 49C and E). There were no significant delay-induced effects for the Sham-Vehicle (Figure 49A) or the Ovx-SGA Low (Figure 49D) groups.

Morris Water Maze

For the MWM, there was a main effect of Day collapsed across all groups for swim distance to the platform [$F_{(4,172)} = 139.09$, $p < 0.0001$], with swim distance decreasing across days, thereby indicating learning on this reference memory task. For the Ovx-Vehicle vs. Ovx-MPA planned comparison, there was a main effect of Treatment for swim distance to the platform [$F_{(1,17)} = 14.58$, $p < 0.01$] (Figure 50A), with MPA-treated Ovx rats showing impairment via swimming a greater distance to the platform than vehicle-treated Ovx rats. The Ovx-Vehicle vs. Ovx-SGA High planned comparison revealed a Treatment main effect for swim distance to the platform [$F_{(1,17)} = 4.57$, $p < 0.05$] (Figure 50A), whereby the Ovx group treated with the high dose of SGA had a greater swim distance to the platform compared to the Ovx group treated with vehicle. There were no significant effects of Treatment for any other set of planned comparisons.

Analyses of the probe trial revealed an effect of Quadrant for each treatment group [Sham-Vehicle ($F_{(1,8)} = 79.98$, $p < 0.0001$); Ovx-Vehicle ($F_{(1,8)} = 175.73$, $p < 0.0001$); Ovx-MPA ($F_{(1,9)} = 115.62$, $p < 0.0001$); Ovx-SGA Low ($F_{(1,9)} = 142.36$, $p < 0.0001$); Ovx-SGA High ($F_{(1,9)} = 94.85$, $p < 0.0001$)] (Figure 50B), whereby a greater percentage of total swim distance for each group was spent in the Target Quadrant (NE)

as compared to the Opposite Quadrant (SW), indicating that all groups were able to spatially localize to the platform.

Visible Platform

Rats were able to perform the necessary procedural components to complete a water-escape task, as evidenced by the general decreasing latency to the platform across trials on the VP task (Figure 51). By the final testing trial, no animal took more than 18 seconds to reach the platform, and rats on average took only 3.20 seconds to locate the platform. For this final trial, across all sets of planned comparisons, there was no significant effect of Treatment [$F_{(4,43)} = 1.15$, $p > 0.05$], suggesting that all treatment groups performed similarly in successfully completing the task, demonstrating the visual and motor acuity needed to solve a water-escape task.

Open Field Test

Rats administered the low dose of SGA following Ovx had greater locomotor activity than those that received the vehicle following Ovx [$F_{(1,17)} = 4.66$, $p < 0.05$] (Figure 52A). None of the other planned comparisons revealed a significant effect of Treatment for total distance traveled within the OFT.

For distance travelled in the corners of the open field, there was no effect of Treatment for any planned comparison (Figure 52B). Analyses of distance travelled in the center of the open field revealed a main effect of Treatment for the Ovx-Vehicle vs. Ovx-SGA Low planned comparison [$F_{(1,17)} = 5.16$, $p < 0.05$], with low dose treatment of SGA increasing distance travelled in the center of the maze compared to treatment with vehicle (Figure 52C). When evaluating distance travelled in the small center, there was a

marginal main effect of Treatment for the Sham-Vehicle vs Ovx-Vehicle planned comparison [$F_{(1,16)} = 4.50$, $p < 0.10$], with Ovx reducing the distance travelled in the small center (Figure 52D). For the Ovx-Vehicle vs Ovx-SGA High planned comparison, there was a main effect of Treatment [$F_{(1,17)} = 6.74$, $p < 0.05$], whereby administration of the high dose of SGA obviated the Ovx-induced reduction of distance in the small center (Figure 52D).

For time spent in the corner (Figure 52E), as well as time spent in the center (Figure 52F) on the OFT, there was no effect of Treatment for any planned comparison. There were effects of Treatment for time spent in the small center for the Sham-Vehicle vs. Ovx-Vehicle [$F_{(1,16)} = 5.38$, $p < 0.05$], Ovx-Vehicle vs. Ovx-SGA Low [$F_{(1,17)} = 6.33$, $p < 0.05$], and the Ovx-Vehicle vs. Ovx-SGA High [$F_{(1,17)} = 6.53$, $p < 0.05$] planned comparisons (Figure 52G). These effects indicated that Ovx yielded less time spent overall in the small center, and that SGA administration at either dose obviated this Ovx-induced decrease.

Body Weights at Euthanasia

Body weights collected immediately before euthanasia yielded a main effect of Treatment for the Sham-Vehicle vs. Ovx-Vehicle planned comparison, [$F_{(1,16)} = 30.18$, $p < 0.0001$], demonstrating weight gain following surgical removal of the ovaries (Figure 53A). Additionally, the Ovx-Vehicle vs. Ovx-MPA planned comparison for final body weight revealed a Treatment effect [$F_{(1,17)} = 6.01$, $p < 0.05$], such that rats administered MPA following Ovx weighed less than those receiving vehicle following Ovx (Figure 53A). For the Ovx-Vehicle vs. Ovx-SGA Low, and Ovx-Vehicle vs. Ovx-SGA High,

planned comparisons, there was no significant effect of Treatment for body weight at euthanasia.

Uterine Horn Weights

For the planned comparison between Sham-Vehicle and Ovx-Vehicle groups, there was a main effect of Treatment [$F_{(1,16)} = 25.09$, $p < 0.001$], such that surgical removal of the ovaries resulted in a significant reduction in uterine horn weight (Figure 53B). For the Ovx-Vehicle vs. Ovx-MPA planned comparison, the Treatment effect [$F_{(1,17)} = 171.59$, $p < 0.0001$] revealed that administration of MPA following Ovx increased uterine horn weights relative to rats receiving vehicle following Ovx (Figure 53B). For the planned comparison between Ovx-Vehicle and Ovx-SGA Low, as well as for the comparison between Ovx-Vehicle and Ovx-SGA High, there was no effect of treatment on uterine horn weight.

Western Blot Protein Analysis

There were no significant effects of Treatment for any planned comparison regarding relative expression of GAD65, GAD67, or IGF-1R, normalized to the loading control beta-actin, for any brain region.

Correlations between WRAM Performance & Normalized GAD 65, GAD 67, and IGF-1R Expression

Correlations between WRAM performance and western blot analyses revealed a negative relationship between hippocampal IGF-1R expression and WMC errors made on the WRAM during the Acquisition Phase for the Sham-Vehicle group [r (7) = -0.82, $p <$ 0.01], with increased IGF-1R expression correlating with fewer working memory errors

during learning (Figure 54A). This correlative relationship was not seen in any other group. Likewise, again in only the Sham-Vehicle group, there was a significant negative relationship between hippocampal IGF-1R expression and WMI errors during the Acquisition Phase of WRAM testing [r (7) = -0.79, $p < 0.01$], whereby increased IGF-1R expression correlated with fewer working memory errors made during learning (Figure 54B). There were no other significant correlations for IGF-1R, nor any significant correlations for GAD65 or GAD67 for any treatment group.

Discussion

The current study evaluated the cognitive effects of a daily subcutaneous regimen of SGA, a progestin with a selective affinity for the progesterone receptor, which has not been well-characterized in the context of HT and cognition. Given that the majority of preclinical rodent work evaluating the cognitive effects of HT has focused on estrogen administration (Acosta, Mayer, Talboom, Zay, et al., 2009; Black et al., 2018; Daniel et al., 2006; Gibbs, 1999; Hiroi et al., 2016; Korol & Kolo, 2002; Prakapenka et al., 2020) or combined therapies (Chisholm & Juraska, 2012; C. A. Frye et al., 2010; Lowry et al., 2010; Prakapenka et al., 2018), our laboratory sought to uniquely address the potential for optimal outcomes associated with progestin administration. The results outlined above demonstrate that SGA has dose- and task- dependent detrimental effects on spatial working and reference memory when administered at two different doses in a surgical menopause model. Notably, neurobiological evaluations of relative GAD65, GAD67, and IGF-1R expression found a disruption of relationships between IGF1-R and working memory outcomes following Ovx that was not reinstated with progestin administration. These findings provide some insight into the relationship between differing progestin

administration and learning and memory outcomes as observed with HT, and how these outcomes may differ across cognitive domains.

WRAM performance demonstrated working memory impairment during task acquisition across two different error types (WMC and WMI errors) for the low dose of SGA relative to Ovx-Vehicle controls. Furthermore, these effects were evident at the highest working memory load trial, Trial 4, demonstrating that SGA-induced impairments emerged with a high degree of working memory burden; this pattern has been shown previously in our laboratory when evaluating other progestins, such as MPA (Braden et al., 2011). In the current study, rats treated with MPA did not demonstrate significant working memory impairments during baseline WRAM testing, as previously reported in our laboratory (Braden et al., 2011). However, it should be noted that these effects were observed with a tonic administration of MPA, and not the cyclic daily injections as used in the current study. Prior work has shown the role that tonic vs. cyclic treatment administration plays in outcomes associated with estrogens (Koebele, Nishimura, et al., 2020; Lowry et al., 2010), underscoring the idea that route of administration for exogenous HT is a key factor in cognitive outcomes. Such findings are relevant for clinical translation in optimizing health outcomes with HT use. Notably, while marginal trial by treatment interactions demonstrated a change in the pattern of working memory impairment with the high dose of SGA relative to Ovx-Vehicle rats across testing trials on the WRAM, analyses of each trial individually did not reveal differences between groups during learning. It is possible that the learning curve of rats treated with the high dose of SGA is shifted from that of the low dose, resulting in some indications for treatment differences without specific trial effects. Future research should

evaluate potential non-linear relationships between SGA dose and subsequent memory outcomes and consider divergent treatment-specific learning curves.

Analyses of WRAM performance following a 4-hour delay demonstrated impairments for the MPA-treated and high dose SGA-treated Ovx groups, as well as a marginal delay-induced impairment for the vehicle-treated Ovx group. The marginal Ovx-induced impairment following a delay aligns with prior work suggesting that surgical removal of the ovaries, the primary source of estrogens and progesterone, results in delay-induced impairments to spatial working memory (Koebele et al., 2019, 2022; Koebele, Quihuis, et al., 2021). Likewise, findings with MPA administration are concordant with prior work in our laboratory demonstrating working memory deficits in Ovx MPA-treated rats following a 4-hour delay (Braden, Andrews, et al., 2017). The impairments observed with the high dose of SGA, but not the low dose, further suggest that there may be dose-dependent effects of SGA associated with working memory processes.

Reference memory impairments were observed in the MWM for Ovx rats treated with MPA as well as Ovx rats treated with the high dose of SGA relative to Ovx rats without subsequent hormone treatment. Such findings align with our prior findings that MPA, when administered to Ovx rats, resulted in overnight forgetting (Braden et al., 2010) or early reference memory impairment (Braden, Andrews, et al., 2017) on the MWM. This also corresponds to findings from other laboratories, whereby the addition of MPA to chronic estrogen treatment impaired MWM performance in middle-aged Ovx rats (Lowry et al., 2010). Notably, while the current study found working memory impairments with the low dose of SGA on the WRAM, reference memory impairments

were observed with the high dose of SGA on the MWM. This replicates prior work with the progestin MPA, whereby reference memory deficits were observed for rats treated with the high dose of MPA, but not with the low dose of MPA, relative to Ovx rats without subsequent hormone treatment (Braden et al., 2010). Notably, it is possible that the dose of progestin sufficient for impairment is dependent upon the type of memory being evaluated. This would imply then that there may be differences in the response of specific brain regions critical to learning and memory to progestin administration and progesterone receptor activation. Indeed, preclinical research has shown that, particularly in middle-age, better performance on a given task may correlate with higher progesterone levels within one brain region, but correlate with lower levels in another (Paris et al., 2011). Future work could characterize the nature of these patterns for reference memory and working memory deficits with increasing SGA dose and corresponding changes within relevant brain regions in an effort to elucidate the potential neurobiological mechanisms underlying behavioral outcomes.

OFT results reveal an interesting pattern of effects on locomotive and anxiety-like behavior with SGA administration. Surgical removal of the ovaries without subsequent hormone treatment resulted in a decrease in time spent in the small center of the arena, indicating an increase in anxiety-like behavior with ovarian hormone depletion. Prior preclinical work has demonstrated increased anxiety-like behavior following Ovx in rats that is sustained for weeks after surgery as evaluated on the EPM (Puga-Olguín et al., 2019). This corresponds with clinical literature, where natural and surgical menopause are commonly associated with depressed mood and increased anxiety (NAMS, 2014; Rocca et al., 2018; Weber et al., 2014). Both the clinical and preclinical literature indicate

estrogen-containing HT could reverse these mood shifts observed with menopause (Gleason et al., 2015; Hiroi et al., 2016; Puga-Olguín et al., 2019). Indeed, one such preclinical study demonstrated that administration of 17β-estradiol resulted in increased center time in Ovx rats on the OFT, interpreted as a reduction in anxiety-like behavior (Hiroi & Neumaier, 2006), while the addition of progesterone reversed these effects. In contrast, the current project found amelioration of these anxiety-like effects with SGA administration, at both the low and high doses, but not with MPA administration; indeed, rats treated with either dose of SGA spent more time in the small center of the arena than their Ovx-Vehicle counterparts, performing similarly to ovary-intact rats that underwent sham surgery. Such effects with SGA but not MPA suggest that different progestins can produce distinct anxiety-like behavioral profiles, possibly due to their differing activities at receptors other than the progesterone receptor, such as the glucocorticoid receptor, androgen receptor, etc. Notably, prior work evaluating the spironolactone-derived progestin drospirenone displayed no effects of administration on anxiety-like behavior on the OFT for Ovx rats (Koebele et al., 2022). This yields the possibility for a progestin that augments, rather than opposes, estrogen-induced benefits to anxiety-like outcomes in preclinical HT evaluations. Such findings follow recently published work in our laboratory in a transitional model of menopause, where the combined administration of 17β-estradiol and levonorgestrel had the most beneficial outcomes for anxiety-like behaviors as evaluated on the OFT (Koebele, Hiroi, et al., 2021). It is possible that progestins have a previously underexplored potential to reverse mood and anxiety shifts associated with menopause, particularly those with high progesterone receptor activity and minimal peripheral activity, such as SGA.

Body and uterine weights provide insight into the physiological effects of these progestins. Ovx-Vehicle animals had elevated body weights compared to Sham controls, as previously observed following the removal of the ovaries (Bimonte & Denenberg, 1999; Koebele et al., 2019; Zeibich et al., 2021) without subsequent estrogen administration (Chisholm & Juraska, 2012); notably, MPA administration attenuated this Ovx-induced body weight increase. This change in weight, where SGA treatment showed no effect, is potentially due to MPA's higher glucocorticoid and androgenic activity (Louw-du Toit et al., 2017; Sitruk-Ware, 2006). Likewise, an Ovx-induced decrease in uterine weight was observed, as previously reported in the literature (Acosta, Mayer, Talboom, Tsang, et al., 2009; Chisholm & Juraska, 2012; Galea et al., 2018; Koebele et al., 2019); MPA reversed this decrease, suggesting some uterine stimulation following MPA administration. Future work should further characterize the differing physiological effects of various progestins to provide insight into which HT formulation might be best to combat the myriad indications associated with menopause.

In the current experiment, Western blot analyses did not reveal significant changes in GAD 65, GAD 67, or IGF-1R following surgical menopause with or without either tested progestin treatment. The lack of GABAergic effects with SGA administration suggest that the neurobiological mechanism related to SGA's detrimental cognitive effects is distinct from that of endogenous progesterone, where administration of the $GABA_A$ receptor antagonist bicuculline obviated memory deficits (Braden et al., 2015). While prior work in our laboratory suggests that MPA administration would lead to regionally-specific changes in GAD levels (Braden et al., 2010), the current study uses a cyclic hormone administration paradigm, while our prior work administered MPA

tonically. Work with estrogens has shown distinct memory effects due differing administration paradigms (Bimonte-Nelson et al., 2006; Koebele, Nishimura, et al., 2020; Mennenga, Gerson, Koebele, et al., 2015); such differences could also be crucial to progestogen outcomes. Additionally, the current study did not find changes in cortical IGF-1R with cyclic SGA administration in Ovx rats, but others have reported that tonic high doses of SGA result in increases in IGF-1 and IGF-1R expression within the frontal cortex in ovary-intact mice (S. Chen et al., 2018). Further research is needed to assess whether tonic administration of SGA may result in differential brain and behavioral outcomes in the female Ovx rat.

Correlations between brain and behavioral outcomes potentially provide insight into the role that ovarian hormones play in maintaining these relationships, and the disruption that may occur with ovarian hormone cessation or disruption. Indeed, for Sham-Vehicle rats, strong relationships existed between working memory performance during learning on the WRAM and expression of IGF-1R within the dorsal hippocampus, such that higher hippocampal IGF-1R levels were associated with improved working memory performance across both working memory error types. The link demonstrated here between IGF-1R expression and working memory performance is not surprising, as IGF signaling plays a critical role in neuroplasticity. IGF-1 is essential for dendritic growth and can increase dendritic arborization, thought to be driven by its modulation of the neuronal inhibitory/excitatory balance (Dyer et al., 2016). Such neuroplasticity is likely involved in the updating of associations for successful working memory performance, thereby suggesting that greater IGF-1R expression would correspond with greater working memory capacity, as shown in the current experiment. In our study,

when the ovaries were removed, this association was disrupted. These findings relate to prior work showing downregulation in hippocampal IGF-1 gene expression following Ovx that corresponded with memory deficits as observed on the MWM (Habibi et al., 2017). Indeed, the abrupt loss of circulating ovarian hormone and instigation of surgical menopause likely disrupts brain relationships with IGF signaling previously held intact by functioning of the hypothalamic-pituitary-gonadal (HPG) axis (Koebele & Bimonte-Nelson, 2017), as has been demonstrated for other neurobiological systems, such as the cholinergic system (Gibbs, 2003). Notably, administration of either progestin to Ovx rats did not reinstate the relationship seen in ovary-intact rats. Like that of MPA and SGA in the current study, prior work evaluating the administration of drospirenone in Ovx rats found no relationship between IGF-1R expression and hippocampal memory outcomes (Koebele et al., 2022); this indicates that progestogens alone do not dictate the relationship between hippocampal memory and IGF system expression. However, it is of note that when drospirenone was paired with an estrogen, such relationships were observed (Koebele et al., 2022), indicating that a combination of estrogens and progestogens could reinstate relationships that emerge with endogenously circulating ovarian hormones. It should also be considered based on the collective findings that estrogens play the dominant role in maintaining such brain-behavior relationships in ovary-intact rats. Indeed, preclinical work in Ovx rats has demonstrated that prior E2 exposure led to increased IGF-1R expression, and such increases resulted in memory benefits (Witty et al., 2013). Additional research is needed to decipher the role of hippocampal IGF-1R profiles in regulating cognitive outcomes observed with SGA

administration, as well as whether estrogen treatment could re-instate relationships seen in the ovary-intact milieu.

Conclusion

The prior literature evaluating SGA has focused on characterizing its pharmacological profile (Kuhl, 2005; Kumar et al., 2000), as well as its clinical effectiveness as a hormonal contraceptive (Brache et al., 2015; Kumar et al., 2017; Sitruk-Ware & Nath, 2010; Sivin et al., 2005). While there are a handful of papers evaluating its impact on the brain (El-Etr et al., 2015; Lenzi et al., 2009; L. Liu et al., 2010), including neuronal outcomes in vitro (Jayaraman & Pike, 2014), few have evaluated SGA's effect on brain function, with most focusing on motor tasks (Fréchou et al., 2021; Garay et al., 2014; Lee et al., 2022; A. Liu et al., 2012). Here, we address the impact of SGA on the brain and its function, specifically regarding cognitive and anxiety-like behaviors.

Evaluations of SGA are not only important in the context of HT and surgical menopause; notably, progestins are also administered to ovary-intact women who undergo a non-surgical, natural menopause transition, or to cycling intact women in the form of hormonal contraceptives. These profiles can be modeled preclinically using the 4-vinylcyclohexene diepoxide (VCD) model of transitional menopause, which is an ovary-intact model that results in ovarian follicular depletion, or by using young adult ovary-intact female rodents to model reproductive cyclicity. Just as preclinical work has shown different cognitive outcomes depending upon ovarian status when evaluating estrogens commonly found in hormonal contraceptives, such as ethinyl estradiol (Mennenga, Gerson, Koebele, et al., 2015; Simone et al., 2015) as well as with estrogens

used in HT, including conjugated equine estrogens and 17ß-estradiol (Acosta et al., 2010; Koebele, Hiroi, et al., 2021; Prakapenka et al., 2018), SGA should be evaluated in different translational contexts to determine whether cognitive effects differ with variations in menopause etiology and HTs. Evaluations that combine SGA with different estrogens experimentally will be especially useful, as progestins are most commonly paired with estrogens in both HT and oral hormonal contraceptives. Notably, the FDA has recently approved a contraceptive vaginal ring, called Annovera™, containing SGA and ethinyl estradiol (A. L. Nelson, 2019), prompting evaluations of SGA beyond putative HT formulations.

Given the SGA-induced anxiolytic effects observed here, our results suggest that SGA may be a candidate for HT when a progestin is needed in women who have a profile of increased anxiety following oopherectomy. These preclinical findings are important, as to our knowledge, Annovera™ has yet to be evaluated for mood or anxiety outcomes. Clinically, the findings testing HTs' effects on anxiety and mood are mixed, but indicate that certain formulations may provide benefits when taken early in menopause (Fischer et al., 2014). Furthermore, there is clinical evidence that hormonal contraceptives can modulate anxiety and affective outcomes, as oral contraceptives containing drospirenone and ethinyl estradiol have been shown result in reduced anxiety in reproductively-aged women (Paoletti et al., 2004), particularly in those with premenstrual dysphoric disorder (Yonkers et al., 2005). Further work should be done to evaluate SGA with a more expansive anxiety-like and depressive-like behavioral battery to determine whether its benefits persist across multiple domains. Ultimately, SGA may benefit cognitive and behavioral outcomes under some parameters, but not others. Much of the research

surrounding HT formulations has revealed such complexity when optimizing menopausal outcomes, where combination of a beneficial estrogen with a beneficial progestogen may obviate cognitive benefits in a surgical menopause model (Koebele et al., 2022; Prakapenka et al., 2018), but not in a transitional menopause model (Koebele, Hiroi, et al., 2021). Indeed, when thinking about the future of menopausal HT, it is possible that a personalized medicine approach, whereby individual medical history and risk factors are taken into account to strategically create a HT profile that has the highest likelihood of being most efficacious, provides the best solution to personalizing and thereby optimizing HT formulation. Toward this end, clinical and preclinical research, comprehensively evaluating the neurobiological, physiological, and behavioral effects of various progestogens mapped onto the background of various menopausal and reproductive histories, is critical to the future of healthcare in the aging female.

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CHAPTER 8

The data presented in this chapter are currently unpublished observations.

AN EVALUATION OF THE MEMORY, ANXIETY-LIKE, AND INFLAMMATORY EFFECTS OF 17-BETA ESTRADIOL ADMINISTRATION IN RODENT MODELS OF SURGICAL MENOPAUSE

Contribution: I was the graduate student principal investigator for this experiment under the mentorship of Dr. Heather Bimonte-Nelson.

ABSTRACT

Hysterectomy is the second most common gynecological procedure in women, often occurring before menopause onset. Hysterectomized women are able to take a unique hormone therapy (HT) formulation: without a uterus, these individuals may take an estrogen without the need for a concomitant progestogen to protect against uterine hyperplasia and cancer. While combination HT has been evaluated preclinically using the ovariectomy (Ovx) model of surgical menopause via ovarian removal, the behavioral and physiological outcomes associated with estrogen-only HT are understudied. Our laboratory has recently developed a novel preclinical rodent model of hysterectomy, which displays cognitive deficits in line with clinical findings of increased dementia risk. This model is key to translationally addressing the long-term outcomes associated with estrogen-only HT administration. The current study used the Ovx and hysterectomy rodent models of surgical menopause to evaluate the effects of 17-beta-estradiol (E2) HT. Middle-aged female rats received either Sham surgery, Ovx, or Hysterectomy; three weeks later, rats received tonic Vehicle (polyethylene glycol; all 3 surgical groups) or E2 (Ovx and Hysterectomy) administration via osmotic pump. Rats were then evaluated on a behavioral battery that assessed spatial working and reference memory, cognitive flexibility, and anxiety-like behavior. Ovx resulted in working and reference memory deficits, where E2 rescued these working memory deficits. Ovx also decreased anxiety-like behavior. Hysterectomy showed a tendency for detrimentally modulating working memory responsivity, and E2 opposed this effect. Finally, E2 in the absence of other circulating ovarian hormones decreased serum levels of interleukin 6 (IL-6), a pro-inflammatory cytokine, relative to ovary-intact rats. These findings, along with unique

treatment-specific correlations to serum tumor necrosis factor alpha (TNF-alpha), suggest that E2 plays a beneficial role in outcomes associated with both Ovx and hysterectomy; however, patterns of effects are distinct across these two models of surgical menopause, highlighting the need for greater clinical work that evaluates the role of the uterus.

Introduction

Menopause, which is defined retrospectively by one year of amenorrhea, occurs naturally on average at age 52 (Pinkerton et al., 2017). However, the experience of menopause, as well as its etiology, is more diverse than this definition may indicate; individuals can experience gynecological surgery before the menopause transition, resulting in a different trajectory with unique indications and treatment plans. For example, oophorectomy, or the surgical removal of the ovaries, performed before natural menopause onset will result in the abrupt decline in circulating estrogens and progesterone, as opposed to the more gradual ovarian hormone decline experienced across the menopause transition (Koebele & Bimonte-Nelson, 2016). Coincident with these sharp declines in ovarian hormone levels is the onset of menopause symptoms that include hot flashes, mood alterations, and memory disturbances and increased risk of osteoporosis (El Khoudary et al., 2019; Pinkerton et al., 2017). Not only are there short-term behavioral and physiological changes with oophorectomy, but also long-term impacts of this form of surgical menopause; indeed, researchers have also found that women that undergo oophorectomy have sustained chronic inflammation post-surgery (Pacifici et al., 1991), and decades later, have an increased risk for dementia (Rocca et al., 2007). To combat some of these early menopausal symptoms, women experiencing natural or surgical menopause can be prescribed hormone therapy (HT). HT contains an estrogen, such conjugated equine estrogens (CEE) or 17-beta estradiol (E2), the most potent endogenous estrogen in women, and can include a progestogen for those with a uterus to combat estrogen-induced endometrial hyperplasia and cancers (Shifren et al., 2019). HT can reduce hot flashes, improve bone health, and has been shown to improve

mood and memory outcomes (Pinkerton et al., 2017). Notably, when prescribed early after oophorectomy, HT can also improve oophorectomy-induced inflammatory outcomes, with reductions in inflammatory markers such as tumor necrosis factor alpha (TNF-alpha) (Pacifici et al., 1991), as well as decrease associated dementia risk (Rocca et al., 2014). Preclinical research has extensively evaluated the cognitive and physiological effects of HT following ovariectomy (Ovx), or surgical removal of the ovaries in the rodent, demonstrating unique cognitive outcomes dependent on key variables such as estrogen/progestogen type, regimen, and route of administration (Braden, Andrews, et al., 2017; Gibbs, 1999; Hiroi et al., 2016; Koebele, Hiroi, et al., 2021; Koebele, Nishimura, et al., 2020). Such work has been a great benefit to the clinic to optimize outcomes for this population.

While it has been a main focus of clinical and preclinical research, oophorectomy is not the only form of surgical menopause. Hysterectomy, or surgical removal of the uterus, is the second most common gynecological surgery, with 1/3 of US women experiencing this procedure by age 60 (Carlson et al., 1993; Whiteman et al., 2008). Hysterectomy may be performed alone, or in conjunction with oophorectomy. The experience of menopause following hysterectomy can be difficult to operationally define in those that do not undergo concurrent oophorectomy surgery. Following hysterectomy, menstruation does not occur, as the physical source of menstrual bleeding has been surgically removed; clinical guidelines for menopause rely up on definition of one year without menstrual bleeding for menopause onset (NAMS, 2014). However, if the ovaries are retained following hysterectomy, ovarian hormones still circulate, sometimes for decades following surgery, until nearing the age at natural menopause. While circulating

levels of estrogens or other ovarian hormones may appear to be a reliable indicator of menopause, research shows that, across this transition, hormone levels can vary widely across women and across peri-menopausal menstrual cycles (Burger et al., 1999; Ferrell et al., 2005; O'Connor et al., 2009). Indeed, even with repeated within-subjects measures, endocrine evaluations can be poor predictors of the menopause transition (Prior, 2005). Rather than the standard clinical criteria, menopause in hysterectomized women is more often indicated by the presence of associated symptoms, such as those listed above (Koebele & Bimonte-Nelson, 2016; The Practice Committee of the American Society of Reproductive Medicine, 2004). It is during this transitional period that hysterectomized individuals are often prescribed HT (Haney & Wild, 2007). Notably, this population is able to take a unique HT formulation containing only an estrogen (NAMS, 2014). This is because hysterectomy surgery negates concerns for the risk of endometrial hyperplasia with unopposed estrogen administration for this population, making progestogen administration obsolete (Haney & Wild, 2007).

Both clinically and preclinically, hysterectomy has been somewhat understudied in the context of menopause and healthy aging, with even less research being conducted to evaluate potential cognitive or physiological outcomes with HT use in these individuals. In clinical work, hysterectomized and non-hysterectomized ovary-intact individuals are often pooled together, as much work as focused on the role of the ovaries in long-term health outcomes (Wharton et al., 2011; Wroolie et al., 2011). However, some clinical studies evaluating the impact of hysterectomy have highlighted the role of the uterus in governing endocrine, cardiovascular, and neurobiological outcomes. There are some reports that hysterectomy may result in earlier menopause (Moorman et al.,

2011), indicating an accelerated ovarian failure following surgery. Additionally, hysterectomized women appear to have an increased risk of both heart disease (Ingelsson et al., 2011) and dementia, with increasing risk for those undergoing surgery earlier in life (Phung et al., 2010; Rocca et al., 2012). Such findings merit further investigation into how hysterectomy might alter the experience of menopause, and whether interventions such as HT might mitigate such long-term risks. Evaluations of hysterectomy in the context of HT have also provided insight into the role of the uterus. Indeed, evaluations of uterus-intact vs hysterectomized individuals have found divergent outcomes with estrogen-alone vs estrogen + progestogen HT administration (Coker et al., 2010; Maki, 2013; Resnick et al., 2009). While clinical research cannot easily elucidate to what extent the addition of a progestin or surgical removal of the uterus modulate these effects, preclinical rodent work is able to provide further insight with its increased experimental controls (Bimonte-Nelson et al., 2021; Koebele & Bimonte-Nelson, 2016). Only recently has a preclinical rodent model of hysterectomy been developed to address such critical questions. We have shown learning and memory deficits using our novel rodent model of hysterectomy compared to sham controls (Koebele et al., 2019) that persist over time (Koebele, 2019), and have demonstrated unique cognitive outcomes dependent upon age at surgery and ovarian status (see Chapters 4 and 6). Where the preclinical literature has found that estrogen-containing HT can be beneficial for memory and anxiety-like outcomes in models of ovariectomy (Acosta, Mayer, Talboom, Zay, et al., 2009; Bimonte & Denenberg, 1999; Koebele, Nishimura, et al., 2020), memory effects have been shown to differ depending upon menopause etiology (Acosta et al., 2010; Koebele, Mennenga, et al., 2020). Notably, these evaluations all include uterus-intact subjects, making the

clinical translation of these observed outcomes somewhat limited. Critically, there has yet to be such determination with HT administration following hysterectomy.

The current study evaluated the cognitive and anxiety-like behavioral effects of E2 administration in two preclinical rodent models of surgical menopause: ovariectomy and hysterectomy. Middle-aged female rats underwent Sham, Ovx, or Hysterectomy surgery and, after a period of three weeks, received E2 or Vehicle treatment for 21 days before being evaluated on a behavioral battery that assessed spatial working and reference memory, cognitive flexibility, and anxiety-like behaviors. Additional physiological measures, including ovary and uterus weights, were performed at euthanasia to assess the impacts of E2 treatment on remaining reproductive organs. Blood serum collected at euthanasia was evaluated for levels of two critical inflammatory markers, TNF-alpha and interleukin 6 (IL-6), to better characterize the impact of two surgical menopause variants, with or without subsequent E2 treatment, on peripheral inflammation. Finally, relationships between inflammatory and memory outcomes were assessed to characterize the potential role of the immune system in modulating behavioral outcomes associated with surgical menopause.

Methods

Subjects

Sexually-inexperienced female Fischer-344 CDF rats (N=50), aged 11-12 months, arrived at the vivarium facilities in Arizona State University within the Department of Psychology from the National Institute on Aging colony at Charles Rivers Laboratories (Raleigh, NC). Upon arrival, rats were pair-housed randomly, and provided with food and water *ad libitum*. Rats were kept on a 12-hour light/dark cycle (lights on 7:00AM)

throughout the duration of the study. Before the initiation of the experiment, rats were given one week to acclimate to the vivarium facility. After the experiment began, weekly body weights were collected to track animal health, as well as to evaluate the impact of subsequent ovarian hormone and surgical interventions. All procedures were performed following the standards set by the National Institutes of Health, and with the approval of the ASU Institutional Animal Care and Use Committee. For a full experimental timeline, see Figure 55.

Surgical Models of Menopause

One week after arrival, all rats underwent one of three surgeries: Sham (n=9), Hysterectomy (Hyst; n=20), and Ovariectomy (Ovx; n=21), which followed established laboratory protocols (Koebele et al., 2019). For all surgical procedures, rats were anesthetized using inhaled isoflurane. For pain management, all rats received 1.0mg/kg meloxicam and 1.2mg/kg buprenorphine SLR; additionally, all rats received post-surgical hydration in the form of 5.0ml isotonic solution. All surgeries involved a midline incision through the skin and peritoneum; for sham surgeries, this was the end of the surgical manipulation. Rats undergoing hysterectomy surgery had uterine horns ligated and removed below the ovary, preserving the oviduct, before being detached from the abdominal fat down the length of the horn. At the utero-cervical junction, the uterus was ligated and cut above the cervix at the base of the uterine body. Rats undergoing Ovx surgery had ovaries removed by separating the abdominal rat around the ovary before the uterine horn was ligated and cut to separate the ovary from the body cavity on each side. For all surgical groups, the muscle incisions were closed via dissolvable Vicryl suture, and bupivacaine was applied to the muscle surface before skin closure via surgical

staples. Following surgery, rats recovered under a heat lamp and were placed into single-housed cages; rats remained single-housed for 7-10 days following surgery. Following this period, animals were re-pair housed with their original cage mates. Three rats in the Ovx group and one rat in the Hysterectomy group died following surgery. An additional Hysterectomy-treated rat died in the week following surgery, but from a surgically-unrelated eye ulcer as confirmed by the staff veterinarian.

Vaginal Cytology – Part I

Two weeks after surgery, vaginal smears were conducted each day across a period of eight days to characterize the impact of these gynecological surgeries on estrous cyclicity. Vaginal smears were classified according to published protocols (Goldman et al., 2007) and done in prior work from our laboratory (Koebele et al., 2019; Prakapenka et al., 2018). There were 4 stages of smear classification: proestrus, estrus, metestrus, or diestrus. Proestrus smears were classified by the presence of clustered round epithelial cells, with or without the presence of cornified cells. Estrus smears were classified by the presence of cornified cells. Metestrus was classified by a mixed presence of cornified cells, round cells, leukocytes, and needle-like cells. Diestrus was classified by the presence of leukocytes, with possible cornified cells or round cells. In Ovx rats, a lack of cells was quantified as “blank”.

Osmotic Pump Surgeries

Three weeks following initial surgeries, rats underwent surgeries to receive osmotic pumps (Alzet; 2006 model) filled with either Vehicle (polyethylene glycol; n=30) or E2 (n=20). E2 levels in these osmotic pumps were scheduled to release 3ug daily across a period of six weeks; this dose was evaluated in prior work (Prakapenka et

al., 2018), where cyclic administration via subcutaneous injection resulted in cognitive benefits following Ovx. Rats were anesthetized via isoflurane before an incision was made into the skin on the animals left side just below the neck scruff. A pocket was made within the subcutaneous space before Vehicle or E2 pumps were inserted, with the end that released drug pointing away from the incision site. The skin was then closed using 3-4 interrupted Vicryl sutures. Pain from surgery was managed with 5mg/kg Rimadyl, and animals were given 2ml saline post-operatively for hydration. Following surgery, animals were dingle-housed for 48 hours, before being re-pair housed with their original cage mates. With surgical pumps in place, the experiment was comprised of the following treatment groups: Sham-Vehicle (n=9), Ovx-Vehicle (n=9), Ovx-E2 (n=9), Hysterectomy-Vehicle (n=10), and Hysterectomy-E2 (n=8).

Vaginal Cytology – Part II

Two weeks after surgery, rats underwent additional analyses of vaginal cytology for 4 days to determine the impact of Vehicle or E2-contiaining osmotic pumps on estrus cyclicity. Procedures followed the same as those listed above in "Vaginal Cytology – Part I".

Behavioral Battery

Three weeks after insertion of the osmotic pumps, rats underwent behavioral testing to evaluate spatial working and reference memory performance, as well as cognitive flexibility and anxiety-like behaviors.

Water radial-arm maze (WRAM). The win-shift version of the WRAM was used to evaluate spatial working and reference memory performance (Bimonte-Nelson et al., 2015). The WRAM is an eight-arm apparatus, filled with 18-20°C water made opaque

with black nontoxic paint. The room that the WRAM is situated is surrounded by robust extra-maze cues to aid in spatial navigation. In the maze, within 4 arms, platforms are submerged under the surface. These platform locations are semi-randomized across subjects, but remained fixed for a given subject across all testing days.

To initiate a trial, a rat is released from the starting arm and given three minutes to locate a platform. If a rat did not find a platform within the allotted time, it was led to the nearest platform location. Once a platform is located, the rat remains there for 15 seconds to spatially localize before being placed into its heated testing cage. This initiates a 30 second inter-trial-interval (ITI), where the recently-found platform is removed, and the maze is swept for odor cues and debris. After the ITI, the rat is placed back into the starting arm, and navigates to find one of the remining platforms. Trials continue until all four platforms are located, resulting in an incremental increase in working memory load across all four trials each day, where animals need to remember not to visit arms where platforms were previously found. Testing continued in this fashion for 12 days.

WRAM performance was quantified for each trial by totaling errors, or entries into non-platformed arms. Entries were counted if the rat's snout passed 11cm into the opening of the arm, clearly designated on the outside of each maze arm. Errors were divided into three subtypes to better characterize working and reference memory performance separately, as done previously in our laboratory (Bernaud et al., 2021; Bimonte-Nelson, 2015d; Braden, Andrews, et al., 2017; Koebele et al., 2019). An entry into a previously-platformed arm was counted as a working memory correct (WMC) error. Entries into non-platformed arms for the first time within a testing day were

quantified as reference memory (RM) errors. Finally, all subsequent entries into non-platformed arms within a day are counted as working memory incorrect (WMI) errors.

Morris water maze (MWM). Following a one-day break in behavior testing after the completion of WRAM evaluation, rats were tested on the MWM, a measure of spatial reference memory performance and cognitive flexibility (Bimonte-Nelson, 2015b; Braden et al., 2011; Morris et al., 1982). The MWM apparatus consists of a large circular tub (diameter = 188 cm) filled with 18-20°C water obscured with black non-toxic paint. In the northeast (NE) quadrant of the maze, submerged under the water's surface, is a hidden platform. Across the first four days of testing, the platform remained in this location for all baseline trials. In the room, robust extra-maze cues were placed around the maze to aid in spatial navigation.

To begin the task, a rat is dropped off at one of the starting locations (North, South, East, West), which are semi-randomized across testing days, and given one minute to navigate to the hidden platform. If the rat did not find the platform within the allotted time, the rat was guided to the platform. Spending 15 seconds on the platform to spatially localize, the rat was then placed into its heated testing cage. The maze was then swept for debris and odor cues before the next rat began its testing trial, making each rat's ITI approximately 10 minutes. Rats were tested in this manner for 4 trials each day across 4 days. A fifth probe trial was added to the 4th day, where the platform was removed, and animals were allowed to freely swim for 60 seconds; this assessed spatial localization to the platform. Another sixth trial was evaluated with the platform placed back into the NE quadrant to eliminate the possibility of extinction. An additional 5th day of MWM testing was performed, but with the platform moved from the NE quadrant to the SW quadrant.

Rats were tested for 4 trials with this new platform location to assess cognitive flexibility in spatially localizing to the novel platform location. An additional 5th trial was evaluated, this time with the probe for the novel SW location. For each trial, swim distance (cm) and latency (s) were tracked using Ethovision (Noldus Instruments).

Elevated plus maze (EPM). The day following the completion of MWM testing, rats were evaluated on the EPM, which assesses anxiety-like behavior (Camp et al., 2012; Hiroi et al., 2016). The EPM apparatus consists of four arms, two opposing arms enclosed by large opaque walls, and two opposing arms exposed and open to the environment. A curtain enclosed the maze, obscuring it from extra-maze cues. To begin a trial, rats were placed at the center of the maze, semi-randomized in the direction of the open arms they faced. Rats were allowed to freely explore the apparatus for 5 minutes. The maze was cleaned between rats. Time spent (s) in the open arms, closed arms, and maze center was tracked via Ethovision (Noldus Instruments).

Euthanasia

Rats were euthanized following a one-day break in testing. At euthanasia, final body weights were collected. Then, rats were deeply anesthetized via isoflurane, and cardiocentesis was performed to collect blood samples. Brains were extracted, and brain regions related to learning and memory outcomes, including right hemisphere Frontal Cortex (FC), were raw dissected according to plate designations (Paxinos & Watson, 1998). In addition, uteri and ovaries for all intact rats were removed, trimmed of excess fat, and individually weighed to assess the effects of surgical menopause and hormone exposure (Koebele et al., 2019; Prakapenka et al., 2018).

Serum Assays

At euthanasia, blood samples were placed into collection tubes and stored on ice for a minimum of 30 minutes before serum was separated via centrifugation at 10,000prm for 10min at 4°C. Serum samples were then aliquoted and stored at -20°C until ELISAs were performed to evaluate IL-6 (R & D Systems; CAT#SR6000B) and TNF-alpha (R & D Systems; CAT#SRTA00). All serum samples were run neat (1:1) across two plates, counterbalanced across the plates by treatment group. Assay protocols provided by the manufacturer were followed for both kits. Color change was detected for each plate using a plate reader (BioTek), set to 450nm, and subsequent data were analyzed using Gen5 software. ELISA results were then transformed into log (concentration + 1) values before statistical analysis was carried out. Intra-assay CVs for IL-6 kits were 20.30% and 65.07%, and the inter-assay CV was 10.26%. For TNF-alpha, intra-assay CVs were 45.98% and 72.29%, and the inter-assay CV was 1.16%.

Statistical Analyses

All statistical analyses were completed using StatView software. For all behavioral and physiological analyses, except those from the MWM probes and serum ELISAs, the following set of planned comparisons were carried out to best characterize the effects of surgical menopause models and subsequent E2 administration: Sham-Vehicle vs. Ovx-Vehicle, Sham-Vehicle vs. Hyst-Vehicle, Ovx-Vehicle vs. Ovx-E2, and Hyst-Vehicle vs. Hyst-E2. All analyses were two-tailed, wherein the alpha level was set to 0.05. Results were designated as marginal if the p value was between 0.05 and 0.10.

WRAM data were separated into three separate testing blocks to distinguish the Early Acquisition Phase (Days 2-4), the Late Acquisition Phase (Days 5-9), and the Asymptotic Phase (Days 10-12), as done in prior work in our laboratory (Bimonte-

Nelson et al., 2015; Koebele et al., 2019; Prakapenka et al., 2018). Data were analyzed using a series of repeated measures ANOVA, with Treatment as the independent variable, Trials within Days as the repeated measures, and WMC, WMI or RM errors as the dependent variable.

MWM data for all platformed trials on Days 1-4 were analyzed using a repeated measures ANOVA, where Treatment served as the independent variable, Trials within Days were the repeated measures, and Swim Distance (cm) or Latency (s) to the Platform served as the dependent variable. For the platformed trials on the 5th day of MWM testing, the same form of repeated measures ANOVA was performed. Finally, for both sets of probe trials, ANOVA were performed for each group individually, where percent Swim Distance served as the dependent measure, and the Target (Platformed) vs. Opposite Quadrants served as the repeated measure.

From the EPM, data were analyzed using ANOVA, where Treatment served as the independent variable, and Time (s) spent in the Open Arms, Closed Arms, and Center served as the dependent variable.

Body weight data collected at euthanasia were analyzed using ANOVA, with Treatment serving as the independent variable, and Body Weight (g) serving as the dependent variable.

Ovary weight data collected from all rats in the Sham or Hyst groups at euthanasia were analyzed using ANOVA, with Treatment serving as the independent variable, and Summed Weight (g) of each ovary (R+L) serving as the dependent variable.

Uterine weight data collected from all Sham- and Hyst-treated rats were analyzed as ANOVA, where Treatment served as the independent variable, and Wet Weight (g) served as the dependent variable

Data from serum ELISAs were analyzed via ANOVA, with Treatment as the independent variable and log (concentration + 1) (pg/mL) as the dependent variable. Due to the exploratory nature of these analyses, a post hoc decision was made to additionally analyze a comparison between Sham-Vehicle and Ovx-E2 groups.

Pearson r correlations were conducted with Fisher's r-to-z transformations to determine statistical significance of relationships between serum inflammatory markers, as evaluated via ELISA, and WRAM performance for each treatment group. Corrected serum concentrations of TNF-alpha and IL-6 (pg/ml) expression were correlated with WMI and WMC errors, averaged across trials, for each phase of WRAM testing. These correlations were performed within each treatment group (i.e., intraclass correlations). Corrections for multiple comparisons were not performed for these correlations due to their exploratory nature, but correlations were only deemed significant if they had values of $p < 0.01$.

Results

Vaginal Cytology – Part I and Part II

During the first set of vaginal smears, all Sham rats displayed normal estrous cycling. Hysterectomy-treated rats similarly displayed a normal 4-5 day estrous cycle, as shown in prior literature (Koebele et al., 2019). All Ovx-treated rats had blank smears, or few leukocytes and small cornified cells if any cells, as established in prior work

following surgical ovary removal and ovarian hormone cessation (Koebele et al., 2019; Prakapenka et al., 2018).

Following osmotic pump insertion, all animals that received pumps containing E2 (i.e. Ovx-E2 and Hyst-E2) displayed cornified cells, indicating persistent estrus smears, as seen in prior work evaluating estrogen treatment outcomes (Prakapenka et al., 2018). Rats in the Sham-Vehicle and Hysterectomy-Vehicle groups displayed typical estrous cyclicity, indicating that pump insertion did not disrupt ovarian cyclicity. Ovx-Vehicle rats continued to display blank smears.

WRAM

During the Early Acquisition Phase of the WRAM (Days 2-4), planned comparisons between the Ovx-Vehicle and Ovx-E2 groups for WMC errors revealed a main effect of Treatment [$F_{(1,16)} = 5.364$, $p < 0.05$; Figure 56A], with E2 administration resulting in fewer working memory errors in Ovx rats. Additionally, there was a significant Trial x Treatment interaction for this planned comparison [$F_{(2,32)} = 3.725$, $p < 0.05$; Figure 56A], as well as the comparison between Sham-Vehicle and Ovx-Vehicle groups [$F_{(2,34)} = 4.084$, $p < 0.05$; Figure 56A]. When evaluating the highest working memory load trial, Trial 4, there was a marginal effect of Treatment for the Sham-Vehicle vs. Ovx-Vehicle planned comparison [$F_{(1,17)} = 4.375$, $p < 0.10$; Figure 56B], with Ovx resulting in marginal deficits in working memory during task acquisition. Additionally, an effect of Treatment was found for Ovx-Vehicle and Ovx-E2 groups [$F_{(1,16)} = 5.535$, $p < 0.05$; Figure 56B], with E2 rescuing Ovx-induced working memory deficits.

When evaluating WMI errors during the Early Acquisition Phase, planned comparisons of Sham-Vehicle and Ovx-Vehicle rats showed a Trial x Treatment

interaction [$F_{(3,51)} = 3.287$, $p < 0.05$; Figure 57A]. When evaluating Trial 4, the high working memory load trial, there was a marginal effect of Treatment [$F_{(1,17)} = 3.465$, $p < 0.10$; Figure 57B], where there was a tendency for Ovx-induced memory deficits.

RM evaluations during the Early Acquisition Phase of the WRAM revealed a main effect of Treatment for the Sham-Vehicle vs. Ovx-Vehicle planned comparison [$F_{(1,17)} = 6.922$, $p < 0.05$; Figure 58], where surgical removal of the ovaries resulted in reference memory deficits during task learning. There were no other Treatment effects or Trial x Treatment interactions across any error type for any other planned comparison during the Early Acquisition Phase.

Analyses for the Late Acquisition Phase did not reveal any Treatment effects or Trial x Treatment interactions for any error type.

During the Asymptotic Phase of the WRAM, analyses for the Sham-Vehicle vs. Hyst-Vehicle planned comparison revealed a marginal Trial x Treatment interaction for WMC errors [$F_{(2,34)} = 2.896$, $p < 0.10$; Figure 59A]; no subsequent effects of Treatment were found for any individual trial. For WMI errors, there was a Trial x Treatment interaction for the Hyst-Vehicle vs. Hyst-E2 planned comparison [$F_{(3,45)} = 2.839$, $p < 0.05$; Figure 59B]; again, no individual trial analyses revealed a Treatment effect. There were no other Treatment effects or Trial x Treatment interactions for this phase of testing.

MWM

MWM performance across Days 1-4, where the platform remained in the NE Quadrant, there was a main effect of Day for all planned comparisons [Sham-Vehicle vs. Ovx-Vehicle: $F_{(3,51)} = 22.555$, $p < 0.0001$; Sham-Vehicle vs. Hyst-Vehicle: $F_{(3,51)} = 24.646$, $p < 0.0001$; Ovx-Vehicle vs. Ovx-E2: $F_{(3,48)} = 22.545$, $p < 0.0001$; Hyst-Vehicle

vs. Hyst-E2: $F_{(3,45)} = 8.951$, $p < 0.0001$], with decreasing swim distance to the platform across testing days, indicating learning on this spatial reference memory task (Figure 60A). However, there was no effect of Treatment for any planned comparison for either swim distance to the platform or swim latency.

Analyses of the probe trial for the NE platform location revealed an effect of Quadrant for each treatment group [Sham-Vehicle: $F_{(1,9)} = 54.126$, $p < 0.0001$; Ovx-Vehicle: $F_{(1,8)} = 70.164$, $p < 0.0001$; Hyst-Vehicle: $F_{(1,8)} = 108.167$, $p < 0.0001$; Ovx-E2: $F_{(1,8)} = 19.047$, $p < 0.01$; Hyst-E2: $F_{(1,7)} = 29.066$, $p < 0.01$], where rats spent a greater percent of swim distance in the Target Quadrant (NE) than the Opposite Quadrant (SW) (Figure 60B); this indicates spatial localization of the platform.

Analyses of performance on Day 5, whereby the platform was re-located to the SW Quadrant, reveal no significant effects of Treatment (Figure 61A). Probe trial evaluations at the conclusion of Day 5 demonstrate significant Quadrant effects in the Ovx-Vehicle group [$F_{(1,8)} = 21.258$, $p < 0.01$], and marginal effects in the Sham-Vehicle group [$F_{(1,9)} = 5.003$, $p < 0.10$] and Hyst-E2 group [$F_{(1,7)} = 4.710$, $p < 0.10$]; however, these effects demonstrate more time spent in the location of the original platform location (NE) than the new platform location (SW) (Figure 61B).

EPM

When evaluating time spent in the open arms on the EPM, there was an effect of Treatment for the Sham-Vehicle vs. Ovx-Vehicle planned comparison [$F_{(1,17)} = 4.507$, $p < 0.05$], where Ovx rats spent more time in the open arms of the maze than their Sham counterparts, an indication of reduced anxiety-like behavior (Figure 62A). There were no other effects for any planned comparison for time spent within the open arms of the EPM.

Evaluating time spent in the closed arms of the EPM likewise revealed a marginal Treatment effect for the Sham-Vehicle vs. Ovx-Vehicle planned comparison [$F_{(1,17)}$ = 3.335, $p < 0.10$], where Ovx rats tended to spend less time in the closed arms of the maze (Figure 62B). There were no other Treatment effects across any planned comparisons for time spent within the closed arms of the maze.

There were no Treatment effects for time spent in the center of the maze for any planned comparison (Figure 62C).

Body, Ovary, and Uterine Weights at Euthanasia

Analyses of body weights collected at euthanasia revealed significant effects of Treatment for the Sham-Vehicle vs. Ovx-Vehicle planned comparison [$F_{(1,17)} = 9.621$, $p < 0.01$] as well as the Ovx-Vehicle vs. Ovx-E2 planned comparison [$F_{(1,16)} = 15.188$, $p < 0.01$]; E2 administration reversed the Ovx-induced weight gain, relative to Sham rats (Figure 63A).

For the summed weight of ovaries collected at euthanasia from Sham and Hyst-treated rats, there were no main effects of Treatment across any planned comparison (Figure 63B).

Uterine weighs from rats that underwent Sham or Ovx surgery revealed a main effect of Treatment for the Sham-Vehicle vs. Ovx-Vehicle [$F_{(1,17)} = 25.168$, $p < 0.001$; Figure 63C] and Ovx-Vehicle vs. Ovx-E2 [$F_{(1,16)} = 40.098$, $p < 0.0001$; Figure 63C] planned comparisons, where Ovx resulted in a significant decline in uterine weight, and E2 resulted in a sharp increase in uterine weight due to hormonal stimulation.

Serum Inflammatory Markers

Serum ELISAs for IL-6 revealed a main effect of Treatment for the Sham-Vehicle vs. Ovx-E2 planned comparison [$F_{(1,17)} = 6.097$, $p < 0.05$; Figure 64A], whereby E2 with the absence of other ovarian hormones resulted in a decrease in levels of this pro-inflammatory cytokine relative to ovary-intact, cycling rats. Serum analyses for TNF-alpha levels did not reveal any Treatment effects (Figure 64B).

Correlations between Serum Inflammatory Markers and WRAM Performance

Correlations between WRAM performance serum IL-6 revealed no significant relationships across any treatment group, for any error type or testing phase. Analyses of relationships between WRAM performance and serum TNF-alpha revealed a significant positive correlation between WMI errors made during the Early Acquisition Phase and TNF-alpha levels for Sham-Vehicle rats [r (8) = 0.75, $p < 0.01$], such that increasing levels of this pro-inflammatory marker were associated with greater working memory errors made during task learning (Figure 65A). Correlation analyses also revealed a significant negative correlation between serum TNF-alpha levels and WMI errors made during the Late Acquisition Phase for E2-treated hysterectomized rats [r (6) = -0.86, $p < 0.01$], with increasing TNF-alpha levels associated with improved working memory performance (Figure 65B).

Discussion

The current study evaluated the cognitive, anxiety-like, and inflammatory effects of E2 administration in two preclinical rodent models of surgical menopause. While preclinical surgical menopause research up to this point has emphasized use of the Ovx model to characterize the effects of E2 administration (Abu-Taha et al., 2009; Bimonte & Denenberg, 1999; Daniel et al., 2006; Gibbs, 1999; Suzuki et al., 2007), this study

expands upon prior work to additionally assess the role of E2 in modulating outcomes following hysterectomy. Furthermore, inclusion of Ovx groups allows for a comparison across these two surgical menopause models with and without E2 administration. The results illustrate E2's ability to reverse Ovx-induced working memory deficits, but not modulation of anxiety-like behaviors with Ovx. Furthermore, analyses show marginal hysterectomy-induced deficits in response to an increasing working memory demand, with E2 ameliorating these effects. Finally, inflammatory analyses revealed a unique impact of E2 following Ovx compared to the milieu of circulating ovarian hormones. These findings provide insight into the beneficial effects of E2 for learning and memory outcomes across both models, and can inform the future of clinical work surrounding HT outcomes with differing menopause etiologies.

Overall, rats in the current experiment demonstrated adequate performance of spatial learning and memory tasks on both the WRAM and MWM. Indeed, analyses of the first MWM probe trial revealed that all rats were able to learn to spatially localize to the platform, and trial effects on the WRAM suggest that rats were responsive to the increasing working memory demand, as observed in prior literature from our laboratory evaluating models of surgical menopause (Koebele, 2019; Koebele et al., 2019) as well as E2 treatment (Bimonte & Denenberg, 1999). Furthermore, body, ovarian, and uterine weights at euthanasia revealed a consistent pattern of effects observed in prior work, where E2 attenuated Ovx-induced body weight increases, and compensated for Ovx-induced uterine atrophy (Bimonte & Denenberg, 1999; Koebele et al., 2019; Prakapenka et al., 2018). Hysterectomy, consistent with prior work, did not alter body or ovarian weight outcomes (Koebele et al., 2019; see Chapter 4).

For the Early Acquisition Phase of the WRAM, it was clear that Ovx resulted in a pattern of both working memory and reference memory impairments. This finding is a replication of some prior work using the Ovx model (Bimonte & Denenberg, 1999). However, others have reported Ovx-induced facilitation of learning and memory outcomes (Bimonte-Nelson et al., 2003; Koebele et al., 2019) or no such memory effects (see Chapter 7); much of this work appears to be dependent upon age at Ovx, time since surgery, and memory type (Koebele & Bimonte-Nelson, 2016, 2017). While WRAM results indicate reference memory impairment with Ovx, no MWM effects on reference memory performance were observed between Ovx and Sham groups. This seeming inconsistency could be due to the ordering of these tasks, as prior work in our laboratory has shown that the first task in a behavioral battery can impact performance on subsequent tasks (Koebele, Quihuis, et al., 2021). In addition, other work in our laboratory has shown interactions between working and reference memory constructs on the WRAM (Bernaud et al., 2021), suggesting that the two reference memory measures across the WRAM and MWM may not mirror one another when a working memory deficit is observed. Notably, E2 administration rescued the Ovx-induced working memory impairments observed in learning; this effect has been widely established in the literature with the Ovx model (Bimonte & Denenberg, 1999; Koebele, Nishimura, et al., 2020; Prakapenka et al., 2018), particularly when E2 is administered shortly after surgical menopause onset (Daniel et al., 2006; Hiroi et al., 2016). These findings add to the literature suggesting that E2 administration provides cognitive benefits in the Ovx model of surgical menopause, reflecting seminal preclinical estrogen-based HT evaluations.

While E2 ameliorated Ovx-induced cognitive deficits during learning, the impact of E2 on hysterectomy-induced cognitive effects was observed at the end of WRAM testing. For the Asymptotic Phase of testing, there were indications of hysterectomy-induced impairments to handle the increasing working memory load. While prior work has found significant impairment with hysterectomy (Koebele, 2019; Koebele et al., 2019), particularly at this middle-aged timepoint (see Chapter 4), the blunted treatment effects observed here may be the result of experimental strains in Sham subjects. Indeed, it is possible that the additional surgery, including pump insertion, modulated behavior in hysterectomy and sham rats such that behavioral impairment was somewhat obscured. Tonic administration of E2, however, resulted in an improved ability to handle the increasing working memory load, as with increasing trials, E2-treated hysterectomized rats had a divergent pattern of working memory errors. These findings suggest that HT, or specifically E2, may provide cognitive benefits to those that experience hysterectomy before menopause onset. Future work should evaluate whether extended treatment paradigms provide lasting cognitive benefit in hysterectomized rats, as cognitive deficits have been shown to persist for up to one year following surgery (Koebele, 2019).

Reference memory performance evaluations on the MWM revealed no significant effects for either surgical menopause model, with or without E2 treatment. This reflects prior evaluations of both surgical menopause models (Koebele et al., 2019; Chapters 4 and 7) and E2 treatment (Prakapenka et al., 2018) from our laboratory, which did not reveal effects on MWM. However, all groups did demonstrate learning of the MWM task for the first four days, with a main effect of Day and significantly greater time spent on the first probe trial in the platform location. When considering the cognitive flexibility

element added on the final day of MWM testing, it appears that animals did not sufficiently learn to update their search from the NE quadrant to the SW quadrant for the platform. There is no clear trial effect for this final day of testing, indicating that rats did not learn to search for the platform in its new location. At the second probe trial, no group swam a greater percent distance within the newly platformed quadrant; instead, several groups favored the quadrant that used to contain the platform (NE). In prior work evaluating cognitive flexibility, 8 trials were given with this novel platform location before the final probe trial (Braden et al., 2011); future work may need a longer training period to elucidate cognitive flexibility in rats following surgical menopause, particularly at the middle-age timepoint.

Similar to the MWM, anxiety-like behavior evaluations via the EPM revealed an unexpected pattern of effects. EPM outcomes in prior literature would suggest either increased anxiety-like behavior (Campos et al., 2020) or no significant effects on anxiety-like behavior (Marcondes et al., 2001; Nomikos & Spyraki, 1988) following Ovx; however, the current study demonstrated a lower anxiety profile with surgical ovary removal, where Ovx rats spent greater time in open arms and marginally less time in closed arms as compared to Sham rats. These effects were unexpected, but may be related to the duration of ovarian hormone cessation prior to evaluation. Prior work has shown that time since Ovx is a critical variable that explains the differential learning and memory outcomes associated with Ovx, particularly observed with aged rats (Bimonte-Nelson et al., 2003). There are some indications that time since Ovx plays a critical role in EPM outcomes (Puga-Olguín et al., 2019; Rodríguez-Landa, 2022), but these findings generally suggest that Ovx results in increased anxiety-like behavior with longer periods

of hormone deprivation. Instead, the observed Ovx effects could also be attributable to changing hormonal profile with reproductive aging. There is considerable evidence that the background milieu plays an important role when contextualizing Ovx effects. Indeed, while young adult rats have shown Ovx induced working memory deficits on the WRAM (Bimonte & Denenberg, 1999), the oldest rats have shown working memory enhancements when evaluated following an extended period after surgery (Bimonte-Nelson et al., 2003). This study suggests that the removal of progesterone and androstenedione with Ovx, which is higher in aged rodents undergoing estropause, can be facilitative for learning and memory outcomes (Bimonte-Nelson et al., 2003). As higher progesterone levels are associated with increased anxiety-like behaviors (C. A. Frye et al., 2000), it is possible that removal of higher endogenously circulating progesterone with Ovx in middle-age results in overall reduction in anxiety-like behaviors. Analysis of serum ovarian hormone levels could support this finding and better characterize the role of the hormonal profile in midlife in subsequent cognitive and affective outcomes. Additionally, future work should expand the battery of anxiety-like and depressive-like behavioral evaluations to provide a more comprehensive picture, or integrate more complex and translational modelling, such as social defeat (Duman, 2010). These additions would aid to better assess mood changes with differing menopause etiologies, as well as whether HT can combat these symptoms and provide protection against risk of depression and anxiety disorders.

Serum inflammatory evaluations provide unique insight into the role of ovarian hormones in modulating immune functioning. Where prior work has demonstrated increases in serum pro-inflammatory markers with surgical ovary removal, both

preclinically and clinically (Abu-Taha et al., 2009; Pacifici et al., 1991; Pfeilschifter et al., 2002), the current study found no such effect for serum levels of IL-6 or TNF-alpha. As stated for EPM results, it is possible that evaluations in middle-age, with a changing hormonal profile as Sham rats begin estropause, resulted in differential inflammatory outcomes. However, it is also possible that other inflammatory markers are being affected, and require further investigation. Results for Ovx rats treated with E2 appear to demonstrate that hormonal milieu is important when considering inflammatory outcomes, as E2 in the absence of other ovarian hormones reduced serum IL-6 compared to ovary-intact rats. Indeed, it appears that the presence of other hormones, such as elevated progesterone and androgens, may obscure E2's anti-inflammatory effects in the middle-aged rat; with ovarian removal and E2 supplementation, these effects are made clear. There is some evidence that estrogens and progestogens can have anti-inflammatory actions alone (Hall & Klein, 2017), but unique interactions may be present across reproductive cycling and with aging, requiring further evaluation. The fact that hysterectomy, with or without E2 administration, did not result in modulation of serum inflammatory markers follows the small amount of literature that has evaluated the inflammatory impact of hysterectomy, showing no long-term changes in peripheral inflammation following surgery (Pacifici et al., 1991). However, further work should be done to characterize the role of the immune system in long-term outcomes with various menopause etiologies, both in the periphery as well as in the brain. The immune system is a complex network of defense, made up of various types of cells that interact to produce both anti- and pro-inflammatory actions on various tissues (Spiering, 2015). Given the novel nature of this work, other more exploratory analyses, such as RNA sequencing of

brain, ovarian, and uterine tissues, could provide specific immune cell targets or pathways for further investigation.

Correlational analyses form the current study reveal interesting relationships between pro-inflammatory cytokine TNF-alpha and working memory outcomes for distinct treatment groups. Analyses in Sham-Vehicle rats revealed a relationship between greater proinflammatory cytokine levels in blood serum and poorer working memory outcomes; both increases in basal inflammation (Gameiro et al., 2010) and a decline in spatial working and reference memory capacity (Bernaud et al., 2021; Bimonte et al., 2003) are commonly observed at later ages in rats, and support the hypothesized link between immunosenscence and cognitive dysfunction with aging (M. A. Erickson & Banks, 2019). Furthermore, the lack of a relationship for any other group suggests that the endogenous ovarian hormone profile at midlife may play a role in establishing such ties between immune function and cognition, as suggested in prior literature (Gameiro et al., 2010). The correlation found for E2-treated hysterectomized rats between increased serum levels of TNF-alpha and improved working memory outcomes was unexpected. This relationship displays a critical shift from that of Sham rats, and suggests that HT for this unique form of surgical menopause should be further explored. The uterus is known to play a key role in estrogen signaling (Yu et al., 2022) and immune signaling (Agostinis et al., 2019) across reproductive cycling and in pregnancy; uterine removal may result in a disruption of behavioral-immune relationships that allows for differing re-organization with E2. Further work must be done to understand this relationship in the broader perspective of inflammation and cognition with HT use in this unique population.

The current study provides insight into the impact of E2 administration on memory, inflammatory, and anxiety-like outcomes with two preclinical menopause models: Ovx and hysterectomy. Given the unique effects observed here, further investigation into other menopause models is warranted. Indeed, the Ovx+Hysterectomy model of menopause, wherein the uterus and ovaries are concurrently removed, provides a better translational model for the population of women without ovaries that are able to take an estrogen-only HT. Prior work has shown unique cognitive effects in this model at the middle-age timepoint (see Chapter 4), but further evaluations should expand to explore the affective and immune outcomes associated with this form of surgical menopause. Additionally, evaluation of E2 effects in hysterectomized rats would be made more translational through the use of the 4-vinylcyclohexene diepoxide (VCD) model of transitional menopause. VCD administration in the female rat results in ovarian follicular depletion and alteration in the ovarian hormone profile that more closely models transitional menopause (Koebele & Bimonte-Nelson, 2016), as compared to our current ovary-intact evaluations. Through the use of these two models of menopause, outcomes associated with E2 administration may more closely model those of hysterectomized women taking HT during menopause. Beyond evaluations of alternative menopause models, assessments of different estrogens found in HT would also be insightful. Estrogens such as CEE, commonly found in HT and evaluated clinically in the WHI, have been evaluated preclinically, where differing cognitive outcomes were observed across the Ovx and VCD models of menopause (Acosta et al., 2010). Such work, along with findings from the current study, can provide key insights to clinical researchers

evaluating HT outcomes in hysterectomized women, with the potential to optimize care for this population through menopause and beyond to optimize aging outcomes.

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CHAPTER 9

Selections of this chapter are inspired by published review articles (Bimonte-Nelson et al., 2021, Climacteric) or articles under review:

GENERAL SUMMARY AND CONCLUSIONS

Rodents have been used for decades as preclinical models to aid characterization of human anatomy, physiology, and behavior. Considering the trajectory of female reproductive aging, rodents in some ways make an excellent model, with many similarities. Indeed, female rodents experience ovarian cyclicity that has discrete stages, with a similar endocrine profile to that of women (Bimonte-Nelson et al., 2021; Koebele & Bimonte-Nelson, 2016). Their comparable reproductive and brain anatomies allow for apt assessments of various forms of interventions, from surgical to pharmacological (Koebele & Bimonte-Nelson, 2016), and the rodent's ability to engage in complex cognitive processes allow for nuanced behavioral assessments (Bimonte-Nelson, 2015a). However, one key difference between these two species presents a challenge for those evaluating the latter phases of reproductive aging in females: their differing experience of reproductive senescence. Humans experience female reproductive senescence in the form of menopause, which occurs on average at age 52 (N. Santoro et al., 2021), and is clinically defined as one year of amenorrhea. Menopause is characterized by the breakdown of the hypothalamic-pituitary-gonadal (HPG) axis, with circulating estrogen and progesterone levels declining to near-undetectable levels coincident with increasing follicle stimulating hormone (FSH) and luteinizing hormone (LH), as well as a reduction in ovarian follicle count, leading to a state of ovarian failure (Bimonte-Nelson et al., 2021; Gracia & Freeman, 2018). Female rodents experience a distinct form of reproductive senescence, termed estropause. Estropause occurs at 10-12 months of age (Lu et al., 1979), and is characterized by HPG axis dysregulation, as well as a persistent estropause cycle phase, with moderate circulating estrogen levels and moderate to high circulating progesterone levels (Koebele & Bimonte-Nelson, 2016). Key to estropause,

the ovarian follicular pool is conserved, and rodents do not enter a state of ovarian failure (Koebele & Bimonte-Nelson, 2016).

While such differences may seem to make preclinical modeling of menopause ineffective, preclinical rodent models can provide opportunities for scientific insights not afforded by studying humans or in vitro. As rodents do not experience ovarian failure at midlife or coincident with chronological aging, researchers can use this to their advantage to initiate menopause through the use of various experimental models at any age necessary for the design of the experiment or the questions that it is attempting to answer (Bimonte-Nelson et al., 2021). Such models are an immense benefit to the field of menopause research. Indeed, menopause can be modeled surgically through the use of the ovariectomy (Ovx) model of menopause, wherein the ovaries are surgically excised; this model reflects oophorectomy, or surgical ovarian removal in women (Koebele & Bimonte-Nelson, 2016; Prakapenka et al., 2018). An additional surgical menopause model, hysterectomy, has been recently developed in our laboratory, wherein uterine removal has been shown to confer unique, long-term cognitive deficits (Koebele, 2019; Koebele et al., 2019). Finally, transitional menopause can even be modeled in the rodent using 4-vinylcyclohexene diepoxide (VCD), which instigates ovarian follicular depletion and results in an ovarian hormone profile more comparable to that of women undergoing the menopause transition (Acosta, Mayer, Talboom, Tsang, et al., 2009; Mayer et al., 2002, 2004).

Bearing in mind the various animal models of menopause, we now have fortuitous opportunities abound to experimentally induce follicular depletion, or surgically manipulate parts of the reproductive tract, at any age. In addition, we can

systematically combine experimental methodologies in animal models. This permits us to ask how particular reproductive structures interact to produce unique cognitive profiles or confer specific long-term health risks. For example, we can determine the impact of simultaneous surgical removal of the uterus and ovaries in combination, as compared to no surgical reproductive tract manipulation, or removal of only one of these structures alone (see Chapter 4). We also have the opportunity to ask questions systematically combining transitional and surgical menopause models; for instance, it can be determined whether the cognitive profile of surgical uterine removal changes depending on whether transitional menopause has occurred before surgery (see Chapter 6). In animal models, methodical, incremental, and stepwise assessments of putative influences can be implemented in individual or combined experimental protocols of menopause induction. Therein lies the beauty of preclinical menopause modeling: with a high degree of experimental control and the unique ability to initiate reproductive senescence at any point across the aging spectrum, we can feasibly assess questions pertaining to long-term cognitive outcomes that would take decades and thousands upon thousands of participants to similarly address in clinical research.

This dissertation has evaluated each of the preclinical models of menopause, spanning surgical, transitional, and interactive menopause etiologies, to better characterize the behavioral and physiological trajectory of female aging. Chapters 2 and 3 achieved this by evaluating learning and memory outcomes across key timepoints in chronological aging, both in models of neurologically typical aging (Chapter 2) as well as in a model of neurodegenerative disease (Chapter 3). Such work lays a foundation for understanding effects of reproductive senescence independent of aging outcomes.

Chapters 4-6 assessed different models of menopause, including Ovx, hysterectomy, and VCD. Chapter 4 explored how chronological aging might interact with the newest model of surgical menopause, hysterectomy, dependent upon whether the ovaries are concomitantly excised. Chapter 5 investigated three common methods for VCD administration to determine putative similarities and differences in cognitive and physiological profiles. Chapter 6 explored potential interactions between these two models, assessing whether prior ovarian follicular depletion alters hysterectomy-induced cognitive outcomes. Chapters 7 and 8 used these menopause models to more translationally assess outcomes related to hormone therapy (HT), the most common therapeutic used to treat menopausal symptoms (Shifren et al., 2019). Chapter 7 assessed the cognitive and neurobiological profile associated with administration of a novel progestin that was highly selective for the progesterone receptor in the Ovx model of surgical menopause. Chapter 8 evaluated similar outcomes, but with administration of 17-beta-estradiol (E2), a common estrogen found in HT, in both the Ovx and hysterectomy models of surgical menopause. Findings from this collection of work will help to contextualize patterns of symptoms or indications observed within the clinic, establish putative mechanisms behind such observations, and highlight critical variables for further clinical research. The following sections will summarize the consistent findings within this dissertation, and contextualize the most important variables discovered within this dissertation to need further research, both within the clinical and preclinical realms.

Effects of Chronological Aging versus Menopause on Learning and Memory Performance

Rodents have long been used to evaluate the cognitive and physiological outcomes associated with aging in humans (Barnes, 1979; Bettio et al., 2017; Finch, 2014). Whether assessing typical aging or the altered aging trajectory of neurodegenerative disease, spatial mazes can be useful tools in assessing learning and memory (Bimonte-Nelson, 2015a), generally showing age-related declines in both working memory and reference memory performance (Bizon et al., 2009; Frick et al., 1995). While a variety of mazes can be used to assess different cognitive domains, the water radial-arm maze (WRAM) is immensely useful, in that the WRAM can simultaneously measure spatial working and reference memory performance, as well as responsivity to an increasing working memory load (Bimonte-Nelson et al., 2015). Working and reference memory on the WRAM have been previously suggested to be orthogonal constructs (Bimonte-Nelson et al., 2003; Jarrard et al., 1984, 2012; Olton, 1979), but prior work in our laboratory indicates that the two memory types may interact (Mennenga, Gerson, Koebele, et al., 2015; Mennenga, Koebele, et al., 2015; Prakapenka et al., 2018).

Chapter 2 systematically evaluated whether the presence of a reference memory component on the WRAM impacts handling of an increasing working memory load, and how aging might further alter responsivity of these two memory systems. Results showed an age-dependent deficit in performance when solely working memory was evaluated; With the addition of a reference memory component via a series of unplatformed arms, young rats behaved similarly to aged rats, showing a similar working memory deficit in visiting previously platformed arms. However, in the combined evaluation of reference and working memory, aged rats showed deficits when compared to young rats by

repeatedly re-visiting the reference memory, or unplatformed, arms. Further establishing the relationship between working and reference memory performance was a correlation for aged rats, which demonstrates that worse performance in visiting reference memory arms in early trials correlated with revisiting of working memory, or previously platformed, arms at later trials. In addition to these behavioral analyses, brain assays were carried out to assess cholinergic activity within the frontal cortex and ventral hippocampus. Analyses revealed age-dependent changes in cholinergic activity that correlated with working memory outcomes, reflecting the complexities of cholinergic functioning within structures critical to memory and attentional processes across trajectory of female aging. Overall, this work suggests that reference memory and working memory processes do overlap within simultaneous evaluations like that of the WRAM, and that aging might heighten this interaction to produce a more profound profile of cognitive deficits. These findings are key for future work in considering how to interpret results from the WRAM in light of other purely working or reference memory specific tasks, such as the MWM or the Barnes maze. Findings from cholinergic functioning assays suggest that the aged brain is in a compensatory state, increasing activity to combat increasing cognitive load. These results highlight the need for further understanding of key brain changes across aging, as well as how these impact subsequent behaviors.

Using this knowledge of the complexity of reference memory and working memory assessments with aging in the WRAM, this maze can be used to evaluate neurodegenerative aging models. Indeed, the mechanisms driving various neurodegenerative diseases and their putative therapeutics are of primary concern to the

aging population, particularly for females. Alzheimer's disease (AD), the most common form of dementia, currently afflicts over 6.2 million individuals in the US; of those, two-thirds are women (Alzheimer's Association, 2020). AD is a progressive disease, characterized by marked memory loss and cognitive decline, which is preceded over decades by pathological hallmarks including amyloid plaques, neurofibrillary tangles, and neuronal loss (Forloni, 2020). Given the progressive nature of the disease, clinical research is limited in finding interventions before pathology becomes too advanced; in this manner, transgenic (Tg) animal models of AD can be quite useful. In the last decade, a rat model of AD has been developed, which expresses the human amyloid precursor protein (APP) with the Swedish mutation and the mutant human presenilin-1 lacking exon 9 (PSEN1dE9) (R. M. Cohen et al., 2013). TgF344-AD rats have the neuropathology characteristic of AD, including soluble amyloid-beta (Aβ) oligomers that later aggregate into Aβ plaques and endogenous hyperphosphorylated tau leading to neurofibrillary tangles, as well as increased neuroinflammation and frank neuronal loss (R. M. Cohen et al., 2013). While some other aspects of the model have been characterized, the behavioral phenotype of TgF344-AD rats, particularly in females, has been underexplored.

Chapter 3 evaluated the trajectory of spatial learning and memory outcomes for female TgF344-AD at three distinct time points from young adulthood to middle-age: 6, 9, and 12-months of age, and seeks to better characterize putative relationships between these behavioral changes and physiological changes, including amyloid brain pathology. Results showed indications of working and reference memory deficits on the WRAM by 6 months of age in Tg rats; at 12 months old, Tg impairments were observed for two

working memory measures on this task. Notably, no impairments were observed at the 9-month timepoint on this maze. On the Morris water maze (MWM), a reference memory task, Tg rats displayed deficits relative to age-matched WT rats at all three timepoints. Such findings age differential patterns of cognitive deficits that are further modulated by task and memory type are key to better characterization of this Tg model. The early indications of working and reference memory deficits indicate that even younger ages should be evaluated to determine the emergence of cognitive and physiological pathologies. The lack of cognitive effects in 9-month-old rats may be compensatory in nature; indeed, prior work has shown that female rats can switch to using striatal-dependent processing of spatial tasks if the hippocampus is compromised (Korol & Wang, 2018). Further supporting the 9-month timepoint as a unique age within the TG-F344 AD model is the emergence of significant heart weight differences, with Tg rats having greater heart weight. Additionally, at 9-months specifically, significant negative correlation was found between body weight and amyloid beta expression within the frontal cortex, suggesting that this period may involve a critical physiological and neurobiological shift within AD progression.

With knowledge of the patterns of learning and memory outcomes across the trajectory of both normal and neurodegenerative aging, preclinical menopause models can be utilized at any age, allowing for exploration of putative interactions. While Ovx has for decades served as the standard for preclinical menopause modeling, the abrupt cessation in circulating ovarian hormones with this Ovx model does not allow for a longitudinal evaluation of menopause as it occurs naturally in women. The VCD model of transitional menopause has provided the field with a more comprehensive tool for

assessing the behavioral and physiological changes associated with the menopause transition while maintaining a high degree of experimental control in instigating ovarian failure across a series of injections (Koebele & Bimonte-Nelson, 2016). Indeed, prior work in our laboratory has shown that the VCD model reflects the cognitive symptoms associated with this transitional phase (Acosta, Mayer, Talboom, Tsang, et al., 2009; Koebele et al., 2017), and is a good model for mapping on additional elements of the menopausal experience, such as HT (Acosta et al., 2010; Koebele, Hiroi, et al., 2021; Koebele, Mennenga, et al., 2020). However, the lack of consistency in protocols used for administering VCD limits the translational power of these findings; without consistent understanding that the variety of methodologies truly leads to similar cognitive and ovarian profiles, the field cannot assimilate findings and develop new questions surrounding this critical time in midlife.

Chapter 5 addressed these differing methodologies by evaluating the effect of VCD administration using different vehicles (50% DMSO/50% Saline vs. Sesame Oil) and intraperitoneal (IP) versus subcutaneous (SubQ) routes of administration commonly found in the literature on ovarian and cognitive outcomes in middle-aged female rats. Results showed that, regardless of vehicle or route of delivery, all VCD methodologies resulted in consistent profiles of ovarian follicular depletion and modest working memory deficits. However, the previously unreported or underreported methodological constraints associated with differing VCD administration paradigms must be made clearer within the literature going forward. Overall, the restraint associated with IP injections, along with the IP route of VCD administration, resulted in substantial weight loss, where the SubQ VCD injections resulted in painful skin lesions. Such knowledge is critical for researchers

seeking to use VCD as a model of menopause to optimally select an administration method that minimizes potential confounds or design challenges according to the variable of interest. Further work should be done to characterize why these adverse reactions take place within the VCD model, as greater knowledge could lead to a better understanding of the mechanisms that underlie VCD's impact on the ovaries and brain.

Impact of Hysterectomy on Cognitive and Physiological Outcomes: Interactions with Aging and Ovarian Status

Preclinical menopause modeling has been an important tool in tackling translational questions that can improve the lives of women experiencing this critical stage in the lifespan. However, until recently, only ovary-dependent models were available for assessing cognitive and physiological outcomes. While the ovaries are a substantial driver of transitional menopause, and oophorectomy results in an abrupt entrance into menopause, hysterectomy is the second most common gynecological surgery (Carlson et al., 1993). While the ovaries can remain intact, the removal of the uterus results in a lack of menstruation, which is the primary indication used to mark the entrance into post-menopausal life (Haney & Wild, 2007). For decades, the uterus was thought to play a minimal role in broader physiological outcomes outside of pregnancy (Navot & Williams, 1991); however, more recent clinical research has highlighted the role of the uterus in long-term ovarian and cognitive outcomes (Laughlin-Tommaso et al., 2020; N. F. Santoro, 2019; Stewart et al., 2012). Indeed, hysterectomy alone has been shown to increase risk of dementia, with earlier surgery resulting in an increased risk (Phung et al., 2010). The extent to which these effects are attributable to chronological aging patterns or reproductive staging is unclear. Such clinical findings merit a model of

hysterectomy within the rodent, wherein the mechanisms behind such health risks can be further probed. Our laboratory recently developed a model of hysterectomy, which showed learning and memory deficits (Koebele et al., 2019) that lasted up to one-year post-surgery (Koebele, 2019). Notably, these studies were conducted with young adult rats, with normally cycling ovaries. Evaluating the role of age, as well as reproductive stage, at hysterectomy in modulating cognitive outcomes is vital to the understanding of this model as a translational tool that reflects patterns observed within the clinic. Chapters 4 and 6 evaluated chronological age and ovarian stage effects, respectively, to address these questions.

In Chapter 4, young adult and middle-aged female rats underwent sham or hysterectomy surgery, with or without concomitant ovarian removal, before being evaluated on a behavioral battery that included the WRAM and MWM. Ovarian tissues collected at euthanasia were evaluated to determine follicle counts, and blood serum was likewise evaluated to detect levels of ovarian hormones and gonadotropins. Analyses of working memory performance revealed that hysterectomy, regardless of age and ovarian status, impaired learning on the WRAM, but that effects were most prominent for middle-aged rats. This differs from clinical patterns with dementia risk, but follow-up work is required to make determinations concerning long-term effects of hysterectomy at this age. Notably, ovarian and endocrine profiles differed with hysterectomy uniquely at the middle-age timepoint, with a reduction in antral follicles and increased androstenedione and E2 relative to age-matched sham-treated rats. Such effects seem to point to the unique experience of estropause in the middle-age rodent as a modulator of the hysterectomy-induced cognitive, ovarian, and endocrine effects observed here. This

notion further highlights the need for an evaluations of hysterectomy outcomes resulting from reproductive stage, and not chronological age, as explored in Chapter 6.

This chapter explored whether cognitive outcomes following hysterectomy are altered if preceded by ovarian follicular depletion. Young adult female rats were treated with VCD or vehicle injections before ovarian follicular depletion occurred over a period of 90 days. Following this period, rats received either sham or hysterectomy surgery; six weeks later, all subjects were evaluated on the WRAM and MWM, as well as the Open Field Test (OFT), which assesses anxiety-like and locomotive behaviors. Results demonstrated that rats that underwent hysterectomy plus VCD showed a marginal learning deficit on the WRAM as compared to hysterectomized rats that received vehicle injections. Notably, on later evaluations of WRAM performance, hysterectomized rats, regardless of VCD injection, demonstrated working memory facilitation, but impairment following a delay on the last testing day. Physiological outcomes revealed the dominating impact of VCD, with reduced ovarian weights and increased body weights regardless of surgery. The study highlights some unique and unexpected effects of combining the VCD and hysterectomy models of menopause. Further work should be done to evaluate serum hormone levels and ovarian morphological changes in these subjects, as it is possible that extended VCD treatment at a young age may have introduced early life stress as an unexpected moderating variable for some of the outcomes highlighted above. Future work could modulate this experimental design and ask a similar, equally-translational question pertaining to hysterectomy and transitional menopause by performing hysterectomy or sham surgery, then adding on the VCD model of transitional menopause. Such an experiment would assess whether the experience of hysterectomy in young

adulthood, with healthy cycling ovaries, impacts cognitive, ovarian, and endocrine outcomes as subjects experience ovarian follicular depletion, modelling the menopause transition.

Developing More Translational Models of Hormone Therapy Provides Unique Insights into Cognitive and Physiological Outcomes

With menopause, whether experienced through transitional or surgical means, individuals can be prescribed HT to combat associated symptoms such as night sweats and hot flashes (Pinkerton et al., 2017). HT contains an estrogen, usually in the form of either E2 or conjugated equine estrogens (CEE) (N. Santoro et al., 2021). For uterus-intact individuals, HT also contains a progestogen to combat estrogen-induced endometrial hyperplasia; hysterectomized individuals can therefore take as estrogen alone as menopausal HT (Haney & Wild, 2007; Pinkerton et al., 2017). The long-term effects of HT use have been of much interest to the menopause research community; large clinical trials such as the Women's Health Initiative (WHI), including its Memory Study (WHIMS), and Kronos Early Estrogen Prevention Study (KEEPS) have explored cardiovascular, bone, mood, and cognitive outcomes associated with both estrogen-only and combined HT use (Chester et al., 2018; Coker et al., 2010; Gleason et al., 2015; Shumaker et al., 2003, 2004). Preclinical research has likewise explored cognitive and physiological outcomes associated with HT use. In brief, estrogens such as E2 have been shown to be largely beneficial to learning and memory outcomes, and such effects are dependent upon menopause etiology (Acosta et al., 2010; Bimonte & Denenberg, 1999; Koebele, Mennenga, et al., 2020). In contrast, progestogens have been shown to be detrimental to cognitive outcomes, either when administered alone (Braden, Andrews, et

al., 2017; Braden et al., 2010, 2011) or when combined with an estrogen representing combination HT (Koebele, Hiroi, et al., 2021; Prakapenka et al., 2018). Given the preclinical literature surrounding HT, two issues emerge: (1) there has yet to be a progestin identified as cognitively beneficial or neutral, when given alone as well as when paired with an estrogen, and (2) estrogen HT evaluations have been limited in clinical translation due to their being carried out in uterus-intact rodents. The final two research chapters seek to address these issues.

Chapter 7 evaluated the cognitive, anxiety-like, and neurobiological effects of administration of segesterone acetate (SGA), a progestin with a high affinity for the progesterone receptor and minimal affinity for other hormone receptors. It was theorized that the selective nature of SGA would result in a cognitively beneficial profile. Middle-aged rats underwent sham or Ovx surgery before receiving daily SubQ injections of sesame oil vehicle, medroxyprogesterone acetate (MPA), a progestin characterized as having cognitively detrimental effects, or SGA, given at a low or high dose. Following week of treatment, rats were evaluated on the WRAM, MWM, and OFT. At euthanasia, brain tissues critical to learning and memory were collected and processed for relative GAD 65 and GAD 67 expression, as well as relative IGF-1R expression. Results demonstrated that both the low and high doses of SGA impaired learning and memory outcomes, but these deficits were different depending upon dose. As expected, MPA also impaired learning and memory ability. In evaluations of anxiety-like behaviors, however, SGA at both doses reversed Ovx-induced anxiogenic effects. This demonstrates the complex nature of progestogens: where one domain may greatly benefit following administration menopause, the same progestogen may result in deficits within another

domain. Such patterns of effects should be further explored, as this may relate back to the mechanism behind the detrimental memory effects of certain hormones, and point towards an optimal HT formulation. Finally, correlations between behavioral and neurobiological outcomes revealed a significant relationship for ovary-intact rats between increasing IGF-1R and improved working memory outcomes; these relationships were obviated by Ovx, and not reinstated by either progestin. Further evaluations into the role that IGF-1R plays in cognitive outcomes should be performed, as HT formulations that reinstate these relationships may hold the key to optimizing cognitive and affective outcomes for menopausal women.

While Chapter 7 evaluated progestin administration in clinically-relevant groups, Chapter 8 pioneered evaluations of learning and memory outcomes associated with estrogen-alone HT in clinically-relevant models. This study used the Ovx and hysterectomy rodent models of surgical menopause to evaluate the cognitive, anxiety-like, and inflammatory outcomes associated with E2 administration as a model of HT. Middle-aged female rats received either sham, Ovx, or hysterectomy surgery. Three weeks following surgery, rats were given tonic administration of either vehicle (polyethylene glycol) or E2 (Ovx and hysterectomy groups only) via Alzet osmotic pumps. Three weeks into E2 administration, rats were evaluated on the WRAM, MWM, and elevated plus maze (EPM), a measure of anxiety-like behavior. As observed in prior work, Ovx resulted in working and reference memory deficits, where E2 rescued working memory deficits on the WRAM. Hysterectomy-treated rats showed a tendency for increased sensitivity to an increasing working memory load, where E2 reversed this effect. Such work suggests that E2 may hold the same benefits for hysterectomy as

traditionally observed in Ovx; however, further work evaluating the long-term effects of HT would provide greater context as to whether these benefits are lasting. Finally, unique inflammatory outcomes were observed following E2 administration in Ovx rats, relative to sham-treated rats. Indeed, E2 in the absence of other circulating ovarian hormones decreased serum levels of interleukin 6 (IL-6), a pro-inflammatory cytokine, relative to ovary-intact rats. Such work highlights the role of the background hormonal milieu in determining behavioral and physiological outcomes.

Future Directions for Preclinical Menopause Modeling

Given the comprehensive nature of this dissertation work, with many different critical variables addressed and key findings presented, there are countless areas for further research. For example, greater care should be taken within the field of preclinical menopause research to integrate the novel model of hysterectomy, both with ovaries preserved and with concomitant Ovx, into future research questions. So much is currently unknown concerning hysterectomy, as even clinical research begins to address the role of the uterus in long-term health outcomes (Stewart et al., 2012); preclinical research could provide an early window into addressing these questions through the including of such surgical groups in menopause-related evaluations. Not only are these models of surgical menopause more translational, as hysterectomy is often performed in conjunction with bilateral salpingo-oophorectomy (BSO) in women (Novetsky et al., 2011), but uterine removal also allows for a more translational evaluation of estrogen-alone HT (Haney & Wild, 2007). Indeed, given that several chapters within this dissertation highlight the role of the background hormonal milieu as critical to behavioral and physiological outcomes

(see correlations in Chapter 7 and inflammatory effects in Chapter 8), the inclusion of Ovx+Hysterectomy groups in estrogen work is critical.

Similarly, future work evaluating cognitive outcomes in rodent models of menopause should expand behavioral batteries to include more longitudinal designs and alternative assessments of outcomes critical to menopause. While much of the work in this dissertation evaluated short-term consequences following experimental manipulation, much more work is needed to assess long-term outcomes more akin to clinical evidence of cognitive changes such as dementia risk. Some longitudinal work has been done (Koebele, 2019; Koebele et al., 2017), with immensely impactful findings and interpretations to the clinic, but more must be done to parallel research designs from the clinic. Additionally, assessments should include more nuanced anxiety-like and depressive-like evaluations. Depressed mood is a commonly reported symptom in menopause (Gracia & Freeman, 2018), and certain menopause etiologies, such as incidence of hysterectomy or BSO, has been shown to increase risk of anxiety or depressive disorders (Laughlin-Tommaso et al., 2020; Rocca et al., 2018; Wilson et al., 2018). Preclinical research could integrate models of chronic anxiety or depression into menopausal research by assessing putative risk factors that relate to risk versus resiliency (Duman, 2010) that can help to identify mechanisms and provide clinicians with tools for a more personalized approach to menopausal care. Alternatively, preclinical work could assess more nuanced affective-like outcomes associated with experimental manipulation through the use of a more comprehensive behavioral battery that includes tasks such as EPM, OFT, forced swim test, sucrose preference test, or novelty suppressed feeding (Wang et al., 2017). Such work would provide greater context to the uncommon and

often-conflicting reports of anxiety-like and depressive-like behavioral profiles across different models of menopause and with HT administration.

Finally, future work should probe new neurobiological targets to account for the nuanced patterns of cognitive effects observed across these differing menopause etiologies and with differing HT formulations. While the serum inflammatory evaluations in Chapter 8 indicate that further exploration of immune system changes within the brain may be responsible for the cognitive deficits with Ovx and hysterectomy, and reversal with E2, challenges arose in using ELISAs to evaluate these measures. Indeed, levels of IL-6 and tumor necrosis factor alpha (TNF-alpha) appeared to be too low to adequately evaluate in regionally-specific samples of brain tissue. Rather than finding another technique to evaluate these inflammatory markers in brain tissues, future work might be best suited in taking a different approach altogether to measuring neuroinflammatory changes. For example, by probing activation of different immune cells, such as microglia, the resident macrophages of the brain, through measures of protein expression such as cluster of differentiation 68 (cd68) (Jurga et al., 2020), researchers can assess a critical element of neuroinflammation that is relevant to aging and menopause (Mishra et al., 2020; Mishra & Brinton, 2018). An alternative strategy for further evaluation would be to move downstream from inflammatory factors to evaluating proteins that interact with immune cells and inflammatory processes within the brain, but also engage in other neurobiological functions relevant to cognition. Evaluated in Chapter 7, insulin-like growth factor 1 receptor (IGF-1R) showed sensitivity to endogenous ovarian hormone modulation in its relationship to working memory outcomes. Other work has shown that the IGF-1 system plays a role in learning and memory processes with aging (Sonntag et

al., 2005), and well as in neuroinflammatory processes (Labandeira-Garcia et al., 2017). Alternatively, a more unbiased exploration of relationships between the immune system and cognition across various menopause etiologies could be achieved through the use of techniques such as RNA sequencing. Evaluations of gene expression in brain, ovarian, and uterine tissue across these models of menopause could point to signaling pathways or proteins relevant to immune and cognitive function that can be further researched.

How Preclinical & Clinical Research Together Can Optimize Menopausal Outcomes

This dissertation has discussed the importance of understanding menopause- and ovarian hormone- related cognitive outcomes by reflecting on the rich history of preclinical and clinical work in this field, as well as by enhancing preclinical understanding of these variables. Indeed, by exploring the roles of chronological aging, menopause etiology, and hormonal milieu in altering brain and behavioral outcomes at midlife, critical variables of interest have been identified to guide research towards a promising future of therapeutic discovery. The work in these chapters has integrated perspectives surrounding the menopause question from a variety of viewpoints and disciplines, ranging from exploration of Alzheimer's disease pathology, to an exploration of menopause as an inflammatory event. As the chapters above have utilized various preclinical models of menopause, acknowledging their strengths and limitations, the case has been made for preclinical rodent modelling as an important tool for investigating questions surrounding the experience of menopause alongside the clinic.

Indeed, the nature of preclinical rodent modeling is to identify, acknowledge, and contend with its strengths and weaknesses. It is accepted that no animal model can

exactly recapitulate human health conditions, including menopause models. The limitations of rodent modeling, including differences in lifespan, reproductive cycling and senescence, and limitations in modeling various environmental factors, must be recognized. However, the knowledge and proper use of such limitations, with careful experimentation, can actually serve as an asset. The ability to study aging in an abbreviated time frame, independent from reproductive aging, in a controlled environment can lead to meaningful work that can contribute to the body of knowledge surrounding menopause and aging outcomes. This is also true of each model of menopause, explored in the chapters above. Ovary-intact aged rodents can provide context about broader aging, but the background hormonal milieu at estropause cannot be ignored. Where Ovx provides a clean hormonal slate for investigation of HT, it is limited in its ability to explore transitional menopause stages. Likewise, VCD allows for such transitional staging at any age, but has methodological constraints that may limit evaluation of certain outcomes. Each facet of preclinical menopause modeling is a careful balance of these strengths and limitations to optimize translation to the clinic. For this reason, preclinical researchers must fully understand that which they are modeling. Just as preclinical work can provide greater insight into relationships observed in the clinic, preclinical researchers must have a thorough understanding of the latest clinical findings.

Ultimately, consistent communication and collaboration across both the clinical and preclinical realms of women's health research is best for optimizing the trajectory of cognitive aging across the menopausal transition and into postmenopausal life, and with endogenous and exogenous hormone exposures. The recent rise in the number of collaborative health centers is a key step to critical information exchange, which will

allow for preclinical researchers to design experiments that further investigate variables of interest identified by clinical work, and will allow clinical researchers to design studies that reflect relationships seen in preclinical literature. This will aid preclinical researchers in designing models and experiments that further investigate variables of interest identified by clinical work, and will allow clinical researchers to design studies that capitalize on and reflect relationships seen in the preclinical literature. This exchange of experimental questions and insights across species, disciplines, and skillsets will ultimately create an increasingly more efficient and defined experimental framework which will propel understanding of hormone-brain-cognition relationships, optimizing care for individuals at midlife and beyond.

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APPENDIX A

DISSERTATION FIGURES

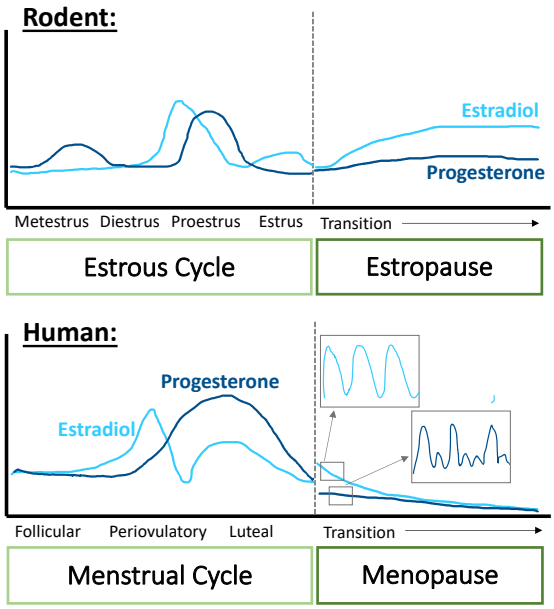
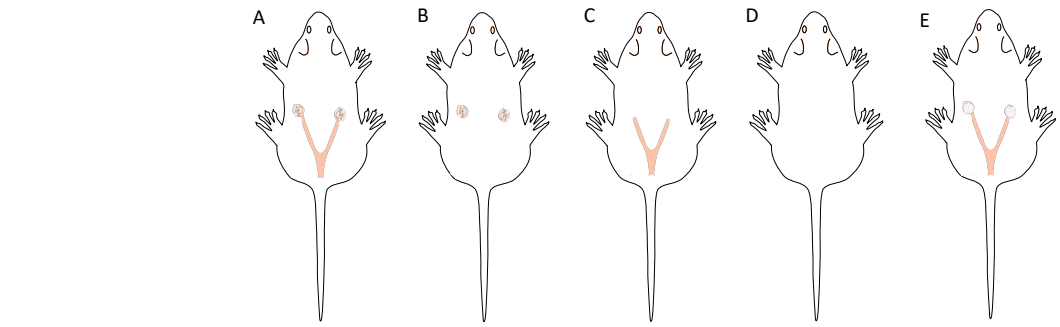


Figure 1: Ovarian Hormone Profiles in Female Rats and Humans. Relative shifts in 17b-estradiol (E2) and progesterone levels in female rodents and humans during reproductive cyclicity and reproductive senescence.



	A	B	C	D	E
Model of Menopause:	Sham (Control) The control procedure depends on the other experimental groups in the study	Hysterectomy Variation of a surgical menopause model including removal of the uterus	Ovx Variation of a surgical menopause model including removal of both ovaries	Ovx + Hysterectomy Variation of a surgical menopause model including removal of the uterus and ovaries	VCD Transitional menopause model with induced ovarian follicular depletion
Question Being Modeled:	Control (baseline), no experimental manipulation of reproductive tracts	How does the outcome change after removal of the uterus?	How does the outcome change after removal of the ovaries?	How does the outcome change after removal of the ovaries plus uterus?	How does the outcome change during or after after the menopause transition, independent of aging?

Example Translational Research Questions Addressed By Combining Menopause Models Within an Experiment or Within a Subject:

Do outcomes differ if the ovaries are removed along with or without the uterus? (Compare Models C and D)

Does surgical menopause via ovarian removal have a different effect than transitional menopause (follicular depletion)? (Compare Models C and E)

Does hysterectomy have a different outcome if transitional menopause (follicular depletion) previously occurred? (Compare Subjects Given E then B to Subjects Given B alone)

Figure 2: Variations in Menopause Etiology Modelled in the Female Rodent. The Sham, Hysterectomy, Ovx, Ovx+Hysterectomy, and VCD models of menopause, with example translational questions addressed using one or several of these models listed below.

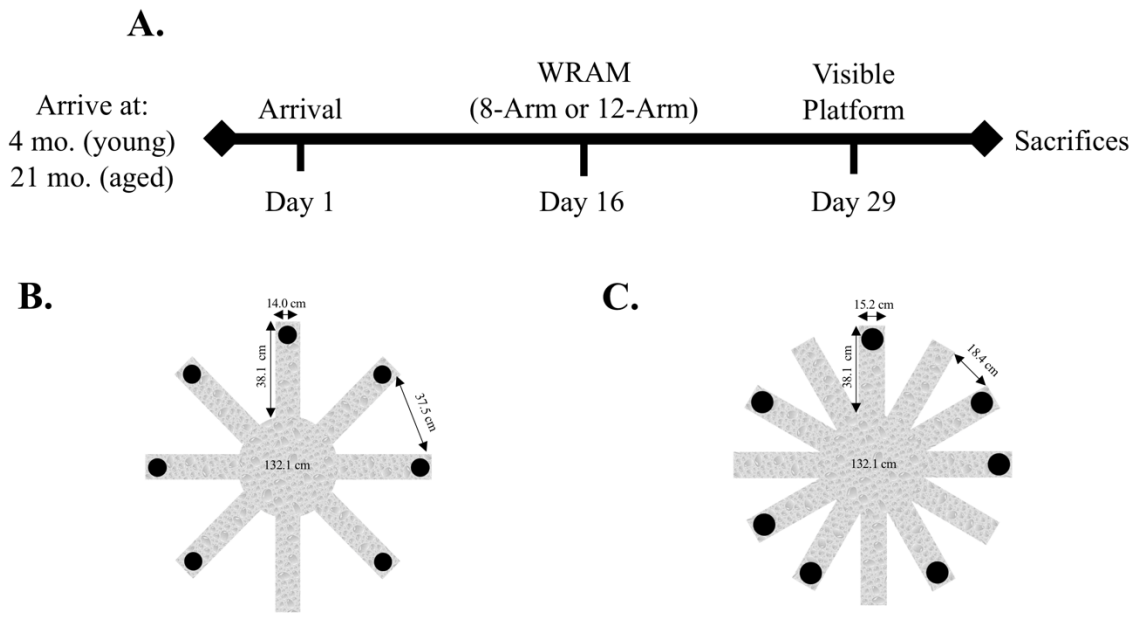


Figure 3: Study Timeline and WRAM Configurations. A) The study timeline depicts the age at arrival and the commencement of behavioral testing, including WRAM and VP. B) The 8-Arm WRAM apparatus, with seven of the eight arms platformed. Besides the starting arm, all other arms in the 8-Arm WRAM are spatial working memory arms. C) The 12-Arm WRAM apparatus, with seven of the 12 arms platformed, making for a total of seven working memory arms, like that of the 8-Arm WRAM. This maze, however, has five reference memory arms, allowing for the analysis of both spatial reference and working memory performance on the WRAM.

Total errors: The sum of errors across trials within a day

- 8-Arm: WMC entries (with negligible start-arm entries)
- 12-Arm: WMC entries plus non-platformed arm entries (RM & WMI)

WMC errors: Entries into an arm that previously contained a platform

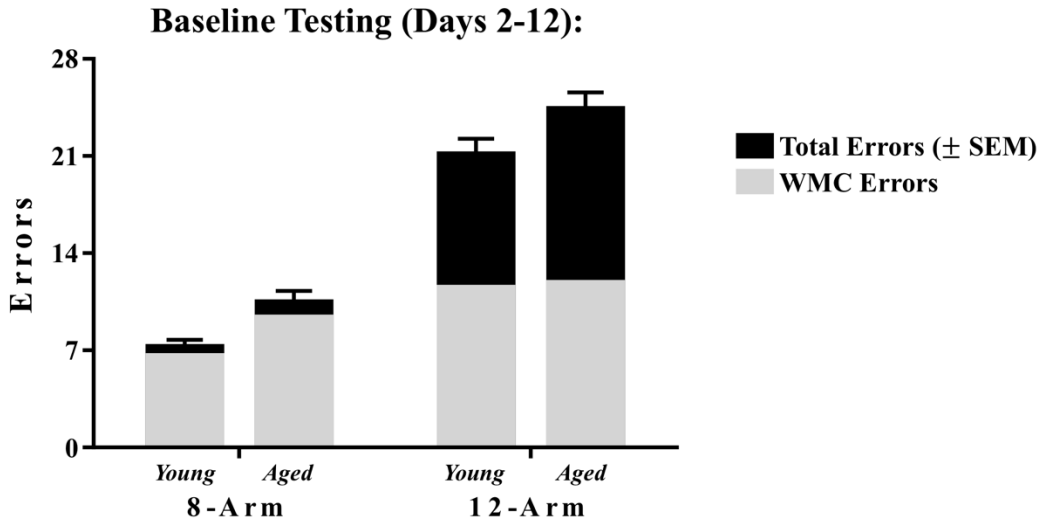


Figure 4: WRAM Performance Represented as WMC Errors and Their Contribution to Total Errors. The proportion of Total errors (±SEM) comprised of WMC errors for both mazes across Acquisition and Asymptotic Phases of testing. For the 8-Arm WRAM, other than start arm errors, all errors must be WMC errors; however, in the 12-Arm WRAM, rats can make WMC errors, as well as RM and WMI errors. While the rats tested on the 8-Arm WRAM rarely made entries into the only arm without a platform i.e., the start arm, rats tested on the 12-Arm WRAM made a large number of unplatformed arm entries. This further demonstrates the separation of errors made when a reference memory component is added to the working memory portion of the WRAM.

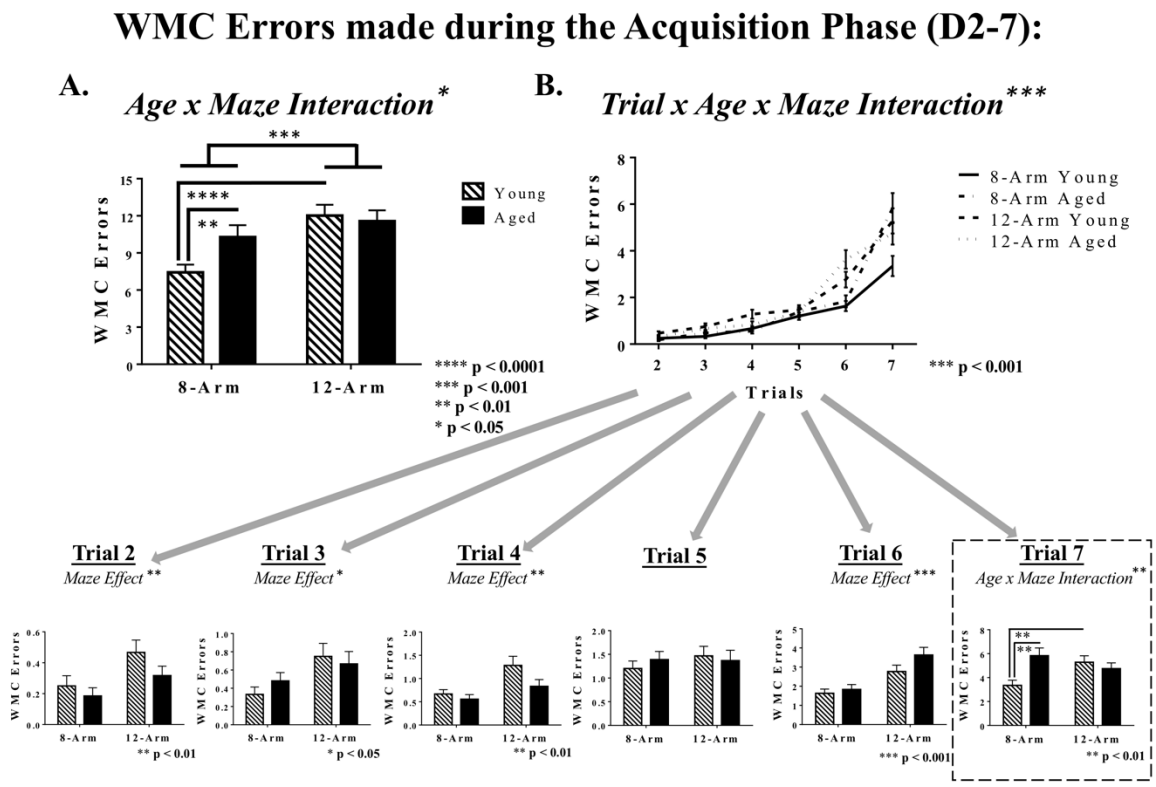


Figure 5: WMC Errors During the Acquisition Phase of WRAM Testing. A) WMC errors for the Acquisition Phase of WRAM testing (Days 2–7). With an effect of Maze and an Age × Maze Interaction, it was found that within the 8-Arm WRAM there was an effect of Age, but not for the 12-Arm WRAM; additionally, young rats tested on the 8-Arm WRAM performed better than those tested on the 12-Arm WRAM, but performance across maze did not differ for aged rats. B) Working memory performance as measured by WMC errors made across all testing trials (Trials 2–7). At the highest working memory load (Trial 7), there was an Age × Maze interaction, where aged rats performing on the 8-Arm WRAM made more errors than their younger counterparts, and the rats performing on the 12-Arm WRAM did not differ by age. Additionally, for young rats there was an effect of Maze, where rats tested on the 8-Arm WRAM made fewer errors than their counterparts tested on the 12-Arm WRAM, with no effect amongst aged rats.

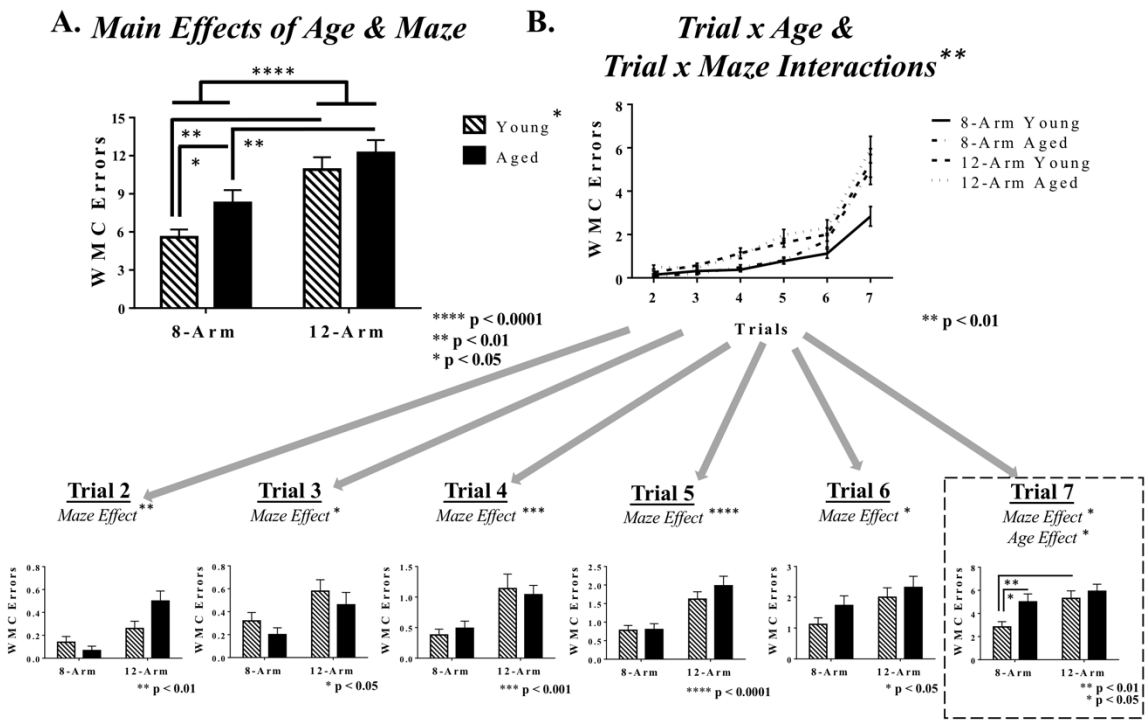


Figure 6: WMC Errors During the Asymptotic Phase of WRAM Testing. A) WMC errors for the Asymptotic Phase of WRAM testing (Days 8–12). With an effect of Maze and an effect of Age, it was found that within the 8-Arm WRAM there was an effect of Age, but not for the 12-Arm WRAM; notably distinct from the Acquisition Phase, both young and aged rats tested on the 8-Arm WRAM performed better their counterparts tested on the 12-Arm WRAM. B) Working memory performance as assessed *via* WMC errors made across all testing trials (Trials 2–7). At the highest working memory load (Trial 7), there was a main effect of Maze and a main effect of Age, where aged rats performing on the 8-Arm WRAM made more errors than their younger counterparts, and the rats performing on the 12-Arm WRAM did not differ by age. Additionally, there was an effect of Maze for young rats exclusively, where young rat performance on the 8-Arm WRAM was better than that of the 12-Arm WRAM, with no effect of Maze for aged rats.

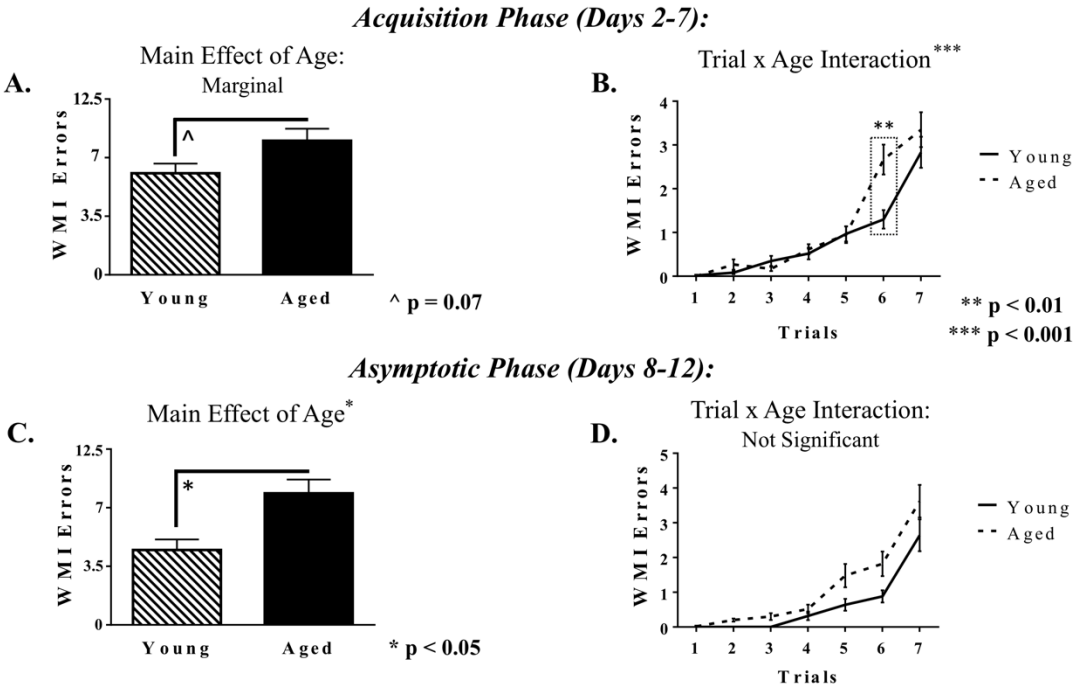


Figure 7: WMI Errors within the 12-Arm WRAM. A) The marginal main effect of Age on WMI errors within the 12-Arm WRAM for the Acquisition Phase (Days 2–7), where aged rats made marginally more WMI errors than young rats. B) WMI errors across Trials 1–7 for Acquisition Phase (Days 2–7) of testing. C) The main effect of Age on WMI errors within the 12-Arm WRAM for the Asymptotic Phase of testing (Days 8–12), where aged rats made significantly more WMI errors than young rats. D) WMI errors across Trials 1–7 for the Asymptotic Phase (Days 8–12) of testing. For the Acquisition Phase, age effects were particular to a high working memory load trial, whereas by the end of testing, in the Asymptotic Phase, the age effects were not trial-dependent.

Reference memory performance on a low cognitive burden trial correlates with working memory performance on the trial with the highest working memory load, solely in aged subjects

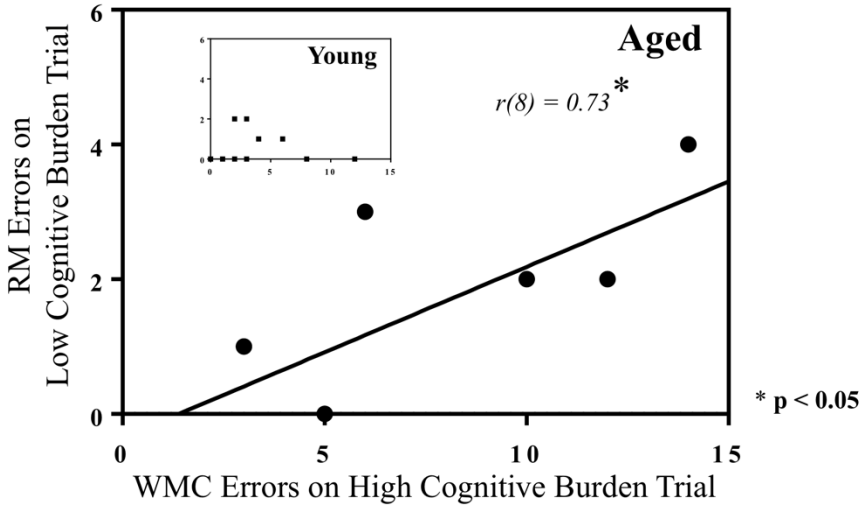


Figure 8: Correlations between RM and WMC Errors on the WRAM. A significant positive correlation was found between RM errors made on an early trial (Trial 2), with a relatively low cognitive load, and WMC errors made on the last trial (Trial 7), or the highest cognitive load trial, for aged rats performing on the 12-Arm WRAM on the last day of testing, but not for young rats [$R^2 = 0.53$, $r(8) = 0.73$, $*p < 0.05$]. Several rats in the aged group made the same number of RM and WMC errors for this figure, resulting in the overlap of data points represented.

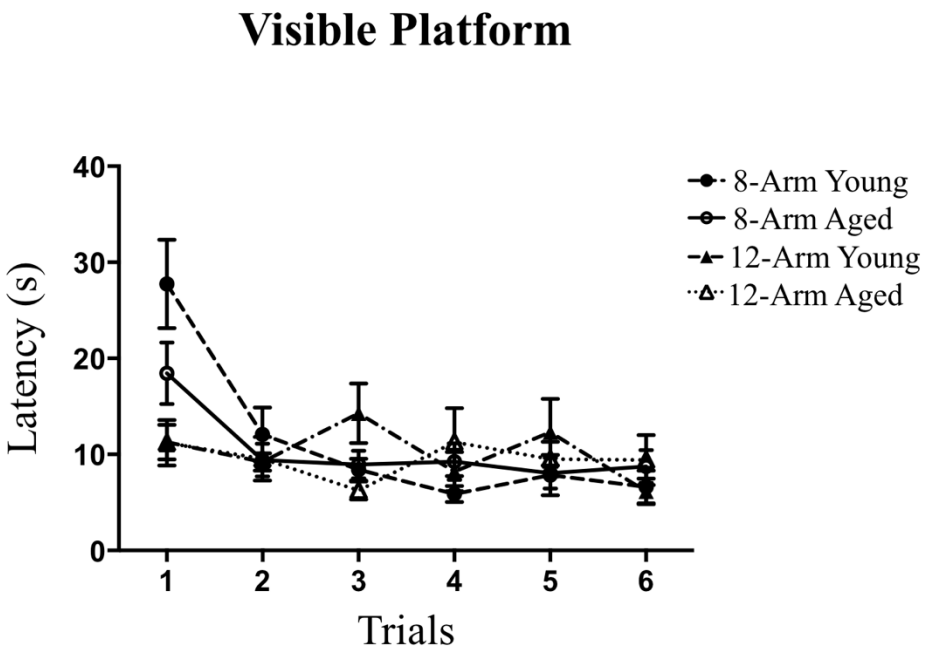


Figure 9: Performance on the Visible Platform (VP) Task. Latency (in seconds) across Trials 1–6 on the VP task, where groups did not differ in ability to perform the procedural components of a water escape task.

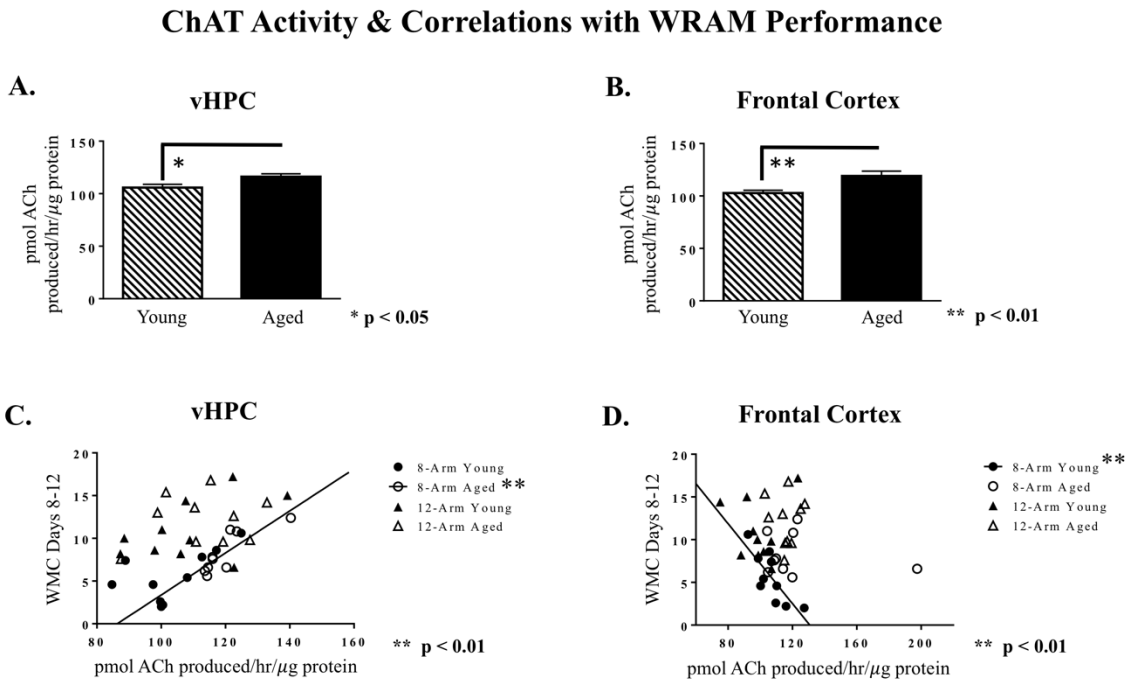


Figure 10: ChAT Activity in Various Brain Regions, and Correlations with WRAM Performance. A) Average ChAT activity levels in the ventral hippocampus, where aged rats had greater ChAT activity. B) Average ChAT activity levels in the frontal cortex, where aged rats had greater ChAT activity. C) A significant positive correlation was found between ChAT activity in the ventral hippocampus and WMC errors made during the Asymptotic Phase for aged rats performing on the 8-Arm WRAM specifically [R^2 = 0.70, $r(7) = 0.84$, $^{**}p < 0.01$). D) A significant negative correlation was found between ChAT activity in the frontal cortex and WMC errors made during the Asymptotic Phase for young rats tested on the 8-Arm WRAM [$R^2 = 0.61$, $r(8) = -0.78$, $^{**}p < 0.01$].

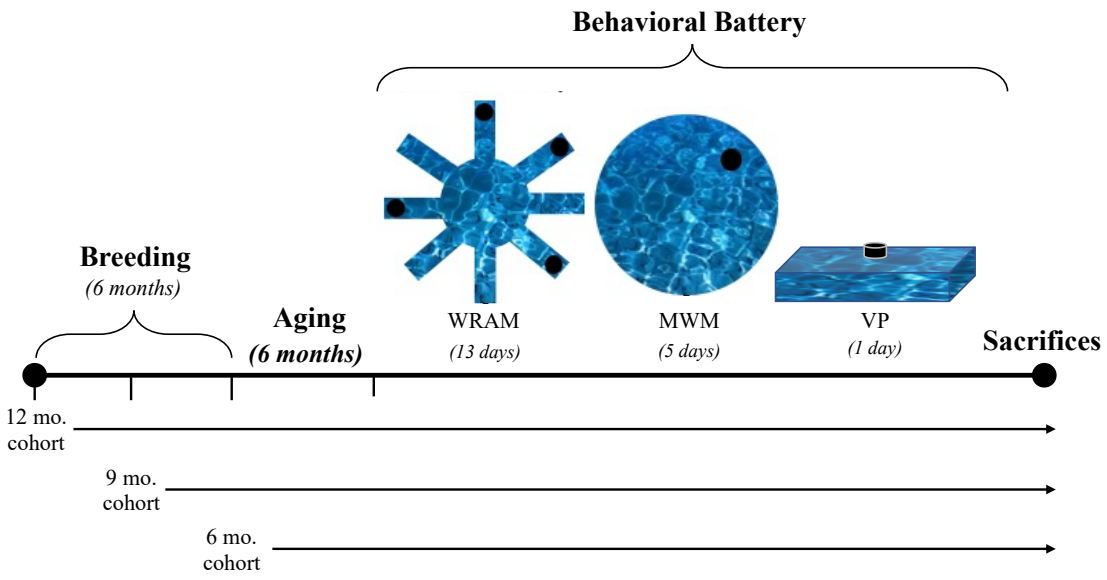


Figure 11: Study Timeline and Behavioral Assessments in This Novel TgF344 Rat Model of AD. The study timeline depicts the breeding schedule for each age cohort and the commencement of behavioral testing, including WRAM, MWM, and VP, before euthanasia at the conclusion of the study.

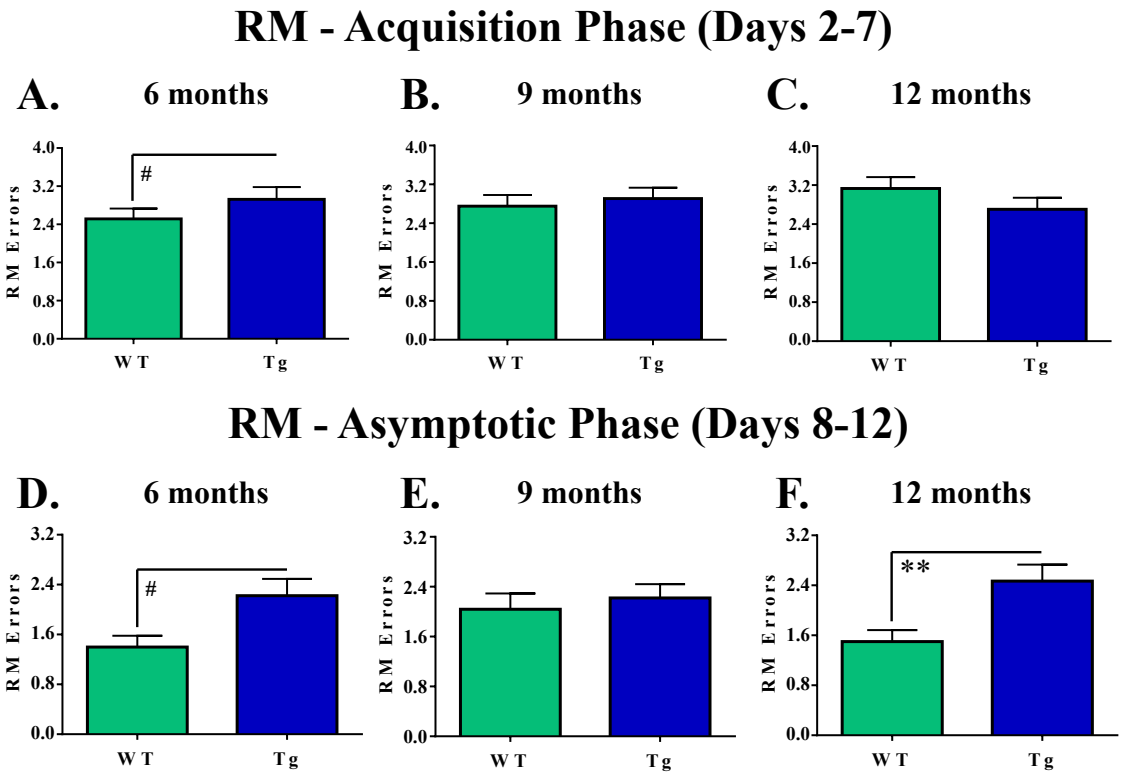


Figure 12: Distinct Patterns of Reference Memory Deficits in TgF344-AD Rats as Evaluated on the WRAM. (A-C) During task acquisition, 6-month Tg rats tended to make more errors than their WT counterparts; there were no effects for RM errors in the 9-month or 12-month groups. (D-F) During task maintenance, 6-month Tg rats tended to make more errors than their WT counterparts. While there were no differences amongst the 9-month groups, at the 12-month timepoint, Tg rats made significantly more errors than WT rats. #$p < 0.10$, **$p < 0.01$.

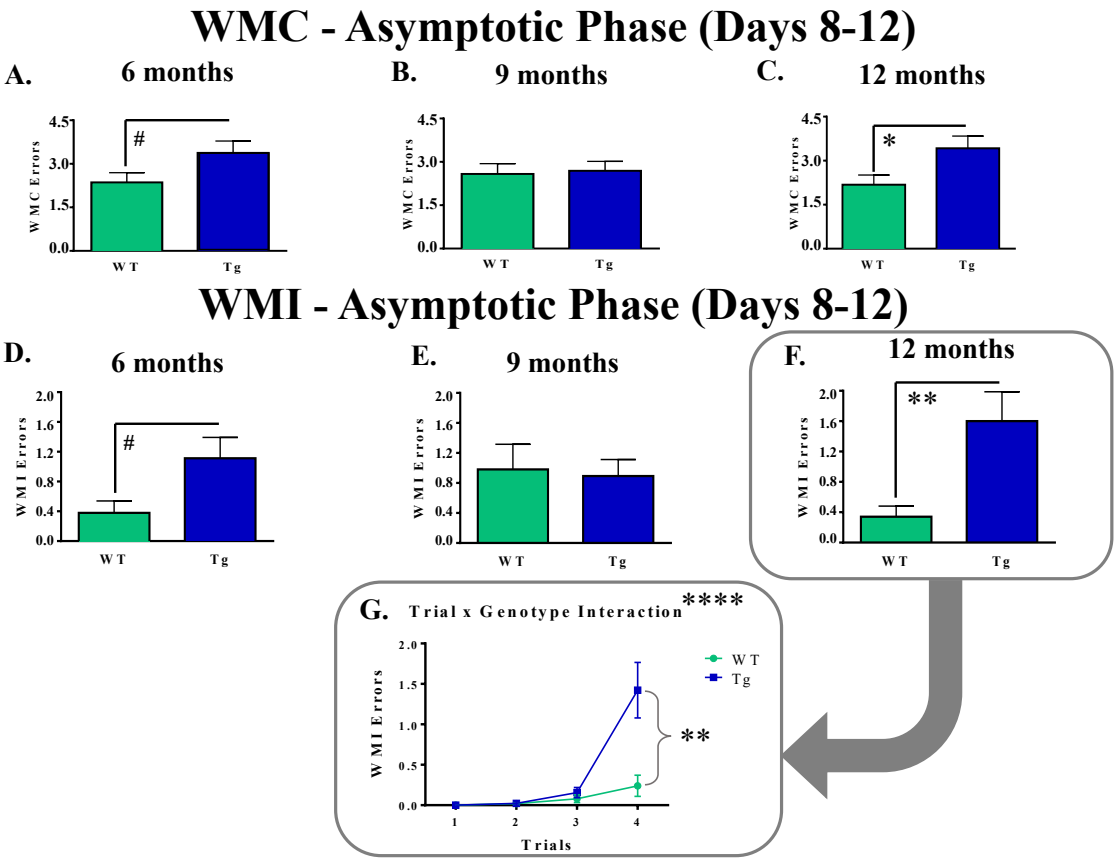


Figure 13: Distinct Patterns of Working Memory Deficits in TgF344-AD Rats as Evaluated on the WRAM. (A-C) During the Asymptotic Phase of WRAM testing, 6-month Tg rats made marginally more WMC errors than WT rats. Tg rats at the 12-month timepoint made significantly more WMC errors than WT counterparts, indicating working memory impairment. (D-F) Likewise, in the Asymptotic Phase of the WRAM, Tg rats in the 6-month cohort made marginally more WMI errors than WT rats of the same age. At the 12-month timepoint, Tg rats overall made significantly more WMI errors than WT rats. (G) Following up on a Trial x Treatment interaction, a genotype effect was found during the highest working memory load trial, with Tg rats making more WMI errors than WT rats at 12-months of age. #$p < 0.10$, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

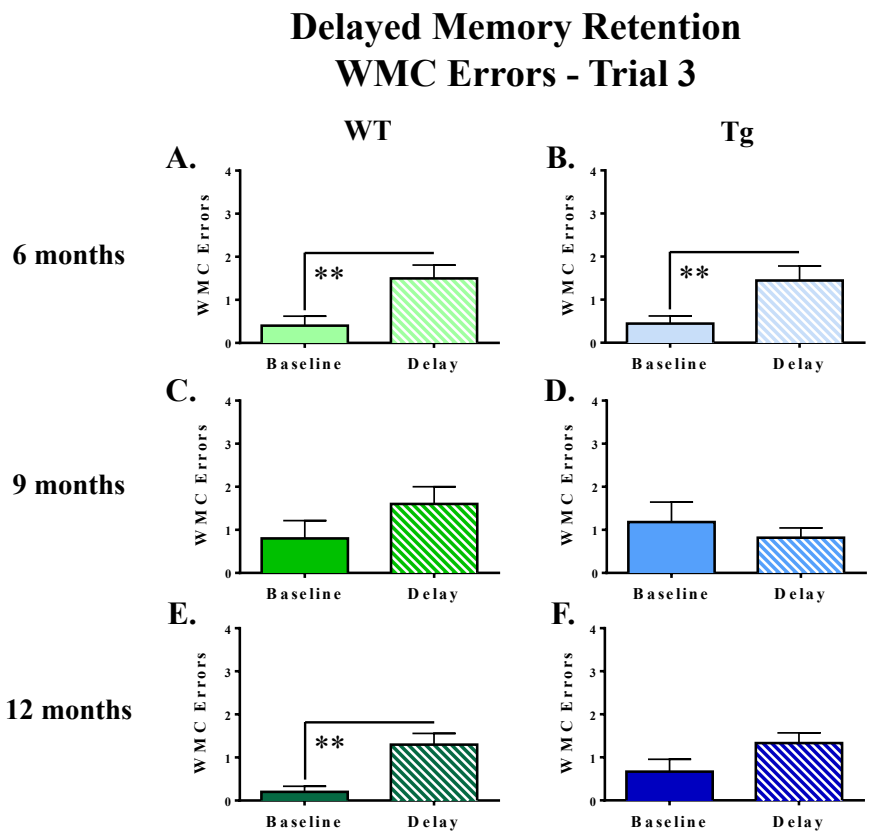


Figure 14: Assessments of Delayed Memory Retention on the WRAM. (A-F) Following implementation of a 6-hour delay on Day 13 of the WRAM, analyses of performance in WMC errors for Trial 3 on Day 12 (Baseline) and Day 13 (Delay) revealed a significant effect of the delay for rats in the 6-month Tg group, the 6-month WT group, and the 12-month WT group. $^{**}p < 0.01$.

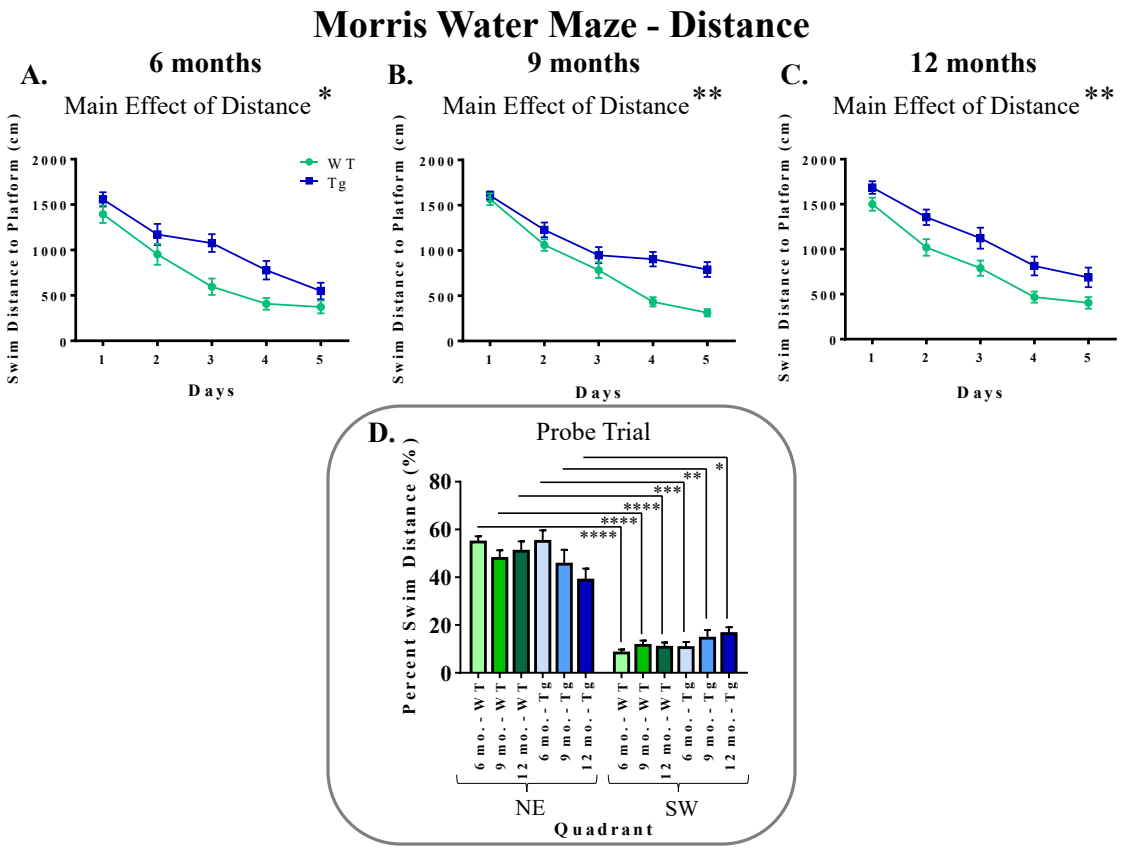


Figure 15: Reference Memory Performance as Evaluated on the MWM – Consistent Deficits in TgF344-AD Rats across Each Age for Swim Distance to the Platform. (A-C) When evaluating swim distance to platform (cm) for each age-point across Days 1-5, there was a significant effect of Genotype, whereby Tg rats swam greater distances to locate the hidden platform than WT rats, indicating spatial reference memory impairment. *$p < 0.05$, **$p < 0.01$. (D) For the probe trial, analyses of percent of total swim distance in the target quadrant (NE) as compared to the opposite quadrant (SW) for each group showed that all groups were successful in spatially localizing the platform by swimming a greater proportion of their total distance in the NE as opposed to the SW. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

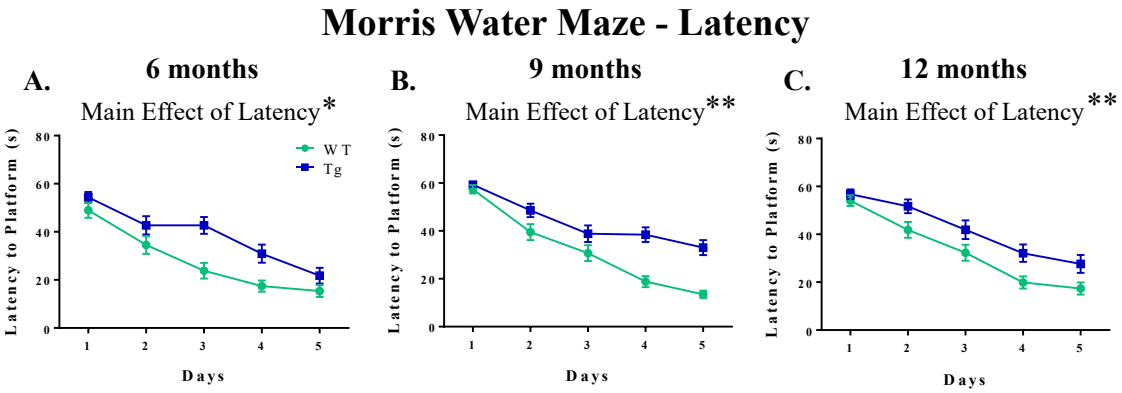


Figure 16: Reference Memory Performance as Evaluated on the MWM – Consistent Deficits in TgF344-AD Rats across Each Age for Latency to the Platform. (A-C) When evaluating latency to the platform (s) for each age-point across Days 1-5, there was a significant effect of Genotype, whereby Tg rats swam longer to locate the hidden platform than WT rats, indicating spatial reference memory impairment. *$p < 0.05$, **$p < 0.01$.

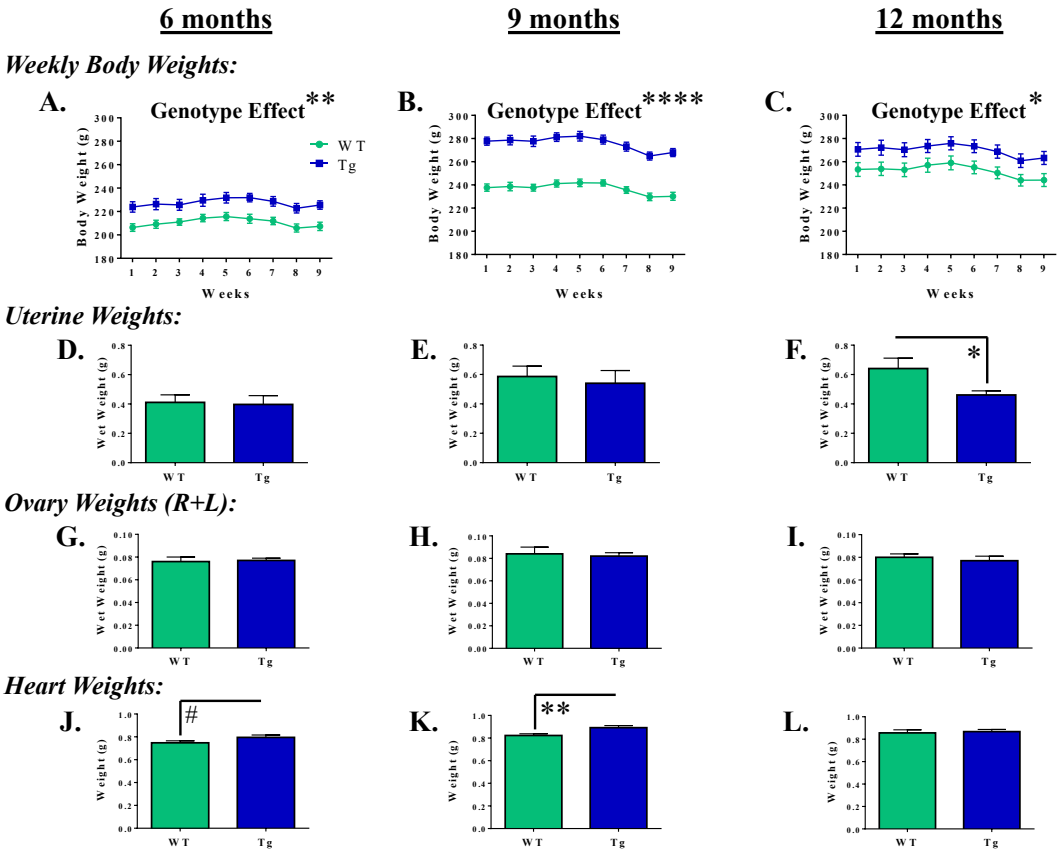


Figure 17: Distinct Age-Dependent Physiological Profiles in the TgF344-AD Rat. (A-C) Weekly body weights collected across the course of the study indicate that, regardless of age, Tg rats weighed more than their WT counterparts. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. (D-F) Analyses of uterine weights collected at sacrifice demonstrated that, uniquely at the 12-month timepoint, Tg rats had reduced uterine weights as compared to WT controls. *$p < 0.05$. (G-I) Analyses of ovary weight (R+L) at sacrifice revealed no effect of Genotype at any age-point. (J-L) Analyses of heart weight demonstrated a marginal effect of Genotype at 6 months and a significant effect at 9 months, where Tg hearts weighed more than WT hearts. #$p < 0.10$, **$p < 0.01$.

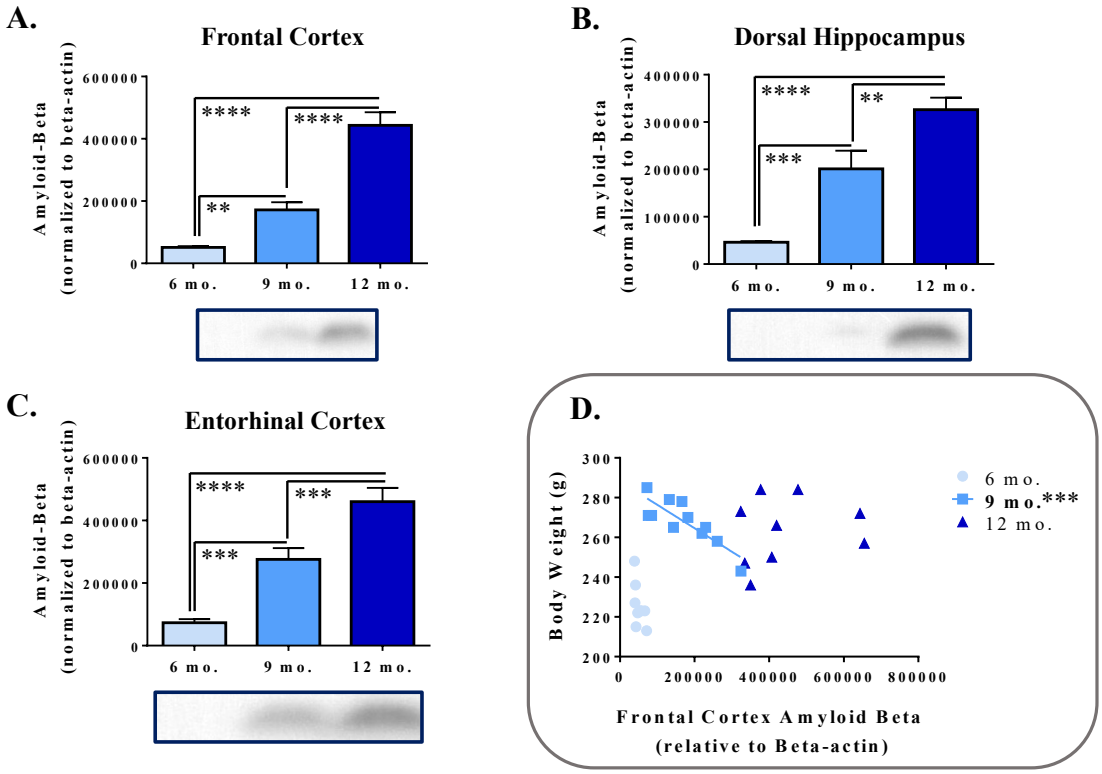


Figure 18: Relative Expression of A$\beta_{1\text{-}42}$ in Frontal Cortex, Dorsal Hippocampus, and Entorhinal Cortex Follows an Age-Dependent Trajectory, and Relationships with Body Weight are Unique to the 9-Month-Old TgF344-AD Rat. Western blot protein analyses demonstrated increasing relative normalized A$\beta_{1\text{-}42}$ expression with increasing age in (A) frontal cortex, (B) dorsal hippocampus, and (C) entorhinal cortex. Cropped representative blots with bands of A$\beta_{1\text{-}42}$ expression (4kDa) and beta-actin expression (45kDa) for each age point are demonstrated below each graph for a given brain region. Post hoc comparisons are represented in this figure – main effects of age are listed within the Results section. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. (D) For the 9-month timepoint, a unique negative correlation exists between body weight and relative normalized A$\beta_{1\text{-}42}$ expression in the frontal cortex, such that increasing A$\beta_{1\text{-}42}$ expression was associated with a reduction in body weight. $^{***}p < 0.001$.

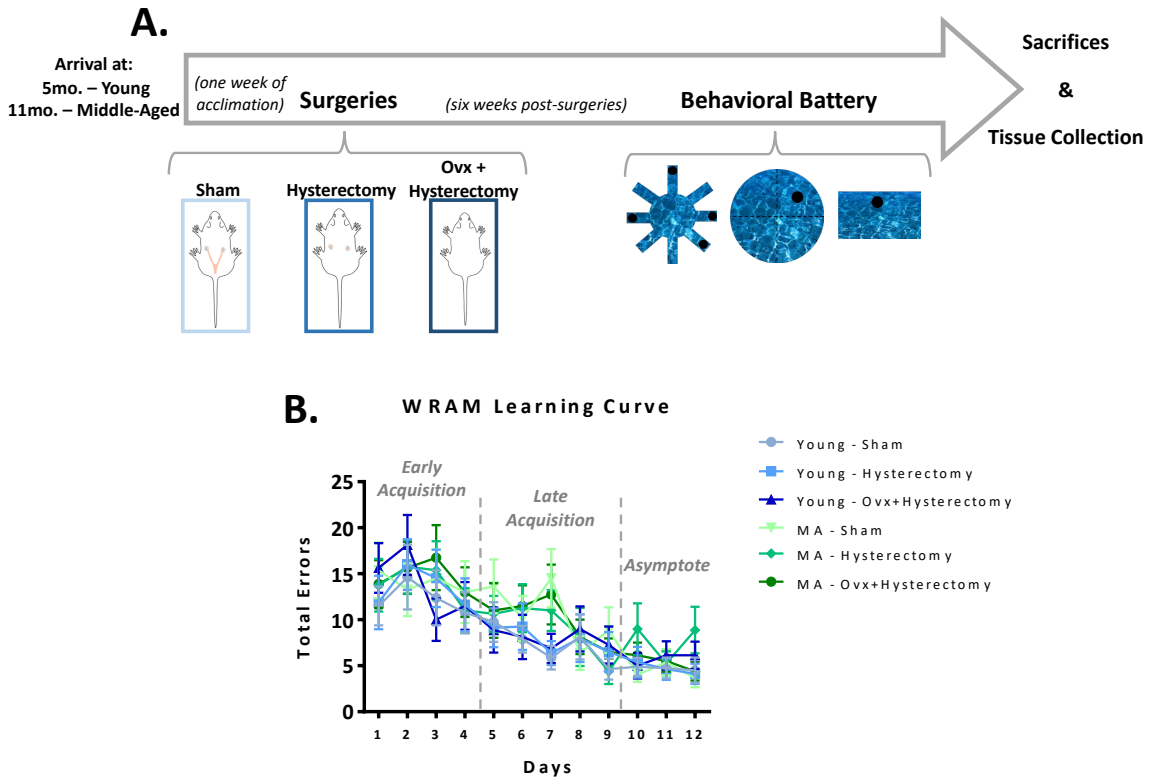


Figure 19: Experimental Timeline. (A) Surgery occurred for the young adult and middle-aged Fischer 344-CDF rats one week after arrival. Six weeks after surgery, animals underwent a behavioral battery that evaluated spatial working and reference memory performance and included the Water Radial-Arm Maze (WRAM), the Morris Water Maze (MWM), and the Visible Platform (VP) Task. Following the conclusion of the behavioral battery, animals were euthanized, where blood, as well as brain and ovarian tissues were collected for further processing. (B) The WRAM learning curve, with total errors made across all baseline testing days represented for each treatment group. Testing was blocked into the Early Acquisition Phase (Days 2-4), the Late Acquisition Phase (Days 5-9), and the Asymptotic Phase (Days 10-12) for further analyses to determine putative differences in task acquisition and maintenance.

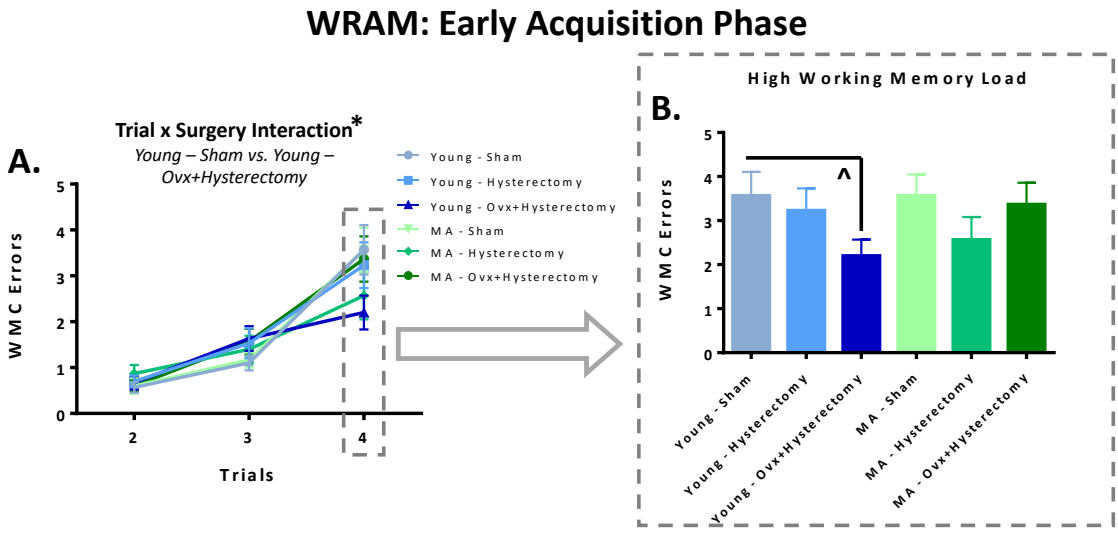


Figure 20: WRAM – Early Acquisition Phase WMC Errors. (A) Following a significant Trial x Surgery interaction for the Young-Sham vs. Young-Ovx+Hysterectomy planned comparison, it was found that (B) for the moderate working memory load trial, Trial 3, rats that had their uterus and ovaries removed tended to perform better relative to Sham rats during learning on the WRAM. Data are presented as the mean ± SEM. * $p < 0.05$, ^ $p < 0.10$.

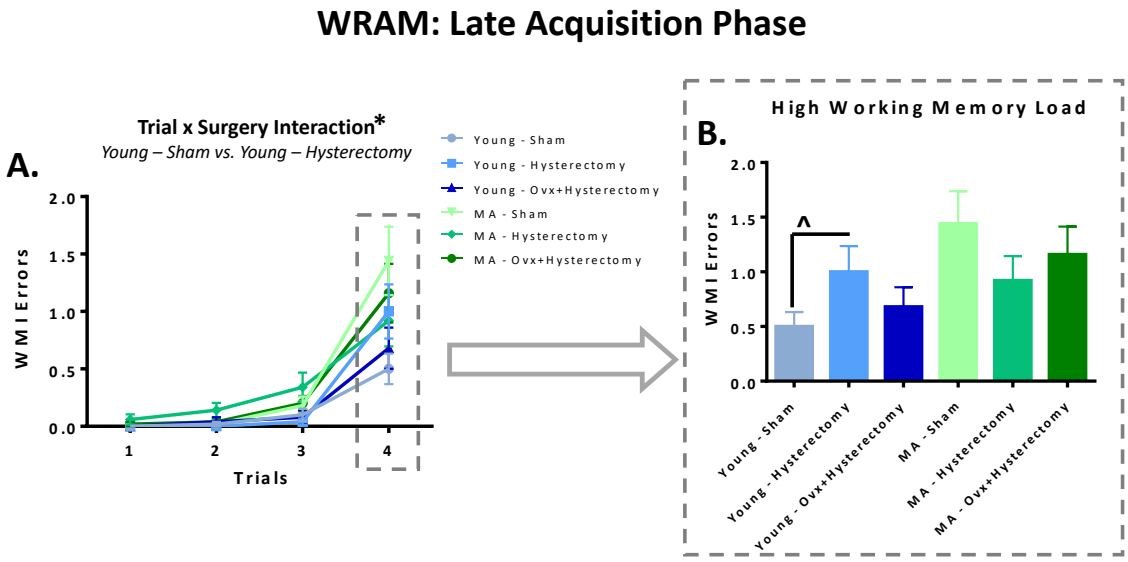


Figure 21: WRAM – Late Acquisition Phase WMI Errors. (A) For the Young-Sham vs. Young-Hysterectomy planned comparison, there was a significant Trial x Surgery interaction. (B) Analyses of the highest working memory load trial showed that hysterectomized young adult rats tended to demonstrate working memory impairment relative to Sham rats; this marginal effect of Surgery was not present for animals that received surgery in middle-age. Data are presented as the mean ± SEM. * $p < 0.05$, ^ $p < 0.10$.

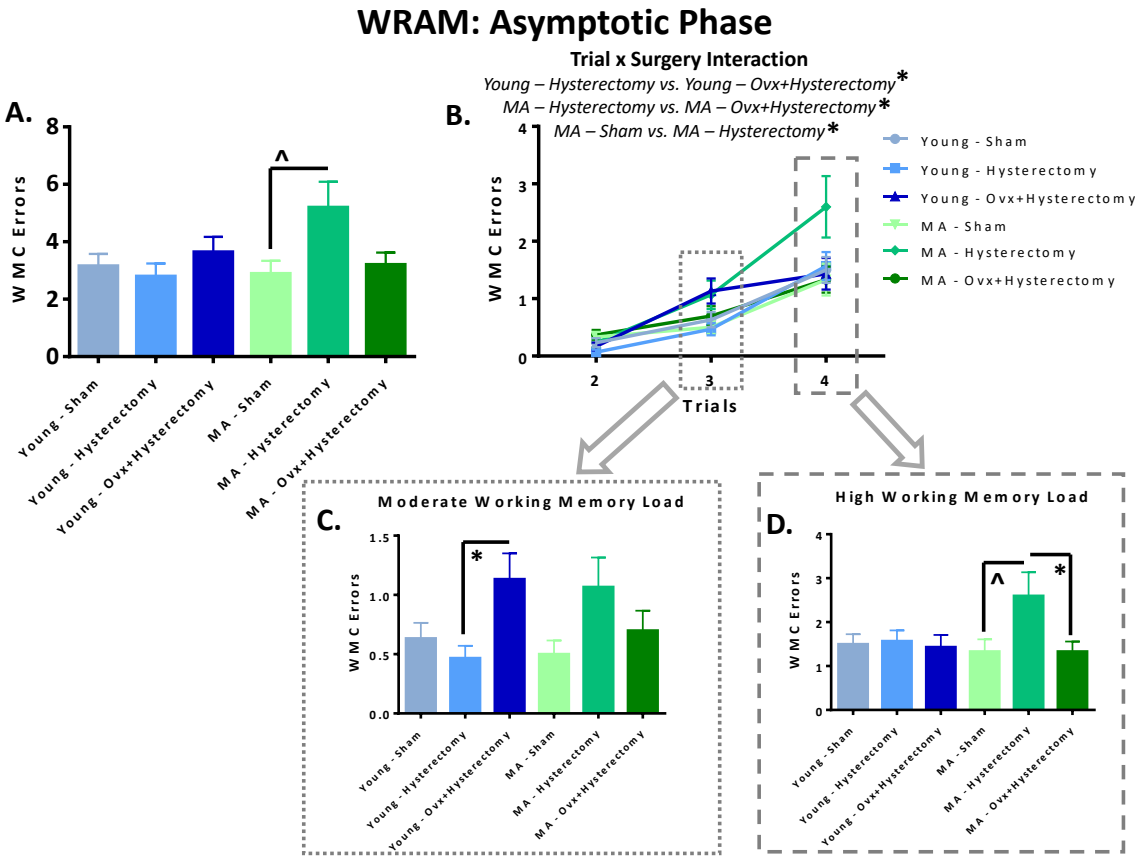


Figure 22: WRAM – Asymptotic Phase WMC Errors. (A) Across all testing trials, there was a marginal working memory impairment for rats that received Hysterectomy surgery in middle-age relative to Sham rats. (B) There were significant Trial x Surgery Interactions for the Young-Hysterectomy vs. Young-Ovx+Hysterectomy planned comparison, as well as for the Middle-Age-Hysterectomy vs. Middle-Age-Ovx+Hysterectomy planned comparison, and for the Middle-Age-Sham vs. Middle-Age-Hysterectomy planned comparison. (C) Subsequent analyses found that for the moderate working memory load trial, rats that received Hysterectomy plus Ovx surgery in young adulthood were impaired relative to age-matched Hysterectomy rats. (D) At the highest working memory load, rats that received Hysterectomy surgery in idle-age tended to make more WMC errors than Sham rats, and made significantly more WMC errors than Ovx+Hysterectomy rats. Data are presented as the mean ± SEM. * $p < 0.05$, ^ $p < 0.10$.

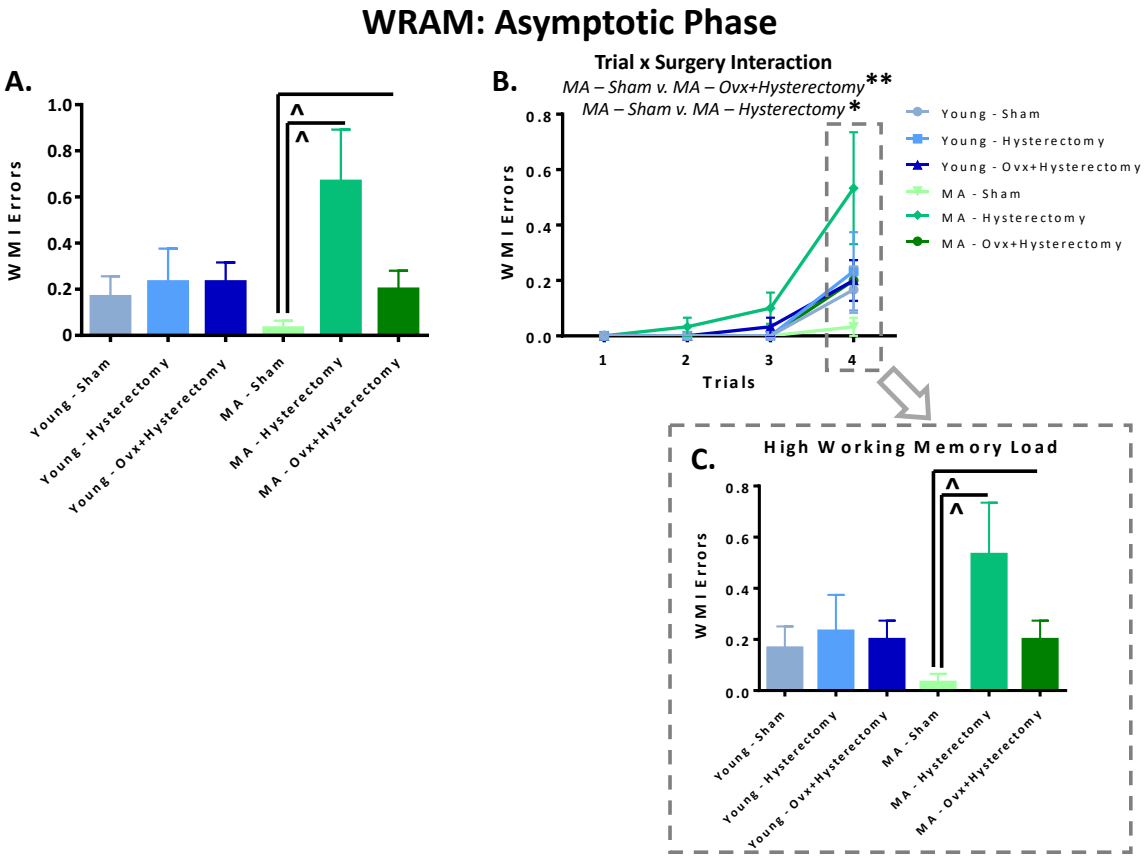


Figure 23: WRAM – Asymptotic Phase WMI Errors. (A) Across all testing trials, there was a marginal working memory impairment for rats that received Hysterectomy surgery in middle-age, regardless of ovarian status, relative to Sham rats. (B) For the Middle-Age-Sham vs. Middle-Age-Hysterectomy planned comparison, as well as for the Middle-Age-Sham vs. Middle-Age-Ovx+Hysterectomy planned comparison, there was a significant Trial x Surgery interaction. (C) Subsequent analyses found that for the highest working memory load trial, rats that received Hysterectomy surgery in middle-age, both with and without ovaries, were marginally impaired relative to Sham animals. Data are presented as the mean ± SEM. ** p <0.01, * p < 0.05, ^ p < 0.10.

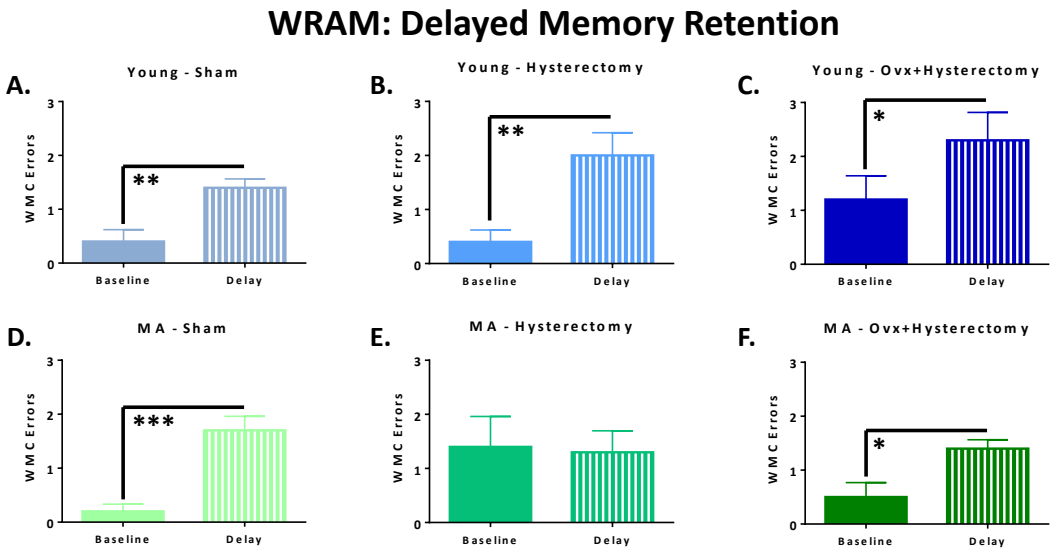


Figure 24: WRAM – Delayed Memory Retention. WMC errors for each treatment group on Trial 3 of the last day of baseline testing, Day 12 (Baseline) compared with the WMC errors made on Trial 3 of the delay day, Day 13 (Delay). All groups (A-D, F) demonstrated delay-induced impairments in working memory performance except the Middle-Age- Hysterectomy group (E). Data are presented as the mean ± SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

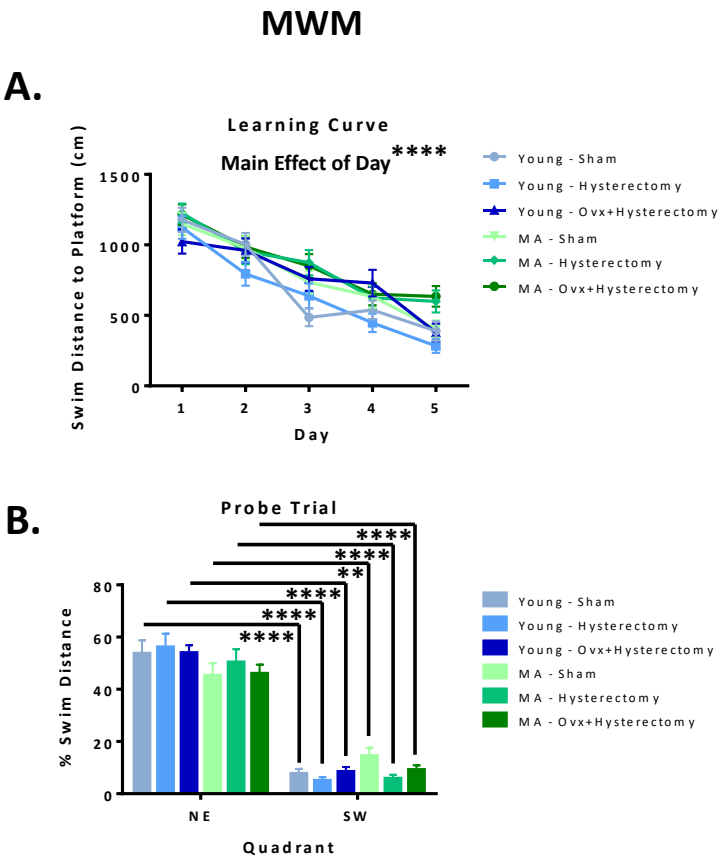


Figure 25: Morris Water Maze. (A) There were no effects of Surgery for any of the planned comparisons across Days 1-5 of MWM testing. (B) All treatment groups spatially localized to the target quadrant more than the opposite quadrant during the probe trial. Data are presented as the mean ± SEM. **** $p < 0.0001$, ** $p < 0.01$.

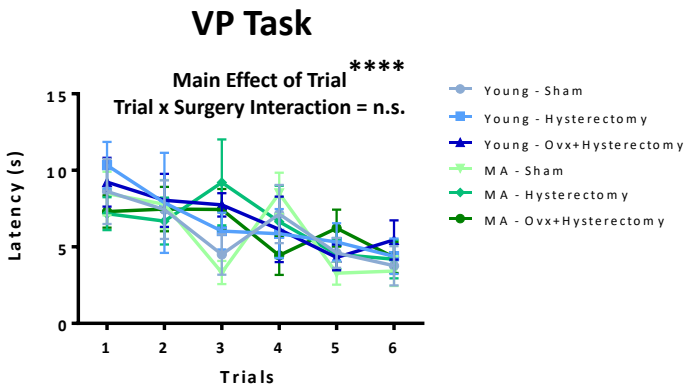


Figure 26: Visible Platform Task. There was a significant effect of Trial, where latency decreased across trials. There was no interaction of Trial with Age or Surgery. No animal had a swim latency that exceeded 15 seconds for the final trial, demonstrating that all animals were capable of completing the procedural components necessary to solve a water-escape task. Data are presented as the mean ± SEM. **** $p < 0.0001$.

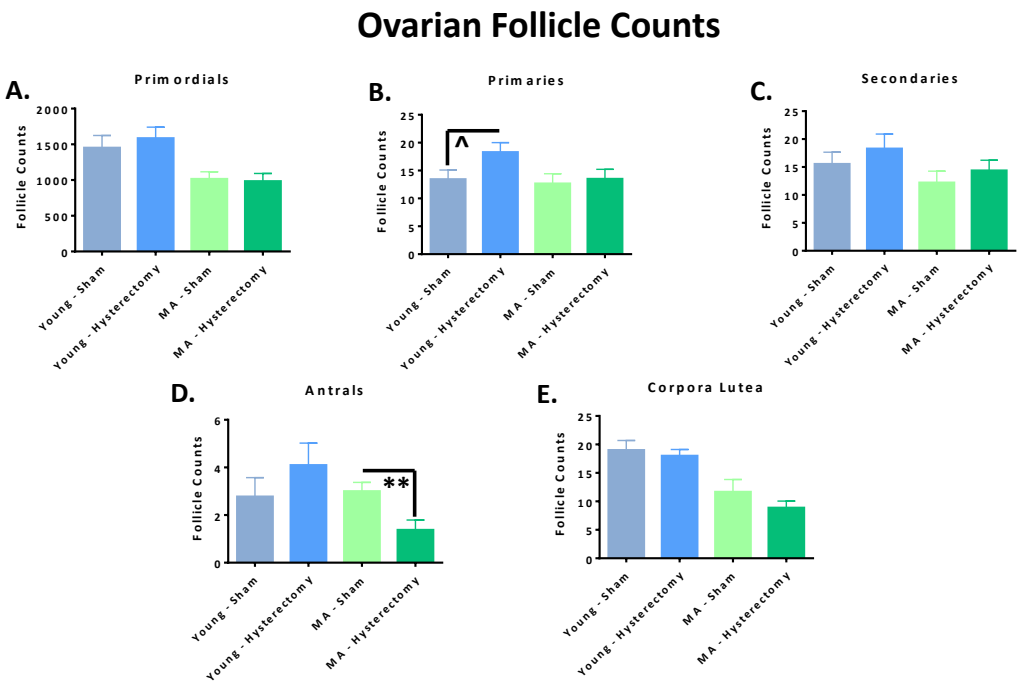


Figure 27: Ovarian Follicle Counts. Ovaries collected at euthanasia for Sham- and Hysterectomy-treated rats were stained such that follicles could be quantified. There were no effects of Surgery in young adulthood or middle-age for (A) primordial follicles. There was a marginal increase in (B) primary follicles with Hysterectomy in young adulthood relative to Sham rats, but not in middle-age. There were no differences in levels of (C) secondary follicles, but for (D) antral follicles, there was a significant decrease in levels following hysterectomy in middle-age relative to Sham rats, but not in young adulthood. There were no differences in levels of (E) corpora lutea following hysterectomy at either age. ** $p < 0.01$, ^ $p < 0.10$.

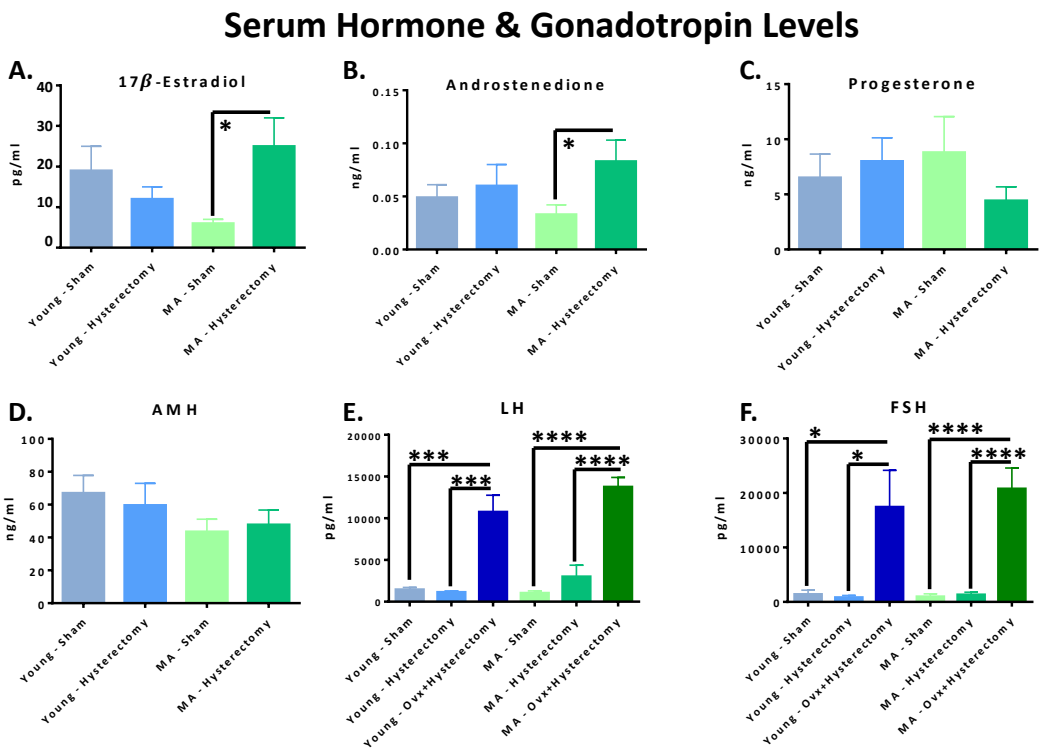


Figure 28: Serum Hormone and Gonadotropin Evaluations. Analyses of serum concentrations of E2 (A) found that hysterectomy resulted in increased levels relative to Sham rats, but only in middle age. Likewise, evaluations of androstenedione (B) found significant increase with hysterectomy in middle-age alone. While there were no effects of hysterectomy for levels of progesterone (C) or AMH (D), levels of LH (E) and FSH (F) were significantly increased in Ovx+Hysterectomy treated rats relative to both Hysterectomy and Sham groups at middle-age and in young adulthood. **** $p < 0.0001$, *** $p < 0.001$, * $p < 0.05$.

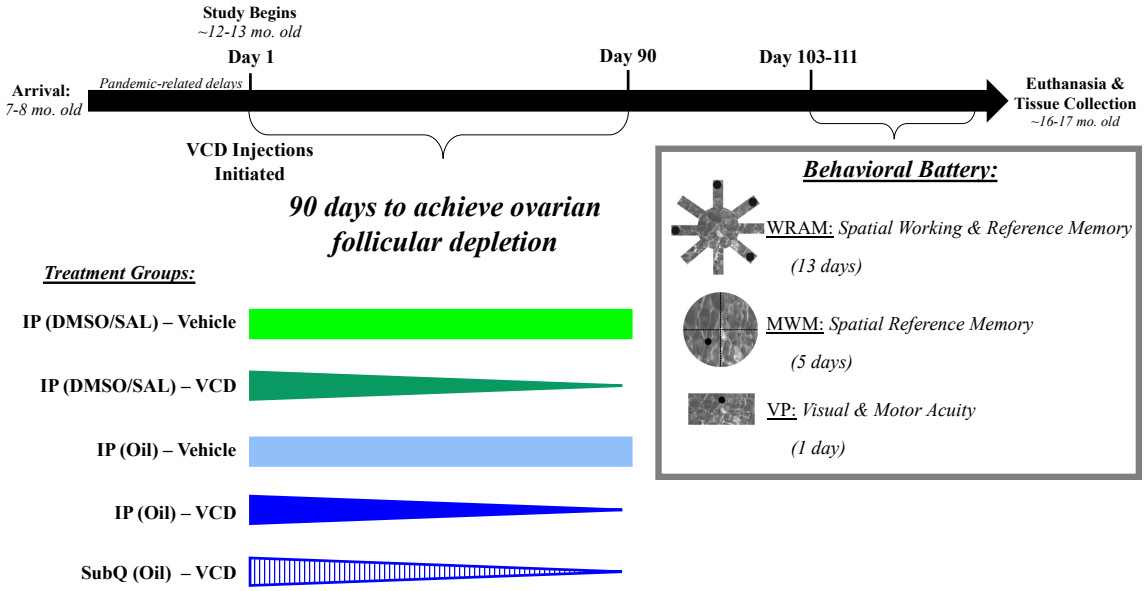


Figure 29: Experimental Timeline. The experimental timeline displays the period of VCD administration and treatment group differences before commencement of the behavioral battery, which included WRAM, MWM, and VP, followed by euthanasia and tissue collection at the conclusion of the study.

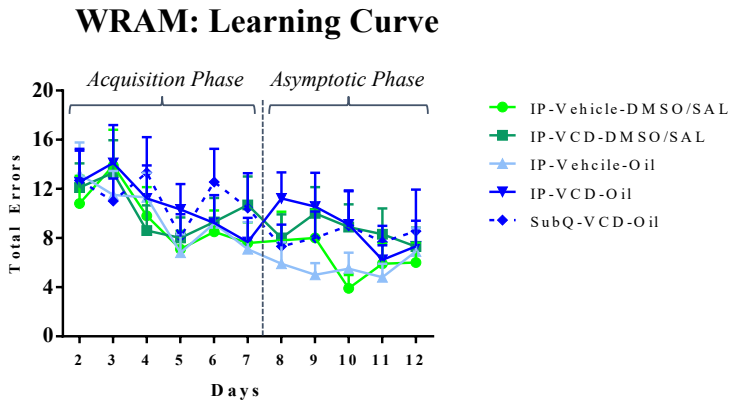


Figure 30: Learning Curve across Baseline Days of WRAM Testing. Demonstration of the learning curves across testing Days 2-12 by treatment group, with a decrease in Total errors across days of testing demonstrating overall learning of the WRAM task in all treatment groups. The graph is separated with a dashed line to demonstrate blocking of the Acquisition and Asymptotic Phases of testing.

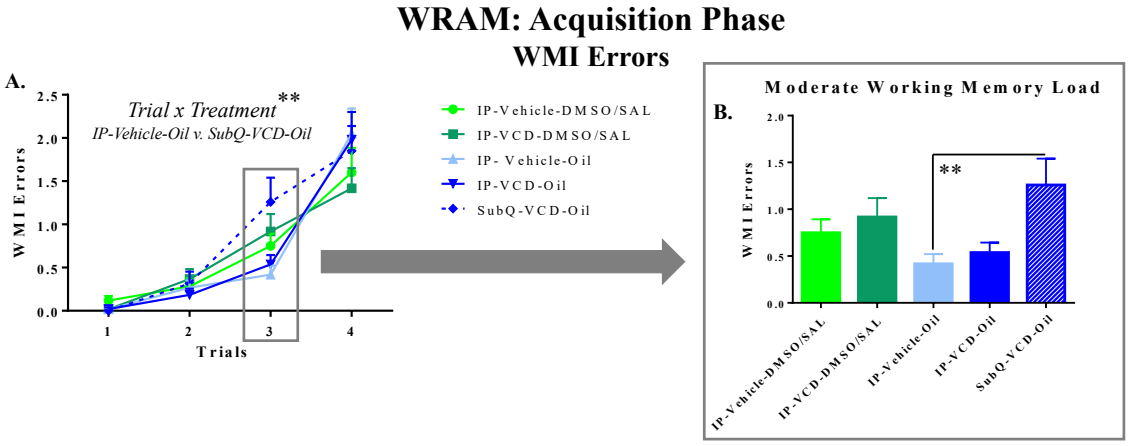


Figure 31: WRAM Performance During the Acquisition Phase – WMI Errors. (A) WMI errors made during the Acquisition Phase of the WRAM across all four trials, demonstrating a Trial by Treatment interaction for the IP-Vehicle-Oil vs. SubQ-VCD-Oil planned comparison. (B) WMI errors made on the moderate working memory load trial (Trial 3) across the Acquisition Phase, where rats in the SubQ-VCD-Oil group displayed working memory impairments as compared to those in the IP-Vehicle-Oil group. $^{**}p<0.01$.

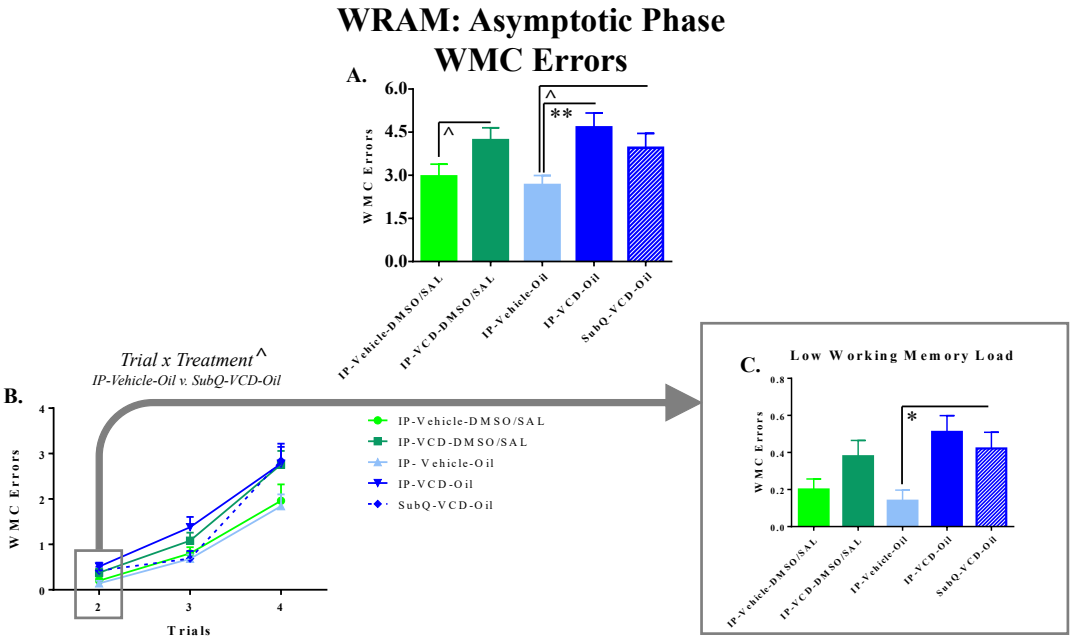


Figure 32: WRAM Performance During the Asymptotic Phase – WMC Errors. (A) WMC errors made during the Asymptotic Phase of the WRAM, demonstrating either marginal or significant impairments with VCD treatment relative to Vehicle-treated rats. (B) Evaluations across trials revealed a marginal Trial by Treatment interaction for the IP-Vehicle-Oil vs. SubQ-VCD-Oil planned comparison for WMC errors. (C) WMC errors made on the low working memory load trial (Trial 2) across the task maintenance phase of the WRAM, where rats in the SubQ-VCD-Oil group displayed working memory impairments as compared to those in the IP-Vehicle-Oil group. $^{**}p<0.01$, $^{*}p<0.05$, $\hat{}p<0.10$.

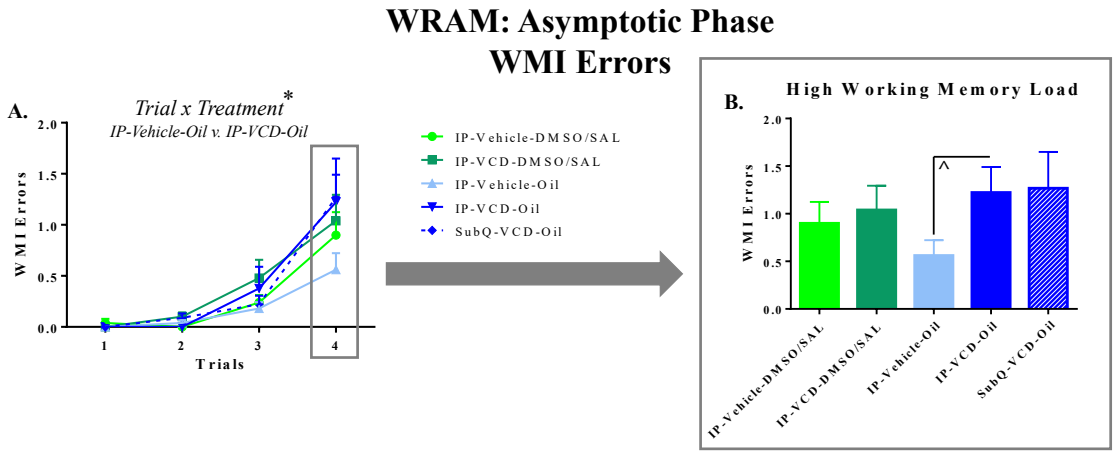


Figure 33: WRAM Performance During the Asymptotic Phase – WMI Errors. (A) WMI errors made during the Asymptotic Phase of the WRAM across all four trials, demonstrating a Trial by Treatment interaction for the IP-Vehicle-Oil vs. IP-VCD-Oil planned comparison. (B) WMI errors made on the high working memory load trial (Trial 4) across the Asymptotic Phase, where rats in the IP-VCD-Oil group displayed a tendency for working memory impairments as compared to those in the IP-Vehicle-Oil group. *p<0.05, ^p<0.10.

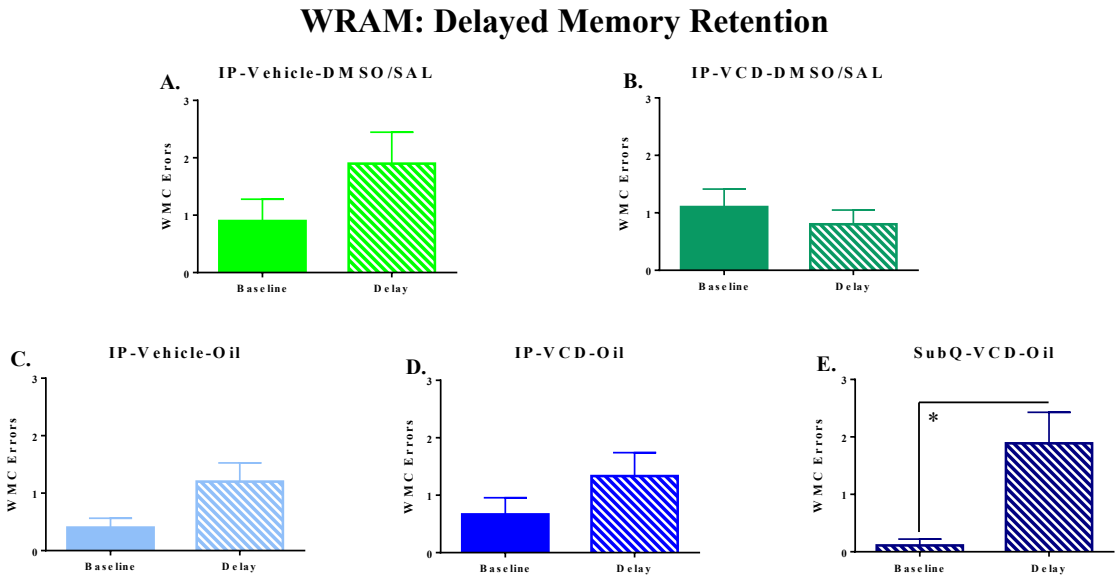


Figure 34: WRAM Performance – Delayed Memory Retention. Following the implementation of a delay between Trials 2 and 3 on the WRAM on Day 13 of testing, none of the groups given Vehicle or VCD via the IP route showed delay-induced deficits (A-D). However, the SubQ-VCD-Oil group (E) showed impairments relative to performance on the same trial from the prior day. *$p<0.05$

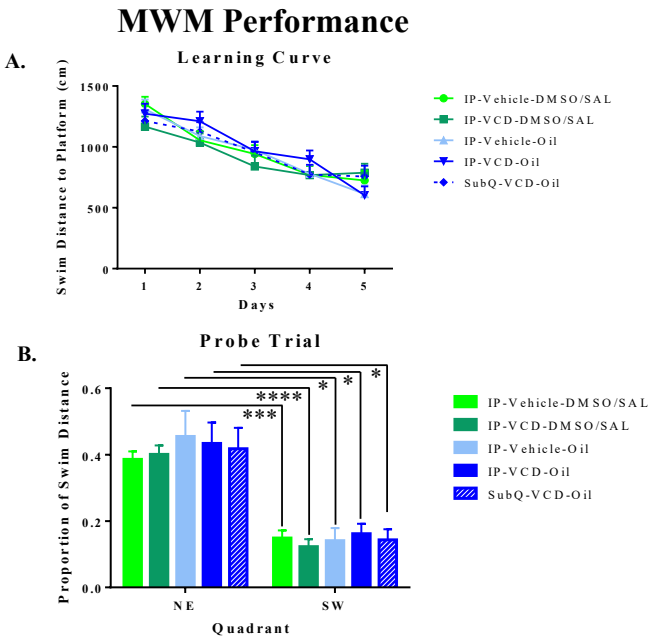


Figure 35: Reference Memory Performance as Evaluated on the MWM. (A) Across all five days of MWM testing, all groups showed a decrease in swim distance to the platform, demonstrating an ability to complete a reference memory task, with no differences between VCD and Vehicle groups. (B) The probe trial on the MWM indicates that all groups had the ability to spatially localize to the platform, given that they spent a significantly greater proportion of distance in the target quadrant (NE) as opposed to the opposite quadrant (SW). ****p<0.0001, ***p<0.001, *p<0.05.

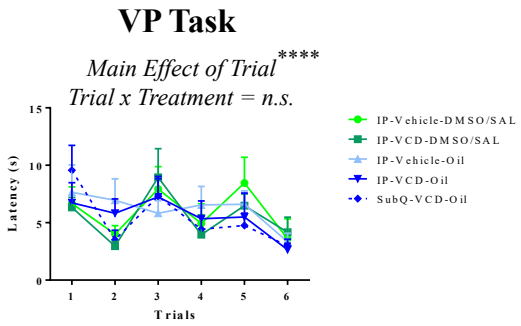


Figure 36: Performance on the VP Task. Analyses of the VP task suggest that rats were able to complete the procedural elements necessary to solve a water-escape task, and had strong visual and motor acuity, given the decrease in swim latency across trials. There were no differences in this ability between groups. ****p<0.0001.

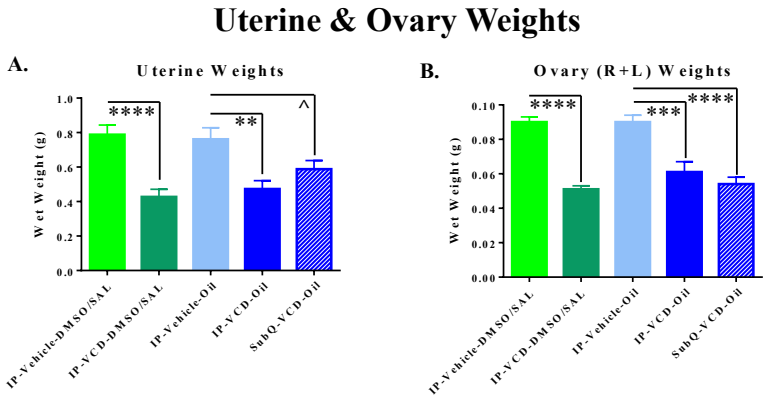


Figure 37: Uterine and Ovary Weights at Euthanasia. (A) Uterine weights collected at sacrifice were reduced for rats treated intraperitoneally with VCD relative to Vehicle controls, but only marginally so in rats in the SubQ-VCD-Oil group. (B) Ovary weights were significantly reduced following VCD treatment for all groups relative to Vehicle controls. ****p<0.0001, ***p<0.001, **p<0.01, ^p<0.10.

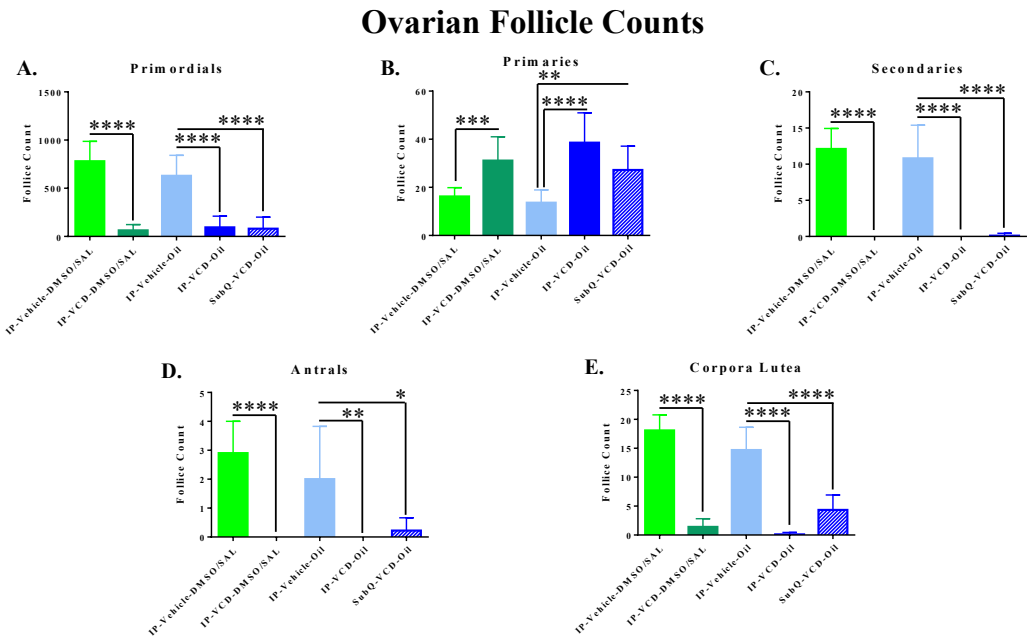


Figure 38: Assessment of Ovarian Follicular Reserves. All VCD-treated groups showed reduced primordial (A) and elevated primary follicles (B), as well as reduced secondary follicles (C), antral follicles (D), and corpora lutea (E) relative to Vehicle-treated groups, a hallmark of the VCD model of transitional menopause. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

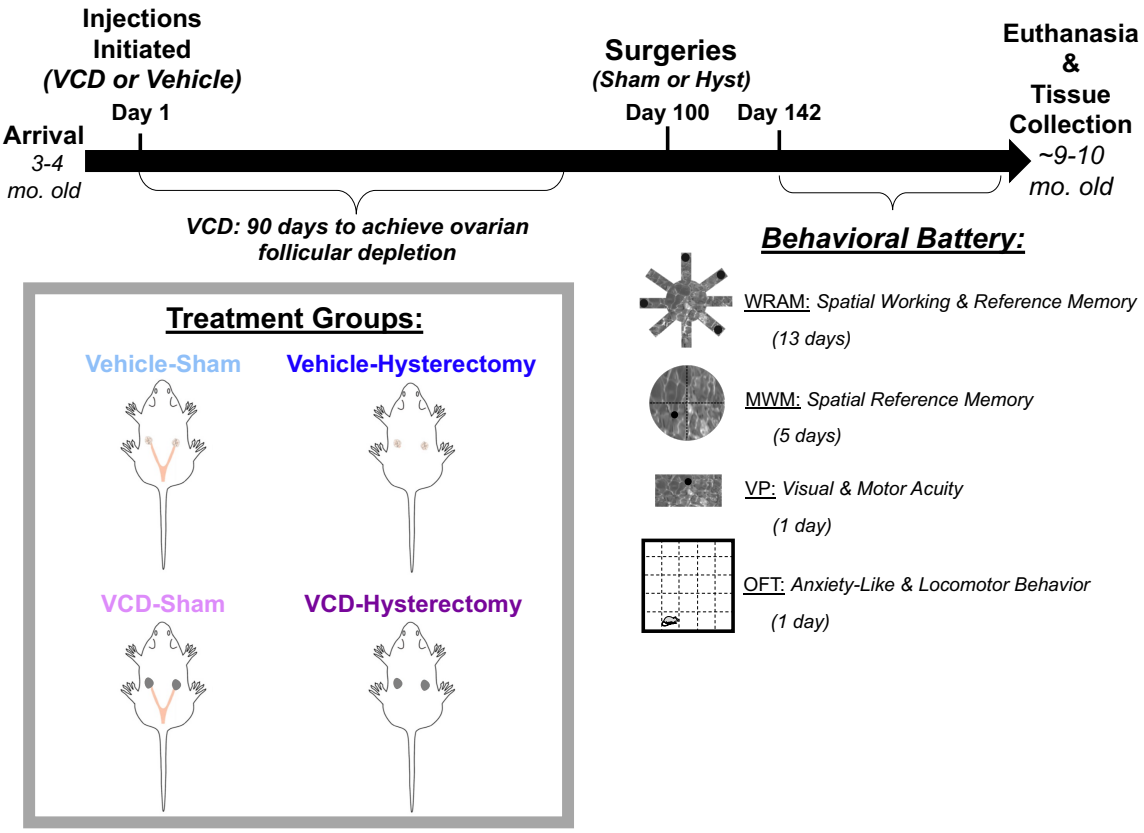


Figure 39: Experimental Timeline. The experimental timeline displays the period of VCD or Vehicle administration, Hysterectomy or Sham surgery, and the behavioral battery, which included WRAM, MWM, VP, and OFT, followed by euthanasia and tissue collection. Treatment groups with surgical and injection manipulations are displayed in the lower left of the figure.

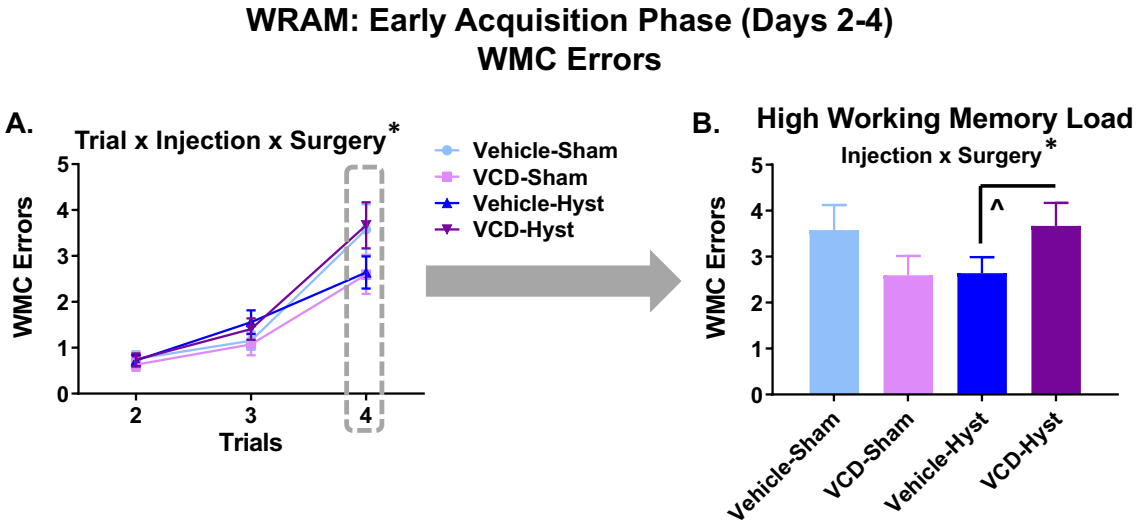


Figure 40: WRAM Performance During the Early Acquisition Phase. (A) A Trial x Injection x Surgery interaction was present for WMC errors during this early learning portion of the task, where (B) for the highest working memory load trial, there was an Injection x Surgery interaction such that VCD induced cognitive deficits in hysterectomized rats, but not in Sham rats. *p<0.05, ^p<0.10.

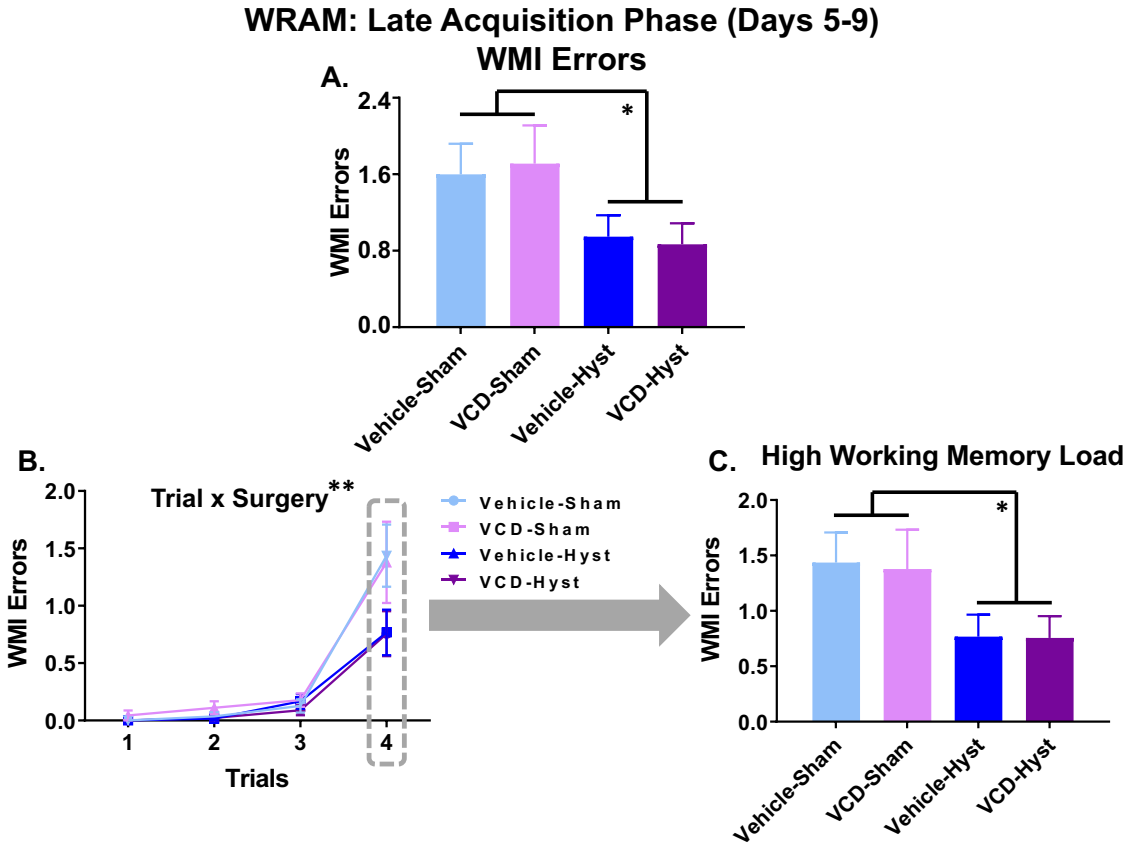


Figure 41: WRAM Performance During the Late Acquisition Phase. (A) During the latter portion of learning on the WRAM task, Hysterectomy-treated rats made fewer WMI errors than their Sham counterparts, regardless of ovarian status. (B) A Trial x Surgery interaction was also revealed, where on the highest working memory load trial (C) hysterectomized rats made fewer errors than Sham rats. **p<0.01, *p<0.05.

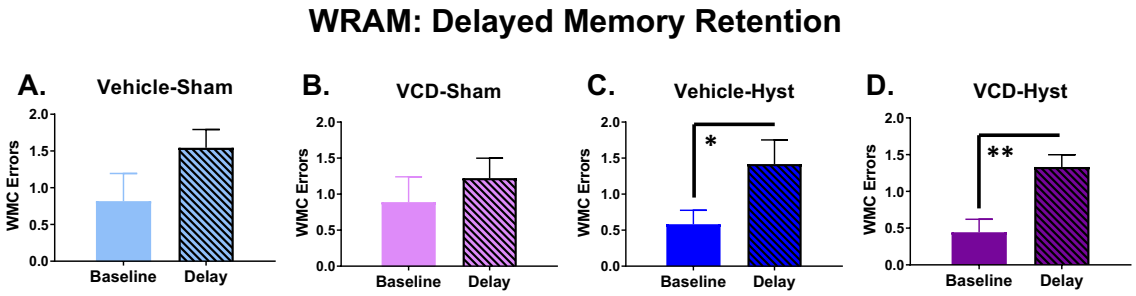


Figure 42: WRAM Performance – Delayed Memory Retention. On Day 13 of WRAM testing, a 6-hour delay placed between Trials 2 and 3 on the WRAM resulted in no difference in WMC errors on Trial 3 (Delay) as compared that of the day prior (Baseline) for the Vehicle-Sham (A) or VCD-Sham (B) groups. However, the Vehicle-Hyst (C) and VCD-Hyst (D) groups showed delay-induced memory impairments. $^{**}p<0.01$, $^{*}p<0.05$.

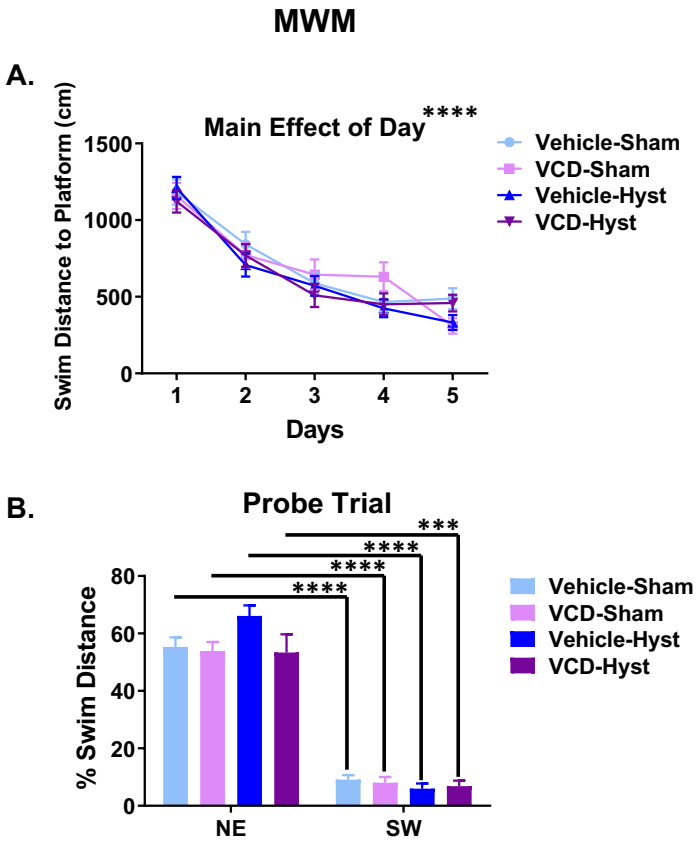


Figure 43: Reference Memory Performance as Evaluated on the MWM. (A) Across all five days of MWM testing, all groups showed a decrease in swim distance to the platform, demonstrating an ability to complete a reference memory task. (B) The probe trial on the MWM indicates that all groups were able to spatially localize to the platform, as they spent a significantly greater proportion of distance in the target quadrant (NE) as opposed to the opposite quadrant (SW). ****p<0.0001, ***p<0.001.

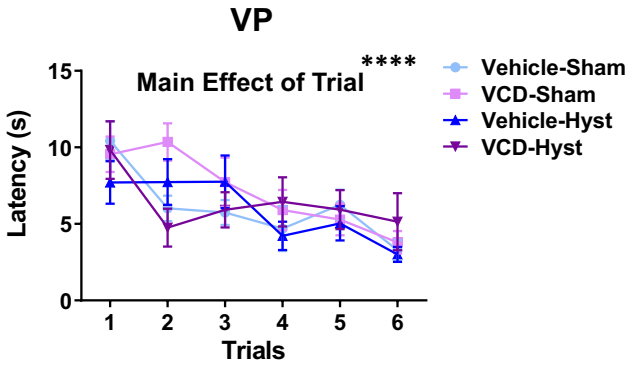


Figure 44: Performance on the VP Task. Analyses of the VP task suggest that rats were able to complete the procedural elements necessary to solve a water-escape task, and had strong visual and motor acuity, given the decrease in swim latency across trials. ****p<0.0001.

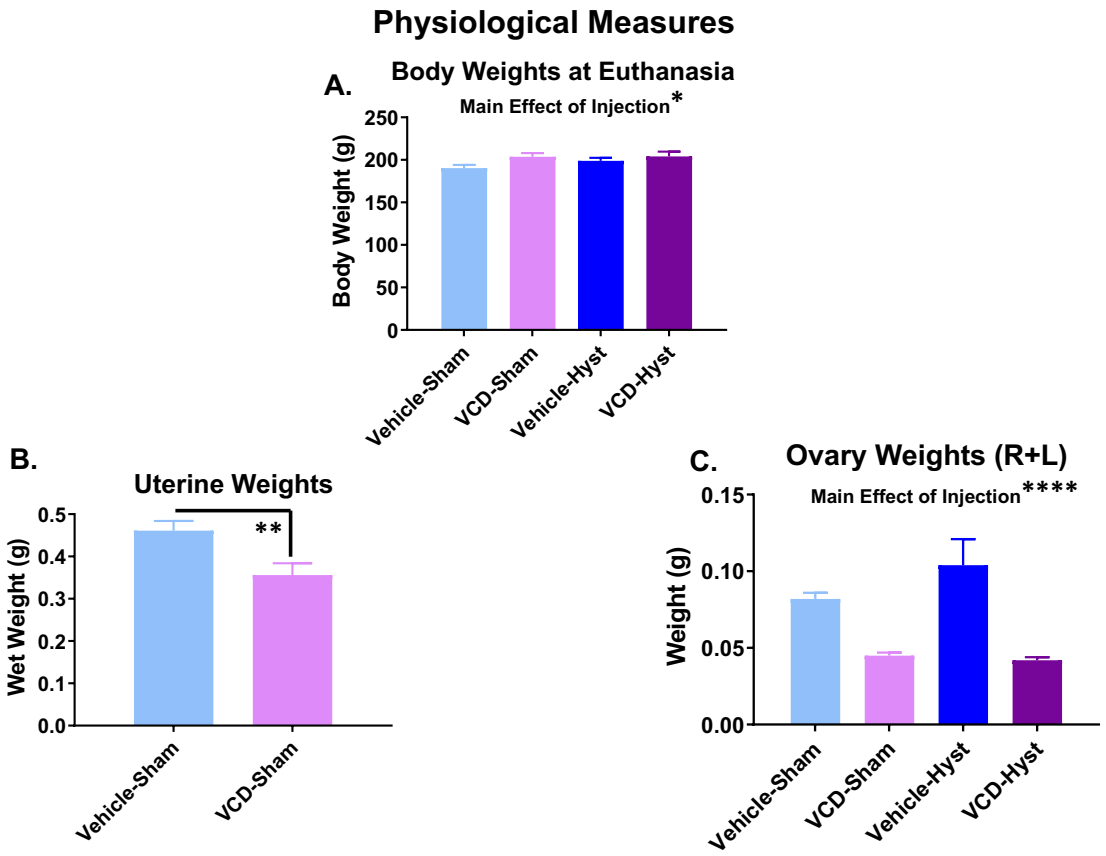


Figure 45: Body, Uterine, and Ovary Weights Collected at Euthanasia. (A) Body weights collected at sacrifice were elevated for rats treated with VCD relative to Vehicle controls, regardless of uterine status. (B) Uterine weights were significantly reduced following VCD treatment for all Sham subjects. (C) Ovary weights were also significantly reduced for VCD-treated rats, regardless of surgery. ****p<0.0001, **p<0.01, *p<0.05.

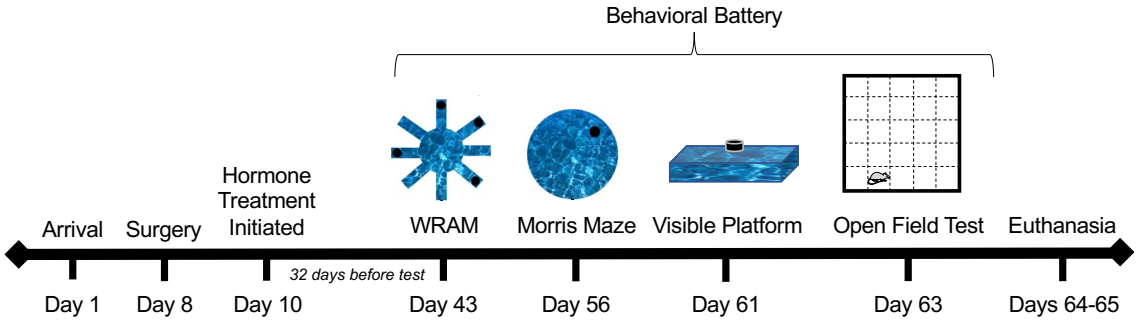


Figure 46: Experimental Timeline. The study timeline, depicting surgeries, hormone administration, and completion of the behavioral battery, which included the WRAM, MWM, VP, and OFT, before euthanasia and tissue collection.

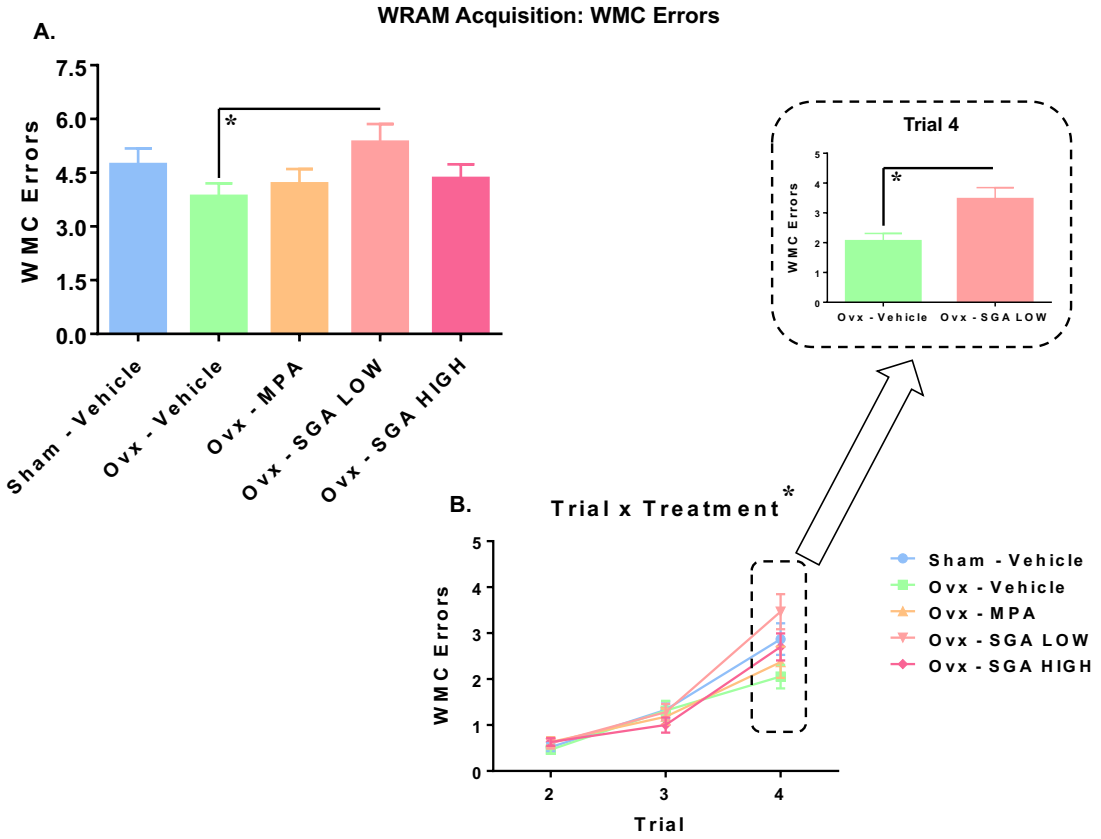


Figure 47: WMC Errors during Acquisition on the WRAM. (A) During task acquisition, Ovx rats administered the low dose of SGA made more WMC errors than Ovx-Vehicle rats. (B) A Trial x Treatment interaction for this comparison, and SGA-induced impairments were present (C) at the highest working memory load trial, Trial 4. *p < 0.05. Sham-Vehicle $n = 9$, Ovx-Vehicle $n = 9$, Ovx-MPA $n = 10$, Ovx-SGA Low $n = 10$, Ovx-SGA High $n = 10$.

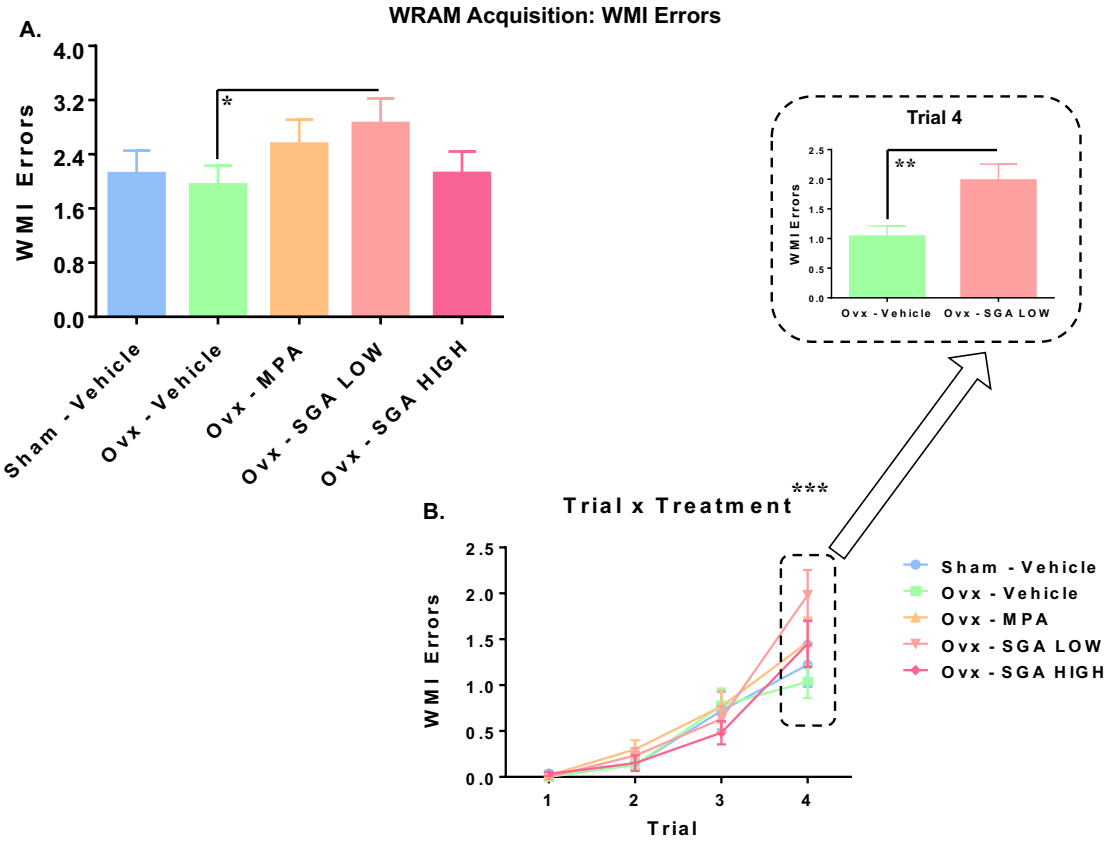


Figure 48: WMI Errors during Acquisition on the WRAM. (A) During task acquisition, rats administered the low dose of SGA made more WMI errors than Ovx-Vehicle rats. (B) The Trial x Treatment interaction for this comparison was significant, revealing SGA-induced impairments (C) at the highest working memory load trial, Trial 4. *p < 0.05, **p < 0.01, ***p < 0.001. Sham-Vehicle $n = 9$, Ovx-Vehicle $n = 9$, Ovx-MPA $n = 10$, Ovx-SGA Low $n = 10$, Ovx-SGA High $n = 10$.

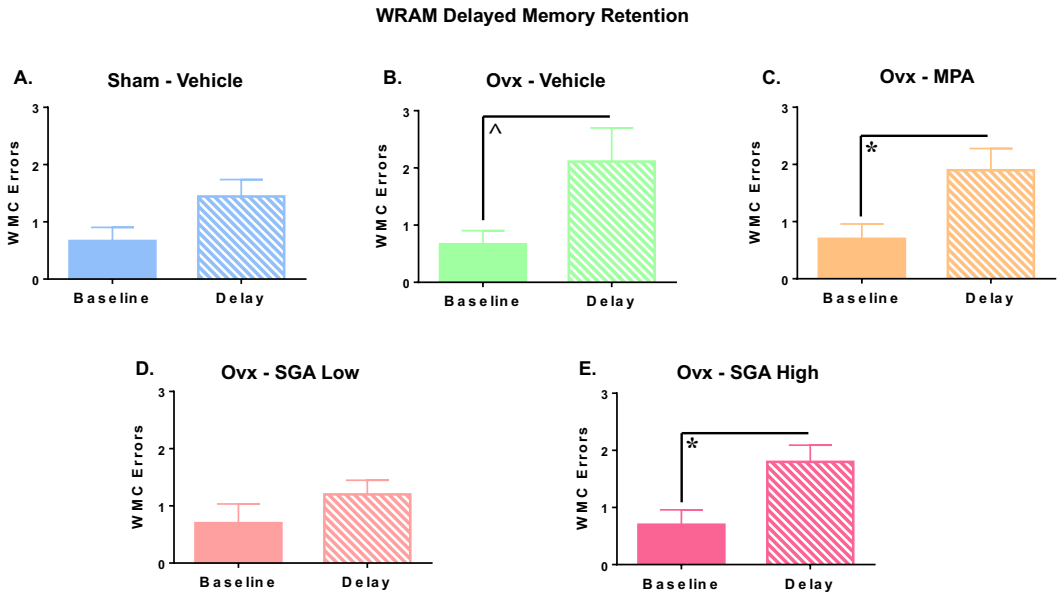


Figure 49: Delayed Memory Retention on the WRAM. Where Sham-Vehicle rats (A) did not show delay-induced impairment, Ovx-Vehicle rats (B) demonstrated marginal deficits following the delay. Ovx-MPA (C) and Ovx-SGA High (E) rats demonstrated delay-induced working memory impairments, where Ovx-SGA Low rats (D) did not show an increase in WMC errors following the delay. ^$p < 0.10$, *$p < 0.05$. Sham-Vehicle $n = 9$, Ovx-Vehicle $n = 9$, Ovx-MPA $n = 10$, Ovx-SGA Low $n = 10$, Ovx-SGA High $n = 10$.

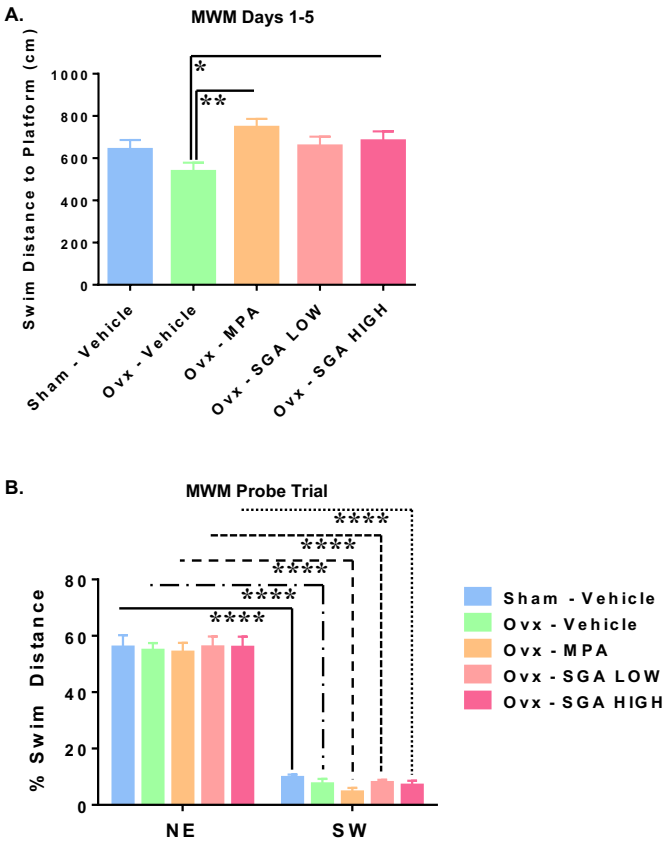


Figure 50: Reference Memory Performance on the MWM. (A) Rats treated with the high dose of SGA, as well as rats treated with MPA, demonstrated reference memory deficits on the MWM compared to Vehicle-treated rats across all 5 baseline days of testing. (B) The probe trial revealed that all groups were able to spatially localize to the platform. *p < 0.05, **p < 0.01, ****p < 0.0001. Sham-Vehicle $n = 9$, Ovx-Vehicle $n = 9$, Ovx-MPA $n = 10$, Ovx-SGA Low $n = 10$, Ovx-SGA High $n = 10$.

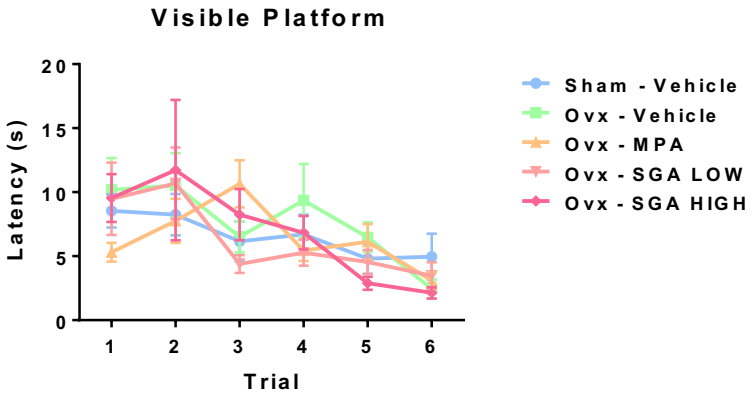


Figure 51: Performance on the VP Task. Rats demonstrated visual and motor acuity, as well as the ability to complete the procedural elements of a water-escape spatial navigation task, as trial latency decreased across the 6 testing trials. Sham-Vehicle $n = 9$, Ovx-Vehicle $n = 9$, Ovx-MPA $n = 10$, Ovx-SGA Low $n = 10$, Ovx-SGA High $n = 10$.

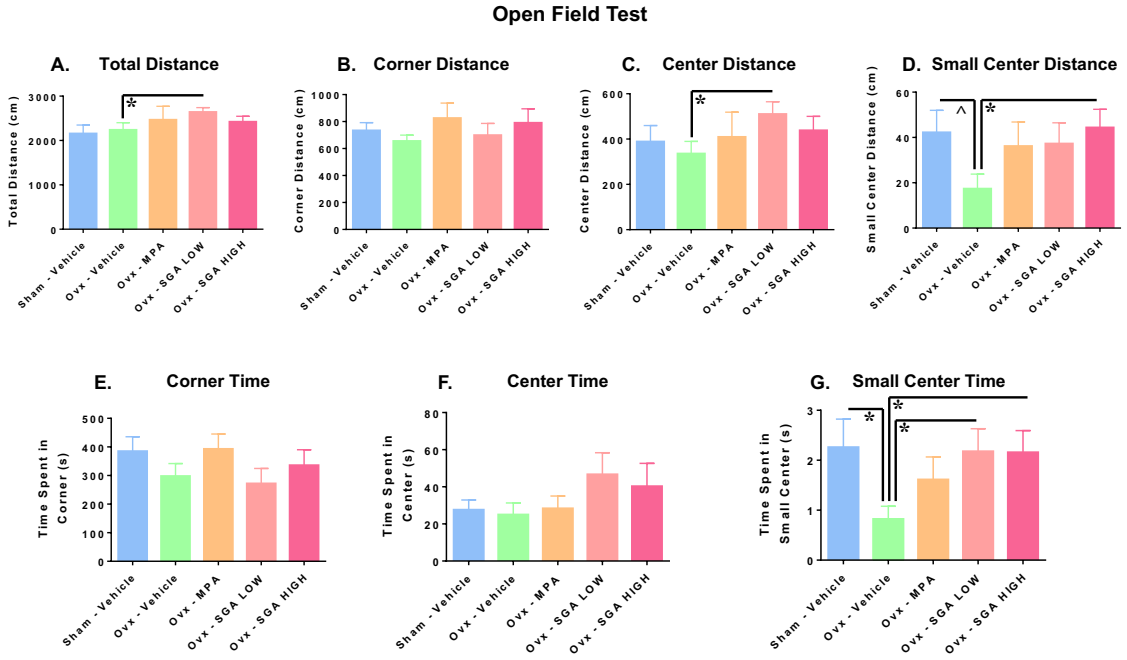


Figure 52: Anxiety-Like and Locomotive Behavior on the OFT. (A) Rats treated with the low dose of SGA demonstrated increased locomotion overall compared with Ovx-Vehicle rats. While there were no effects for corner distance (B) on the OFT, evaluations of distance travelled in the center (C) and small center (D) revealed that Ovx-Vehicle rats demonstrated a tendency towards elevated anxiety-like behavior, while SGA at the low dose and the high dose prevented Ovx-induced elevation in anxiety-like profile. There were no effects of Treatment for time spent in the corners or center of the OFT (E and F). Findings for time spent in the small center (G) reflected a similar pattern observed in distance analyses on the OFT, where SGA reversed the Ovx-induced decreased time in the small center. ^$p < 0.10$, *$p < 0.05$. Sham-Vehicle $n = 9$, Ovx-Vehicle $n = 9$, Ovx-MPA $n = 8$, Ovx-SGA Low $n = 10$, Ovx-SGA High $n = 10$.

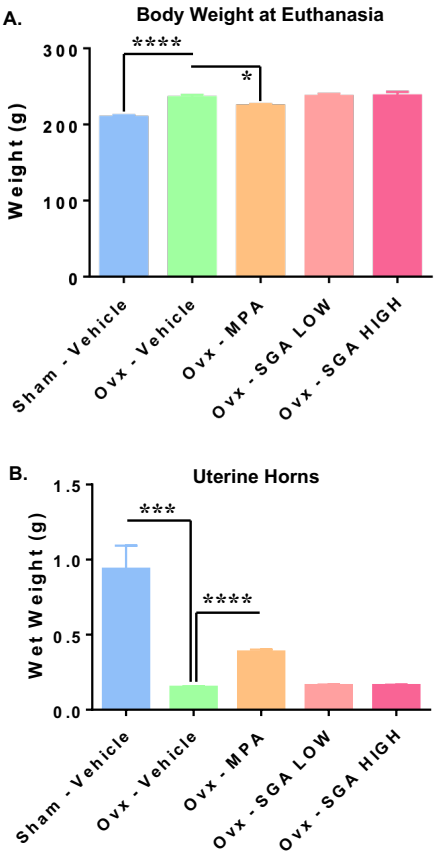


Figure 53: Body and Uterine Weights Collected at Euthanasia. (A) Ovx-Vehicle rats had elevated body weights as compared to Sham-Vehicle rats; administration of MPA attenuated Ovx-induced weight gain. (B) Ovx led to a significant reduction in uterine weight collected at euthanasia relative to Sham rats, but MPA attenuated this Ovx-induced decrease, suggesting some uterine stimulation as a result of MPA administration. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. Sham-Vehicle $n = 9$, Ovx-Vehicle $n = 9$, Ovx-MPA $n = 10$, Ovx-SGA Low $n = 10$, Ovx-SGA High $n = 10$.

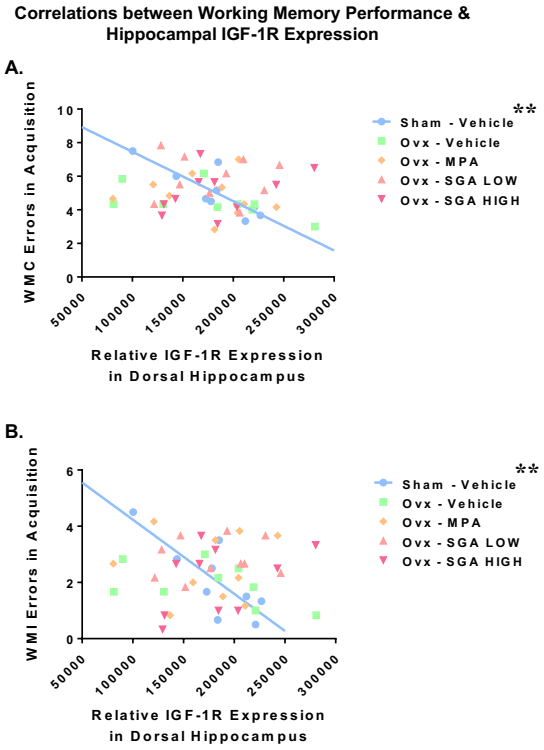


Figure 54: Correlations between Neurobiological and Behavioral Outcomes. Assessment of relationships between WRAM performance and neurobiological outcomes demonstrated significant negative correlations for the Sham-Vehicle rats between relative IGF-1R expression within the dorsal hippocampus and (A) WMC or (B) WMI errors made during learning on the WRAM. **p < 0.01. Sham-Vehicle *n* = 9, Ovx-Vehicle *n* = 9, Ovx-MPA *n* = 10, Ovx-SGA Low *n* = 10, Ovx-SGA High *n* = 10.

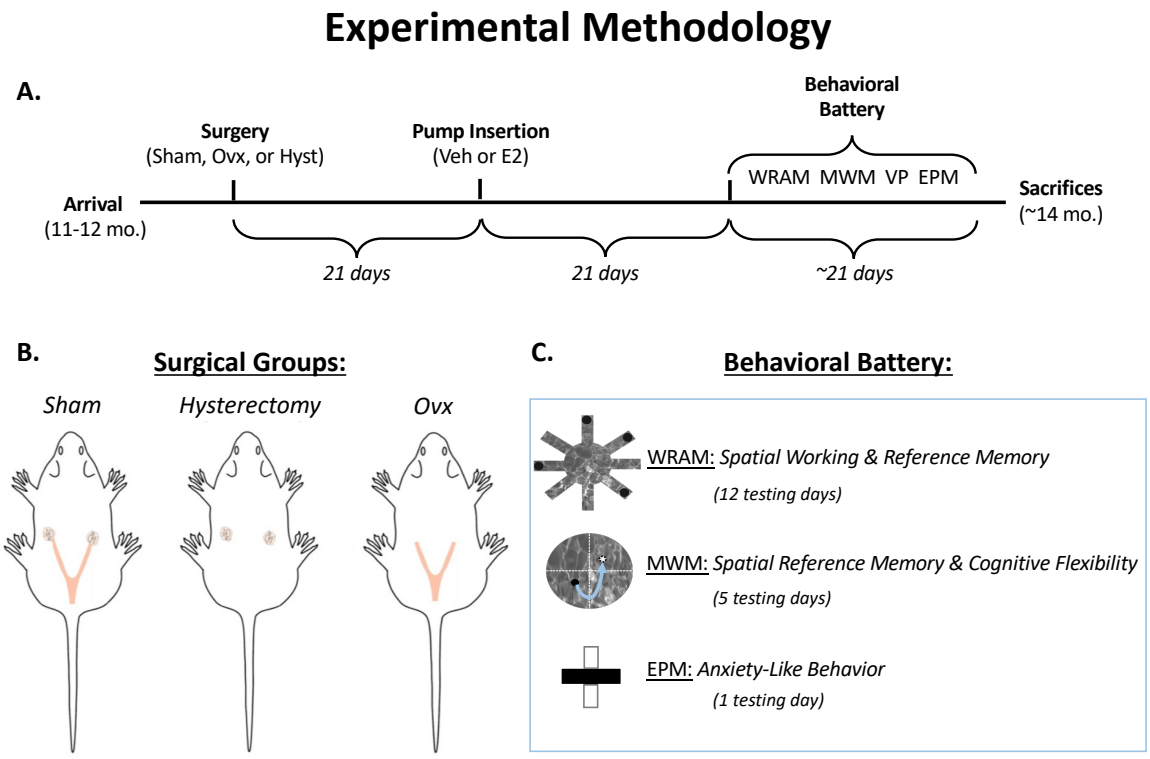


Figure 55: Experimental Timeline. (A) Rats arrived at middle-age (11-12mo.) and received Sham, Ovariectomy (Ovx), or Hysterectomy (Hyst) surgery (B) one week after arrival. Three weeks after surgery, rats received either E2 or Vehicle osmotic pumps. Three weeks later, rats underwent a behavioral battery (C) that evaluated spatial working and reference memory performance on the Water Radial-Arm Maze (WRAM) and the Morris Water Maze (MWM), as well as anxiety-like behaviors as assessed on the Elevated Plus Maze (EPM). Following the conclusion of the behavioral battery, animals were euthanized, where blood, as well as brain, uterine, and ovarian tissues were collected.

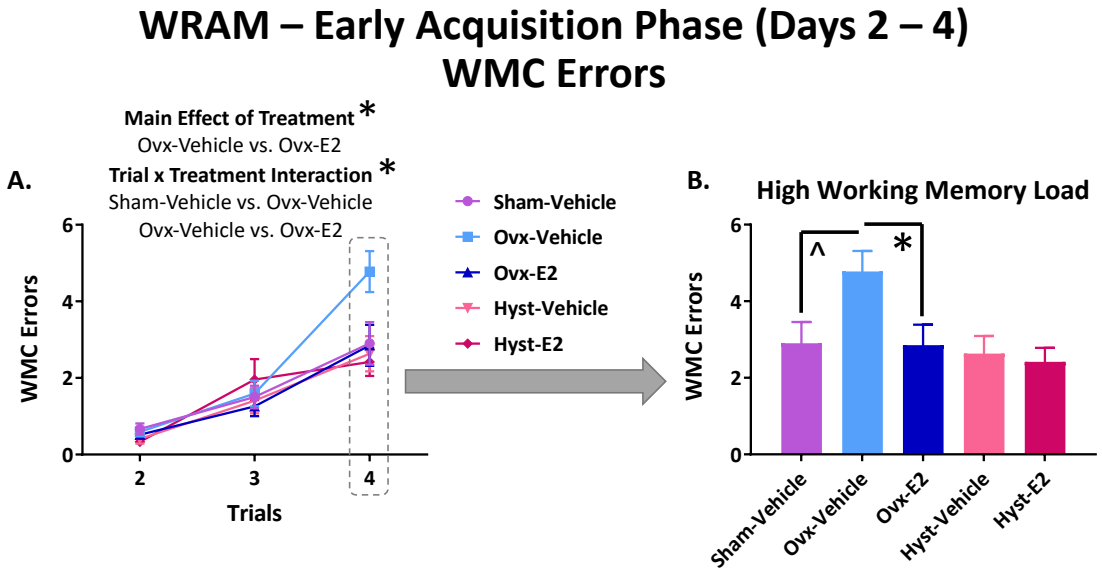


Figure 56: WRAM Early Acquisition Phase – WMC Errors. (A) Across all trials, E2 resulted in a decrease in WMC errors in Ovx rats. Following a significant Trial x Surgery interaction for the Sham-Vehicle vs. Ovx-Vehicle and Ovx-Vehicle vs. Ovx-E2 planned comparisons, it was found that (B) Ovx resulted in marginal working memory impairments, and E2 rescued this impairment in task learning. Data are presented as the mean ± SEM. * $p < 0.05$, ^ $p < 0.10$.

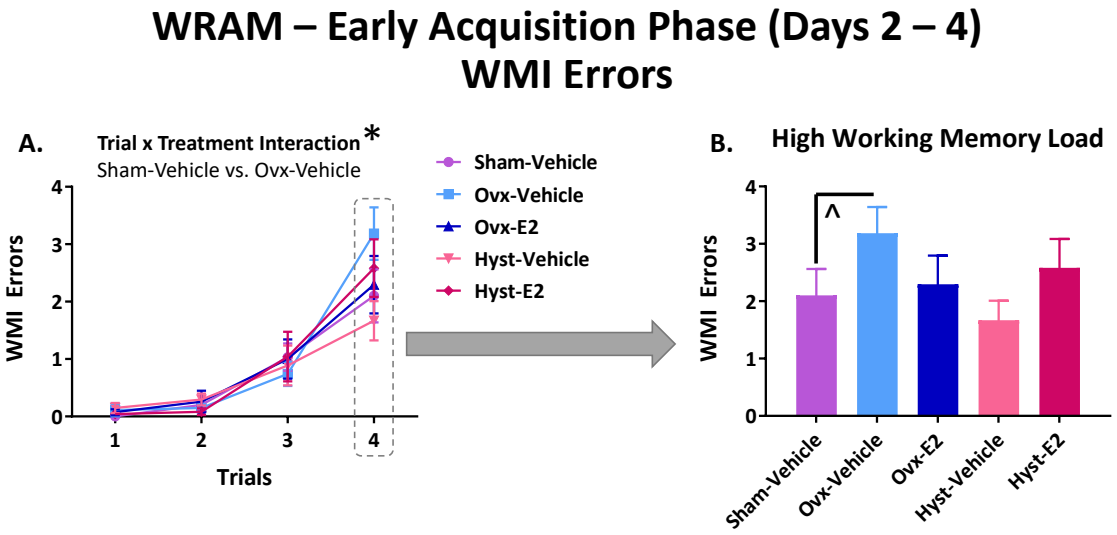


Figure 57: WRAM Early Acquisition Phase – WMI Errors. (A) With a significant Trial x Surgery interaction for the Sham-Vehicle vs. Ovx-Vehicle planned comparison, the high working memory load was evaluated. (B) Analyses revealed that was a marginal working memory impairment for Ovx on Trial 4. Data are presented as the mean ± SEM. * $p < 0.05$, ^ $p < 0.10$.

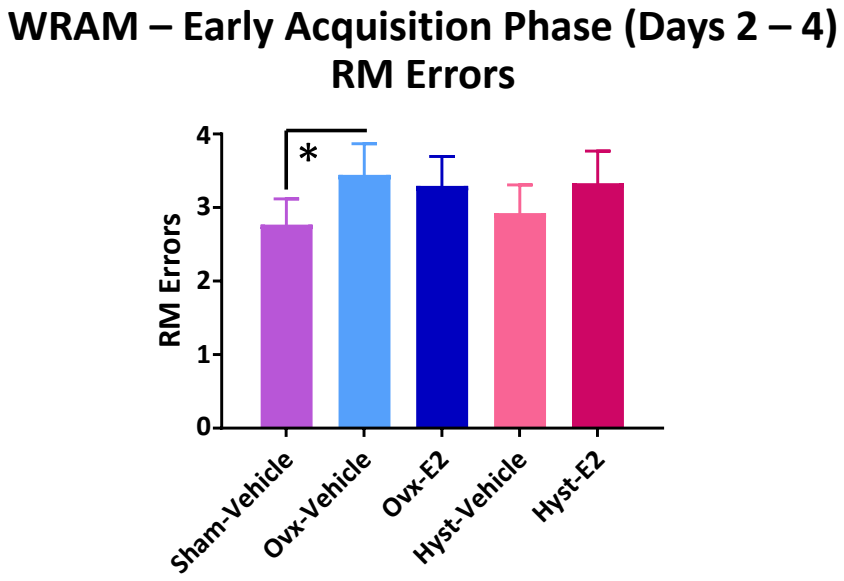


Figure 58: WRAM Early Acquisition Phase – RM Errors. E2 treatment resulted in reference memory improvements during learning in Ovx rats. Data are presented as the mean ± SEM. * $p < 0.05$.

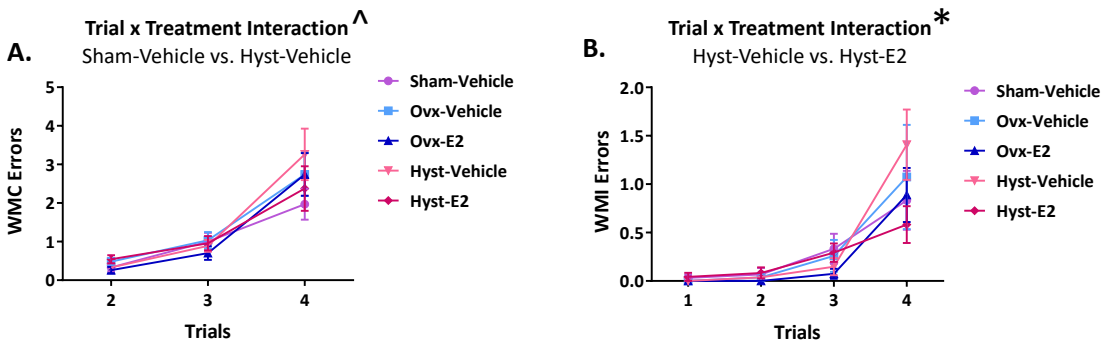


Figure 59: WRAM Asymptotic Phase – WMC & WMI Errors. (A) Hysterectomy results in a tendency for a differential response to a changing working memory load, where hysterectomized rats appeared to make more errors with increasing working memory burden. (B) E2 administration in hysterectomized rats also modulated working memory responsiveness, suggesting estrogen may be protective against a high working memory load. Data are presented as the mean ± SEM. * $p < 0.05$, ^ $p < 0.10$.

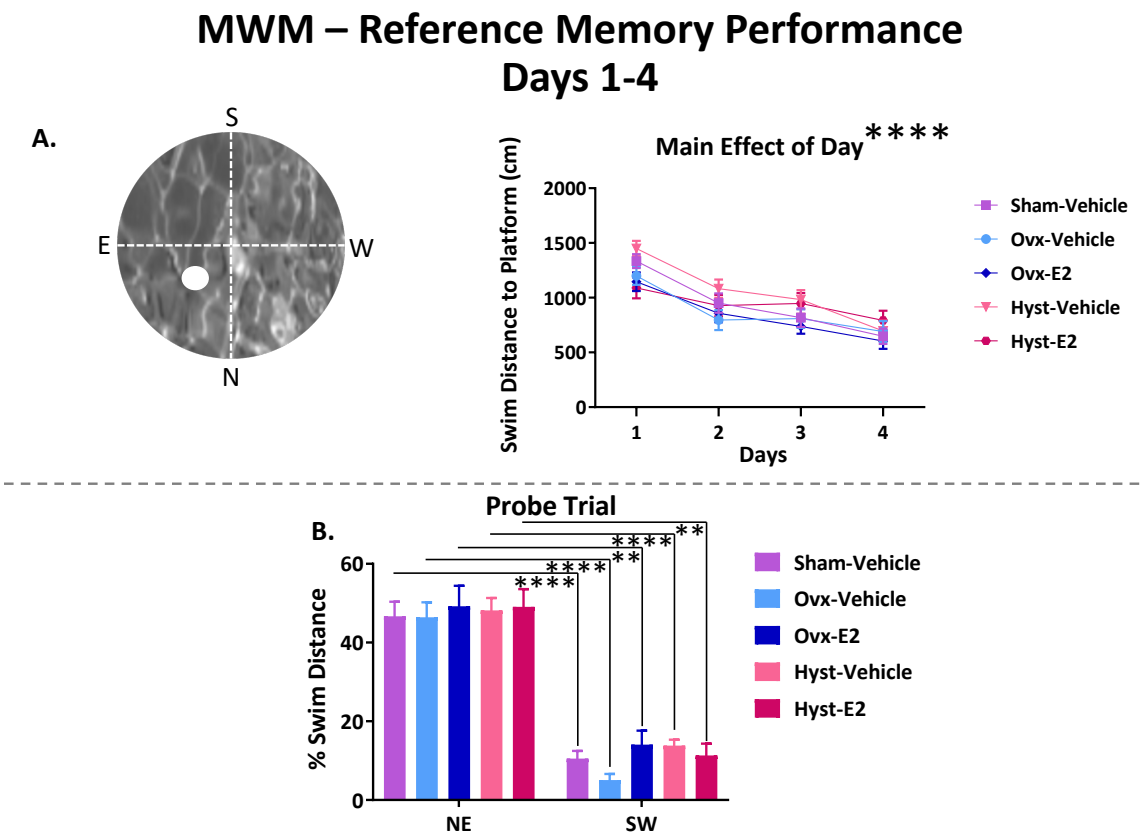


Figure 60: MWM – Reference Memory Deficits. (A) Rats demonstrated learning of the reference memory task, demonstrated as an effect of Days for each planned comparison, with reductions in swim distance to platform across testing days. (B) The first probe trial analysis revealed a main effect of Quadrant for each treatment group, where animals spatially localized the platform in the NE Quadrant. Data are presented as the mean ± SEM. **** $p < 0.0001$, ** $p < 0.01$.

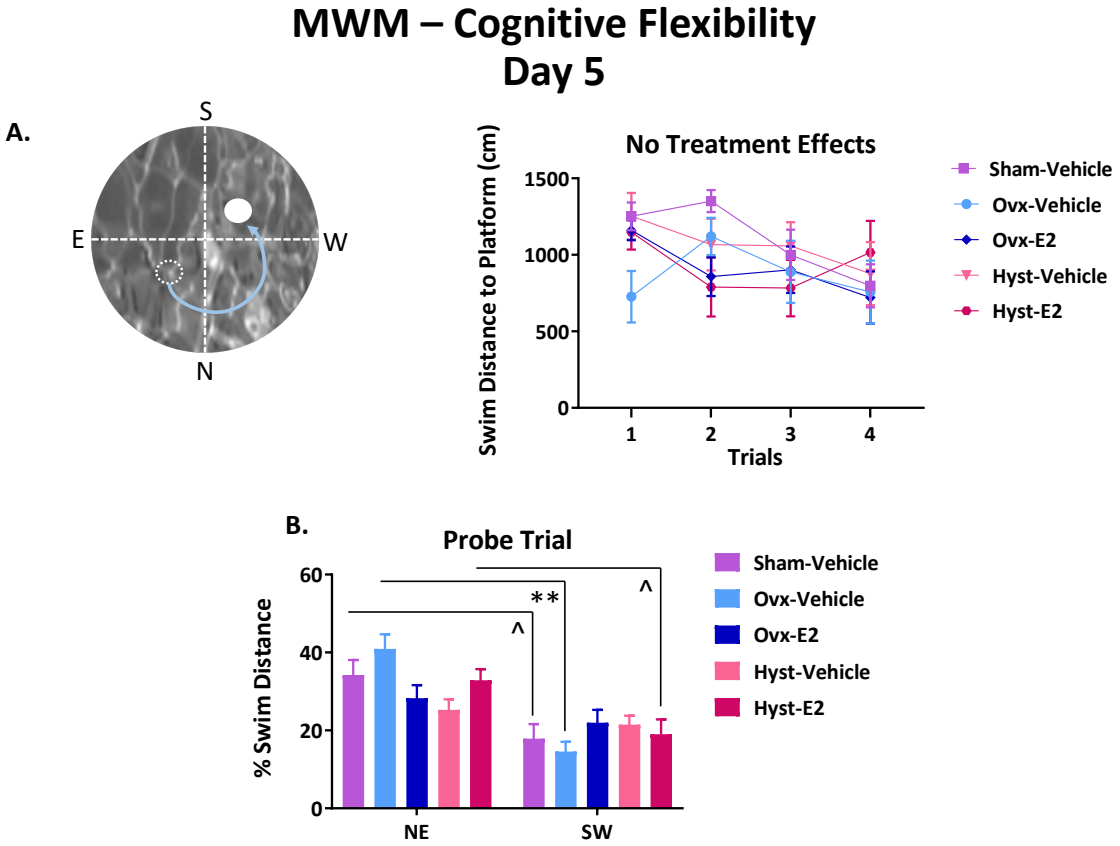


Figure 61: MWM – Cognitive Flexibility. (A) Rats did not differ in their response to the cognitive flexibility portion of the MWM task, where the platform location was switched to the SW Quadrant on the 5th testing day. (B) Probe trial analyses for this novel platform location revealed rats did not spend more time in the SW Quadrant, where the platform was recently hidden, but rather did not differ across the two quadrants (Hyst-Vehicle, Ovx-E2), or spent more time in the NE Quadrant (Sham-Vehicle, Ovx-Vehicle, Hyst-E2). Data are presented as the mean ± SEM. ** $p < 0.01$, ^ $p < 0.10$.

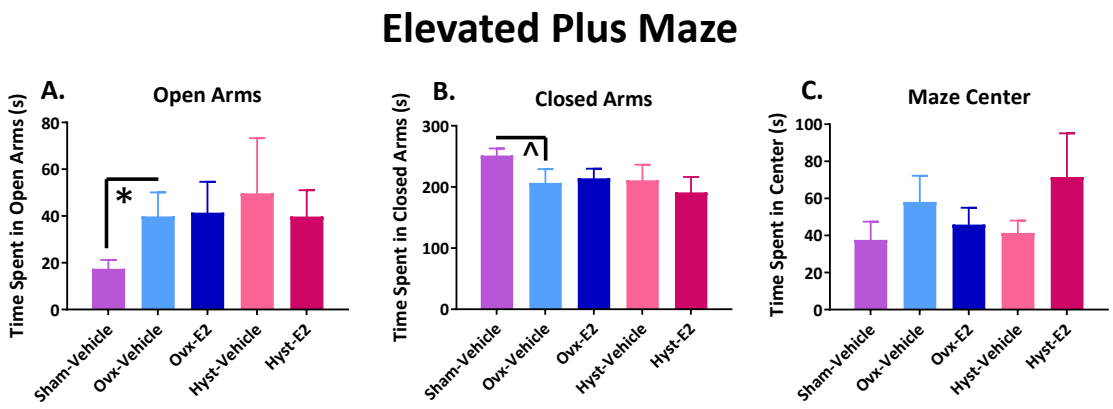


Figure 62: EPM – Anxiety-like Behaviors. (A) Ovx-Vehicle rats spent more time in open arms of the EPM than Sham-Vehicle rats, demonstrating reduced anxiety-like behavior. (B) Likewise, Ovx-Vehicle rats demonstrated a reduced anxiety profile by spending marginally less time in the closed arms of the EPM relative to Sham-Vehicle rats. (C) Rats did not differ across any planned comparison in time spent in the center of the EPM. Data are presented as the mean ± SEM. * $p < 0.05$, ^ $p < 0.10$.

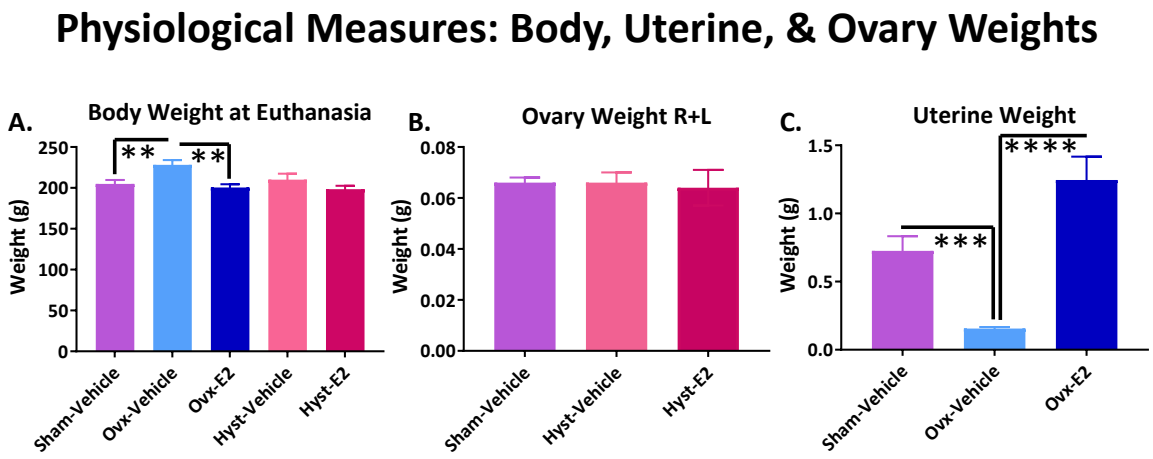
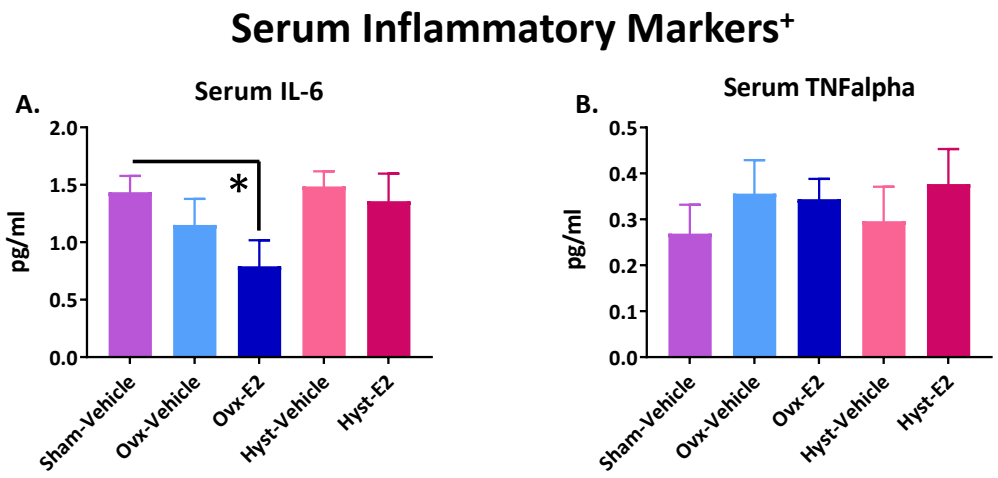


Figure 63: Physiological Measures Collected at Euthanasia. (A) Body weights evaluated at euthanasia showed that Ovx elevated weight, where E2 administration attenuated this weight increase. (B) Ovary weights did not show any differences with hysterectomy or E2 treatment. (C) Uterine weights decreased with Ovx, and E2 resulted in subsequent uterine weight increases, demonstrating stimulation with estrogen supplementation. Data are presented as the mean ± SEM. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$.



+*Values are represented are Log(Conc+1)*

Figure 64: Serum Assays Evaluating Inflammatory Cytokines. (A) Serum ELISAs evaluating IL-6 demonstrated that E2, in the absence of other ovarian hormones, resulted in a decrease in levels of this proinflammatory cytokine relative to Sham rats. (B) There were no Treatment effects for serum TNF-alpha. Data are presented as the mean ± SEM. * $p < 0.05$.

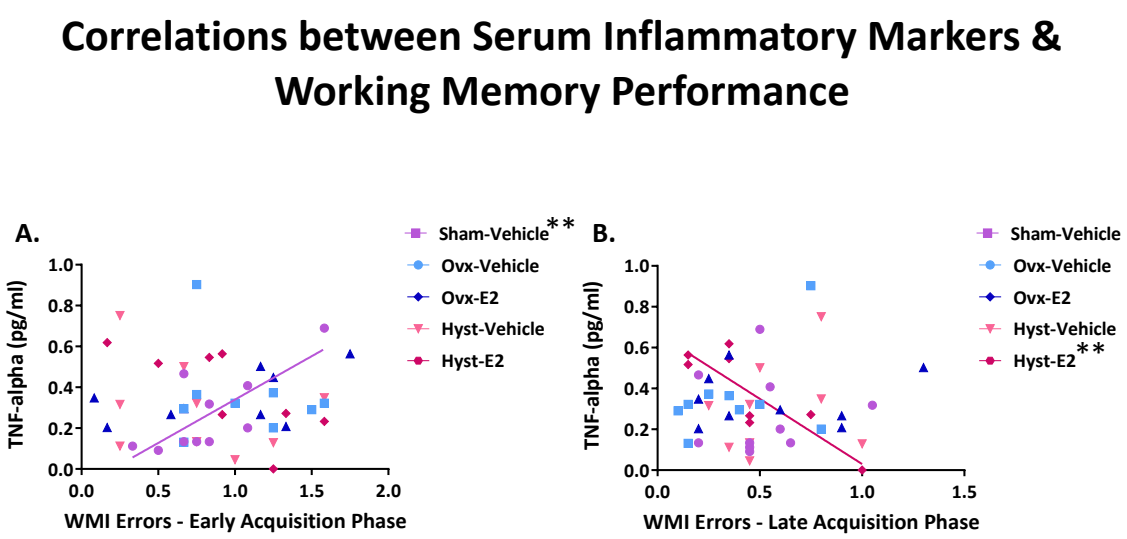


Figure 65: Correlations between Inflammatory and Working Memory Outcomes. Assessment of relationships between WRAM performance and serum inflammatory outcomes demonstrated a significant positive correlation (A) for the Sham-Vehicle rats between TNF-alpha levels and WMI errors made during the Early Acquisition Phase of the WRAM, as well as a significant negative correlation (B) for Hyst-E2 rats between TNF-alpha levels and WMI errors made during the Late Acquisition Phase of the WRAM. ** $p < 0.01$.

APPENDIX B

GIN MARTINI WITH A TWIST

GIN MARTINI WITH A TWIST

Ingredients

1 ½ oz Gin (something that has a complex flavor profile – "The Botanist" is preferred)

¾ oz White Vermouth

Lemon

Jarred Green Olives (optional)

Instructions

Place a martini glass in the freezer at least 15 minutes before beginning your cocktail. In a glass with a large cube of ice, add gin and vermouth. Stir until the outside of the glass feels cold. Strain the liquid into the frozen martini glass. Finally, peel a strip from the lemon, and add the "twist" to the cocktail, expressing the oils before placing it into the drink.

If you like a more savory cocktail ("Hyrum-Style"), add a jarred green olive on a cocktail skewer to the drink in addition to the lemon peel.

I hope you enjoy! ☺