Variations in Menopause Etiology Affect Cognitive Outcomes: How Age, Menopause Type, and Exogenous Ovarian Hormone Exposures Across the Lifespan Impact the Trajectory of Brain Aging

by

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ABSTRACT

Reproductive hormones are recognized for their diverse functions beyond reproduction itself, including a vital role in brain organization, structure, and function throughout the lifespan. From puberty to reproductive senescence, the female is characterized by inherent responsiveness to hormonal cyclicity. For most women, a natural transition to menopause occurs in midlife, wherein the endogenous hormonal milieu undergoes significant changes and marks the end of the reproductive life stage. Although most women experience natural menopause, many women will undergo gynecological surgery during their lifetime, which can lead to an abrupt surgical menopause. It is of critical importance to better understand how endogenous and exogenous reproductive hormone exposures across the lifespan influence cognitive and brain aging, as women are at a greater risk for developing a variety of diseases after menopause, including dementia. Using rodent models, this dissertation explores how the etiology of reproductive senescence, that is, whether it is transitional or surgical, influences the female phenotype to result in divergent cognitive outcomes dependent upon a variety of factors, with an emphasis on age at the time of intervention playing a key role in brain outcomes. Furthermore, the impact of exogenous hormone therapy on cognition is evaluated in the context of surgical menopause. A novel rat model of hysterectomy is also presented, with results demonstrating for the first time that the nonpregnant uterus, which is typically considered to be a quiescent organ, may play a unique, direct role in modulating cognitive outcomes. Neurobiological mechanisms associated with reproductive hormones and aging are assessed to better recognize neural correlates underlying the observed behavior changes. The overarching goal of this

dissertation was to elucidate novel factors contributing to cognitive aging outcomes in females. Collectively, the data presented herein indicate that the age at the onset of reproductive senescence has significant implications for learning and memory outcomes, and that variations in gynecological surgery can have unique, long-lasting effects on the brain and cognition. Translationally, this series of experiments moves the field forward toward the goal of improving the health and quality of life for women throughout the lifespan.

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CHAPTER 1

GENERAL INTRODUCTION

From early in embryonic development to the end of the lifespan, hormones play a vital role in regulating many functions in the body, without which an individual could not survive. The functional role of hormones —particularly reproductive hormones— transcends species, highlighting their necessity and importance. The intricate interplay among hormones acting on many body systems results in particular phenotypes that influence the health and survival of the organism. It is now known that the role of reproductive hormones goes beyond reproduction itself, and these hormones have crucial functions in many systems, including the brain. Understanding how reproductive hormones influence human development, reproduction, the brain, and cognition has been of substantial interest in the last several decades. This dissertation details investigations utilizing rodent models into how endogenous and exogenous reproductive hormone exposures across the lifespan influence cognitive and brain aging, with an emphasis on reproductive senescence. I explore how the etiology of reproductive senescence, that is, whether it is transitional or surgical, can influence the female phenotype to result in divergent cognitive outcomes dependent upon a variety of factors, with an emphasis on age at the time of intervention playing a key role in brain outcomes.

A Brief Primer on Mammalian Sexual Differentiation

For a behavioral endocrinologist, it all begins with sex. According to the long-standing model based on a plethora of research, for mammals, sexual differentiation of

the gonads results from a cascade of events initiated by the chromosomal constitution of the animal (Figure 1a). This model is traditionally linear: chromosomal constitution leads to gonadal constitution which leads to phenotypic constitution. If the Y chromosome is present, testes develop, testosterone and other hormones are secreted, and male internal and external genitalia develop. If no Y chromosome is present, ovaries develop, no testosterone is secreted, and female internal and external genitalia develop. Thus, under this tenet female genital development is thought to develop by "default" – in the absence of gonadal hormone stimulation. This widely accepted model of default genital development for the female has been updated as the field garners new information. A series of gene transcription factors have been implicated in a more active process of ovarian development. For example, the FOXL2 gene is a notable contender to be an ovary-determining gene. As the earliest known marker, FOXL2 is necessary to differentiate testes development from ovary formation, and it plays a key role in actively suppressing SOX9, a downstream target through which the sex-determining-region Y (SRY) gene induces testes formation (Georges et al., 2014; Kalfa et al., 2008; Schmidt et al., 2004; Uhlenhaut et al., 2009). Furthermore, female mice without *Foxl2* (*Foxl2*$^{-/-}$ mutants) do not undergo normal ovarian follicle development, experience pervasive neonatal follicular atresia, and are sterile (Ottolenghi et al., 2005; Schmidt et al., 2004; Uda et al., 2004; Uhlenhaut et al., 2009). Depleting *Foxl2* can trigger a cascade of events including upregulation of genes that produce male phenotypic gonad development (Garcia-Ortiz et al., 2009; Ottolenghi et al., 2005; Uhlenhaut et al., 2009). *Foxl2* may not only be critical during prenatal development, but also across the lifespan (Uhlenhaut et al., 2009). In fact, experimentally-induced *Foxl2* loss in eight-week-old mice resulted in

ovarian granulosa cells morphing into Sertoli-like cells, and thecal cells beginning to upregulate an enzyme controlling testosterone biosynthesis (Uhlenhaut et al., 2009). There are likely other genomic processes acting in concert with *Foxl2*, but this recent evidence provides novel pathways to explore with regard to sexual differentiation and active development of the female phenotype. In addition, there is fascinating ongoing work in the field of sexual differentiation to better elucidate the role of gonadal hormones, as well as the newly recognized unique impact of genetic sex, as direct factors in the phenotypic outcome of an individual (A. P. Arnold et al., 2004; Bakker & Baum, 2008). Indeed, non-linearity may exist in the genetics-determines-gonads-determines-phenotype model, such that the resulting phenotype is *directly* influenced by genomic mechanisms, which likely work in synergy with gonadal hormones and even epigenetic effects (McCarthy & Arnold, 2011; McCarthy, Arnold, Ball, Blaustein, & De Vries, 2012). These intriguing new discoveries continue to modify more traditionally-accepted models and will aid in elucidating the complex nature of sex differences and steroid hormone effects on multiple systems spanning early development to reproductive senescence and beyond, impacting the phenotype throughout life.

Brain Sexual Differentiation in Mammals

In mammals, the traditional model of sexual differentiation of the brain parallels that of the genitalia, maintaining that if testosterone exposure occurs early in life during a defined critical window, it exerts permanent effects resulting in a male phenotype. This traditional model also holds the tenet that if the brain is not exposed to gonadal steroids during this critical window, this will result in a female phenotype. Therefore, in this

model the "normal" female phenotype has been considered to be organized by default. The updated model builds upon abundant research and the traditional framework, incorporating new evidence and views (Figure 1b). Accumulating behavioral and brain data have demonstrated that in fact, normal female brain development and organization depends on estrogen exposure, and that female brain organization is an active process that is *not* by default.

A flow diagram depicting sexual differentiation of the mammalian brain and phenotype is presented in Figure 2. For the purposes of discussion here, masculinization is defined as the induction of the male phenotype, feminization as the induction of the female phenotype, demasculinization as removing the potential for the development of male traits, and defeminization as removing the potential for the development of female traits. A phenotypically "normal" female is both feminized and demasculinized, while a phenotypically "normal" male is masculinized and defeminized. Much of the research leading to our understanding and defining of these processes has been done in rodents; hence, the work discussed in this section is largely based on rodent experimental evaluations. Regarding the process of sexual differentiation of the mammalian brain, testosterone is released from the gonads, and is thought to lead to masculinization in the male via direct and indirect routes. Indeed, testosterone readily crosses the blood brain barrier and is converted in the brain to 17β-estradiol via the aromatase enzyme. Numerous rodent studies have shown that high doses of 17β-estradiol can be masculinizing to the brain and behavior, including reproductive and non-reproductive brain areas and behaviors. Why then do female rodents not experience the same masculinization from estrogen exposure during gestation? Alpha-fetoprotein (AFP), a

transient plasma protein circulating during fetal development, has a high binding affinity and capacity for the estrogens rodents are exposed to during gestation (likely maternal in origin) (Figure 3). Once bound to AFP, the estrogen compound is sufficiently large such that it can no longer cross the blood brain barrier, effectively preventing estrogen-induced brain masculinization and defeminization in females during the early perinatal timeframe (Figure 3) (Attardi & Ruoslahti, 1976; Benno & Williams, 1978; McEwen, Plapinger, Chaptal, Gerlach, & Wallach, 1975). Bakker et al. assessed the principle that AFP prevents estrogens from exerting masculinizing and defeminizing effects in the brain by testing female mice without AFP (*Afp*$^{-/-}$ mutant); consequently estrogen could enter the brain in the *Afp*$^{-/-}$ mutants (Bakker et al., 2006). These *Afp*$^{-/-}$ mutant animals did not exhibit female-typical sex behavior and had increased male-typical sex behavior compared to wild-type female mice, supporting the tenet that when AFP is not present, estrogens enter the brain and subsequently masculinize and defeminize (Bakker et al., 2006). Moreover, females lacking AFP had male-like quantities of tyrosine hydroxylase expression in the anteroventricular nucleus of the preoptic region, an area important for female reproductive function, and administering treatment of an aromatase inhibitor to the mother during pregnancy (effectively attenuating estrogen production and exposure in the fetus) produced a normal female phenotype for the measured variables in the female *Afp*$^{-/-}$ mice that was similar to that of wild-type females (Bakker et al., 2006). This research provides further support that estrogens exert brain masculinizing and defeminizing properties during development, and that the presence of AFP can prevent these actions in the female. The system is really quite remarkable. Indeed, in rodents, AFP levels become undetectable in the brain after post-natal day (P) 7 (Ali, Kaul, &

Sahib, 1981), around the same time that the ovaries increase in activity and produce detectable levels of gonadal hormones (Mannan & O'Shaughnessy, 1991; Picut et al., 2015; Sokka & Huhtaniemi, 1995; Weniger, Zeis, & Chouraqui, 1993). Because the female brain seems to have an extended period of sensitivity to gonadal hormones beyond the neonatal period (discussed in more detail in the section: "On the framework of organizational and activational effects of ovarian hormones on the brain"), these estrogens of ovarian origin are likely necessary for normal female brain development, and they can now access the brain during this time since AFP no longer attenuates brain access (for review, see: Bakker and Baum, 2008; Toran-Allerand, 1984).

We would be remiss if we did not note that the questions of sexual differentiation of the brain in humans are difficult to address due to the obvious ethical considerations of systematic experimental manipulation. However, some work, including in human brain extracts and cord serum, has questioned whether AFP binds to estrogens in humans in a manner similar to rodents; thus, the role of AFP in human sexual differentiation is still highly controversial (Ali, Balapure, Singh, Shukla, & Sahib, 1981; Nunez, Vallette, Benassayag, & Jayle, 1974; Swartx & Soloff, 1974). Nonetheless, sexual differentiation and the role of AFP has been extensively studied and characterized in the rodent model, providing a solid foundation and framework for understanding the role of gonadal hormones in sexual differentiation of the brain.

On the Framework of Organizational and Activational Effects of Ovarian Hormones on the Brain

The turn of the 20th century was abound with empirical evaluations in the field of sex differences and behavior. In the early years of this research, Parkes described "internal secretions of the ovary" (Parkes, 1927, p. 1663) as critical to development, the menstrual and estrous cycles, and pregnancy maintenance, and that injections of "extracts of corpus luteum" (Parkes, 1927, p. 1665) disrupt the normal estrous cycle (Parkes, 1927). Young and Beach began investigating these internal secretions, later known as gonadal hormones, and their relationship to sex behaviors in the 1930's and 1940's. Beach found that gonadal hormones had sex-specific effects on the presence or absence of phenotypic mating behaviors, and that these behaviors could be manipulated by altering hormone levels. For example, his laboratory found that testosterone propionate induced male-typical sex behaviors, in addition to female receptivity sex behaviors, in female rats that underwent ovariectomy (surgical removal of the ovaries; Ovx) prior to the onset of puberty (Beach, 1942), and that gonadally intact males demonstrated female-typical sex behaviors, in addition to male-typical sex behaviors, when given large doses of androgen in adulthood (Beach, 1941).

In 1959, Phoenix, Goy, Gerall, and Young posited the theory of organizational and activational effects of gonadal hormones (Phoenix, Goy, Gerall, & Young, 1959; Young, Goy, & Phoenix, 1964). This dichotomy is based primarily on the parameters of gonadal differentiation and on androgenic actions in the male. A myriad of research has ensued in the area of sex differences and sexual differentiation of the body and brain. Many of these investigations are based on the classic concept of critical periods in which

gonadal hormone exposure must occur at just the right time during development to effectively and permanently organize an organism to respond normally upon sexual maturity. In the 1970's, Jost and others studied embryonic development of the gonads, postulating that testes develop earlier in gestation and by an active process, while ovaries were considered to develop in the absence of testes formation (for discussion, see Jost et al., 1973). These classic and landmark studies were critical to the field, driving further research and contributing to the establishment of the long-held dogma that many female systems develop by "default."

Organizational effects of hormones have varied definitions depending on the discussant, but they are typically operationally defined as permanent and occurring early in development. *Activational effects* of hormones are typically operationally defined as transitional, depending on the immediate presence of the hormone for the effect, and occurring later in development. Under the tenet of this traditional model, sex differences reflect the organizing and permanent actions of sex steroids present during an early development critical period, while activational effects "activate" underlying previously-organized substrates specifically in adulthood. In the landscape of traditional as well as newer models, organizational and activational effects of sex steroids work in concert. In fact, although early sex hormone exposure plays a significant role in organizing brain substrates driving sexually differentiated behaviors, many of these effects are realized only with exposure to the activating sex hormones; in many cases, the "male" or "female" phenotype is expressed normally only with the activational hormone present (Beatty, 1992). As a result of this synergy of organizational and activational effects, "male" and "female" brains are not likely to respond to the same circulating/activating

hormones in an equivalent fashion because the structures being activated have had different prior organizational hormone exposures and organizational consequences (Beatty & Beatty, 1970).

Evidence accumulating over the last few decades has blurred the traditional operational definitions of the organizational and activational dichotomy, especially regarding the temporal distinction wrapped into the definitions. More recent views of the organizational/activational tenet suggest that the temporal parameters should not be so strictly defined, and that the distinction between effects is not necessarily linearly related in time. Rather, whether "the induced changes represent permanent or transient effects, whenever in life they occur" (Fitch and Denenberg, 1998, p. 312) should provide the distinction. Examples of findings blurring the traditional dichotomy include activation of ear wiggling sexual behavior in female rat pups by estrogen, when pups were as young as six days of age (Williams, 1987), and non-transient alterations in brain morphology following post-pubertal sex hormone manipulations (Bimonte, Fitch, & Denenberg, 2000b, 2000a; Bimonte, Mack, Stavnezer, & Denenberg, 2000; Bloch & Gorski, 1988; Pappas, Diamond, & Johnson, 1979; Rodriguez-Sierra, 1986).

Nearly 30 years ago, researchers were already rethinking the idea that organizational effects occur only early in development. A variety of brain regions and behaviors are differentially impacted by exposure to estrogens and/or androgens, with females showing extended sensitivity to hormonal manipulations and changes in anatomical and behavioral outcomes (for review see: Fitch and Denenberg, 1998). For example, Rodriguez-Sierra showed that estradiol benzoate administration on P25 (prior to puberty but after neonatal brain organization) modified synaptogenesis in several brain

regions within days after estrogen treatment, and that these estrogen effects may be sex-specific to females (Rodriguez-Sierra, 1986) Research from the Denenberg laboratory found that ovarian hormones actively feminized the corpus callosum, and that this effect was not reversible within the extended timeframes into adulthood that were assessed. These data indicate that the permanent, potentially "organizational" effects of ovarian hormones can extend into adulthood for females (Bimonte et al., 2000). Moreover, in females there is flexibility in the window of sensitivity for normal feminized brain organization. There has been extensive research on the corpus callosum that nicely illustrates this idea. It is known that in the normally developing rat the organizing effects of androgen on the male corpus callosum end by P1 (Bimonte, Fitch, et al., 2000b; Fitch, Berrebi, Cowell, Schrott, & Denenberg, 1990; Fitch, Cowell, Schrott, & Denenberg, 1991; Mack et al., 1996), but the female corpus callosum is sensitive to ovarian hormone input at least up to P70 (adulthood). Pre-pubertal Ovx enlarges, or defeminizes, the corpus callosum; ovary transfer post-puberty on P70 can counteract this effect, resulting in a smaller (feminized) corpus callosum in adulthood (Bimonte, Fitch, et al., 2000a). In addition, there is evidence that neonatal estrogen exposure is necessary for brain feminization later in life. Exposure to the estrogen receptor blocker tamoxifen from birth until Ovx on P25 resulted in a larger corpus callosum size even after estradiol was given starting at P70, whereas animals that had normal ovarian hormone exposure until P25 Ovx responded as would be expected to exogenous estradiol administration beginning on P70 — the size of the corpus callosum was smaller in a feminized fashion. One could interpret these findings as meaning that the presence of ovarian hormones through the first 25 postnatal days of life allowed estradiol to later feminize the corpus callosum. If

estrogens were not impacting the brain during this early timeframe, attenuating estrogen receptor stimulation would have had no impact on the response to the activating effects of estrogens when given later. Collectively, these studies implicate an active role of ovarian hormones in organizing the female brain, and an overall extended period of sensitivity in females relative to the period of sensitivity in males.

(Re)organizational Events Across the Lifespan

It is possible that non-transient organizational hormone events in the brain can continue across the lifespan, challenging the long-held dogma that organizational effects are limited to the neonatal period of development, and supporting the updated distinction of the organizational and activational dichotomy depending on whether the effects are permanent or transient, irrespective of the life timeframe during which they occur (e.g. see Arnold and Breedlove, 1985; Fitch and Denenberg, 1998; Rodriguez-Sierra, 1986; Stewart and Kolb, 1988). For example, Bloch and Gorski found that in gonadectomized male rats, sexually dimorphic structures within the preoptic area are impacted by exogenous estrogen and progesterone administration when given in adulthood, with results indicating some feminization since the response was in the female direction (Bloch & Gorski, 1988). Moreover, while it is clear that gonadal hormones play a significant role in organizing the brain during early development, there are other hormonal exposures that can result in seemingly permanent effects on the brain, even if they occur later in life. An extended and systematic series of studies from the Juraska laboratory has beautifully shown that puberty marks another period of architectural remodeling in the brain, including alterations in neurons and glia, and that ovarian

hormones play active roles in these events (see Juraska et al., 2013, for review), which could be considered reorganizational effects. Evidence from our laboratory extends the idea of reorganizational effects to exogenous and synthetic hormone exposures, testing the permanence of effects of hormones that are taken by women. We have recent data suggesting that exposure to the synthetic progestin medroxyprogesterone acetate (MPA) in adulthood — even if that administration is discontinued — negatively impacts female rats' working memory performance with detrimental effects persisting up to four months after transient exposure is terminated (Braden et al., 2011). While the impact of MPA on cognition could be considered activational because the brain was organized early on to respond to hormones, even a transient exposure appears to have a long-lasting, and potentially permanent, impact on the trajectory of cognitive performance with aging. Hence, this pattern of effects resulting from the adult transient exposure to this synthetic hormone might be considered a reorganizational event.

Pregnancy is another life event marked by potent steroid hormone changes; some of these changes appear to result in long-term brain and behavioral effects in females such that they respond differently to other hormonal exposures later in life compared to females who have never experienced pregnancy-related hormone changes. Might this be considered a reorganizational hormone event? Parity not only impacts maternal behaviors in rats, but it also induces changes in spatial learning and memory performance, stress behaviors, and alterations in related brain measurements (Gatewood et al., 2005; Kinsley et al., 1999; Macbeth & Luine, 2010; Macbeth, Scharfman, Maclusky, Gautreaux, & Luine, 2008; Pawluski, Walker, & Galea, 2006). For example, there was a reduction in number of amyloid precursor protein immunoreactive cells in the hippocampi of parous

rats compared to nulliparous rats (Gatewood et al., 2005), and a noted increase in monoaminergic activity in the olfactory bulb, and BDNF in the hippocampal CA1 region and medial septum, in multiparous versus nulliparous animals (Macbeth et al., 2008). Additionally, an acute (10μg) injection of 17β-estradiol, 17α-estradiol, or estrone increased the number of BrdU-immunoreactive cells in the dentate gyrus of middle-aged Ovx multiparous rats compared to age-matched virgin rats, indicating that previous sexual or reproductive experience impacts responsiveness to estrogens later in life (Barha & Galea, 2011). Of note, parity (i.e., pregnancy, lactation, and pup experience [Kinsley et al., 1999]) involves other hormone fluctuations, including a significant and extended increase in progesterone levels accompanying the increase in estrogen levels, and these gonadal hormones likely act in concert to result in the observed long-term changes in the brain and behavior associated with motherhood. Less is known about the long-term effects of pregnancy experience in humans, but research suggests that there are changes associated with hormonal fluctuations during and after pregnancy that affect future brain morphology and behavior (Glynn, 2010; for discussion, see Macbeth and Luine, 2010).

Clearly, gonadal hormones act upon a variety of systems and brain structures that work together to result in the observable outcomes of hormone actions. Many of these observations have been carried out at adolescent or young adult time points, and we note that these hormone exposures likely influence the trajectory of aging such that the brain has "activational" responses dependent not only on the organization that occurs very early in life, but also to reorganizational events that occur across the whole lifespan, including very late in life. These long lasting (potentially permanent) effects of estrogen exposures, such as from pregnancy, exogenous hormone administration (e.g.,

contraceptives, hormone therapy), and menopause, may be considered "reorganizational" rather than "activational" as their effects appear to persist long after the initial exposure is terminated, and they have been found to change the trajectory of responsiveness later in life. This is not mutually exclusive of the waxing and waning in sensitivity to estrogens across the lifespan; indeed, a decrease in sensitivity does not mean activational or reorganizational effects cannot occur. McCarthy notes that the developing and the reproductively senescent time periods may have more in common than previously recognized (McCarthy, 2011). Research elucidating how steroid hormones organize the brain throughout life can yield insight into optimizing the hormone milieu during menopause, including providing perspectives and considerations for menopause trajectories and hormone therapy options.

Reproductive Hormones and the Brain

Ovarian hormones, and particularly estrogens, are potent modulators of the brain, including in regions known to affect learning and memory. All steroid hormones are derived from the cholesterol molecule. Three types of estrogen are present in humans, as well as in rodents: 17β-estradiol, estrone, and estriol (Kuhl, 2005). Of these, 17β-estradiol is the most potent and abundant naturally circulating estrogen during the reproductive life stage. In addition to many peripheral tissues, the brain contains ample estrogen receptors (ERs), including both α and β subtypes. 17β-estradiol binds to estrogen receptor (ER) ERα and ERβ with the strongest affinity as compared to estrone and estriol. Estrone binds these receptors with the next strongest affinity, and estriol binds these receptors with the weakest affinity of the three estrogens (Kuhl, 2005; Kuiper

et al., 1997). Moreover, 17β-estradiol and estrone bind ERα and ERβ with about equal preference, while estriol, though a very weak estrogen in comparison, has a slight preference for ERβ compared to ERα (Kuiper et al., 1997). The hippocampus, a key brain structure for learning and memory, contains both α and β ERs, with greater levels of ERβ than ERα in humans and rats (Foster, 2012). Because ERα transcription processes are more active than ERβ, ERβ may, in part, serve as a regulator for ERα, at least in the hippocampus (Bean, Ianov, & Foster, 2014; Han et al., 2013). Indeed, the ratio of ERα to ERβ may play a chief role in how estrogens ultimately impact behavior. Estrogens may act through traditional signaling pathways, as well as exert rapid non-genomic changes associated with a third receptor type referred to as GPR30/GPER1, a membrane coupled receptor (Bean et al., 2014). Many of these cognitive brain regions that contain ERs are sensitive to changes as aging ensues. Thus, estrogens can impact the brain and its functions across the entire lifespan in various contexts.

The Female Reproductive Cycle and Menopause

During the reproductive life stage, the ovary is the main synthesis site of circulating sex steroid hormones, including estrogens, progesterone, and androgens. Estrogens are primarily synthesized by growing ovarian follicles, progesterone predominantly by the corpus luteum after ovulation and in small amounts by growing follicles, and androgens by both ovarian interstitial tissue and the adrenal glands. The production and regulation of these hormones are mediated by the hypothalamic-pituitary-gonadal (HPG) axis. The feedback loop begins with the hypothalamus, which produces and releases gonadotropin releasing hormone (GnRH) into the anterior pituitary gland,

initiating the synthesis and secretion of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH are two key hormone regulators of ovarian follicle development and the ovarian cycle. The human female reproductive cycle is approximately 28 days in length and is divided into the follicular, ovulation, luteal, and menstrual phases. During the follicular phase, estrogen levels progressively rise as the ovarian follicles mature as a result of signaling from FSH. Immature ovarian follicles undergo various developmental stages over the course of several months and are classified by size and the different cell types that comprise the growing follicle (Figure 4) (Gougeon, 2010; Gougeon & Chainy, 1987). Once estrogen levels reach a critical threshold, the LH surge occurs and triggers ovulation of one mature ovum per month. The luteal phase begins after ovulation, wherein the remaining follicle develops into the corpus luteum and begins to produce progesterone in preparation for a potential pregnancy. If the mature egg is not fertilized after ovulation, it degenerates and the menstrual phase occurs in women. FSH signaling from the pituitary gland stimulates maturation of ovarian follicles, thus beginning the follicular phase again.

It is a well-accepted tenet that women are born with a finite pool of immature ovarian follicles. Histological follicle counts and mathematical models of ovarian follicle reserves estimate an average of 295,000 follicles per ovary at birth to 180,000 follicles per ovary at puberty; as the menopause transition approaches, estimates are reduced to between 100-1000 immature follicles per ovary (Gougeon, 2010; W. H. B. Wallace & Kelsey, 2010). At puberty, the ovulatory cycle begins as a result of the maturation of the hypothalamic-pituitary-gonadal (HPG) feedback loop. Women experience regular ovulation, and therefore a consistent menstrual cycle, for about 40 years. Approximately

400 eggs are ovulated across a woman's reproductive life stage; the remainder — and vast majority — of ovarian follicles undergo atresia, or programmed cell death (Baker, 1963; Hsueh, Billig, & Tsafriri, 1994).

Around the fifth decade of life, once the follicle pool is depleted through natural atresia and ovulation, the ovaries do not generate a sufficient amount of estrogens and progesterone to sustain the normal uterine cycle. Although the natural transition to reproductive senescence is not completely understood, it is clear that it is not an abrupt event; rather, it is thought that when a critical threshold of remaining ovarian follicles is reached, women begin to experience the transitional phase to menopause, which involves intermittent ovulatory cycles, significant fluctuations in ovarian hormone levels, and variable but rising FSH and LH levels. This process culminates with an eventual cessation of menses and infertility, referred to as menopause, and occurs at the average age of 51-52 in women (Hoffman et al., 2012; NAMS, 2014). This gradual menopause transition may last up to ten years prior to the final menstrual period and thus the end of the reproductive life stage (Harlow et al., 2013). In addition to these physiological changes, women often report undesirable symptoms including hot flashes, genitourinary symptoms, and changes in sleep, mood, and memory during the menopause transition (Mitchell & Woods, 2001; Weber, Maki, & McDermott, 2014; Weber & Mapstone, 2009). As a result, women may opt to take hormone therapy (HT) to attenuate some of these undesirable symptoms. Estrogens have been shown to have beneficial effects on a myriad of body systems, including cardiovascular, bone, and brain health. However, estrogens' actions are complex, and often exert beneficial effects only when given at a fitting dose, through the ideal route of administration, and with appropriate timing and

duration of the treatment (Koebele & Bimonte-Nelson, 2015; Turgeon, Carr, Maki, Mendelsohn, & Wise, 2006; Wise, Suzuki, & Brown, 2009).

In the early 2000's, findings from the Women's Health Initiative Memory Study (WHIMS) challenged the tenet that estrogen-containing HT has protective effects against global cognitive decline, mild cognitive impairment, and probable dementia risks when the large, double-blind, placebo-controlled clinical trials indicated that some forms of HT increase the risk of probable dementia development when administered post-menopause (Coker et al., 2010; Espeland et al., 2004; Shumaker et al., 2004, 2003). The WHIMS findings propelled the field forward; the window of opportunity and other hypotheses for therapeutic benefits of estrogen-including HT arose, with a particular emphasis on cognitive aging (Daniel, 2013; Maki, 2006, 2013; McCarrey & Resnick, 2015; Rocca, Grossardt, & Shuster, 2010). Specifically, the window of opportunity is thought to be a critical period, likely during the early stages of the menopause transition wherein estrogenic HT may confer a benefit to memory systems and delay the onset of cognitive decline or dementia (Maki et al., 2011). Thus, the field is focused on furthering understanding of the menopause transition to help identify a critical window for therapeutic benefits and possible intervention to lower risk factors associated with aging and menopause.

Of note, the window of opportunity is likely not a singular factor, but could be thought of as a profile that encompasses several constitutive elements associated with aging and menopause, such as the age at which a woman begins to transition, the length of time since the impetus of the transition, exposures to both exogenous hormone administration (e.g., oral contraceptives) and transient endogenous changes in ovarian

hormone milieu (e.g., pregnancy), as well as maternal family history of menopause length and symptomology, and gynecological surgical interventions. This broad spectrum of factors is often difficult to assess in the healthy aging woman; indeed, much of our insight into human factors comes from studying disease states, which may be a confounding factor itself. Rodent models help us to systematically explore brain and behavioral effects associated with timing in this critical window (Daniel, Hulst, & Berbling, 2006; Foster, Sharrow, Kumar, & Masse, 2003; Gibbs, 2000b; McLaughlin, Bimonte-Nelson, Neisewander, & Conrad, 2008; Talboom, Williams, Baxley, West, & Bimonte-Nelson, 2008). These models can teach us the optimal parameters for timing, duration, dose, formulation, and routes of administration by allowing direct manipulation, while also permitting control over factors such as parity and previous exogenous hormone exposures. Furthermore, rodent models are useful to assess the cognitive effects of variations in menopause etiology, including a transitional model of menopause using 4-vinylcyclohexene-diepoxide (VCD), and surgical models of menopause, including ovariectomy and/or hysterectomy models, all of which will be discussed in detail below.

Rodent Aging and Reproductive Senescence

Before we can fully appreciate rodents as valuable and appropriate models for menopause and aging research (Brinton, 2012; Van Kempen, Milner, & Waters, 2011), we must first acknowledge some key differences between human and rodent reproductive senescence (Downs & Wise, 2009; Finch, 2014). Rodents have an estrous cycle rather than a menstrual cycle. The rodent estrous cycle differs in several ways, including that rodents' uterine lining is reabsorbed rather than shed via menstruation. The estrous cycle

occurs every four to five days and consists of four phases — proestrus, estrus, metestrus, and diestrus — that involve similar ovarian hormone fluctuations to the human menstrual cycle (Figure 5A,B) (Burger, 2006; Goldman, Murr, & Cooper, 2007). Around 9-12 months of age, rats and mice typically experience irregular estrous cycles, aptly termed estropause. A constant/persistent estrus state is common, wherein the estrus phase is prolonged and anovulatory cycles may occur (Figure 6A). Animals may then transition into an anestrous state, where ovulatory cycles halt and low levels of gonadal steroids are present (Clemens & Meites, 1971; Huang, Steger, Bruni, & Meites, 1978; Lu, Hopper, Vargo, & Yen, 1979). Rats, more often than mice, will also pass through a phase of repetitive pseudopregnancy prior to transitioning into an anestrous state, or they may stay in a pseudopregnant state for their remaining lifespan, wherein they ovulate irregularly (and sometimes supraovulate), resulting in corpora lutea that are maintained for an extended period of time producing high progesterone levels (Finch, 2014; Lu et al., 1979). Rodents also experience dysregulated HPG axis activity in estropause, including decreased LH release responsiveness to estrogen signaling in middle-aged rats (Scarborough & Wise, 1990; Wilkes, Lu, Fulton, & Yen, 1978). A chief difference between humans and rodents is the presence of potentially mature ovulatory follicles in the aged rodent. Rodents experience some natural ovarian follicular depletion and are also suspected to have a finite follicle pool (Gosden, Laing, Felicio, Nelson, & Finch, 1983); however, some preliminary evidence for adult neo-oogenesis in rodents has challenged this idea (J. Johnson et al., 2005; J. Johnson, Canning, Kaneko, Pru, & Tilly, 2004). Because a hallmark of human menopause is complete ovarian failure, it is important to keep these differences in mind when choosing an appropriate animal model

of menopause. Several rodent models of reproductive aging are implemented in the laboratory to aid in understanding of brain and body aging and the menopause transition, considered in detail below.

Ovary-Intact: A Model of the Aging Hypothalamic-Pituitary-Gonadal Axis

Similar to women, rodents experience regular reproductive cycles in adulthood, as well as age-related dysregulation of this cycle and the HPG axis, ovarian changes, and gonadal hormone fluctuations. Many researchers have utilized the ovary-intact female rat as a model of human menopause to evaluate the changing brain, pituitary, and ovary interactions with age (Downs & Wise, 2009; Kermath & Gore, 2012; Wise et al., 2002). Phyllis Wise's research, beginning in the 1970's, demonstrated that as aging occurs in ovary-intact rats, changes occur in the rhythmicity of hypothalamic GnRH release that initiates the LH surge. These alterations, originating in the brain, precede observable changes in normal estrous cyclicity in aging rodents (Wise, 1982b, 1982a). These alterations are particularly evident during the proestrus phase, wherein a LH and FSH surge precedes ovulation. Wise's laboratory found that middle-aged animals had lower pre-ovulatory circulating estradiol levels, and that before the LH surge, there was a temporal delay in rising GnRH concentrations in the median eminence, which connects the hypothalamus to the pituitary gland and facilitates gonadotropin release via the hypophysial portal blood stream; decreased serum LH, estradiol, and progesterone levels following the LH surge in middle-aged animals point to an age-related disruption in circadian rhythm function that consequently alters normal estrous cycle patterns (Wise, 1982b). Wise and others have also found that the amplitude of the LH, FSH, and

prolactin surges was reduced in middle-aged, ovary-intact animals (Cooper, Conn, & Walker, 1980; Wise, 1982a). These landmark studies gave rise to the theory of aging of neural pacemakers, which suggests both chronological and endocrine aging are crucial factors for understanding the transition to reproductive senescence, and that the brain plays a significant role in these processes. Using the ovary-intact rat model, the Gore laboratory has evaluated aging and its relationship to neuroendocrine control and feedback mechanisms. This team found increased GnRH mRNA levels in middle-aged and aged rats compared to young control subjects; yet, gene transcription for GnRH decreased with age, pointing to a potential posttranscriptional mechanism for the age-related increase in GnRH mRNA (Gore, Oung, Yung, Flagg, & Woller, 2000). GnRH and LH release are, in part, mediated by excitatory glutamate signaling via input from N-methyl-D-aspartate (NMDA) receptors; alterations in these inputs likely disrupt positive and negative feedback mechanisms of the HPG axis regulation (Gore, Oung, et al., 2000). Although the number of GnRH neurons does not appear to change with age, there are age- and reproductive senescence- related changes in the gene expression of the subunits that constitute the NMDA receptor (Gore, Oung, et al., 2000; Gore, Yeung, Morrison, & Oung, 2000). Gore and others have suggested that during aging, there is attenuated NMDA receptor-mediated glutamatergic activation of GnRH-producing neurons in brain regions important for HPG feedback (Arias et al., 1996; Gore, Oung, et al., 2000; Zuo, Mahesh, Zamorano, & Brann, 1996). This disrupted balance between excitatory and inhibitory neurotransmission is related to the transition to reproductive senescence (Kermath & Gore, 2012). These alterations in neuroendocrine function may occur prior to animals displaying outward behavioral or estrous cycle abnormalities, and would likely

be difficult to target studying human clinical populations. Therefore, utilizing the ovary-intact rat model to evaluate the natural age-related cellular and molecular changes in brain regions involved in normal reproductive functioning and feedback is crucial to understanding neurological changes that occur in women, and to the development of novel therapeutic targets to mitigate negative symptoms associated with age and reproductive senescence.

A drawback to modeling menopause in the ovary-intact rodent is the difference in the ovarian follicle reserve and steroid hormone changes as aging ensues in the rat compared to women. Because rodents do not typically exhibit follicle-deplete ovaries by the onset of reproductive senescence like women do, circulating gonadal hormone levels in an aged ovary-intact rodent do not closely resemble those of menopausal women. Women in the post-menopausal state have very low circulating levels of 17β-estradiol and progesterone and significantly elevated FSH and LH levels (Figure 6B) (Burger, 2006; Timiras, Quay, & Vernadakis, 1995). Rodents, on the other hand, experience several reproductive-aging states and typically maintain moderate circulating 17β-estradiol levels, despite HPG axis dysregulation (Lu et al., 1979). Variations in the onset and expression of HPG axis changes in the ovary-intact female rat could add undesirable variability to this menopause model; however, as we gain understanding of these changes, and if we acknowledge these variations when interpreting findings, we can use this natural variation to our experimental advantage. Vaginal cytology monitoring is a common, minimally invasive way to monitor estrous cycle activity (Figure 7). Yet, vaginal cytology can become erratic in middle age due to dysregulated HPG axis activity. If a researcher intends to use a particular hormone profile of a middle-aged rodent, such

as constant estrous, for an experiment, it is likely that only a subset of animals in a colony will fall under this profile at the appropriate age and timing for the experiment. While studying the natural diversity that occurs in the ovary-intact model and taking these estrous cycle variations into account benefits correlation data evaluations, it is limiting in causal-type inferences. It is preferable to methodically control reproductive status and age at evaluation in experimental animals in order to dissociate the specific effects of reproductive senescence from advanced aging, since confounding factors due to age alone may muddle results.

Some stocks or strains of laboratory rodents are considered better models than others for aging research. One example is the National Institute on Aging's (NIA) recommendation to use the Fischer-344 (F344) inbred strain for aging research due to their longevity and well-defined aging patterns. Of note, Fischer-344-NIH rats were initially bred at the National Institutes of Health breeding facility and used for a number of years. As of 2016, due to breeding challenges, the F344-NIH strain was shifted to the Charles River-derived Fischer rat background (F344-CDF). The F344-CDF strain has minor genetic variations from the F344-NIH strain, but overall has significant overlap in genetic identity. Thus, as of the present time, F344-CDF rats are the standard strain utilized for much of NIA-supported research (National Institute on Aging, 2019). Sprague Dawley and Long Evans are also commonly utilized rat strains in aging research. Most laboratory rodent colonies do not have aging animals in stock; those that are available are often limited to retired breeders. In menopause research, reproductive history is a variable of chief importance – indeed, several laboratories have demonstrated that sexual experience and parity influence cognitive and brain outcomes (Barha &

Galea, 2011; Cost, Lobell, Williams-Yee, Henderson, & Dohanich, 2014; Gatewood et al., 2005; Macbeth & Luine, 2010; Macbeth et al., 2008). For example, these studies suggest a history of pregnancy is related to enhanced spatial memory performance compared to nulliparous animals, and brain responsiveness to estrogen treatment may be influenced by reproductive history. Thus, when employing the ovary-intact rodent model for aging and menopause work, scientists must be carefully consider the stock or strain of rodent as well as their age and life history to obtain an appropriate aging model for their research questions regarding the transition to reproductive senescence.

Ovariectomy: A Model of Surgical Menopause

The gold standard in the preclinical field for evaluating gonadal hormone effects in female animal models is the ovariectomy, or surgical removal of the ovaries, often abbreviated Ovx. Rodent anatomy differs from women in that rodents have a bifurcated uterus, called the uterine horn, which accommodates large litters (Figure 8). The standard Ovx procedure is to bilaterally excise the ovaries, oviducts (i.e., the fallopian tubes), and tips of the uterine horn from the peritoneal cavity, leaving the ligated uterine horns intact (Olson & Bruce, 1986). Full recovery from Ovx occurs within one week. Ovx most accurately models surgical menopause in women. Ovx is an ideal model to evaluate specific effects of gonadal hormone deprivation and subsequent exogenous hormone treatments on the brain and periphery because it creates a "blank gonadal hormone slate." As an example, estrogens may be administered after Ovx, alone or in combination with other ovarian hormones, with variations in the type of estrogen administered, dose, route of administration, and treatment duration while controlling for interactions with

endogenous levels of sex steroid hormones. Combined exogenous therapies, such as 17β-estradiol plus progesterone, may be used to evaluate their interactions on a particular system, more closely modeling HT regimens that many menopausal women take. Our laboratory and many others have utilized the Ovx model to evaluate the potential for neuroprotective effects of exogenous ovarian hormone administration on the brain. For example, administering HT to Ovx females has been shown to enhance performance on mazes evaluating spatial memory compared to Ovx females that were not receiving estrogen treatment (Bimonte-Nelson, Francis, Umphlet, & Granholm, 2006; Bimonte & Denenberg, 1999; Daniel, Fader, Spencer, & Dohanich, 1997; Daniel et al., 2006; Markowska & Savonenko, 2002a; McLaughlin et al., 2008; Talboom et al., 2008). Higher circulating 17β-estradiol levels have been associated with better spatial memory performance for both young and middle-aged Ovx rats (Talboom et al., 2008), and chronic, long-term exogenous estrogen plus progesterone treatment enhances radial-arm maze performance in middle age (Gibbs, 2000b). Importantly, the Ovx model has also shown limitations of efficacy in gonadal hormones, including some estrogens. For example, when estrone, a weaker metabolite of 17β-estradiol and the main component in Premarin, was given at a tonic dose to middle-aged Ovx rats, it had a negative impact on spatial memory at a high dose (Engler-Chiurazzi et al., 2012).

A drawback to the Ovx model in the context of translational research is that the majority of women retain their whole reproductive tract throughout the menopause transition. While removing the ovaries prior to the onset of reproductive senescence allows researchers the control to evaluate the impact of particular hormones without aging as a confounding factor, the age at which Ovx occurs in rodents may also result in

divergent effects (Chakraborty & Gore, 2004; Diz-Chaves et al., 2012), particularly for cognition (Foster et al., 2003). Additionally, the sudden loss of ovarian steroids is not characteristic of the majority of transitionally menopausal women; compounded with this, the post-menopausal ovary continues to release androgens and low levels of other steroids (Mayer, Devine, Dyer, & Hoyer, 2004; Mayer et al., 2002). Consequently, the Ovx model results in a considerably different hormone profile, as well as an altered HPG axis, compared to animals with an intact reproductive tract. Nonetheless, the Ovx model is a classic technique that merits much praise for its utility in evaluating novel phenomena in the context of aging and menopause research. While it is imperative to understand the impact of specific hormone and drug effects on the brain and body systems with a "blank gonadal hormone slate," in the context of the menopause transition, these factors associated with reproductive senescence do not operate in a vacuum, and understanding how exogenous hormone formulations interact with intact systems (i.e., a more representative model of human menopause) is translationally important.

4-Vinylcyclohexene Diepoxide: A Model of Transitional Menopause

The drug 4-vinylcyclohexene diepoxide, or VCD, a metabolite of 4-vinylcyclohexene, is produced when the industrial chemical butadiene is utilized to manufacture various plastic-based goods (Chhabra, 1989; Hoyer, Devine, Hu, Thompson, & Sipes, 2001). VCD is used commercially to reduce fecundity and species proliferation of rodent pests without the use of hazardous poisons that endanger the environment (Dyer et al., 2013). In the early 2000's, VCD was introduced to biobehavioral animal research

as a rodent model of transitional menopause because it was found to selectively target and deplete the non-growing ovarian follicle pool in rodents via atresia, resulting in accelerated follicular depletion and eventual ovarian failure in rodents (Borman et al., 1999; Devine, Sipes, & Hoyer, 2001; Flaws, Doerr, Sipes, & Hoyer, 1994; Hirshfield, 1991; Hoyer et al., 2001; Hu, Christian, Thompson, Sipes, & Hoyer, 2001; Hu, Christian, Sipes, & Hoyer, 2001; Kao, Sipes, & Hoyer, 1999; Mayer et al., 2004; Mayer, Dyer, Eastgard, Hoyer, & Banka, 2005; Mayer et al., 2002; L. N. Springer, Flaws, Sipes, & Hoyer, 1996; L. N. Springer, McAsey, et al., 1996; L. N. Springer, Tilly, Sipes, & Hoyer, 1996). VCD is thought to function through accelerating atresia in primordial and primary ovarian cells —but not disrupting growing follicles — by altering the expression and distribution of the Bcl-2 family of proteins that regulate apoptosis, including Bcl-xL, Bax, and Bak proteins within ovarian follicles, thus initiating cytochrome c release into the cell cytosol, triggering downstream caspase signaling activity, all of which are related to accelerated follicle atresia (Hu, Christian, Thompson, et al., 2001; Hu, Christian, Sipes, et al., 2001; L. N. Springer, Flaws, et al., 1996; L. N. Springer, McAsey, et al., 1996; L. N. Springer, Tilly, et al., 1996; Van Kempen et al., 2011).

The introduction of VCD as a model of transitional menopause has been an integral contribution to science. This innovative model more closely mimics the human menopause transition compared to the ovary-intact or Ovx models by effectively depleting the resting follicle pool but allowing for the retention of follicle-deplete ovarian tissue, resulting in an ovarian and hormone profile more similar to a vast majority of women undergoing the natural menopause transition who retain their reproductive organs in the post-reproductive life stage. In rodents, VCD is typically administered via a series

of intraperitoneal injections ranging from 80 mg/kg-160 mg/kg (Devine et al., 2001; Frye et al., 2012; Hoyer et al., 2001; Mayer et al., 2004, 2002; L. E. Wright et al., 2011). Recently, an oral bait has been developed for rodents that contains a combination of VCD and triptolide, a Chinese herb thought to accelerate the follicular depletion of later-stage ovarian follicles (e.g., pre-ovulatory antral follicles) (Dyer et al., 2013), which could be used both in the laboratory to decrease the number of necessary VCD injections and to target later-stage follicle development, as well as commercially to reduce wild rodent pest populations by further decreasing fertility. This model is still undergoing development but is an excellent addition to the VCD-related models (Dyer et al., 2013). The chief drawback of the VCD model is that it is toxic in high doses (Chhabra, 1989). However, the use of VCD in the laboratory has been refined as a model of transitional menopause and no significant effects have been indicated on other organs under appropriate administration regimens (Devine et al., 2001; Frye et al., 2012; Hoyer et al., 2001), although it may be an acute stressor to the animal during drug administration. Animals may decrease in body weight during injections, but generally return to baseline weights after drug administration.

Resulting serum hormone and gonadotropin profiles after VCD-induced follicular depletion have been shown to be similar to the hormone profiles observed in ovary-intact menopausal women (Acosta et al., 2010, 2009; Mayer et al., 2004, 2002; Timiras et al., 1995); it is noteworthy that gonadotropin and sex steroid hormone profiles from VCD-induced follicle depletion may vary based on species, dose, age, duration of administration, and length of time since VCD administration (Frye et al., 2012). In addition to the circulating hormone profile produced by VCD, this is an excellent model

of follicle atresia in women. Ovarian follicle counts are considered a reliable marker of reproductive capacity. In clinical research, antral follicle count (AFC) is often used as a biomarker for reproductive capacity in the context of fertility (The American College of Obstetrics and Gynecology, 2015), and more recently, in the transition to menopause (Hansen, Craig, Zavy, Klein, & Soules, 2012; Hansen, Hodnett, Knowlton, & Craig, 2011; Hansen et al., 2008). AFC may be a more accurate marker than circulating serum gonadal hormone and gonadotrophin levels, as these levels fluctuate erratically in the human menopause transition (Hansen et al., 2011). Because the VCD model allows for the retention of post-menopausal ovarian tissue, researchers can now evaluate how ovarian follicle count relates to reproductive senescence in rodents. This model can also inform our understanding of the impact of intact post-menopausal ovarian tissue with its continued release of androgens, particularly androstenedione (Mayer et al., 2004) and the relationship to other low levels of circulating steroid hormones from extra-ovarian sources, such as the adrenals, breast, and adipose tissues in the post-menopausal life stage. Overall, the VCD menopause model has a substantial advantage over the ovary-intact and Ovx models in that it results in an ovarian follicle and circulating steroid hormone profile more similar to the majority of women transitioning into the post-menopausal life stage.

The VCD model is growing in popularity in the field of menopause and aging research. Our laboratory has implemented the VCD model in the context of spatial memory performance, and showed that transitional menopause differentially impacts spatial memory as well as the efficacy of the estrogenic HT, conjugated equine estrogens (CEE; tradename: Premarin) compared to Ovx animals (Acosta et al., 2010, 2009).

Others have shown VCD-induced reproductive senescence is associated with insulin resistance, bone loss, atherosclerotic lesion development, and changes in anxiety-like behaviors (Mayer et al., 2005; Reis et al., 2014; Romero-Aleshire, Diamond-Stanic, Hasty, Hoyer, & Brooks, 2009; L. Wright et al., 2008), all of which can be negative health risks related to aging and the menopause transition in women. These studies point to the benefits and utility of the VCD model, and suggest that it should be further tapped in the context of menopause research in order to elucidate novel therapies in an intact system.

Hysterectomy: A Novel Model of Gynecological Surgery and Menopause in Rats

Hysterectomy, or the surgical removal of the uterus, is a common gynecological surgery performed in women, second only to cesarean section (Carlson, Nichols, & Schiff, 1993; Centers for Disease Control and Prevention, 2010). Although hysterectomy rates have declined in recent years, an estimated 600,000 surgeries occur in the United States annually, mostly for benign reasons such as endometriosis, pelvic inflammatory disease, uterine fibroids, cysts, or abnormal bleeding (Carlson et al., 1993; Corona et al., 2015; J. D. Wright et al., 2013). In approximately 50% of hysterectomy procedures, the ovaries are removed at the time of hysterectomy. This is usually considered a prophylactic measure to avoid an increased risk of developing ovarian cancer or other complications following hysterectomy, but results in an abrupt, surgical menopause (Hoffman et al., 2012; Whiteman et al., 2008). The majority of these procedures are completed in women prior to the onset of menopause (J. D. Wright et al., 2013). Thus, if a woman retains her ovaries at the time of hysterectomy, which is true in about half of

hysterectomy cases, the ovaries are thought to continue to function normally until the age of natural menopause onset. Evidence from research at Mayo Clinic and other laboratories indicate that there is an association between hysterectomy, both with and without ovarian conservation, and an increased risk of dementia development later in life; this risk further increases with a younger age at surgery (Phung et al., 2010; Rocca et al., 2007; Rocca, Grossardt, Shuster, & Stewart, 2012).

There have been few evaluations of hysterectomy using a rodent model. Those that have been completed are focused on physiological measures such as serum hormone levels and ovarian follicles. Early investigations into the role of hysterectomy on endocrine function suggested that the uterus may alter pituitary RNA activity and play a role in regulating hormone and gonadotropin synthesis and release from the anterior pituitary (Biró, 1979; Biró & Eneroth, 1989; Biró, Eneroth, & Ritzén, 1983, 1988; Biró, Ritzén, Hall, & Eneroth, 1984; Biró, Ritzén, & Eneroth, 1987; H. Spies, Hilliard, & Sawyer, 1968). Another experiment where hysterectomy was performed in rats at six weeks of age found increased ovulation approximately three weeks later, with an increase in ovarian homogenate-derived 17β-estradiol levels and aromatase activity (Tanaka et al., 1994). Other evaluations 50 or 100 days after hysterectomy showed no changes in serum ovarian steroid hormones or FSH levels, but evidence of accelerated ovarian aging (Tapisiz et al., 2008), while a longer interval after hysterectomy surgery indicated increased serum FSH levels as well (Özdamar, Ülger, Sorkun, & Müderris, 2005). To date, there have been no systematic evaluations of the role of hysterectomy on cognition. For this dissertation, I created a rodent model of hysterectomy to methodically test the impact of the surgery on spatial learning and memory. In this surgical model, the uterus

(i.e. the uterine body and uterine horns) are surgically removed, while the ovaries and oviducts remain intact. In the ovariectomy + hysterectomy model, the reproductive tract, including the ovaries, oviducts, and uterus, are removed. This model fills an important gap in the literature regarding the effects of uterus removal on cognition, and more closely models the most common surgical procedures that occur in women. That is, ovariectomy with uterine preservation is the gold standard in rodent models of menopause; however, this surgical procedure, although growing in popularity, is not as commonly performed in women as uterus removal with or without ovary removal. Chapters 7, 8, and 9 will detail the results from this novel rodent model of variations in surgical menopause and the implications of these findings for women's health and healthy brain aging in the post-reproductive life stage.

Reproductive Hormones and Cognition

Estrogen and Progesterone as the Traditional Key Ovarian Hormone Players

As noted above, female mammals typically experience a cessation of reproductive capacity in mid- to end- of life. Most animals are not long-lived following reproductive senescence; humans are one of the unique exceptions to this rule. People are living longer than ever before, with the average female lifespan surpassing 81 years in developed countries such as the United States (Murray et al., 2015). However, the age at menopause does not seem to be changing with increased longevity (Sherwin, 2003). This means that women are now living in a postmenopausal state, with significantly reduced circulating ovarian hormone levels, for a substantial part of their lives. This underscores the need to

understand the effects of aging and related hormone loss on the body, including the brain and its function.

There has been ample research, both in basic science and human realms, suggesting that the loss of ovarian hormones has a negative impact on a variety of body systems. These adverse effects are especially robust when an abrupt hormone loss occurs, such as that associated with ovariectomy (Ovx; the surgical removal of the ovaries). Ovarian hormone loss is associated with a decline in cognitive function both in humans (Nappi et al., 1999; Rocca et al., 2007; Sherwin, 2003) as well as in animal models (Daniel, 2013; Frick, 2015; Koebele and Bimonte-Nelson, 2015; Korol and Pisani, 2015; Luine, 2014). Animal models have provided an excellent framework to elucidate the effects of ovarian hormones on the brain and behavior. For example, seminal preclinical work in the field of neuroendocrinology, aging, and cognition has shown that Ovx impairs spatial memory performance, and that subsequent estrogen treatment can improve memory performance following Ovx, at least for a period of time (Bimonte & Denenberg, 1999; Bohacek, Bearl, & Daniel, 2008; Savonenko & Markowska, 2003; Talboom et al., 2008; M. Wallace, Luine, Arellanos, & Frankfurt, 2006). Interestingly, transient 17β-estradiol treatment after Ovx can enhance memory performance, as well as increase hippocampal choline acetyltransferase (ChAT; the synthesizing enzyme for acetylcholine) and ERα levels, even after the estrogen treatment has been terminated (Rodgers, Bohacek, & Daniel, 2010). However, timing of 17β-estradiol replacement is critical; spatial memory performance was improved only when hormone treatment is initiated immediately after Ovx, but not after five months of hormone deprivation (Daniel et al., 2006). It is also notable that estrogen replacement following Ovx is more

efficacious in young and middle-aged animals than in old age (Diz-Chaves et al., 2012; Savonenko & Markowska, 2003), and that chronic estrogen treatment can improve cognitive performance, but only after priming with a cyclic regimen of 17β-estradiol injections (Markowska & Savonenko, 2002a). Moreover, animals' responsiveness to the enhancing effects of 17β-estradiol or estradiol benzoate changes with age (Foster et al., 2003; Talboom et al., 2008), which may be related to estrogen receptor expression in the hippocampus (Foster, 2012). ERα and ERβ are associated with a range of intracellular signaling molecules that are rapidly activated in the presence of estrogens. Remarkably, Ovx animals receiving hippocampal lentivirus injection of ERα, which increases ERα expression, displayed enhanced spatial working memory, even in the absence of high circulating estrogen levels (Foster, Rani, Kumar, Cui, & Semple-Rowland, 2008; Witty, Foster, Semple-Rowland, & Daniel, 2012). Lentiviral delivery of ERα to the hippocampus also increased phosphorylated extracellular regulated kinases (ERK1/2; discussed in more detail below) in rats, suggesting that signal transduction pathways important for learning and memory are, in part, moderated by estrogen receptor expression and activity (Witty et al., 2012). Taken together, these novel findings indicate that serum estrogen levels alone cannot necessarily dictate or predict cognitive outcomes; they are part of a complex and interactive system involving many cellular and molecular mechanisms that impact memory performance in a collaborative fashion.

Of note, estrogens do not operate on the brain and body in isolation. Progestogens are a class of steroid hormones that include endogenous progesterone and synthetic progestins that bind to the progesterone receptor. Progesterone is an important component of the reproductive cycle, and is especially critical for the maintenance of pregnancy. In a

non-pregnant female, the main release occurs during the endogenous female reproductive cycle via the corpus luteum after ovulation; with follicular depletion and ensuing menopause, corpora lutea formation is attenuated and therefore there is a lack of elevated progesterone. In a broad context, the scientific study regarding the impact of the shifts in progesterone across the female lifespan is important because of the systematic and rapid alterations in progesterone levels across the regular reproductive cycle in adulthood, markedly elevated levels with pregnancy, as well as the decreased levels that occur into old age. The effects of progestogens specifically on the brain and its functions is also a crucial growing area of research; in fact, the work is yielding strong evidence that progestogens have marked impacts on brain areas integral to many reproductive and non-reproductive behaviors, including translating effects to cognition.

Interestingly, our laboratory has found that the beneficial effects of estrogen treatment on spatial memory can be reversed by concomitant progesterone administration (Bimonte-Nelson et al., 2006), and that administering the synthetic progestin medroxyprogesterone acetate (MPA) to Ovx rats impaired performance on a spatial working memory task (Braden et al., 2011, 2010). However, it seems that progestins are not unequivocally harmful to cognition. Our laboratory and others have recently demonstrated that different classes of synthetic progestins commonly used in HT formulations, including levonorgestrel and norethindrone acetate, can have differential effects on spatial memory performance compared to MPA (Gambacciani et al., 2003; Simone et al., 2015; Tierney et al., 2009). While MPA administration has been shown to produce detrimental, long-lasting cognitive impairments (Braden et al., 2011, 2010), and norethindrone acetate dose-dependently impaired spatial memory, levonorgestrel has

been shown to have a null or even enhancing effect on spatial memory performance (Braden et al., 2016; Prakapenka et al., 2018). In the context of translational research, finding a null effect of a progestin is a better outcome than the generally detrimental effects of MPA; that is, it is preferable for women to use a progestin that will have no effect or a beneficial effect on memory, rather than utilize a known cognitively impairing option like MPA. Thus, these novel findings regarding the differential effects of progestogens on cognition warrant further investigation into progestin type, dose, and timing of treatment to produce an optimal brain aging profile while maintaining the important protective effects that progestogens provide for other body systems.

Androstenedione: Long Ignored but not Unimportant

Androstenedione is an androgen synthesized by the adrenal glands and interstitial ovarian tissue, as well as by the thecal cells of maturing follicles. The aromatase enzyme converts androstenedione to estrone and 17β-estradiol; androstenedione can also be converted to testosterone via the enzyme 17β-HSD; both of these androgens and their metabolites can impact the brain and cognition (Bimonte-Nelson, Singleton, Nelson, et al., 2003; Camp et al., 2012; Mennenga, Koebele, et al., 2015). In the context of menopause, research shows that, while estrogen and progesterone production declines substantially with reproductive senescence, the postmenopausal ovary continues to produce androgens in rodents (Mayer et al., 2004) and in humans (Fogle, Stanczyk, Zhang, & Paulson, 2007). In fact, it has been estimated that in the postmenopausal state, the ovaries continue to produce about 30 percent of the circulating androstenedione levels and 50 percent of total testosterone levels (Vermeulen, 1976). Recent research in

postmenopausal women shows that exogenous testosterone can enhance memory (Davis et al., 2014; Davison et al., 2011), but endogenous testosterone levels may differentially impact cognition, such that a lower testosterone to estrogen ratio is better for memory performance (Joanne Ryan et al., 2012). Thus, the cognitive effects of androgens likely depend on a woman's background hormone profile in the postmenopausal state, and should be taken into consideration when interpreting the effects of ovarian hormones on cognitive outcomes.

Although the effects of endogenous and exogenous estrogens and progestins have been the focus of research related to cognitive function, the role of androgens (particularly androstenedione) in learning and memory remains somewhat elusive. Our laboratory has shown that in a transitional menopause rat model, animals with higher naturally circulating androstenedione levels tended to make more working memory errors on the water radial-arm maze (Acosta et al., 2009). Given that androgens can convert to estrogens, we recognized this novel finding could inform important innovations in the realm of hormone therapy options for women. Thus, intrigued by this correlation, we continued to explore the role of androstenedione on memory in the middle-aged female rat. We found that in middle-aged Ovx rats, a high dose of androstenedione impaired spatial working memory as well as reference memory on the Morris water maze (Camp et al., 2012). Androstenedione can be aromatized to estrone, an estrogen that we have shown to impair memory in the Ovx model (Engler-Chiurazzi et al., 2012).

Consequently, our laboratory systematically evaluated whether the apparent detrimental effects of androstenedione on memory were due to binding to the androgen receptor or androstenedione's conversion to estrone. Results indicated that blocking

aromatase enzymatic activity via anastrozole reversed spatial memory impairments in young Ovx rats, but blocking the androgen receptor did not prevent detrimental effects on memory, suggesting that the conversion of androstenedione to estrone influences cognitive performance (Mennenga, Koebele, et al., 2015). Collectively, these findings point to a crucial role of androgens, a long ignored yet ostensibly critical factor in understanding the role of the hormone milieu on cognition in the menopausal woman. Future research should continue to focus on understanding how circulating androgen levels impact the brain and body of aging women, and how maintaining a "golden ratio" of androgens to estrogens may be key to preserving cognition in the postmenopausal life stage.

What About Those Gonadotropins? Cognitive Effects of LH and FSH During Menopause and Aging

The reproductive system in females is regulated by communication and interactions of numerous hormones from the hypothalamus, pituitary, and ovaries. Thus, ovarian-derived steroids, such as estrogens, progesterone, and androgens, are not the only hormones to become dysregulated with age and reproductive senescence. Research in recent years has indicated that changes in gonadotropin levels, namely FSH and LH, have a major role in cognitive changes and risk factors for developing age-related neurodegenerative disorders. FSH and LH are glycoprotein hormones released from the anterior pituitary, and they each have critical effects on body growth and maturation, as well as reproductive functions. FSH is released to stimulate the growth of immature ovarian follicles, resulting in a gradual rise in circulating estrogen levels during the first

half of the cycle. Once estrogen levels reach a certain threshold, an LH surge occurs, which triggers ovulation and concurrently initiates corpus luteum development from the remaining ovarian follicle, which produces progesterone in preparation for egg fertilization. Once the follicle pool falls below a critical threshold, typically in midlife, the normal feedback from the ovaries back to the hypothalamus and pituitary becomes disrupted. Thought to be a compensatory mechanism, increased FSH and LH levels are released in the system's attempt to stimulate normal follicular growth and ovulation. Seminal work from the laboratories of Dr. Andrea Gore and Dr. Phyllis Wise has provided evidence that perturbations in the cyclic release of GnRH and subsequent release of gonadotropins occur before alterations in regular estrous or menstrual cyclicity becomes apparent, and that changes in NMDA receptor function may play a key role in disrupted GnRH release and feedback (Gore et al., 2000a; Gore et al., 2000b; Scarborough and Wise, 1990; Wise, 1982).

Some human research suggests that it is the alterations in gonadotropin levels, over and above declines in circulating ovarian hormone levels, that result in the cognitive changes observed during aging (Webber et al., 2005). In fact, higher circulating FSH and LH levels have been associated with neurodegenerative disease and pathologies in clinical populations (Bowen, Isley, & Atkinson, 2000; Bowen et al., 2002; Short, Bowen, O'Brien, & Graff-Radford, 2001). Basic science research using rodent models has further substantiated this tenet. For example, Dr. Gemma Casadesus and colleagues found that transgenic mice that overexpress LH receptors performed more poorly on hippocampal-dependent Y-maze task, while LH receptor knock out mice were not impaired, despite increased circulating LH levels (Casadesus et al., 2007). Furthermore, this group has

shown that pharmacologically down-regulating serum LH improves cognitive performance after Ovx in wildtype and a triple transgenic mouse model of Alzheimer's disease (Blair et al., 2016; Palm et al., 2014); in wild type animals, decreasing LH serum levels beneficially affects memory performance, even after exogenous 17β-estradiol treatment is no longer effective in enhancing memory following Ovx (Blair et al., 2016). In addition, our laboratory has shown an inverted U association between LH levels and cognitive performance in middle-aged female rats. Specifically, for animals with their ovaries (sham and follicle-deplete), higher LH levels were associated with poorer memory performance. Conversely, for Ovx animals, higher LH levels tended to be associated with better memory performance (Acosta et al., 2009). It is clear that in addition to circulating ovarian steroid hormone levels, gonadotropins also play a part in mediating cognitive performance, and these effects likely depend on background hormone milieu. Overall, these exciting findings point to novel pathways that are necessary to explore to fully understand the impact of a dysregulated hypothalamic-pituitary-ovarian feedback loop, especially regarding the transition to reproductive senescence as related to the trajectory of cognitive aging.

Aging, Ovarian Hormones, and Altered Neural Systems

The brain is a highly plastic organ. It adapts and changes throughout the lifespan, constantly revising and redacting information in order to adjust to an organism's ever changing environment. Neural systems and biochemical mediators are affected by many factors that are modified with age and interactions with the environment. A fundamental factor influencing the brain beginning early in life is sex steroid hormones. It is well

established that androgens and estrogens play a key role in organizing the developing brain and set it up to respond in a particular way following sexual maturity of an organism. Many of these neural systems and molecular pathways that are impacted by age and reproductive hormones are also associated with learning and memory processes. Here, we have focused upon the cholinergic and GABAergic systems, which are two of the most well-studied neural systems critical for learning and memory processes that are concomitantly impacted by age and ovarian hormones. The effects of age and altered ovarian hormone levels on dendritric morphology, as well as ERK1/2 signaling, a ERα-linked signaling pathway, are also discussed.

The Cholinergic System

Age and ovarian hormones, both endogenously circulating and exogenously administered, impact a myriad of factors in the brain, including, but not limited to, growth factors (e.g., neurotrophins), inflammatory response, immune response, mitochondrial function, and the cholinergic system. The latter involves the neurotransmitter acetylcholine, which also has diverse functions in the brain. One of acetylcholine's significant functions is to act as a key regulator of learning and memory consolidation. The basal forebrain is a primary synthesis site for acetylcholine in the mammalian forebrain. It is known that there are long-range projections from the basal forebrain to frontal cortex as well as the hippocampus, crucial brain structures for learning and memory consolidation. Beginning in the 1980's, landmark research has indicated that age impacts morphology and functionality of the brain's cholinergic system. For example, aged animals showed a decline in ChAT and acetylcholinesterase

(AChE; the enzyme that breaks down acetylcholine) activity in the basal forebrain and hippocampus (J. E. Springer, Tayrien, & Loy, 1987). Recently, the Bizon laboratory reported a decreased number of ChAT-immunoreactive (ChAT+) basal forebrain neurons in aged males rats compared to young adult male rats (Bañuelos et al., 2013). By examining p75NTR expression, a growth factor receptor that often co-localizes with cholinergic neurons, Veng and colleagues reported a reduction in density and presence of healthy cholinergic neurons in both aged male and female rats compared to younger animals (Veng, Granholm, & Rose, 2003). Aged males exhibited smaller cholinergic neuron somas compared to younger males, while aged females did not show a reduction in mean soma size (Veng et al., 2003). In addition to age-related alterations in cholinergic neurons, Ovx has been associated with a decline in ChAT activity, while subsequent 17β-estradiol administration restored ChAT activity in the female rat basal forebrain and projection sites into the frontal cortex and CA1 region of the hippocampus (Gibbs, 1994; Luine, 1985; M. Singh, Meyer, Millard, & Simpkins, 1994). Further, lesions to the medial septum and vertical/diagonal bands of the basal forebrain resulted in impaired spatial memory performance and prevented the memory enhancing effects of 17β-estradiol (Gibbs, 2002; Hagan, Salamone, Simpson, Iversen, & Morris, 1988). It is important to note that age and ovarian hormone loss do not necessarily affect the number of ChAT-producing neurons in the basal forebrain, but do impact the functional integrity of the cholinergic system (Gibbs, 2003). Recently, it has been shown that GPR30, a membrane bound G-protein coupled estrogen receptor distinct from ERα and ERβ, exhibits co-localization with basal forebrain cholinergic neurons and likely mediates some of the estrogens' effects on both basal forebrain cholinergic integrity and resulting

cognitive outcomes (Hammond & Gibbs, 2011; Hammond, Nelson, & Gibbs, 2011; Ping, Trieu, Wlodek, & Barrett, 2008). Thus, ovarian-derived hormones likely play a significant role in the neuroendocrine modulation of the cholinergic-hippocampal pathway. Most research on this subject has been evaluated in the Ovx model, where the ovaries, which are the endogenous source of circulating gonadal hormones, are surgically removed, and subsequent exogenous hormone therapy is administered. The effects of estrogens on cholinergic neurons in the basal forebrain are not always consistent. For example, many studies have shown that after Ovx, exogenous administration of 17β-estradiol can increase ChAT+ neurons in the basal forebrain (Engler-Chiurazzi et al., 2012; Gibbs, 1997); it is of note that other estrogen types initiate varied effects on this measurement. For example, tonic administration of estrone, a weaker metabolite of 17β-estradiol, failed to impact ChAT+ neurons in the basal forebrain (Engler-Chiurazzi et al., 2012), and the synthetic estrogen used in oral contraceptives, ethinyl estradiol, decreased the number of ChAT+ neurons in the basal forebrain following chronic administration in an Ovx model (Mennenga, Gerson, et al., 2015). These diverse effects of estrogens on one system highlight the complexity of estrogens' actions in the brain, and underscore the importance of taking multiple factors into account when assessing estrogens' effects on the brain and other body systems, such as type of estrogen, dose, route of administration, and timing of administration (for review, see Koebele and Bimonte-Nelson, 2015).

The GABAergic System

To add complexity to understanding the system, many cholinergic projections from the basal forebrain synapse onto ɣ-aminobutyric acid (GABA)ergic cortical

neurons; GABA is the primary inhibitory neurotransmitter in the brain and an important neuromodulator for normal cognitive processes, including hippocampal and cortical function. Acetylcholine release onto these GABAergic neurons in the hippocampus may modulate hippocampal theta wave oscillations through both direct and indirect pathways; these hippocampal theta rhythms play a role in regulating memory consolidation and synaptic plasticity (Dannenberg et al., 2015). The basal forebrain also has long-range GABAergic projections to the frontal cortex and hippocampus, both of which are thought to play a regulatory role in normal neural activity. Among its many roles, GABA signaling in the brain is a key regulatory factor in normal memory formation and maintenance (Kalueff & Nutt, 1997; Katz & Liebler, 1978). Inhibitory GABAergic neurons and signaling appear to become dysregulated with aging (Shetty & Turner, 1998; Stanley & Shetty, 2004). Animal models with altered GABAergic signaling, both systematically and with normal aging, show altered cognition with changes to the system, both in relation to cognitive aging and other psychiatric disorders (Bañuelos et al., 2013; McQuail, Frazier, & Bizon, 2015). For example, the Bizon laboratory found that younger animals had better performance on the probe trial of the spatial reference memory Morris water maze compared to aged rats. The basal forebrain was immunohistochemically processed for ChAT and glutamate decarboxylase 67 (GAD67; a synthesizing enzyme for GABA). For GAD67-immunoreactive (GAD67-IR) neurons, there was no overall difference between young and aged rats. However, when aged rats were sub-classified into spatially- unimpaired and impaired groups, aged-spatially-impaired rats were found to have significantly more GAD67-IR neurons compared to both young and aged-spatially-unimpaired animals. Further, this group showed a negative correlation with

spatial memory performance in aged rats, such that a greater number of GAD67-IR neurons was associated with poorer memory performance (Bañuelos et al., 2013). This laboratory also recently found that aged male rats have impaired performance on a set-shifting task, and that poorer performance on this task was associated with fewer GABA(B) receptors in the medial prefrontal cortex of the aged animals, but not the young animals. Directly infusing a GABA(B) receptor agonist into this brain region enhanced performance on the set shifting task for the aged animals (Beas, McQuail, Bañuelos, Setlow, & Bizon, 2017), suggesting that cognitive changes with age are in part modulated by GABAergic signaling.

In addition to age-related changes in the GABAergic system and subsequent memory performance, ovarian hormones influence the GABAergic system. While some research suggests 17β-estradiol can influence GABAergic signaling in the hippocampus (Wójtowicz & Mozrzymas, 2010), the majority of studies thus far have focused on the role of progesterone and GABAergic functioning. For example, our laboratory and others have shown that progesterone decreased GAD65+67 protein levels in the hippocampus and increased GAD65+67 protein levels in the entorhinal cortex (Braden et al., 2010) as well as decreased GAD activity in several brain regions, including the dorsal hippocampus, as measured by kinetic studies (Wallis & Luttge, 1980). Furthermore, an *in situ* hybridization study revealed that 12 hours after treatment, progesterone, but not MPA, reduced hippocampal mRNA expression of the α4 subunit of the GABA(A) receptor (Pazol, Northcutt, Patisaul, Wallen, & Wilson, 2009), suggesting that different progestogens can have variable impacts on the GABAergic system. We recently showed that in a middle-age Ovx rat model, progesterone administration resulted in transient

working memory impairments on a spatial memory task, but concomitant delivery of bicuculline, a GABA(A) receptor antagonist, obviated these memory impairments (Braden et al., 2015). Finally, the recent finding that cholinergic neurons may also co-release GABA adds an additional level of complexity wherein the full impact on cognitive function has yet to be determined (Tritsch, Granger, & Sabatini, 2016). Nonetheless, whether there are sex differences in how GABAergic circuitry and signaling are affected by aging, as well as how endogenous alterations and exogenous administrations of other sex steroid hormone levels impact this system, remains somewhat elusive and warrants further investigation.

MAPK/ERK1/2 Signaling Pathway

A wide range of intracellular pathways and kinases are known to be important for normal learning and memory processes (Giese & Mizuno, 2013), many of which are recruited downstream of estrogen receptor activation. One pathway in particular, the extracellular signal-regulated kinases, known as ERK1/2, p44/42, and classical mitogen-activated protein kinases (MAPKs; in humans, ERK1=MAPK3), has diverse functions in regulating learning and memory (Atkins, Selcher, Petraitis, Trzaskos, & Sweatt, 1998; Bozon et al., 2003; Fasano & Brambilla, 2011). Estrogen receptors are thought to activate ERK1/2 via production of cyclic adenosine monophosphate (cAMP) and/or interactions with growth factor receptors. Age-related brain changes in ERK1/2 signal transduction have not yet been extensively studied; however, one experiment found that aged male rats had decreased ERK1/2 activity in the cortex compared to younger rats (Zhen, Uryu, Cai, Johnson, & Friedman, 1999). Further investigations into how aging alters ERK1/2

signaling are necessary to elucidate whether aberrant signal transduction has functional consequences on cognitive outcomes across the lifespan. Given that ERK1/2 is ubiquitously expressed throughout the brain and other organs, it is important to evaluate potential age-related changes in multiple cognitive brain regions, as well as consider sex, age, and hormone status as factors influencing ERK1/2 expression and signaling. Indeed, in recent years, 17β-estradiol has been proposed to regulate ERK1/2 activity in both in vitro and in vivo studies. Dr. Karyn Frick's laboratory has demonstrated that intraperitoneal injections and intracerebroventricular or hippocampal infusions of 17β-estradiol activated ERK2 and enhanced object recognition memory (Fernandez et al., 2008; Frick, 2015; Lewis, Kerr, Orr, & Frick, 2008). Temporal parameters may impact the outcome of estrogen effects on this system; in aged Ovx rats, the amount of time since Ovx (and therefore ovarian hormone deprivation) impacted subsequent effects of 17β-estradiol on ERK1/2 phosphorylation, which were dependent upon brain region and time since Ovx (Pinceti, Shults, Rao, & Pak, 2016). Additionally, findings from Dr. Thomas Foster's laboratory revealed that ERα lentivirus injection directly into the hippocampus of middle-aged Ovx rats (i.e. animals with low endogenous estrogen levels) increased ERK1/2 phosphorylation and enhanced memory performance (Witty et al., 2012), suggesting that estrogen receptor activity, and possibly brain-derived estrogens, can activate and alter signal transduction pathways critical for learning and memory formation. These novel findings point to ERK1/2 signaling as another important biochemical mediator to investigate in the context of aging- and menopause- related brain changes. The interactions between ERK1/2 and the multitude of other hormone-linked pathways on learning and memory processes are only beginning to be explored. Further

investigations in this newer field of how ovarian hormone fluctuations and aging impact these biochemical signaling pathways are currently underway.

Delta (Δ) FosB: A Proposed Biological Marker of Long-Term Brain Changes

Immediate early genes (IEGs) are genes that are rapidly and transiently activated following extracellular activity. IEGs do not require new protein synthesis to exert meaningful effects on the brain and periphery (J. I. Morgan & Curran, 1989; Sheng & Greenberg, 1990). While IEGs are not limited to the brain, they have been found to play important roles in many neurobiological processes, including synaptic plasticity and memory (Minatohara, Akiyoshi, & Okuno, 2016; Sheng & Greenberg, 1990). Among the various IEGs, IEGs in the fos family hetereodimerize with jun family IEGs to eventually up- or down-regulate gene expression (Nestler, Barrot, & Self, 2001; Ruffle, 2014). Though c-fos is one of the best-characterized IEGs, it has a short half-life of approximately 60 minutes and expression rapidly declines afterwards, making it difficult to evaluate long-term effects in the brain (Carle et al., 2007). In recent years, it has been discovered that ΔFosB, a transcription factor encoded by the IEG FOSB, has unusual stability compared to other IEG transcription factors due to its lack of degron domains on the C-terminus that are present in the full-length FosB (Carle et al., 2007). The absence of these domains prevents rapid ubiquitination and degradation of the protein (Carle et al., 2007). In fact, ΔFosB has been described as a "sustained molecular switch" because it can be maintained at detectable levels in neurons for weeks to months following initial activation; indeed it has been touted as the "longest-lived adaptation known to occur in [sic] adult brain" (Nestler et al., 2001) and has a long half-life compared to other

transcription factors (Carle et al., 2007; J. Chen, Kelz, Hope, Nakabeppu, & Nestler, 1997; Ulery-Reynolds, Castillo, Vialou, Russo, & Nestler, 2009).

Delta FosB has primarily been investigated in the context of addiction, such that ΔFosB is upregulated in the nucleus accumbens with repeated drug exposures and remains elevated even after drug cessation, suggesting a role for ΔFosB in drug dependency (Nestler et al., 2001; Ruffle, 2014). This work indicates that ΔFosB can have long-term behavioral effects. Indeed, ΔFosB has been shown to be upregulated with stress, indicating a potential role for ΔFosB in hypothalamic-pituitary-adrenal axis function (Ruffle, 2014). This sustained IEG also increases gene expression of CDK5 in the hippocampus and other regions, a gene that is related to phosphorylation of synaptic proteins, and thus synaptic plasticity (Ruffle, 2014). Notably, overexpression of ΔFosB in mice has been shown to result in an upregulation of genes within the nucleus accumbens that are also upregulated with cAMP response element binding protein (CREB) expression (McClung & Nestler, 2003), which is another transcription factor implicated in many processes, including its crucial role in memory formation. Interestingly, an extended overexpression of ΔFosB in mice results in an opposite up- and down-regulation the gene expression seen with short-term ΔFosB overexpression, at least in the context of drug reward pathways (McClung & Nestler, 2003). More recently, overexpression of ΔFosB was found to transiently increase CREB protein levels in the striatum of male mice and *in vitro* (Enwright et al., 2010).

It is plausible that CREB and ΔFosB expression may work in concert to produce long-term changes in gene expression and behavior not only in the context of addiction and reward, but also in other brain regions that regulate learning and memory processes.

Recently, ΔFosB was shown to increase expression in the hippocampus after learning in the spatial Morris Water Maze (MM) compared to maze-naïve control rats and rats that experienced a non-spatial version of the MM (Eagle et al., 2015). This study also found the AAV- and HSV- mediated silencing of ΔFosB, as well as AAV-mediated overexpression of ΔfosB impaired learning and memory performance (Eagle et al., 2015). It was recently reported that in a ΔFosB knock-out mouse model confined to the subgranular zone of the dentate gyrus ($NtsR2^{Cre/+}FosB^{lox/lox}$ mice) neurogenesis and learning were impaired compared to wildtype littermates (Manning et al., 2019), further suggesting an important role for ΔFosB in learning and memory processes.

Evidence from the addiction literature suggests that ΔFosB can decrease the expression of an endogenous opioid neuropeptide called dynorphin (Nestler et al., 2001). Dynorphin-producing neurons have diverse roles in the brain; of relevance to the work presented herein, 17β-estradiol has been shown to regulate dynorphin expression in the arcuate nucleus of the hypothalamus (Kanaya, Iwata, & Ozawa, 2017). Indeed, dynorphin-producing neurons colocalize with other neuropeptides in the arcuate nucleus, which are collectively referred to as kisspeptin/neurokininB/dynorphin (KNDy) neurons that are abundantly expressed in the hypothalamus and play a key role not only in gonadotropin synthesis and release from the hypothalamus and pituitary, but are also thought to regulate the feedback loop of the hypothalamic-pituitary-ovarian axis and thus influence the female reproductive cycle (Lehman, Coolen, & Goodman, 2010). As such, it is plausible, and indeed likely, that alterations to the female reproductive tract, including surgical manipulations of the ovaries and uterus, interact with this system. Given the nature of ΔFosB expression, this IEG may be a candidate biomarker for long-term

changes in brain areas important to cognition following surgical menopause and hormone replacement, and merits further investigation into this novel area of research.

Parameters for Understanding the Impact of Hormones on the Brain and Cognition: Evaluating Learning and Memory in the Rodent Model

Mazes and Memory

Since the early 20th century, a variety of innovative tasks have been implemented to study learning and memory in the rodent (Bimonte-Nelson, Daniel, & Koebele, 2015), and these maze tasks have been utilized to gain understanding of hormone effects on the brain and cognition. The radial-arm maze and Morris maze are two apparatuses often used to study estrogen effects on cognition; therefore, their histories and methods will be highlighted in brief here to set the backdrop for later discussion in the following chapters. In the 1940's, Edward Tolman and colleagues created a "sunburst" maze, which was comprised of several arms radiating from a circular arena, to evaluate rodent navigation and formation of a cognitive map (Tolman, 1948; Tolman, Ritchie, & Kalish, 1946). These groundbreaking experiments sparked a revolution in the use of mazes to study learning and memory for spatial navigation in laboratory rodents. Spatial navigation is defined as utilizing distal or extra-maze cues to navigate in an environment. In the 1970's, David Olton pioneered an eight-armed radial arm maze, based on Tolman's sunburst design, to test rodent cognition. Each arm was baited with a food reward, and once a food reward was obtained it was not replaced within a day, requiring the animals to remember and update which spatial locations they had already visited. Olton and

colleagues noted that animals most often relied on spatial cues to efficiently solve the radial-arm maze (Olton & Samuelson, 1976). The radial-arm maze continues to be one of the most utilized maze paradigms in animal models of learning and memory to evaluate *reference memory*, a form of long-term memory wherein information stays constant, and *working memory*, a form of short-term memory that is updated within a testing session (Bimonte-Nelson et al., 2015). Many studies using the land version of this task have contributed significantly to our understanding of ovarian hormone effects on cognition (e.g. Daniel et al., 1997, 1999; Fader et al., 1999; Gibbs and Johnson, 2008; Holmes et al., 2002; Witty et al., 2013), and the land version has also been used for notably translational working and reference memory assessments using Premarin, a widely used hormone therapy in women (Barha & Galea, 2013). One caveat to the land version of the radial-arm maze is that animals are typically food deprived, and food reward is given upon successful choices, to motivate rodents to perform in the maze. Using appetitive motivation can add complexity to interpretations of hormone research, as longer-term food restriction can impact cyclicity and alter circulating levels of LH, FSH, and estradiol (Ahmed et al., 2012) in the female rodent. As a solution to this concern, a water version of the radial-arm maze was developed in the Denenberg laboratory in the late 1990's; of note, others were utilizing water escape in other maze tasks as well (for review see: Bimonte-Nelson et al., 2015). The water version of this task relieves the need for food deprivation by using hidden escape platforms (thereby removing the animal from the water, which is the negative reinforcer) and returning the animal to a warm, heated home cage as a positive reinforcer across all trials (Bimonte & Denenberg, 1999). Working memory load is taxed in the water version of the radial-arm maze by placing platforms in

the end of each arm, and removing each platform without replacement once the animal locates it within a day. A reference memory component can be added to this paradigm by placing escape platforms in a subset of the arms. In this maze testing protocol, the animal not only has to utilize working memory to remember where it has been within a day, but it also must use reference memory to remember where it should never go (i.e. arms that never contain a platform) (Bimonte-Nelson et al., 2015). Figure 9a provides a schematic of a common radial-arm maze setup.

Another invaluable task for studying spatial navigation and cognition in the rodent model is the Morris water maze, developed by Richard Morris in the early 1980's. This simple, yet cleverly designed, water escape task involves a circular tub of water in which a hidden platform is submerged just beneath the surface of the water; rodents are tasked with navigating to the hidden escape platform, typically using spatial cues placed around the room (Figure 9b) (Morris, 1981, 1984, 2015; Morris, Garrud, Rawlins, & O'Keefe, 1982). This task often (and optimally, should) include a probe trial, where the platform is removed from the maze, and animals are allowed to swim freely for 60 seconds. The probe trial is critical to evaluate the use of spatial localization to the hidden platform. The Morris water maze is traditionally a measure of spatial reference memory, but many laboratories have altered the paradigm to evaluate working memory, the use of distal and proximal cues, and room geometry and cue learning (Clark, Broadbent, & Squire, 2007; Pearce, Roberts, & Good, 1998). Other tasks including, but not limited to, the T maze, Y maze, delayed-match-to-sample, novel object recognition, and place recognition have also been used to quantify hormone effects on learning and memory performance in rodents (Bimonte-Nelson et al., 2015; Chisholm & Juraska, 2013; Daniel,

Hulst, & Lee, 2005; Foster et al., 2003; Frick & Gresack, 2003; Gibbs, 2002; Gibbs, Mauk, Nelson, & Johnson, 2009; D. A. Johnson, Zambon, & Gibbs, 2002; Nelson, Witty, Williamson, & Daniel, 2012; Orr, Lewis, & Frick, 2009). Many of the experiments discussed in this dissertation utilize a battery of these tasks to evaluate the effects of gonadal hormones on rodent cognition, and thus it is critical to provide a foundation to understand the history and utility of mazes in investigating rodent cognition in the context of menopause and aging.

Summary and Dissertation Goals

The reproductive system is indisputably a fundamentally essential body system. Without it, a species fails to survive. This intricate and complex system is carefully regulated via the hypothalamic-pituitary-gonadal axis. In recent decades, it has become increasingly evident that the reproductive system influences the body far beyond reproduction itself. Ovarian hormones are essential to the health and function of many systems, including cardiovascular, gastrointestinal, and skeletal systems, and importantly, the brain. Reproductive hormone receptors are abundantly present throughout the mammalian brain and influence not only brain-mediated reproductive behaviors, but also have regulatory roles for mood, sleep, eating behavior, and, notably, cognition, including learning and memory processes. This dissertation explores how alterations to the reproductive system, including variations in surgical and transitional menopause, as well as exogenous hormone administration, impact cognition in adult and aging rats. Specifically, Chapter 2 and Chapter 3 describe an evaluation of longitudinal cognitive changes throughout follicular depletion using a transitional model of menopause (Chapter

2), with an exploration of relationships among endocrine, brain, and behavior factors in these subjects (Chapter 3). Chapter 4 and Chapter 5 detail investigations into exogenous hormone therapy following surgical menopause and subsequent cognitive effects, which were found to be dependent upon the hormone(s) administered, dose, and behavioral task order. Chapter 6 and Chapter 7 evaluate a novel rat model of hysterectomy and cognitive effects of variations in gynecological surgery both in the short-term time point after surgery (Chapter 6), as well as for extended time frames after surgery/during aging (Chapter 7). Chapter 8 evaluates a potential neurobiological mechanism underlying the cognitive changes noted in Chapters 6 and 7. The age at which these collective interventions occur is emphasized as a critical variable for cognitive outcomes, and highlights the necessity for investigating "re"-organizational events across the lifespan that influence longitudinal outcomes for the brain and behavior.

CHAPTER 2

This chapter was published in Hormones and Behavior *in 2017 and is titled:*

COGNITIVE CHANGES ACROSS THE MENOPAUSE TRANSITION: A LONGITUDINAL EVALUATION OF THE IMPACT OF AGE AND OVARIAN STATUS ON SPATIAL MEMORY

Contribution: I was the first author on this manuscript and was the graduate student principal investigator for this experiment. This chapter encompasses my master's thesis work under the mentorship of Dr. Heather Bimonte-Nelson. My co-authors are acknowledged for the invaluable contributions to carrying out the experimental data collection and manuscript preparation.

ABSTRACT

Cognitive changes that occur during mid-life and beyond are linked to both aging and the menopause transition. Studies in women suggest that the age at menopause onset can impact cognitive status later in life; yet, little is known about memory changes that occur during the transitional period to the post-menopausal state. The 4-vinylcyclohexene diepoxide (VCD) model simulates transitional menopause in rodents by depleting the immature ovarian follicle reserve and allowing animals to retain their follicle-deplete ovarian tissue, resulting in a profile similar to the majority of perimenopausal women. Here, Vehicle or VCD treatment was administered to ovary-intact adult and middle-aged Fischer-344 rats to assess the trajectory of cognitive change across time with normal aging and aging with transitional menopause via VCD-induced follicular depletion, as well as to evaluate whether age at the onset of follicular depletion plays a role in cognitive outcomes. Animals experiencing the onset of menopause at a younger age exhibited impaired spatial memory early in the transition to a follicle-deplete state. Additionally, at the mid- and post- follicular depletion time points, VCD-induced follicular depletion amplified an age effect on memory. Overall, these findings suggest that the age at the onset of menopause is a critical parameter to consider when evaluating learning and memory across the transition to reproductive senescence. From a translational perspective, this study illustrates how age at menopause onset might impact cognition in menopausal women, and provides insight into time points to explore for the window of opportunity for hormone therapy during the menopause transition period. Hormone therapy during this critical juncture might be especially efficacious at

attenuating age- and menopause- related cognitive decline, producing healthy brain aging profiles in women who retain their ovaries throughout their lifespan.

Introduction

Women typically begin to experience a natural transition to menopause, or the post-reproductive state, during the fifth decade of life (NAMS, 2014; Soules et al., 2001). This transitional stage, often referred to in the clinic as "perimenopause" or "climacteric," is characterized by irregular menstrual cycles and erratic, fluctuating ovarian hormone levels that can last up to ten years before the final menstrual period (Hoffman et al., 2012; NAMS, 2014). Consequently, a range of physiological indicators often accompany the menopause transition, including vasomotor symptoms (e.g., hot flashes, night sweats), dyspareunia, genitourinary issues, sleep and mood alterations, and memory complaints (Al-Safi & Santoro, 2014; NAMS, 2014; Weber et al., 2014) Indeed, menopause and normal aging have each been associated with memory impairment (Mitchell & Woods, 2001; Tulving & Craik, 2000). Both the type of menopause a woman experiences (transitional or surgical), and the age at which she experiences this climacteric change, may impact cognitive function later in life. Research in women suggests that oophorectomy, the surgical removal of the ovaries, detrimentally impacts cognition, and that this effect depends on the relationship to menopause status. Indeed, there is evidence that oophorectomy before the onset of the transition to menopause may result in a more negative impact on verbal memory and an increased risk of developing dementia than retaining the ovaries throughout the menopause transition, or compared to oophorectomy after the menopause transition is complete (Farrag, Khedr, Abdel-Aleem, & Rageh, 2002; Nappi et al., 1999; Rocca et al., 2007; Rocca, Grossardt, & Shuster, 2011; Rocca et al., 2012). Thus, understanding the cognitive effects during and after transitional menopause

could be fundamentally important to women who may need to undergo surgical menopause.

Menopause typically occurs around age 51; however, some women experience early-onset menopause, defined as the final menstrual period occurring before the age of 45. In addition, spontaneous premature ovarian insufficiency, wherein the final menstrual period occurring before age 40, affects a small percentage of women (Pal & Santoro, 2002; Shuster, Rhodes, Gostout, Grossardt, & Rocca, 2010; Simpson & Rajkovic, 1999). Evaluating whether age at the onset of premature, early, and normal menopause impacts cognition may provide insight into the divergent effects observed for memory and other age-related health factors associated with menopause and the post-reproductive life stage.

In preclinical research, the gold standard for evaluating the impact of gonadal hormone loss and exogenous administration of hormone therapy on memory performance is ovariectomy (Ovx), or the surgical removal of the ovaries, which results in an abrupt loss of ovarian hormones. However, in the context of translational preclinical research, this classic technique only models a small percentage of women who undergo surgical menopause via bilateral oophorectomy (Centers for Disease Control and Prevention, 2010; M. J. Hall, DeFrances, Williams, Golosinskiy, & Schwartzman, 2010). Most women experience a gradual, natural menopause transition and typically maintain their reproductive organs into the post-menopausal life stage. While rodents are animal models often utilized in hormone research, rats and mice do not experience menopause; they undergo estropause (Finch, 2014; Meites & Lu, 1994). In contrast to human menopause, whereby immature follicles in the ovaries are depleted via natural atresia, rodents do not experience follicular loss to the same extent as women. Rather, the driving mechanism

underlying reproductive senescence in rodents is a significant dysregulation of the hypothalamic-pituitary-gonadal (HPG) axis in middle age (for review, see: Downs & Wise, 2009; Finch, 2014; Wise et al., 1997, 1996, 1999, 2002, 1989). Thus, the introduction of 4-vinylcyclohexene diepoxide (VCD) as a rodent model of transitional menopause has given researchers another tool to model menopause in the preclinical laboratory (for review, see: Koebele & Bimonte-Nelson, 2016). VCD targets primordial and primary ovarian follicles by initiating accelerated atresia, or programmed cell death, in the ovary (Hoyer et al., 2001; L. N. Springer, McAsey, et al., 1996) resulting in follicular depletion and eventual ovarian failure in rodents (Borman et al., 1999; Flaws et al., 1994; Hirshfield, 1991; Hoyer et al., 2001; Hu, Christian, Thompson, et al., 2001; Hu, Christian, Sipes, et al., 2001; Kao et al., 1999; Mayer et al., 2004, 2005, 2002; L. N. Springer, Flaws, et al., 1996; L. N. Springer, McAsey, et al., 1996; L. N. Springer, Tilly, et al., 1996). As such, VCD provides a translational tool for evaluating ovarian and hormone changes that occur across the transition to a follicle-deplete state. Indeed, compared to Ovx animals, VCD-treated animals exhibit hormone profiles more similar to transitionally menopausal women, and allow for the retention of follicle-deplete ovarian tissue like most women who transition to menopause without surgical intervention (Burger, 2006; Frye et al., 2012; Timiras et al., 1995). In rats, VCD treatment uniquely models perimenopause, in which follicular depletion is accelerated and gonadal hormone levels fluctuate over time (Frye et al., 2012; Mayer et al., 2002). As such, the VCD-induced menopause model is ideal for studying aging and the menopause transition, including the early stages of the transition when a multitude of physiological and affective symptoms begin to present in many women (Hale, Robertson, & Burger, 2014).

Our laboratory has previously shown that middle-aged rats that experienced VCD-induced follicular depletion demonstrated impaired working and recent memory in the post-depletion time point compared to animals that did not have this VCD-induced accelerated follicular depletion, and compared to animals that underwent Ovx or VCD treatment followed by Ovx (Acosta et al., 2009). However, spatial memory performance has not yet been evaluated during the transition to a follicle-deplete state using a rat model.

Here, we aimed to elucidate the longitudinal cognitive effects of transitional menopause via VCD-induced follicular depletion in ovary-intact Fischer-344 rats, and whether there were differences in cognition depending on the age at which accelerated follicular depletion was experimentally initiated, with VCD treatment beginning at either six or twelve months of age. In addition, we also longitudinally assessed performance in vehicle-treated ovary-intact rats at two age cohorts, evaluating the younger group as they aged from adulthood (6 months) to middle age (12 months), and the older group as they aged from middle age (12 months) to aged (18 months). Thus, the goals of evaluation for each age cohort were manifold: (1) to longitudinally investigate learning and memory as experimentally-induced follicular depletion ensues, (2) to longitudinally evaluate learning and memory in the normally aging rat, (3) to assess the hormonal and ovarian changes that occur during VCD-induced follicular depletion that may relate to behavioral outcomes, and (4) to evaluate the hormonal and ovarian changes that occur with normal aging that may relate to behavioral outcomes. Given that some research in women suggests that the loss of ovarian hormones earlier in life (including non-surgical, naturally premature or early transitional menopause, as well as surgical menopause) may

be detrimental to cognition (Rocca et al., 2007; J. Ryan et al., 2014), we hypothesize that if transitional menopause is induced at an earlier age, there will be a greater negative impact on cognition than if transitional menopause is induced at a later age. The VCD model affords us the opportunity to methodically test this hypothesis. Further, we predict that vehicle-treated, regularly aging animals will exhibit impaired memory performance as aging ensues; vehicle-treated controls were included to address this question. This inclusion of vehicle-treated, regularly aging animals also allows for a direct comparison to the VCD-treated animals with induced menopause across the transition to a follicle-deplete state.

Beginning at either six or twelve months of age, animals were trained on a water radial-arm maze, a complex spatial task requiring both working and reference memory. Of note, spatial memory necessitates the use of distal, extra-maze cues to solve a task, working memory is a type of short-term memory that needs to be updated, and reference memory is a form of long-term memory that stays constant (Bimonte-Nelson et al., 2015). Following training, subjects were administered VCD or vehicle treatment, and then repeatedly tested on the same water radial-arm maze task over a four-month period during the VCD-induced menopause transition. We examined the impact of altered hormone profiles on memory, how memory ability changes across this transition, and whether the age at follicular-depletion initiation influences cognitive performance. Animals were also tested on a task requiring cognitive flexibility, as well as a reference memory task following the water radial-arm maze evaluations to assess performance on unfamiliar tasks in the post-follicular depletion time point prior to sacrifice.

Methods

Animals

Animals used for this study were 56 female virgin Fischer-344 rats obtained from the National Institute on Aging colony at Harlan Laboratories (Indianapolis, IN). At the beginning of the study, rats were either 6 months (n=28) or 12 months (n=28) of age. Upon arrival, rats were pair-housed, given food and water *ad libitum*, and maintained on a 12-hour light/dark cycle for the entirety of the study. Animals were given one week to acclimate in the vivarium prior to the commencement of the experiment. All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards.

Experimental Timeline

The following methods are described in chronological order of occurrence during the experiment. Prior to treatment, all animals were trained on the water radial-arm maze (WRAM), described in detail below. Pre-treatment training on the WRAM was conducted at six or twelve months of age and prior to VCD-induced menopause in order to obtain a baseline measure of learning and memory performance. Pre-treatment training also served to familiarize the animals with the task, allowing future evaluations of memory for a familiar task. Animals were randomly assigned to one of two treatments (balanced across age groups): Vehicle or VCD (to induce follicular depletion). After Vehicle or VCD treatment was administered, animals were subsequently given a two-day refresher session on the WRAM, followed by a two-day retest every other week for four months to obtain a behavioral profile across the early- , mid- , and post- follicular

depletion time points. A subset of animals (n=3/group) were randomly selected for sacrifice during early follicular depletion. Following the final WRAM retest, the remaining animals were evaluated on several other tasks (described below) prior to sacrifice, including a flexibility measure where parameters had to be relearned for a revised WRAM task, the Morris water maze task, and the Visible Platform task. An overview of the study timeline can be found in Figure 10.

Water Radial-Arm Maze Training

One week after arrival, and before any experimental hormonal manipulation, animals were trained for 12 days on the win-shift WRAM, evaluating spatial working and reference memory, as previously described (Figure 11A; Bimonte-Nelson, Hunter, Nelson, & Granholm, 2003; Bimonte-Nelson, Singleton, Hunter, et al., 2003; Bimonte-Nelson, Singleton, Williams, & Granholm, 2004; Bimonte & Denenberg, 1999; Bimonte, Hyde, Hoplight, & Denenberg, 2000a; Bimonte, Nelson, & Granholm, 2003). Briefly, the WRAM is an eight-arm apparatus (each arm 39.37 x 13.97 cm) filled with room temperature water (18-20°C) tinted with non-toxic, powdered black tempera paint. Four out of eight arms contained a hidden platform (11 cm diameter) just beneath the surface of the water. Each subject was assigned to a set of unique platform locations that remained fixed across all days of testing, including the training period and all subsequent retests. The room contained salient extra-maze spatial cues to aid in spatial navigation. All animals received a total of four trials per day of testing. Each rat was released from the start arm and had three minutes to find a hidden platform. If the platform was not located within the allotted three-minute trial, the experimenter led the animal to the

nearest platform. Once the platform was located, the rat remained on the platform for 15 seconds before the experimenter returned the animal to its heated testing cage for an inter-trial interval (ITI) of 30 seconds. During the ITI, the just-found platform was removed from the maze, and the experimenter cleaned the water to remove any debris and to interrupt potential olfactory cues. The rat was placed back into the start arm and given three minutes to locate the next platform. Because one platform was removed after each trial, the working memory system was increasingly taxed as trials progressed. The daily testing session ended when the animal had located all four hidden platforms. Errors were quantified as an entry into a non-platformed arm; an arm entry was counted when the tip of the rat's snout crossed a mark delineated on the outside of the arm (not visible from inside the maze; 11 cm into the arm).

VCD Injections

Animals in each age cohort were randomly assigned to either the Vehicle or VCD group. Animals were classified as "Young Vehicle" (n=13), "Young VCD" (n=15), "Middle-Aged Vehicle" (n=13), or "Middle-Aged VCD" (n=15) to signify whether animals were young (6 mo) or middle-aged (12 mo) at the beginning of the experiment, and whether they underwent VCD-induced follicular depletion. VCD was administered for a total of 15 days via intraperitoneal injection at 160 mg/kg/day (Senestech Inc., Flagstaff, AZ) in 47% dimethyl sulfoxide (DMSO)/saline vehicle (Sigma-Aldrich, St. Louis, MO). The vehicle injection was 0.5 ml of 47% DMSO/saline solution. Daily VCD injection volume was determined by the animal's weight. Animals were not injected if they decreased below 90% of their initial body weight; injections were resumed when

weight was regained. VCD injections were administered on Monday, Tuesday, Thursday, and Friday; injections were not administered Wednesday, Saturday, or Sunday to allow animals to recover any weight lost, and were completed over the course of one month.

Water Radial-Arm Maze Refresher and Retests

Following the initial training on the WRAM task and subsequent VCD or Vehicle administration, all animals were given a two-day refresher on the WRAM, with procedures and platform locations identical to the initial 12 days of training. Immediately following two days of refresher practice, the first WRAM retest took place. Each retest lasted two days. A total of nine retests occurred every other week for four months. Because ovarian follicular depletion via VCD takes approximately three months from the first injection (Lohff, Christian, Marion, Arrandale, & Hoyer, 2005; Mayer et al., 2004, 2002), these retests captured behavioral snapshots throughout the transition to the follicle-deplete state, as well as throughout aging independent of VCD-induced follicular depletion in Vehicle-treated groups.

Water Radial-Arm Maze Flexibility Task

Following the final WRAM retest, the animals were evaluated for cognitive flexibility. That is, we tested cognitive flexibility by examining the animals' ability to update previously learned information in the WRAM task. Specifically, we shifted the reward locations within the WRAM task so that the animals had to update formerly learned escape locations with new escape locations to successfully solve the task. Subjects were tested in the same room and apparatus as the previous WRAM retests, but

two of their four previously-assigned platform locations were changed to new platform locations for each animal, as schematically represented in Figure 11b. Altering two of the platform locations required animals to update previously learned information about navigating to a particular arm to find a platform and escape the maze. All other maze procedures were identical to previous WRAM testing.

Morris Water Maze

After one day of rest, all of the animals were evaluated for spatial reference memory using the win-stay Morris water maze (MM; Figure 11c). The apparatus was a large round tub (188 cm diameter) filled with black-tinted water maintained at 18-20°C. One platform (11 cm diameter) was hidden just below the surface of the water in the northeast quadrant of the maze. This platform location remained constant across all days and trials. Salient spatial cues were present around the room to aid in spatial navigation to the platform (Morris et al., 1982). Each animal received four trials per day for five days. At the beginning of each trial, animals were dropped off from one of four starting points (north, south, east, or west). Drop-off points varied semi-randomly across days. Animals had 60 seconds to locate the platform before the experimenter led them to it. Once the platform was found, the rat remained on the platform for 15 seconds to allow for spatial localization before the experimenter returned it to the heated testing cage for an ITI of five to eight minutes. On the final day of MM, animals were given an additional probe trial in which the submerged platform was removed from the maze and animals swam freely in the maze for 60 seconds. The probe trial was implemented to evaluate whether the animals had spatially localized to the platform by quantifying the swim distance in the

target quadrant versus the opposite quadrant. A video camera and tracking system (Ethovision; Noldus Instruments; Wageningen, The Netherlands) were utilized to measure each rat's swim path (distance in cm) across all days and trials, as well as on the probe trial.

Visible Platform

Animals were tested on the Visible Platform (VP) task as a measure of visual and motor competency to solve a water-escape task at the end of the behavioral assay battery. This is a non-spatial adaptation of the cue-navigation version of the spatial MM, which has been used to dissociate visual and motor acuity from place memory (Morris et al., 1982). The apparatus was a rectangular tub (100 x 60 cm) filled with clear water maintained at 18-20°C. A black platform (10 cm diameter) was placed 4 cm above the surface of the water. Opaque curtains were hung around the room to block out any potential spatial or geometric cues (Figure 11d). The rats were given six trials in one day. Animals were dropped off from a fixed location, while the platform location varied semi-randomly in three possible locations across trials. Each subject had 90 seconds to locate the platform, and was allowed to remain on the platform for 15 seconds before being returned to its heated home cage for an ITI of five to eight minutes.

Sacrifices

Subset sacrifice. A subset of animals (n=3/group) was sacrificed on Day 52 of follicular depletion (i.e. approximately halfway to a follicle-deplete state). This subset is representative of the early menopause transition time point, wherein immature ovarian

follicles are undergoing extensive atresia leading to a follicle-deplete state (Mayer et al., 2004, 2002). Procedures for the subset sacrifice and end sacrifice were identical and are described below.

End sacrifice. Three additional animals died over the course of the study (one subject from the Middle-Aged-Vehicle group, one subject from Middle-Aged-VCD group, and one subject from the Young-VCD group). The remaining animals (N=41) were sacrificed one day after the final behavioral measure to obtain blood, ovaries, and uterine weights. Rats were deeply anesthetized with isoflurane anesthesia. Blood was collected via cardiocentesis prior to decapitation. Uterine horns and ovaries were dissected from the body cavity. Ovaries were removed from the tips of the uterine horn, trimmed of excess fat, and fixed in 4% paraformaldehyde until analysis. Uterine horns were trimmed of visible fat and wet weight was obtained as a marker of gonadal hormone stimulation.

Serum hormone measurements. At sacrifice, blood was collected via cardiocentesis and allowed to clot at 4°C (Vacutainer 367986, Becton Dickinson and Company, Franklin Lakes, NJ, USA). Serum was collected after centrifugation for 20 minutes at 2,000 rpm at 4°C and stored at -20°C until measurement by radioimmunoassay. Steroid hormone levels for 17β-estradiol, estrone, androstenedione, and progesterone were determined by radioimmunoassay using previously described methods (Acosta et al., 2010; Camp et al., 2012; Mennenga, Gerson, et al., 2015; Mennenga, Koebele, et al., 2015). Briefly, 17β-estradiol was determined using a double antibody liquid-phase radioimmunoassay purchased from Beckman Coulter (Brea, CA), which employs estradiol-specific antibodies along with an ^{125}I-labeled estradiol as the

tracer. Interassay coefficients of variation for the assay average 8% at a mean value of 6 pg/ml. Functional sensitivity of the assay is 4 pg/ml. Estrone was determined using a double antibody liquid-phase radioimmunoassay purchased from Beckman Coulter (Brea, CA), that employs estrone-specific antibodies along with an ^{125}I-labeled estrone as the tracer. Interassay coefficients of variation for the assay average 11% at a mean value of 90 pg/ml. Functional sensitivity of the assay is 16 pg/ml. Androstenedione was determined using a solid-phase radioimmunoassay purchased from Siemens (Los Angeles, CA), based on androstenedione-specific antibodies that are immobilized to the wall of polypropylene tubes and ^{125}I-labeled androstenedione as the tracer. Interassay coefficients of variation for the assay average 3% at a mean value of 2.80 ng/ml. Functional sensitivity of the assay is 0.1 ng/ml. Progesterone was determined using a solid-phase radioimmunoassay, based on progesterone-specific antibodies that are immobilized to the wall of polypropylene tubes and ^{125}I-labeled progesterone as the tracer. Interassay coefficients of variation for the assay average 4% at a mean value of 3.3 ng/ml. Functional sensitivity of the assay is 0.1 ng/ml.

Ovarian follicle counts. After post-fixing ovaries in 4% paraformaldehyde at sacrifice, one ovary from each subject was randomly selected for processing and quantification of primordial, primary, secondary, and antral follicles, as well as corpora lutea. Primordial follicles are considered to be non-growing, or resting-state, follicles within the ovary; these are the follicles targeted by VCD. Figure 12a provides a schematic of ovarian follicle types and corpora lutea, and 3b shows representative ovary micrographs from each group.

Ovarian tissues were processed for paraffin embedding, sectioned at 5 μm, mounted, and stained with hematoxylin and eosin Y-phloxine B. Primordial, primary, secondary, and antral follicles were counted for every 20th section. Corpora lutea were counted at a magnification of 10x (Spencer compound microscope; American Optical, Buffalo, NY). The total number of follicles was calculated using the following formula: $Nt = (N_0 \times S_t \times t_s) / (S_0 \times d_0)$, where Nt = total calculated number of follicles, N_0 = number of follicles observed in the ovary, S_t = total number of sections in the ovary, t_s = thickness of the section (μm) S0 = total number of sections observed, and d_0 = mean diameter of the nucleus (Gougeon & Chainy, 1987). Ovarian follicle stage was determined using criteria from Haas et al., 2007. Briefly, primordial cells were denoted by the presence of a single layer of squamous granulosa cells surrounding an oocyte. Primary follicles presented with a single layer of cuboidal granulosa cells. Secondary follicle classification required several granulosa cell layers. Antral follicles were defined as having two or more layers of granulosa cells as well as a fluid-filled antral space within the follicle (Haas, Christian, & Hoyer, 2007).

Statistical Analyses

All data analyses were completed using SPSS 23 software. For all analyses following Vehicle or VCD treatment assessments, two group comparisons were set *a priori* to compare treatment effects within each age group to determine if younger animals respond differently than older animals to the onset of VCD-induced follicular depletion. That is, an effect of Follicular Depletion was determined by comparing VCD

and Vehicle treatments only in the young animals, as well as VCD and Vehicle treatments only in middle-aged animals.

Additionally, planned comparisons were set to compare age effects within each treatment group to understand, in both young and middle-aged animals, the impact of menopause induction versus normal aging. Specifically, an Age effect was evaluated within the VCD-treated group only, as well as within the Vehicle-treated group only. Unless otherwise noted, two-tailed tests were used with an alpha level set at 0.05. The Huynh-Feldt correction was applied to repeated measures analyses to account for potential violations of sphericity by altering degrees of freedom (but not the F-ratio) to reduce Type I error rate as a result of repeated measures analysis (Huynh & Feldt, 1976). For all planned comparison repeated measures ANOVAs, effect sizes are reported as generalized eta squared (η_G^2) (Bakeman, 2005; Olejnik & Algina, 2003). For all planned comparison ANOVAs with only one between-subjects independent variable (i.e., serum hormone levels and ovarian follicle counts), effect sizes are reported as eta squared (η^2). These effect sizes are interpreted by standard guidelines for η^2 outlined by Cohen, whereby 0.02 is a small effect, 0.13 is a medium effect, and 0.26 is a large effect (Bakeman, 2005; Cohen, 1988, 1992). Cohen's d is reported for all pairwise comparisons as a measure of effect size, and is interpreted by the standard guidelines specified by Cohen, where 0.2 indicates a small effect, 0.5 indicates a medium effect, and 0.8 indicates a large effect (Cohen, 1988, 1992). Four animals were excluded for health-related concerns, and three animals were excluded from all analyses due to significant outlying scores that were greater than two standard deviations from the mean of the group's performance when compared to other animals for WRAM performance.

Behavior data.

Water radial-arm maze. Raw error scores from the WRAM were log transformed to account for extreme scores and the negative skew of error distribution as trials progress (Cohen, Cohen, West, & Aiken, 2003). WRAM training data prior to the Vehicle or VCD treatments were analyzed using a nested repeated-measures ANOVA design with Age as the independent variable, and Days and Trials as repeated measures. The dependent measure was Total Errors committed. WRAM refresher, retest, and flexibility data following Vehicle or VCD treatments were also analyzed for Total Errors. Each retest (averaged across the two-day block) was assessed separately utilizing a nested repeated-measures ANOVA design. Days and Trials were repeated measures within each two-group *a priori* comparison, with four trials per day within each of the two-day retests.

Morris water maze. Data were analyzed using a nested repeated-measures ANOVA. Days and Trials were repeated measures in all comparisons, with four trials per day within each of the five days. The dependent measure assessed was Swim Distance (cm) to the platform. The probe analysis used percent of total Swim Distance (cm) in the Target versus Opposite Quadrant as the dependent measure.

Visible platform task. Visible platform data were analyzed using repeated-measures ANOVA with latency to platform (s) as the dependent measure and trials as the repeated measure.

Ovarian follicle and serum hormone level data. Ovarian follicle and serum hormone level data were analyzed using ANOVA. Quantification and analysis of ovarian

follicles were divided into the following stages: primordial, primary, secondary, and antral follicles; corpora lutea were also assessed. Dependent variables for serum analyses included 17β-estradiol, androstenedione, progesterone, and estrone levels. Of note, for the estrone hormone assay, we did not collect a sufficient quantity of serum from seven animals at the end time point to complete the assay, and for the androstenedione hormone assay, we did not collect a sufficient quantity of serum from three animals at the end time point to complete the assay. Animals that were excluded for behavioral analyses were also excluded from serum and ovarian follicle analyses.

Results

Water Radial-Arm Maze Training

Evaluating age effects: Do young and middle-aged ovary-intact rats differ in performance on the water radial-arm maze task? The ANOVA for Total Errors (ln) including all young and middle-aged animals prior to treatment administration across all days of training revealed a main effect of Age [$F_{(1,44)}$=4.12, p<0.05, η_G^2=0.003], wherein Middle-Aged animals made more errors than Young animals collapsed across all days of testing (Figure 13a). There was also a main effect of Day [$F_{(10,445)}$=29.83, p<0.01, η_G^2=0.14], such that animals decreased in errors across days of testing, and a main effect of Trial [$F_{(3,129)}$=277.64, p<0.01, η_G^2=0.35], wherein errors increased as trials progressed and working memory load increased.

Previous studies examining the impact of gonadal hormones on spatial memory have observed marked effects on the WRAM when working memory load is taxed (Bimonte-Nelson et al., 2004; Bimonte & Denenberg, 1999; Bimonte et al., 2003; Braden

et al., 2010). Working memory load effects often present in the latter part of testing, or the asymptotic phase, after animals have learned the rules of the task (Bimonte & Denenberg, 1999; Bimonte et al., 2000, 2003; Hyde, Sherman, & Denenberg, 2000; Mennenga, Gerson, et al., 2015). Therefore, we assessed performance for the asymptotic phase, comprised of the last six days of testing (Days 7-12), based on previous WRAM findings from our laboratory (e.g., Bimonte & Denenberg, 1999; Braden et al., 2011; Mennenga, Gerson, et al., 2015); of note, asymptotic performance is operationally defined as animals approaching their optimal performance. For this block, there was a marginal main effect of Age [$F_{(1,44)}$=3.88, p=0.06, η_G^2=0.01]. Each trial for the Day 7-12 block was assessed separately, and we found that there was a main effect of Age on Trial 3 [$F_{(1,44)}$=5.58, p<0.05, η_G^2=0.04], where the Middle-Aged group made more errors than the Young group (Figure 13b). However, by Trial 4, there was no longer an Age effect [$F_{(1,44)}$=1.354, p=NS, η_G^2=0.01], indicating that the working memory load on Trial 4 was sufficiently difficult to challenge both young and middle-aged animals.

Water Radial-Arm Refresher Practice

Does forgetting occur for the water radial-arm maze task across a one-month interval? After initial WRAM training and then one month of Vehicle or VCD injections, animals in the Young group were eight months old, and animals in the Middle-Aged group were fourteen months old (Figure 10). Animals received two days of refresher practice on the WRAM, utilizing the same respective platform locations each animal previously learned during training, one month prior to starting the retest sessions. Performance was evaluated within each treatment group from the last day of training

(prior to treatment administration) to the first day of the refresher practice block (after treatment) to assess forgetting over the one-month interim. For the Young Vehicle-treated group, there was no effect of Day [$F_{(1,11)}$=2.72, p=NS, η_G^2=0.05], suggesting that during the one-month interval where these animals received Vehicle injections and were not tested, they retained memory for their four assigned platform locations. However, the Young VCD-, Middle-Aged Vehicle-, and Middle-Aged VCD- treated groups had a main effect of Day (Young-VCD: [$F_{(1,10)}$=8.76, p<0.05, η_G^2=0.09; Middle-Aged Vehicle: [$F_{(1,9)}$=15.72, p<0.01, η_G^2=0.14]; Middle-Aged VCD: [$F_{(1,12)}$=13.69, p<0.01, η_G^2=0.11]) with errors increasing across the training to refresher interval, indicating forgetting across the month in which they received their VCD or Vehicle injections (Figure 14). The collected results from the refresher indicate that initiation of follicular depletion impacted the Young group's ability to remember platform locations learned a month ago as compared to Vehicle-treated counterparts, and that Middle-Aged animals exhibited forgetting across the month delay regardless of treatment.

Water Radial-Arm Maze Retests

Evaluating follicular depletion effects: Does follicular depletion across the menopause transition impact young and middle-aged animals differently? To evaluate how each age group responded to VCD-induced follicular depletion compared to age-matched Vehicle-treated animals, planned comparisons between VCD and Vehicle-treated animals were performed within the Young cohort and within the Middle-Aged cohort. Retests 1 and 2 were combined into a single block representing the early follicular depletion period. During this block, two group (Vehicle vs. VCD) planned comparisons

for Young animals revealed a main effect of Follicular Depletion [$F_{(1,21)}$=10.32, p<0.01, η_G^2=0.07], with Young-VCD animals making more errors than Young-Vehicle animals. We also found a Trial x Follicular Depletion interaction [$F_{(3,63)}$=3.56, p<0.05, η_G^2=0.05], with Young-VCD animals making disproportionately more errors as trials and, respectively, working memory load increased, as compared to Young-Vehicle animals. On Trial 4, the highest working memory load trial, the Young VCD-treated group made more errors than the Young Vehicle-treated group [$F_{(1,21)}$=15.93, p<0.01, d=1.66] (Figure 15a). Planned comparisons for the Middle-Aged cohort showed neither a main effect of Follicular Depletion [$F_{(1,21)}$=0.61, p=NS, η_G^2=0.004] nor a Trial x Follicular Depletion interaction [$F_{(2,46)}$=0.07, p=NS, η_G^2=0.002]. Moreover, Middle-Aged Vehicle- and Middle-Aged VCD-treated groups did not differ in performance on Trial 4 [$F_{(1,21)}$=0.22, p=NS, d=0.196] (Figure 15b).

Following this initial early menopause transition block, no Follicular Depletion effects were observed in the planned comparisons within the Young cohort and within the Middle-Aged cohort on retests three through nine for Total Errors, indicating that VCD-induced follicular depletion had a transient impairing effect on spatial memory for young animals, but not middle-aged animals, early in the menopause transition.

Evaluating age effects: Does follicular depletion influence age effects on cognition? Another goal of the study was to evaluate whether menopause exacerbates age-related changes in spatial memory performance. Therefore, we compared Young and Middle-Aged animals within the Vehicle-treated cohort and within the VCD-treated cohort. During the early follicular depletion block (Retest 1 and 2), a marginal main effect of Age was observed for Vehicle-treated animals [$F_{(1,20)}$=3.56, p=0.07, η^2=0.02],

where Middle-Aged Vehicle-treated animals made marginally more errors than Young Vehicle-treated animals (Figure 15c). For VCD-treated animals, an age effect was not observed, indicating that Young VCD-treated animals performed similarly to Middle-Aged VCD-treated animals (Figure 15d).

No differences were observed on Retest 3. However, on Retest 4, as the VCD-treated cohort transitioned to a mid-follicle deplete state, we observed that for VCD-treated animals, there was a main effect of Age [$F_{(1,16)}$=5.38, p<0.05, η_G^2=0.04] and a marginal Age x Trial interaction [$F_{(3,48)}$=2.77, p=0.05, η_G^2=0.06]. On Trial 4, for animals that received VCD, Middle-Aged animals, which were 15 months old at this time point, made more errors than Young animals, which were 9 months old at this time point [$F_{(1,16)}$=8.75, p<0.01, d=1.40], indicating that VCD-induced follicular depletion imparted an age effect (Figure 15f). On the other hand, for the Vehicle-treated cohort, there was neither a main effect of Age [$F_{(1,14)}$=2.21, p=NS, η_G^2=0.03] nor an Age x Trial interaction [$F_{(2,23)}$=1.11, p=NS, η_G^2=0.03]. On Trial 4, there was no Age effect for animals that received Vehicle treatment (Figure 15e). Thus, menopause induction revealed an age effect that was not seen in Vehicle-treated animals.

No age effects were observed on Retests 5, 6, 7, or 8 within each treatment cohort. On Retest 9 – a post-follicular depletion time point – for VCD-treated animals, there was a marginal main effect of Age [$F_{(1,16)}$=4.34, p=0.05, η_G^2==0.06]. On Trial 4, there was a marginal effect of Age for animals that received VCD-induced follicular depletion, whereby Middle-Aged animals, now 18 months old, trended toward making more errors than Young animals, now 12 months old [$F_{(1,16)}$=3.42, p=0.08, d=0.88], indicating that VCD-induced follicular depletion marginally imparted an age effect in the

post-follicular depletion time point (Figure 15h). For Vehicle-treated animals, there was no main effect of Age [$F_{(1,14)}$=1.27, p=NS, η_G^2=0.02] and no observed differences in performance on Trial 4 (Figure 15g).

Water Radial-Arm Maze Flexibility Task

After Retest 9, platform location assignments were altered for all animals; two of their assigned locations remained the same, and two locations were changed to different arms, such that there were two familiar and two novel platform locations for this task (Figure 11b). Animals that were Young animals at VCD or Vehicle treatment were now 12 months of age, and Middle-Aged animals were now 18 months of age at the flexibility task assessment.

Evaluating follicular depletion effects: Does follicular depletion impact cognitive flexibility in young and middle-aged animals differently? From the last day of Retest 9 to the first day of the WRAM Flexibility task, there was a main effect of Day for the Young cohort [$F_{(1,15)}$=80.14, p<0.01, η_G^2=0.39] as well as for the Middle-Aged cohort [$F_{(1,15)}$=30.97, p<0.01, η_G^2=0.27] wherein all animals made more errors on the test day of the platform location flip as compared to the last day of WRAM testing with all platforms in the familiar spatial location; since there was no interaction with Follicular Depletion for either age group, the platform location flip impaired performance in both ages regardless of Vehicle or VCD treatment (Figure 16a). Across the five days of flipped testing, there was a main effect of Day within each age (Young: [$F_{(3,52)}$=12.96, p<0.01, η_G^2=0.15]; Middle-Aged: [$F_{(4,60)}$=13.28, p<0.01, η_G^2=0.13]), where errors decreased across days, indicating that all animals learned across the testing period (Figure

16b-c); again, a lack of interaction with Follicular Depletion within each age indicated that learning occurred similarly regardless of vehicle treatment or VCD-induced follicular depletion. On the final day of the flexibility task, for the Middle-Aged cohort, there was a marginal main effect of Follicular Depletion [$F_{(1,15)}$=3.97, p=0.07, η_G^2=0.06], where VCD-treated animals trended toward making more errors than Vehicle-treated counterparts, suggesting that follicular depletion in middle-aged animals results in poorer performance compared to Vehicle-treated counterparts for a flexibility measure (Figure 16c); however, the Young cohort did not differ in performance, regardless of ovarian status.

Evaluating age effects: Does follicular depletion influence age effects for cognitive flexibility? From the last day of Retest 9 to the first day of the WRAM Flexibility task, there was a main effect of Day for the Vehicle cohort [$F_{(1,14)}$=50.28, p<0.01, η_G^2=0.32] as well as for the VCD cohort [$F_{(1,16)}$=51.64, p<0.01, η_G^2=0.34], wherein all animals made more errors on the day of the flip, regardless of age (Figure 16a). Across the five days of testing, there was a main effect of Day (Vehicle: [$F_{(3,56)}$=14.55, p<0.01, η_G^2=0.15]; VCD: [$F_{(4,64)}$=10.54, p<0.01, η_G^2=0.13]) indicating that all animals learned across the testing period. On the final day of the flexibility task, in the VCD cohort, there was a marginal main effect of Age [$F_{(1,16)}$=3.52, p=0.08, η_G^2=0.05] where older animals tended to make more errors than younger animals; of note, this age effect did not occur in the Vehicle cohort. Again, we see that follicular depletion exacerbated memory deficits in older, but not younger, animals, while there were no age effects in animals that did not undergo follicular depletion (Figure 16d-e).

Morris Water Maze

Evaluating follicular depletion effects: Does follicular depletion impact young and middle-aged animals differently for a novel reference memory task? For the Young cohort, across the five days of MM testing, there were no Follicular Depletion effects. There was a main effect of Day [$F_{(4,60)}$=74.85, p<0.01, η_G^2=0.49] indicating learning across days for all Young animals (Figure 17a). Planned comparisons for Middle-Aged animals revealed a main effect of Follicular Depletion [$F_{(1,15)}$=5.24, p<0.05, η_G^2=0.04], where VCD-treated animals had a lower swim distance compared to Vehicle-treated counterparts, indicating that ovarian follicle depletion occurring in middle-age may aid reference memory for a novel task as compared to non-follicle deplete animals of the same age. There was also a main effect of Day [$F_{(4,60)}$=37.62, p<0.01, η_G^2=0.44] demonstrating learning across days for all Middle-Aged animals (Figure 17b).

Overnight forgetting was assessed as a measure of memory retention for a reference memory task across an overnight interval. We examined performance on the last trial of each day (Trial 4) and the first trial of each day (Trial 1). When collapsed across overnight intervals, for Middle-Aged animals, there was a marginal Trial x Follicular Depletion interaction [$F_{(1,15)}$=3.52, p=0.08, η_G^2=0.03], where on the first trial of the day, although it did not reach significance, Middle-Aged VCD-treated animals had a lower swim distance to the platform than Vehicle-treated counterparts [$F_{(1,15)}$=4.32, p=0.06, d=1.02], suggesting better memory retention during the overnight interval for older follicle-deplete animals compared to their Vehicle-treated counterparts. There were no effects of follicular depletion between Young groups for overnight forgetting.

Evaluating age effects: Does follicular depletion influence age effects for a novel reference memory task? For the Vehicle-treated cohort, across the five days of MM testing, there were no Age effects. There was a main effect of Day [$F_{(4,56)}$=41.98, p<0.01, η_G^2=0.41] indicating learning across days for all Vehicle-treated animals (Figure 17c). Planned comparisons for the VCD-treated cohort revealed a main effect of Age [$F_{(1,16)}$=5.42, p<0.05, η_G^2=0.02], where Young animals had less swim distance compared to Middle-Aged animals, indicating that older follicle-deplete animals were impaired compared to their younger counterparts. There was also a main effect of Day [$F_{(4,64)}$=63.75, p<0.01, η_G^2=0.53] indicating learning across days for all VCD-treated animals (Figure 17d). Overnight forgetting planned comparisons did not reveal effects of Age in either Vehicle- or VCD- treated cohorts.

Probe trial performance. Each group was assessed separately for performance on the probe trial. Results indicated that there was a main effect of Quadrant for each group (Young-Vehicle: [$F_{(1,8)}$=35.09, p<0.01, η_G^2=0.77], Young-VCD: [$F_{(1,7)}$=49.60, p<0.01, η_G^2=0.85], Middle-Aged-Vehicle: [$F_{(1,6)}$=29.43, p<0.01, η_G^2=0.81], Middle-Aged-VCD: [$F_{(1,9)}$=79.64, p<0.01, η_G^2=0.83]), indicating that all animals, regardless of follicular depletion status or age, had a greater percent of total swim distance in the target quadrant; that is, they all localized to the northeast quadrant where the platform was previously located versus the opposite, southwest quadrant (Figure 18).

Visible Platform

Evaluating follicular depletion effects: Does follicular depletion impact young and middle-aged animals differently for visual and motor competency? Among Young animals as well as Middle-Aged animals, there were no main effects of Follicular Depletion. The mean escape latency for the last trial was 6.13±1.90 seconds for Young animals, and 5.31±1.15 seconds for Middle-Aged animals, indicating the ability to successfully perform the procedural components of a water escape task for both younger and aged cohorts (Figure 19).

Evaluating age effects: Does follicular depletion influence age effects for visual and motor competency? There was no effect of Age within Vehicle-treated animals, or within VCD-treated animals. The mean escape latency for the last trial was 4.88±0.92 seconds for Vehicle-treated animals, and 6.56±1.77 seconds for VCD-treated animals, indicating the ability to successfully perform the procedural components of a water escape task for both Vehicle- and VCD- treated cohorts (Figure 19).

Serum Hormone Levels

Serum hormone levels for 17β-estradiol, estrone, androstenedione, and progesterone were obtained for animals at the subset sacrifice time point and at the end sacrifice time point. Tables 1-4 include the mean ± SE, range, and median values for each hormone at each time point.

Subset sacrifice. Young animals (VCD: n=3; Vehicle: n=3) were 8 months old and Middle-Aged animals (VCD: n=3; Vehicle: n=3) were 14 months old at the time of serum analysis for the subset sacrifice.

Evaluating follicular depletion effects for the subset sacrifice: Does follicular depletion impact circulating ovarian hormone levels differently in young and middle-aged animals at the early follicular depletion time point? For the subset sacrifice, there were no Follicular Depletion effects within either age group for 17β-estradiol, estrone, or progesterone levels (Figures 20a, b, d, respectively), indicating that follicular depletion had no impact on these hormones within the Young cohort or within the Middle-Aged cohort early in the menopause transition (52 days after initiating VCD-induced follicular depletion). For androstenedione levels in the Young cohort, there was a marginal main effect of Follicular Depletion [$F_{(1,4)}$=4.71, p=0.096, η^2=0.53], where Vehicle-treated animals had marginally more androstenedione than VCD-treated animals. No differences in androstenedione levels were found in the Middle-Aged cohort at this time point (Figure 20c).

Evaluating age effects for the subset sacrifice: Does follicular depletion influence age effects on circulating ovarian hormone levels at the early follicular depletion time point? There were no Age effects within either treatment group for 17β-estradiol or estrone (Figures 20a, b, respectively), indicating that the age did not impact estrogen levels within the Vehicle cohort or within the VCD cohort early in the menopause transition. For androstenedione levels in the VCD-treated cohort, there was a marginal main effect of Age [$F_{(1,4)}$=7.50, p=0.05, η^2=0.65], wherein Middle-Aged animals had marginally higher androstenedione levels than Young animals (Figure 20c). For the Vehicle-treated cohort, there were no Age effects for androstenedione levels. For progesterone in the VCD-treated cohort, there was a main effect of Age [$F_{(1,4)}$=11.24, p<0.05, η^2=0.74], where Middle-Aged animals had more progesterone than Young

animals (Figure 20d); however, there were no differences in progesterone levels for the Vehicle cohort at the subset sacrifice time point.

End sacrifice. Animals that were excluded for behavioral analyses were also excluded from serum analyses. Young animals (VCD: n=8; Vehicle: n=9) were 12 months old, and Middle-Aged animals (VCD: n=10; Vehicle: n=7) were 18 months old, at the end sacrifice time of serum analysis.

Evaluating follicular depletion effects for the end sacrifice: Does follicular depletion impact circulating ovarian hormone levels differently in young and middle-aged animals at the post-follicular depletion time point? There were no Follicular Depletion effects within either age group for 17β-estradiol or androstenedione levels at the end sacrifice (Figures 20e, g, respectively), indicating that follicular depletion did not impact these hormones within the Young cohort or within the Middle-Aged cohort in the post-follicular depletion time point. For estrone levels, within the Young cohort as well as within the Middle-Aged cohort, there was a marginal main effect of Follicular Depletion (Young: [$F_{(1,11)}$=3.62, p=0.08, η^2=0.25]; Middle-Aged: [$F_{(1,12)}$=4.31, p=0.06, η^2=0.26]; Figure 20f), whereby VCD-treated animals had marginally less estrone compared to Vehicle-treated animals within both age groups. For progesterone levels within the Young cohort as well as within the Middle-Aged cohort, there was a main effect of Follicular Depletion (Young: [$F_{(1,15)}$=6.98, p<0.05, η^2=0.32]; Middle-Aged: [$F_{(1,15)}$=12.35, p<0.01, η^2=0.45]; Figure 20h) wherein VCD-treated animals had less progesterone than Vehicle-treated animals within both age groups. Because the majority of progesterone is produced by the corpus luteum after ovulation, these latter results

suggest that the VCD-induced follicular depletion was effective in initiating ovarian failure.

Evaluating age effects for the end sacrifice: Does follicular depletion influence age effects on circulating ovarian hormone levels at the post-follicular depletion time point? There were no Age effects within either treatment group for 17β-estradiol, estrone, or progesterone levels (Figures 20e, f, h, respectively) indicating that age did not impact these serum hormone levels within the Vehicle cohort or within the VCD cohort in the post-follicular depletion time point. For androstenedione levels within the Vehicle cohort, there was a marginal main effect of Age [$F_{(1,12)}$=3.97, p=0.07, η^2=0.25] wherein Middle-Aged animals had marginally more androstenedione than Young animals (Figure 20g). However, within the VCD cohort, there were no age differences in androstenedione levels (Figure 20g), such that younger VCD-treated animals had similar androstenedione levels to older VCD-treated animals.

Ovarian Follicle Counts

Ovarian follicle counts were obtained for both subset and end sacrifices (Figure 21).

Subset sacrifice.

Evaluating follicular depletion effects at the subset sacrifice: Does follicular depletion impact ovarian follicle counts differently in young and middle-aged animals at the early follicular depletion time point? Within the Young cohort there was a main effect of Follicular Depletion for primordial follicles [$F_{(1,4)}$=26.40, p<0.01, η^2=0.87, Figure 21a], primary follicles [$F_{(1,4)}$=14.30, p<0.05, η^2=0.78, Figure 21b], secondary

follicles [$F_{(1,4)}$=10.98, p<0.05, η^2=0.73, Figure 21c], and antral follicles [$F_{(1,4)}$=28.80, p<0.01, η^2=0.88, Figure 21d], where VCD-treated animals had fewer ovarian follicles of each subtype than Vehicle-treated counterparts. Within the Middle-Aged cohort there was a main effect of Follicular Depletion for primordial follicles [$F_{(1,4)}$=27.01, p<0.01, η^2=0.87, Figure 21a] and a marginal main effect of Follicular Depletion [$F_{(1,4)}$=6.42, p=0.06, η^2=0.62, Figure 21b] for primary follicles, with VCD-treated animals tending to have fewer cells than Vehicle-treated counterparts. The Middle-Aged cohort did not have an observed difference in secondary or antral follicles at the subset time point (Figures 21c, d respectively). There were no observed differences in the number of corpora lutea in either the Young cohort or the Middle-Aged cohort at this time point (Figure 21e).

Evaluating Age effects at the subset sacrifice: Does follicular depletion influence age effects on ovarian follicle loss at the early follicular depletion time point? Within the Vehicle cohort, there was a marginal main effect of Age [$F_{(1,4)}$=5.50, p=0.08, η^2=0.58], where Young animals had more primordial follicles than Middle-Aged animals (Figure 21a). There were no differences in primary, secondary, or antral follicles for the Vehicle cohort (Figures 21b, c, d, respectively). Within the VCD cohort there were no differences in any ovarian follicle subtype (Figures 21a-d). Neither the Vehicle cohort nor the VCD cohort exhibited differences in corpora lutea at this time point (Figure 21e).

End sacrifice. Animals that were excluded for behavioral analyses were also excluded from ovarian follicle count analyses.

Evaluating follicular depletion effects at the end sacrifice: Does follicular depletion impact ovarian follicle counts differently in young and middle-aged animals

at the post-depletion time point? Within the Young cohort, there was a main effect of Follicular Depletion for primordial follicles [$F_{(1,15)}$=25.12, p<0.01, η^2=0.63, Figure 21f], primary follicles [$F_{(1,15)}$=34.72, p<0.01, η^2=0.70, Figure 21g], secondary follicles [$F_{(1,15)}$=27.44, p<0.01, η^2=0.65, Figure 21h], antral follicles [$F_{(1,15)}$=16.31, p<0.01, η^2=0.52, Figure 21i], and corpora lutea [$F_{(1,15)}$=200.89, p<0.01, η^2=0.93, Figure 21j], where VCD-treated animals had fewer ovarian follicles and corpora lutea than Vehicle-treated counterparts. Within the Middle-Aged cohort, there was also a Follicular Depletion effect observed for primordial follicles [$F_{(1,15)}$=4.79, p<0.05, η^2=0.24, Figure 21f], primary follicles [$F_{(1,15)}$=63.57, p<0.01, η^2=0.81, Figure 21g], secondary follicles [$F_{(1,15)}$=61.45, p<0.01, η^2=0.80, Figure 21h], antral follicles [$F_{(1,15)}$=41.36, p<0.01, η^2=0.73, Figure 21i], and corpora lutea [$F_{(1,15)}$=241.00, p<0.01, η^2=0.94, Figure 21j], where VCD-treated animals had fewer ovarian follicles and corpora lutea than Vehicle-treated counterparts. In sum, VCD treatment effectively reduced ovarian follicle number in both age groups compared to Vehicle treatment.

Evaluating age effects at the end sacrifice: Does follicular depletion influence age effects on ovarian follicle loss at the post-depletion time point? Within the Vehicle cohort, there was a marginal effect of Age [$F_{(1,14)}$=3.80, p=0.07, η^2=0.21, Figure 21f] for primordial follicles, where Young animals had marginally more primordial follicles than Middle-Aged animals. In addition, for Vehicle-treated animals a main effect of Age was revealed for primary follicles [$F_{(1,14)}$=10.00, p<0.01, η^2=0.42, Figure 21g] and secondary follicles [$F_{(1,14)}$=5.34, p<0.05, η^2=0.28, Figure 21h], where Young animals had more of these follicle subtypes than Middle-Aged animals. However, there were no effects of Age for antral follicles or corpora lutea in the Vehicle cohort (Figures 21i, j, respectively).

Within the VCD cohort, there were no effects of Age on primordial follicles, primary follicles, secondary follicles, or antral follicles (Figures 21f, g, h, i, respectively), suggesting that VCD reduced the number of follicles in the ovary without interactions with age. It is of note that none of the VCD-treated animals had quantifiable antral follicles at this time point, suggesting that these animals were no longer experiencing a normal ovarian cycle. However, within the VCD-treated animals, there was a marginal main effect of Age for corpora lutea counts [$F_{(1,16)}$=3.66, p=0.07, η^2=0.19, Figure 21j] where Middle-Aged VCD-treated animals had marginally more corpora lutea than Young VCD-treated animals. These collective results provide evidence for age-related follicle decline in Vehicle-treated rats, as well as substantial atresia of ovarian follicles in VCD-treated animals.

Uterine Horn Weights

There were no main effects of Age or Follicular Depletion on uterine horn wet weight (g) at either sacrifice time point. All uterine horns were similar in weight regardless of treatment. Given that estrogens have a well-known stimulatory effect on uterine tissue growth (Brody & Wiqvist, 1961; Yuan Hsu Kang, Anderson, & DeSombre, 1975) and there were no differences in circulating 17β-estradiol levels, this result was expected.

Discussion

Here, we performed a longitudinal study systematically evaluating the effects of age and ovarian status on spatial memory performance across the transition to menopause

in a rodent model. The collective results indicate that: (1) age at the onset of transitional menopause impacts cognitive performance, with the onset of follicular depletion at a younger age negatively impacting spatial memory, and (2) follicular depletion exacerbates age-related memory changes later in the menopause transition. Furthermore, the impact of age and follicular depletion on spatial memory become evident when the working memory system was highly taxed, as demonstrated by the differences on trial four of the WRAM, when working memory load was the highest.

Inducing Transitional Menopause at a Younger Age Impairs Spatial Memory Early in Follicular Depletion

Early in the transition to menopause (Retests 1 and 2), VCD treatment impaired WRAM performance in the young animals, but not the middle-aged animals, compared to their respective age-matched Vehicle-treated controls (Figure 15a, b). In fact, at this time point, VCD-induced transitional menopause rendered younger animals' performance on the WRAM similar to that of middle-aged animals (Figure 15d). Because only the Young cohort was impaired with VCD treatment, this age-specific impairment suggests that undergoing the transition to menopause prior to middle-age is detrimental to memory performance, at least in the early stages of follicular depletion. This early stage of the VCD menopause model involves substantial ovarian follicle decline, particularly for the young animals, which is analogous to the beginning of the human menopause transition when women tend to report some memory complaints (Woods, Mitchell, & Adams, 2000).

It is particularly interesting that these changes occur in adult animals undergoing follicular depletion, because advanced age is not a confounding factor for these animals due to the nature of our experimental design. Indeed, their age-matched Vehicle-treated counterparts performed better on the WRAM at the highest working memory load, indicating that follicular depletion and the associated hormonal and ovarian changes have a unique impact on adult animals that are not yet at the age that they would be naturally transitioning to a reproductively senescent state. Dissociating the effects of aging and follicular depletion is a tremendous benefit gained from using animal models. These variables are difficult to evaluate independently in women, because the onset of transitional menopause is typically concomitant with aging. Our results, if translated to women, suggest that an earlier onset of transitional menopause may result in disrupted cognition. It is notable that aging affects the functional connectivity and structure of brain areas important for learning and memory (Barnes, 1979; Poe et al., 2000) and these changes may impact strategy selection for solving behavioral tasks (Samson et al., 2015). As such, it is a possibility that younger animals relied more heavily on an allocentric spatial strategy to solve the WRAM compared to the strategies used by older animals. If induced transitional menopause impacted the capacity to use a hippocampal-dependent strategy or attend to the extramaze cues, this could, in part, explain their poor performance compared to age-matched counterparts. In addition, strategy selection for some spatial tasks has been shown to vary in female rats depending on estrous cycle phase (Korol, Malin, Borden, Busby, & Couper-Leo, 2004), which may also have influenced subjects' performance and maze-solving strategy.

Currently, there are no definitive clinical markers that identify the onset of the menopause transition. Menopause is confirmed retrospectively following one year without a menstrual period, and its onset is difficult to determine prospectively. Research suggests that changes in hypothalamic-pituitary-gonadal (HPG) axis function are detectable prior to the onset of menstrual or estrous cycle irregularities (Downs & Wise, 2009; Wise et al., 1997, 1996, 1999, 1989). It is possible that perturbations in HPG axis activity prior to the average onset of reproductive senescence — as is the case for early and premature onset of menopause — may result in unique cognitive and brain changes that ultimately impact the trajectory of cognitive and brain aging. Developing reliable measures to establish the impetus of the transition to reproductive senescence in women could provide the opportunity to intervene during a critical window early in the transition, which may be necessary to ascertain the beneficial effects of hormone therapy on menopause-related memory changes.

For Transitionally Menopausal Animals, Detrimental Age Effects Present in Mid- and Post- Follicular Depletion

The series of retests administered here throughout the transition into a follicle-deplete state allowed us to capture memory performance at several key time points in the menopause transition as well as across normal aging. Following the early menopause time point, as the transition to menopause progressed, an age-related effect of follicular depletion became apparent. During Retest 4 on the WRAM, during mid-follicular depletion, this age-related deficit was only evident in animals undergoing VCD-induced follicular depletion; Middle-Aged VCD-treated animals made more errors than Young

VCD-treated animals, particularly at the highest working memory load trial. In contrast to the age-related change in the VCD cohort, there was no age-related change in the Vehicle-treated animals during Retest 4. Therefore, the detrimental cognitive effect of aging was only apparent in the transitional menopause model, but not in the naturally aging group.

Post-follicular depletion, at Retest 9, we saw a similar age-related impairment only in the VCD-treated animals, although the effect reached only marginal significance at this time point. Thus, during (at Retest 4) and after (at Retest 9) the transition to follicular depletion, older animals tended to perform worse than younger animals undergoing accelerated follicular depletion. Perhaps repeated testing on the WRAM (i.e., cognitive practice) resulted in a beneficial effect for memory maintenance, albeit only for animals aging independently of accelerated follicular depletion — a finding which has previously been shown in our and other laboratories (Markowska & Savonenko, 2002b; Talboom et al., 2014). Given that age-related impairment remained in the VCD cohort, this result could indicate that undergoing follicular depletion in middle age may obviate the beneficial effects of cognitive practice for a task that taxes the working memory system. Translationally, because the average lifespan in women is increasing (Central Intelligence Agency, 2015; Murray et al., 2015; G. K. Singh, Kochanek, & MacDorman, 1996; Xu, Kochanek, Murphy, & Arias, 2014), it is potentially of interest to slow the rate of ovarian follicle reserve loss in order to prolong the benefits of follicle-replete ovaries and normal circulating sex steroid hormone levels on memory as well as on a myriad of other body systems, such that age effects seen with follicular depletion would be delayed until later in life.

All Animals Showed Cognitive Flexibility to Learn a Revised Task, With Some Marginal Impairments of Transitional Menopause

After initial training on the WRAM task, half of the reinforced locations were switched so that we could assess cognitive flexibility. All animals were able to update previously learned information by shifting to new escape locations for the altered WRAM task, as indicated by improved performance across the five days of this WRAM flexibility task. On the final day, older VCD-treated animals performed marginally worse than their age-matched Vehicle controls, indicating that in a later stage of follicular depletion, the loss of ovarian follicles had a minor impact on performance on a novel flexibility task. Within the VCD-treated cohort, a marginal age effect was observed, with older animals tending to make more errors than younger animals; this effect did not occur within the Vehicle-treated cohort, indicating that follicular depletion may initiate age-related impairments for cognitive flexibility, similar to the impact of follicular depletion on the WRAM task in the mid- and post- menopausal time points.

Follicular Depletion Improves Reference Memory Performance for Older Animals, but Impairs Performance Compared to Younger Transitionally Menopausal Animals

Morris water maze results indicated that the younger animals learned the task similarly regardless of ovarian status. On the other hand, VCD treatment improved MM performance in middle-aged animals, as measured by decreased swim distance to the platform on the last day of testing. Ovarian follicular depletion in older animals bestowed a beneficial effect on learning and memory retention for a novel spatial reference

memory task, providing evidence that the negative memory effects often noted in the post-menopausal state may not extend to all learning and memory domains. It is noteworthy that the VCD-treated animals had been in a post-follicle deplete state for several months at this time point and likely had a more stable circulating hormone profile, including significantly lower progesterone levels that could benefit spatial reference memory performance (discussed below). Of note, within the VCD-treated cohort, middle-aged animals had a greater swim distance to the platform, interpreted as poorer performance, compared to younger animals. Since a concordant age-related difference was not seen in Vehicle-treated animals, this suggests again that VCD induced follicular depletion revealed age-related effects. It is noteworthy that all groups were effectively utilizing a spatial strategy to solve the MM, regardless of ovarian status or age, as indicated by the probe trial. Additionally, the Visible Platform task verified that, after six months of testing, the animals included in these analyses were still able to see as well as perform the motor-related components of the task, even with advanced age.

Endocrine and Ovarian Markers

VCD-Induced Follicular Depletion Alters Ovarian Hormone Profiles. A hallmark of the human transition into a post-menopausal state is a marked decrease in circulating ovarian hormone levels. During the transitional phase, serum hormone levels may be erratic due to irregular ovulation patterns and disrupted hypothalamic-pituitary-ovarian communication. VCD treatment models this perimenopausal time point during which gonadal hormone levels are in a state of flux resulting from accelerated follicular depletion. Measurement of the four primary ovarian hormones, 17β-estradiol, estrone,

androstenedione, and progesterone at the subset sacrifice time point (that is, at the early follicular depletion time point), revealed that older VCD-treated animals had marginally increased androstenedione as well as significantly increased progesterone levels compared to younger VCD-treated animals. These results possibly indicate that the younger animals experienced a more rapid decline in ovarian follicle reserve, even though estrogen levels were not different at this time point. At the post-menopausal time point, both Young and Middle-Aged VCD-treated cohorts had less progesterone and marginally less estrone than Vehicle-treated counterparts. A key characteristic of menopause is the decline in circulating progesterone levels resulting from anovulation and, consequently, no formation of the corpora lutea to secrete progesterone. Furthermore, exogenous progesterone alone (Bimonte-Nelson et al., 2006, 2004), as well as exogenous estrone alone (Engler-Chiurazzi et al., 2012), have been implicated in impaired spatial memory performance in middle-aged Ovx rats. Perhaps the elevated progesterone levels, as well as the marginally elevated estrone levels, in the Vehicle-treated animals observed at the end time point can partially explain the older VCD-treated animals' advantage over their Vehicle-treated counterparts in the MM task discussed above.

The initial, foundational research using VCD-induced follicular depletion as a model of transitional menopause was completed in mice; VCD treatment was initiated early in life and resulted in a decline in progesterone and androstenedione, as well as undetectable 17β-estradiol levels, compared to age-matched controls (Mayer et al., 2004). Other VCD studies in both rats and mice have found no differences in 17β-estradiol levels at multiple time points compared to age-matched controls (Frye et al., 2012; Lohff

et al., 2005; Mayer et al., 2002). These experiments consistently report increased FSH levels, reduced ovarian follicle counts, and extended estrous cycles following VCD treatment. Mice tended to transition into persistent diestrus (acyclicity) following VCD treatment (Lohff et al., 2005; Mayer et al., 2004), while rats had extended periods of persistent estrous (and detectable 17β-estradiol levels) during VCD-induced disrupted cyclicity (Frye et al., 2012; Mayer et al., 2002). Thus, this suggests that in rats, VCD-induced follicular depletion is a unique model of the perimenopause time point, during which ovarian cyclicity is disrupted and ovarian hormone levels may be fluctuating and at some time points elevated prior to the eventual decline of these circulating hormones. Indeed, our serum hormone levels with VCD-induced follicular depletion reported here are consistent with the observations reported in the Frye et al. (2012) and Mayer et al. (2002) publications using the same VCD dosing regimen. A limitation to this study is that we did not collect blood at multiple points across time within the same animal. It is possible that we may have observed fluctuating patterns of ovarian hormones that are similar to women's circulating ovarian hormone patterns during the perimenopause. Human research suggests that it is not necessarily the final decline in circulating ovarian hormones, but the erratic fluctuations during the transition, in conjunction with changes in affect, stress, and physical health during midlife, that are associated with perceived cognitive changes as identified by self-reports (Mitchell & Woods, 2001; Weber et al., 2014; Weber, Mapstone, Staskiewicz, & Maki, 2012; Weber, Rubin, & Maki, 2013). Indeed, within the time constraint of the experimental design, all subjects, regardless of ovarian status, showed stable 17β-estradiol levels. Had we continued our assessments until a later time point or completed blood draws to evaluate hormone fluctuations across

time, we may have observed altered 17β-estradiol profiles. Thus, this is an important future direction to pursue in order to fully understand the VCD model of transitional menopause in rats and its relationship to what is known about menopause in women.

VCD Successfully Induced Follicular Depletion Across Time and Normally Aging Rats Experience Some Follicular Atresia in Midlife

Recent clinical literature suggests that ovarian follicle counts are a contender as a reliable biomarker to determine reproductive status, over and above other measurements such as serum hormone levels alone. Indeed, serum hormone levels may not be a reliable measurement across the transition to menopause because they are in constant flux. More stable markers, such as antimüllerian hormone levels to estimate the non-growing follicle population (i.e., the ovarian follicle reserve), as well as antral follicle count, may be important in determining individual trajectories into the post-menopausal state (Anderson, Nelson, & Wallace, 2012; Broer et al., 2011; Fleming, Kelsey, Anderson, Wallace, & Nelson, 2012). Ovarian follicle counts quantified here were an excellent guide in determining the rats' progression into reproductive senescence and verifying the effectiveness of VCD-induced follicular depletion. At the subset sacrifice during the early menopause transition, a snapshot of the progression into follicle depletion was obtained. For primordial (non-growing) follicles, the VCD-treated animals had fewer follicles than Vehicle-treated animals in both age cohorts; for primary follicle counts, Young VCD-treated animals had fewer cells than age-matched counterparts, and Middle-Aged VCD-treated animals had marginally fewer. These results verify that VCD treatment effectively targets the resting ovarian follicle pool by initiating accelerated depletion of the pool.

Interestingly, Young Vehicle-treated animals had marginally more primordial follicles than Middle-Aged Vehicle-treated counterparts, indicating that rats naturally experience some follicle depletion during aging; this loss of ovarian follicles is clearly not as extreme as is the case with VCD-induced ovarian changes, as evidenced by the fact that both VCD-treated age groups had significant follicular depletion of non-growing follicles at the time of the subset analysis. The antral follicle count, which is most often used in the clinical setting as a reliable marker of reproductive capacity, differed within the Young cohort, such that VCD-treated animals had fewer antral (pre-ovulatory) follicles compared to age-matched Vehicle-treated counterparts. The Middle-Aged cohort did not exhibit the same effect – VCD- and Vehicle- treated animals did not differ in antral follicle count at this subset snapshot. At the subset time point, within the Young and within the Middle-Aged cohorts, corpora lutea did not differ depending on treatment; this null effect was also seen within the VCD cohort and within the Vehicle cohort. Evidence of corpora lutea in the ovary at this early follicular depletion time point indicates that even animals undergoing accelerated follicular depletion still experience intermittent ovulatory cycles – akin to women's irregular ovulatory cycles in the early menopause transition (O'Connor et al., 2009).

At the end of the study, within the Young cohort and within the Middle-Aged cohort, there were main effects of VCD-induced follicular depletion for all ovarian follicle types, as well as for corpora lutea, wherein animals that had undergone VCD-induced follicular depletion had fewer ovarian follicles and corpora lutea than Vehicle-treated animals. Indeed, VCD-treated animals had no quantifiable antral follicles at analysis, indicating the termination of normal ovulatory cycles and follicle maturation in

these animals after the transition to a post-menopausal state. Although older VCD-treated animals had marginally more corpora lutea than younger VCD-treated counterparts, there was no difference in circulating progesterone levels between these groups at this time point. These results indicate that, despite VCD-treated animals experiencing intermittent ovulation, the few corpora lutea produced were not sufficient to generate a significant impact on resulting spatial memory performance. Similar to the results seen in the subset sacrifice, older Vehicle-treated animals had fewer primordial, primary, and secondary follicles than their younger counterparts, corroborating the idea that as aging ensues, rodents experience a natural decline in the ovarian follicle pool over time (for review, see: Finch, 2014), though not to the same extent as the profile of a VCD-induced follicle-deplete rat, or that of a menopausal woman. Within the VCD cohort, ovarian follicle counts did not differ between the ages, suggesting that overall, follicular depletion was successfully induced in both age groups with VCD treatment.

Broad Interpretations, Translational Implications, and Conclusions

To our knowledge, this is the first study to perform a longitudinal, repeated measures evaluation of ovary intact female rats comparing animals with or without VCD-induced follicular depletion at different ages to model human menopause. Several key findings are presented here: (1) undergoing the transition to menopause at a younger age is detrimental to memory performance compared to age-matched, reproductively competent subjects, at least during the early stages of the transition to reproductive senescence; (2) follicular depletion amplifies age-related differences in cognitive performance in the mid- and late- transitional menopause phases; and (3) in a post-

follicle deplete state, ovarian follicular depletion in older animals may confer an enhanced capacity to solve a novel spatial memory task.

Of note, other laboratories have conducted cross-sectional studies looking at age-related memory decline in males (Bizon et al., 2009; Frick, Baxter, Markowska, Olton, & Price, 1995; Markowska, 1999b) and females (Markowska, 1999a; Markowska & Breckler, 1999). Ovx and sham female Fischer-344 rats have also been longitudinally evaluated for cognitive performance over a nine-month period, beginning in middle-age (Markowska & Savonenko, 2002a). Our laboratory recently demonstrated that cognitive practice can attenuate age-related decline in males and females by enhancing performance on the practiced task when tested at a later age compared to naïve, age-matched animals; additionally, cognitive practice bestows a sex-specific benefit for females to transfer enhanced performance capacity to a novel cognitive task (Talboom et al., 2014). It is possible that the animals' previous, repeated experience on the complex WRAM task (i.e. cognitive practice) improved performance on the MM later in life. Similar findings for cognitive practice in females have also been seen in non-human primates (Lacreuse et al., 2005).

Many factors, including the appropriate timing, duration, hormone formulation, dose, and route of administration, are involved in developing hormone therapy regimens that will result in the attenuation of physiological menopause symptoms as well as provide potential beneficial effects for cognition. To add complexity to this puzzle, what is optimal for one woman may not have the same effect in another woman (for review, see: Koebele & Bimonte-Nelson, 2015). Our findings provide further insight into the window of opportunity for hormone therapy by revealing that the age at the onset of the

menopause transition matters for memory outcomes. The current study's finding that early follicular depletion is detrimental to cognition corroborates clinical findings that younger women who experienced oophorectomy prior to the onset of natural menopause have poorer short-term verbal scores than those who experienced oophorectomy later in life (Nappi et al., 2009). Taken together, these findings indicate that age at menopause onset is an important parameter that impacts cognitive outcomes. Whether the age at onset of menopause, and further, the age at the onset of perimenopause, influences efficacy of hormone therapy is an important consideration as a next research step. Indeed, in the future, it will be important to experimentally evaluate whether the sensitive window(s) of follicular depletion and hormone loss map onto the sensitive window(s) for hormone therapy exposures to result in beneficial cognitive effects. Elucidating the impact of age, ovarian status, and hormone changes on spatial memory across the transition to a reproductively senescent state is key in developing personalized hormone therapy regimens that can be translated to the clinic for women experiencing the menopause transition. Understanding the dynamic nature of the menopausal hormone milieu and potential interactions with subsequent hormone therapy is key to deciphering parameters for optimal healthy brain aging profiles in women throughout their lifespans.

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CHAPTER 3

The data presented in this chapter are currently unpublished observations culminating from the brains acquired from the experiment described in Chapter 2. These data were, in part, presented with correlations to behavior findings at the Society for Neuroscience conference in 2016.

EVALUATING RELATIONSHIPS AMONG NORMAL AGING, FOLLICULAR DEPLETION, AND OVARIAN HORMONE LEVELS WITH CHOLINE ACETYLTRANSFERASE-IMMUNOREACTIVE NEURONS IN THE BASAL FOREBRAIN AND GLUTAMIC ACID DECARBOXYLASE PROTEIN EXPRESSION IN THE HIPPOCAMPAL FORMATION

Contribution: I was the graduate student principal investigator for this experiment. These data presented in this chapter are a continuation of my master's thesis work under the mentorship of Dr. Heather Bimonte-Nelson. I completed the slicing, staining, stereological quantification, western blot, and densitometry assessments of the brains. Shruti Patel is acknowledged for her assistance with tissue slicing and preparation for stereology. Bryanna Hadder is acknowledged for her assistance in completing western blot protein analyses.

ABSTRACT

During the menopause transition, ovarian hormones fluctuate irregularly prior to a chronic decline in serum levels. This altered hormonal milieu impacts many body systems, including the brain. Long-range cholinergic projections originating from the basal forebrain are the primary cholinergic input into the hippocampal formation, including connections to hippocampal GABAergic interneurons, which are important regulators for spatial learning and memory. These neuronal populations and their synaptic connections are each altered with age, endogenous ovarian hormone changes, and exogenous hormone treatment. The fluctuation in ovarian hormones levels during the menopause transition may also be related to midlife memory changes regulated by these systems. Here, the 4-vinylcyclohexene diepoxide (VCD) transitional menopause rat model, which accelerates ovarian follicular depletion, was utilized to explore menopause- and age- related changes in the cholinergic system and GABAergic systems, and relationships among these systems with serum hormone levels and cognitive outcomes. Specifically, stereology was used to quantify ChAT-immunoreactive (IR) neurons in the medial septum and vertical-diagonal bands of the basal forebrain in early- and late-middle-aged Fisher-344 rats tested on a behavioral battery for spatial working and reference memory. Western blot protein analysis was used to assess GAD65 and GAD67 protein expression in the dorsal hippocampus, entorhinal cortex, perirhinal cortex, and ventral CA1/2 hippocampus of the right hemisphere. Correlations encompassing serum hormone levels, ovarian follicle reserve, ChAT-IR cell count estimates, GAD65/GAD67 protein expression, and spatial memory are presented here. Overall, the relationships observed suggest that age and ovarian status are both important factors contributing to the

complex relationships among these systems, and provide insight into their influence on learning and memory during the menopause transition.

Introduction

Neural systems are dynamic across the lifespan. The aging brain is susceptible to a number of alterations to important regulatory systems that can, in turn, influence health outcomes and determine whether an individual will have a normal cognitive and brain aging profile, or whether neuropathological aberrations will occur. A critical life stage during which alterations likely ensue is during the transition to menopause, or reproductive senescence, which ovary-intact women naturally undergo around the fifth decade of life. The finite ovarian follicle pool reaches a critical threshold of natural depletion, the menstrual cycle halts, and systemic estrogens and progesterone decline substantially, while androgens become the predominantly circulating ovarian hormones for the remainder of the post-menopausal lifespan. Natural menopause is not an abrupt event, but rather a transitional stage that can last up to ten years before the final menstrual period. The menopause transition is marked by irregular fluctuations in circulating ovarian hormone levels and variable menses resultant from dysregulation of the normal female reproductive cycle (Harlow et al., 2013; Soules et al., 2001). Symptoms associated with the menopause transition include hot flashes, night sweats, vaginal dryness, dyspareunia, and alterations in mood, sleep, and cognition (Al-Safi & Santoro, 2014). Specifically, up to two-thirds of women report subjective changes in memory, especially within the domain of working memory (Im et al., 2019; K. N. Morgan, Derby, & Gleason, 2018; Unkenstein, Bryant, Judd, Ong, & Kinsella, 2016; Weber et al., 2014, 2013; Weber & Mapstone, 2009; Woods et al., 2000). These symptoms can negatively impact quality of life for women and warrant further investigation.

It is well known that steroid hormone receptors are present in many brain regions, including those important for learning and memory. The irregular fluctuations in ovarian hormone levels during the menopause transition likely cause dysregulation within these neural circuits essential for cognition. It is challenging, however, to determine whether the fluctuation and eventual decline in ovarian hormone levels result in transient changes to these systems, or if the menopause transition initiates reorganization in neural circuitry with consequential long-term changes to the synthesis and release of neurotransmitters necessary for normal learning and memory. Exploring possible relationships among known neural systems important for cognition and serum hormone levels is one way to further understanding of the variables at play associated with cognitive changes during the menopause transition and aging in general.

One of the neurotransmitters that can be altered by aging and ovarian hormone levels is acetylcholine. Among acetylcholine's diverse roles in the brain and periphery, this neurotransmitter is intricately linked to learning and memory. The basal forebrain is the primary synthesis site for acetylcholine in the mammalian brain, and long-range cholinergic projections exist from the medial septum and vertical/diagonal bands of the basal forebrain to the hippocampus and cortical regions, including the frontal cortex. Cholinergic neurons in the basal forebrain produce choline acetyltransferase (ChAT), the enzyme necessary for acetylcholine synthesis. Impairments in the cholinergic neuron function and loss of these neurons can occur with aging and neurodegenerative disease. Estrogens have also been heavily implicated in maintaining normal functioning of cholinergic neurons and activity (Gibbs, 2010; Luine, 1985). Furthermore, the cholinergic hypothesis suggests that the loss of estrogen with menopause at midlife could play a

crucial role in the degradation of the underlying neural circuitry, leading to subsequent cognitive decline, including the occurrence of neurodegenerative disease like Alzheimer's disease (Bañuelos et al., 2013; Gibbs, 1997, 1998, 2000c, 2000a, 2000b; Gibbs & Aggarwal, 1998).

Cholinergic neurons from the basal forebrain are the primary cholinergic input into the hippocampal formation; they project to many subtypes of hippocampal neurons, including inhibitory interneurons (Pelkey et al., 2017). The hippocampal formation includes several key structures beyond the hippocampus itself, including the surrounding entorhinal and perirhinal cortices, which act as gateways or hubs for information transfer related to temporal and object recognition components of memory, respectively (Kitamura, MacDonald, & Tonegawa, 2015; Suzuki & Naya, 2014). Receptors for acetylcholine can be found pre- and post- synaptically on hippocampal γ-aminobutyric acid-(GABA)ergic interneurons (Pelkey et al., 2017). Although GABAergic interneurons make up a small subpopulation of the total neural population in the hippocampus (10-15%), they play a specific and critical role in regulating many critical brain functions and have a central role in regulating memory formation (Kalueff & Nutt, 1997; Katz & Liebler, 1978; Pelkey et al., 2017). Glutamic acid decarboxylase (GAD) is the rate-limiting enzyme for GABA synthesis present in these neurons, and has two distinct isoforms, GAD65 and GAD67, which are products of two independent genes (Soghomonian & Martin, 1998). Although GAD65 and GAD67 are generally co-expressed in GABAergic neurons and both work to synthesize the neurotransmitter GABA, each isoform has distinct biochemical features and thus the possibility of functionally different roles regarding synaptic transmission of GABA in the central

nervous system (Soghomonian & Martin, 1998). GAD67 has also been detected in excitatory granule cells in the rat hippocampus under normal conditions, further complicating the interpretation of the role of these isoforms in regulating neurotransmission (Sloviter et al., 1996). Like acetylcholine signaling, GABA signaling is altered with age (Shetty & Turner, 1998; Stanley & Shetty, 2004), and ovarian hormones have been implicated in regulating GAD65 and GAD67 production (McCarthy, Kaufman, Brooks, Pfaff, & Schwartz-Giblin, 1995; Wójtowicz & Mozrzymas, 2010). Furthermore, our laboratory has shown that the synthetic progestin medroxyprogesterone acetate (MPA) significantly decreased GAD65+GAD67 expression in the hippocampus and significantly increased GAD65+GAD67 expression in the entorhinal cortex of middle-aged Ovx rats compared to Ovx-Vehicle treated rats; Ovx rats treated with natural progesterone also tended to have lower GAD65+GAD67 in the hippocampus, and had significantly increased GAD65+GAD67 expression in the entorhinal cortex, compared to the Ovx-Vehicle group (Braden et al., 2010).

Cholinergic and GABAergic signaling are tightly linked to one another (Lawrence, 2008) and are each independently altered with age and ovarian hormone levels (Koebele & Bimonte-Nelson, 2017). In order to explore some of the complex relationships among cholinergic and GABAergic neurons in the context of menopause and aging, we investigated ChAT-immunoreactive (IR) neuron counts in the basal forebrain, as well as GAD65 and GAD67 protein expression in the hippocampal formation (including Right Dorsal Hippocampus, Entorhinal Cortex, Perirhinal Cortex, and Ventral CA1/2 Hippocampus) in the brains of early- to late- middle-aged ovary-intact rats treated with 4-vinylcyclohexene diepoxide (VCD), a rodent model of transitional

menopause, or Vehicle control. These rats were longitudinally tested on cognitive tasks, detailed in Chapter 2 of this dissertation and published in Koebele et al., 2017. The brains of the subjects used in that behavioral experiment were analyzed, and correlations were performed with previously published serum, ovarian follicle count, and behavioral data to provide insight into the complex relationships among aging, menopause, and neural circuits important for learning and memory, and to inform future research directions on neurobiological factors involved in altered learning and memory with hormone changes and age.

Methods

Subjects

Fifty-six female virgin Fischer-344 rats acquired from the National Institute on Aging colony at Harlan Laboratories (Indianapolis, IN) were utilized in this experiment. Rats were either Young (6 months, n = 28) or Middle-Aged (12 months, n = 28) upon arrival to the Arizona State University animal facility. Rats were pair-housed, provided with free access to food and water, and maintained on a 12-hour light/dark cycle for the entirety of the study. Half of the rats in each age cohort were assigned to Vehicle or VCD treatment to induce follicular depletion. Rats were periodically behaviorally tested on a series of water maze tasks testing spatial working and memory (for detailed procedures, see Chapter 2) across six months in order to encompass the menopause transition period in the VCD-treated rats. A subset of rats in each age and treatment group (n=3/group) were randomly selected for a subset sacrifice about half way through follicular depletion to obtain a snapshot of circulating serum hormone levels, ovarian follicle counts, and

corresponding brain changes at this time point. All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards. For the purpose of the data presented here, only rats included in the behavioral analyses of the published data were included in the brain analyses and correlations present in this chapter.

Tissue Collection

At the subset sacrifice and at the end of the experiment, rats were heavily anesthetized using inhaled isoflurane anesthesia. Following cardiocentesis, brains were rapidly removed. The anterior portion of the brain encompassing the basal forebrain was blocked and fixed in 4% paraformaldehyde for 48 hours. Then, post-fixed brain tissues were transferred to 0.1 M phosphate buffered solution until histological analysis. The posterior right hemisphere of the brain was rapidly raw dissected for the dorsal hippocampus, entorhinal cortex, perirhinal cortex, and ventral CA1/2 hippocampus. Wet weight was obtained for each region and then immediately frozen at -70°C until analysis.

Immunohistochemistry

All brains from the subset and end sacrifices were processed for immunohistochemistry. Two to three days prior to sectioning, post-fixed brains were transferred to a 30% sucrose solution until fully saturated. Using a freezing microtome (Leica SM 2010R), 40 μm coronal sections were obtained throughout the basal forebrain (plates 12-18, Paxinos and Watson) and transferred to 0.1 M phosphate buffered saline (PBS, pH 7.4). Every third section was selected for choline acetyltransferase (ChAT)

immunohistochemistry and stereology. Tissue sections were washed in PBS, incubated for 15 minutes in 0.03% Triton (Triton100X) in PBS (PBST), blocked for 30 minutes at room temperature using horse serum in PBST, and incubated on a shaker overnight at 4°C with the primary antibody (1:1000 goat anti-ChAT, Sigma-Aldrich/Millipore #AB144P). The following day, sections were washed with PBS and incubated with the secondary antibody (1:200 donkey anti-goat IgG, JacksonImmuno Research, #705-065-147) for 45 minutes at room temperature. Following secondary incubation, tissues were washed in PBS and incubated in quenching solution for 30 minutes at room temperature. Again tissues were washed with PBS and then transferred to ABC solution (Vector Laboratories, #PK-6100) for 45 minutes at room temperature. Tissues were washed with PBS and transferred to DAB solution (Vector Laboratories, #SK-4100) for several minutes until color was optimal. Tissues were removed from the DAB and transferred to PBS solution. Tissue sections were then mounted on subbed slides and allowed to dry overnight in a fume hood. Once dry, tissues were dehydrated and cover-slipped using DPX (VWR, #100503-834). Slides were allowed to dry for at least 48 hours prior to cleaning in preparation for stereology. Experimental treatment groups were counterbalanced and equally represented across plates, and two controls were included for each staining run; one control did not receive primary antibody (resulting in no cellular staining) and the second control did not receive secondary antibody (resulting in a lack of DAB reaction).

Stereology

Unbiased stereology using the optical fractionator method in StereoInvestigator software (MBF Bioscience, Williston, VT) was completed to quantify ChAT-IR cells in the medial septum (MS) and vertical/diagonal bands (VDB) of the basal forebrain, as these regions innervate the hippocampus and frontal cortex. All subjects from the subset sacrifice (n=3/group) were counted; however, one Middle-Aged Vehicle subject from the subset sacrifice was excluded due to error in blocking the brain at sacrifice, and thus the whole basal forebrain was not collected. In addition, five subjects from each experimental group from the end sacrifice that were processed via immunohistochemistry were randomly selected for stereological analysis. One researcher who was blind to treatment groups completed all stereological cell counts. The optical fractionator method used allows for an accurate estimate of a particular cell population by uniformly randomly sampling within regions of interest and, using the known number of cells counted within the samples, mathematically estimates the total number of cells in that region (West, 2002, 2012; West, Slomianka, & Gundersen, 1991), as previously done in our laboratory (Engler-Chiurazzi et al., 2012; Mennenga, Gerson, et al., 2015).

Western Blot Protein Analysis

All brains from the subset and end sacrifices were processed for western blot protein analysis. Dorsal Hippocampus, Entorhinal Cortex, Perirhinal Cortex, and Ventral (CA1/2) Hippocampus from the right hemisphere of each brain were analyzed for GAD65 expression and GAD67 expression via Western blots. Frozen raw tissue samples were suspended in a 1:50 weight-to-volume 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 subsequently kept on ice at all times. Tissues were homogenized using a probe sonicator (Ultrasonic Processor, Cole Parmer, IL, USA), and then centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatant was collected, aliquoted, and frozen at 70°C until analysis. Protein concentration for each sample was determined using a bicinchoninic acid protein assay (Thermo-Fisher Scientific, Pittsburg, PA, USA).

Treatment groups from both sacrifice time points were counterbalanced and equally represented on each gel run. The NuPAGE PowerEase electrophoresis system was utilized for tissue processing. Samples for each region were loaded at an equal protein concentration and were run on a 4-12% NuPAGE Bis-Tris gel in an XCell SureLock Mini-Cell (Invitrogen, Carlsbad, CA, USA) using MOPS running buffer. After transferring to an Immobilon polyvinylidene difluoride membrane, the blot was blocked in 5% nonfat milk for one hour at room temperature. Following blocking, the membrane was washed in 1xTBST and then incubated overnight with primary antibodies for anti-GAD65 (1:5000; Abcam, ab26113), anti-GAD67 (1:10,000; Abcam 26116) and loading control primary antibody anti-beta-actin (1:20,000: Cell Signaling, #4970S) in 5% nonfat milk on a shaker at 4°C. The following day, the membrane was washed in 1xTBST and then incubated with secondary antibodies anti-mouse HRP (1:2000; Cell Signaling #7076S) for GAD65 and GAD67, and anti-rabbit HRP (1:2000; Cell Signaling #7074) for beta-actin for one hour at room temperature in 5% nonfat milk. The membrane was washed again, and developed using chemiluminescence (Lumiglo and peroxide, Cell

Signaling, #7003S) in a film developer (Konica SRX-101A Film Processor, Tokyo, Japan). Films were scanned in as JPEG files at 600 dpi. Densitometry analyses were completed using ImageJ software (Gallo-Oller, Ordoñez, & Dotor, 2018). GAD65 and GAD67 levels were normalized to beta-actin levels on each gel run. A total of four gels per region were run.

Statistical Analyses

All statistical analyses were performed using Statview software. ANOVA was utilized to analyze densitometry data, with Age and Follicular Depletion as independent variables, and GAD65 expression, GAD67 expression, or ChAT-IR cell count estimates as the dependent variables. Pearson r correlations were completed between densitometry and stereological cell counts with ovarian hormone levels, ovarian follicle counts, and cognitive scores, which have been reported in a prior publication (Koebele et al., 2017) and are detailed in Chapter 2 of this dissertation. Rats that received VCD treatment beginning at 6 months of age were referred to as "Young" groups, and rats that received VCD treatment beginning at 12 months of age were referred to as "Middle-Aged" groups. Brain evaluations took place when the Young groups were 8-12 months old (late adulthood to early middle-age), and the Middle-Aged groups were 14-18 months old (middle-age to aged). All analyses were two-tailed and had an alpha level set to 0.05. The correlations presented here were exploratory in nature to investigate whether associations existed between the examined variables; therefore, corrections for multiple comparisons were not made (Michael Edwards, personal communication, June 4, 2019).

As noted above, only rats included in published behavioral analyses (Koebele et al., 2017) were included in brain evaluations. Due to a processing error during tissue homogenization, 11 subjects were excluded from the Dorsal Hippocampus GAD65 and GAD67 western blot analyses and correlations (7 subjects from the subset sacrifice and 4 subjects from the end sacrifice). One subject from the Middle-Aged Vehicle group was excluded from all 17β-estradiol (E2) analyses due to a significant outlying value for serum E2.

Results

GAD65 and GAD67 Western Blots

For rats in the end sacrifice group, there was a marginal effect of Follicular Depletion [$F_{(1,28)}$=3.21, p=0.08] for GAD65 expression in the Dorsal Hippocampus, such that VCD treatment tended to increase GAD65 expression compared to Vehicle-treated rats (Figure 22A). There was also a marginal effect of Follicular Depletion [$F_{(1,32)}$=3.84, p=0.06] for GAD67 expression in the Entorhinal Cortex, where VCD tended to decrease GAD67 expression compared to Vehicle-treated rats (Figure 22B). No differences for Follicular Depletion or Age were found for GAD65 or GAD67 expression in the Perirhinal Cortex or Ventral Hippocampus.

Basal Forebrain ChAT-IR Stereology

For the subset sacrifice only, there was a marginal main effect of Follicular Depletion [$F_{(1,9)}$=3.61, p=0.09], such that VCD-treated rats tended to have fewer ChAT-IR cells in the VDB compared to Vehicle-treated rats, collapsed across age. Given the

low number of subjects per group in the subset sacrifice (n=2-3/group), these findings should be interpreted with caution. There were no Follicular Depletion or Age differences in ChAT-IR cells for the end sacrifice in the VDB or MS.

Correlations

Because there were no significant differences for stereology or western blot protein analyses, subjects from the subset and end sacrifices were combined for correlation analyses. Subjects within each treatment group that were part of the subset sacrifice are denoted in graphs with a triangle symbol. Only significant correlations are presented in graph form.

Region-specific correlations between serum hormone levels and GAD65 and GAD67. Relationships between GAD65 expression and GAD67 expression were assessed with serum E2 (pg/mL), Progesterone (ng/mL), Androstenedione (ng/mL), and Estrone (pg/mL) levels. There was a marginal positive correlation between Estrone levels and GAD67 expression [r(9)=0.606, p=0.09] as well as with GAD65 expression [r(9)=0.600, p=0.09] in the Dorsal Hippocampus for Middle-Aged-Vehicle rats, where higher levels of Estrone tended to be associated with higher GAD67 expression and higher GAD65 expression in the Dorsal Hippocampus. The interclass correlation, which evaluated relationships collapsed across treatment and age, between Dorsal Hippocampus GAD67 expression and serum Estrone was also marginal [r(30)=0.353, p=0.06], where higher levels of Estrone tended to be associated with higher GAD67 expression in the Dorsal Hippocampus. There were no significant or marginal correlations between

GAD65 or GAD67 expression with E2, Androstenedione, or Progesterone in the Dorsal Hippocampus.

In the Entorhinal Cortex, GAD67 expression negatively correlated with serum Estrone for the Young-VCD group, such that increased Estrone levels were associated with lower GAD67 expression [$r(10)=-0.635$, $p<0.05$] (Figure 23A). However, there was a marginal positive interclass correlation between GAD67 levels in the Entorhinal Cortex and Estrone levels [$r(40)=-0.277$, $p=0.08$]. There were no significant correlations between GAD65 expression or GAD67 expression with E2, Androstenedione, or Progesterone in the Entorhinal Cortex.

Within the Ventral Hippocampus, there was a positive correlation between E2 and GAD65 expression [$r(12)=0.705$, $p<0.01$] as well as with GAD67 expression [$r(12)=0.763$, $p<0.01$] for Young-Vehicle treated rats, where higher serum E2 levels were associated with increased GAD65 and GAD67 expression in the Ventral Hippocampus (Figure 23B-C). There was also a marginal positive correlation between serum Estrone levels and GAD65 expression for the Middle-Aged VCD group in the Ventral Hippocampus [$r(11)=0.538$, $p=0.09$]. No significant or marginal correlations were found between GAD65 or GAD67 expression and Androstenedione or Progesterone in the Ventral Hippocampus.

There were no significant or marginal correlations between any of the measures serum hormone levels and GAD65 expression or GAD67 expression in the Perirhinal Cortex (data not shown).

Region-specific correlations between serum hormone levels and ChAT-IR cell counts. Regarding ChAT-IR cell counts, within the MS, there was a marginal positive correlation between serum E2 levels and MS ChAT-IR cell counts for the Young-VCD group, such that higher serum E2 levels tended to be associated with a greater number of ChAT-IR cells in the MS [$r(8)=0.672$, $p=0.07$]. There was a significant positive correlation between serum Androstenedione levels and MS ChAT-IR cell counts for the Middle-Aged VCD group [$r(7)=0.796$, $p<0.05$] (Figure 24) and a marginal positive correlation between serum Androstenedione levels and MS ChAT-IR cell counts for the Young-Vehicle group [$r(8)=0.671$, $p=0.06$], such that higher Androstenedione levels were associated with more ChAT-IR cells in the MS.

Within the VDB of the basal forebrain, there was a significant positive correlation between VDB ChAT-IR cell counts and Androstenedione levels [$r(8)=0.851$, $p<0.01$], Progesterone levels [$r(8)=0.869$, $p<0.01$], and Estrone levels [$r(7)=0.798$, $p<0.05$] for the Young-Vehicle group, such that higher VDB ChAT-IR cell counts were associated with higher levels of Androstenedione, Progesterone, and Estrone levels (Figure 25A-C). Interestingly, the associations between serum ovarian hormone levels and ChAT-IR cell counts were not present in the Young-VCD group, suggesting VCD alters the association between circulating serum hormone levels and cholinergic neurons in the VDB of the basal forebrain. There were no significant relationships among circulating ovarian hormone levels and ChAT-IR counts in the VDB for the middle-aged groups, regardless of follicular depletion status.

It is notable that there were positive correlations between the number of ChAT-IR cells in the MS and ChAT-IR cells in the VDB for Young-Vehicle [$r(8)=0.729$, $p<0.05$],

Young-VCD [r(8)=0.961), $p<0.0001$] (Figure 26A), and Vehicle-Middle-Aged [r(6)=0.975, $p<0.001$] groups. Although this association did not reach significance in the VCD-Middle-Aged group [r(8)=0.283, p=NS] (Figure 26B), the interclass correlation between ChAT-IR cells in the two regions of interest was significant [r(30)=0.824, $p<0.0001$].

Ovarian follicle counts correlate with ChAT-IR cell counts. There was a positive correlation between primordial ovarian follicles (considered a marker of ovarian reserve) and ChAT-IR cells in the VDB for the Young-Vehicle group [r(8)=0.903, $p<0.001$] (Figure 27); this association was not present in Young-VCD rats or Middle-Aged groups. For growing follicle correlations, there was a marginal positive correlation between primary follicles and VDB ChAT-IR cell counts [r(7)=0.720, p=0.07] and MS ChAT-IR cell counts [r(7)=0.713, p=0.07] for the VCD-Young group.

Correlations for spatial memory performance, ovarian hormone levels, and brain measures. Performance on the highest working memory load trial (Trial 4) on the last water radial-arm maze (WRAM) re-test completed before sacrifice as well as average swim distance to the platform on the last day of the Morris water maze (MM) were correlated with serum hormone levels, GAD65 expression, GAD67 expression, and ChAT-IR cell counts.

High working memory load measures in the WRAM. There was a significant negative correlation for the Young-VCD group between serum E2 levels and WRAM performance, where higher levels of serum E2 were associated with fewer errors on the highest working memory load trial of the WRAM [r(11)=-0.694, $p<0.05$] (Figure 28A).

There was a marginal positive correlation between Dorsal Hippocampus GAD67 expression and WRAM performance for the Young-VCD rats [r(10)=0.578, p=0.08], where higher GAD67 expression was associated with more errors on Trial 4, and a marginal negative correlation between Dorsal Hippocampus GAD67 expression and WRAM performance for the Young-Vehicle rats [r(10)=-0.574, p=0.08], where higher GAD67 expression was associated with fewer errors on Trial 4.

Morris water maze performance. There was a marginal positive correlation between GAD67 expression in the Entorhinal Cortex and average swim distance on the last day of MM [r(9)=0.601, p=0.09] and a significant correlation between GAD65 expression in the Entorhinal Cortex and average swim distance on the last day of MM [r(9)=0.764, p<0.01] (Figure 28B) for Young-Vehicle rats, where higher GAD67 expression tended to be associated with greater swim distance, and higher GAD65 expression was associated with a greater swim distance, and thus, poorer performance. A negative correlation was present for GAD67 expression in the Perirhinal Cortex and average swim distance on the last day of MM within the Young-Vehicle group [r(9)=-0.717, p<0.05] (Figure 28C), where higher GAD67 expression was associated with less average swim distance on the last day of MM. There were no significant correlations between ChAT-IR cell counts and behavioral outcomes.

Discussion

The menopause transition is a pivotal period that marks the end of the female reproductive life stage. The reproductive years are characterized by regular, cyclic fluctuations across the 28-day menstrual cycle in women. During the menopause

transition, however, these regular fluctuations become altered. Production and release of ovarian hormones becomes irregular and eventually declines to low, sometimes undetectable, levels. From early in life, the female brain is organized to respond to the presence of ovarian hormones; the brain and many peripheral body systems contain receptors for ovarian hormones and rely on their regular production for normal processes. The menopause transition and post-reproductive life stage can be thought of as a reorganizational event for the female, where the systems that utilize ovarian hormones must adapt to the post-menopausal hormone milieu (Koebele & Bimonte-Nelson, 2015).

Ovarian hormones play a role in neuromodulation and cognitive processes. There is evidence that the cholinergic system and estrogens have a synergistic influence on normal learning and memory. For example, lesions to basal forebrain cholinergic neurons prevent estrogen from benefiting acquisition of a spatial memory task (D. A. Johnson et al., 2002), and dose and duration of estrogen treatment have short-term and long-term effects on basal forebrain cholinergic function in Ovx rats (Gibbs, 1997). Basal forebrain cholinergic neurons project to the hippocampal formation and surrounding cortical areas, including GAD-producing hippocampal interneurons, which also have a crucial role in memory formation and maintenance (McQuail et al., 2015). Normal neural circuitry that promotes GABAergic signaling within the hippocampus and between the basal forebrain and hippocampus becomes disrupted with age and ovarian hormone loss (Bañuelos et al., 2013; Gibbs, 1998; McQuail et al., 2015) and can negatively impact cognitive outcomes. Thus, it is important to explore the complex relationships among ovarian hormones, cholinergic neurons in the basal forebrain, and GABAergic neurons in the hippocampus and surrounding structures to elucidate possible mechanisms underlying neural changes

in relation to menopause and aging. Until now, most pre-clinical evaluations have utilized an Ovx rodent model with or without ovarian hormone replacement to evaluate cognitive effects associated with menopause. The VCD model of transitional menopause allows us to better mimic the hormonal milieu during the transition to menopause, rather than the abrupt loss of ovarian hormones with the Ovx model (Koebele & Bimonte-Nelson, 2016). Additionally, few studies have explored interactions among these variables under endogenous conditions.

In this evaluation herein, we discovered that the relationships of serum ovarian hormone levels, including estrogens, progesterone, and androstenedione with the cholinergic system and GABAergic system appear to be dependent upon follicular depletion status and age. Many of the observed relationships were marginal; the reason for this is likely two-fold. First, with this longitudinal study design, some natural attrition of subjects occurred, and thus the analyses may have been underpowered for correlations. Second, the experiment was designed to evaluate basal stereological cell estimates of cholinergic neurons and GAD expression in relationship to endogenous circulating ovarian hormone levels in normally aging and VCD-treated rats to best model the normally aging female; as such, there was no acute treatment that could result in a drastic change in brain chemistry, and the longitudinal nature of this experiment lends itself to the brain's natural homeostatic processes of adaption to the hormonal milieu. However, some relationships were still evident, and provide insight into the complex relationships of ovarian hormones and basal forebrain-hippocampus circuitry in the context of learning and memory during normal aging. Although effects are subtle, these associations will

provide direction for designing future experiments to systematically evaluate, and potentially manipulate, these variables.

When collapsing across age, VCD-induced follicular depletion tended to decrease GAD65 expression in the Dorsal Hippocampus and increase GAD67 expression in the Entorhinal Cortex, two crucial areas for normal memory processing, compared to Vehicle controls. It is possible that altered hormone profiles associated with VCD-induced follicular depletion cause a disruption in normal GABAergic processes. Although these effects did not reach statistical significance, it is notable that the direction of these effects is opposite from previously reported findings in our laboratory using an Ovx rat model given progestin treatment. In the previous report, Ovx rats exhibited higher hippocampal GAD65+GAD67 expression in the hippocampus and lower GAD65+67 expression in the entorhinal cortex compared to Ovx + progesterone and Ovx + medroxyprogesterone acetate-treated groups (Braden et al., 2010). As such, it is important to acknowledge that the menopause model used in a given experiment may differentially affect brain areas important to learning and memory.

With regard to correlations, for the Young-VCD group, higher Estrone levels were associated with lower GAD67 expression in the Entorhinal Cortex. Our laboratory has previously shown that exogenous Estrone administration can impair spatial memory in middle-aged Ovx rats (Engler-Chiurazzi et al., 2012); the current findings point to the idea that higher Estrone levels are associated with disrupted GABAergic regulation in ovary-intact, follicular-deplete females as well. In Young-Vehicle rats, higher E2 levels were associated with higher GAD65 expression and GAD67 expression in the Ventral Hippocampus, suggesting that E2 may facilitate hippocampal GABAergic processes in

non-follicular-deplete middle-aged rats. Older, middle-aged rats (14-18 mo) did not exhibit correlations between serum ovarian hormone levels and GAD65 or GAD67 expression, suggesting that relationships may shift after a particular time point in middle-age.

We demonstrate that the Young-Vehicle group (8-12 mo) showed positive correlations between ChAT-IR cells in the VDB of the basal forebrain with Androstenedione, Progesterone, and Estrone serum levels, where higher circulating ovarian hormone levels were associated with an increased number of ChAT-IR neurons in the VDB. A positive correlation was also observed between the ovarian follicle reserve and ChAT-IR VDB neuron counts. These associations were not observed within the Young-VCD group. These observations indicate that the relationships among ovarian hormone levels and ChAT-IR counts in the basal forebrain are altered with follicular depletion status in this age range. We previously reported (see Chapter 2) that Progesterone levels were significantly lower in Young-VCD rats compared to their Vehicle-treated counterparts, and Estrone levels tended to be decreased as well (Koebele et al., 2017). It is possible that the endogenous hormonal milieu associated with follicular depletion may alter the relationships between ChAT-producing cells in the basal forebrain compared to the hormonal milieu of a normally aging rat. Furthermore this relationship was not present in older rats regardless of follicular depletion status, again indicating that this middle-aged time point may be a critical reorganizational period for rats undergoing a menopause-like transition. Although the traditional cholinergic hypothesis emphasizes the role of E2 in altered cholinergic function with age and hormone loss (Gibbs, 1998, 2000d, 2010), these associations reported herein extend the

potential role for ovarian hormones to interact with normal cholinergic system function, and indicate that premature follicular depletion alters the relationship between cholinergic neurons and ovarian hormone levels in the transitionally menopausal rat.

Lastly, brain measures were correlated with spatial working and reference memory performance, results of which are discussed in detail in Chapter 2. A significant negative correlation between E2 levels and errors on the highest working memory load trial of the water radial-arm maze, a task that measures spatial working and reference memory, was observed for the Young-VCD group, where higher serum E2 was associated with fewer total errors when working memory load was maximally taxed in follicle-deplete rats. These findings corroborate prior findings from our laboratory indicating that higher serum E2 levels from hormone therapy treatment were associated with enhanced performance on the MM in young and old Ovx rats (Talboom et al., 2008). Collectively, these findings provide support for the idea that E2, whether endogenous or exogenous in form, can benefit cognitive performance in the working and reference memory domains during the menopause transition when the hormonal milieu is changing during follicular depletion. Higher GAD65 expression in the Entorhinal Cortex was found to positively correlate with a greater swim distance to the platform on the final baseline testing day of the spatial reference memory MM for Young-Vehicle rats; however, higher GAD67 expression in the Perirhinal Cortex was associated with less swim distance to the platform in this group. These region- and GAD isoform- specific associations highlight the complexity of relationships between spatial memory, multiple memory-related neural circuits, and neurotransmitter systems working together to produce behavioral outcomes. Furthermore, this exploratory analysis provides insight for

systematic manipulation within these cortical regions and the GABAergic system to further parse out specific functions for these factors in cognition.

Overall, this exploratory correlational investigation highlights the multifaceted relationships among circulating serum hormone levels during aging and follicular-depletion with neural circuits important for spatial learning and memory, including the cholinergic system within the basal forebrain and the GABAergic system within the hippocampus and surrounding cortical areas. The reported associations between hormone levels and ChAT-IR neuron counts, as well as with GAD65 expression and GAD67 expression, afford valuable insight into how these systems are affected by follicular-depletion in the aging female, and point to middle-age as a critical time of influence for changing ovarian hormone levels for these circuits. The findings provide a foundation for understanding central factors in cognitive outcomes when investigating memory changes in the context of menopause and aging. Further, these results will aid in the development of novel experiments methodically evaluating these variables, and translationally, for developing ideal hormone therapy options for midlife women undergoing a natural transition to menopause.

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CHAPTER 4

Preliminary data from this experiment was presented at the Society for Neuroscience conference in 2015 and at the International Learning and Memory Conference at University of California Irvine in 2018.

MAZE COMPLEXITY AND COGNITIVE EXPERIENCE AFFECT MEMORY PERFORMANCE IN ESTROGEN-TREATED RATS

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

ABSTRACT

Aging and reproductive senescence in women have been linked to cognitive decline. Surgical menopause can induce or exacerbate menopause symptoms, including memory complaints. Estrogen-containing hormone therapy is commonly prescribed after surgical menopause to treat symptoms such as hot flashes, and may ameliorate reported memory changes. Clinical and preclinical research suggest that performing cognitive exercises are neuroprotective during aging. Cognitive task complexity and prior experience with cognitively-challenging tasks are important factors to consider when evaluating cognitive outcomes. Whether these outcomes interact with estrogen status is unknown. Using a rat model of surgical menopause, we systematically assessed whether the level of cognitive demand involved in performing maze tasks, and order of task presentation, impacted learning and memory when receiving Vehicle, Low-17β-estradiol (E2), or High-E2 treatment. Low-E2 treated rats with low cognitive demand experience made fewer high-working-memory-load errors during the latter half of the subsequent high cognitive demand task compared to Low-E2 treated rats that were naïve. Low-E2 treated rats with high cognitive demand experience made fewer errors while learning the low cognitive demand task compared to Low-E2 treated rats that were naïve. High-E2 treated rats with high cognitive demand experience had some learning enhancements for the low cognitive demand task compared to Naïve High-E2 treated rats; however, prior low cognitive demand experience did not impart benefits for the subsequent high demand task for this E2 treatment. Prior maze experience did not have a substantial effect on subsequent task performance for Vehicle treated rats regardless of task demand level during the first exposure to maze experience or final testing. Of the three treatment

regimens given mapped onto a surgically menopausal background, a lack of estrogen treatment yielded the least benefit from prior cognitive experience, the high-dose estrogen a variable benefit, and the low-dose estrogen the most benefit. Specifically, the low-dose estrogen treatment broadly enhanced learning and memory in rats that had prior experience on another task, regardless of task complexity level. These collective results highlight the importance of understanding how the complexity of cognitive experience impacts cognition, and indicate that estrogen milieu influences the impact of prior cognitive experience on cognition during aging.

Introduction

Aging can be associated with a normal decline in cognitive function. A diverse number of variables, including genetic, epigenetic, and lifestyle factors contribute to the extent and severity of cognitive changes in an aging individual. Collectively, these factors can impact whether an individual undergoes normal healthy brain aging, or develops pathological and cognitive changes associated with neurodegenerative disease and dementia. While women typically live longer than men, this does not account for the disproportionate number of women affected by the most common form of dementia, Alzheimer's disease (Alzheimer's Association, 2018; Riedel, Thompson, & Brinton, 2016). The significant decline in ovarian hormones all women experience by midlife with menopause is also associated with cognitive changes, especially those related to working memory and attention (Mitchell & Woods, 2001; Weber & Mapstone, 2009). In fact, up to two-thirds of women report subjective memory problems during the transition to menopause (Henderson, 2009; Weber et al., 2012). The mechanisms underlying these cognitive changes are an area of substantial interest and are still being investigated. It is now recognized that ovarian hormones, and estrogens in particular, impact a wide variety of peripheral body systems as well as the brain, and further exploration into the complex interplay between the endocrine system and cognition is necessary to elucidate a clearer understanding of how these factors work together to impact brain health outcomes during midlife and beyond.

That estrogens are neuroprotective molecules remains a popular tenet that has received significant scientific support and attention in the past few decades. Basic science research utilizing animal models has shown that estrogen-containing hormone therapy

can enhance memory in surgically menopausal, or ovariectomized (Ovx), rodents (Bimonte & Denenberg, 1999; Heikkinen, Puoliväli, Liu, Rissanen, & Tanila, 2002; Heikkinen, Puoliväli, & Tanila, 2004; M. Singh et al., 1994; M. Wallace et al., 2006). The beneficial effects of exogenously administered estrogens are thought to be dependent upon a variety of factors, including dose, duration of treatment, administration route, and administration timing regarding time since ovarian hormone deprivation as well as age at onset of administration (Koebele & Bimonte-Nelson, 2015). The appropriate parameters for optimally efficacious estrogen treatment effects remain difficult to pinpoint in humans because menopause is retrospectively determined after one year without menses (Harlow et al., 2013). Initial findings from the Women's Health Initiative Memory Study (WHIMS) suggested initiating exogenous estrogen-containing hormone therapy (conjugated equine estrogens, CEE) given with or without the progestin medroxyprogesterone acetate (a synthetic form of progesterone) in post-menopausal women aged 65 and older could be detrimental to cognition and increased the relative risk of developing dementia (Coker et al., 2010; Espeland et al., 2004; Shumaker et al., 2003). However, more recent evaluations regarding variations in hormone therapy use have been underway, including: the Kronos Early Estrogen Prevention Study (KEEPS), the Early versus Late Intervention Trial with Estrogen (ELITE), the Women's Health Initiative Memory Study-Younger (WHIMS-Y), the Women's Health Initiative Study of Cognitive Aging (WHISCA), and the Study of Women's Health Across the Nation (SWAN). This research has been undertaken to better understand cognitive aging, the use of estrogen-containing hormone therapy in perimenopausal or menopausal women, and the impact hormone therapy has on the body and brain acutely and longitudinally

(Gleason et al., 2015; Greendale et al., 2009; Kantarci et al., 2016; Maki & Henderson, 2012; Vaughan et al., 2013; Wharton, Gleason, Miller, & Asthana, 2013). Indeed, there is evidence that initiating CEE-containing hormone therapy closer to menopause onset (age 50-54) does not result in cognitive impairments or benefits when tested 6-7 years after the initial WHIMS study was terminated (Espeland et al., 2017, 2013). Furthermore, initiating hormone therapy earlier in the transition to menopause has been reported to enhance verbal memory, and increase hippocampal activation during task performance compared to women who never used hormone therapy (Greendale et al., 2009; Maki et al., 2011). Thus, estrogen-containing hormone therapy has merit for its capacity to alleviate many unfavorable symptoms associated with menopause, including vasomotor symptoms as well as vaginal dryness and atrophy (Pinkerton et al., 2017), and the possibility to delay cognitive changes in aging women given the optimal parameters.

Even with the plethora of research indicating that estrogen can induce positive effects on the brain and its functioning, it is important to acknowledge that estrogen-containing hormone therapy is not the golden ticket to ensure healthy brain aging. In fact, currently the clinical recommendation is to *not* prescribe hormone therapy for the primary indication of memory complaints (Pinkerton et al., 2017). There are conflicting reports that exist in the scientific literature regarding factors that impact the efficacy of hormone therapy for cognition (for review, see: Koebele & Bimonte-Nelson, 2017; Korol & Pisani, 2015; Turgeon et al., 2006). Some lifestyle factors have been shown to impact cognitive aging outcomes, including diet, exercise, and cognitive practice. Both clinical and preclinical research suggest that performing complex cognitive tasks can be neuroprotective during aging. Basic science research indicates that neurogenesis occurs

throughout adulthood in many species (Kempermann, Hongjun, & Gage, 2015; Lim & Alvarez-Buylla, 2016; Paredes, Sorrells, Garcia-Verdugo, & Alvarez-Buylla, 2016), and that experience in complex environments can lead to enhanced dendritic complexity in rodents (Diamond, 2001; Green, Greenough, & Schlumpf, 1983), collectively implicating greater plasticity of the brain throughout the lifespan with enriching experiences. Several decades of research in humans also associates a higher educational attainment level with a decreased incidence of developing dementia (Crimmins et al., 2018; Mortimer & Graves, 1993; Schoenhofen Sharp & Gatz, 2011). Numerous reports in the literature indicate a significant relationship between education and dementia risk. It is important to acknowledge that there are many psychosocial and demographic factors that mediate and contribute to this association, including socioeconomic status, access to education and healthcare, nutrition, emotional wellbeing, and lifetime exposures to infections and other illnesses (Goveas et al., 2016; Schoenhofen Sharp & Gatz, 2011). Research from the Nun Studies have reported an increased incidence of dementia associated with lower education levels (Tyas et al., 2007), and a similar finding has been recently reported in a more heterogeneous population, representative of U.S. adults over age 50, as well (Crimmins et al., 2018). Indeed, higher educational attainment is associated with a greater life expectancy, as well as with good cognition while living longer lives (Crimmins & Saito, 2001; Crimmins et al., 2018). Whether these cognitive outcomes interact with estrogen status across the lifespan remains unknown and merits further investigation.

Because of the inability to methodically control for many lifestyle, sociodemographic, and hormone exposure factors, there can be limitations to research in humans when attempting to systematically investigate the specific contributions of each

of these factors on cognitive outcomes. Rat models aid in elucidating key factors associated with exogenous estrogen exposures in conjunction with life factors including cognitive practice and reserve, which we propose may be roughly equated to educational attainment measures. Rodents are often tested on a battery of maze tasks to evaluate different memory types (Bimonte-Nelson et al., 2015). The complexity of these tasks vary, and the order in which animals experience or learn these tasks could impact performance as well as the ability to shift task rule learning from one task to another. Indeed, our laboratory has shown that cognitive practice earlier in life benefits memory for the familiar task longitudinally for both sexes, and that females in particular have the capacity to transfer the benefits of earlier cognitive practice to novel tasks, even when they are aged (Talboom et al., 2014). As such, it is critical to evaluate whether the order in which mazes of varying complexity are presented contributes to cognitive outcomes, and if so, to acknowledge how prior maze experience might influence the interpretation of behavioral data.

The overarching goal of this experiment was to elucidate how prior cognitive demand experience alters subsequent learning and memory during aging, and whether 17β-estradiol (E2) treatment influences the effects of cognitive experience. We utilized two spatial memory tasks of varying cognitive demand and complexity. The delayed match-to-sample water maze task (DMS) is considered a low cognitive demand task that requires animals to learn the new location of a single hidden platform within a day; the platform location changes each day, thus requiring the animals to update that platform location daily. The water radial-arm maze task (WRAM) is considered a high cognitive demand task because it involves an increasing working memory load within a day, such

that animals have to remember several items of information in order to effectively solve the task each day. We tested whether prior low- and/or high- cognitive demand experience using spatial maze learning and memory altered learning and memory performance for subsequent tasks with or without tonic E2 treatment in middle-aged, surgically menopausal rats.

Methods

Subjects

Sixty female, virgin Fischer-344-CDF rats were obtained from the National Institute on Aging colony at Charles River Laboratories (Raleigh, NC, USA). Rats were 11 months old upon arrival. All animals were pair-housed, had free access to food and water, and were maintained on a 12-hour light/dark cycle for the entirety of the experiment. Rats were given two weeks to acclimate in the vivarium prior to the commencement of the experiment. All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards.

Ovariectomy and Hormone Replacement

Surgeries were completed in two waves. Ovx occurred two weeks after arrival to the facility. Under inhaled isoflurane anesthesia, dorsolateral incisions were made in the skin and peritoneum. The ovaries and tips of the uterine horns were exposed, ligated, and excised. Muscle and skin incisions were sutured closed with dissolvable Vicryl suture. All subjects received a subcutaneous injection of 5 mg/mL/kg Rimadyl (carprofen, a

nonsteroidal anti-inflammatory drug) for pain management and 2 mL sterile saline for post-surgical hydration. Rats were single-housed for a 48-hour recovery period before being re-pair-housed with a cage mate.

Two weeks after Ovx, Alzet osmotic pumps (2006 model with a constant release rate of 0.15 μl of solution per hour for six weeks) were implanted subcutaneously to deliver vehicle (polyethylene glycol; PEG) or E2 treatment. Rats were anesthetized using inhaled isoflurane anesthesia and a small incision was made in the skin of the scruff of the neck. A small pocket was made in the subcutaneous space and osmotic pumps were inserted. The skin incision was sutured closed with dissolvable Vicryl suture. Rats from each wave of surgery were randomly assigned to Vehicle (PEG), Low-E2 (2μg/day dissolved in PEG), or High-E2 (4μg/day dissolved in PEG) treatment groups. The E2 doses were based on previous studies in our laboratory that produced cognitive-behavioral effects of exogenous estrogen treatment when administered via osmotic pumps (Engler-Chiurazzi et al., 2011, 2012; unpublished observations). Rats were given a 48-hour recovery period before being re-pair-housed with their cage mate until sacrifice.

Rats were randomly assigned to the high cognitive demand WRAM-first condition (Vehicle n = 10, Low-E2 = 10, High-E2 = 9) or the low cognitive demand DMS-first condition (Vehicle n = 11, Low-E2 = 10, High-E2 = 10).

Vaginal Cytology

Vaginal smears were obtained one day prior to osmotic pump insertion to confirm that Ovx was successful. One day of smears was also completed one week after pump

insertion to verify successful vehicle treatment or hormone replacement in the estrogen-treated animals. Smears were classified as proestrus- , estrus- , metestrus- , or diestrus-like smears (Goldman et al., 2007; Koebele and Bimonte-Nelson, 2016). Prior to osmotic pump insertion, all rats exhibited diestrus-like smears characterized by leukocytes but with more scarcity of cells compared to an ovary-intact diestrus smear, indicating successful ovary removal. As anticipated after pump insertion, estrogen-treated rats exhibited estrus-like smears containing cornified cells, while vehicle-treated animals continued to display diestrus or blank vaginal smears.

Behavior Testing

Two weeks after osmotic pump surgeries, subjects began behavioral testing with either WRAM or DMS water mazes. Half of the rats were assigned to learn the high cognitive demand WRAM first, followed by the low cognitive demand DMS second. The other half were assigned to learn the low cognitive demand DMS first, followed by the high cognitive demand WRAM second. Rats were 12 months old at the start of behavior testing.

Water radial-arm maze. The win-shift 8-arm WRAM was a water escape task that measured spatial working and reference memory (Bimonte-Nelson et al., 2015, 2004; Bimonte-Nelson, Singleton, Hunter, et al., 2003; Bimonte & Denenberg, 1999; Bimonte, Granholm, Seo, & Isacson, 2002; Bimonte et al., 2000, 2003). The WRAM was constructed with 8 evenly-spaced and equally-sized arms radiating out from the circular center (each arm 39.37 cm x 13.97 cm). Salient spatial cues were placed on the walls and around the room to aid in spatial navigation. The maze was filled with room temperature

water (maintained at 18-20°C) and made opaque with black non-toxic powdered paint. Each rat was assigned a pre-determined set of platform location combinations such that four of the eight arms contained a hidden escape platform at the end of the arm, submerged 2-3 cm beneath the water's surface. Platform location combinations remained constant across days for each rat, but varied amongst rats. Each subject received four trials per day for 14 consecutive days. The first 12 days were considered baseline testing. At the beginning of each testing session, the rat was placed in the non-platformed start arm of the maze and was given a maximum of 3 minutes to locate a platform on each trial. If the rat did not find the platform within the allotted 3-minute time limit, the experimenter guided the rat to the nearest platform. Once a platform was located, the rat remained on it for 15 seconds before being returned to its heated testing cage for a 30 second inter-trial-interval (ITI). During the ITI, the experimenter removed the just-found platform from the maze (not replaced with a daily testing session), removed any debris, and stirred the water to distribute potential olfactory cues. Following 12 days of baseline testing, on days 13 and 14, a four-hour delay was implemented between trials two and three to evaluate memory retention after a delay. Arm entry errors were recorded for each trial on the testing sheet by the experimenter, defined as the tip of the rat's snout passing a line 11 cm into the arm (visible to the experimenter outside of the maze, but not visible to the rat). Three error types were recorded. Working memory correct (WMC) errors were entries into an arm that previously contained a platform within a day (trials 2-4). Reference memory (RM) errors were first entries into an arm that never contained a platform. Working memory incorrect (WMI) errors were subsequent entries within a day into an arm that never contained a platform. The WRAM is defined as a *high cognitive*

demand spatial working and reference memory task because the number of locations needed to be recalled increases as trials progress, thus progressively taxing working memory load capacity across trials each day.

Delayed match-to-sample water maze. The DMS was a water escape task that evaluated spatial working and recent memory (Bimonte-Nelson et al., 2015). The apparatus had 4 equally sized and spaced arms (each arm 39.37 cm x 13.97 cm), configured as a plus shape, radiating out from a circular center. Salient spatial cues were placed on the walls and around the room to aid spatial navigation. A single platform was submerged 2-3 cm below the surface of the water in 1 of the 4 arms. The platform remained in the same location within a day for all rats, but the platform location changed across days. With this protocol, rats must update their working memory to go to the newly-learned location within each day, thus breaking the association with the previous day's rewarded place in space. However, working memory was not increasingly taxed in DMS because there was only 1 item of information (i.e., the constant daily location of the one platform) to retain in a given testing session. Each rat experienced 6 trials per day for 10 days. The first 8 days were considered baseline testing. The first trial of each day was operationally defined as an information trial in which the rats learn the new platform location. Trial 2 was considered the working memory trial, and trials 3 through 6 were recent memory trials, where rats must retain information about the current platform escape location. Rats were dropped off in 1 of 2 alternating non-platformed start arms that were not directly across from the platform location, such that the rat must make a series of left and right turns to locate the platform. The order in which drop-off location points occurred was semi-random so that there was no consistent pattern of left and right

turns within a day or across days. Each trial had a maximum 90-second swim time before the experimenter led the rat to the platform. Once found, the rat remained on the platform for 15 seconds before being returned to its heated testing cage for a 30 second ITI, during which the experimenter removed any debris from the maze and stirred the water to obscure potential olfactory cues. After 8 days of baseline training was completed, a four-hour delay was implemented between trials 1 and 2 for days 9 and 10 to test delayed memory retention for the new platform location each day. An arm entry was recorded when the tip of rat's snout passed the 11 cm mark into a given arm. Total entry errors into non-platformed arms on each trial prior to locating the platform were calculated. The DMS is defined as a *low cognitive demand* spatial memory task because, while information needs to be updated across days (requiring working memory), the task does not involve an increasing working memory load component within or across days.

Body Weights

Body weights (grams) were recorded at baseline (i.e., prior to Ovx surgery), at pump insertion surgery (i.e., 2 weeks after Ovx), prior to behavior (i.e., 2 weeks after pump insertion), and at euthanization.

Euthanization

Rats were euthanized 1 day after the completion of their respective maze tasks, and were approximately 13 months of age at this time point. Subjects were deeply anesthetized using inhaled isoflurane anesthesia. After confirming successful Ovx, the uterine horns were removed from the body cavity, trimmed of excess fat, and wet weight

was collected for each subject. Osmotic pumps were removed from the dorsal subcutaneous space and inspected for pump integrity.

Statistical Analyses

All data analyses were completed using Statview statistical software. Separate analyses were completed for Vehicle, Low-E2, and High-E2 treatment groups. Repeated measures ANOVAs were utilized for WRAM and DMS data. The independent variable within each analysis was Low- or High- Demand Experience. For WRAM data analyses, "Low-Demand-Experience" refers to prior testing on the DMS before exposure to WRAM testing, and "Naïve" refers to the respective groups' first exposure to a maze task. For DMS data analyses, "High-Demand-Experience" refers to prior testing on the WRAM before exposure to DMS testing, and "Naïve" refers to the respective groups' first exposure to a maze task. The repeated measures were Trials nested within Days. The dependent variable was Errors; for WRAM, this included analyses for WMC, WMI, and RM errors, and for DMS this comprised a Total error analysis. ANOVAs were utilized to assess body weights and uterine weights, with Treatment as the independent variable and Body Weight or Uterine Weight as the dependent variable, respectively. All analyses were two-tailed and the alpha level was set at 0.05. Results were deemed marginal if the p value was between 0.05 and 0.10.

Results

Water Radial-Arm Maze

WRAM data were divided into two blocks: Days 1-6 were considered the Learning Phase of the task wherein the rats were learning the rules of the task, and Days 7-12 were considered the Asymptotic Phase of the task, wherein rats were performing to the best of their ability. These blocks were chosen based on the trajectory of the subjects' learning curves in this experiment; additionally, we have previously reported cognitive effects when WRAM data were evaluated using these blocks with rats of the same age (Koebele et al., 2017). Furthermore, this data blocking was the optimal way to indirectly compare WRAM and DMS data in this experiment. That is, in DMS, Day 1 was analyzed separately, whereby each rat had 6 exposures to the same platform location. Because WRAM requires that subjects find a combination of four platform locations per day, assessing performance on Days 1-6 for WRAM provides a comparable six-exposures to each platform location as was completed on Day 1 of DMS. Similarly, the latter half of testing (Days 7-12) was blocked after rats learned the rules of the task, similar to the Days 2-8 blocking structure in the DMS. There were no main effects or interactions of Low-Demand-Experience for the Vehicle treated rats, the Low-E2 treated rats, or the High-E2 treated rats for WMC, WMI, or RM errors during the Learning Phase of WRAM (Days 1-6, data not shown).

For the Asymptotic Phase (Days 7-12), there was a marginal main effect of Low-Demand-Experience for WMC for the Vehicle rats [$F_{(1,19)} = 3.23$, $p = 0.09$] (Figure 29A) and the High-E2 rats [$F_{(1,17)} = 3.69$, $p = 0.07$] (Figure 29C), whereby the animals with prior Low-Demand-Experience tended to make more errors than their naïve counterparts.

There was no main effect or interaction for WMC errors for the Low-E2 treated rats during the Asymptotic Phase collapsed across all trials (Figure 29B). Prior findings in our laboratory have shown that group differences often become evident on the WRAM when working memory load is highly taxed, during moderate and high working memory load trials. Therefore, we evaluated WMC errors on Trials 3 and 4 alone, the moderate and highest working memory load trials respectively, as done previously (Braden et al., 2015; Camp et al., 2012; Koebele et al., 2017, 2019; Mennenga, Gerson, et al., 2015; Prakapenka et al., 2018). There was no difference between Naïve rats and Low-Demand-Experience rats within the Vehicle treated rats for WMC errors on Trial 3 (Figure 29D). However, there was a main effect of Low-Demand-Experience for the Low-E2 treated rats on Trial 3 [$F_{(1,18)} = 10.69$, $p < 0.01$], whereby Low-Demand-Experience Low-E2 treated rats made fewer WMC errors than Naïve Low-E2 treated rats, indicating that Low-E2 treatment enhanced memory performance on the moderate working memory load trial, only when subjects had prior Low-Demand-Experience (Figure 29E). Interestingly, for the High-E2 treated rats, there was a marginal main effect of Low-Demand-Experience on Trial 3 [$F_{(1,17)} = 3.59$, $p = 0.08$]; Low-Demand-Experience High-E2 treated rats tended to make more WMC errors than Naïve High-E2 treated rats, such that High-E2 treatment tended to have an impairing effect on the moderate working memory load trial when subjects had Low-Demand-Experience, suggesting a dose-dependent effect of estrogen when prior experience is taken into account (Figure 29F). There were no main effects for Trial 4 alone, indicating the WRAM task was sufficiently difficult on the highest working memory load trial to impair all groups, an effect we have previously observed with middle-aged rats (Koebele et al., 2017). There were no main

effects or interactions across all trials for WMI errors, nor were there high-working memory load trial-specific effects for WMI. Likewise, there were no main effects of Low-Demand-Experience for RM errors in any treatment group.

On days 13 and 14 of WRAM testing, rats were given a four-hour delay between trials 2 and 3. Each treatment group was assessed separately to evaluate individual group performance on Trial 3 of day 12 (the last day of baseline WRAM testing) versus the average of Trial 3 following both four-hour delays (days 13 and 14). For WMC errors, there was a main effect of Delay Day for the Naïve Vehicle group [$F_{(9,1)} = 12.86$, $p < 0.01$], the Low-Demand-Experience Vehicle group [$F_{(10,1)} = 23.07$, $p < 0.001$], the Naïve Low-E2 group [$F_{(9,1)} = 7.11$, $p < 0.05$], the Low-Demand-Experience Low-E2 group [$F_{(9,1)} = 14.23$, $p < 0.01$], the Naïve High-E2 group [$F_{(8,1)} = 18.18$, $p < 0.01$], and the Low-Demand-Experience High-E2 group [$F_{(9,1)} = 16.20$, $p < 0.01$], such that all subjects, regardless of treatment or prior low cognitive demand maze experience, made more WMC errors on trial 3 after a four-hour delay in the WRAM (Figure 30A-F).

Delayed Match-to-Sample Water Maze

As noted above, Day 1 of DMS was analyzed separately so that we could evaluate the effect of High-Demand-Experience (that is, prior testing experience on the WRAM) on the first exposure to the DMS, a low cognitive demand working memory task, within each treatment group. For each treatment group, performance on the recent memory trials, Trials 3-6, were analyzed on Day 1, in order to assess performance after 2 exposures to reinforcement at the same location in space (i.e., the rat is removed from the water and placed into the heated testing cage as a result of locating the platform). For the

Vehicle treated rats, there was no difference between Naïve rats and High-Demand-Experience rats on Trials 3-6 [$F_{(1,19)} = 3.00$, p = NS] (Figure 31A). There was a main effect of High-Demand-Experience for Low-E2-treated rats [$F_{(1,18)} = 11.12$, $p < 0.01$] (Figure 31B) as well as for High-E2-treated rats [$F_{(1,17)} = 4.49$, $p = 0.049$] (Figure 31C), with Low- or High- E2 treated High-Demand-Experience rats making fewer errors than their Naïve counterparts on Trials 3-6 of Day 1 of DMS testing. It is notable that upon visualizing errors on each trial (Figure 31D-F), Vehicle treated rats with high demand experience did appear to perform better than Vehicle treated naïve rats on Trials 4-6. Therefore, we made a post-hoc decision to evaluate Trials 4-6 on Day 1 for the Vehicle treated group; there was a significant effect of High-Demand-Experience [$F_{(1,19)}$=4.65, p <0.05], where High-Demand-Experience Vehicle treated rats made fewer errors than Naïve Vehicle treated rats on Trials 4-6 (Figure 31G).

Days 2-8 of DMS were analyzed within each vehicle or hormone treated group for Trials 2-6 (working plus recent memory trials). Performance of Vehicle treated rats with High-Demand-Experience did not differ from Naïve Vehicle treated rats for the remainder of the baseline days, indicating that High-Demand-Experience did not impact performance in Ovx rats administered Vehicle treatment [$F_{(1,19)} = 0.55$, p = NS] (Figure 32A,D). There was a main effect of High-Demand-Experience for the Low-E2 treated rats [$F_{(1,18)} = 6.00$, $p < 0.05$]; a Fisher's PLSD post hoc test indicated that High-Demand-Experience rats treated with Low-E2 made fewer errors than Naïve rats treated with Low-E2 ($p < 0.05$, Figure 32B,E), suggesting that prior high demand experience enhanced performance on the DMS when Low-E2 treatment was administered. There was a marginal main effect of High-Demand-Experience for the High-E2 group [$F_{(1,17)} = 3.69$, p

= 0.07], where High-Demand-Experience rats treated with High-E2 tended to make fewer errors than Naïve rats treated with High-E2, suggesting a subtle enhancement in performance on the DMS for High-E2 treated rats with prior high demand cognitive experience (Figure 32C). Furthermore, there was a significant High-Demand-Experience x Trial interaction for the E2-High group [$F_{(4,68)} = 2.93$, $p < 0.05$] (Figure 32F), where the benefit of high demand experience appeared to be most prominent on Trial 2, when the platform location is being updated using working memory. Collectively, these results indicate that high demand cognitive experience was most beneficial to subsequent DMS performance when E2 was present after Ovx, particularly with the Low-E2 dose. Of note, we separately evaluated Trials 3-6 on for Days 2-8 to assess whether these effects were carried by Trial 2, the working memory trial. When Trial 2 was excluded from the analysis, the same effects remained for each group, concluding that the effects were not specifically carried by the working memory trial (data not shown).

On Days 9 and 10 of DMS testing, rats were given a four-hour delay between the information trial (Trial 1) and the working memory trial (Trial 2). Each treatment group was analyzed separately to assess performance on Trial 2 of day 8 (the last day of baseline testing) as compared to the average of trial 2 following both four-hour delays (days 9 and 10). There were no differences between baseline and delay days for the Naïve Vehicle group, the High-Demand-Experience Vehicle group, the Naïve Low-E2 group, or the High-Demand-Experience Low-E2 group (Figures 33A-D). However, there was a main effect of Delay Day for the Naïve High-E2 rats [$F_{(9,1)} = 11.25$, $p < 0.01$] and for the High-Demand-Experience High-E2 rats [$F_{(8,1)} = 10.67$, $p < 0.01$], such that number of total errors committed in the DMS task by High-E2 treated rats increased with this

elevated four-hour long temporal retention requirement; this impairment was apparent regardless of whether rats had prior high demand cognitive experience (Figure 33E-F).

Body Weights

Across time, there was a main effect of Measurement Time Point [$F_{(3,171)} = 83.38$, $p < 0.0001$], as well as a Measurement Time Point x Treatment interaction [$F_{(6,171)} = 30.04$, $p < 0.0001$]. At baseline, prior to Ovx surgery, there were no group differences in weight [$F_{(2,58)} = 0.18$, p = NS], nor were there group differences prior to pump insertion [$F_{(2,58)} = 0.35$, p = NS]. However, body weight increased in all rats between Ovx and Pump Insertion time points [$F_{(1, 58)} = 161.18$, $p < 0.0001$]. Prior to commencement of behavior testing, 2 weeks after pump insertion, there was a main effect of Treatment [$F_{(2,58)} = 9.72$, $p < 0.001$]; a Fisher's PLSD post hoc test revealed that the Low-E2 group ($p < 0.0001$) and the High-E2 group ($p < 0.0001$) weighed less than the Vehicle-treated group. Body weights in the Low-E2 and High-E2 groups did not differ from each other. At sacrifice, there was also a main effect of Treatment [$F_{(2,57)} = 9.93$, $p < 0.001$]; a Fisher's PLSD post hoc test revealed that the Low-E2 group ($p < 0.0001$) and the High-E2 group ($p < 0.0001$) weighed less than the Vehicle-treated group, but the estrogen-treated groups did not differ from each other (Figure 34).

Uterine Weights

Uterine wet weights were collected at sacrifice. As expected, there was a main effect of Treatment [$F_{(2,54)} = 26.28$, $p < 0.0001$]. Fisher's PLSD post hoc tests indicated that uteri from the Vehicle-treated group weighed significantly less than the Low-E2

group ($p < 0.0001$) and the High-E2 group ($p < 0.0001$) (Figure 35). Uterine weight did not differ between Low- and High- E2 treated groups.

Discussion

Cognitive practice is considered neuroprotective and can impart benefits for acquiring and remembering novel information. Whether estrogen milieu interacts with prior cognitive experience to influence outcomes is an area of substantial interest from both basic science and translational clinical perspectives. In the current study, we evaluated whether prior experience on a low- or high- cognitive demand maze task impacted the ability to learn a subsequent cognitive task, and whether these effects were influenced by circulating E2 milieu, during middle age. We utilized a model of surgical menopause and hormone therapy administration in middle-aged rats, evaluating whether E2 administration impacted learning and memory on a series of mazes. We used two mazes of varying complexity: the water radial-arm maze, a high cognitive demand task that tests spatial working and reference memory and requires the maintenance of an increasing working memory load, and the delayed match-to-sample water maze, a low cognitive demand task that tests spatial working memory without a memory load component. Overall, we found that prior experience on tasks of varying complexity can alter learning and/or memory for the subsequent maze task, and these effects appear to be moderated, at least in part, by exogenous E2 treatment.

Low Demand Experience Impacts Subsequent High Demand Working Memory Performance Only with Estrogen Supplementation

We report here that prior experience on a low complexity maze task does not impart benefits while subsequently *learning* a more cognitively demanding task, regardless of hormone milieu. Specifically, the experience of learning the low cognitive demand task, DMS, first did not alter performance during the learning phase of a high cognitive demand task, WRAM, regardless of Vehicle or E2 treatment. However, after experiencing the low cognitive demand task, the presence of Low-E2 did enhance *memory* performance for the more complex task after task rules had been learned. This is evidenced by the observation that during the asymptotic phase of the WRAM, the Low-E2 treated rats with prior low demand task experience made significantly fewer errors than the naïve Low-E2 treated rats when working memory load was taxed. When E2 was not present, prior low demand experience on the DMS somewhat impaired memory performance on the high demand task in control rats given vehicle. Specifically, Vehicle treated rats with prior low demand experience trended toward worse performance across all trials during the asymptotic phase of the WRAM compared to Vehicle treated rats naïve to the current task. However, when working memory load was taxed, both the Naïve-Vehicle rats and Low-Demand-Experience Vehicle rats performed similarly, indicating that prior low demand experience had no effect on WRAM performance for Ovx vehicle-treated rats when the current task became demanding. Of note, the High-E2 treated rats with prior low demand experience on the DMS also showed a trend toward impaired performance compared to the High-E2 treated rats that were naïve to the task when working memory load was taxed on the WRAM, indicating that low demand

experience had a somewhat negative impact on the subsequent ability to handle an increased working memory load for rats with a hormone milieu of high dose E2 supplementation. Collectively, these findings suggest that the presence of a low dose of E2 in the hormonal milieu of rats with prior experience on a low cognitive demand task can impart beneficial effects on a subsequent more complex task after the initial learning period of this subsequent task. It is notable that there may be a dose-dependent estrogen response for this enhanced spatial working memory performance on the high demand task, whereby too high of a dose of E2, perhaps resulting in supraphysiological circulating levels of the hormone, can impair cognitive performance, an effect that has been previously reported (Barha, Dalton, & Galea, 2010; Holmes et al., 2002; Korol & Pisani, 2015).

When delayed working memory retention was evaluated with a four-hour delay between trials 2 and 3 in the WRAM, all rats made more errors in the post-delay trials compared to baseline performance. These results suggest that the four-hour delay was sufficiently difficult to disrupt working memory in middle-aged Ovx rats, regardless of whether E2 was present in the hormonal milieu or whether rats had prior low cognitive demand task experience.

High Demand Experience Enhances Subsequent Low Demand Learning and Recent Memory Performance with Estrogen Supplementation

Here, we showed that prior experience on a cognitively challenging task can bestow beneficial effects for learning and memory on a subsequent low demand cognitive task, and that these performance benefits were particularly evident when E2 was present.

First, we evaluated Day 1 of DMS separately as a measure of learning for Naïve versus High-Demand-Experience rats within each hormone treatment group. An analysis of DMS performance on Day 1 only was completed because on this task the rats have 6 exposures to the single daily platform location across trials within a day; thus, daily platform location learning can be observed within a day. For Day 1, Trials 3-6 were evaluated, such that both the information trial and working memory trial were considered training. Rats treated with Low-E2 or with High-E2 with prior high demand experience on the WRAM performed significantly better than Low- and High- E2 treated rats naïve to the DMS task on Day 1. While High-Demand-Experience and Naïve Vehicle-treated rats did not differ in error scores when assessed on Trials 3-6, the pattern of performance across trials indicated that the Vehicle-treated groups did learn the rules of the task within the first day, and this was confirmed via statistical analysis. Indeed, an analysis of Trials 4-6 on Day 1 for the Vehicle-treated groups revealed enhanced performance for the Vehicle treated rats with high demand experience group compared to Vehicle treated rats that were naïve. It is notable that for High-Demand-Experience rats treated with low or high doses of E2, enhanced performance on the DMS was apparent at an earlier trial within the first training day than for Vehicle treated rats, suggesting that E2 treatment after Ovx may aid in acquiring new task rules faster, or after fewer exposures, than if circulating E2 is not present in measurable quantities, as in the Ovx-Vehicle rats. The beneficial effects of prior high demand experience in the Low-E2 treated rats continued to be present throughout the remainder of DMS testing, where Low-E2 treated rats with prior high demand task experience made fewer total errors across Days 2-8 of DMS than their naïve counterparts. The High-E2 treated rats with prior High-Demand-Experience

also tended to make fewer total errors than their naïve counterparts for Days 2-8, although this effect did not reach statistical significance. On the other hand, both the Naïve Vehicle treated rats and the High-Demand-Experience Vehicle treated rats performed similarly to each other across Days 2-8 of DMS, regardless of experience profile. Overall, rats that had prior experience on a high cognitive demand task and were also given E2 treatment, particularly at a low dose, exhibited enhanced learning and memory for a subsequent low cognitive demand task. When a four-hour memory retention delay was implemented between trials 1 and 2 on the low cognitive demand task, only the rats receiving High-E2-treatment — both naïve and those that had high cognitive demand task experience — made more errors, again suggesting that there are dose-dependent effects of E2 for the impact of prior cognitive experience on subsequent cognitive performance. Specifically, low E2 dose treatment yielded beneficial effects for learning and memory on a low cognitive demand task and intact memory retention following a delay when there was a background of prior high demand experience. On the other hand, high E2 dose treatment imparted learning and memory benefits on the low demand task, but impaired memory retention following a delay, even with a background of prior high demand experience. Overall, the benefits of prior cognitive task experience were most pronounced with low dose E2 administration; indeed, the low E2 dose treatment utilized in this experiment yielded the broadest beneficial cognitive effects for subsequent learning and memory performance on mazes of varying complexity.

Body and Uterine Weights

As expected, body weights decreased in E2-treated rats, regardless of test experience order, following E2 supplementation after Ovx, an effect consistently observed in our (Mennenga & Bimonte-Nelson, 2013; Prakapenka et al., 2018), and other (Diz-Chaves et al., 2012; Geary, Trace, McEwen, & Smith, 1994; McLaughlin et al., 2008) laboratories. Vehicle-treated rats gained weight following Ovx (Kakolewski, Cox, & Valenstein, 1968; Rogers, Li, Strissel, Obin, & Greenberg, 2009) and maintained a higher average weight than both E2-treated groups at sacrifice.

Uterine weights were increased in both the Low-E2 treated rats and the High-E2 treated rats compared to the Vehicle treated rats. This confirms that the currently utilized E2 treatment via Alzet osmotic pumps was successful through the duration of the study, as estrogen stimulation of uterine tissue results in growth and therefore increased weight, whereas Vehicle-treated rats without E2 experienced uterine atrophy following Ovx surgery (Engler-Chiurazzi et al., 2012; Prakapenka et al., 2018; Westerlind, Gibson, Malone, Evans, & Turner, 1998).

Conclusions

Estrogen-containing hormone therapy administration following surgical menopause in middle-age has been reported to have mixed effects in the literature, including beneficial, detrimental, or null effects for cognition in both animal and human studies. There are likely many factors contributing to these outcomes, including the dose, route of administration, timing of treatment initiation, and duration of hormone treatment. Hormone milieu does not impact cognitive outcomes in isolation, but undoubtedly

interacts with a number of environmental factors, including exposure to novel task learning and cognitive reserve capacity. This current study highlights variables related to these factors, including maze task complexity and maze order presentation, to consider when assessing the cognitive effects of E2 on learning and memory in rodent models. Many laboratories, including our own, implement a battery of tasks to evaluate various types of learning and memory, as well as other behaviors, such as measures of anxiety-like, depressive-like, and exploratory behaviors. While one resolution could be to counterbalance the order in which rats in each group experience the tasks, our findings indicate that, depending on the treatment administered, prior experience on maze tasks of varying complexity alters performance on subsequent tasks, and counterbalancing task order may therefore actually muddle treatment effects if task counterbalancing is implemented. Our laboratory has shown that for gonadally intact rats, prior experience on a simple cognitive task can confer benefits to learning future, novel tasks. Specifically, benefits of prior experience were especially notable for female rats compared to male rats, as the females with prior cognitive practice showed enhanced performance on a task assessing a different type of memory, even a year after the first behavioral experience (Talboom et al., 2014). In addition, this effect was not accounted for by the procedural components of testing, but was only noted when cognitive demand was utilized (Talboom et al., 2014). These prior findings, in conjunction with the current report, indicates that it is crucial to describe any previous experience animals may have on tasks prior to reporting experimental results, as previous cognitive experience can be a significant factor in performance on a given task.

Furthermore, the current experiment demonstrates a potential dose-dependent effect of E2 that interacts with prior maze experience, highlighting the importance of estrogen dose in the context of investigating cognition. Here, low and high doses of E2 were administered tonically via Alzet osmotic pumps, such that rats received a steady rate of E2 (0.15 μl of solution per hour, resulting in 2μg or 4μg of E2 per day for Low and High groups, respectively) and treatment was initiated 2 weeks after Ovx surgery and continued until the study's end. This regimen compliments the current standard recommendations for estrogen-containing menopausal hormone therapy in women, which is the lowest effective dose of estrogen possible, often in a non-oral administration route (such as a hormone-containing transdermal patch, vaginal applications, or intrauterine devices) to produce physiologically relevant circulating levels of E2 that concomitantly minimizes health risks (Pinkerton et al., 2017). It is also notable that the E2 used in the current study is considered a bioidentical hormone, rather than a synthetic substrate such as ethinyl estradiol, or a natural but non-bioidentical, non-human derived compound such as conjugated equine estrogens (CEE, tradename Premarin), which are commonly used in hormonal contraceptives and menopausal hormone therapy, respectively. The use of endogenously available E2, conferring beneficial effects herein, may yield different effects than CEE or ethinyl estradiol, which have been shown to have mixed cognitive outcomes (Acosta et al., 2010; Mennenga, Gerson, et al., 2015).

Collectively, this experiment found that prior cognitive experience of varying complexity can alter subsequent learning and memory prowess, and that the beneficial effects of prior cognitive experience are, in part, dependent upon the presence of circulating estrogen milieu when in a surgically menopausal aging background. These

results provide important insight into the role of estrogen and cognitive practice during aging. Our findings underscore that acknowledgement and careful consideration of task order are critical when testing a behavioral battery and when interpreting the often mixed results of basic science evaluations of hormone efficacy. As the behavioral endocrinology field moves forward with the goal to translate preclinical data outcomes to better the cognitive health of women during aging, it is crucial to consider these types of effects as putative mediating factors for determining the efficacy of estrogen treatments, rather than secondary nuances that arise from the idiosyncrasies of preclinical rodent behavioral testing. Taking such considerations into account when interpreting outcomes will yield clarity and help dictate new pathways to discovery of how factors such as enrichment, educational attainment, cognitive status, and cognitive reserve histories influence outcomes of hormone therapies and healthy brain aging in menopausal women.

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CHAPTER 5

The data presented in this chapter are currently unpublished observations. Preliminary data from this experiment were, in part, presented at the Society for Neuroscience conference in 2017.

EVALUATING THE COGNITIVE IMPACT OF DROSPIRENONE, A FOURTH-GENERATION PROGESTIN, INDEPENDENTLY AND IN COMBINATION WITH ETHINYL ESTRADIOL IN OVARIECTOMIZED ADULT RATS

Contribution: I was the graduate student principal investigator for this experiment. Undergraduate research assistant Mallori Poisson contributed significantly to this project, as this two-part experiment fulfilled her requirements for an undergraduate honor's thesis through Barrett, the Honors College at ASU, under the mentorship of Dr. Heather Bimonte-Nelson.

ABSTRACT

Oral contraceptives and hormone therapies administered to uterus-intact women require a progestogen component to prevent estrogen-induced uterine hyperplasia and reduce the risk of gynecological cancers. Because natural progesterone is not readily orally bioavailable, there are diverse classes of synthetic progestogens, called progestins, that can be used in these formulations as an alternative to natural progesterone. Although progestins have similar efficacy peripherally, prior research has found that many progestins have a negative impact on cognition and can reverse neuroprotective effects of estrogen. Give that most women need a progestin component with estrogen-containing oral contraceptives and hormone therapies, it is important to discover a progestin that does not detrimentally affect cognition. Here, we investigate drospirenone, a fourth-generation progestin derived from spironolactone. Currently, drospirenone is the progestin component in several FDA-approved oral contraceptives (e.g. Yaz®) and hormone therapies for menopausal women (e.g. Angeliq®). Unlike other progestins, drospirenone is comparatively similar in its pharmacological profile to natural progesterone, and, notably, has anti-androgenic and anti-mineralocorticoid properties without concomitant glucocorticoid activity, making this a unique progestin of interest to investigate for its effects on cognition, including spatial memory and anxiety-like behaviors. Study 1 assessed several doses of drospirenone in young adult, ovariectomized (Ovx) rats, and found that a moderate dose of drospirenone had a beneficial effect on spatial working memory. To investigate potential neural mechanisms underlying this observed effect, brains were probed for GAD65, GAD67, and IGF1-R expression in the hippocampus, entorhinal cortex, and perirhinal cortex using western blot. Study 2

investigated this optimal drospirenone dose with and without concomitant ethinyl estradiol (EE) treatment, the most common synthetic estrogen prescribed in oral contraceptives. Results suggest that the addition of EE to drospirenone administration reversed the beneficial effects of drospirenone on cognition. It is essential to better understand how these hormones work in concert with one another, and why they produce unique cognitive profiles when administered together compared to those observed when each hormone is administered separately.

Introduction

Most women use some form of contraception in their lifetime (Daniels & Abma, 2018). There has been a rise in the popularity of hormone-containing methods, including oral contraceptives, intrauterine devices, vaginal rings, and subcutaneous implants due to their reliability not only in preventing pregnancy, but also for their value in treating a range of other health-related indications such as endometriosis, acne, and premenstrual dysphoric disorder (PMDD; Dayal and Barnhart, 2001). It is currently estimated that 79.3% of women have used the oral contraceptive pill at some point during their life, and 13.9% of U.S. women ages 15-44 report current use of the pill between 2015 and 2017; this percentage increases to 19.5% for users in the 20-29 year old age range (Centers for Disease Control and Prevention, 2019). Likewise, some women undergoing the menopause transition opt to take hormone therapy to alleviate unwanted symptoms including hot flashes, dyspareunia, and vaginal dryness (Pinkerton et al., 2017). Although the reported rates of hormone therapy use have declined substantially in the last 15 years (Crawford et al., 2018; Sprague, Trentham-Dietz, & Cronin, 2012), it is important to understand the long-term effects hormone-containing contraceptives and menopausal hormone therapy have on health outcomes in women.

Women who have a uterus and utilize-estrogen containing formulations require a progestin component to prevent endometrial hyperplasia and alleviate gynecological cancer risk, making combined oral contraceptives (COC) the most popular form of oral contraceptive use (K. S. Hall & Trussell, 2012). Given the low oral bioavailability of natural 17β-estradiol and progesterone, synthetic forms of estrogen and progesterone are most often utilized in contraceptives and hormone therapies. Ethinyl estradiol (EE) is the

synthetic estrogen used in nearly all COC formulations. However, there is a wide range of synthetic forms of progesterone, called progestins. Progestins are derived from a variety of parent molecules structurally related to either testosterone (derivatives of 19-nortestosterone, called estranes and gonanes), or to progesterone (17α-hydroxyprogesterone [pregnanes]; 17α-hydroxynorprogesterone or 19-norprogesterone [norpregnanes]), thus resulting in different pharmacological and pharmacokinetic profiles, including variable affinities to receptors for progesterone, androgens, estrogens, as well as to glucocorticoids receptors and mineralocorticoid receptors (Schindler et al., 2003; Sitruk-Ware & Nath, 2010). Progestins are often referred to by generation (i.e., first through fourth), although these designations can be arbitrary and refer to when the drugs were introduced to the market, or to their structure or pharmacological actions (Apgar & Greenberg, 2000; Davtyan, 2012; Petitti, 2003). Although progestins have similar efficacy peripherally, prior research has found that many of the earlier generation progestins have a negative impact on cognition and can reverse neuroprotective effects of estrogen (Bimonte-Nelson et al., 2006, 2004; Braden et al., 2011, 2015, 2010; Chesler & Juraska, 2000; Harburger, Bennett, & Frick, 2007; Harburger, Saadi, & Frick, 2009; Lowry, Pardon, Yates, & Juraska, 2010; Rosario, Ramsden, & Pike, 2006), while others have beneficial or neutral effects when administered alone (Braden et al., 2016; Prakapenka et al., 2018). Given the prevalence of progestin use in COCs and hormone therapies, it is of critical importance to better understand how these synthetic hormones impact the brain and behavior beyond their prescribed use.

While most progestins on the market are chemically similar to testosterone and progesterone, the fourth-generation progestin drospirenone is derived from a novel

source: spironolactone, an anti-androgenic aldosterone antagonist (Archer, Ahrendt, & Drouin, 2015; Krattenmacher, 2000). This makes the molecular structure and function of drospirenone unique. Aldosterone is an adrenal-derived hormone that regulates water retention and blood pressure; thus, beyond drospirenone's capacity to bind to the progesterone receptor with high affinity and its molecular structure more similar to natural progesterone than other clinically-available progestins (Fuhrmann, Krattenmacher, Slater, & Fritzemeier, 1996; Muhn, Krattenmacher, Beier, Elger, & Schillinger, 1995), it may also better modulate fluid retention (i.e. bloating) that naturally occurs during the menstrual cycle (Bitzer & Paoletti, 2009; Fenton, Wellington, Moen, & Robinson, 2007; Foidart, 2005). Drospirenone possesses these spironolactone-derived anti-androgenic and anti-mineralocorticoid receptor properties without concomitant glucocorticoid receptor activity (Schindler et al., 2003). As such, drospirenone-containing COCs are the only COCs approved by the Food and Drug Administration to treat acne vulgaris and PMDD, a mood disorder different from premenstrual syndrome (PMS) that affects approximately 5% of women (Fenton et al., 2007; Hofmeister & Bodden, 2016). Furthermore, drospirenone-containing COCs are a popular choice for younger women, making up 17% of all COC prescriptions in 2010 (K. S. Hall & Trussell, 2012). Although drospirenone was reported to increase deep vein thrombosis, this finding has since been refuted, and its safety profile is similar to other clinically-used progestins (Larivée et al., 2016).

Ovarian hormones have a well-established impact on cognition. Little attention has been dedicated to methodically understanding drospirenone’s impact on spatial learning and memory and anxiety-like behaviors. Given drospirenone’s unique

pharmacological properties and potential for alleviating cognitive symptoms associated with PMDD, it is of significant interest to evaluate its effects on cognition alone and in combination with the synthetic estrogen EE. Our laboratory has demonstrated unique cognitive effects of progestins and estrogens depending on whether the drugs are administered alone or in combination with each other. Specifically, when levonorgestrel, a second-generation progestin that has null or beneficial effects on spatial working memory in rodent models when administered alone (Braden et al., 2016; Prakapenka et al., 2018), was paired with 17β-estradiol in middle-aged ovariectomized rats, its beneficial effect was attenuated compared to levonorgestrel alone (Prakapenka et al., 2018). Furthermore, the presence of progesterone and synthetic progestins can impact multiple neural systems to influence learning and memory processes. Our laboratory has reported that the synthetic progestin medroxyprogesterone acetate (MPA), a first-generation pregnane-derived progestin, had detrimental effects on spatial memory, even after drug cessation, when it was no longer detectable in blood serum (Braden et al., 2011, 2010). Furthermore, MPA impacted the GABAergic system, which is the primary inhibitory neurotransmitter system in the brain and a critical modulator of normal learning and memory. This was evidenced by altered GAD65+GAD67 expression in the hippocampus and surrounding cortical areas of Ovx rats treated with MPA (Braden et al., 2010), which could point to an underlying mechanism for the observed cognitive effects with MPA treatment. Another recent study showed that the norpregnane-derived progestin segesterone acetate increased expression of insulin-like growth factor-1 receptor (IGF1-R), which is known to be important for neurogenesis, in the frontal cortex of mice (S. Chen, Kumar, Mao, Sitruk-Ware, & Brinton, 2018). The frontal cortex is

heavily involved in working memory, and thus these recent findings implicating progestins in the neurogenesis process provides an interesting route to explore for understanding neural correlates of progestin-induced behavioral changes. Additionally, our laboratory showed that there are dose-dependent effects of EE on memory, where a higher dose impaired memory (Mennenga, Gerson, et al., 2015), so evaluating these commonly prescribed hormones is imperative to parse out cognitive effects of the drugs alone and in combination with one another.

Elucidating whether drospirenone may differentially impact cognition in contrast to earlier generation progestins is an important step toward discovering novel pharmacotherapies that can provide long-term health benefits, including neuroprotective properties. In order to assess the cognitive impact of drospirenone on cognition, two experiments were performed. Using adult, ovariectomized (Ovx) Fischer-344-CDF rats, Study 1 evaluated a range of doses of drospirenone to determine an optimal dosing regimen that impacted cognition. Study 2 incorporated the optimal dose determined from Study 1 and combined this dose with EE to investigate cumulative effects of the drugs on cognitive performance.

Methods

Subjects: Study 1

Forty three-month-old Fischer-344 (F344-CDF) rats were obtained from Charles-River Laboratories (Raleigh, NC). Upon arrival to the animal facility, all rats were pair-housed, provided with free access to food and water for the duration of the experiment, and were maintained on a 12-hour light/dark cycle for the entirety of the study.

Procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards.

Subjects: Study 2

Sixty three-month-old F344-CDF rats were obtained from Charles-River Laboratories (Raleigh, NC). Upon arrival to the animal facility, all rats were pair-housed, provided with free access to food and water for the duration of the experiment, and were maintained on a 12-hour light/dark cycle for the entirety of the study. Procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards.

Ovariectomy

Procedures were identical for Study 1 and Study 2. After 9-10 days of acclimation to vivarium, all rats underwent Ovx, or surgical removal of the ovaries. Ovx results in a "blank ovarian hormone slate" that allows us to evaluate the specific effect of the hormone treatment given without interactions with endogenously-circulating hormones. Rats were anesthetized via inhaled isoflurane anesthesia. Five mg/mL/kg of the NSAID Rimadyl (carprofen) was administered to prevent post-surgical pain. Following sterilization of the surgical area, all rats received bilateral dorsolateral incisions through the skin, muscle, and peritoneal cavity. Ovaries and tips of the uterine horns were ligated and excised on each side. Muscle was sutured with dissolvable Vicryl suture and a local anesthetic, bupivacaine (Marcaine; Pfizer Pharmaceutical, Hospira Inc., Lake Forest, IL) was applied topically to the incision site. Skin was sutured with dissolvable Vicryl suture.

All rats received 2 mL of sterile saline subcutaneously to prevent post-surgical dehydration.

Hormone Treatment: Study 1

Drospirenone will be abbreviated as DRSP in reference to treatment groups. Rats were randomly assigned to one of the following treatment groups (n=10/group): Vehicle (sesame oil, control), DRSP-Low (12.5 μg/day), DRSP-Medium (30 μg/day), or DRSP-High (300 μg/day). Forty-eight to 72 hours after Ovx surgery, daily subcutaneous treatment injections began. Each treatment was administered in a 0.1 mL volume in the scruff of the neck, and continued throughout the entirety of the experiment until euthanasia. Each treatment was dissolved into sesame oil and sonicated for 2.5 hours at 50°C. The DRSP-High dose was sonicated for an additional 2.5 hours at 50°C. Sonication of the drug vials continued weekly for 2 hours throughout the experiment to ensure that the drug stayed in solution. All treatments were stored at room temperature. The low dose was based on the most common dose prescribed to women in a COC (3 mg/day), adjusted for an average weight (250 g) Ovx rat. Clinically, the ratio of DRSP to EE in a typical COC is 100:1, so the medium dose reflected this ratio when considering the range of EE doses used in Study 2. The high dose was a replication of the dose used in a prior study outside of our laboratory (Muhn et al., 1995) that resulted in ovulation inhibition in ovary-intact adult rats.

Hormone Treatment: Study 2

Rats were randomly assigned to one of the following treatment groups (n=10/group): Vehicle (control, sesame oil); DRSP (30 µg/day); EE-Low (0.125 µg/day), EE-High (0.3 µg/day), EE-Low + DRSP (0.125 µg/day EE + 30 µg/day DRSP), or EE-High + DRSP (0.3 µg/day EE + 30 µg/day DRSP). All treatments were administered daily in 0.1 mL of sesame oil in the scruff of the neck beginning 2-3 days after Ovx and continued throughout the experiment until euthanasia. The EE doses utilized here were based on prior research from our laboratory; the Low-EE dose represented a typical dose of EE in a modern-day oral contraceptive (30-35 µg/day), and the High-EE dose represented the higher doses of EE prescribed in earlier generations of oral contraceptives (75-80 µg/day), each adjusted for rat body weight (Mennenga, Gerson, et al., 2015). The DRSP-Medium dose (30 µg/day) was chosen for use in Study 2 based on Study 1 results.

Vaginal Smears

Eighteen days after the first hormone injection, vaginal smears were performed for three consecutive days. Vaginal cytology was assessed to confirm successful Ovx and understand how DRSP treatment could impact vaginal epithelial cells. Cytology was defined based on specifications in Goldman et al., 2007 and Koebele and Bimonte-Nelson, 2016, where diestrus smears contained leukocytes with or without the presence of cornified cells, proestrus smears contained round, nucleated epithelial cells and cornified cells present in clusters, estrous was defined by the presence of cornified cells, and metestrus contained a combination of cornified cells, leukocytes, round cells, and

keratonized, needle-like cells (Goldman et al., 2007; Koebele & Bimonte-Nelson, 2016). Procedures were identical for Study 1 and Study 2.

Body Weights

Beginning at Ovx surgery, weekly weights (grams) were recorded for all rats in Study 1 and Study 2 until the end of the experiment.

Cognitive Behavioral Battery

One month after daily hormone treatment initiation, rats were administered a battery of cognitive tasks evaluating spatial working and reference memory, including the water radial-arm maze (WRAM) and Morris water maze (MM). Additionally, rats were tested on the open field task (OFT) to assess locomotor activity and anxiety-like behavior. Procedures were identical for Study 1 and Study 2.

Water Radial-Arm Maze. The water radial-arm maze was an eight arm apparatus used to test spatial working and reference memory in rodents, as previously described (Bimonte-Nelson et al., 2015; Bimonte & Denenberg, 1999; Bimonte et al., 2000). Working memory was defined as memory that required updating within a session. Reference memory was defined as memory that remained constant through the entirety of the task. Each arm was identical in size (39.37 cm long x 13.97 cm wide) and evenly spaced, radiating out from the circular center of the maze. Water was maintained at 18-20°C. Black non-toxic powdered paint was used to make the water opaque. Four out of the eight arms contained hidden platforms, 2 cm below the water's surface, at the beginning of each daily testing session. The specific locations of the platforms were

constant within a rat, but varied across rats and were counterbalanced across treatment groups. Salient spatial cues were placed on the walls around the testing room to assist the rats with learning to spatially navigate to the hidden platforms.

Rats underwent baseline WRAM testing for 12 consecutive days, with four trials administered per daily testing session (one for each hidden platform). Once the rat was placed in the non-platformed start arm, it had 3 minutes to locate a hidden platform. If the rat did not locate a platform in the maximum allotted time of 3 minutes, the experimenter led the rat to the nearest hidden platform. Once a platform was located, the rat was permitted to stay on it for 15 seconds, and then the experimenter removed the rat from the maze and placed it back into a heated testing cage for a 30 second inter-trial-interval (ITI). During those 30 seconds, the experimenter removed the just-found platform from the maze for the remainder of the daily testing session and gently stirred the water using a net to obscure potential olfactory cues and remove any debris from the water. The rat was placed back into the maze for the remaining three trials in an identical fashion. Following 12 days of baseline testing, a six-hour delay was implemented between trials 2 and 3 to test delayed memory retention. Cognitive performance on the WRAM was quantified by the number of non-platformed arm entries—called errors—committed prior to locating a platform on each trial. An arm entry was quantified when the rat's nose passed a designation mark 11 cm into the arm that was visible to the experimenter but not visible to the rat. Errors were defined in 1 of 3 categories: working memory correct (WMC) errors were entries into a previously platformed arm on trials 2-4, reference memory (RM) errors were entries into a never-platformed arm for the first time within a daily

testing session, and working memory incorrect (WMI) errors were defined as repeat entries into never-platformed arms within a daily testing session.

Morris Water Maze. The MM was a large round tub (diameter = 188 cm) filled with 18-20°C black-painted water used to assess spatial reference memory (Bimonte-Nelson et al., 2015; Morris, 2015; Morris et al., 1982). One hidden platform was submerged within the northeast quadrant of the maze, where it remained for all days and trials. Salient spatial cues were placed on the walls in the testing room to aid in spatial navigation. Each rat received four trials per day for five days. On each trial, a rat was dropped off from one of the four cardinal directions (north, east, south, and west). The order of drop-off locations was the same for all rats within a day, but changed across days. The maximum trial time was 60 seconds. If a rat did not locate the hidden platform within the maximum allotted time, the experimenter led the rat to the platform. Once the rat found the platform it remained on it for 15 seconds prior to being returned to its heated testing cage for an ITI of approximately 10 minutes. The rats' swim paths and latency to platform were recorded using Ethovision software (Noldus Instruments, Wageningen, The Netherlands). On the fifth testing day, an additional trial was implemented following the four baseline trials. During this probe trial, the platform was removed from the maze and the rats were allowed to swim freely for 60 seconds.

Open Field Task. The OFT measured locomotor activity and anxiety-like behavior. This task has been shown to be sensitive to the presence and absence of ovarian hormones (Hiroi & Neumaier, 2006). The OFT was a 100 cm x 100 cm x 30 cm black Plexiglas arena. Although some paradigms use a bright light in the center of the maze, this assay was completed in red light (i.e., darkness for rats), as rats with significant

anxiety-like phenotypes tend not to move at all if the center of the arena is lit. Rats were acclimated to the anteroom of the testing area for at least 30 minutes. Each rat was placed in the arena on the north wall. The experimenter quietly exited the room while the rat was allowed to explore the arena freely for a 10 minute trial. The rat was then placed back in its testing cage and removed from the room. The experimenter counted and removed any fecal boli from the area, cleaned the arena with water, and dried it with paper towel prior to the next subject's trial. Dependent variables assessed in the OFT were total distance moved, distance moved and time spent in the arena center, small center, and arena corners.

Euthanasia

One day following behavioral assay completion, all rats were deeply anesthetized with inhaled isoflurane anesthesia. Brains were removed and blocked posterior to the basal forebrain. This anterior portion was post-fixed in 4% paraformaldehyde for 48 hours and then transferred to 0.1 M phosphate buffered solution for storage. The dorsal hippocampus, entorhinal cortex, and perirhinal cortex of the right hemisphere were rapidly raw dissected, weighed, and frozen until western blot analysis. Ovx status was verified at necropsy and the uterine horns were removed from the body cavity, trimmed of visible fat, and wet weight was recorded. Procedures were identical for Study 1 and Study 2.

Western Blot Protein Analysis

Right hemisphere dorsal hippocampus, entorhinal cortex, and perirhinal cortex from Study 1 were analyzed for GAD65 expression, GAD67 expression, and IGF1-R expression via Western blots. Frozen raw tissue samples were suspended in a 1:25 weight-to-volume 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 subsequently kept on ice at all times. Tissues were homogenized using a probe sonicator (Ultrasonic Processor, Cole Parmer, IL, USA), and then centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatant was collected, aliquoted, and frozen at 70°C until analysis. Protein concentration for each sample was determined using a bicinchoninic acid protein assay (Thermo-Fisher Scientific, Pittsburg, PA, USA).

Treatment groups were counterbalanced and equally represented on each gel run. The NuPAGE PowerEase electrophoresis system was utilized for tissue processing. Samples for each region were loaded at an equal protein concentration and were run on a 4-12% NuPAGE Bis-Tris gel in an XCell SureLock Mini-Cell (Invitrogen, Carlsbad, CA, USA) using MOPS running buffer. After transferring to an Immobilon polyvinylidene difluoride membrane, the blot was blocked in 5% nonfat milk for one hour at room temperature. Following blocking, the membrane was washed in 1xTBST and then incubated overnight with primary antibodies for anti-GAD65 (1:5000; Abcam, ab26113), anti-GAD67 (1:10,000; Abcam 26116), IGF1-R (1:1000; Cell Signaling, 9750S) loading control primary antibody anti-beta-actin (1:20,000: Cell Signaling, 4970S) in 5% nonfat milk on a shaker at 4°C. The following day, the membrane was washed in 1xTBST and

then incubated with secondary antibodies anti-mouse HRP (1:2000; Cell Signaling #7076S) for GAD65 and GAD67, and anti-rabbit HRP (1:2000; Cell Signaling #7074) for beta-actin for one hour at room temperature in 5% nonfat milk. The membrane was washed again, and developed using chemiluminescence (Lumiglo and peroxide, Cell Signaling #7003S) in a film developer (Konica SRX-101A Film Processor, Tokyo, Japan). Films were scanned in as JPEG files at 600 dpi. Densitometry analyses were completed using ImageJ software (Gallo-Oller et al., 2018). GAD65, GAD67, and IGF1-R expression was normalized to beta-actin expression on each gel run. A total of 3 gels per region were run for Study 1.

Statistical Analyses

Behavior analyses were identical in Study 1 and Study 2. *A priori* two-group comparisons between each DRSP group and Vehicle were completed using repeated measures ANOVA for Study I. For Study II, each DRSP, EE, and DRSP+EE group was compared to Vehicle using two-group comparisons. Additionally, we planned to compare EE-Low and EE-High groups to each other to assess dose-dependent effects, as well as EE-Low, EE-High, and DRSP groups to the combinations of EE+DRSP to evaluate effects of the hormones alone versus in combination on spatial working memory. Alpha level was set to 0.05 and all comparisons were two-tailed.

WRAM data were separated into 3 separate blocks based on the learning curve. Day 1 was considered training and was excluded from the analysis. Block 1 (Days 2-5) was considered the Early Acquisition Phase, Block 2 (Days 6-9) was considered the Late Acquisition Phase, and Block 3 (Days 10-12) was considered the Asymptotic Phase.

WMC, RM, and WMI errors were analyzed separately for each of the blocks. Treatment group was the independent variable, and trials nested within days were the repeated measures. Prior research from our laboratory indicates that hormone treatment differences become evident when working memory load is taxed, so *a priori* analyses of Trial 3 alone and Trial 4 alone were completed, as previously done (Braden et al., 2010; Koebele et al., 2017, 2019; Mennenga, Gerson, et al., 2015; Prakapenka et al., 2018).

MM data were analyzed using repeated measures ANOVA, with Treatment as the independent variable and Swim Distance to Platform (cm) as the dependent variable for baseline trials within each planned comparison. Performance on the probe trial was analyzed separately for each treatment group with Quadrant (%NE, %SW) as the dependent variable.

OFT data were analyzed using ANOVA, with Treatment group as the independent variable, and Total Distance Moved (cm), Center Distance, Center Time, Small Center Distance, Small Center Time, Corner Distance, and Corner Time as dependent variables for each planned comparison.

Western blot protein analyses for Study 1 were completed using ANOVA, with Treatment as the independent variable and normalized GAD65, GAD67, and IGF1-R expression as the dependent variable for each planned comparison.

Body weight and uterine weight data were also analyzed using ANOVA, with Treatment as the independent variable and Weight (g) as a dependent variable for each planned comparison.

Results

Study 1: Water Radial-Arm Maze

Early Acquisition Phase. There was a main effect of Treatment for WMC errors on Trial 3 for each planned comparison (Vehicle versus DRSP-Low: $F_{(1,18)} = 5.69$, $p < 0.05$; Vehicle versus DRSP-Medium: $F_{(1,18)} = 10.50$, $p < 0.01$; and Vehicle versus DRSP-High: $F_{(1,18)} = 7.57$, $p < 0.05$), indicating that all DRSP-treated rats made fewer errors than Vehicle controls on the moderate working memory load trial (Figure 36A). A main of effect of Treatment was also present for WMI on Trial 3 for the Vehicle group compared to the DRSP-Low group ($F_{(1,18)} = 7.83$, $p < 0.05$) and compared to the DRSP-Medium ($F_{(1,18)} = 5.13$, $p < 0.05$) group, where DRSP-treated groups made fewer WMI errors compared to Vehicle-treated rats (Figure 36B). There were no effects of RM for the Early Acquisition Block.

Late Acquisition Phase. There were no main effects of Treatment during the Late Acquisition Phase for WMC, WMI, or RM errors.

Asymptotic Phase. There was a main effect of Treatment for WMI errors on Trial 4 for the Vehicle versus DRSP-Medium comparison [$F_{(1,18)}=4.47$, $p < 0.05$], such that the DRSP-Medium rats made fewer WMI errors on the maximum working memory load trial compared to the Vehicle treated rats (Figure 36C). There were no effects of RM in the Asymptotic Phase.

Delayed Memory Retention. WMC errors on Trial 3 from the last day of baseline testing (day 12) were compared to Trial 3 after the 6 hour delay (first post-delay trial) for each treatment group. Post-delay errors on Trial 3 were increased for Vehicle rats [$F_{(9,1)}=5.44$, $p < 0.05$] and DRSP-Low rats [$F_{(9,1)}=10.79$, $p < 0.01$] compared to the

previous day's performance (Figure 37A,B). DRSP-Medium rats [$F_{(9,1)}$=3.65, p = 0.09] and DRSP-High rats [$F_{(9,1)}$=4.37, p = 0.07] tended to make more WMC errors on Trial 3 after a delay, although these analyses did not reach statistical significance (Figure 37C,D).

Study 1: Morris Water Maze

Day 1 was considered a training day and was not included in the analyses. For Days 2-5, the Learning Curve can be seen in Figure 38A. There was a main effect of Treatment for the Vehicle versus DRSP-Medium comparison [$F_{(1,18)}$ = 8.03, p < 0.01], where DRSP-Medium rats swam less distance to the platform compared to Vehicle-treated rats (Figure 38C). Each treatment group was analyzed separately on the probe trial. There was a main effect of Quadrant for each group (Vehicle: [$F_{(9,1)}$ = 60.17, p < 0.0001]; DRSP-Low: [$F_{(9,1)}$ = 174.65, p < 0.0001]; DRSP-Medium: [$F_{(9,1)}$ = 78.85, p < 0.0001]; DRSP-High: [$F_{(9,1)}$ = 56.32, p < 0.0001]). All rats had a greater percent of total swim distance in the target quadrant that previously contained the platform compared to the opposite quadrant (Figure 38E).

Study 1: Open Field Task

There was no main effect of Treatment for Total Distance in any planned comparison, indicating that DRSP treatment did not impact overall locomotor activity. Although there were no significant effects of Treatment, the Vehicle versus DRSP-Medium comparison revealed that for Small Center Distance [$F_{(1,18)}$ = 3.59, p = 0.07] and Time [$F_{(1,18)}$ = 4.12, p = 0.06], a marker of anxiolytic behavior, there was a marginal main

effect of Treatment, where the DRSP-Medium-treated rats tended to have increased distance covered and time spent in the Small Center of the OFT compared to Vehicle-treated rats. There was also a marginal effect of Corner Time for the DRSP-High rats versus Vehicle rats [$F_{(1,18)} = 3.14$, $p = 0.09$], where DRSP-High rats tended to spend more time in the Corners than Vehicle rats.

Study 1: Peripheral Markers of Hormone Stimulation

Body Weight. There was no main effect of Treatment on body weight at sacrifice for any two-group comparison, indicating the drospirenone administration did not influence body weight after Ovx compared to Vehicle treatment in this experiment (Figure 39A).

Vaginal Smears. All vaginal smears were diestrus-like or blank, regardless of treatment group, indicating that daily drospirenone at any dose did not impact vaginal cytology following Ovx.

Uterine Weights. There were no main effects of Treatment for uterine weights for any two-group comparison, such that daily drospirenone administration at Low, Medium, or High doses did not alter uterine weight compared to Vehicle-treated rats (Figure 39B).

Study 1: Western Blot Protein Analysis

There were no main effects of Treatment present for GAD65 expression, GAD67 expression, or IGF1-R expression in the dorsal hippocampus, entorhinal cortex, or perirhinal cortex for any two-group comparison (Figure 40A-C).

Study 2: Water Radial-Arm Maze

Early Acquisition Phase. For the Vehicle versus DRSP comparison, there was a main effect of Treatment for WMI errors on Trial 3 [$F_{(1,18)} = 7.03$, $p < 0.05$] where DRSP-treated rats made fewer errors than Vehicle-treated counterparts, replicating findings from Study 1 (Figure 41A). DRSP alone versus combination treatment comparisons revealed that on Trial 3, DRSP+EE-Low rats [$F_{(1,18)} = 7.79$, $p < 0.05$] and DRSP+EE-High rats [$F_{(1,18)} = 10.23$, $p < 0.01$] each made more WMI errors than DRSP-treated rats, indicating that the addition of EE at either dose impaired working memory during the Early Acquisition block of testing (Figure 41A). No significant Treatment effects were detected for WMC errors or RM errors for the planned comparisons during the Early Acquisition Phase.

Late Acquisition Phase. For Trial 3, there was a main effect of Treatment for WMI errors between the EE-High versus EE-High+DRSP groups [$F_{(1,18)} = 5.65$, $p < 0.05$], where EE-High+DRSP made fewer WMI errors on Trial 3 compared to EE-High alone (Figure 41B). For Trial 4 (the maximum working memory load trial), there was a main effect of Treatment for WMC errors between the EE-Low versus EE-Low+DRSP groups, where the EE-Low+DRSP-treated rats made more WMC errors than the DRSP alone group [$F_{(1,18)} = 5.17$, $p < 0.05$; Figure 41C].

Asymptotic Phase. There were no main effects of Treatment during the Asymptotic Phase for WMC, WMI, or RM errors for any planned comparison.

Delayed Memory Retention. WMC errors on Trial 3 from the last day of baseline testing (day 12) were compared to Trial 3 after the 6 hour delay (first post-delay trial) for each treatment group. Post-delay errors on Trial 3 were increased for the EE-

Low group [$F_{(9,1)}$=19.29, $p < 0.01$; Figure 42B], the EE-High group [$F_{(9,1)}$=16.20, $p < 0.01$; Figure 42C], and the EE-Low+DRSP group [$F_{(9,1)}$=12.52, $p < 0.01$; Figure 42E] compared to the previous day's performance. The Vehicle group tended to make more WMC errors on Trial 3 after a delay, although these analyses did not reach statistical significance [$F_{(9,1)}$=4.97, $p = 0.05$; Figure 42A]. The DRSP group [$F_{(9,1)}$=0.43, p = NS; Figure 42D] and the EE-High+DRSP group [$F_{(9,1)}$=2.44, p = NS; Figure 42F] did not exhibit a significant increase in WMC errors on the post-delay trial compared to Trial 3 performance on the last day of baseline testing.

Study 2: Morris Water Maze

Day 1 was considered a training day and was excluded from the analysis. For Days 2-5, there were no main effects of Treatment for any two-group planned comparisons (Figure 43A).

Each treatment group was analyzed separately on the probe trial. There was a main effect of Quadrant for each group (Vehicle: [$F_{(9,1)} = 63.43$, $p < 0.0001$]; DRSP: [$F_{(9,1)} = 40.74$, $p < 0.0001$]; EE-Low: [$F_{(9,1)} = 191.53$, $p < 0.0001$]; EE-High: [$F_{(9,1)} = 76.74$, $p < 0.0001$]; EE-Low+DRSP: [$F_{(9,1)} = 92.29$, $p < 0.0001$]; EE-High+DRSP: [$F_{(9,1)} = 63.36$, $p < 0.0001$]). In other words, all rats had a greater percent of total swim distance in the target quadrant that previously contained the platform compared to the opposite quadrant (Figure 43B).

Study 2: Open Field Task

Two-group comparisons were completed for OFT data. Total Distance moved (cm) in the arena was a marker of locomotor activity (Figure 44A); main effects of Treatment were observed for the EE-Low versus EE-High comparison [$F_{(1,18)} = 10.77$, $p < 0.01$], with EE-High rats having a greater distance covered in the 10 minute trial. Additionally the DRSP versus EE-High+DRSP groups differed in Total Distance moved [$F_{(1,18)} = 4.96$, $p < 0.05$], where DRSP+EE-High rats covered a greater distance than DRSP-treated rats. Center Distance (cm), an indicator of anxiolytic behavior (Figure 44B), showed that a main effect of Treatment between the Vehicle group and the EE-High group [$F_{(1,18)} = 5.92$, $p < 0.05$], where EE-High rats covered more distance in the center of the arena compared to Vehicle treated rats. Additionally there was a main effect of Treatment for EE-Low versus EE-High groups [$F_{(1,18)} = 9.14$, $p < 0.01$], where EE-High-treated rats moved more in the Center. Small Center Distance (cm) was distance traveled in the immediate center of the arena, and can be an additional marker of anxiolytic behavior (Figure 44C). There was a main effect of Treatment for EE-Low versus EE-High groups [$F_{(1,18)} = 4.71$, $p < 0.05$], where EE-High treated rats traveled more distance in the Small Center, although this may be an artifact of this group covering overall greater distance across the 10-minute trial. Corner Distance analyses, a measure of anxiety-like behavior (Figure 44D), revealed a main effect of Treatment for the Vehicle versus EE-Low comparison [$F_{(1,18)} = 5.41$, $p < 0.05$], as well as for the Vehicle versus EE-Low + DRSP comparison [$F_{(1,18)} = 6.99$, $p < 0.05$], where the Vehicle group traveled more distance in the corner than the EE-Low group and the EE-Low+ DRSP group, respectively, for Corner Distance.

In addition to distance, time measures were evaluated for the OFT. For Time Spent in the Center (s; Figure 44E), there was a main effect of Treatment for the planned comparison between Vehicle versus EE-High groups $F_{(1,18)} = 7.02$, $p < 0.05$], EE-Low versus EE-High groups [$F_{(1,18)} = 8.40$, $p < 0.01$], and EE-High versus EE-High+DRSP [$F_{(1,18)} = 4.69$, $p < 0.05$] groups, where the EE-High group spent more time in the Center than each respective group. Small Center time did not differ for any comparison (Figure 44F). Corner Time measures of anxiety-like behavior were also evaluated (Figure 44G); there was a main effect of Treatment for the Vehicle versus EE-High comparison [$F_{(1,18)} = 9.05$, $p < 0.01$] and the Vehicle versus EE-Low+DRSP comparison [$F_{(1,18)} = 10.42$, $p < 0.01$], where Vehicle-treated rats spent more time in the corner in each case. There was also a main effect of Treatment for the EE-Low versus EE-Low+DRSP comparison [$F_{(1,18)} = 5.21$, $p < 0.05$], where EE-Low spent more time in the corner than rats receiving the combination of EE-Low+DRSP.

Study 2: Peripheral Markers of Hormone Stimulation

Body Weights. Two-group comparisons were completed for body weight at euthanasia (Figure 45A). The Vehicle group weighed more than the EE-Low group [$F_{(1,18)} = 36.26$, $p < 0.0001$], EE-High group [$F_{(1,18)} = 26.24$, $p < 0.0001$], EE-Low+DRSP group [$F_{(1,18)} = 40.02$, $p < 0.0001$], and EE-High+DRSP group [$F_{(1,18)} = 36.61$, $p < 0.0001$]. The Vehicle group tended to weigh less than the DRSP group [$F_{(1,18)} = 3.69$, $p = 0.07$], although this effect did not reach statistical significance. EE-Low and EE-High groups did not differ from one another. Additionally, the EE-Low group did not differ from the EE-Low+DRSP group, nor did the EE-High group differ from the EE-

High+DRSP group. However, the DRSP group weighed more than the EE-Low+DRSP group [$F_{(1,18)} = 53.94$, $p < 0.0001$] and the EE-High+DRSP group [$F_{(1,18)} = 51.22$, $p < 0.0001$]. Collectively, EE-treated groups, with and without concomitant DRSP administration, did not differ from one another, suggesting that EE administration decreased body weight in Ovx rats compared to Vehicle-treated Ovx rats, and DRSP treatment did not further alter body weight when combined with EE, at least at this time point and the doses given.

Vaginal Smears. The Vehicle group and the DRSP group exhibited blank or diestrus-like smears for all 3 days evaluated, indicating successful Ovx and a lack of stimulation from daily drospirenone treatment alone, replicating results from Study 1. For rats treated with EE-Low, EE-High, EE-Low+DRSP, and EE-High+DRSP, all animals exhibited cornified cells in vaginal smears across all days, indicating EE-induced stimulation of the vaginal epithelium.

Uterine Weights. Two-group comparisons were completed for uterine weights at euthanasia (Figure 45B). Wet uterine weight (g) was lower in the Vehicle group compared to the EE-Low group [$F_{(1,18)} = 246.14$, $p < 0.0001$], EE-High group [$F_{(1,18)} = 153.73$, $p < 0.0001$], EE-Low+DRSP group [$F_{(1,18)} = 351.73$, $p < 0.0001$], and EE-High+DRSP group [$F_{(1,18)} = 114.6$, $p < 0.0001$]. Uterine weights from the Vehicle group and DRSP group did not differ from one another, replicating findings from Study 1. The DRSP group also had significantly lower uterine weights compared to the EE-Low+DRSP group [$F_{(1,18)} = 386.84$, $p < 0.0001$] and the EE-High+DRSP group [$F_{(1,18)} = 118.16$, $p < 0.0001$]. Uterine weights from the EE-High group weighed more than the EE-Low group [$F_{(1,18)} = 7.60$, $p < 0.01$]. The EE-Low group did not differ from the EE-

Low+DRSP group, nor did the EE-High group differ from the EE-High+DRSP group. Overall this indicated that unopposed EE-High treatment significantly increased uterine weights, while drospirenone treatment did not have a significant influence on uterine weights compared to Vehicle or when combined with EE.

Discussion

Hormone containing contraceptives —including COCs— are commonly utilized by women of reproductive age for a variety of indications including not only contraceptive use, but also as management of other hormone-related conditions such as endometriosis, severe acne, and PMDD. Although the estrogen component, EE, is consistent across most preparations, the progestin component of COCs varies widely. Progestins in these formulations are derived from a variety of parent molecules that result in variable pharmacokinetic profiles and biological activity. Drospirenone, a fourth-generation progestin derived from spironolactone, has a unique pharmacological profile and is structurally more similar to natural progesterone than other earlier-generation progestins. Ovarian hormones are known modulators of the brain and cognition, and it is crucial to understand how this commonly-prescribed progestin influences learning and memory processes alone and in combination with EE, especially given that progestins evaluated in our laboratory and others often result in detrimental cognitive effects. Drospirenone's unique properties merited investigation into its potential impact on cognition.

In Study 1, a range of drospirenone doses were evaluated in a young adult, Ovx rat model to determine if there was a dose-dependent response to drospirenone for spatial

learning and memory compared to Vehicle-treated rats. We found that the Medium dose of drospirenone, which most closely models the typical ratio of drospirenone to EE used in COC formulations, had beneficial effects on spatial working memory when memory load was taxed compared to Vehicle-treated rats. The Low and High doses of drospirenone also showed benefits for working memory performance during the Early Acquisition Phase, but the Medium dose was the only dose of drospirenone that continued to exhibit benefits to spatial working memory in the Asymptotic Phase of the WRAM on the maximum working memory load trial. A six-hour delay on the WRAM significantly impaired working memory performance for Vehicle and DRSP-Low treated groups, but only marginally impaired DRSP-Medium and DRSP-High doses on the post-delay trial, suggesting the potential for a dose-dependent effect for delayed memory retention. In the MM, a test of spatial reference memory, again the DRSP-Medium dose showed benefits for reference memory performance compared to Vehicle-treated rats, such the DRSP-Medium group swam less distance, on average, to the platform than Vehicle-treated rats across days 2-5 of the MM. The probe trial indicated that each treatment group spatially localized to the quadrant where the platform was previously located. Although several proteins of interest associated with spatial learning and memory, including GAD65, GAD67, and IGF1-R were evaluated in the dorsal hippocampus as well as the entorhinal and perirhinal cortices, no differences in protein expression were revealed for any two-group comparison. It is possible that the behavioral effects observed in this experiment are regulated by different neural systems. Alternatively, the chronic nature of Ovx and daily hormone administration could have led to reorganizational processes in the systems of interest by the time brains were evaluated;

another possibility is that alterations to these neural systems are not evident at the level of protein expression, at least at the time point assessed. Evaluations for other proteins and signaling cascades known to change with ovarian hormone loss and replacement, as well as other brain regions involved in spatial working memory, should be investigated to establish a possible underlying neurobiological mechanism for the cognitive effects observed here.

In Study 2, the Medium dose of drospirenone was administered alone and in combination with two doses of EE. The ratios of drospirenone to EE reflected commonly prescribed doses of hormone in COCs. In the Early Acquisition phase of the WRAM, we replicated the finding that drospirenone enhanced spatial working memory when memory load was taxed compared to Vehicle-treated rats. DRSP-treated rats also had enhanced performance during the Early Acquisition Phase compared to both combinations of DRSP + EE, suggesting that concomitant EE administration attenuated the beneficial effects of drospirenone alone. This result supports and extends prior findings in our laboratory using another progestin, levonorgestrel, wherein the progestin was beneficial when administered alone, but resulted in spatial working memory impairments when administered in combination with the endogenous estrogen, 17β-estradiol, in middle-aged Ovx rats (Prakapenka et al., 2018). During Late Acquisition, the EE-High dose impaired working memory compared to EE-High+DRSP treatment, indicating a potential benefit of combined hormone treatment in relation to estrogen-only treatment, at least at a high dose. However, Low-EE treated rats made fewer working memory errors than combined Low-EE+DRSP treated rats during Late Acquisition, suggesting that working memory outcomes could be dependent on both dose and combination of hormones in this Ovx

model. A six-hour delay impaired working memory performance for EE-Low, EE-High, and EE-Low+DRSP groups. The Vehicle group trended toward a delay-induced impairment, while the DRSP group and the EE-High+DRSP group did not exhibit poorer performance following the delay. These findings points to a potential protective effect of drospirenone on delayed memory retention alone and in combination with EE-High treatment. There were no differences on the MM task in Study 2; each treatment group swam more distance in the previously-platformed quadrant versus the opposite quadrant during the probe trial, indicating that all subjects spatially localized to the platform location. Differences in the OFT were evident in Study II, with the EE-High group having overall increased locomotor activity compared to Vehicle and EE-Low groups. Differences between EE-High and other treatment groups for other measures in the OFT may have been due to an overall change in locomotor activity, making these findings difficult to interpret clearly in the context of anxiety-like behaviors. EE treatment also affected body weight, where all rats treated with EE, alone and in combination with drospirenone, weighed less than Vehicle-treated rats at euthanasia. In addition, the DRSP group weighed more than the EE-Low+DRSP and EE-High+DRSP groups, and tended to weigh more than the Vehicle group. This was surprising, given drospirenone's anti-mineralocorticoid receptor properties as it pertains to water retention and metabolic effects. However, it is important to note that all rats in this experiment were Ovx, and therefore it is possible that the metabolic effects of Ovx impacted body weight over and above any potential effect drospirenone could have on body weight. To this end, an evaluation of drospirenone administration on weight maintenance or gain in ovary-intact rats would be informative in the future. Uterine weight was increased for all EE-treated

rats at sacrifice, supporting the idea that EE stimulates uterine tissue growth, a finding we have previously reported (Mennenga, Gerson, et al., 2015). The dose of drospirenone given in combination with EE treatments did not attenuate uterine weight at sacrifice, suggesting the dose given was insufficient to counter EE-induced uterine stimulation in this Ovx model. This is a primary role of progestins in COCs, and thus different ratios of drospirenone to EE will be important to investigate in the future.

Overall, this series of experiments found that the fourth-generation progestin drospirenone had beneficial effects for spatial working memory performance in a young adult Ovx rat model. The Medium dose of drospirenone, which is a dose modeled after the ratio of drospirenone to EE prescribed in COCs, was consistently beneficial for working memory performance across early and late phases of the WRAM, as well as for reference memory in the MM in Study 1, compared to Vehicle-treated rats. When this dose of drospirenone was combined with low and high doses of EE in Study 2, the combination treatment attenuated the beneficial cognitive effects of drospirenone compared to drospirenone treatment alone during learning. Because drospirenone did not decrease uterine weights for the combination treatment groups, it is possible that a higher dose of drospirenone would be necessary to counteract the proliferative effects of EE, especially at the high estrogen dose. It is notable that our laboratory has previously reported that High-EE treatment impaired spatial working memory (Mennenga, Gerson, et al., 2015); again, a higher dose of drospirenone could be necessary to result in beneficial cognitive effects when combined with EE. Future investigations using an ovary-intact rat model with these combination therapies would be beneficial to understand how combination treatment impacts cognitive function in a normally-cycling

reproductive system, as the majority of women prescribed COCs have an intact uterus and ovaries. Additionally, further investigations into neurobiological mechanisms underlying these cognitive changes are necessary to aid our understanding of how different progestins affect spatial learning and memory. While drospirenone treatment alone has promise for beneficial cognitive effects, the search continues for an estrogen-progestin combination therapy that results in null or even beneficial cognitive outcomes. Discovering these optimal hormone combinations are necessary to provide information to the millions of women taking hormone-containing COCs and hormone therapy in order for individuals to have an informed understanding of the risks and benefits of utilizing hormone-containing drugs for contraceptive use and other hormone-related health conditions that impact quality of life.

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CHAPTER 6

The data presented in this chapter were published in Endocrinology *in 2019 and is titled:*

HYSTERECTOMY UNIQUELY IMPACTS SPATIAL MEMORY IN A RAT MODEL: A ROLE FOR THE NON-PREGNANT UTERUS IN COGNITIVE PROCESSES

Contribution: I was the graduate student principal investigator of this experiment under the mentorship of Dr. Heather Bimonte-Nelson. I primarily designed the experiment and developed the surgical procedure protocol with Dr. Bimonte-Nelson, Dr. Dale DeNardo, and the veterinary team at Arizona State University. Coauthors are thanked for their important contributions to data collection and preparation of the manuscript for submission.

ABSTRACT

Approximately one-third of women experience hysterectomy, or the surgical removal of the uterus, by age 60, with most surgeries occurring prior to the onset of natural menopause. The ovaries are retained in about half of these surgeries while, for the other half, hysterectomy occurs concurrently with oophorectomy. The dogma is that the non-pregnant uterus is dormant. There have been no preclinical assessments of surgical variations in menopause including hysterectomy, with and without ovarian conservation, on potential endocrine and cognitive changes. We present a novel rat model of Hysterectomy alongside Sham, Ovariectomy (Ovx), and Ovx-Hysterectomy groups to assess effects of surgical menopause variations. Rats without ovaries learned the working memory domain of a complex cognitive task faster than those with ovaries. Moreover, uterus removal alone had a unique detrimental impact on the ability to handle a high-demand working memory load. The addition of Ovx, that is, Ovx-Hysterectomy, prevented this hysterectomy-induced memory deficit. Performance did not differ amongst groups in reference memory-only tasks, suggesting that the working memory domain is particularly sensitive to variations in surgical menopause. Following uterus removal, ovarian histology and estrous cycle monitoring demonstrated that ovaries continued to function, and serum assays indicated altered ovarian hormone and gonadotropin profiles by two months post-surgery. These results underscore the critical need to further study the contribution of the uterus to the female phenotype, including effects of hysterectomy with and without ovarian conservation, on the trajectory of brain and endocrine aging to decipher the impact of common variations in gynecological surgery in women. Moreover, findings demonstrate that the nonpregnant uterus is not dormant, and indicate that there is

an ovarian-uterus-brain system that becomes interrupted when the reproductive tract has been disrupted, leading to alterations in brain functioning.

Introduction

One of the most common gynecological surgical interventions in women, second only to cesarean section, is hysterectomy, or the surgical removal of the uterus (Carlson et al., 1993; United States Center for Disease Control and Prevention, 2010). Approximately half of the women who undergo hysterectomy retain their ovaries. This approach is taken to alleviate adverse symptoms associated with benign uterine conditions prompting hysterectomy while maintaining the ovarian tissue, thus preventing an abrupt menopause onset and other deleterious health effects if the woman is of reproductive age at the time of surgery (Whiteman et al., 2008). Despite the number of hysterectomy surgeries declining in recent years, it is estimated that currently, by age 60, one-third of women have undergone hysterectomy, with most women undergoing hysterectomy before age 50 (J. D. Wright et al., 2013). Thus, the majority of hysterectomies are performed before the average onset of natural menopause, which typically occurs between ages 51-52 (Hoffman et al., 2012; NAMS, 2014). Some research in premenopausal women suggests that hysterectomy disrupts normal ovarian function and initiates ovarian failure earlier than transitionally menopausal women who maintain their uterus, potentially due to localized ovarian blood flow disruption (Chan, Ng, & Ho, 2005; Kaiser, Kusche, & Würz, 1989; Kritz-Silverstein, Goldani Von Muhlen, & Barrett-Connor, 2000; Moorman et al., 2011; Read, Edey, Hapeshi, & Foy, 2010), while others report little to no change in ovarian function following hysterectomy (Beavis, Brown, & Smith, 1969; Chalmers et al., 2002; Findley et al., 2013). Of note, the measures used to operationally define normal ovarian function versus ovarian failure are not always consistent. Within the primary operational definition of menopause—

amenorrhea for twelve consecutive months (Hoffman et al., 2012; NAMS, 2014) — women who undergo hysterectomy with ovarian conservation could be considered menopausal in clinical practice despite continued ovarian function following surgery. Thus, determining the onset of the menopause transition in women who have undergone hysterectomy can be challenging, and may be defined as the onset of menopausal symptoms (e.g., hot flashes) rather than menstrual irregularity (for discussion, see Chalmers et al., 2002), both of which may not occur simultaneously, or may not occur in a sequential fashion where one precedes the other, in reproductively-intact women (Wise et al., 1999). The timing and etiology of menopause may prove to be critical to aging outcomes, and for determining an optimal time point for initiating individualized hormone therapy intervention to obtain the most favorable quality of life outcomes.

Pre- and post- menopausal circulating reproductive hormone profiles are significantly different from one another. There is abundant evidence that endogenous ovarian hormones — particularly estrogens — have neuroprotective properties and beneficial effects on the health and functioning of several body systems, including cardiovascular, skin, bone, and urogenital systems (Hoffman et al., 2012). Disruptions to the reproductive hormone feedback loop via gynecological surgery likely have differential effects on the function of these systems depending on age and menopause status at the time of surgery, as well as whether or not the ovaries are retained. Both human and pre-clinical animal research indicate that an abrupt loss of circulating ovarian hormones from oophorectomy (the surgical removal of the ovaries; in non-human animals, termed ovariectomy [Ovx]) prior to natural reproductive senescence can be detrimental to many aspects of health, including cognition (Bimonte & Denenberg, 1999;

Farrag et al., 2002; Gibbs & Johnson, 2008; Nappi et al., 1999; Rocca et al., 2007, 2011, 2012, 2009; Talboom et al., 2008; M. Wallace et al., 2006). Animal model studies of surgical menopause via Ovx confirm a significant decrease in serum ovarian steroid hormones and an increase in gonadotropin levels following surgical ovary removal (Acosta et al., 2010; Gibbs & Johnson, 2008). When ovaries are conserved during hysterectomy, they have the potential to function normally following hysterectomy if the surgery occurs during the pre-menopausal years. Although there has been limited study of ovarian structure and function following hysterectomy in women, some reports indicate that hysterectomy with ovarian conservation does not result in similar drastic changes in circulating ovarian hormones and gonadotropins as occurs with oophorectomy, because the steroid-producing ovaries remain intact (Chalmers et al., 2002; Corson, Levison, Batzer, & Otis, 1981). Nonetheless, whether ovarian structure and function are altered following hysterectomy, particularly when the surgery occurs during reproductive years, has been understudied. There is evidence that transient post-hysterectomy steroid hormone changes can occur. Vuorento and colleagues assessed daily salivary progesterone levels pre- and post- operatively in pre-menopausal women who underwent hysterectomy with ovarian conservation, and reported that 39% of the women had decreased progesterone levels during the luteal phase in at least one menstrual cycle within six months of the surgery. Of these women, most irregular serum progesterone levels occurred one month after hysterectomy; the majority of these women returned to preoperative progesterone levels by six months post-surgery, suggesting that alterations in ovulatory function and steroidogenesis following hysterectomy could be transient (Vuorento, Mäenpää, & Huhtaniemi, 1992). Other clinical studies have reported

no changes in circulating ovarian hormone levels up to 21 years post-hysterectomy when the ovaries were conserved (Beavis et al., 1969; Souza, Fonseca, Izzo, Clauset, & Salvatore, 1986), or variable estrogen levels up to 10 years after hysterectomy which result in inconclusive evidence for altered hormone profiles following the surgical removal of the uterus alone (Kaiser et al., 1989). Collectively, there remains a lack of comprehensive, longitudinal assessments in the clinical literature to conclusively determine the short- or long- term impact of hysterectomy on ovarian structure and function.

The uterus and ovaries are part of an intricately connected system including the hypothalamus, pituitary, and female reproductive tract. Over the past few decades, it has become clear that this system —and the hormone milieu produced by it— influences domains reaching far beyond reproduction alone with actions on many body systems, including the brain (for review, see Dubal & Wise, 2002; Turgeon, Carr, Maki, Mendelsohn, & Wise, 2006). For example, research in animal models shows that experimental manipulations to the endogenous ovarian hormone milieu impacts cognition (Daniel, 2013; Frick, 2015; Koebele & Bimonte-Nelson, 2015; Korol & Pisani, 2015; Luine, 2014). While the steroid-producing ovaries have been the main focus of these and the majority of related basic science investigations, the uterus is also tightly linked to the hypothalamic-pituitary-ovarian axis loop and the potentially wide-reaching effects of this system. Indeed, despite the long-held dogma that the non-pregnant uterus is a dormant and "useless organ" (p. 294) (Navot & Williams, 1991), there is evidence that the uterus contains gonadotropin and steroid hormone receptors (Lessey et al., 1988; Reshef et al., 1990; Stilley et al., 2014), as well as direct sensory and autonomic innervation from the

central and peripheral nervous systems (Aguado & Ojeda, 1984; Dyer & Erickson, 1985; Gnanamanickam & Llewellyn-Smith, 2011), even in a non-pregnant state. Thus, it is biologically plausible, and in fact likely, that hysterectomy itself, with or without ovarian conservation, sufficiently alters the hypothalamic-pituitary-female reproductive tract system and, as a result, plays a role in altering the brain and cognition. Along these lines, several recent retrospective clinical studies have reported an increased relative risk of early-onset dementia for women who underwent hysterectomy compared to women with no history of hysterectomy, particularly when the surgery occurred prior to menopause onset (Phung et al., 2010; Rocca et al., 2007, 2012). In contrast, another group reported a small decrease in the relative risk for developing Alzheimer's disease associated with oophorectomy, hysterectomy, and oophorectomy with hysterectomy; notably, the majority of the women in this study were >51 at the time of surgery, and were therefore likely post-menopausal (Imtiaz et al., 2014), with studies collectively suggesting that menopause status at the time of gynecological surgery could impact cognitive outcomes. In order to elucidate the impact of variations in surgical menopause on the brain and cognition during aging, we must acknowledge and appreciate the complexity of this multifaceted system and include the examination of the influence of uterine tissues into experimental models.

Few studies have assessed hysterectomy in a pre-clinical animal model. A handful of endocrine studies using hysterectomy in a rodent model demonstrated that hysterectomy with ovarian conservation resulted in unique endocrine changes, such as increased ovarian aromatase activity, elevated follicle stimulating hormone (FSH) levels, and potentially accelerated ovarian failure via follicle atresia (Biró et al., 1984; Özdamar

et al., 2005; Tanaka et al., 1994; Tapisiz et al., 2008). Hysterectomy provides a novel model for investigating variations in surgical menopause in the context of studying cognition. Specifically, the additional factor of the presence or absence of uterine tissue provides an ancillary perspective to Ovx menopause models by more closely modeling the surgical procedures that occur most often in clinical practice. Indeed, it is not common practice to remove only the ovaries in women, as does the classic Ovx model used in basic science research.

The cognitive effects of hysterectomy with and without ovarian conservation have not yet been fully explored in a systematic experimental context. Given the large number of women who undergo variations in gynecological surgeries, it is essential to understand how ovarian morphology and function may be altered following hysterectomy, as well as to elucidate how hysterectomy with and without ovarian preservation relates to the trajectory of brain aging and cognitive decline. For a truly translational approach, it is crucial to establish and methodically assess a pre-clinical rodent model of the most common surgical practices performed in women to obtain a complete understanding of how these surgical manipulations affect endocrine as well as cognitive and brain aging. Additionally, using a rodent model allows for a controlled manipulation of the type of surgical procedures and the age at which they occur, which is one of the primary complexities in interpreting outcomes when evaluating these phenomena in humans. The rodent reproductive system is well-defined and is in many ways functionally similar to the human; as such, the rat provides an excellent pre-clinical model to investigate the impact of hysterectomy on the brain and cognition. Of note, the uterine body of the rat bifurcates into two uterine horns, which will collectively be referred to as the uterus

hereafter. We herein aimed to methodically investigate how hysterectomy in adulthood (i.e., prior to the onset of reproductive senescence), with and without ovarian conservation, impacts cognition using a rat model. Utilizing this novel surgical model of hysterectomy, we evaluated spatial learning and memory performance in adult rats that received variations in surgical menopause, including ovariectomy, hysterectomy, ovariectomy plus hysterectomy and sham operations (Figure 46). Our systematic experimental design also allowed for simultaneous evaluation of endocrine and ovarian profiles known to change following variations in gynecological surgery, including putative biomarkers of menopause. Specifically, we assessed serum hormone levels of ovarian-derived hormones and the gonadotropins and ovarian follicle morphology, as well as monitored estrous cyclicity and body weight changes, in order to gain a comprehensive understanding of the far-reaching impact variations in gynecological surgeries have on the body's reproductive anatomy, physiology, and function, and how these factors ultimately may lead to cognitive changes.

Methods

Subjects

Sixty virgin, reproductively-intact female Fischer-344-CDF rats were obtained from the National Institute on Aging colony at Charles Rivers Laboratories (Raleigh, NC, USA). Subjects arrived at the animal facility at 5 months of age, were provided food and water ad libitum, and were maintained on a 12-hour light/dark cycle for the duration of the study. Rats were given 1 week to acclimate to the vivarium prior to beginning the experiment. All procedures in this experiment were approved by the Arizona State

University Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards.

Surgical Procedures

Rats were randomly assigned to 1 of 4 treatment groups: Sham, Ovariectomy (Ovx), Hysterectomy, or Ovx plus Hysterectomy (Ovx-Hysterectomy). Surgeries were performed 1 week after arrival. All rats were anesthetized with inhaled isoflurane anesthesia and received 1.0 mg/kg meloxicam (NSAID), 1.2 mg/kg buprenorphine-SR-LAB, and 5.0 mL of an isotonic solution used to ensure post-surgical hydration. All subjects received a ventral midline incision through the skin and peritoneum. The sham surgery group received skin and peritoneum incisions only. In the Ovx group, both ovaries were exposed, ligated along with the oviducts and tips of the uterine horns, and excised. In the Hysterectomy group, each uterine horn was ligated and cut below the ovary and oviduct. The uterus was then separated from the adjacent fat, and the uterocervical junction was ligated and cut above the cervix, at the base of the uterine body. In the Ovx-Hysterectomy group, the ovaries and uterus were separated from the internal fat, and the uterocervical junction was ligated and cut above the cervix. All groups' muscle incisions were sutured with dissolvable Vicryl suture, and bupivacaine (Marcaine, Pfizer Pharmaceutical, Hospira Inc., Lake Forest, IL) was applied to the muscle incision prior to skin closure for all subjects. The skin incision was closed with surgical staples. Rats were allowed to recover under a heat lamp and were single-housed for 7 days following the surgery. Two rats died after surgery. As a result, 1 Hysterectomy rat and 1 Ovx rat were single-housed; the remaining subjects were pair-housed for the

entirety of the study. The final n's per group used in the statistical analyses are the following, unless otherwise noted: Sham n = 15, Hysterectomy n = 14, Ovx n = 14, and Ovx-Hysterectomy n = 15.

Weights and Vaginal Cytology

Baseline weights were recorded at surgery, and weekly weights were recorded until behavior testing began. Two weeks after surgery, daily vaginal smears were evaluated for eight consecutive days to monitor estrous cyclicity following surgery. Smears were classified according to Goldman et al. (Goldman et al., 2007) and Mennenga and Bimonte-Nelson (Mennenga & Bimonte-Nelson, 2015), as proestrus, estrus, metestrus, or diestrus phases. Proestrus was characterized by the presence of round epithelial cells and some cornified cells. Estrus was classified by the presence of primarily cornified cells. Metestrus included the presence of cornified cells, round cells, needle-like cells, and leukocytes. Diestrus was characterized by leukocytes with or without cornified cells.

Behavioral Testing

Figure 47 illustrates the timeline for the experiment.

Water radial-arm maze. Behavior testing began 6 weeks after surgery. The water radial-arm maze (WRAM) is a win-shift, 8-arm water escape task that tests spatial working and reference memory in rodents (Bimonte-Nelson et al., 2015, 2004; Bimonte-Nelson, Singleton, Hunter, et al., 2003; Bimonte & Denenberg, 1999; Bimonte et al., 2002, 2000, 2003) The maze had 8 evenly spaced arms radiating out from the circular

center (each arm 39.37 cm x 13.97 cm). Salient spatial cues were located around the room to assist in spatial navigation. Room temperature water (maintained at 18-20°C) was made opaque with black non-toxic powdered paint. Platforms were hidden in the ends of four of the eight arms, submerged just beneath the water surface. Each subject was assigned a unique set of platform locations, which remained constant for that animal across all days of testing. Platform combinations varied between subjects and treatment groups. Each subject had four trials per day. At the beginning of each trial, the rat was placed in the platform-free start arm of the maze and had 3 minutes to locate a platform. Once the rat climbed onto a platform, the rat remained on it for 15 seconds to localize to its spatial location before being returned to a heated testing cage for a 30-second inter-trial-interval (ITI). During the ITI, the experimenter removed the just-located platform from the WRAM. Once found, platforms were not replaced within a daily testing session. The experimenter then removed any debris and stirred the water to distribute olfactory cues prior to the start of the next trial. If the rat did not find the platform within the allotted 3-minute trial time, the experimenter guided the rat to the nearest platform. Rats received four trials per day for 12 days. On day 13, a six-hour delay was implemented between trials 2 and 3 to evaluate delayed memory retention. Errors, defined as an entry into an arm that did not contain a platform, were recorded for each trial; an arm entry was defined as the rat's snout passing a line 11 cm into the arm (only visible on the outside of the maze, but not visible to the rat). Three error types were quantified. Working memory correct (WMC) errors were entries into an arm on trials 2-4 that previously contained a platform within a day. Reference memory (RM) errors were first entries into an arm that

never contained a platform. Working memory incorrect (WMI) errors were repeated entries within a day into an arm that never contained a platform.

Morris water maze. After 1 day of rest following the WRAM delay day, a subset of subjects began testing on the Morris water maze (MM), a task that assesses spatial reference memory. The apparatus was a large round tub (188 cm diameter) filled with water maintained at 18-20°C and made opaque with non-toxic black powdered paint. One platform (11 cm diameter) was hidden just below the surface of the water in the northeast quadrant of the maze. The location of the platform remained constant across all days and trials. Salient spatial cues were present around the room to aid in spatial navigation to the platform (Morris et al., 1982). Rats received four trials per day for five consecutive days. At the beginning of each trial, rats were dropped off from one of four starting points (north, south, east, or west). The order in which the rats were exposed to the drop off points changed across days, but was the same for all rats within a day. Rats were given a maximum trial time of 60 seconds to locate the platform before the experimenter terminated the trial and led the rat to the platform. Once the platform was found, the rat remained on it for 15 seconds to allow for spatial localization before the experimenter returned the rat to its heated testing cage for an ITI of approximately 8-10 minutes. On the last day of MM, a probe trial was given. Following the four regular trials, rats were administered a fifth trial in which the hidden platform was completely removed from the maze, and rats swam freely for the standard allotted trial time of 60 seconds. The probe trial was implemented to evaluate whether the rats had spatially localized to the platform by quantifying the percent of total swim distance in the target quadrant versus the opposite quadrant. A video camera and tracking system (Ethovision; Noldus Instruments;

Wageningen, The Netherlands) were utilized to measure each rat's swim path (distance in cm) across all days and trials, including the probe trial.

Delayed match-to-sample win-stay task. The remaining subset of rats was tested on a delayed match-to-sample (DMS) win-stay task that evaluated spatial reference memory. The WRAM apparatus was utilized for this task, but rats were tested in different rooms and with novel spatial cues compared to the WRAM task. One platform was placed in an arm of the WRAM, where it remained across all days and trials. Rats received 6 trials per day for 4 days. For each trial, subjects were dropped off from 1 of the 6 different start arm locations (excluding the platform-containing arm and the arm directly across from the platform). The order in which the drop-off locations occurred varied across days, but stayed the same within a day for all rats. There was no consistent pattern of left or right turns from the start arm to the platform within or across days. Once the rat found the platform, it was allowed to remain on it and spatially localize for 15 seconds before being returned to its heated testing cage for a 30-second ITI. There was a 90-second maximum trial time; if the rat did not find the platform within the allotted time, the experimenter led the rat to the platform. An arm entry was quantified when the snout of the rat passed an 11 cm mark denoted on each arm of the maze that was visible to the experimenter, but not to the rat. The dependent variable for this task was the total number of errors committed on each trial. Total errors were defined as any entry into a non-platformed arm within a trial prior to locating the platform.

Visible platform. Following the last day of the reference memory tasks, all subjects were tested on the Visible Platform (VP) task to confirm that rats could perform the visual and motoric components of water maze tasks. The apparatus was a rectangular

tub (100 x 60 cm) filled with clear water (18-20°C). A black platform (10 cm diameter) was placed in the tub and protruded about 4 cm above the water's surface. Opaque curtains were hung in a circular fashion around the room to block potential spatial or geometric cues. All rats received 6 trials in 1 day. For each trial, the rat was dropped off from a fixed start location. The visible platform escape location varied semi-randomly in 3 possible locations across trials. The maximum trial time was 90 seconds, and, once the rat located the visible platform, it was allowed to remain on it for 15 seconds before being returned to its heated testing cage for an ITI of 8-10 minutes. Latency (in seconds) from drop off to the platform was recorded for each trial.

Euthanasia

Rats were euthanized 1 week after the completion of the behavioral battery. Subjects were approximately 7 months old at euthanization. Rats were deeply anesthetized with inhaled isoflurane anesthesia. Blood was collected via cardiocentesis and allowed to clot at 4°C (Vacutainer 367986, Becton Dickinson and Company, Franklin Lakes, NJ, USA). Blood samples were then centrifuged at 2,000 rpm at 4°C for 20 minutes. Serum was collected and stored at -20°C until analysis. Ovaries from the Sham and Hysterectomy groups were collected, trimmed of excess fat, and fixed in 10% buffered formalin for 48 hours before being transferred to 70% ethanol until analysis. Uteri were collected from the Sham and Ovx groups, trimmed of excess fat, and wet weight was obtained.

Serum Hormone Assays

A double antibody liquid-phase RIA (Beckman Coulter; Brea, CA, USA) was used to determine 17β-estradiol levels. This RIA utilized estradiol-specific antibodies with an ^{125}I-labeled estradiol as the tracer. Interassay coefficients of variation for this assay averaged 10% at a mean value of 28 pg/ml. Functional sensitivity for this assay was 5 pg/ml. Androstenedione was assessed using an ELISA assay (ALPCO, Salem, NH, USA), based on the typical competitive binding scenario between unlabeled antigen (present in standards, controls, and unknowns) and the enzyme-labeled antigen (conjugate) for a limited number of antibody binding sites on the microwell plate. Interassay coefficients of variation for this assay averaged 9% at a mean value of 0.5 ng/ml. Functional sensitivity of the assay was 0.1 ng/ml. Progesterone levels were determined using an ELISA assay (ALPCO, Salem, NH, USA). Interassay coefficients of variation for the progesterone assay averaged 13% at a mean value of 2.6 ng/ml. Functional sensitivity of this assay was 0.3 ng/ml.

A sensitive two-site sandwich immunoassay was utilized to measure serum LH (Fallest, Trader, Darrow, & Shupnik, 1995; Haavisto et al., 1993) using monoclonal antibodies against bovine LH (no. 581B7; RRID:AB_2665514) and against the human LH-beta subunit (no. 5303: Medix, Kauniainen, Finland; RRID:AB_2665513) as previously described (Haavisto et al., 1993). The tracer antibody (no. 518B7) was kindly provided by Dr. Janet Roser (Department of Animal Science, University of California, Davis) (Matteri, Roser, Baldwin, Lipovetsky, & Papkoff, 1987), iodinated by the chloramine T method, and purified on Sephadex G-50 columns. The capture antibody (no. 5303) was biotinylated and immobilized on avidin-coated polystyrene beads (7mm;

Epitope Diagnostics, Inc., San Diego, CA). The standard was a Mouse LH reference prep (AFP5306A; provided by Dr. A.F. Parlow and the National Hormone and Peptide program). The assay had a sensitivity of 0.04 ng/ml.

RIA was used to assay serum FSH levels utilizing reagents provided by Dr. A.F. Parlow and the National Hormone and Peptide Program, as described previously (Gay, Midgley, & Niswender, 1970). Mouse FSH reference prep AFP5308D was used for assay standards. The primary antibody was a Mouse FSH antiserum (guinea pig; AFP-1760191; RRID:AB_2665512) diluted to a final concentration of 1:400,000. The secondary antibody was purchased from Equitech-Bio, Inc. and diluted to a final concentration of 1:25. The assay had a sensitivity of 2.0 ng/ml, and less than 0.5% cross-reactivity with other pituitary hormones.

A commercially available ELISA kit (ANSH Labs, Webster, TX, AL-113) was used to determine Anti-Müllerian Hormone (AMH) levels. The sensitivity of this assay was 0.011 ng/ml, and the reportable range spanned between 3.36 – 215.0 ng/ml.

Ovarian Follicle Counts

One ovary from each Sham and Hysterectomy subject (i.e., all rats that had ovaries at the end of the experiment) was randomly selected for histological evaluation and quantification of healthy primordial, primary, secondary, and antral follicles, as well as corpora lutea. Following fixation, ovarian tissues were paraffin embedded and sectioned at 5 μm. Every 10th section was mounted on a slide and stained with hematoxylin and eosin Y-phloxine B. Primordial, primary, secondary, and antral follicles were counted at 20x magnification for every 20th section. Corpora lutea were counted at

a lower magnification (Pannoramic DESK; 3D Histech, Budapoest, Hungary). The following formula was used to calculate the total number of follicles in the ovary: $N_t = (N_0 \times S_t \times t_s) / (S_0 \times d_0)$, where N_t = total calculated number of follicles, N_0 = number of follicles observed in the ovary, S_t = total number of sections in the ovary, t_s = thickness of the section (μm), S_0 = total number of sections observed, and d_0 = mean diameter of the nucleus (Gougeon & Chainy, 1987). Ovarian follicles were classified using criteria from Haas et al., 2007 (Haas et al., 2007). Primordial cells were counted when there was a single layer of squamous granulosa cells surrounding the oocyte. Primary follicles contained a single layer of cuboidal granulosa cells. Secondary follicles required several granulosa cell layers surrounding the oocyte. Antral follicles were counted when the follicle contained two or more layers of granulosa cells and had a fluid-filled antral space in the follicle (Haas et al., 2007).

Statistical Analyses

Data analyses were completed using Statview software. The independent variable for all analyses was Surgery type (Sham, Ovx, Hysterectomy, Ovx-Hysterectomy). Repeated measures ANOVAs were utilized for WRAM data, with Errors as the dependent variable, and Trials within Days as the repeated measures. Separate repeated measures analyses were completed for each error type (WMC, WMI, and RM errors). WRAM data were blocked into 3, 4-day blocks, as we have previously published (Mennenga, Koebele, et al., 2015). Block 1, Days 1-4, was the Early Acquisition Phase; Block 2, Days 5-8, was the Late Acquisition Phase; Block 3, Days 9-12, was the Asymptotic Phase. For MM data, Swim Distance to Platform (cm) across repeated Days

and Trials was evaluated. In the DMS win-stay task, Total errors were evaluated, repeated across Days and Trials. Visible platform data was evaluated for Latency to platform (seconds), repeated across Trials. Serum hormone, ovarian follicle, body weight, and uterine weight data were analyzed using ANOVA, with Surgery as the independent variable and Serum Hormone Concentration, Follicle Estimate, Body Weight, and Uterine Weight as the dependent variables, respectively. All analyses were two-tailed with an alpha level set to 0.05; results were deemed marginal if the p value fell between 0.05 and 0.10. Effect size for repeated measures ANOVA analyses were reported as generalized eta squared (η_G^2) (Bakeman, 2005; Olejnik & Algina, 2003). For all other ANOVAs (non-repeated measures), effect sizes were reported as eta squared (η^2). Effect sizes were interpreted using Cohen's general guidelines for η^2, where 0.02 is a small effect, 0.13 is a medium effect, and 0.26 is a large effect (Bakeman, 2005; Cohen, 1992). All effect sizes for post hoc tests were reported using Cohen's d and are interpreted by Cohen's standard guidelines, where 0.2 indicates a small effect, 0.5 indicates a medium effect, and 0.8 indicates a large effect (Cohen, 1992).

Results

Vaginal Cytology

Vaginal smears were performed for eight consecutive days, beginning two weeks after surgery. Rats that received Ovx or Ovx-Hysterectomy showed blank or diestrus-like smears, indicating a lack of estrogen stimulation of the vaginal epithelium and therefore successful surgical removal of the ovaries. Sham and Hysterectomy groups showed

normal 4-5 day estrous cycles, suggesting continued ovarian function and ovulatory patterns following surgery (Figure 48).

Water Radial-Arm Maze

Early acquisition phase (days 1-4). The Early Acquisition Phase best captures initial task-rule acquisition and learning, and its associated exploratory behavior in the WRAM. During the Early Acquisition Phase, a main effect of Surgery [$F_{(3,54)}$=2.90, p < 0.05, η_G^2=0.02] was observed for WMC errors. Fischer's PLSD indicated that Ovx rats made fewer WMC errors than did Sham rats (p < 0.01, d=0.33), and Ovx-Hysterectomy rats tended to make fewer WMC errors than did Sham rats (p = 0.06, d=0.20) (Figure 49A). For this block of testing, there was also a Surgery x Trial interaction [$F_{(6,108)}$=2.84, p < 0.01, η_G^2=0.03]. Given that previous studies in our laboratory have shown age- , hormone- , and menopause- mediated differences in memory when working memory load is taxed (Acosta et al., 2010; Bimonte-Nelson, Hunter, Nelson, et al., 2003; Bimonte-Nelson, Singleton, Hunter, et al., 2003; Bimonte-Nelson et al., 2004; Bimonte & Denenberg, 1999; Bimonte et al., 2003; Koebele et al., 2017; Mennenga, Gerson, et al., 2015; Mennenga, Koebele, et al., 2015), we further probed this effect and assessed WMC errors for Trials 3 and 4 only, which are the high working memory load trials. There was a main effect of Surgery [$F_{(3,54)}$=2.84, p < 0.05, η_G^2=0.03] such that Sham rats made more errors than Ovx rats (p < 0.01, d=0.42) and Ovx-Hysterectomy rats (p < 0.05, d=0.28) on the high working memory load trials (Figure 49B). There was also a Surgery x Trial interaction for WMC errors on high working memory load trials ($F_{(3,54)}$=3.15, p < 0.05, η_G^2=0.03) (Figure 49C). Thus, we further probed this interaction. There were no

differences amongst groups on Trial 3. However, there was a main effect of Surgery on Trial 4, the maximum working memory load trial [$F_{(3,54)}$=3.76, $p < 0.05$, η_G^2=0.07], with the post hoc again indicating the same effect wherein Sham rats made more errors than did Ovx rats ($p < 0.01$, d=0.70) and Ovx-Hysterectomy rats ($p < 0.05$, d=0.45) (Figure 49D). For WMI errors, there was a Surgery x Trial interaction [$F_{(9,162)}$=3.10, $p < 0.01$, η_G^2=0.03] (Figure 50A). Upon probing the high working memory load trials (Trials 3 and 4), a marginal main effect of Surgery [$F_{(3,54)}$=2.51, $p = 0.07$, η_G^2=0.02] and a significant Surgery x Trial interaction [$F_{(3,54)}$=3.02, $p < 0.05$, η_G^2=0.02], (Figure 50B) were revealed. Post hoc analyses indicated that Sham rats made more errors than Ovx ($p < 0.05$, d=0.26), Hysterectomy ($p < 0.05$, d=0.27), and Ovx-Hysterectomy ($p < 0.05$, d=0.30) groups. Because there was a significant Surgery x Trial interaction for the high working memory load trials, we further investigated these effects. There were no differences amongst groups during Trial 3, but there was a main effect of Surgery during Trial 4 [$F_{(3,54)}$=3.45, $p < 0.05$, η_G^2=0.05], with Sham-treated rats making more errors than Ovx ($p < 0.01$, d=0.47), Hysterectomy ($p < 0.05$, d=0.38) and Ovx-Hysterectomy ($p < 0.01$, d=0.54) groups (Figure 50C). There were no main effects or interactions with Surgery for RM errors during the Early Acquisition phase (data not shown).

Late acquisition phase (days 5-8). The Late Acquisition Phase captures mid-task learning when rats tend to decrease in errors compared to the Early Acquisition Phase, but continue to exhibit variable performance as they learn to handle the working memory load associated with the WRAM task. There were no main effects or interactions with Surgery during the Late Acquisition phase. However, there was a marginal main effect of Day for WMC errors [$F_{(3,162)}$=2.30, $p = 0.08$, η_G^2=0.01] and a main effect of Day for

WMI [$F_{(3,162)}$=5.66, $p < 0.001$, η_G^2=0.02] and RM errors [$F_{(3,162)}$=3.25, $p < 0.05$, η_G^2=0.01] such that errors decreased across days, suggesting learning of the task (data not shown).

Asymptotic phase (days 9-12). The Asymptotic Phase of the WRAM is most crucial to understanding spatial working memory performance, as this phase occurs when the rats have learned the rules of this win-shift task and are approaching peak memory performance, where errors are decreased compared to earlier phases and remain at a consistent level of performance within the block of days, here 9-12. For WMC errors in the Asymptotic Phase, there was a marginal Surgery x Trial interaction [$F_{(6,108)}$=1.93, p = 0.08, η_G^2=0.02] (Figure 51). For WMI errors, there was a main effect of Surgery [$F_{(3,54)}$=3.06, $p < 0.05$, η_G^2=0.03]. Fisher's PLSD indicated that the Hysterectomy group made significantly more WMI errors than did the Sham ($p < 0.05$, d=0.27), Ovx ($p <$ 0.05, d=0.28), and Ovx-Hysterectomy groups ($p < 0.01$, d=0.40) (Figure 52A). There was also a marginal Surgery x Trial interaction for WMI errors [$F_{(9,162)}$=1.75, p = 0.08, η_G^2=0.02] (Figure 52B). When only high working memory load trials (Trials 3 and 4) were probed, a main effect of Surgery [$F_{(3,54)}$=2.93, $p < 0.05$, η_G^2=0.05] was again shown. Fisher's PLSD showed that the Hysterectomy group made significantly more WMI errors than did the Sham group ($p < 0.05$, d=0.37), such that hysterectomy alone (i.e. with ovarian conservation) impaired memory compared to reproductively unaltered rats. Rats that had only their uterus removed made more WMI errors than any group of rats that had their ovaries removed, whether or not that ovarian removal was concomitant with uterus removal, as indicated by the Hysterectomy group making more errors than the Ovx group ($p < 0.05$, d=0.39) and the Ovx-Hysterectomy group ($p < 0.01$, d=0.56) when working

memory load was taxed in the Asymptotic Phase of WRAM (Figure 52C). Notably, once the ovaries were removed, the additional removal of the uterus had no further impact on cognitive effects (Fisher's PLSD Ovx vs. Ovx-Hysterectomy: $p = 0.64$). There was no interaction between Surgery and Trial for this analysis; thus, effects were not further probed. These findings suggest that, 6 weeks after surgery, the surgical removal of the uterus alone, conserving the ovaries, impairs memory when working memory load is taxed in the latter half of testing. In addition, this detrimental impact of hysterectomy with ovarian conservation appears to be prevented if Ovx accompanies the hysterectomy. There were no main effects or interactions with Surgery for RM errors during the Asymptotic Phase, indicating that the spatial memory deficit observed for the Hysterectomy group is likely specific to working memory.

Six hour delay. On day 13 of WRAM testing, a six-hour delay was given between trials 2 and 3 to test delayed memory retention. Each treatment group's performance was assessed separately using a repeated measures ANOVA to evaluate group performance on trial 3 of the last day of baseline testing (day 12) compared to trial 3 following the six-hour delay on day 13. Sham-treated rats did not show impaired performance between baseline and delay testing, suggesting that there was not a delay-induced impairment (Figure 53A). There was a main effect of Delay Day for WMC errors on trial 3 for Ovx rats [$F_{(1,13)}=7.00$, $p < 0.05$, $\eta_G^2=0.35$] (Figure 53B). For Hysterectomy-treated rats, there was a marginal main effect of Delay Day for WMC errors on trial 3 [$F_{(1,13)}=3.96$, $p = 0.07$, $\eta_G^2=0.23$], such that WMC errors somewhat increased as a result of a six-hour memory retention delay (Figure 53C). There was also a main effect of Delay Day for WMC errors on trial 3 for Ovx-Hysterectomy rats

[$F_{(1,14)}$=47.25, $p < 0.0001$, η_G^2=0.77] (Figure 53D). Thus, both groups without ovaries showed significantly impaired delayed memory retention following a delay in WRAM trials, an effect our laboratory has previously reported (Hiroi et al., 2016; Prakapenka et al., 2018).

Reference Memory Tasks

A subset of rats (n=5/group) underwent training in the spatial reference memory MM task. All rats learned across days [$F_{(4,64)}$=33.48, $p < 0.0001$, η_G^2=0.38], with no differences in learning among surgery types. Furthermore, there was a main effect of Quadrant on the Probe trial [$F_{(1,16)}$=129.08, $p < 0.0001$, η_G^2=0.87] and no interaction with surgery type, suggesting that all rats successfully spatially localized to the target (previously platformed) quadrant (data not shown).

All other subjects were trained on a win-stay version of the DMS spatial reference memory task. Again, there was a main effect of Day [$F_{(3,102)}$=69.25, $p < 0.0001$, η_G^2=0.16] and no interaction with Surgery, such that all rats learned to navigate to the platform and decreased in the total number of errors committed across the 4 days of the task (data not shown). Given that no differences in reference memory performance were found on either of these reference memory-only tasks, nor the reference memory measure of the WRAM, findings support the tenet that the memory impairment induced by Hysterectomy is specific to the working memory domain.

Visible Platform

There was a main effect of Trial [$F_{(5,270)}$=5.53, $p < 0.0001$, η_G^2=0.07] that did not interact with Surgery type, such that all rats decreased escape latency to the visible platform across six trials. The average escape latency on Trial 1 was 13.55s, and averaged 6.91s on Trial 6 (data not shown). This control task verifies that all subjects could perform the procedural components of the water maze tasks, including visual and motoric capacities.

Body Weights

Two weeks after surgery, there was a main effect of Surgery on body weight [$F_{(3,54)}$=11.92, $p < 0.0001$, η^2=0.40]. Fischer's post-hoc analyses revealed that Ovx-treated rats weighed more than Sham ($p < 0.0001$, d=1.88) and Hysterectomy ($p < 0.0001$, d=1.61) rats; Ovx-Hysterectomy rats also weighed more than Sham ($p < 0.0001$ d=1.48) and Hysterectomy ($p < 0.0001$, d=1.25) rats. This indicates that surgical removal of the ovaries increased body weight compared to rats that retained their ovaries. This difference in body weight between subjects with and without their ovaries continued to diverge across week 3 [$F_{(3,54)}$=21.37, $p < 0.0001$, η^2=0.54], week 4 [$F_{(3,54)}$=37.35, $p < 0.0001$, η^2=0.68], week 5 [$F_{(3,54)}$=44.02, $p < 0.0001$, η^2=0.71], and through the euthanization time point, [$F_{(3,54)}$=43.77, $p < 0.0001$, η^2=0.71]. At euthanization, Ovx and Ovx-Hysterectomy rats weighed more than Sham (Ovx vs Sham: $p < 0.0001$, d=3.62; Ovx-Hysterectomy vs Sham: $p < 0.0001$, d=2.47). Ovx and Ovx-Hysterectomy rats also weighed more than Hysterectomy rats (Ovx vs Hysterectomy: $p < 0.0001$, d=3.82; Ovx-Hysterectomy vs Hysterectomy: $p < 0.0001$, d=2.61) rats (Figure 54).

Uterine Weights

Uterine weights of Sham and Ovx rats (i.e., subjects with uterine conservation) were assessed at euthanasia. Ovx rats had decreased uterine weight compared to Sham-operated rats [$F_{(1,27)}$=72.61, $p < 0.0001$, η^2=0.73] (Figure 55). This confirms that Ovx was successful, and that the uteri of Sham rats continued to receive stimulation from endogenously circulating estrogen.

Ovarian Follicle Counts

Ovaries were analyzed in rats within the Sham and Hysterectomy groups; that is, the rats that retained their ovaries throughout the experiment. Figure 11 depicts ovary micrographs of representative subjects from the Sham (56A) and Hysterectomy (56B) groups. No differences in early-stage follicles were found between Sham and Hysterectomy groups (Primordials: $F_{(1,25)}$=0.39, p = NS, η^2=0.03, Figure 57A; Primaries: $F_{(1,25)}$=0.80, p = NS, η^2=0.003, Figure 57B; Secondaries: $F_{(1,25)}$=0.38, p = NS, η^2=0.03, Figure 57C). While there was a trend toward Hysterectomy subjects having fewer antral follicles as compared to Sham-treated counterparts, this analysis did not reach significance [$F_{(1,25)}$=3.12, p = 0.09, η^2=0.11] (Figure 57D). Furthermore, corpora lutea counts were not different from each other [$F_{(1,25)}$=0.63, p = NS, η^2=0.01] (Figure 57E), suggesting that following Hysterectomy surgery, the ovaries of Hysterectomy rats did not exhibit morphological changes in healthy follicle counts compared to Sham rats, at least up to 2 months after surgical intervention. Two subjects were excluded from the follicle

and corpora lutea analyses due to comprised tissue quality after processing (Sham n = 13, Hysterectomy n = 14).

Serum Hormone Levels

The 17β-estradiol analysis revealed a main effect of Surgery [$F_{(3,53)}$=15.30, p < 0.0001, η^2=0.46]. Fisher's PLSD indicated that the Ovx group had lower 17β-estradiol levels than the Sham group (p < 0.0001, d=2.85) and the Hysterectomy group (p < 0.001, d=1.74); the Ovx-Hysterectomy group also had lower 17β-estradiol levels than the Sham group (p < 0.0001, d=2.79) and the Hysterectomy group (p < 0.001, d=1.70), indicating successful surgical removal of the ovaries in rats that were Ovx. Additionally, the Hysterectomy group trended toward having lower 17β-estradiol levels compared to the Sham group (p = 0.06, d=0.52) (Figure 58A). One serum sample was excluded from the 17β-estradiol analysis due to an issue with the sample during processing (Sham n = 15, Ovx n = 14, Hysterectomy n = 14, Ovx-Hysterectomy = 14) .

For androstenedione, there was a main effect of Surgery [$F_{(3,54)}$=94.23, p < 0.0001, η^2=0.84]. Fisher's PLSD revealed that Sham rats had higher androstenedione levels than Ovx (p < 0.0001, d=6.08) and Ovx-Hysterectomy groups (p < 0.0001, d=6.08). Hysterectomy rats also had higher androstenedione levels than Ovx (p < 0.001, d=6.42) and Ovx-Hysterectomy groups (p <0.0001, d=6.42); indeed, neither Ovx nor Ovx-Hysterectomy rats had detectable levels of androstenedione at euthanization. The Hysterectomy group had lower androstenedione levels compared to the Sham-operated group (p < 0.01, d=0.70). (Figure 58B).

Progesterone analyses revealed a main effect of Surgery [$F_{(3,54)}$=8.79, $p < 0.0001$, η^2=0.33], and Fisher's PLSD indicated that Sham rats had higher progesterone levels compared to Ovx rats ($p < 0.05$, d=1.09) and Ovx-Hysterectomy rats ($p < 0.05$, d=1.23). The Hysterectomy group had higher progesterone levels compared to the Ovx group ($p < 0.05$, d=1.37) and the Ovx-Hysterectomy group ($p < 0.05$, d=1.45). Additionally, the Hysterectomy group had elevated progesterone levels compared to the Sham group ($p < 0.05$; d=0.68) (Figure 58C).

Analysis of AMH, which is produced by ovarian granulosa cells and is considered to be a correlate of ovarian follicle reserve (Hansen, 2013; Hansen et al., 2011), revealed a main effect of Surgery [$F_{(3,53)}$=93.61, $p <0.0001$, η^2=0.84]. Fischer's PLSD showed that Sham rats had higher AMH levels than Ovx rats ($p < 0.0001$, d=5.26) and Ovx-Hysterectomy rats ($p < 0.0001$, d=5.26). Hysterectomy rats also had higher AMH levels than Ovx rats ($p < 0.0001$, d=10.01) and Ovx-Hysterectomy rats ($p < 0.0001$, d=10.01). Indeed, AMH levels were undetectable in both the Ovx and Ovx-Hysterectomy groups. Because AMH is produced by the ovarian follicles, the ovary-intact groups (i.e. Sham and Hysterectomy groups) were compared to each other in an additional analysis. No statistical difference was seen for AMH levels between Sham and Hysterectomy rats, corroborating ovarian follicle count results wherein there were no differences in primordial follicle count, the ovarian follicle reserve (58D).

LH analyses showed a main effect of Surgery [$F_{(3,53)}$=27.51, $p < 0.0001$, η^2=0.61], with Fischer's PLSD indicating that Ovx rats had higher LH levels than Sham rats ($p < 0.0001$, d=2.50) and Hysterectomy rats ($p < 0.0001$, d=2.42). Ovx-Hysterectomy rats also had higher LH levels than Sham rats ($p < 0.0001$, d=7.09) and Hysterectomy rats ($p <$

0.0001, d=6.39). These results indicate that surgical removal of the ovaries resulted in higher circulating LH levels, as has been previously observed in rodents (Gay & Midgley, 1969; Wise & Ratner, 1980) and women (Yen & Tsai, 1971). LH levels were compared between ovary-intact groups only (Sham and Hysterectomy rats) separately; no differences were observed between ovary-intact groups, suggesting that hysterectomy with ovarian conservation does not alter serum LH levels, at least up to 2 months post-surgery as tested here (58E).

FSH analyses also revealed a main effect of Surgery [$F_{(3,53)}$=535.57, p <0.0001, η^2=0.97], with Fischer's PLSD indicating that Ovx rats had higher FSH levels than Sham rats (p < 0.0001, d=14.16) and Hysterectomy rats (p < 0.0001, d=12.92); Ovx-Hysterectomy rats had higher FSH levels than Sham rats (p < 0.0001, d=11.372) and Hysterectomy rats (p < 0.0001, d=10.49) as well, corresponding with LH results suggesting that Ovx increases circulating FSH levels, an effect previously observed in rats (Elias & Blake, 1983; Wise & Ratner, 1980) and women (Yen & Tsai, 1971). FSH levels were compared between ovary-intact groups only; there was a main effect of Surgery for ovary-intact groups [$F_{(1,27)}$=4.97, p < 0.05, η^2=0.16], such that FSH levels were increased in Hysterectomy rats compared to Sham-operated rats, suggesting a response by the hypothalamus-pituitary axis induced by uterus removal (58F).

Discussion

To the best of our knowledge, the current report is the first systematic investigation of variations in surgical menopause including hysterectomy in an adult rat model testing relationships amongst reproductive profiles and cognition. A multi-systems

approach simultaneously evaluated ovarian morphology, endocrine physiology, and cognition within the same subjects. Collectively, results showed that common variations in surgical menopause yield distinct effects on spatial working memory performance across early acquisition to asymptotic phases, and that hysterectomy with ovarian conservation – a novel surgical model—has unique, detrimental effects on spatial working memory 2 months after surgery.

During the Early Acquisition phase of the WRAM, wherein animals were acquiring task rules through maze exploration, rats with Ovx alone made fewer WMC errors compared to Sham rats across all trials. Ovx-Hysterectomy rats also made fewer errors than Sham rats on high working memory load trials. For WMI errors, the Sham group made more errors during the Early Acquisition Phase compared to all other experimental groups. These findings may be interpreted as enhanced WRAM task rule acquisition for subjects without their ovaries. This enhancement during acquisition could be related to maze solving strategy or attentional processes. For example, estrogen milieu can impact learning strategies, as shown with exogenous ovarian hormone administration (Korol & Kolo, 2002), and across estrous cycle phases (Korol et al., 2004). In accordance with these findings, our laboratory has reported Ovx-induced spatial memory enhancements for aged rats tested on the WRAM as compared to Sham-operated rats (Bimonte-Nelson, Singleton, Hunter, et al., 2003; Bimonte-Nelson et al., 2004), and other laboratories have concordant findings showing long-term Ovx yields benefits for spatial memory compared to age-matched reproductively-intact controls in rhesus macaques (Lacreuse, Herndon, & Moss, 2000) and mice (Heikkinen et al., 2004). Of note, there are reports of no effects of Ovx in middle-aged rats, and some work has shown memory

impairments after Ovx in young adult and middle-aged rats, effects which occurred after animals had learned the task or after extended temporal delays to evaluate high-demand mnemonic retention, indicating that task phase and difficulty impacts outcomes of Ovx (Bimonte-Nelson, Acosta, & Talboom, 2010). Another interpretation of the increased errors in the Sham group during the early acquisition phase is related to an increase in exploratory behavior or activity levels in this group, rather than a relative learning impairment per se. Indeed, previous studies have found that Ovx decreases activity levels (Blizard, Lippman, & Chen, 1975) and increases anxiogenic behaviors (Hiroi and Neumaier, 2006) in the open field, and that that presence of ovarian hormones restores these activity levels and decreases anxiogenic behavior (Blizard et al., 1975; Hiroi & Neumaier, 2006). Thus, it is possible that the rats without ovaries are making fewer arm entries during the first several days of the task, which is operationally defined as better performance, due to an artifact of decreased exploratory or locomotor activity during task acquisition.

During the Asymptotic Phase of the WRAM (Days 9-12) when rats have learned the task rules, Hysterectomy with ovarian conservation had a unique, detrimental effect on spatial memory when working memory load was taxed compared to all other variations in surgical menopause tested here, as well as compared to Sham-operated rats. Interestingly, concomitant surgical removal of the ovaries with the uterus (i.e., Ovx-Hysterectomy) prevented the detrimental memory effects of Hysterectomy alone on spatial working memory. Rats that received Sham surgery or Ovx surgery with uterine conservation did not show cognitive impairments at a high working memory load, suggesting that the presence or absence of ovaries alone does not dictate the observed

working memory effects. Taken together, these results indicate that during the Asymptotic Phase of WRAM, when subjects are performing to the best of their ability, there is a unique impact of uterus removal alone that detrimentally impacts the spatial working memory domain.

Following the WRAM, all subjects were exposed to 1 of 2 win-stay reference memory tasks: the MM or the DMS win-stay maze. Compared to the WRAM, these are both lower cognitive demand tasks that do not involve a working memory load component. In each reference memory task, all subjects learned the task, effectively decreasing swim distance to the platform in the MM or decreasing errors committed across days in the DMS task, regardless of surgery condition. This lack of difference among surgery conditions is notable in that the effects of these variations in surgical menopause are likely a domain-specific, rather than a global, change in cognitive function. Translationally, this is a crucial factor to keep in mind, as deficits in a particular cognitive area may not lead to an overall global decline in cognitive function. Furthermore, the order in which the rats were exposed to the task is likely important when interpreting cognitive outcomes. Specifically, following a high cognitive demand task like the WRAM, lower cognitive demand tasks may be easier to acquire since the subjects has experienced a taxing spatial memory task first. In the future, it will be important to explore whether maze task order matters for spatial learning and memory in the context of hormones and aging.

It remains controversial whether the ovaries continue to function normally long-term following hysterectomy in the premenopausal state. Some evidence from human literature where women in their reproductive years underwent hysterectomy with ovarian

conservation implies that the ovaries continue to function normally for many years (Beavis et al., 1969; Chalmers et al., 2002; Findley et al., 2013); this is evidenced in that these women are not prescribed estrogen-containing hormone therapy following their surgery until they may opt to take it in midlife, around the average age of natural menopause onset, when circulating ovarian hormone levels become erratic and symptoms of menopause, such as hot flashes, begin to present (Kritz-Silverstein et al., 2000). However, other clinical literature reports that women who undergo hysterectomy with ovarian conservation during reproductive years may transition to menopause earlier in life than women who have an intact uterus and ovaries throughout the menopause transition (Kaiser et al., 1989; Moorman et al., 2011; Read et al., 2010). Whether this is directly related to the hysterectomy procedure, or whether these women may be predisposed to an earlier menopause related to a comorbid condition, remains uncertain. Furthermore, recent evidence suggesting that a younger age at hysterectomy is associated with an increased relative risk of developing dementia compared to age-matched intact women (Phung et al., 2010; Rocca et al., 2007, 2012) points to a crucial role for uterine tissue in a trajectory of healthy aging. The data presented in the current study reveal that rats that received a hysterectomy with ovarian conservation had normal estrous cyclicity 2 weeks after surgery, gained body mass at a rate similar to that of sham-operated controls, and did not show alterations in ovarian follicle morphology 2 months post-surgery. However, alterations in circulating ovarian steroid hormones and gonadotropin levels were apparent two months after surgery. Whether changes in ovarian function and steroidogenesis are a transient effect of hysterectomy, or whether these are long-term changes in ovarian function remains an open, but critical, question.

Altered ovarian- and pituitary- derived hormone synthesis and release following hysterectomy are likely key factors to understanding the progression of morphological, physiological, and cognitive changes associated with hysterectomy surgery. It is notable, however, that serum AMH, a putative marker of ovarian follicle reserve, is not different between Sham and Hysterectomy rats, suggesting that hysterectomy with ovarian conservation did not significantly impact ovarian follicle reserve, at least in this short-term 2 month time point after surgery. Furthermore, serum LH levels, the gonadotropin that regulates ovulation and corpus luteum function, were not different between Hysterectomy rats and ovary-intact Sham rats; indeed, low LH levels in the Hysterectomy group indicate continued ovarian function following surgery, supporting the idea that localized ovarian dysfunction is not the primary contributing factor to the cognitive detriments seen in the Hysterectomy rats 6 weeks after surgery, and that uterus itself likely has a unique impact on cognition, with or without additional impacts or interactions from other factors not yet determined. Notably, 2 months after surgery, FSH levels were significantly increased in Hysterectomy rats compared to ovary-intact Sham rats. Given that FSH is currently the gold standard in clinical settings to determine a woman's menopause status and has been previously reported to increase following hysterectomy with ovarian conservation in women (Chan et al., 2005; Kaiser et al., 1989), it is of particular interest to further investigate this increase in FSH in Hysterectomy rats in relation to hypothalamic-pituitary-reproductive tract communication and dysfunction. That FSH was one of the hormones altered two months after hysterectomy with ovarian conservation in our study implicates a potential accelerated disruption of hypothalamic-pituitary-reproductive tract communication when the uterus

alone is removed, which, when considering translational implications, could yield an earlier menopause in women who undergo hysterectomy. One potential contributing factor to this specific change in FSH is altered secretion of inhibins, which are peptide hormones belonging to the transforming growth factor-β family produced by growing ovarian follicles. FSH stimulates inhibin production, and in turn, inhibins selectively regulate FSH release and feedback from the pituitary gland across the female reproductive cycle (Norris & Lopez, 2011; Welt, Sidis, Keutmann, & Schneyer, 2002). The only ovarian change we found in our current evaluations was a marginal decrease in antral follicles, which are one of the primary sources of inhibin-B secretion (Nahás et al., 2003). Indeed, declines in inhibins have been proposed as an early marker of ovarian follicular depletion following hysterectomy in women (Nahás et al., 2003). The role of inhibins is of interest to explore in future studies. In our current study, hysterectomy alone yielded changes in several circulating hormone levels produced by the ovaries and pituitary, which could be a result of altered hormone biosynthesis processes, changes in bioenergetics metabolism, and/or steroid release from the ovary prior to any gross morphological changes in the ovarian follicle structure or onset of follicular atresia, which may occur following a longer period after surgical intervention. We would be remiss if we did not also acknowledge that sex steroid hormones can be derived in non-negligible quantities from extra-ovarian sources, including adipose tissue, skin, hair follicles, liver, and the adrenal glands (Rosen & Cedars, 2007), and that changes in these non-reproductive systems could also be induced by the surgical removal of the uterus. As such, the observed reproductive hormone changes following surgery can currently be interpreted as a potential mediating factor of cognitive change, rather than the primary

cause. Overall, it is crucial to evaluate the long-term effects of hysterectomy with ovarian conservation on ovary structure and function to better understand how a local change such as removing the uterus can ultimately result in a systemic alteration of neurobiological factors important for learning and memory. Given recent findings indicating autonomic and sensory innervation to the non-pregnant rat uterus (Gnanamanickam & Llewellyn-Smith, 2011), one possibility is that disturbance to this uterine innervation via hysterectomy is sufficient to alter neuroendocrine and neurotransmitter signaling in the central and peripheral nervous systems, in turn disrupting hormone production and secretion involved in brain-ovary-uterine communication. Furthermore, there is evidence that adrenergic innervation of the ovaries can influence ovarian hormone secretion during the estrous cycle in the rat (Aguado & Ojeda, 1984), and several neurotransmitters within the catecholamine class have been shown to induce androgen production in cultured ovarian theca-interstitial cells (Dyer & Erickson, 1985). These collected findings implicate a direct role of central and peripheral nervous system regulation of the non-pregnant uterus and the ovaries, and provide a starting point to investigate neurobiological mechanisms associated with the observed cognitive changes in future studies. Additionally, it is imperative to investigate how exogenous estrogen therapy affects this variant of surgical menopause in a rat model, as researchers in the field have historically investigated cognitive effects of estrogen-containing hormone therapy in an Ovx model, where ovaries are removed, but the uterus remains intact. In sum, these findings are a fundamental next step in elucidating how variations in surgical menopause can impact cognitive and brain aging. Specifically, this is the first preclinical study to methodically investigate the impact of hysterectomy with

and without ovarian conservation on learning and memory. Furthermore, using this novel experimental design, we report that the nonpregnant uterus itself is not a quiescent organ. Rather, uterus removal with or without concomitant ovarian removal can have significant effects on physiology and cognition, opening new doors for future investigations into the role of the uterus in behavioral outcomes across the lifespan. In the future, it will be critical to experimentally evaluate the role of age at surgery and time since surgical manipulation to systematically decipher how variations in surgical menopause impact brain aging. Translationally, these findings are impactful in that they can inform clinical understandings of, and lead to additional human studies testing, the intricate connections between the brain and the female reproductive system. This will provide fundamental stepping stones to initiate further exploration into how common variations in gynecological surgery impact quality of life, as well as cognitive and brain aging, in women throughout their lifetimes.

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CHAPTER 7

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INVESTIGATING LONG-TERM COGNITIVE EFFECTS OF VARIATIONS IN GYNECOLOGICAL SURGERY DURING ADULTHOOD IN A RAT MODEL

Contribution: I was the graduate student principal investigator of this experiment under the mentorship of Dr. Heather Bimonte-Nelson. I designed the experiment, and developed the surgical procedure protocol with Dr. Bimonte-Nelson, Dr. Dale DeNardo, and the veterinary team at Arizona State University. This work was supported by the National Institute on Aging (1F31AG056110).

ABSTRACT

A significant number of women undergo gynecological surgery for benign reasons during their lifetime. A majority of these surgeries occur during a woman's reproductive years, prior to the onset of natural menopause in midlife. Hysterectomy, or the surgical removal of the uterus, is a common gynecological surgery. If a woman is pre-menopausal at the time of surgery and has a low cancer risk, ovaries are typically retained in order to prevent an abrupt surgical menopause; however, surgical ovary removal occurs alongside hysterectomy in about 50% of total cases. Thus, a woman can live a substantial proportion of her life without a uterus and/or ovaries. A premature loss of ovarian hormones negatively impacts cognition in women and animal models. Interestingly, hysterectomy with and without ovary removal is associated with a relatively higher risk of developing dementia, and this risk increases with a younger age at surgery. Our laboratory recently reported that two months after surgery, hysterectomy with ovarian conservation resulted in a unique, detrimental working memory impairment compared to other variations in gynecological surgery and to control subjects. Here, we aimed to determine whether this observed cognitive impairment was transient after surgery, or whether it was the onset of a long-term, and possibly global, impairment that persisted throughout aging. Three cohorts of rats received sham, ovariectomy, hysterectomy, or ovariectomy-hysterectomy in adulthood and were tested on a battery of behavior tasks evaluating spatial working memory, spatial reference memory, and anxiety-like behavior. The first cohort was tested six weeks after surgery based on the timing of our initial study, in adulthood; the second cohort was tested seven months after surgery, in middle-age; the third cohort was tested twelve months after surgery, in old

age. Findings revealed that spatial working memory deficits with hysterectomy were present at all time points, suggesting that hysterectomy in adulthood results in a long-term detrimental impact on working memory compared to other variations in gynecological surgery and intact controls. Serum progesterone levels were decreased in Ovx groups compared to ovary-intact groups at all time points. Androstenedione, progesterone, and 17β-estradiol serum levels did not differ between Sham and Hysterectomy rats at any time point. Furthermore, ovarian follicle counts were not different between Sham and Hysterectomy rats at any time point, indicating that the ovaries did not undergo divergent morphological changes following hysterectomy compared to age-matched controls. Collectively, findings point to a primary role for the uterus itself in regulating cognition that is not secondary to ovarian changes. This is the first report of long-term effects of hysterectomy with and without ovarian conservation on cognition, as well as endocrine and ovarian profiles associated with variations in gynecological surgery in a rat model. Translationally, these findings highlight the critical need to better understand how variations in gynecological surgery that occur in adulthood impact the trajectory of cognitive aging in women in order to develop strategies to prevent or postpone cognitive decline in at-risk women.

Introduction

Rates of hysterectomy have been decreasing worldwide during the last several decades as alternative therapies have become available for benign uterine conditions, such as uterine leiomyomas, endometriosis, persistent abnormal bleeding, or pelvic organ prolapse (Corona et al., 2015; J. D. Wright et al., 2013). Yet, hysterectomy remains the most common non-obstetrical gynecological surgical procedure (Carlson et al., 1993; Centers for Disease Control and Prevention, 2010; McPherson, Gon, & Scott, 2013). By age 60, an estimated one-third of US women will have experienced hysterectomy (J. D. Wright et al., 2013), and in 2008, it was projected that lifetime prevalence of hysterectomy neared 48% (Merrill, 2008). This surgery is often performed in adulthood prior to age 52, which is the average age of natural menopause onset (Hoffman et al., 2012; NAMS, 2014). Although some women undergo a complete ovariohysterectomy in adulthood, to avoid premature surgical menopause, the ovaries are retained in about half of hysterectomy cases, especially when women are at a low-risk for certain cancers (Asante et al., 2010; Lowder et al., 2010; Pocobelli et al., 2012; The American College of Obstetrics and Gynecology, 2008; Whiteman et al., 2008). The ovaries are thought to continue to function normally following hysterectomy if the surgery occurs when a woman is still of reproductive age (Beavis et al., 1969; Chalmers et al., 2002; Corson et al., 1981; Findley et al., 2013). This idea has been contested, however, given clinical reports that women who undergo hysterectomy with ovarian conservation tend to experience the transition to menopause three to four years earlier than uterus-intact women, possibly due to compromised ovarian blood flow following the hysterectomy procedure (Chan et al., 2005; Gallicchio et al., 2006; Kaiser et al., 1989; Kritz-Silverstein

et al., 2000; Moorman et al., 2011; Read et al., 2010). Operationally defining the onset of the menopause transition in women with hysterectomy is challenging, as vasomotor symptoms or elevated follicle stimulating hormone (FSH) levels are the primary indicators for menopause in women with hysterectomy, while irregular menstrual patterns are often the first indication of the menopause transition in uterus-intact women. Further methodical evaluation of menopause symptomology is needed to understand whether hysterectomy impacts the trajectory of reproductive system aging. Indeed, given that the average onset of menopause occurs around age 52—and potentially at a younger age in women with hysterectomy—and the average lifespan in the US for females is 82, women who undergo variations in gynecological surgery in adulthood may live many decades in a post-surgical, and eventually post-menopausal state. How hysterectomy with or without ovarian conservation impacts health risk factors longitudinally remains an open, yet imperative, question that necessitates further exploration.

Ovarian hormones are intricately linked with the normal function and regulation of many peripheral body systems, including cardiovascular, skeletal, muscular, integumentary, and gastrointestinal systems, as well as with the central nervous system. A premature loss of ovarian hormones, as is the case with surgical ovary removal, is known to negatively impact cognition in women and animal models (Bimonte & Denenberg, 1999; Bove et al., 2014; Farrag et al., 2002; Gibbs & Johnson, 2008; Nappi et al., 1999; Rocca et al., 2007, 2011, 2012, 2010, 2009; Talboom et al., 2008; M. Wallace et al., 2006). The dogma in the field is that any cognitive changes that present following gynecological surgery are resultant of alterations in the ovaries because the non-pregnant uterus is thought to be a quiescent organ as an independent structure. However, research

from the Mayo Clinic and other laboratories has suggested that *hysterectomy* –with and without ovarian conservation– can increase the relative risk of developing neurodegenerative diseases such as dementia (Phung et al., 2010; Rocca et al., 2007, 2012) and Parkinson's disease (Benedetti et al., 2001) compared to reproductive-tract-intact women of a similar age. The magnitude of this increased relative risk of dementia rises with a younger age at the time of surgery (Phung et al., 2010).

The association between hysterectomy and neurodegenerative disease has piqued interest into how the non-pregnant uterus —which is typically considered an endocrine target but otherwise dormant without direct communication the ovaries— may influence non-reproductive functions, including cognitive processes. The standard model for evaluating effects of surgical menopause in rodents is the ovariectomy (Ovx) model, a procedure which surgically removes the ovaries but retains the uterine tissue (Koebele & Bimonte-Nelson, 2016). However, in clinical practice, this type of procedure is rarely performed; rather, women either undergo a hysterectomy with ovarian conservation or a complete ovariohysterectomy. Thus, a significant gap in the basic science literature existed regarding the effects of hysterectomy in the context of gynecological surgery, menopause, and cognition. A few investigations using rodent models to evaluate the physiological effects of hysterectomy on the ovaries and serum hormone levels do exist. For example, a series of early experiments using crude uterine extracts in rats suggested that the uterus may alter pituitary RNA activity as well as regulate hormone and gonadotropin synthesis and release from the hypothalamus and anterior pituitary (Biró, 1979; Biró & Eneroth, 1989; Biró et al., 1983, 1988, 1984, 1987; H. G. Spies, Hilliard, & Sawyer, 1968). Another experiment where six-week-old rats underwent hysterectomy

found increased ovulatory activity approximately three weeks later, as well as increased ovarian homogenate-derived 17β-estradiol levels and aromatase activity during diestrus, but no change in plasma FSH or ovarian testosterone levels (Tanaka et al., 1994). More recent reports demonstrated that up to four months after hysterectomy, no changes were observed in serum FSH levels, but there was evidence of accelerated ovarian follicular atresia (Tapisiz et al., 2008); a longer interval after hysterectomy surgery indicated higher serum FSH levels in the hysterectomy rats in addition to increased follicular atresia compared to controls (Özdamar, Ülger, Sorkun, & Müderris, 2005). Despite these cumulative findings, no systematic evaluations of the role of hysterectomy on cognition existed. To address this knowledge gap, our laboratory recently developed a novel model of hysterectomy to systematically evaluate effects of variations in gynecological surgery on cognition. Surgery manipulations included Sham (intact control), Ovx, Hysterectomy, and Ovx-Hysterectomy surgeries in the adult rat. Six weeks after surgery, rats were cognitively tested on a battery of spatial memory tasks assessing working and reference memory. We reported that hysterectomy with ovarian conservation resulted in a unique, detrimental effect on spatial working memory two months after surgery (Koebele et al., 2019). These findings should be interpreted in the context of a short-term time point after surgery. The goal of the current experiment was to extend these findings by investigating whether the cognitive effects following hysterectomy in adulthood were transient and reverse with time, or if the observed cognitive changes were the beginning of a long-term, possibly global, cognitive impairment. Three cohorts of rats received Sham, Ovx, Hysterectomy, or Ovx-hysterectomy at 5 months of age. The first cohort was tested 6 weeks after surgery, in adulthood; the second cohort was tested 7 months after surgery, in

middle age; the third cohort was tested 12 months after surgery, in old age. Rats underwent cognitive testing on a series of behavior tasks evaluating spatial working memory, spatial reference memory, and anxiety-like behavior to elucidate the long-term impact of variations in gynecological surgery on cognition. Serum hormone levels and ovarian follicle estimates were also assessed to understand how ovarian steroidogenesis and follicular development could be impacted by gynecological surgery manipulations.

Methods

Subjects

Three cohorts of forty 5-month-old female, virgin, reproductively-intact Fischer-344-CDF rats from the National Institute on Aging colony at Charles Rivers Laboratories (Raleigh, NC) were utilized in this experiment (N=120). Upon arrival to the Arizona State University animal facility, rats were pair-housed, provided free access to food and water, and were maintained in a temperature- and humidity- controlled environment on a 12-hour light/dark cycle (lights on at 7 am, lights off at 7 pm). Experimental procedures commenced after allowing 1 week of acclimation to the facility. All experimental procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards.

All rats were 5 months old at the beginning of the experiment. The Adult cohort was tested 6 weeks after surgery, when rats were approximately 7 months of age. The Middle-Aged cohort was tested 7 months after surgery, when rats were approximately 12 months of age. The Aged cohort was tested 12 months after surgery, when rats were approximately 18 months of age.

Surgical Procedures

For each cohort of rats (N=40/cohort), 10 subjects were randomly assigned to one of the following surgical conditions: Sham, Ovx, Hysterectomy, or Ovx-Hysterectomy. Surgeries were performed one week (±1 day) after arriving to the animal facility. Isoflurane, a vaporized inhaled anesthetic was used. Each rat received subcutaneous injections of 1.0 mg/kg meloxicam (a non-steroidal anti-inflammatory drug), 1.2 mg/kg buprenorphine SR-LAB (an extended-release opioid analgesic), and 5.0 mL of Lactated Ringer's Solution to maintain hydration post-surgery. Following sterilization of the surgical field, all rats received a ventral midline incision through the skin and muscle to expose the peritoneal cavity. The Sham group only underwent these incisions; abdominal viscera was not further manipulated. For the Ovx group, each ovary was exposed, ligated with dissolvable Vicryl suture along with the oviduct and tip of the uterine horn, and removed. For the Hysterectomy group, each uterine horn was exposed, ligated, and cut below the ovary and oviduct. Then, the uterine body was separated from the abdominal fat and ligated at the utero-cervical junction prior to excision. For the Ovx-Hysterectomy group, the ovaries, oviducts, and uterus were separated from the abdominal fat, and the uterine body was ligated at the utero-cervical junction, which was then removed. Following each surgical procedure, the muscle incision was sutured with dissolvable Vicryl suture. Topical 0.25% bupivicane (Marcaine, Pfizer Pharmaceutical, Hospira Inc., Lake Forest, IL), was applied to the incision prior to closing the skin with surgical staples. Rats recovered under an escapable heat lamp following surgery. All subjects were single-housed for one week after surgery prior to being re-pair-housed with a cage mate for the remainder of the study. Two rats died following surgery from the Middle-

Aged Cohort (2 Ovx-Hysterectomy rats), and 3 rats died following surgery from the Aged Cohort (2 Ovx rats and 1 Hysterectomy rat). Over the course of this year-long experimental design, some natural attrition occurred in the Middle-Aged and Aged cohorts as well. The final n/group for each cohort was as follows: Adult (6 week cohort): Sham = 10, Ovx = 10, Hysterectomy = 10, Ovx-Hysterectomy = 10; Middle-Aged (7 month cohort): Sham = 10, Ovx = 10, Hysterectomy = 9, Ovx-Hysterectomy = 8; Aged (12 month cohort): Sham = 10, Ovx = 8, Hysterectomy = 7, Ovx-Hysterectomy = 9.

Weekly Weights and Vaginal Cytology Monitoring

Baseline weight was recorded for each rat at the time of surgery. Body weights were then recorded by an experimenter weekly for the entirety of the experiment, as well as on the day of euthanization for each cohort.

Rats in each cohort underwent vaginal cytology monitoring two weeks after surgery for eight consecutive days. The Adult (6 week) cohort was smeared 24 hours prior to euthanization and on the day of euthanization. The Middle-Aged and Aged cohorts were smeared again for eight consecutive days six months after surgery, prior to the Middle-Aged cohort's behavior testing. The Middle-Aged (7 month) cohort was then smeared 24 hours prior to euthanization and on the day of euthanization. Finally, the Aged cohort was smeared 12 months after surgery for eight consecutive days, prior to starting behavior testing. Finally, the Aged cohort was smeared 24 hours prior to euthanization and on the day of euthanization.

Vaginal cytology classification was completed in order to assess normal estrous

cyclicity in rats with intact ovaries (i.e. the Sham and Hysterectomy groups) as well as confirm Ovx status in rats without intact ovaries (i.e. the Ovx and Ovx-Hysterectomy groups). Smear cytology was classified as proestrus, estrus, metestrus, or diestrus, according to Goldman et al. (2007) and Koebele and Bimonte-Nelson (2016). Proestrus smears were characterized by round nucleated epithelial cells with or without some cornified cells. Estrus was classified as cornified cells. Metestrus was considered a combination of cornified, needle-like, leukocytes, and round epithelial cells. Diestrus consisted of the presence of leukocytes, with or without cornified cells (Goldman et al., 2007; Koebele & Bimonte-Nelson, 2016).

Behavioral Tasks

Water Radial-Arm Maze. The water radial-arm maze (WRAM) was a water-escape, win-shift task that evaluated spatial working and reference memory, as previously described (Bimonte-Nelson et al., 2015; Bimonte & Denenberg, 1999; Koebele et al., 2019). The maze apparatus had a circular center with eight evenly-sized arms (38.1 cm long x 12.7 cm wide) radiating from the center arena in a circular fashion. The maze was filled with room temperature water maintained at 18-20°C throughout the testing period. Water was made opaque with black nontoxic powdered paint. Four out the eight arms had platforms submerged ~2 cm below the surface of the water. The pattern of arm locations for the hidden platforms stayed the same within a rat across all testing days, but varied among rats, and platform location patterns were counterbalanced across treatment groups. The testing rooms had clear spatial cues placed around the room to facilitate spatial navigation to the platforms. Rats were given four trials per day for 12 baseline days.

During each daily testing session, at the beginning of the first trial, the rat was released into the non-platformed start arm and allotted 3 minutes to locate a platform. If a platform was not discovered after the maximum allotted trial time, the experimenter gently guided the rat to the nearest platform. Once the rat found or was led to the platform, it was allowed to remain on it for 15 seconds before the experimenter gently removed the rat from the maze and placed it back into its testing cage for a 30 second inter-trial-interval (ITI). During those 30 seconds, the experimenter removed the just-found platform from the maze and used a net to stir the water to distribute potential olfactory cues and remove any debris from the water. The rat was then placed back in the water for trial 2 with the 3 remaining platforms remaining. This procedure was repeated until all four platforms were located within the daily testing session. The experimenter manually recorded arm entries during all testing trials. An arm entry was quantified when the rat's snout passed an 11 cm mark on the outside of a given arm, which was only visible to the experimenter and not visible the rat. On the thirteenth day of testing, a six-hour delay was incorporated between trials 2 and 3 in order to assess delayed working memory retention. Across all days of testing, arm entries made prior to locating a platform on a given trial were considered errors and were further classified into three error subtypes. Working memory correct (WMC) errors were arm entries made on trials 2-4 into arms that previously contained a platform during the daily testing session. Reference memory (RM) errors were the first entries into a never-platformed arm within a day. Working Memory Incorrect (WMI) errors were quantified when a rat subsequently re-entered a never-platformed arm within the daily testing.

Morris Water Maze. Following one day of rest after the WRAM, all rats were tested on the spatial reference memory Morris water maze (MM) task as previously described (Bimonte-Nelson et al., 2015; Morris, 2015; Morris et al., 1982). The apparatus was a circular tub (188 cm in diameter). The tub was filled with 18-20°C water made opaque with nontoxic powdered black paint. One platform (11 cm in diameter) was submerged ~2 cm below the water's surface in the northeast quadrant of the maze, where it remained constant across all days and trials. Obvious spatial cues were placed around the room to aid in spatial navigation. The rats received four trials per day for five days. For each trial within a daily testing session, the rats were each dropped off from one of four possible starting points (north, south, east, or west). The trial drop-off location order varied across days, but was the same for all rats within a day. The rat was allotted a maximum of 60 seconds to locate the submerged platform. If the rat was unable to find the platform after 60 seconds, the experimenter gently guided the rat to the platform with a lead stick, where it remained for 15 seconds before being returned to its heated testing cage for an ITI of approximately 10 minutes. Between each trial the water was stirred and cleared of any debris. On day five of the task, after the four baseline trials were complete, an additional probe trial was administered. During the probe trial, the tester completely removed the platform from the maze, and each rat was allowed to swim for 60 seconds to test whether the rats had successfully spatially localized to the fixed platform location in this maze. A digital video camera and Ethovision tracking system (Ethovision; Noldus Instruments, Wageningen, Netherlands) were utilized to track the rat's swim distance and path (cm) to the platform during each trial.

Visible Platform. The day following the completion of the MM task, all rats were tested on the control Visible Platform task. This task evaluates the motoric and visual competency of the rats to successfully solve a water maze task. The apparatus was a small, rectangular tub (100 cm x 60 cm) filled with 18-20°C clear water. A visible black platform (10 cm in diameter) protruded about 4 cm above the surface of the water. The tub was surrounded by opaque curtains to block any potential extra-maze cues. Each rat underwent six trials within one day. The visible platform location varied across trials (left, middle, or center of the north wall of the tub), but the location within a given trial was the same for all rats. Each rat was dropped off from a fixed start location in the middle of the south wall of the tub and had a maximum of 90 seconds to locate the visible platform. Once located, the rat remained on it for 15 seconds before the experimenter returned the rat to its heated testing cage for an ITI of approximately 10 minutes while the other rats were being tested. The experimenter recorded latency (seconds) to reach the visible platform for each subject and trial.

Open Field Task. After two days of rest following the Visible Platform task, all rats underwent testing in the open field task (OFT), a measure of locomotor activity and anxiety-like behavior. Task performance has been reported to be sensitive to the presence and absence of hormones (Hiroi & Neumaier, 2006). The open field apparatus was a 100 x 100 square arena made of black Plexiglas. Twenty-four hours prior to the task, the arena was thoroughly cleaned with Odormute, an enzyme cleaner used to eliminate odors. A red light visible to the experimenter was used in the room, such that the task was run in darkness for the rats, as has been previously done (Hiroi & Neumaier, 2006). Rats were allowed to acclimate in the anteroom of the testing area for at least 30 minutes in their

individual testing cages prior to OFT testing. Each rat was brought into the testing room individually. The experimenter gently placed the rat in the arena from the center of the north wall and quietly exited the room. The rat was allowed to explore the box freely for a 10-minute trial. Ethovision was used to record and track the rat's movements in the OFT. Twenty-five equally spaced squared were digitally overlaid onto the arena in order to track distance moved and time spent in the small center, center, and corners of the arena. Following the 10-minute trial, the rat was removed from the arena. Any fecal boli were removed from the arena; the experimenter wiped the arena with water and then dried it prior to starting the next rat's trial. This was completed in order to evenly distribute any odors left by a previously tested rat throughout the arena.

Euthanasia

One week following the OFT, rats were euthanized. The Adult cohort was approximately 7 months old, the Middle-Aged cohort was approximately 12 months old, and the Aged cohort was approximately 18 months old at sacrifice. All rats were deeply anesthetized using inhaled isoflurane anesthesia. Blood was obtained via cardiocentesis, and allowed to clot at 4°C for a minimum of 30 minutes (Vacutainer 367986, Becton Dickinson and Company, Franklin Lakes, NJ) prior to centrifugation at 2000 rpm at 4°C for 20 minutes. Serum was extracted and stored at -20°C until analysis. Rats were decapitated and brains were rapidly removed. The right hemisphere of the brain was rapidly raw dissected for frontal cortex, dorsal hippocampus, entorhinal cortex, perirhinal cortex, and ventral CA1/2 areas. The raw dissected tissues were then weighed, snap frozen, and stored at -70°C until analysis. The left hemisphere was post-fixed in 4%

paraformaldehyde for 48 hours and then transferred to 0.1M phosphate buffered solution until analysis. Ovaries were collected from ovary-intact groups (i.e. the Sham and Hysterectomy groups), trimmed of excess fat, weighed, and transferred to 10% buffered formalin for 48 hours prior to being stored in 70% ethanol until analysis. Uteri were collected from uterus-intact groups (i.e. the Sham and Ovx groups), trimmed of excess fat, and wet weight was obtained.

Serum Hormone Assays

All serum hormone assays were completed by the Center for Research in Reproduction Ligand Assay and Analysis Core Laboratory at the University of Virginia. Samples were run in duplicate unless otherwise noted. 17β-Estradiol (E2) levels were determined with a commercially-available ELISA kit (CAT# ES180S-100, CALBIOTECH, Spring Valley, CA). E2 inter-assay coefficients of variation averaged 9.7% (intra-assay 6.7%) with a functional sensitivity of 3 pg/mL and a reportable range of 3 – 300 pg/mL. Progesterone levels were determined with a commercially-available ELISA kit (CAT# IB79105, Immuno-Biological Laboratories Inc., Minneapolis, MN). Inter-assay coefficients of variation averaged 10.5% (intra-assay 6.6%) with a functional sensitivity of 0.045 ng/mL and a reportable range of 0.150 – 40.00 ng/mL. Androstenedione levels were determined with a commercially available ELISA kit (CAT# 11-ANRHU-E01, ALPCO, Salem, NH). Inter-assay coefficients of variation averaged 9.1% (intra-assay 5.9%) with a functional sensitivity of 0.04 ng/mL and a reportable range of 0.1- 10.0 ng/mL. E2 and androstenedione levels were measures for ovary-intact rats only. Progesterone levels were obtained for all subjects.

Ovarian Follicle Counts

All ovarian histology and quantification was completed by Senestech, Inc (Flagstaff, AZ) with methods previously described (Koebele et al., 2017, 2019). For each cohort, one ovary from each Sham and Hysterectomy subject (i.e., all rats that had ovaries at the end of the experiment) was randomly selected for histological evaluation and quantification of healthy primordial, primary, secondary, and antral follicles, as well as corpora lutea. Following fixation, ovarian tissues were paraffin embedded and sectioned at 5 µm. Every 10th section was mounted on a slide and stained with hematoxylin and eosin Y-phloxine B. Primordial, primary, secondary, and antral follicles were counted at 20x magnification for every 20th section. Corpora lutea were counted at a lower magnification (Pannoramic DESK; 3D Histech, Budapoest, Hungary). The following formula was used to calculate the total number of follicles in the ovary: $N_t = (N_0 \times S_t \times t_s) / (S_0 \times d_0)$, where N_t = total calculated number of follicles, N_0 = number of follicles observed in the ovary, S_t = total number of sections in the ovary, t_s = thickness of the section (µm), S_0 = total number of sections observed, and d_0 = mean diameter of the nucleus (Gougeon & Chainy, 1987). Ovarian follicles were classified using criteria from Haas et al., 2007 (Haas et al., 2007). Primordial cells were counted when there was a single layer of squamous granulosa cells surrounding the oocyte. Primary follicles contained a single layer of cuboidal granulosa cells. Secondary follicles required several granulosa cell layers surrounding the oocyte. Antral follicles were counted when the follicle contained two or more layers of granulosa cells and had a fluid-filled antral space in the follicle (Haas et al., 2007). Atretic/unhealthy follicles were not counted.

Statistical Analyses

Statview software was utilized to complete all statistical analyses. The independent variable for all analyses was Surgery type (Sham, Ovx, Hysterectomy, and Ovx-Hysterectomy). All analyses were two-tailed tests with an alpha level set to 0.05 unless otherwise noted. Two-group planned comparisons were completed between Hysterectomy and each other surgery group for all analyses. Results were deemed significant if the *p* value was < 0.05, and marginal if the *p* value fell between 0.05 and 0.10.

WRAM data were analyzed using repeated measures, with trials within days as the repeated measures, and the dependent variable as Errors. WRAM data were divided into two blocks, similarly to how our laboratory has previously reported (Bimonte & Denenberg, 1999; Bimonte et al., 2000; Braden et al., 2016; Koebele et al., 2017; Mennenga, Gerson, et al., 2015; Prakapenka et al., 2018). For all cohorts, Day 1 was considered training and was not included in the analyses. Days 2-7 were considered the Learning Phase and Days 8-12 were considered the Asymptotic Phase. Planned two-group comparisons were completed for high working memory load trials, Trial 3 and Trial 4, for each error type within the Learning Phase and Asymptotic Phase, as previously published (Koebele et al., 2019; Prakapenka et al., 2018). Separate analyses were run for each error type quantified in the WRAM (i.e., WMC, WMI, RM).

MM data were also evaluated using two-group planned comparison repeated measures ANOVA, with trials within days as repeated measures, and the dependent variable as Swim Distance (cm) to Platform for all baseline trials. For the probe trial, Percent Total Swim Distance (cm) was evaluated in the target versus the opposite

quadrant for each treatment group. Visible Platform data were assessed using repeated measures, with trial as the repeated measure and Latency (sec) as the dependent variable.

OFT distance and time measures were analyzed using ANOVA for all planned comparisons. Serum ovarian hormone levels, ovarian follicle counts, body weights, ovary weights, and uterine weights were also analyzed using ANOVA with each of those respective measurements as the dependent variable for all planned comparisons.

Three rats were excluded from all analyses within the Middle-Aged cohort due to consistent floating behavior, and thus failure to perform in the WRAM task. Two rats were excluded from all analyses within the Aged cohort due to deteriorated health. Unless otherwise noted, the final n/group for each cohort was as follows: Adult (6-week cohort): Sham = 10, Ovx = 10, Hysterectomy = 10, Ovx-Hysterectomy = 10; Middle-Aged (7-month cohort): Sham = 10, Ovx = 9, Hysterectomy = 9, Ovx-Hysterectomy = 6; Aged (12-month cohort): Sham = 9, Ovx = 8, Hysterectomy = 6, Ovx-Hysterectomy = 9.

Results

Adult (6 Week) Cohort WRAM

Learning Phase (Days 2-7). Based on recent findings regarding hysterectomy-induced working memory deficits 6 weeks post-surgery (Koebele et al., 2019) and other reports revealing working memory deficits when working memory load becomes taxed in rats, we planned to evaluate working memory performance on Trial 3, the moderate working memory load trial, and Trial 4, the maximum working memory load trial, using two-group comparisons between the Hysterectomy group and each other surgery group.

Performance across trials for WMC errors is demonstrated in Figure 59A and for WMI errors in 59B. For WMC errors on Trial 3, Hysterectomy and Ovx-Hysterectomy groups were significantly different from each other [$F_{(1,18)}$=6.82, $p < 0.05$], such that Hysterectomy rats made more WMC errors than Ovx-Hysterectomy rats (Figure 59C). Furthermore, for WMI errors, two-group comparisons revealed that Hysterectomy rats made more WMI errors on Trial 3 compared to Sham rats [$F_{(1,18)}$=8.82, $p < 0.01$] and trended toward making more WMI errors when compared to Ovx-Hysterectomy rats [$F_{(1,18)}$=3.17, $p = 0.09$], although this latter two-group comparison was marginal (Figure 59D). That Hysterectomy rats made more WMI errors compared to Sham rats six weeks after surgery is a replication of our prior work (Koebele et al., 2019). There were no main effects of Surgery for Trial 4 alone during this block for any planned comparison (Figure 59E-F). There were no effects for RM errors (data not shown), pointing to a working-memory specific impairment in the Hysterectomy group.

Asymptotic Phase (Days 8-12). There were no significant differences in performance for WMC or WMI in the Asymptotic Phase of testing for any planned comparison, suggesting that all surgery groups successfully learned the WRAM task by the latter portion of testing and were able to similarly handle a working memory load, regardless of surgery type (Figure 60A-F). There were no effects for RM errors in the Asymptotic Phase (data not shown).

Delayed Memory Retention. Following the last day of baseline testing on the WRAM, a six-hour delay was introduced between trials 2 and 3 on Day 13. Performance was evaluated on Trial 3 (the first post-delay trial) compared to Trial 3 performance on the last baseline day for each surgery group. A main effect of Delay Day was present for

Ovx rats [$F_{(9,1)}$=15.00, $p < 0.01$], Hysterectomy rats [$F_{(9,1)}$=6.43, $p < 0.05$], and Ovx-Hysterectomy rats [$F_{(9,1)}$=14.23, $p < 0.01$], where more WMC errors were committed in the post-delay trial compared to the previous day's performance. Sham rats did not show a significant delay-induced working memory impairment (Figure 61).

Adult (6 Week) Cohort Morris Water Maze

Learning curves for the MM task are visualized in Figure 62A. Within each planned group comparison, there were no main effects of Surgery. There was a main effect of Day within each comparison (Hysterectomy versus Sham: [$F_{(4,72)}$=37.22, $p < 0.0001$]; Hysterectomy versus Ovx: [$F_{(4,72)}$=30.23, $p < 0.0001$]; Hysterectomy versus Ovx-Hysterectomy: [$F_{(4,72)}$=18.05, $p < 0.0001$]), indicating learning across the five days of baseline testing. Each Surgery group was evaluated independently for probe trial performance. There was a main effect of Quadrant for all groups (Sham: [$F_{(9,1)}$=80.79, $p < 0.0001$]; Ovx: [$F_{(9,1)}$=103.59, $p < 0.0001$]; Hysterectomy: [$F_{(9,1)}$=118.30, $p < 0.0001$]; Ovx-Hysterectomy: [$F_{(9,1)}$=125.63, $p < 0.0001$]), where all subjects spent a greater proportion of total swim distance in the previously platformed quadrant compared to the opposite quadrant, indicating spatial localization to the platform location (Figure B).

Adult (6 Week) Cohort Visible Platform

Each group was evaluated individually for performance on the Visible Platform. The average latency to platform for the Sham group on Trial 1 was 10.62 ± 1.80 seconds, and the average latency to platform on Trial 6 was 4.08 ± 1.06 seconds. For Ovx rats, the

average latency to platform for Trial 1 was 10.16 ± 2.72 seconds, and the average latency to platform on Trial 6 was 6.33 ± 1.73 seconds. For Hysterectomy rats, the average latency to platform on Trial 1 was 10.42 ± 2.87 seconds, and the average latency to platform on Trial 6 was 5.55 ± 1.32 seconds. Ovx-Hysterectomy rats had an average latency to platform of 10.85 ± 2.93 seconds on Trial 1, and an average latency to platform on Trial 6 of 5.94 ± 1.50 seconds. Overall, all groups' average latency to platform was lower by the last trial of Visible Platform testing. By Trial 6, all rats located the visible platform in 20 seconds or less, indicating that all subjects had intact visual and motor procedural skills to perform a water maze task.

Adult (6 Week) Cohort Open Field Task

There was a marginal main effect of Total Distance for the Ovx versus Hysterectomy comparison [$F_{(1,18)}$=3.86, p = 0.06], where Ovx rats tended to move less than Hysterectomy rats overall (data not shown). There were no other significant or marginal main effects in any of the measured variables for any comparison at this time point.

Adult (6 Week) Cohort Vaginal Smears

Vaginal smears were collected beginning two weeks after surgery for eight consecutive days. Ovx and Ovx-Hysterectomy rats all displayed diestrus-like or blank smears, indicating successful ovary removal and a lack uterine stimulation. Sham and Hysterectomy groups exhibited normal cyclic estrous activity consisting of 4-5 day

estrous cycles. Vaginal smears were also collected one day prior to euthanization and on the day of euthanization. Ovx and Ovx-Hysterectomy groups continued to display diestrus-like or blank smears, and Sham and Hysterectomy rats were in various phases of the estrous cycle, such that all phases (proestrus, estrus, metestrus, and diestrus) were each represented in both ovary-intact groups on the day of euthanization.

Adult (6 Week) Cohort Body Weights

Two-group comparisons were made between all Surgery conditions at the euthanasia time point. There was a main effect of Surgery for Body Weight for the Ovx versus Hysterectomy comparison [$F_{(1,18)}$=96.35, $p < 0.0001$], where Ovx rats weighed more than Hysterectomy rats. There was also a main effect of Body Weight for the Ovx-Hysterectomy versus Hysterectomy comparison [$F_{(1,18)}$=72.35, $p < 0.0001$], where Ovx-Hysterectomy rats weighed more than Hysterectomy rats. Sham rats weighed less compared to Ovx rats [$F_{(1,18)}$=47.82, $p < 0.0001$] and compared to Ovx-Hysterectomy rats [$F_{(1,18)}$=37.36, $p < 0.0001$] (Figure 63A).

Adult (6 Week) Cohort Uterine Weights

Only rats that retained their uterus throughout the experiment (i.e., Sham and Ovx rats) were compared. As expected, there was a main effect of uterine weight between Sham and Ovx rats [$F_{(1,18)}$=58.56, $p < 0.0001$], such that uterine weights for Ovx rats were lower than Sham rats due to a lack of estrogen stimulation from the ovaries (Engler-Chiurazzi et al., 2012; Koebele et al., 2019; Mennenga, Gerson, et al., 2015; Prakapenka

et al., 2018; Westerlind et al., 1998). When uterine weights were corrected for body weight, the main effect of Surgery persisted [$F_{(1,18)}$=53.12, $p < 0.0001$] (Figure 63B).

Adult (6 Week) Cohort Ovary Weights and Ovarian Follicle Estimates

Only rats that retained their ovaries throughout the experiment (i.e., Sham and Hysterectomy rats) were compared. When ovary weight was summed, there was no difference in weight between groups at this time point (Figure 63C).

There were no main effects of Surgery for Primordial Follicles [$F_{(1,18)}$=0.06, p = NS], Primary Follicles [$F_{(1,18)}$=0.33, p = NS], Secondary Follicles [$F_{(1,18)}$=0.28, p = NS], Antral Follicles [$F_{(1,18)}$=2.07, p = NS], or Corpora Lutea Counts [$F_{(1,18)}$=0.64, p = NS] at this time point (Figure 64A-E).

Adult (6 Week) Cohort Serum Hormone Levels

One Sham subject and one Hysterectomy subject were excluded for Progesterone analyses due to serum values outside the detectable range of the assay. There were no differences between Sham and Hysterectomy groups [$F_{(1,16)}$=0.50, p = NS]. However, Hysterectomy rats had higher Progesterone levels compared to Ovx rats [$F_{(1,17)}$=11.19, $p < 0.01$] and compared to Ovx-Hysterectomy rats [$F_{(1,17)}$=10.04, $p < 0.01$]. Sham rats also had higher Progesterone levels compared to Ovx rats [$F_{(1,17)}$=10.38, $p < 0.01$] and compared to Ovx-Hysterectomy rats [$F_{(1,17)}$=9.58, $p < 0.01$] (Figure 65A). Ovx groups did not differ from each other.

Three Sham subjects and two Hysterectomy subjects were excluded for Androstenedione analyses due to serum values outside the detectable range of the assay. One value in the Sham group was based on a singlet value, as the other singlet was outside the detectable range. There were no differences in Androstenedione levels between Sham and Hysterectomy rats [$F_{(1,13)}$=0.161, p = NS; Figure 65B].

One Sham subject and one Hysterectomy subject were excluded for E2 analyses due to serum values outside the detectable range of the assay. One value in the Hysterectomy group was based on a singlet value, as the other singlet was outside the detectable range. There were no differences in E2 levels between Sham and Hysterectomy rats [$F_{(1,16)}$=1.20, p = NS; Figure 65C].

Middle-Aged (7 month) Cohort WRAM

Learning Phase (Days 2-7). Performance by Trial during the Learning Phase, averaged across days, is shown in Figure 66A (WMC errors) and Figure 66B (WMI errors). For Trial 3, there was a main effect of Surgery for WMC errors between Hysterectomy and Ovx rats [$F_{(1,16)}$=4.83, $p < 0.05$] and between Hysterectomy and Ovx-Hysterectomy rats [$F_{(1,13)}$=9.49, $p < 0.01$]. There was also a marginal main effect of Surgery on Trial 3 WMC errors between Sham and Hysterectomy rats [$F_{(1,17)}$=3.25, p = 0.09], where Hysterectomy rats made more WMC errors on Trial 3 than each group (Figure 66C). For WMI errors on Trial 3, there was a main effect of Surgery between Sham and Hysterectomy rats [$F_{(1,17)}$=4.62, $p < 0.05$], as well as between Ovx and Hysterectomy rats [$F_{(1,16)}$=11.80, $p < 0.01$] with Hysterectomy rats making more errors than Sham and Ovx rats, respectively (Figure 66D).

There were no effects of Surgery for WMC errors on Trial 4 (Figure 66E). However, for WMI errors on Trial 4, Hysterectomy and Ovx-Hysterectomy rats differed [$F_{(1,13)}$=5.27, $p < 0.05$], with Hysterectomy rats making more errors than Ovx-Hysterectomy rats (Figure 66F). There were no main effects of RM for any comparison (data not shown).

Asymptotic Phase (Days 8-12). Performance by Trial during the Asymptotic Phase, averaged across days, is shown in Figure 67A for WMC errors and Figure 67B for WMI errors. There were no main effects or interactions with Surgery for WMC and WMI errors during the Asymptotic Phase for this cohort, indicating that by the end of WRAM testing, all subjects could similarly handle the working memory load, regardless of Surgery condition (Figure 67C-F). There were no effects for RM for any comparison at this time point (data not shown).

Delayed Memory Retention. Following the last day of baseline testing on the WRAM, a 6 hour delay was introduced between trials 2 and 3 on Day 13. Performance was evaluated on Trial 3 (the first post-delay trial) compared to Trial 3 performance on the last baseline day for each surgery group. A main effect of Delay Day was present for Ovx-Hysterectomy rats [$F_{(5,1)}$=7.35, $p < 0.05$; Figure 68D], where more WMC errors were made on Trial 3 following the six-hour delay. A marginal effect of Delay Day was present for Sham rats [$F_{(9,1)}$=4.37, $p = 0.07$; Figure 68A] and for Hysterectomy rats [$F_{(8,1)}$=5.26, $p = 0.05$; Figure 68C], with the six-hour delay resulting in increased WMC errors on Trial 3 for each group. Ovx rats did not exhibit increased WMC errors on the post-delay trial at this time point (Figure 68B).

Middle-Aged (7 month) Cohort Morris Water Maze

Within each planned comparison, there were no main effects of Surgery. There was a main effect of Day within each comparison (Hysterectomy versus Sham: [$F_{(4,68)}$=12.64, $p < 0.0001$]; Hysterectomy versus Ovx: [$F_{(4,64)}$=8.73, $p < 0.0001$]; Hysterectomy versus Ovx-Hysterectomy: [$F_{(4,52)}$=17.20, $p < 0.0001$]), indicating learning across the five days of baseline testing (Figure 69A). Each Surgery group was evaluated independently for probe trial performance. There was a main effect of Quadrant for all groups (Sham: [$F_{(9,1)}$=29.12, $p < 0.001$]; Ovx: [$F_{(8,1)}$=67.11, $p < 0.0001$]; Hysterectomy: [$F_{(8,1)}$=38.59, $p < 0.001$]; Ovx-Hysterectomy: [$F_{(5,1)}$=14.10, $p < 0.05$]), where all rats spent a greater proportion of total swim distance in the previously platformed quadrant compared to the opposite quadrant, indicating spatial localization to the platform location (Figure 69B).

Middle-Aged (7 month) Cohort Visible Platform

Each group was evaluated individually for performance on the Visible Platform. The average latency to platform for the Sham group on Trial 1 was 9.71 ± 2.19 seconds, and the average latency to platform on Trial 6 was 6.15 ± 0.94 seconds. For Ovx rats, the average latency to platform for Trial 1 was 12.16 ± 4.05 seconds, and the average latency to platform on Trial 6 was 3.12 ± 0.51 seconds. For Hysterectomy rats, the average latency to platform on Trial 1 was 9.99 ± 2.78 seconds, and the average latency to platform on Trial 6 was 3.81 ± 1.24 seconds. Ovx-Hysterectomy rats had an average latency to platform of 10.50 ± 2.44 seconds on Trial 1, and an average latency to platform on Trial 6 of 5.83 ± 2.92 seconds. Overall, all groups average latency to platform was

lower by the last trial of testing. By Trial 6, all rats located the visible platform in 20 seconds or less, indicating that all subjects had intact visual and motor procedural skills to perform a water maze task.

Middle-Aged (7 month) Cohort Open Field Task

There was a marginal main effect of Total Distance Moved [$F_{(1,13)}$=3.79, p = 0.08] and Center Distance [$F_{(1,13)}$=3.61, p = 0.08] for the Ovx-Hysterectomy versus Hysterectomy comparison, where Hysterectomy rats tended to move more overall; the trend toward greater distance traveled in the center is likely an artifact of overall increased movement. There were no other marginal or significant effects for any OFT measure at this time point (data not shown).

Middle-Aged (7 month) Cohort Vaginal Smears

Vaginal smears were collected beginning two weeks after surgery for eight consecutive days, when rats were approximately 6 months of age. All Ovx and Ovx-Hysterectomy rats displayed diestrus-like or blank smears, indicating successful ovary removal and a lack uterine stimulation. Sham and Hysterectomy groups exhibited normal cyclic estrous activity consisting of 4-5 day estrous cycles. Eight consecutive days prior to behavior testing, vaginal smears were collected again for this cohort, when rats were approximately 12 months of age. All Ovx and Ovx-Hysterectomy rats continued to display diestrus-like or blank vaginal smears. All Sham and Hysterectomy rats displayed regular estrous cycle activity at this time point as well. Lastly, after behavior testing was

complete, vaginal smears were also collected one day prior to euthanization and on the day of euthanization. Ovx and Ovx-Hysterectomy groups continued to display diestrus-like or blank smears, and Sham and Hysterectomy rats were in various phases of the estrous cycle, such that all phases (proestrus, estrus, metestrus, and diestrus) were each represented in both ovary-intact groups within 24 hours of euthanization.

Middle-Aged (7 month) Cohort Body Weights

Hysterectomy rats weighed less than Ovx rats [$F_{(1,16)}$=23.13, $p < 0.001$] and Ovx-Hysterectomy rats [$F_{(1,13)}$=31.50, $p < 0.0001$]. Sham rats also weighed less than Ovx rats [$F_{(1,17)}$=28.39 $p < 0.0001$] and Ovx-Hysterectomy rats [$F_{(1,14)}$=45.25, $p < 0.0001$]. Ovx and Ovx-Hysterectomy rats marginally differed from each other at the euthanasia time point [$F_{(1,13)}$=4.13, $p = 0.06$], where Ovx-Hysterectomy rats tended to weigh more than Ovx rats (Figure 70A).

Middle-Aged (7 month) Cohort Uterine Weights

Only rats that retained their uterus throughout the experiment (i.e., Sham and Ovx rats) were compared. As expected, there was a main effect of uterine weight between Sham and Ovx rats [$F_{(1,17)}$=51.76, $p < 0.0001$], such that uterine weights for Ovx rats were lower than Sham rats due to a lack of estrogen stimulation from the ovaries (Engler-Chiurazzi et al., 2012; Koebele et al., 2019; Mennenga, Gerson, et al., 2015; Prakapenka et al., 2018; Westerlind et al., 1998). When uterine weights were corrected for body weight, the main effect of Surgery persisted [$F_{(1,17)}$=51.53, $p < 0.0001$] (Figure 70B).

Middle-Aged (7 month) Cohort Ovary Weights and Ovarian Follicle Estimates

Only rats that retained their ovaries throughout the experiment (i.e., Sham and Hysterectomy rats) were compared. There was no effect of Surgery on ovary weight at this time point (Figure 70C).

There were no main effects of Surgery for Primordial Follicles [$F_{(1,17)}$=0.03, p = NS], Primary Follicles [$F_{(1,17)}$=0.04, p = NS], Secondary Follicles [$F_{(1,17)}$=1.23, p = NS], Antral Follicles [$F_{(1,17)}$=0.68, p = NS], or Corpora Lutea Counts [$F_{(1,17)}$=2.67, p = NS] at this time point (Figure 71A-E).

Middle-Aged (7 month) Cohort Serum Hormone Levels

Only subjects included in behavioral analyses were included in serum assessments. Additionally, two Sham subjects, two Hysterectomy subjects, and one Ovx subject were excluded for Progesterone analyses due to serum values outside the detectable range of the assay. There were no differences in Progesterone levels between Sham and Hysterectomy rats [$F_{(1,13)}$=0.43, p = NS]. However, Hysterectomy rats had more Progesterone compared to Ovx rats [$F_{(1,13)}$=10.62, $p < 0.01$] and compared to Ovx-Hysterectomy rats [$F_{(1,11)}$=12.24, $p < 0.01$]. Sham rats also had more Progesterone compared to Ovx rats [$F_{(1,14)}$=9.62, $p < 0.01$] and compared to Ovx-Hysterectomy rats [$F_{(1,12)}$=8.79, $p < 0.05$] (Figure 72A). Ovx groups did not differ from each other.

One Hysterectomy subject was excluded for Androstenedione analyses due to serum values outside the detectable range of the assay. Two values in the Sham group and one value in the Hysterectomy group were based on a singlet value, as the other

singlet was outside the detectable range. There were no differences in Androstenedione levels between Sham and Hysterectomy rats [$F_{(1,16)}$=0.23, p = NS; Figure 72B].

Six Sham subjects and four Hysterectomy subjects were excluded for E2 analyses due to serum values outside the detectable range of the assay. Additionally, one value in the Hysterectomy group was based on a singlet value, as the other singlet was outside the detectable range. There were no differences in E2 levels between Sham and Hysterectomy rats [$F_{(1,7)}$=0.89, p = NS; Figure 72C].

Aged (12 month) Cohort WRAM

Learning Phase (Days 2-7). Performance by Trial on the Learning Phase, averaged across days, can be seen in Figure 73A (WMC errors) and Figure 73B (WMI errors). For Trial 3, there were no differences in WMC or WMI errors committed in any two-group comparison (Figure 73C,D). For Trial 4, marginal main effects of Surgery for WMC errors were present for the Sham versus Hysterectomy comparison [$F_{(1,13)}$=4.11, p = 0.06] and Ovx versus Hysterectomy comparison [$F_{(1,12)}$=4.20, p = 0.06], where Hysterectomy rats tended to make more WMC errors than each comparison group on Trial 3 (Figure 73E). For WMI errors on Trial 4, there was a main effect of Surgery for the Sham versus Hysterectomy comparison [$F_{(1,13)}$=7.11, p < 0.05] and Ovx versus Hysterectomy comparison [$F_{(1,12)}$=14.36, p < 0.01], where Hysterectomy rats made more WMI errors on the maximum working memory load trial than Sham and Ovx rats, respectively. There was also a marginal main effect of Surgery on WMI errors on Trial 4 for the Ovx-Hysterectomy versus Hysterectomy comparison [$F_{(1,13)}$=4.41, p = 0.06],

where Hysterectomy rats tended to make more errors than Ovx-Hysterectomy rats (Figure 73F). There were no RM effects for any planned comparison (data not shown).

Asymptotic Phase (Days 8-12). Performance by Trial on the Asymptotic Phase, averaged across days, can be seen in Figure 74A (WMC errors) and Figure 74B (WMI errors). There were no main effects or interactions with Surgery for any two group comparison for WMC, WMI, or RM errors during the Asymptotic Phase for this cohort. However, there was a marginal effect of Surgery for the Ovx versus Hysterectomy comparison for Trial 4 [$F_{(1,12)}$=3.78, p = 0.08], where Ovx rats tended to make more errors on this high load trial (Figure 74E).

Delayed Memory Retention. When a six-hour delay was implemented between trials 2 and 3 on Day 13 of WRAM, Sham rats made more WMC errors on the post-delay Trial 3 compared to performance on Trial 3 on the last day of baseline testing [$F_{(8,1)}$=16.00, p < 0.011 Figure 75A]. Ovx, Hysterectomy, and Ovx-Hysterectomy rats performed similarly on the post-delay trial, suggesting a delay did not result in significantly impaired working memory for these subjects at this time point (Figure 75B-D).

Aged (12 month) Cohort Morris Water Maze

Within each planned comparison, there were no main effects of Surgery. There was a main effect of Day within each comparison (Hysterectomy versus Sham: [$F_{(4,52)}$=15.77, p < 0.0001]; Hysterectomy versus Ovx: [$F_{(4,48)}$=24.34, p < 0.0001]; Hysterectomy versus Ovx-Hysterectomy: [$F_{(4,52)}$=18.85, p < 0.0001]), indicating learning

across the five days of baseline testing (Figure 76A). Each Surgery group was evaluated independently for probe trial performance. There was a main effect of Quadrant for all groups (Sham: [$F_{(8,1)}$=11.75, $p < 0.01$]; Ovx: [$F_{(7,1)}$=7.52, $p < 0.05$]; Hysterectomy: [$F_{(5,1)}$=11.25, $p < 0.05$]; Ovx-Hysterectomy: [$F_{(8,1)}$=30.49, $p < 0.001$]), where all subjects spent a greater proportion of total swim distance in the previously platformed quadrant compared to the opposite quadrant, indicating spatial localization to the platform location (Figure 76B).

Aged (12 month) Cohort Visible Platform

Each group was evaluated individually for performance on the Visible Platform. The average latency to platform for the Sham group on Trial 1 was 8.31 ± 1.17 seconds, and the average latency to platform on Trial 6 was 4.62 ± 1.13 seconds. For Ovx rats, the average latency to platform for Trial 1 was 17.92 ± 4.26 seconds, and the average latency to platform on Trial 6 was 5.56 ± 1.57 seconds. For Hysterectomy rats, the average latency to platform on Trial 1 was 12.25 ± 2.66 seconds, and the average latency to platform on Trial 6 was 5.25 ± 1.31 seconds. Ovx-Hysterectomy rats had an average latency to platform of 8.24 ± 0.93 seconds on Trial 1, and an average latency to platform on Trial 6 of 4.94 ± 0.97 seconds. Overall, all groups average latency to platform was lower by the last trial of testing. By Trial 6, all rats located the visible platform in 20 seconds or less, indicating that all subjects had intact visual and motor procedural skills to perform a water maze task.

Aged (12 month) Cohort Open Field Task

There was a marginal effect of Surgery in the Ovx versus Hysterectomy comparison for Time Spent in the Corner [$F_{(1,12)}$=3.64, p = 0.08], where Ovx rats tended to spend more time in the corner than Hysterectomy rats. There was a main effect of Total Distance Moved for the Ovx-Hysterectomy versus Hysterectomy rats [$F_{(1,13)}$=5.14, $p < 0.05$], where Hysterectomy rats moved move total distance than Ovx-Hysterectomy rats. There was also a marginal effect of Surgery between Ovx-Hysterectomy and Hysterectomy rats for Center Distance [$F_{(1,13)}$=4.00, p = 0.07], where Hysterectomy rats traveled more distance in the center than Ovx-Hysterectomy rats, although this may be an artifact of overall increased distance moved for Hysterectomy rats because there was a trend toward Hysterectomy rats having a greater distance traveled in the corners compared to Ovx-Hysterectomy rats as well [$F_{(1,13)}$=3.87, p = 0.07] (data not shown).

Aged (12 month) Cohort Vaginal Smears

Vaginal smears were collected beginning two weeks after surgery for eight consecutive days, when rats were approximately 6 months of age. All Ovx and Ovx-Hysterectomy rats displayed diestrus-like or blank smears, indicating successful ovary removal and a lack uterine stimulation. Sham and Hysterectomy groups exhibited normal cyclic estrous activity consisting of 4-5 day estrous cycles. Vaginal smears were collected for this cohort when rats were approximately 12 months of age for eight consecutive days. All Ovx and Ovx-Hysterectomy rats continued to display diestrus-like or blank vaginal smears. All Sham rats displayed regular estrous cycle activity at this time point. Seven out of nine Hysterectomy rats displayed estrous cyclicity; the remaining two

Hysterectomy rats were observed to be in persistent diestrus across all eight days of vaginal smear collection at this middle-aged time point. Eight consecutive days prior to behavior testing, vaginal smears were collected for this cohort, when they were approximately 18 months of age. Seven out of eight Ovx rats displayed diestrus or blank smears; one Ovx rat displayed a persistent estrous phenotype across all eight days of evaluation. All Ovx-Hysterectomy rats displayed blank or diestrus-like smears. While two Sham rats displayed persistent diestrous smears across all eight days of evaluation, the remaining eight subjects displayed elongated estrous cycles with most days in diestrus and variable/intermittent cycling. Three out of seven Hysterectomy rats displayed persistent diestrus smears; the remaining four exhibited elongated estrous cycling, with most days in diestrus and variable/intermittent cycling. Lastly, after behavior testing was complete, vaginal smears were also collected one day prior to euthanization and on the day of euthanization. Ovx-Hysterectomy groups displayed diestrus-like or blank smears. Seven out of eight Ovx displayed blank or diestrus-like smears, and one Ovx rat (the same rat) remained in persistent estrous. All Sham rats were in diestrus prior to euthanization. Five out of seven Hysterectomy rats were in diestrous, and two Hysterectomy rats displayed estrus or metestrus-like cytology within 24 hours of euthanization.

Aged (12 month) Cohort Body Weights

Ovx rats weighed more than Sham rats at euthanasia [$F_{(1,15)}$=4.78, $p < 0.05$; Figure 77A]. There were no other differences in body weight for any two-group comparison at this time point.

Aged (12 month) Cohort Uterine Weights

Only rats that retained their uterus throughout the experiment (i.e., Sham and Ovx rats) were compared. As expected, there was a main effect of uterine weight between Sham and Ovx rats [$F_{(1,15)}$=68.15, $p < 0.0001$], such that uterine weights for Ovx rats were lower than Sham rats due to a lack of estrogen stimulation from the ovaries (Engler-Chiurazzi et al., 2012; Koebele et al., 2019; Mennenga, Gerson, et al., 2015; Prakapenka et al., 2018; Westerlind et al., 1998). When uterine weights were corrected for body weight, the main effect of Surgery persisted [$F_{(1,15)}$=52.49, $p < 0.0001$] (Figure 77B).

Aged (12 month) Cohort Ovary Weights and Ovarian Follicle Estimates

Only rats that retained their ovaries throughout the experiment (i.e., Sham and Hysterectomy rats) were compared. There was a main effect of Surgery for summed ovary weight [$F_{(1,13)}$=7.35, $p < 0.05$], such that Hysterectomy rats' ovaries weighed more than Sham rats' ovaries at this time point. However, when ovary weight was corrected for body weight, this effect became marginal [$F_{(1,13)}$=3.76, $p = 0.08$] (Figure 77C).

There were no main effects of Surgery for Primordial Follicles [$F_{(1,13)}$=0.11, p = NS], Primary Follicles [$F_{(1,17)}$=0.93, p = NS], Antral Follicles [$F_{(1,13)}$=0.51, p = NS], or Corpora Lutea Counts [$F_{(1,13)}$=1.07, p = NS] at this time point (Figure 78A,B,D,E). There was a marginal main effect of Surgery for Secondary Follicles [$F_{(1,13)}$=3.57, $p = 0.08$; Figure 78C], where Hysterectomy rats tended to have fewer secondary follicles than Sham rats, although this effect did not reach statistical significance.

Aged (12 month) Cohort Serum Hormone Levels

Only subjects included in behavioral analyses were included in serum assessments. Unfortunately, 7/9 Sham rats and 4/6 Hysterectomy rats had values outside of the detectable range of the Progesterone assay. These results should be interpreted with caution, given that n=2 for Sham and Hysterectomy groups. No differences in Progesterone levels between Sham and Hysterectomy rats [$F_{(1,2)}$=1.85, p = NS]. Despite the low n, Hysterectomy rats had higher Progesterone levels than Ovx rats [$F_{(1,8)}$=54.00, $p < 0.0001$] and tended to have higher Progesterone levels compared to Ovx-Hysterectomy rats [$F_{(1,9)}$=5.05, $p = 0.05$]. Sham rats also had higher Progesterone levels than Ovx rats [$F_{(1,8)}$=40.09, $p < 0.001$]. Sham rats did not differ from Ovx-Hysterectomy rats, but this is likely due to the unequal and low n in the Sham group. Ovx groups did not differ from each other [$F_{(1,15)}$=1.14, p = NS] (Figure 79A).

There were no significant differences in serum Androstenedione levels at this time point [$F_{(1,13)}$=2.60, p = NS; Figure 79B].

Three Sham subjects and three Hysterectomy subjects were excluded from the E2 assay due to levels outside of the detectable range. Additionally, three values from the Sham group and three values from the Hysterectomy group were based on a singlet value, as the other singlet value registered outside the detectable range of the assay. There were no significant differences in serum E2 levels at this time point [$F_{(1,8)}$=0.60, p = NS; Figure 79C].

Discussion

Women may undergo gynecological surgery at any point in their lives. A younger age at surgery can increase the risk of developing a multitude of diseases later on, including dementia, coronary heart disease, diabetes, depression, Parkinson's disease, and some cancers (Adelman & Sharp, 2018; Benedetti et al., 2001; Bove et al., 2014; Gierach et al., 2014; Rocca et al., 2011, 2012; Wilson, Pandeya, Byles, & Mishra, 2018a). If a woman is under 35 when oophorectomy occurs, or under 40 when hysterectomy occurs, these surgeries increase the risk of all-cause mortality (for review, see: Adelman and Sharp, 2018; Gierach et al., 2014), although this risk may be mitigated with hormone therapy use (Wilson, Pandeya, Byles, & Mishra, 2018b). Given that women can live a substantial proportion of their lives post-surgery, it is important to elucidate how variations in gynecological surgery, including hysterectomy with and without ovarian conservation, impact cognition longitudinally. There is a significant gap in the basic science literature regarding the cognitive effects of hysterectomy and corresponding neurobiological factors. Chapter 6 of this dissertation was the first methodical evaluation of variations in gynecological surgery using a novel rat model of hysterectomy. Findings suggested that hysterectomy in adulthood resulted in a unique, detrimental impact on spatial working memory compared to reproductively-intact Sham rats, as well as compared to Ovx rats and Ovx-Hysterectomy rats. We also reported altered serum hormone level profiles between Sham and Hysterectomy groups, with no changes to ovarian morphology at the time point assessed. However, it is important to keep in mind that the cognitive evaluation for this experiment occurred six weeks after surgery, making it a relatively short-term time point after surgery. Evidence from human literature

suggests potential short-term effects of hysterectomy on serum hormone levels that return to baseline in longer time frames after surgery (Kaiser et al., 1989; Vuorento et al., 1992). Therefore, it was crucial to understand whether the cognitive and physiological effects of hysterectomy on cognition were transient after surgery, or whether they were indicative of a long-term impairment associated with the procedure. Thus, to address this, the current experiment investigated the longitudinal effects of hysterectomy and other variations of surgical menopause on spatial learning and memory.

Spatial Memory

Three cohorts of rats underwent Sham, Ovx, Hysterectomy, or Ovx-Hysterectomy surgery at 5 months old (adulthood). The first cohort was cognitively tested six weeks after surgery, a replication of experimental methods on Chapter 6; rats were approximately 7 months old (Adult) at euthanasia. The second cohort was assessed 7 months after surgery, when rats were 12 months old (Middle-Aged). The third cohort was assessed 12 months after surgery, when rats were 18 months old (Aged). All cohorts experienced an identical behavioral battery, including the WRAM, MM, and OFT tasks, as well as the control VP task to assess visual and motor skills needed to successfully complete a water maze task. Remarkably, at all three time points assessed, Hysterectomy rats exhibited impaired spatial working memory performance on the WRAM when memory load was taxed. Specifically, during the Learning Phase, Hysterectomy rats in the Adult cohort made more WMC errors on Trial 3 compared to Ovx-Hysterectomy rats; furthermore Hysterectomy rats made more WMI errors compared to Sham rats, and tended to make more errors than Ovx-Hysterectomy rats on Trial 3– a replication of

findings in Chapter 6 (Koebele et al., 2019). Following a test of delayed memory retention in the WRAM, all surgery groups were relatively impaired on the post delay-trial compared to individual group performance on the last baseline day of testing, again replicating prior findings. For the Middle-Aged cohort, which was tested 7 months after surgery, when rats were 12 months old, Hysterectomy rats made more WMC errors on Trial 3 compared to the other groups, and more WMI errors on Trial 3 compared to Sham and Ovx groups during the Learning Phase. Furthermore, on the maximum working memory load trial, Trial 4, Hysterectomy rats made more WMI errors than Ovx-Hysterectomy rats. Delayed memory retention results were more variable for this time point. Ovary-intact groups tended to show impaired memory performance in the post-delay trial. Ovx-Hysterectomy rats were significantly impaired, but interestingly, Ovx rats did not exhibit a significant increase in WMC errors following a six-hour delay. When Ovx surgery is performed in adulthood and animals are tested in old age, null effects of Ovx on working memory have been reported in mice (Heikkinen et al., 2004), and beneficial effects of Ovx on spatial delayed recognition-span task have been shown in rhesus macaques compared to age-matched intact controls (Lacreuse et al., 2000), corroborating our current findings in rats. Furthermore, our laboratory has reported benefits of long-term Ovx when surgery is performed in aged animals compared to age-matched Sham controls; as such, Ovx may enhance spatial working memory in some instances (Bimonte-Nelson, Singleton, Hunter, et al., 2003; Bimonte-Nelson et al., 2004). Similarly to the Middle-Aged time point, spatial working memory impairments were evident during the Learning Phase for the Aged cohort, tested one year after initial surgery, when rats were 18 months old. Impairments became evident on the maximum

working memory load trial for this cohort. Specifically, WMC errors tended to increase in Hysterectomy rats compared to Sham and Ovx rats on Trial 4. WMI errors were significantly increased in Hysterectomy rats compared to Sham and Ovx rats, and marginally increased compared to Ovx-Hysterectomy rats on Trial 4. Although Hysterectomy rats trended toward decreased WMC errors during the asymptotic phase compared to Ovx rats, this effect did not reach statistical significant. In this Aged time point, Sham rats were impaired on the test of delayed memory retention, while surgical groups were not, indicating a potential interaction of delayed memory impairments with age dependent upon surgery status. Collectively, these results provide convincing evidence that Hysterectomy with ovarian conservation results in long-term cognitive detriments for spatial working memory. These findings have significant translational implications for the role of hysterectomy and its effect on cognition and quality of life during aging, particularly when the surgery occurs during a woman's reproductive years.

Physiological Measures

At the Adult and Middle-Aged time points, ovary-intact rats (i.e., Sham and Hysterectomy groups) had lower body weights compared to Ovx rats (i.e., Ovx and Ovx-Hysterectomy groups) at euthanasia. At the Aged time point, all rats had progressively gained weight with age, such that only Sham rats weighed less than Ovx rats. Hysterectomy rats weighed similarly to Ovx and Ovx-Hysterectomy rats. This is particularly interesting, as a prospective research study in women reported greater weight gain in women with hysterectomy one year after surgery (Moorman et al., 2009, but see also: Fitzgerald et al., 2009), indicating uterus removal with ovarian conservation may

have a unique effect on metabolism and/or the microbiome. This association is of interest to systematically investigate in future research.

Uterine weights were increased in Sham rats compared to Ovx rats at all time points, indicating estrogen stimulation of the uterus in ovary-intact rats, an effect that has been consistently reported in the literature (Engler-Chiurazzi et al., 2012; Koebele et al., 2019; Mennenga, Gerson, et al., 2015; Prakapenka et al., 2018; Westerlind et al., 1998). At the 12 month time point, ovaries from Hysterectomy rats tended to weigh more than Sham ovaries after correcting for body weight, although this was not a statistically significant effect.

Ovarian follicle counts estimate the number of healthy follicles present in the ovaries, including non-growing ovarian follicle reserve (Primordial Follicle counts), as well as growing follicles (Primary, Secondary, and Antral Follicles) that are steroidogenic. Corpora Lutea were also quantified as a measure of ovulatory activity. There were no differences between Sham and Hysterectomy groups for any ovarian follicle type or corpora lutea counts at the Adult and Middle-Aged time points. There were no differences in Primordial, Primary, or Antral Follicles at the Aged time point. Hysterectomy rats tended to have fewer Secondary follicles compared to Sham rats at this time point, although the difference did not reach statistical significance. Corpora Lutea counts did not differ at the Aged time point. Overall, this suggests that the ovaries did not undergo divergent morphological changes following hysterectomy compared to age-matched controls at any time point. These findings challenge the notion that ovarian dysfunction or accelerated ovarian aging occurs after hysterectomy in adulthood and

collectively point to a primary role for the uterus itself in regulating cognition that is not secondary to ovarian changes.

Serum progesterone, androstenedione, and E2 levels did not differ between ovary-intact groups at any time point. That ovarian-derived steroid hormone serum levels did not differ between Sham and Hysterectomy groups suggests that the ovaries continue to function in a similar fashion to reproductive-tract-intact ovaries following hysterectomy, at least at the time points assessed. In each cohort assessed, Progesterone was significantly decreased in Ovx groups compared to ovary-intact groups. It is notable that the serum results reported in this current experiment show a different pattern of effects from those reported in Chapter 6. Specifically, we had previously detected a marginal decrease in E2, a decrease in androstenedione, and an increase in progesterone levels between Sham and Hysterectomy rats. In the current report, endogenous E2 levels were overall substantially lower in values compared to the data presented in Chapter 6. Here, samples were analyzed with a commercially available ELISA kit (CAT# ES180S-100, CALBIOTECH, Spring Valley, CA). In Chapter 6, RIA was used to determine E2 levels. However, RIA measurements of E2 may unreliably produce E2 results several fold higher than values obtained via ELISA- or gas chromatography/tandem mass spectrometry (GC/MSMS)- based measurements of E2, which both produce similar results (Haisenleder, Schoenfelder, Marcinko, Geddis, & Marshall, 2011; Nilsson et al., 2015). Androstenedione and progesterone were measured by ELISA kits in both cases, however, kit-to-kit variability (in the case of androstenedione) and different kit use (in the case of progesterone) may be responsible for the variation in serum hormone levels. Serum hormone analyses for luteinizing hormone (LH) and follicle stimulating hormone

(FSH) levels for this experiment are currently underway. It is important to note that steroid hormone levels vary across the normal estrous cycle, and fluctuate irregularly as aging ensues, and are therefore not the best indicator of a given hormonal milieu. This is also true of the erratic fluctuations of serum steroid hormone levels in women undergoing the transition to menopause. In both species, LH and FSH levels rise at a much steadier rate, and may be a better measure overall of ovarian status during the transition to reproductive senescence. Indeed, FSH is the gold standard serum marker for menopause status in clinical settings (Chan et al., 2005; Kaiser et al., 1989). These data will provide meaningful information to the interpretation of the current data set.

Conclusions and Future Directions

This is the first report of long-term cognitive impairments associated with hysterectomy in a rodent model. Hysterectomy with ovarian conservation had a unique, negative impact on spatial working memory 6 weeks after surgery, in adulthood; 7 months after surgery, in middle-age; and 12 months after surgery, in old age. It is well-accepted that intricate feedback loops exist between the reproductive system and the brain. Variations in gynecological surgery likely result in reorganizational processes of important neural circuits, including those associated with learning and memory. Determining specific brain regions impacted by age and gynecological surgery manipulations will aid in developing novel and targeted therapies with the goal of delaying or preventing the onset of deleterious cognitive effects associated with hysterectomy and other variations in surgical menopause. In order to develop these novel interventions for associated cognitive detriments, it is necessary to identify prospective

biomarkers in the brain that are indicative of long-term neural reorganization (see Chapter 8).

In the future it will also be critically important to investigate whether the age at which hysterectomy occurs differentially impacts cognitive outcomes. For example, one study reported that hysterectomy after the natural menopause transition did not affect the relative risk of developing dementia, and may in fact be neuroprotective (Imtiaz et al., 2014). We have previously reported that the age at the onset of follicular depletion (i.e., a model of transitional menopause) is a paramount factor in cognitive outcomes (Koebele et al., 2017); thus, it is of particular interest to evaluate age as a central variable in hysterectomy model outcomes. Furthermore, given that ovarian follicle count estimates and ovarian-derived hormone levels were similar between Sham and Hysterectomy rats at each time point, systematic evaluation into the direct role of the uterus on cognition is a critical next step. There is clear, direct sensory and autonomic nervous system innervation of the nonpregnant rat uterus (Brauer & Smith, 2015; Gnanamanickam & Llewellyn-Smith, 2011). Although this may be unsurprising given the intricate neural control associated with parturition, the collective evidence described herein alludes to a significant role of the uterus-brain connection in addition to factors related solely to pregnancy and birth. Nervous system plasticity occurs naturally throughout the reproductive cycle and is mediated, in large part, by reproductive hormones; this plasticity is undoubtedly interrupted following gynecological surgery. It is plausible, and rather likely, that neural signaling is impacted by hysterectomy, even when ovaries are preserved, resulting in longitudinal effects on the trajectory of brain aging. This novel model of hysterectomy affords significant advancement in the field of behavioral

neuroendocrinology by highlighting the importance of the nonpregnant uterus, opening new doors to many more questions revolving around the ovary-uterus-brain connection, through which we will better our understanding of reproductive hormones, aging, and cognition as a whole.

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CHAPTER 8

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GYNECOLOGICAL SURGERY RESULTS IN AGE- AND SURGERY- DEPENDENT FOSB AND DELTA (Δ)FOSB EXPRESSION IN BRAIN REGIONS IMPORTANT FOR LEARNING AND MEMORY

Contribution: I was the graduate student principal investigator of this experiment under the mentorship of Dr. Heather Bimonte-Nelson and Dr. Jason Newbern. I designed the experiment and completed all brain assays. This work was supported by the NRSA.

ABSTRACT

Gynecological surgery can alter a woman's relative risk of developing cognitive impairment or dementia later in life. Premature menopause following surgical ovary removal (oophorectomy) in adulthood likely results in some neural circuit reorganization, including that related to cognition, and in particular, learning and memory modulation. Hysterectomy with ovarian conservation does not result in a similar drastic drop in ovarian hormones immediately after surgery like oophorectomy, but is nonetheless also associated with a greater relative risk of dementia in women, and has recently been shown to impair working memory in rats. Given the brain's impressive capacity for rapid changes as well as long-term plasticity, adaptation, and reorganization, it is challenging to identify how neural circuitry and signaling become dysregulated and/or acclimate to a long-term environmental change in a given system. Delta (Δ) FosB, an immediate-early gene-derived transcription factor, has been proposed as a long-term marker of change within the brain. Here, FosB and ΔFosB protein expression were evaluated in regions intricately involved in spatial working memory, including the Dorsal Hippocampus, Frontal Cortex, and Entorhinal Cortex, in the brains of rats that received variations of gynecological surgery, including Sham, Ovariectomy, Hysterectomy, or Ovariectomy-Hysterectomy in adulthood. Rats were 7 mo old (Adult), 12 mo old (Middle-Aged), or 18 mo old (Aged) at the time of behavioral testing and subsequent brain analysis. Data revealed that within the Entorhinal Cortex, FosB expression and ΔFosB expression were altered at Middle-Aged and Aged time points in rats that underwent ovary removal in adulthood. When evaluating the brain regions of interest within each group across the three age cohorts, age- and surgery- dependent alterations in FosB and ΔFosB protein

expression were detected in the Dorsal Hippocampus and the Entorhinal Cortex during aging. This is the first systematic evaluation of a prospective marker of long-term change (ΔFosB) in the brains of aging rodents that have undergone variations in gynecological surgery including hysterectomy. Overall, results provide insight into possible ΔFosB-related neural pathways that may be altered with age and following gynecological surgery in brain regions vital to normal learning and memory processes.

Introduction

It is estimated that up to nearly half of women experience some form of gynecological surgery in their lifetime (Merrill, 2008). Approximately one-third of women in the United States will undergo hysterectomy, or surgical removal of the uterus, by age 60 (J. D. Wright et al., 2013). While surgery rates continue to be on the decline as alternative treatment options become available for benign uterine conditions (Corona et al., 2015), hysterectomy remains a common gynecological surgery, second only to cesarean section (Carlson et al., 1993; Centers for Disease Control and Prevention, 2010). Women undergo hysterectomy for a variety of benign indications, including leiomyomas, abnormal bleeding, endometriosis, chronic pain, or uterine prolapse (Carlson et al., 1993). Prophylactic oophorectomy, or the surgical removal of the ovaries, is performed in conjunction with hysterectomy in about half of cases, especially when women are at high-risk for gynecological cancers (Asante et al., 2010; Lowder et al., 2010; Pocobelli et al., 2012; The American College of Obstetrics and Gynecology, 2008; Whiteman et al., 2008). Although it is generally recommended to avoid oophorectomy in reproductive-age women without genetic risk factors (The American College of Obstetrics and Gynecology, 2008), if the surgery occurs prior to the natural onset of menopause, oophorectomy results in surgical menopause, meaning there is an abrupt, drastic drop in serum ovarian hormone levels (NAMS, 2014). The premature loss of ovarian hormones in adulthood can have manifold repercussions on the health and quality of life of women (Faubion, Kuhle, Shuster, & Rocca, 2015; Matthews, 2013; Parker, Jacoby, Shoupe, & Rocca, 2009; Rodriguez & Shoupe, 2015). Given that the majority of hysterectomy procedures occur before age 52, the average age of natural menopause, it is imperative to

understand the full range of long-term health risks associated with variations in gynecological surgery. Indeed, women who experience surgical menopause self-report more severe menopausal symptoms, such as hot flashes, sexual dysfunction, mood, and memory changes compared to women who undergo a natural transition to menopause (Benshushan et al., 2009; Gallicchio et al., 2006; Rodriguez & Shoupe, 2015). As well, observational data suggest that there is an increased relative risk of developing neurodegenerative diseases, including Parkinson's disease (Benedetti et al., 2001) and dementia in women who undergo oophorectomy, hysterectomy, and compete oophorectomy-hysterectomy; this relative risk increases with a younger age at surgery (Phung et al., 2010; Rocca et al., 2011, 2012).

It is challenging to determine specific neurobiological underpinnings of the increased dementia risk in women. Many neural circuits are influenced by ovarian hormones and become disrupted when hormones are no longer present; many of the same circuits are also impacted by aging (for review, see: Koebele & Bimonte-Nelson, 2017). Given the remarkable life-long plasticity of the brain, it is plausible that reorganizational processes ensue to promote continued functionality of these circuits, especially those related to learning and memory. Because the brain likely utilizes homeostatic mechanisms to adapt to changing conditions, and due to the chronic nature of aging and hormone loss following variations in surgical menopause, it is difficult to pinpoint the time course of specific changes within a given neural system; nonetheless, it is imperative to find a prospective indicator of long-term change in the brain.

One candidate marker to evaluate is Delta (Δ) FosB. Delta FosB is a transcription factor encoded by the immediate early gene (IEG) *fosB*. *FosB* can undergo alternative

splicing to produce full-length FosB and a truncated form called ΔFosB, which has unusual stability compared to other IEG transcription factors. This is because ΔFosB lacks proteasomal degradation sites (called degron domains) present on the C-terminus of full-length FosB, consequently preventing rapid protein breakdown (Carle et al., 2007). In general, IEGs are rapidly and transiently activated genes following various extracellular activity or stimulation. IEGs are unique in that they do not require new protein synthesis to exert meaningful effects on the brain and periphery (J. I. Morgan & Curran, 1989; Sheng & Greenberg, 1990). While IEGs are not limited to activity in the brain, they have been found to play important roles in many neurobiological processes, including synaptic plasticity and memory (Minatohara et al., 2016; Sheng & Greenberg, 1990). There are several different families of IEGs with distinctive properties. IEGs in the *fos* family hetereodimerize with *jun* family IEGs to form a complex called the activator protein 1 (AP-1) complex, through which it eventually up- and down- regulates gene expression (Carle et al., 2007; Nestler et al., 2001; Ruffle, 2014). Though *c-fos* is one of the best-characterized IEGs, it has a short half-life of approximately 60 minutes, after which its expression rapidly declines, making it difficult to evaluate for long-term effects in the brain (Carle et al., 2007). Because of the stability associated with ΔFosB expression, it has been described as a "sustained molecular switch," as it can be maintained at detectable levels in neurons for weeks to months following initial activation; in fact, ΔFosB has been touted as the "longest-lived adaptation known to occur in [sic] adult brain" (Nestler et al., 2001) and has an extended half-life compared to other transcription factors (Carle et al., 2007; J. Chen et al., 1997; Ulery-Reynolds et al., 2009).

What we know of ΔFosB primarily comes from research on addiction. Specifically, ΔFosB expression becomes upregulated in the nucleus accumbens with repeated drug exposures (e.g. cocaine) and remains elevated even after drug cessation, implicating ΔFosB in mechanisms of drug dependency (Nestler et al., 2001; Ruffle, 2014). This work furthermore suggests that ΔFosB activation and/or expression can have long-term behavioral effects. For example, ΔFosB has been shown to become upregulated with stress, indicating a potential role for ΔFosB in hypothalamic-pituitary-adrenal axis function (Ruffle, 2014). Sustained ΔFosB expression also increases hippocampal gene expression of cyclin-dependent protein kinase 5 (*cdk5*), a gene related to phosphorylation of synaptic proteins that has many crucial functions in the central nervous system, including acting as a mediator of synaptic plasticity (Ruffle, 2014). Cdk5 also plays a role in regulating extracellular signal-regulated kinase (ERK1/2) signaling (Sharma et al., 2002), an important signaling cascade with a multitude of downstream effects, including on learning and memory processes. When dysregulated, cdk5 increases tau hyperphosphorylation and is associated with neurodegenerative disease, such as Alzheimer's and Parkinson's diseases (Cortés, Guzmán-Martínez, Andrade, González, & Maccioni, 2019). Overexpression of ΔFosB in mice has been shown to result in an upregulation of genes within the nucleus accumbens that also become upregulated with cAMP response element binding protein (CREB) expression (McClung & Nestler, 2003), a transcription factor implicated in many neural processes, including its crucial role in memory formation (Kandel, 2012) as well as a downstream target of ERK1/2 signaling. Interestingly, an extended overexpression of ΔFosB in mice results in opposing up- and down- regulation of gene expression compared to short-term ΔFosB overexpression, at

least in the context of drug reward pathways (McClung & Nestler, 2003). More recently, overexpression of ΔFosB was found to transiently increase CREB protein levels in the striatum of male mice and *in vitro* (Enwright et al., 2010). As such, it is plausible that CREB and ΔFosB work in concert to produce long-term changes in gene expression and behavior not only in the context of addiction and reward, but also in brain regions that regulate learning and memory. Recently, the number of FosB/ΔFosB-immunoreactive (IR) cells was shown to increase in the CA1 region of the hippocampus after spatial Morris water maze (MM) learning compared to maze-naïve control rats and compared to rats that experienced a non-spatial version of the MM (Eagle et al., 2015). Additionally, FosB/ΔFosB-IR cells increased in the dentate gyrus of all mice and rats that experienced behavior testing. AAV- and HSV- mediated silencing of ΔFosB, as well as AAV-mediated overexpression of ΔFosB, impaired learning and memory performance in this experiment (Eagle et al., 2015). Furthermore, it was recently reported that a ΔFosB knock-out mouse model confined to the subgranular zone of the dentate gyrus ($NtsR2^{Cre/+}FosB^{lox/lox}$ mice) exhibited neurogenesis and learning impairments compared to wildtype littermates (Manning et al., 2019), further signifying a role for ΔFosB in learning and memory.

Additional evidence from literature on the neuroscience of addiction suggests that ΔFosB can decrease the expression of an endogenous opioid neuropeptide called dynorphin (Nestler et al., 2001). Dynorphin-producing neurons have diverse roles in the brain; of relevance to menopause and aging, 17β-estradiol—the most potent endogenous estrogen primarily produced by the ovaries—has been shown to regulate dynorphin expression in the arcuate nucleus of the hypothalamus (Kanaya et al., 2017). Indeed,

dynorphin-producing neurons colocalize with other neuropeptides in the arcuate nucleus, which are collectively referred to as kisspeptin/neurokininB/dynorphin (KNDy) neurons. KNDy neurons are abundant in the hypothalamus and play a key role not only in gonadotropin synthesis and release from the hypothalamus and pituitary, but are also thought to regulate the hypothalamic-pituitary-ovarian axis feedback loop, and thus influence the female reproductive cycle (Lehman et al., 2010). Early research into the physiological alterations that occur with hysterectomy and oophorectomy point to an inhibitory role of the uterus on pituitary function, including the synthesis and release of gonadotropins and other hormones (Biró, 1979; Biró & Eneroth, 1989; Biró et al., 1988, 1984, 1987). It is conceivable that alterations to the female reproductive tract, including surgical manipulations of the ovaries and/or uterus, interact with these regulatory neural systems and signaling cascades in order to influence behavioral outcomes, such as those observed in Chapters 6 and 7 of this dissertation.

Given its prolonged nature, ΔFosB expression may be a candidate biomarker for long-term changes in brain areas important to cognition following variations in surgical menopause and with age. To explore this hypothesis, we evaluated full-length FosB and ΔFosB protein expression in the Frontal Cortex, Dorsal Hippocampus, and Entorhinal Cortex of rats that had undergone variations in gynecological surgery, including Sham, Ovariectomy (Ovx), Hysterectomy, or Ovx-Hysterectomy surgery in adulthood. The rats were then aged and were behaviorally evaluated 6 weeks after surgery (Adult time point), 7 months after surgery (Middle-Aged time point) or 12 months after surgery (Aged time point). Following a battery of behavioral tasks evaluating spatial learning and memory, the results of which are described in detail in Chapter 7 of this dissertation, brains were

collected for analysis. We hypothesized that these variations in gynecological surgery would result in long-term changes in areas of the brain important for spatial working and reference memory, as measured by FosB and ΔFosB protein expression.

Methods

Subjects

Three cohorts of 40 female, virgin, reproductively-intact Fischer-344-CDF rats from the National Institute on Aging colony at Charles Rivers Laboratories (Raleigh, NC) were utilized in this experiment (N=120). All rats were 5 months of age upon arrival to the Arizona State University animal facility. Rats were pair-housed, provided free access to food and water, and were maintained in a temperature- and humidity- controlled environment on a 12-hour light/dark cycle (lights on at 7 am, lights off at 7 pm). Experimental procedures commenced after 1 week of acclimation to the facility. All experimental procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards.

Surgical Procedures

For each cohort of rats (N=40/cohort), 10 subjects were randomly assigned to one of the following surgical conditions: Sham, Ovx, Hysterectomy, or Ovx-Hysterectomy. Surgeries were performed 1 week (±1 day) after arriving to the animal facility. Surgical procedures and behavior testing protocols are detailed in Chapter 7 of this dissertation.

Euthanization and Tissue Collection

At the end of the experiment, rats were heavily anesthetized using inhaled isoflurane anesthesia. Following cardiocentesis, brains were rapidly removed. The right hemisphere of the brain was rapidly raw dissected for the Frontal Cortex, Dorsal Hippocampus, and Entorhinal Cortex. Wet weight was obtained for each region and then tissues were immediately frozen at -70°C until analysis.

Western Blot Protein Analysis

Frontal Cortex, Dorsal Hippocampus, and Entorhinal Cortex from the right hemisphere were processed for FosB expression and ΔFosB expression via western blot protein analysis for all subjects that survived until the end of the experiment. Frozen raw tissue samples were suspended in a 1:25 weight-to-volume 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 subsequently kept on ice at all times. Tissues were homogenized using a probe sonicator (Ultrasonic Processor, Cole Parmer, IL, USA), and then centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatant was collected, aliquoted, and frozen at -70°C until analysis. Protein concentration for each sample was determined using a bicinchoninic acid protein assay (Thermo-Fisher Scientific, Pittsburg, PA, USA).

Treatment groups were counterbalanced and equally represented on each gel run. The NuPAGE PowerEase electrophoresis system was utilized for tissue processing. Samples for each region were loaded at an equal protein concentration and run on a 4-

12% NuPAGE Bis-Tris gel in an XCell SureLock Mini-Cell (Invitrogen, Carlsbad, CA, USA) using MOPS running buffer. After transferring to an Immobilon polyvinylidene difluoride membrane, the blot was blocked in 5% nonfat milk for 1 hour at room temperature. Following blocking, the membrane was washed in 1xTBST and then incubated with the primary antibody for anti-FosB (1:1000; Abcam, ab184938) in 5% nonfat milk on a shaker at 4°C overnight. The following day, the membrane was washed in 1xTBST and then incubated with secondary antibody anti-rabbit HRP (1:500; Cell Signaling #7074) for 1 hour at room temperature in 5% nonfat milk. The membrane was washed again, and developed using chemiluminescence (Lumiglo and peroxide, Cell Signaling, #7003S) in a film developer (Konica SRX-101A Film Processor, Tokyo, Japan). The membrane was washed again in 1xTBST 3 times for 5 minutes each, and then incubated with the primary antibody for anti-beta-tubulin (1:20,000; Cell Signaling, #2128S) in 5% nonfat milk on a shaker at 4°C overnight. The following day, the membrane was washed in 1xTBST and then incubated with secondary antibody anti-rabbit HRP (1:2000; Cell Signaling #7074) for 1 hour at room temperature in 5% nonfat milk. The membrane was washed again, and developed using chemiluminescence (Lumiglo and peroxide, Cell Signaling, #7003S) in a film developer (Konica SRX-101A Film Processor, Tokyo, Japan). Films were scanned in as JPEG files at 600 dpi. Densitometry analyses were completed using ImageJ software (Gallo-Oller et al., 2018). FosB expression and ΔFosB expression were normalized to beta-tubulin expression on each gel run. A total of 9 gels per region (3 per age cohort) were completed.

Statistical Analyses

All statistical analyses were completed using Statview software. Data were assessed using an omnibus ANOVA test. Surgery type was the independent variable and normalized FosB expression and ΔFosB expression were the dependent variables. All tests were two-tailed and set at an alpha level of 0.05. Results were deemed marginal if the *p* value fell between 0.05 and 0.10.

Results

Right Dorsal Hippocampus

There were no main effects of Surgery for FosB expression or ΔFosB expression at any time point in the Dorsal Hippocampus.

Right Frontal Cortex

There were no main effects of Surgery for FosB expression or ΔFosB expression at any time point in the Frontal Cortex.

Right Entorhinal Cortex

There were no main effects of Surgery for FosB expression or ΔFosB expression in rats tested 6 weeks after surgery, at the Adult time point when rats were 7 months old (Figure 80A-B). At the Middle-Aged time point, 7 months after surgery when rats were 12 months old, there was a main effect of Surgery for FosB expression [$F_{(3,33)}$=4.15, $p < 0.05$]. Fisher's PLSD post hoc test revealed that Ovx rats had lower FosB expression compared to Sham rats ($p < 0.05$) and Hysterectomy rats ($p < 0.01$) at the 7 month post-

surgery time point. Ovx-Hysterectomy rats also had lower FosB expression compared to Hysterectomy rats ($p < 0.05$), and tended to have lower FosB expression compared to Sham rats ($p = 0.06$), at this time point, while ovary intact groups did not differ from one another, nor did Ovx groups (Figure 80C). There was a marginal main effect of Surgery for ΔFosB expression [$F_{(3,33)}=2.82$, $p = 0.05$]. Fisher's PLSD post hoc test indicated that Hysterectomy rats had higher ΔFosB expression compared to Ovx rats ($p < 0.05$) and Ovx-Hysterectomy rats ($p < 0.05$; Figure 80D) at this time point.

At the Aged time point, 12 months after surgery when rats were 18 months old, there were no Surgery differences in FosB expression in the Entorhinal Cortex (Figure 80E). Although there was no main effect of Surgery for ΔFosB expression, an exploratory two-group comparison between Sham and Ovx rats based on findings at the 7 month post-surgery time point indicated that Ovx rats had lower ΔFosB expression compared to Sham rats at the 12 month post-surgery time point ($p < 0.05$; Figure 80F).

Exploratory Analysis of Age Effects

Acknowledging that each age cohort was behaviorally tested at separate times, we evaluated whether Age impacted FosB expression and ΔFosB expression within each Surgery group. Each brain region was assessed separately using an ANOVA with Age (Adult, Middle-Aged, and Aged at test) as the independent variable. Normalized FosB expression and normalized ΔFosB expression were the dependent variables.

Frontal Cortex. There were no main effects of Age on FosB expression or ΔFosB expression within the Frontal Cortex for any surgery group. (Figure81A-H).

Dorsal Hippocampus. For Sham rats, there was a main effect of Age for ΔFosB expression in the Dorsal Hippocampus [$F_{(2,27)}$=8.97, $p < 0.001$]. Fisher's PLSD post hoc tests indicated that Aged rats had significantly lower ΔFosB expression than Adult rats ($p < 0.001$) and Middle-Aged rats ($p < 0.01$) (Figure 82B). Ovx rats showed a significant effect of Age for ΔFosB expression [$F_{(2,25)}$=4.70, $p < 0.05$], with post hoc analyses indicating that Aged rats had lower ΔFosB expression than Adult rats ($p < 0.05$) and Middle-Aged rats ($p < 0.01$) (Figure 82D). There was a main effect of Age for ΔFosB expression in Hysterectomy rats [$F_{(2,23)}$=8.99, $p < 0.001$], where Fisher's PLSD post hoc analyses indicated that Aged rats had lower ΔFosB expression in the Dorsal Hippocampus compared to Adult rats ($p < 0.001$) as well as compared to Middle-Aged rats ($p < 0.01$) (Figure 82F). For Ovx-Hysterectomy rats, there was a marginal main effect of Age for ΔFosB expression [$F_{(2,24)}$=3.28, $p = 0.06$]. Fisher's PLSD post hoc tests indicated that Aged rats had lower ΔFosB expression than Adult rats ($p < 0.05$) and Middle-Aged rats ($p < 0.05$) (Figure 82H). There were no main effects of Age for FosB expression within any surgery group (Figure 82A,C,E,G).

Entorhinal Cortex. For Sham rats, there was a main effect of Age for FosB expression [$F_{(2,27)}$=5.52, $p < 0.01$] and for ΔFosB expression [$F_{(2,27)}$=7.02, $p < 0.01$]. Fisher's PLSD post hoc tests showed that FosB expression increased from Adult to Middle-Aged rats ($p < 0.05$), and decreased from Middle-Aged to Aged rats ($p < 0.01$), suggesting a transient increase in Entorhinal Cortex FosB expression in midlife (Figure 83A). Delta FosB in the Entorhinal Cortex increased from Adult to Middle-Aged rats (p

< 0.01), and remained elevated in Aged rats compared to Adult rats ($p < 0.01$) (Figure 83B). For Ovx rats, there was a marginal effect of Age for ΔFosB expression [$F_{(2,25)}$=2.66, $p = 0.09$]; Fisher's PLSD post hoc for this analysis indicated that there was an increase in ΔFosB expression from Adult to Middle-Aged rats ($p < 0.05$) (Figure 83D). For Hysterectomy rats, there was a main effect of Age for FosB expression [$F_{(2,21)}$=4.42, $p < 0.05$] as well as for ΔFosB expression [$F_{(2,21)}$=7.01, $p < 0.01$] in the Entorhinal Cortex. Fisher's PLSD post hoc indicated there was a marginal increase in FosB expression from Adult to Middle-Aged time points ($p = 0.07$), and that Aged rats had lower FosB expression compared to Middle-Aged rats ($p < 0.01$) (Figure 83E). Delta FosB expression, on the other hand, significantly increased from Adult to Middle-Aged rats ($p < 0.001$). Aged Hysterectomy rats also tended to have higher ΔFosB expression compared to Adult rats, although this effect was marginal ($p = 0.07$) (Figure 83F). There were no significant Age effects in Entorhinal Cortex FosB expression (Figure 83G) or ΔFosB expression (Figure 83H) in Ovx-Hysterectomy rats.

Discussion

The brain is a highly plastic organ throughout life; it is ever-adapting to changing conditions. Ovarian hormones act as modulators for many neural systems, including those that regulate normal learning and memory processes (Koebele & Bimonte-Nelson, 2017). When systems are disrupted, such as with surgical menopause or with aging, there are likely a number of reorganizational events that occur in order to maintain the function of critical neural communication in a new hormonal milieu (Koebele & Bimonte-Nelson, 2015). It has been established that women who undergo variations in gynecological

surgery, including the removal of the ovaries and uterus, are at a greater risk for neurodegenerative diseases, including dementia and Parkinson's disease, and this risk increases with a younger age at surgery (Benedetti et al., 2001; Phung et al., 2010; Rocca et al., 2007, 2011; Rocca, Grossardt, & Shuster, 2014; Rocca et al., 2012, 2009). Even women who undergo hysterectomy with ovarian conservation, which does not involve an abrupt loss of circulating hormones, are at a greater relative risk of dementia compared to ovary intact women (Benedetti et al., 2001; Phung et al., 2010; Rocca et al., 2012). Given the frequency of gynecological surgery in women, it is of critical importance to form an understanding of the neurobiological underpinnings of this increased risk of neurodegenerative disease in order to potentially prevent or postpone the onset of cognitive changes. These observational findings in women have recently been supported by experimentally randomized and controlled basic science research indicating that hysterectomy with ovarian conservation impairs spatial working memory in adult rats compared to other variations in menopause, at least 6 weeks after surgery (Koebele et al., 2019); these findings were recently extended to show that cognitive impairments observed with hysterectomy are present at long-term time points after surgery, when rats are middle-aged and aged (unpublished observations, Chapter 7).

In this chapter, ΔFosB protein expression was evaluated as a prospective indicator of long-term change in the brains of rats that underwent gynecological surgery in adulthood. Full-length FosB protein expression was also measured. When comparing across Surgery type, results indicated that there were differences in FosB expression and ΔFosB expression within the Entorhinal Cortex 7 months and 12 months after surgery. Specifically, 7 months after surgery, when rats were 12 months old (Middle-Aged), Ovx

rats had less FosB expression compared to ovary-intact groups. Ovx-Hysterectomy rats had less FosB expression compared to Hysterectomy rats, and trended toward having less FosB expression compared to Sham rats. Delta FosB expression tended to be lower in Ovx and Ovx-Hysterectomy rats compared to Hysterectomy rats at this time point as well. One year after surgery, when rats were 18 months old (Aged), ΔFosB expression tended to be lower in Ovx rats compared to Sham rats. There were no significant or marginal differences in FosB expression or ΔFosB expression in the Dorsal Hippocampus or Frontal Cortex at these time points when comparing effects by Surgery type. Given that the Entorhinal Cortex acts as an information gateway between the hippocampus and other cortical areas, the observation that a marker of long-term change such as ΔFosB is decreased in animals that had their ovaries removed indicates a potential role for ovarian hormones in regulating neuronal activity, including FosB and ΔFosB expression, thus subsequently impacting information transfer during spatial learning and memory.

It was also of interest to evaluate FosB expression and ΔFosB expression within each surgical group to understand how protein expression varies with age. There were no changes in expression in the Frontal Cortex for any surgery group at any age time point. However, in the Dorsal Hippocampus, significantly lower ΔFosB expression in Aged rats was observed for Sham, Ovx, and Hysterectomy groups; there was also a marginal decrease with age for the Ovx-Hysterectomy group. FosB was unaltered by age in all groups. Collectively, these results indicate that lower ΔFosB expression in the Dorsal Hippocampus of Aged rats appears to be age-mediated, regardless of surgery type.

Within the Entorhinal Cortex, a different pattern of FosB expression was present across ages in a surgery-dependent manner compared to that observed in the Dorsal

Hippocampus. In ovary-intact groups (i.e. Sham and Hysterectomy groups), there was a transient increase in FosB expression during the Middle-Aged time point that was not present in groups without ovaries. Although full-length FosB undergoes proteasomal degradation after activation much faster than ΔFosB, it continues to be produced at basal levels by the *fosB* gene to some extent, and may provide meaningful information about alternative splicing mechanisms of the gene with age and hormone changes. For example, this transient increase in full-length FosB may be associated with reorganizational factors related to changing ovarian hormone levels as rats approach estropause, or rodent reproductive senescence, in middle-age. Furthermore, ovary-intact groups exhibited greater ΔFosB expression in Middle-Age compared to the Adult time point. This increased expression was sustained at the Aged time point, despite lower expression of full-length FosB at the Aged time point. One possible explanation for this is that *fosB* gene splicing preferentially generates the truncated, long-lasting ΔFosB variant with age in ovary-intact rats. Recent reports that both aging and 17β-estradiol alter alternative gene splicing mechanisms lends credence to this idea (Shults, Pinceti, Rao, & Pak, 2015; Tollervey et al., 2011). Ovx rats also displayed an increase in ΔFosB expression in Middle-Age compared to Adult rats, but this effect did not persist at a statistically significant level into the Aged time point. Ovx-Hysterectomy rats did not have significant changes in Entorhinal Cortex ΔFosB expression with age, suggesting that removal of the entire reproductive tract during adulthood could differentially influence patterns of reorganizational processes in relation to removal of the ovaries alone.

This is the first observation of a prospective biomarker of long-term change in the brain during aging following variations in gynecological surgery including hysterectomy.

The mechanisms underlying these changes that, in turn, regulate behavioral output are complex and multifaceted. Age was the primary factor for altered ΔFosB expression over and above the influence of gynecological surgery when analyzed within a surgery group. It is notable that opposite patterns of age-related ΔFosB expression exist for the Dorsal Hippocampus and Entorhinal Cortex. Specifically, ΔFosB expression declines with age in the Dorsal Hippocampus, but increases with age in the Entorhinal Cortex. This differential pattern of expression across brain regions with aging could indicate a compensatory mechanism that occurs with aging and the transition to reproductive senescence. Alternatively, ΔFosB expression in either region may be dynamic with age; observations at later time points, in advanced age, will be important in future investigations. It is also essential to note that ovary-intact rats undergo estropause, which is characterized by a different hormone profile than menopause in women; because female rats do not experience follicular depletion to the extent that humans do, reproductively senescent rats typically display tonically increased estrogen and/or progesterone levels instead of experiencing a significant decline in these hormones with age (Koebele & Bimonte-Nelson, 2016). Therefore, these age-related findings should be interpreted with caution, and evaluations in follicle-deplete rats with a hormone profile more similar to human menopause will be informative in future research.

It is important to keep in mind that FosB expression and ΔFosB expression do not operate in a vacuum, and are intricately linked to many complex neural systems with both up- and down- stream effectors working together to produce changes in gene expression as well as behavioral outcomes. A premature deprivation of ovarian hormones in adulthood likely alters neural systems shortly after hormone loss occurs, and the

mechanisms by which they reorganize may vary due to the absence of ovarian hormones. In the future it will be necessary to investigate other key regulators, including ERK1/2 and CREB activation, in these rats in order to parse out a more cohesive understanding of the complex regulatory processes that can alter behavioral outcomes. Evaluating the role of ΔFosB expression in relation to KNDy neurons is also of interest to elucidate whether altered ΔFosB expression influences hypothalamic-pituitary-reproductive tract axis regulation and function throughout aging.

Regardless of surgery status, women have an increased risk of developing cognitive impairment and dementia with age compared to men (Alzheimer's Association, 2018; Riedel et al., 2016). Changes in ovarian hormone levels with reproductive senescence may influence this risk. Within the Entorhinal Cortex, which is thought to be the first brain region affected by tauopathy in Alzheimer's disease pathology in humans (Braak & Braak, 1991; Kaufman, Del Tredici, Thomas, Braak, & Diamond, 2018), both ovary intact groups showed a transient increase in full-length FosB expression in middle-age, as well as a sustained increase in ΔFosB expression at the Aged time point that was not seen in groups without their ovaries. Prior research using transgenic mice has shown that ΔFosB expression upregulates cdk5 in the hippocampus (J. Chen et al., 2000). Cdk5 is implicated in increased phosphorylation and inhibition of activated MEK1, leading to a decrease in ERK1/2 phosphorylation, possibly resultant of a negative feedback mechanism (Sharma et al., 2002). Although cdk5 has many important regulatory roles in the normally functioning system, cdk5 dysregulation can have pathological consequences, including a significant role in tau hyperphosphorylation and consequential neurodegenerative disease, including Alzheimer's and Parkinson's diseases, among

others (Cortés et al., 2019). Perhaps the sustained increase in Entorhinal Cortex ΔFosB expression in ovary-intact rats is an early indicator of a disrupted system that could lead to eventual pathological brain aging. Given that ERK1/2 activation and signaling also regulates IEG expression, a disruption to this feedback loop could result in altered behavioral outcomes in the aging female. In the future it will be informative to investigate these other molecular targets to determine if altered expression of ΔFosB with age could initiate downstream changes that alter susceptibility to cognitive decline in ovary-intact animals. This hypothesis, however, does not explain the relative increased risk of cognitive impairment when ovaries are removed in humans. Given that our laboratory has reported beneficial effects of aged Ovx for rats compared to aged intact rats (Bimonte-Nelson, Singleton, Hunter, et al., 2003; Bimonte-Nelson et al., 2004), and other laboratories have shown protective effects of long-term Ovx on spatial memory in rhesus macaques (Lacreuse et al., 2000) and mice (Heikkinen et al., 2004), it is necessary to investigate alternative pathways influencing susceptibility to cognitive impairment following Ovx in the future as well.

Lastly, correlations between brain and behavioral measures will afford meaningful information regarding the relationships among neural signaling, age, and ovarian hormones in the context of variations in gynecological surgery. Translationally, better understanding how neural systems important for regulating learning and memory are impacted by aging and variations in gynecological surgery will provide insight into the increased susceptibility for age-related cognitive changes and risk for neurodegenerative disease in women, as well as possible novel targets for intervention therapies in order to improve quality of life and cognitive health throughout the lifespan.

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CHAPTER 9

GENERAL SUMMARY AND DISCUSSION

Aging can be thought of as a lifelong process. From early development, to puberty, to menopause and beyond, each life stage is comprised of critical events that shape an individual's future responses and health outcomes. Throughout every life stage, the endocrine system—and in particular, reproductive hormones—play an integral role in shaping and shifting an organism's responses. Reproductive hormones are now recognized for their integral role in the functionality of many critical body systems beyond reproduction, including cardiovascular, skeletal, muscular, integumentary, and gastrointestinal health. Perhaps most critically, reproductive hormones are a paramount regulatory factor in the brain, and thus, in cognitive function. The collective research presented in this dissertation expounds upon the influence of endogenous and exogenous hormone exposures across the lifespan in the context of reproductive senescence and cognitive aging. Noteworthy discoveries include the influence of age at the onset of reproductive senescence and how the etiology of reproductive senescence affects cognition and brain aging.

Females are born with a finite pool of immature ovarian follicles that, throughout life, undergoes a normal process of depletion, called follicular atresia (Hsueh et al., 1994; W. H. B. Wallace & Kelsey, 2010). At puberty, the hypothalamic-pituitary-ovarian axis becomes active, and ovarian follicles begin to grow and produce sex steroid hormones including estrogens, progesterone, and androgens. The growth and maturation of ovarian

follicles is stimulated by the release of gonadotropin releasing hormone from the hypothalamus, as well as follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland. This intricate feedback system carefully regulates the female reproductive cycle (in women, the menstrual cycle; in rats, the estrous cycle). The reproductive years –from puberty to menopause– are characterized by monthly cyclic fluctuations in circulating estrogens, progesterone, androgens, and the gonadotropins. As such, this cyclicity is inherent to the female for much of her life. Of the thousands of immature follicles present at birth, only about 400 are selected to become fully mature eggs for ovulation during the reproductive lifespan (Gougeon, 2010). The vast majority of immature follicles become atretic. By midlife, it is thought that the finite ovarian follicle reserve reaches a critical threshold of depletion, in turn initiating the transition to reproductive senescence, or menopause (Hansen et al., 2008; W. H. B. Wallace & Kelsey, 2010). In women, menopause is a natural event that typically happens in a transitional fashion around the fifth decade of life. Ovarian hormone levels —particularly estrogens and progesterone—fluctuate irregularly throughout this transition, which can last up to ten years, and eventually decline to low or undetectable levels for the remainder of the lifespan, while gonadotropins become chronically elevated due to disrupted feedback (Harlow et al., 2013; Soules et al., 2001). The first section of this dissertation, Chapters 2 and 3, investigate the impact of age at the onset of follicular depletion in a rodent model of transitional menopause. In particular, the experiments herein emphasize how age at the onset of the menopause transition was an important factor for cognitive outcomes. Relationships among serum hormone levels and altered neural systems that are linked to learning and memory processes are also

explored with the goal of better understanding associations and interactions between ovarian hormones and brain regions known to be crucial for learning and memory processes. A summary of these findings will be described in detail below.

The transition to menopause is indicated by a variety of physical symptoms, including vasomotor symptoms (i.e., hot flashes, night sweats), urogenital symptoms (e.g., vaginal dryness, urgency and frequency changes in urination), dyspareunia, and disruptions to sleep, mood, and memory (Al-Safi & Santoro, 2014). Depending on severity, these symptoms can have a substantial negative impact on quality of life for women. Women may opt to take estrogen-containing hormone therapy to alleviate these symptoms. The first prescription hormone therapy was FDA-approved in the early 1940's (Stefanick, 2005), and hormone-containing contraceptives came to market in the 1960's (Tyrer, 1999). In this day and age, it is plausible that a woman will experience multiple, extended exposures to exogenous hormones 1) during reproductive years via hormone-containing contraceptives, and 2) during perimenopausal and post-menopausal years, if she opts to take hormone therapy. The popularity of estrogen-containing hormone therapy has dwindled in recent years (Crawford et al., 2018; Sprague et al., 2012); however, given that the cognitive effects of hormone exposures can persist even when the drug is no longer detectable (Braden et al., 2011; Rodgers et al., 2010), it is necessary to understand how hormone-containing therapies can impact brain health. Despite a natural decline in endogenous estrogen and progesterone levels with age, females retain responsiveness to these hormones until the end of the lifespan. The type of hormone(s) administered, dose, route of administration, timing, and duration of hormone use are all factors that can influence long-term health in women who opt to take hormone therapy,

including an individual's susceptibility to cognitive decline (for review, see: Koebele & Bimonte-Nelson, 2015). While prescriptions for hormone-containing contraceptives and menopausal hormone therapy operate under a one-size-fits-all approach in clinical settings, it has become abundantly clear in recent years that this is not an ideal strategy. A more personalized approach that considers life factors and individual risk is necessary to provide optimal treatment to alleviate disruptive symptoms while not compromising long-term health and cognition. Given that the average lifespan is continually increasing, particularly for women, it is important to find solutions that will improve quality of life in the post-menopausal state, which can comprise a third of a woman's life. The second section of this dissertation, Chapters 4 and 5, evaluate cognitive performance on spatial learning and memory tasks in the presence of clinically relevant exogenous hormone treatments to better understand the role of these factors on cognition during aging, and results will be described in detail below.

Although a majority of women undergo natural menopause, there is a non-negligible proportion of women who will undergo gynecological surgery during their lifetime. This surgery can result in abrupt surgical menopause if both ovaries are removed prior to the natural onset of menopause in midlife. Premature ovarian hormones loss has a well-documented negative impact on cognition (Bimonte & Denenberg, 1999; Farrag et al., 2002; Gibbs & Johnson, 2008; Nappi et al., 1999; Rocca et al., 2007, 2011, 2012, 2009; Talboom et al., 2008; M. Wallace et al., 2006) as well as a multitude of other body systems (Faubion et al., 2015; Matthews, 2013; Parker et al., 2009; Rodriguez & Shoupe, 2015). In addition, more recent findings also implicate hysterectomy, the surgical removal of the uterus, in an increased relative risk of developing neurodegenerative

diseases if the surgery is performed during the reproductive years, before natural menopause onset. A substantial gap in the basic science literature exists regarding the effects of hysterectomy on cognition and brain function during aging. The third and final section of this dissertation, Chapters 6, 7, and 8, explore the cognitive effects of variations in surgical menopause, including a novel model of hysterectomy, as well as propose a prospective neurobiological marker of long-term change in the brain following surgical menopause; collective results are discussed in detail below. Overall, this dissertation examines numerous factors contributing to longitudinal cognitive outcomes associated with reproductive senescence, and builds a new framework for understanding how age and menopause etiology influence cognitive outcomes across the lifespan.

Age at the Onset of Follicular Depletion Matters for Memory Outcomes

The average onset of menopause occurs at age 52 in women (NAMS, 2014). The vast majority of species do not live long beyond the end of the reproductive life stage. Even among primates, humans are one of the few exceptions to this rule (Gems, 2014). The age of menopause onset has remained relatively stable, despite a continually increasing lifespan in humans in the 20th century, especially among women (Amundsen & Diers, 1970, 1973; Hawkes & Coxworth, 2013; Mennenga & Bimonte-Nelson, 2013; Murray et al., 2015; Regan & Partridge, 2013; Seifarth, Mcgowan, & Milne, 2012; Sherwin, 2003). Thus, the number of reproductive-age years is not proportionally increasing along with the rising average lifespan, such that women are now living a substantial proportion of their lives in a post-menopausal state (Sherwin, 2003). While most women undergo this natural transition to menopause in the fifth decade of life, there

are some women who undergo early-onset menopause, defined as the final menstrual period occurring before age 45, as well as a small percentage of women who undergo spontaneous premature ovarian insufficiency, wherein the final menstrual period occurs before age 40 (Pal & Santoro, 2002; Shuster et al., 2010; Simpson & Rajkovic, 1999). Because premature deprivation of ovarian hormones in a surgical context is known to impact cognition negatively in humans and animals, we hypothesized that an earlier onset of follicular depletion would be detrimental to cognition.

Utilizing a rodent model of transitional menopause, we found that a younger age at the onset of follicular depletion did indeed impair spatial working memory when memory was taxed compared to age-matched counterparts in the early perimenopausal time point. Middle-aged rats performed similarly on the task, regardless of whether or not they were undergoing follicular depletion. In fact, Young-VCD-treated rats performed similarly to their Middle-Aged-VCD-treated counterparts at this early-depletion time point. At later time points in the post-follicular deplete state, Middle-Aged-VCD-treated rats tended to perform worse than their younger VCD-treated counterparts; however, it is significant that Young-VCD treated rats exhibited poorer spatial working memory performance early in the transition to follicular depletion. Among VCD-treated groups, there was a main effect of Age for the reference memory Morris water maze (MM) task, such that middle-aged animals performed worse than younger animals. However, Middle-Aged-VCD-treated rats exhibited enhanced performance on the MM compared to age-matched Vehicle-treated rats, indicating some potential benefit of follicular depletion for reference memory during aging. There was a significant decline in all ovarian follicle types in VCD-treated rats. Additionally, serum hormone profiles varied among treatment

groups, with VCD-treated rats exhibiting significantly lower progesterone and marginally lower estrone levels at the post-follicular depletion time point compared to their age-matched Vehicle-treated counterparts. Overall, this was the first longitudinal evaluation of cognitive performance across the transition to follicular depletion in rats, and underscores the importance of age at the onset of the menopause as a key factor for interpreting cognitive outcomes. In particular, the working memory deficit observed at the early perimenopause time point may indicate that the critical window for intervention with hormone therapy varies depending on the age when the menopause transition begins. The onset of the transition is challenging to pinpoint in humans, as menopause is retrospectively confirmed one year after the final menstrual period; the transition likely begins many years prior, even before menopausal symptoms present. Therefore, developing reliable biomarkers to more accurately establish the onset of the menopause transition will be an important future endeavor to enhance health outcomes for women, regardless of age at menopause transition onset.

Given these behavioral findings in conjunction with the known intricate links between ovarian hormone levels and cognition, Chapter 3 was an exploratory analysis using the brains of the rats in Chapter 2 to investigate potential correlations among serum hormone levels, the cholinergic system, and the GABAergic system, and whether relationships were dependent on age and ovarian follicle status. The basal forebrain was assessed for choline acetyltransferase (ChAT)-immunoreactive (IR) neurons. ChAT is the enzyme that forms acetylcholine, a neurotransmitter that has diverse functions in the nervous system, including a role in normal learning and memory processes. The basal forebrain is the primary site of acetylcholine synthesis in the brain, and long-range

projections from the basal forebrain to the hippocampus and surrounding areas influence learning and memory circuits. Of relevance to menopause and aging literature, these connections need to be intact in order for estrogen to exert beneficial effects on learning and memory; furthermore, ovarian hormone loss can impair cholinergic function (Gibbs, 1998, 2002, 2003, 2010). Additionally, the GABAergic system, which is the primary inhibitory neurotransmitter system in the brain, is a key player in regulating spatial learning and memory. Expression of GABA-synthesizing enzymes, GAD65 and GAD67, was assessed in the hippocampus and surrounding cortical areas to determine whether age and ovarian follicle status were associated with changes in this system. Findings revealed subtle relationships among endogenous serum hormone levels, ChAT-IR cell counts, GAD expression, and memory performance in normally aging and follicle-deplete rats. VCD tended to increase Dorsal Hippocampus GAD65 expression and decrease Entorhinal Cortex GAD67 expression. The clearest relationships were observed within the Young-Vehicle group (aged 8-12 months at evaluation). A summary these correlations can be found in Figure 84 and Figure 85. For Young-Vehicle rats, serum 17β-estradiol levels were positively correlated with GAD65 and GAD67 expression in the Ventral Hippocampus. Furthermore, there were significant positive correlations among androstenedione, progesterone, and estrone levels with ChAT-IR neuron counts in the vertical diagonal bands (VDB) of the basal forebrain for Young-Vehicle rats. These associations were not observed for Young-VCD rats. However, a negative correlation between Estrone levels and Entorhinal Cortex GAD67 expression was present for Young-VCD rats, such that follicular depletion may reverse the direction of the relationships among GABAergic function and serum hormone levels. Overall, these effects implicate a

role for follicular depletion in altering relationships among circulating ovarian hormone levels and the cholinergic system in adult to early middle-aged rats. To the best of knowledge, this was the first report of age and follicular depletion status affecting relationships among neural systems important for learning and memory. Furthermore, other than a positive correlation between androstenedione levels and ChAT-IR neurons in the medial septum (MS) for the Middle-Aged VCD group, there were no significant correlations among the variables tested for Middle-Aged rats (aged 14-18 months at evaluation), suggesting that a critical window during which relationships among hormone levels and neural systems may exist. This critical window before 12 months of age could point to optimal time points for prevention-based interventions for cognition. Overall, although exploratory in nature, these findings will propel future research forward by providing a fundamental understanding of the key factors associated with cognitive outcomes in the context of transitional menopause and aging, and by offering a foundation for future methodical experimental manipulation of each of these variables to further elucidate the roles of age and follicular depletion on learning and memory systems.

Exogenous Ovarian Hormone Therapy Influences Cognitive Performance on Spatial Working Memory Tasks: Unique Effects of Maze Complexity and Treatment Regimen

As noted above, although the rate of hormone therapy use has declined in the past two decades (Crawford et al., 2018; Sprague et al., 2012), exogenous estrogen-containing hormone therapy is one of the only FDA-approved treatments for menopause

symptomology. Estrogens are generally considered to be neuroprotective, though exogenous estrogen treatment can result in variable effects in rodent models, with many factors influencing outcomes (for review, see Koebele & Bimonte-Nelson, 2017). The experiment in Chapter 4 considered the influence of high- and low- demand cognitive experience in conjunction with estrogen therapy on cognition. Using a middle-aged ovariectomized (Ovx) rat model, results demonstrated that when exogenous, tonic, low-dose 17β-estradiol treatment was administered after Ovx, prior high-demand cognitive experience enhanced learning and memory on a subsequent low-demand task, and prior low-demand cognitive experience had the potential to enhance memory on a subsequent high-demand cognitive task. Prior experience did not significantly impact Ovx Vehicle-treated rats, and rats treated with a high dose of 17β-estradiol exhibited variable benefit, depending on the type of prior cognitive experience. Because many behavioral neuroscientists utilize a battery of cognitive tasks to evaluate experimental manipulations, findings from this experiment emphasize the importance of consistent protocol use in order to reliably replicate effects within and across laboratories. Indeed, the basic science literature reports variable effects of exogenous estrogens on cognitive outcomes. Depending on the experimental conditions, estrogens can have beneficial, neutral, or even detrimental effects on learning and memory. These results highlight novel considerations, including task order and task complexity, in conjunction with hormone dose and route of administration, when interpreting cognitive outcomes.

Chapter 5 explored the cognitive effects of a novel progestin, drospirenone, alone and in combination with a synthetic estrogen commonly prescribed in oral contraceptives. Although it is well-established that endogenous ovarian hormones and their

corresponding exogenously-administered variants affect cognition, less attention has been paid to synthetic forms of progesterone alone and in combination with estrogens in the context of aging and menopause research. In order to address this, the fourth-generation progestin, drospirenone, was evaluated in young adult Ovx rats in a two-part study. The first experiment showed that drospirenone enhanced spatial working memory at a medium dose, which was based on the ratio of progestin-to-estrogen in combined oral contraceptives. However, the second experiment revealed that when this dose of drospirenone was administered in combination with ethinyl estradiol, beneficial effects of drospirenone were attenuated compared to drospirenone alone. These results extended prior findings from our laboratory showing divergent cognitive effects of progestin administration alone versus in combination with estrogen (Prakapenka et al., 2018). Although an optimal combination treatment still remains to be discovered, these findings provide new insight into the role of the commonly-prescribed progestin drospirenone on cognition. This was a particularly timely investigation, given recent FDA approval of a drospirenone-only oral contraceptive pill (US Food and Drug Administration, 2019) in addition to the combination formulations already available on the market.

Hysterectomy: A Novel Rodent Menopause Model and its Longitudinal Effects on Cognition

There are many ways to model the phenomenon of menopause in rodents (for review, see Koebele & Bimonte-Nelson, 2016). The gold standard for studying cognitive effects of hormone loss and exogenous hormone administration in experimental research settings is via Ovx, where the ovaries are surgically removed, but uterine tissue is

conserved. This is a valuable approach to understanding the effects of specific hormones and other drug treatments; indeed, much of what we know about aging and cognition in females comes from this "blank ovarian hormone slate" model in basic science literature. However, translationally, a minority of women undergo surgical menopause in their lifetime, and even fewer experience bilateral ovary removal without concomitant hysterectomy. The vast majority experience a natural, transitional menopause in midlife. This can be modeled with 4-vinylcyclohexene diepoxide (VCD), which initiates ovarian follicular depletion and results in a hormone profile more similar to a woman undergoing the transition to menopause. Natural reproductive senescence is typically concurrent with aging, making it challenging to attribute any cognitive changes observed specifically to ovarian hormone decline versus to aging alone. VCD is an exciting scientific advancement because it affords researchers the possibility to evaluate the separate effects of follicular depletion without the necessary confound of aging in rodent models.

The Ovx and VCD models are indisputably valuable and provide a fundamental understanding of menopause and aging effects. Nevertheless, until now, a large gap in the literature has existed regarding the cognitive effects of hysterectomy, the second most common gynecological surgery, on learning and memory. The dogma in the field is that the non-pregnant uterus is an essentially dormant organ (Navot & Williams, 1991; Rosen & Cedars, 2007); any cognitive changes following gynecological surgery are thought to be driven by ovarian hormones. However, based on a growing body of clinical literature suggesting that hysterectomy with ovarian conservation increases the relative risk of dementia and other diseases later in life in women, we hypothesized that the uterus may play a unique role in regulating cognitive processes. Thus, in this dissertation, a novel

model of hysterectomy in rats was created to test this idea. Results of the proof-of-concept experiment, detailed in Chapter 6, revealed that six weeks after surgery, adult rats with hysterectomy exhibited a working memory impairment when memory load was taxed compared to sham controls, as well as compared to Ovx and Ovx-Hysterectomy groups. Ovarian follicle counts did not significantly differ between ovary-intact groups, but serum ovarian hormone profiles differed between Sham and Hysterectomy rats two months after surgery. This was the first systematic evaluation of variations of surgical menopause including hysterectomy on spatial memory. It was demonstrated that variations in surgical menopause yielded distinct effects on working memory performance. Furthermore, a shift in ovarian hormone levels and FSH levels, but no change in ovarian morphology or corpora lutea counts, was present, suggesting that uterus removal may directly impact steroidogenesis in the ovaries. Broadly, this experiment indicated that the nonpregnant uterus is not quiescent, as its removal had significant effects on physiology and cognition, at least within a short-term time point two months after surgery.

These unprecedented findings led to a subsequent series of experiments investigating the longitudinal effects of hysterectomy; that is, it was necessary to determine whether this observed hysterectomy-induced impairment was transient in the short-term time point after surgery, or if it was a long-lasting effect that impacted memory for the remainder of the lifespan. Findings described in Chapter 7 revealed that hysterectomy with ovarian conservation in adulthood resulted in working memory impairments present 6 weeks after surgery (Adult time point, when rats were 7 months old; a replication of findings in Chapter 6), 7 months after surgery (Middle-Aged time

point, when rats were 12 months old), and 12 months after surgery (Aged time point, when rats were 18 months old). In contrast to the results presented in Chapter 6, no differences in serum progesterone, androstenedione, or 17β-estradiol levels were detected between ovary-intact groups at any time point. Ovx and Ovx-Hysterectomy groups exhibited significantly decreased progesterone levels compared to ovary intact groups, a replication of prior findings (Koebele et al., 2019). Although this lack of replication for ovarian steroid hormone levels is difficult to reconcile, it is possible that a difference in detection method (ELISA versus RIA for 17β-estradiol levels) as well as ELISA kit-to-kit variability could be responsible for these divergent results. Furthermore, it is important to keep in mind that endogenous ovarian hormone levels fluctuate across the estrous cycle and become irregular with age, making any one sample challenging to interpret broadly. For that reason, FSH levels are the gold standard for evaluating reproductive status in women over and above serum hormone levels at any given time point. Thus, serum FSH and LH levels will provide valuable insight into whether hysterectomy resulted in disrupted hypothalamic-pituitary-ovarian feedback compared to sham controls. These serum gonadotropin evaluations are currently underway for this experiment. In addition to the lack of differences in serum sex-steroid hormone measures at all time points, there were no statistically significant differences between Sham and Hysterectomy groups' ovarian follicle and corpora lutea counts at any given time point, a replication of findings in Chapter 6. This is an important observation, as it suggests that ovarian structure and function were not uniquely altered following hysterectomy at any given time point compared to reproductive-tract-intact rats' ovaries. The present understanding in the field is that ovarian function may become compromised following

hysterectomy in women of reproductive age, leading to earlier ovarian failure. These current findings challenge the concept of accelerated ovarian aging after hysterectomy in adulthood, and collectively point to a primary role for the uterus itself in mediating cognition that is not secondary to ovarian changes.

It is noteworthy that there were age-related changes in both ovarian follicle counts and serum ovarian hormone levels, regardless of hysterectomy status, in ovary-intact rats. Acknowledging that this experiment was a cross-sectional design where separate cohorts of rats were tested at different time points, we ran an exploratory analysis of age-related changes in serum hormone levels and follicle counts. There was a main effect of Age for all follicle types, including the ovarian follicle pool [Primordials: $F_{(2,48)}=25.836$, $p < 0.0001$] and growing follicles [Primaries: $F_{(2,48)}=3.65$, $p < 0.05$; Secondaries: $F_{(2,48)}=39.33$, $p < 0.0001$; Antrals: $F_{(2,48)}=8.21$, $p < 0.001$]. Fisher's PLSD post hoc analyses indicated that Adult rats had more primordial follicles than Middle-Aged rats ($p < 0.0001$) and Aged rats ($p < 0.0001$) (Figure 86A), more primary follicles than Middle-Aged rats ($p < 0.01$) and Aged rats ($p < 0.05$) (Figure 86B), more secondary follicles than Middle-Aged rats ($p < 0.0001$) and Aged rats ($p < 0.0001$) (Figure 86C), and more antral follicles than Middle-Aged rats ($p < 0.05$) and Aged rats ($p < 0.001$) (Figure 86D). Additionally, Aged rats had fewer secondary follicles than Middle-Aged rats ($p < 0.0001$) (Figure 86C). There were no significant Age differences for Corpora Lutea counts (Figure 86E). This age-related decrease in ovarian follicle counts, but not corpora lutea, is a replication of prior work in our laboratory (Koebele et al., 2017), supporting the idea that rodents experience some follicular depletion with age (Gosden et al., 1983) in accordance with the onset of estrous acyclicity (for review, see: Finch, 2014). Age-

related serum hormone level changes were also present in ovary-intact rats, regardless of hysterectomy status. There was no main effect of Age for Progesterone levels (Figure 87A); however, when Hysterectomy rats were evaluated separately, there was a marginal main effect of Age [$F_{(2,15)}$=3.48, p = 0.06], with an exploratory Fisher's PLSD post hoc test indicating that Aged Hysterectomy rats had higher progesterone levels than Adult (p < 0.05) and Middle-Aged (p < 0.05) Hysterectomy counterparts. There was a main effect of Age for Androstenedione [$F_{(2,44)}$=18.03, p < 0.0001; Figure 87B], where Aged rats exhibited elevated androstenedione levels compared to Adult rats (p < 0.0001) and Middle-Aged rats (p < 0.0001). Last, there was a main effect of Age for 17β-estradiol levels [$F_{(2,31)}$=3.35, p < 0.05; Figure 87C], where Aged rats had significantly decreased 17β-estradiol levels compared to Adult rats (p < 0.01). These data correspond well with the ovarian follicle data, given the observed decrease in estrogen-producing ovarian follicles in old age, while progesterone-producing corpora lutea did not show significant changes, and androstenedione is primarily produced by ovarian interstitial cells (Dyer & Erickson, 1985). Overall, age appeared to be the primary factor impacting physiological measures of steroid hormone levels and ovarian follicle counts over and above the presence of the uterus, suggesting that the ovaries remain functional following hysterectomy until the onset of natural reproductive senescence.

The last chapter of this dissertation detailed an investigation into a prospective neurobiological marker of long-term change, ΔFosB, in the brains of the aging rats that underwent variations of gynecological surgery in Chapter 7. Delta FosB is a transcription factor and product of the immediate early gene *fosB*, and has been recognized for having unusual long-term stability, making it a putative "sustained molecular switch" (Nestler et

al., 2001) associated with long-term reorganizational processes in the brain. Much of the scientific literature to this point has been focused on the role of ΔFosB expression in addiction and drug dependency (Nestler et al., 2001; Ruffle, 2014), but ΔFosB has recently been implicated in learning and memory as well (Eagle et al., 2015; Manning et al., 2019). Additionally, ΔFosB expression increases hippocampal *cdk5* expression, a gene important for synaptic plasticity and a regulator of extracellular signal-regulated kinase (ERK1/2) signaling, all of which can be affected by hormones and aging, and influence learning and memory. Thus, ΔFosB expression was a prime candidate to explore as a marker of long-term change in the context of variations of surgical menopause and aging. Results revealed that within the Entorhinal Cortex, rats that had their ovaries removed (Ovx and Ovx-Hysterectomy groups) had lower full-length FosB expression compared to ovary-intact groups at the Middle-Aged time point. Ovx rats also tended to have lower ΔFosB expression compared to Hysterectomy rats at this time point, 7 months after surgery. During the Aged time point, 12 months after surgery when rats were 18 months old, Ovx rats had lower ΔFosB expression compared to Sham rats in the Entorhinal Cortex as well. Next, FosB and ΔFosB expression were evaluated across Age time points within each brain region of interest to determine whether age differentially affected FosB and ΔFosB protein expression within each surgery group. For all surgical variations, there was a significant decrease in Dorsal Hippocampus ΔFosB expression at the Aged time point. Within the Entorhinal Cortex, ovary-intact groups (Sham rats and Hysterectomy rats) displayed a transient increase in full-length FosB expression in Middle-Age, whereas Ovx and Ovx-Hysterectomy rats did not. The Sham group and the Hysterectomy group also showed greater ΔFosB expression in Middle-Age compared to

the Adult time point, with sustained expression at the Aged time point. Ovx and Ovx-Hysterectomy groups did not show a statistically significant increase in ΔFosB expression with age. Overall, changes in ΔFosB expression in the Dorsal Hippocampus appeared to be an age-mediated effect, while expression in the Entorhinal Cortex may be influenced by an interaction between age and ovarian hormone milieu. Given that the Entorhinal Cortex is an information gateway between the hippocampus and other cortical areas, this suggests that changes in ovarian hormones with age and surgical menopause variations play a role in influencing long-term neural reorganization and/or adaptation. Furthermore, taking into account the role of ΔFosB expression in relation to other important gene expression changes and signaling cascades involved in learning and memory, this was an important step toward understanding the neural circuits involved in longitudinal behavioral changes associated with surgical menopause variations.

Future Directions

These cumulative findings have contributed to our understanding of the behavioral and brain changes associated with age and surgical menopause, and have paved the way toward many new paths to explore. The question for future research remains: are these observed cognitive and brain changes primarily driven by alterations in the ovaries following hysterectomy, or does the uterus have a definitive, direct impact on learning and memory processes? Direct sensory and autonomic nervous system innervation of the non-pregnant, virgin rat uterus has recently been described (Brauer & Smith, 2015; Gnanamanickam & Llewellyn-Smith, 2011). Estrogens are known to influence sympathetic neurite growth and retraction within the uterus via neurotrophic

factors (Krizsan-Agbas, Pedchenko, Hasan, & Smith, 2003; Wessels, Wu, Leyland, Wang, & Foster, 2014; Zoubina, Mize, Alper, & Smith, 2001). As such, nervous system plasticity occurs naturally throughout the reproductive cycle; it is undoubtedly interrupted following gynecological surgery. Given the direct connections between the uterus and the nervous system, it is plausible that neurotransmitter systems closely associated with sensory and autonomic communication may be affected by the removal of the uterus, even when the ovaries are preserved. For example, using a whole-mount uterine horn preparation, it was demonstrated that the uterine tissue was densely populated with tyrosine hydroxylase-IR axons innervating blood vessels and the different muscle layers (Gnanamanickam & Llewellyn-Smith, 2011). Tyrosine hydroxylase is the rate-limiting enzyme in the synthesis of dopamine, a fundamental neurotransmitter involved in many regulatory systems, including learning and memory. Estrogen is a known dopamine modulator (Yoest, Cummings, & Becker, 2014). Dopamine also plays a key role in cognition and affect, possibly via estrogen regulation (for review, see: Almey et al., 2015; Jacobs and D'Esposito, 2011; Yoest et al., 2014). Furthermore, dopamine dysregulation is a defining characteristic of Parkinson's disease development and progression, and may play a role in Alzheimer's disease as well (Martorana & Koch, 2014). Given the reported increased risk of Parkinson's disease and dementia in women who experienced hysterectomy, the collective evidence makes dopamine an excellent candidate for future investigation in the context of gynecological surgery and cognition. Indeed, disruptions to the ovary-uterus-brain feedback loop following hysterectomy and other variations in gynecological surgery may significantly alter neural signaling initiating from the sympathetic, parasympathetic, and sensory connections in the uterus, resulting in long-

term changes in the trajectory of brain aging. Other prospective neurobiological factors associated with ΔFosB expression, including changes in ERK1/2 signaling, CREB expression, cdk5 expression, and KNDy neurons, are all viable contenders to probe for future evaluations in order to further our knowledge of interactions between variations in gynecological surgery and aging with the brain and behavior. This novel model of hysterectomy affords significant advancement in the field of behavioral neuroendocrinology, opening the door to many more questions revolving around the ovary-uterus-brain connection, through which we will better our understanding of reproductive hormones, aging, and cognition as a whole.

General Conclusions

The female brain and body have a remarkable capacity to adapt to the constant ebb and flow of the reproductive cycle throughout the lifespan. Cyclicity is inherent to the nature of the female. Alterations to this elaborate system —whether via exogenous hormone treatments or by gynecological surgery, as well as through natural reorganizational periods such as puberty, pregnancy, and menopause— create new challenges for adaptation and survival. Since the early twentieth century, rodent models have been instrumental to our current understanding of the reproductive system, hypothalamic-pituitary-gonadal feedback loops, and steroid hormone effects— particularly estrogens— on a wide range of body systems and processes, including cardiovascular disease, bone health, stroke, cancers, affective disorders, and cognition, including spatial learning, memory, and attentional processes. In addition, we have the advantageous capacity to evaluate behavioral and brain changes at the cellular and

molecular level in rodent models much more quickly than would be possible using human subjects. This utility affords basic scientists the opportunity to explore novel brain targets and drug delivery methods for favorable cognitive outcomes and healthy brain aging profiles that have the potential to translate to the clinical research setting. Indeed, rodent models of menopause, including ovary-intact, VCD, Ovx, and now hysterectomy variations, form the basis of our abilities as preclinical scientists to contribute to our understanding of the female reproductive system, as well as brain and behavioral changes that occur with perturbations and alterations to this system, such as removal of steroid hormones, depletion of ovarian follicles, or exogenous hormone therapy treatment. Indeed, these models allow basic scientists to systematically and methodically evaluate longitudinal impacts of hormones and aging, as well as directly assess brain, ovarian, uterine, and other body tissues from which we can glean insight into the physiological correlates associated with the aging female and the menopause transition. All of these elements showcase the utility of rodent models as translational models of human menopause and aid in our understanding of risk factors associated with aging and menopause, providing direction and potential solutions for individualized treatments for women in mid-life, to lower risk factors for disease, and to maintain a high quality of life.

Accumulating evidence points to roles for both age and reproductive hormones on the brain and behavior throughout life. Given the continuously increasing average human lifespan, it is more important than ever for the field of neuroendocrinology and aging to better understand how aging and the long-lasting changes in gonadal hormones and gonadotropins that occur in midlife can affect the neural circuits and molecular mechanisms related to learning and memory. Thus far, discoveries have included

multiple neural systems, domains of function, and biochemical mediators, such as the basal forebrain-hippocampal cholinergic pathway, GABAergic transmission, ERK1/2 signal transduction, ΔFosB expression, and structural brain changes. It is of particular interest to understand how the neurobiological and neurochemical changes associated with menopause and aging alter the underlying circuitry of cognitive pathways, and if these systems compensate by using alternative mechanisms or undergo a rewiring to return to homeostasis as aging occurs. Elucidating the changes in these molecular mechanisms with age and ovarian hormone milieu in a systematic and demonstrable fashion will yield insight into how and when the brain responds to endogenous hormone changes as well as to potential exogenous hormone treatment. This will, in turn, drive progress forward toward development and optimization of opportunities and choices for women undergoing the transition to menopause that not only addresses the undesirable symptoms associated with menopause, but also that potentially prevents, attenuates, or postpones the onset of cognitive or affective changes for at-risk women during aging. In order to move toward this realm of discovery, it should be recognized that ovarian hormones have a powerful impact on many complex and interactive neural systems that influence cognitive outcomes throughout life. It is only through continued methodical scientific evaluations that we will uncover the intricacies of these interconnected systems and transform our understanding of the connections between the reproductive system and the brain, in order to enhance the quality of life and care of women throughout the lifespan. Many will continue to search for the key to understanding reproductive hormones' intricate relationships with cognitive processes; while we have learned much, we still have much to discover and unexplored routes to travel. It is often the most

unexpected findings that can lead down a new path in the grand scientific venture of discovering the truth in nature. Indeed, “still round the corner there may wait, a new road or a secret gate” (Tolkien, 1954).

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Table 1. Mean ± SEM, range, and median of 17β-estradiol levels (pg/ml) at subset and end time points.

Group	Age	Mean of 17β-estradiol levels±SE	Range of 17β-estradiol levels	Median of 17β-estradiol levels
Young-Vehicle	8 months	10.07±1.32	8.6-12.7	8.90
	12 months	12.59±1.66	7.4-24.4	11.90
Young-VCD	8 months	10.47±2.57	6.1-15.0	10.30
	12 months	13.41±1.68	5.5-20.7	14.00
Middle-Aged-Vehicle	14 months	15.70±2.90	12.4-21.5	13.20
	18 months	13.61±4.23	6.1-38.5	10.10
Middle-Aged-VCD	14 months	10.57±1.20	8.7-12.8	10.20
	18 months	12.55±1.23	9.0-20.3	11.45

Table 2. Mean ± SEM, range, and median of Estrone levels (pg/ml) at subset and end time points.

Group	Age	Mean of Estrone levels±SE	Range of Estrone levels	Median of Estrone levels
Young-Vehicle	8 months	52.9±4.74	45.8-61.9	51.00
	12 months	60.33±4.65	48.4-76.8	56.70
Young-VCD	8 months	58.37±2.36	53.9-61.9	59.30
	12 months	50.70±2.51	41.8-63.0	49.50
Middle-Aged-Vehicle	14 months	68.47±7.33	55.0-80.2	70.20
	18 months	60.02±1.80	55.5-66.1	59.35
Middle-Aged-VCD	14 months	65.57±3.50	61.3-72.5	62.90
	18 months	53.19±2.49	43.2-64.6	52.55

Table 3. Mean ± SEM, range, and median of Androstenedione levels (ng/ml) at subset and end time points.

Group	Age	Mean of Androstenedione levels±SE	Range of Androstenedione levels	Median of Androstenedione levels
Young-Vehicle	8 months	0.30±0.03	0.24-0.34	0.32
	12 months	0.36±0.17	Undetectable – 1.27	0.23
Young-VCD	8 months	0.19±0.04	0.11-0.24	0.22
	12 months	0.76±0.30	0.18-2.76	0.49
Middle-Aged-Vehicle	14 months	0.47±0.11	0.29-0.66	0.46
	18 months	0.83±0.16	0.20-1.47	0.70
Middle-Aged-VCD	14 months	1.00±0.29	0.48-1.49	1.02
	18 months	0.81±0.26	Undetectable-2.28	0.55

Table 4. Mean ± SEM, range, and median of Progesterone levels (ng/ml) at subset and end time points.

Group	Age	Mean of Progesterone levels±SE	Range of Progesterone levels	Median of Progesterone levels
Young-Vehicle	8 months	22.77±6.72	18.9-39.2	19.20
	12 months	30.30±7.38	4.8-68.7	34.4
Young-VCD	8 months	11.23±3.41	5.2-17.0	11.50
	12 months	8.41±2.81	2.4-26.7	5.05
Middle-Aged-Vehicle	14 months	38.46±10.73	19.9-54.1	43.40
	18 months	40.04±10.74	9.7-76.8	45.5
Middle-Aged-VCD	14 months	50.67±11.26	29.4-67.7	54.9
	18 months	8.1±1.68	1.0-19.0	7.45

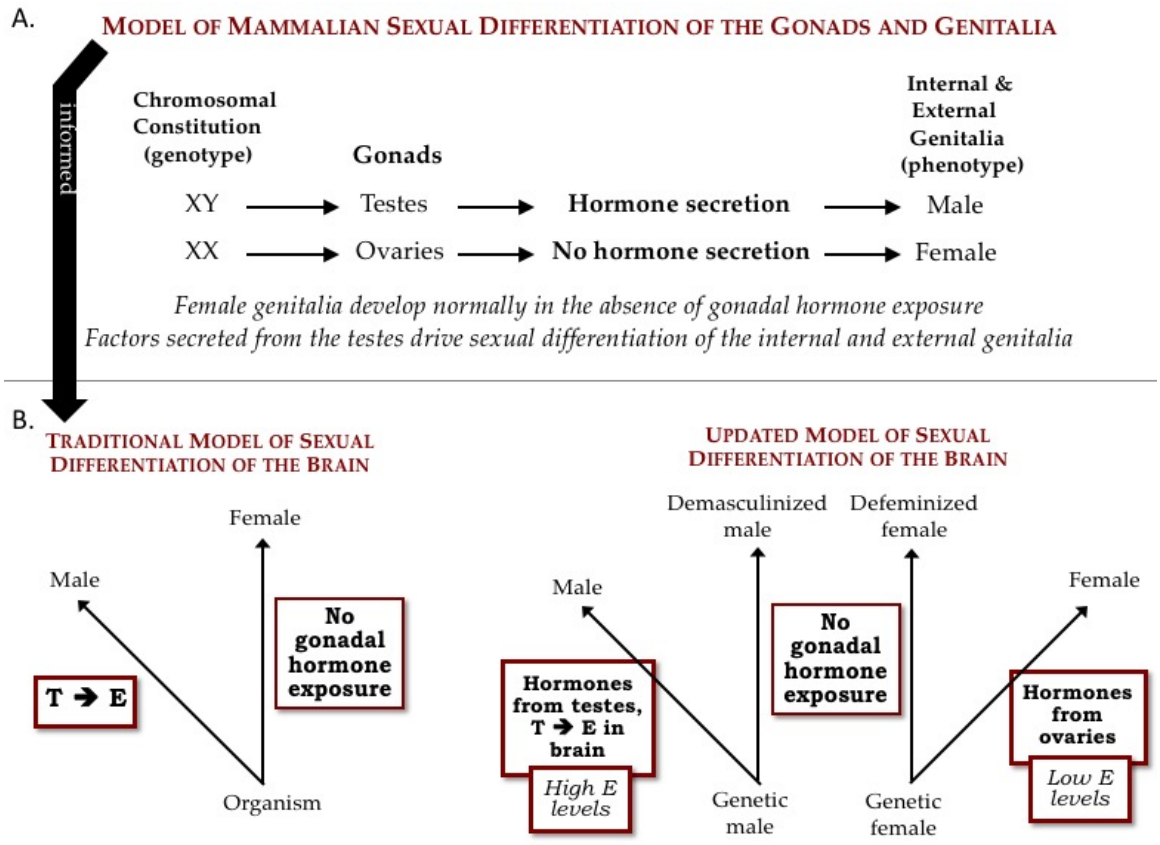

Figure 1a: The standard model of mammalian sexual differentiation holds that male genitalia actively develop in the presence of androgens, while female genitalia develop in the absence of gonadal hormone exposure. 1b: The traditional model of sexual differentiation of the brain holds the same "default" concept for females as the model for genitalia development; the updated model builds on these concepts using new research, and suggests that sexual differentiation of the brain involves active processes of gonadal hormone exposure for both males and females. E = estrogen, T = testosterone

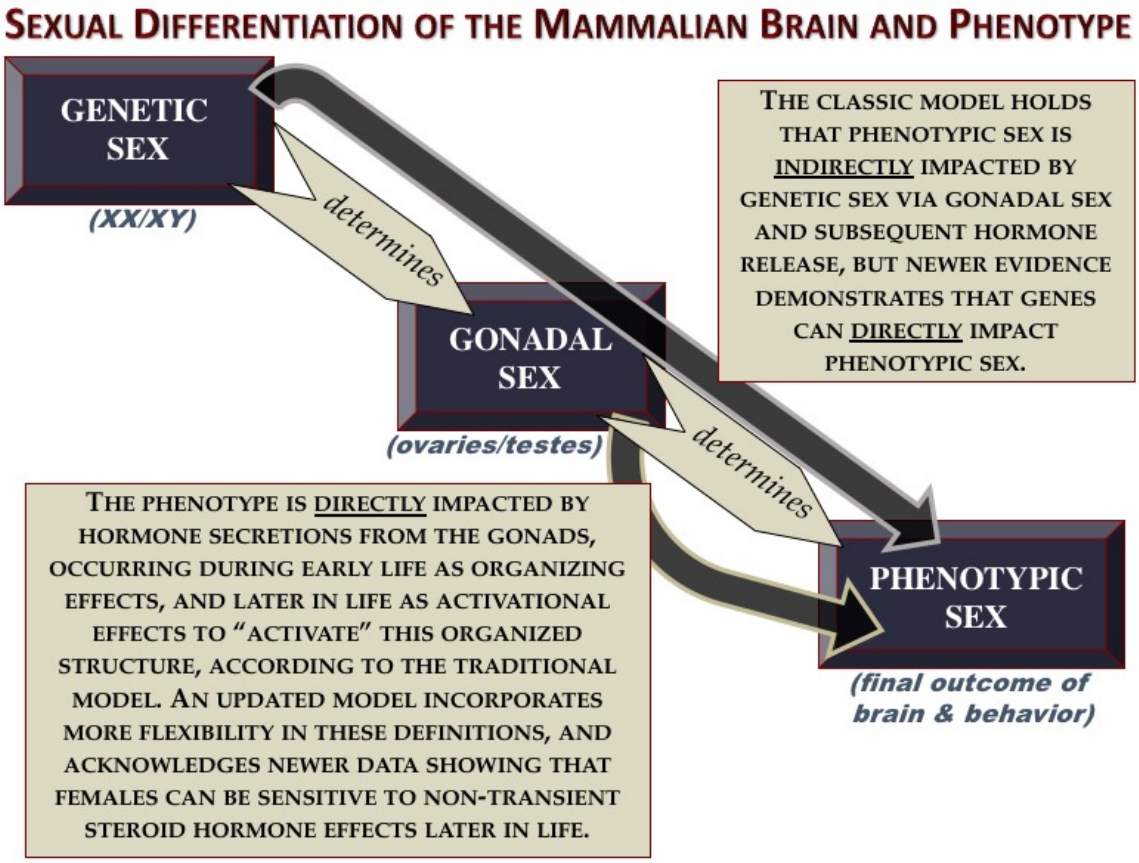

Figure 2: A flow diagram of sexual differentiation of the mammalian brain and phenotype. The classic model holds that genetic sex determines gonadal sex, and that gonadal sex determines phenotypic sex. The phenotype is directly impacted by hormone secretions from the gonads, occurring during early life as organizational effects, and later in life as activational effects. An updated model incorporates more flexibility in these definitions, and acknowledges newer data showing that females can be sensitive to non-transient steroid hormone effects later in life. Further, phenotypic sex is indirectly impacted by genetic sex via gonadal sex and subsequent hormone release, but accumulating evidence demonstrates that genes can directly impact phenotypic sex as well.

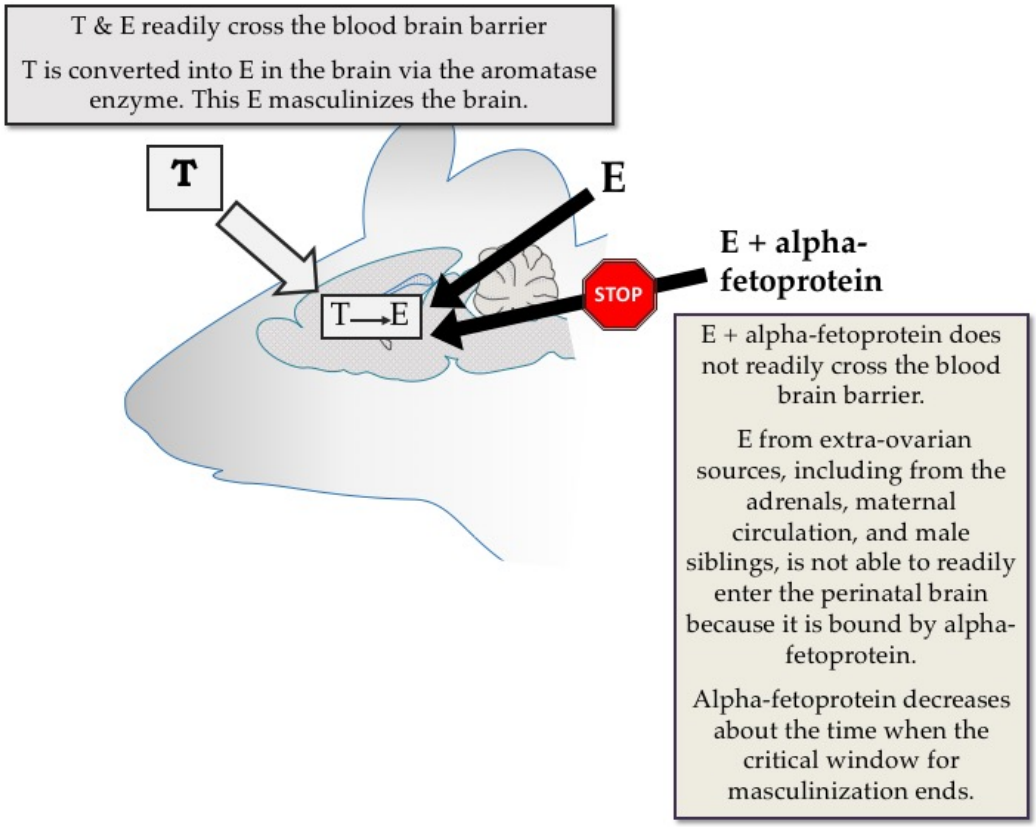

Figure 3: In rodents, testosterone of testes origin indirectly masculinizes the male brain. Testosterone readily crosses the blood brain barrier and is converted in the brain to 17β-estradiol via the aromatase enzyme, and this 17β-estradiol exposure results in masculinization. The plasma protein alpha-fetoprotein has a high binding affinity and capacity for estrogens in rodents. Once bound to alpha-fetoprotein, estrogen can no longer cross the blood brain barrier. This scenario prevents estrogen-induced brain masculinization in females during the early perinatal timeframe, when extra-ovarian estrogens are present. Remarkably, alpha-fetoprotein levels become undetectable in the brain after P7, around the same time that the ovaries increase in activity and produce detectable levels of gonadal hormones. The extended temporal period of brain sensitivity to gonadal hormones in females suggested in many rodent studies corresponds with the work showing that estrogens of ovarian origin are likely necessary for normal female brain development, and that they can access the brain when alpha-fetoprotein declines after the early perinatal period. Of note, temporally, this is after the window for normal brain masculinization has closed. E = estrogen, T = testosterone

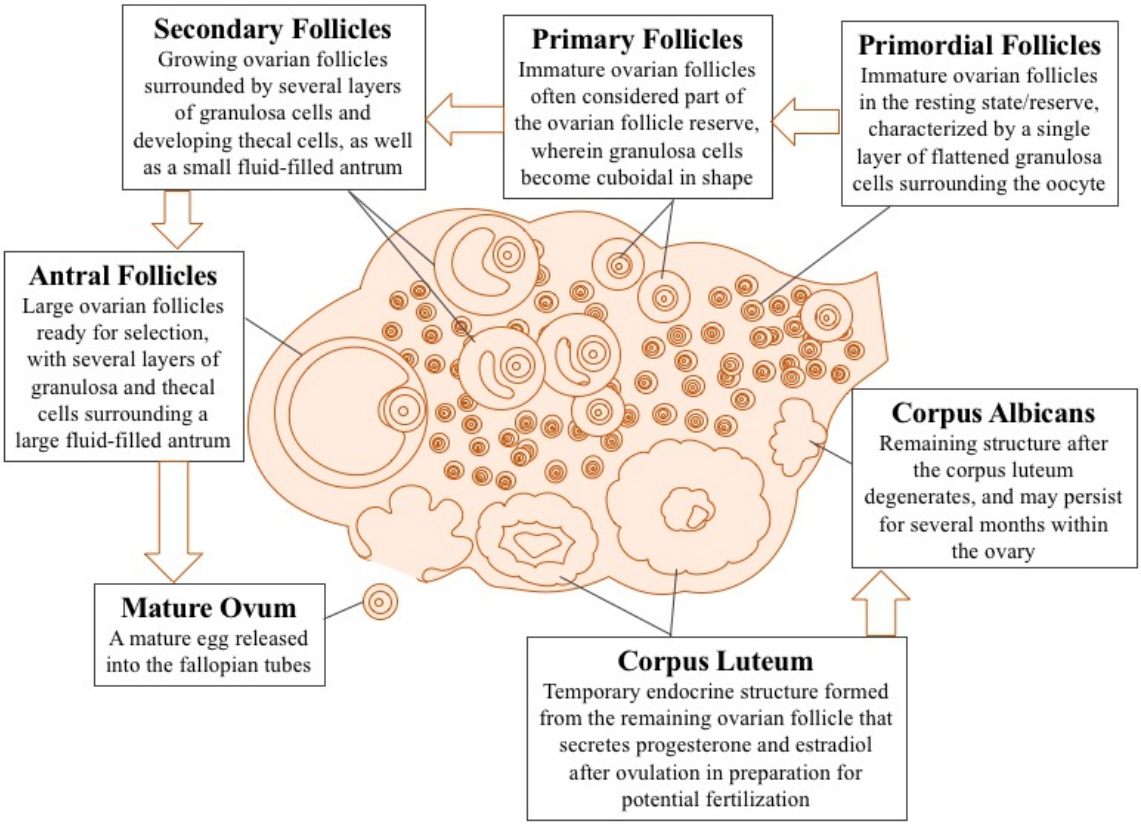

Figure 4: Stages of Ovarian Follicle Development. Stages of ovarian follicle development, beginning at the primordial resting stage and growing in size and shape through the antral (pre-ovulatory) mature follicle. Once the ovum is ovulated, the remaining follicle forms the corpus luteum, a temporary endocrine structure that produces high amounts of progesterone and some estradiol. The corpus luteum degenerates into the corpus albicans if no egg fertilization occurs.

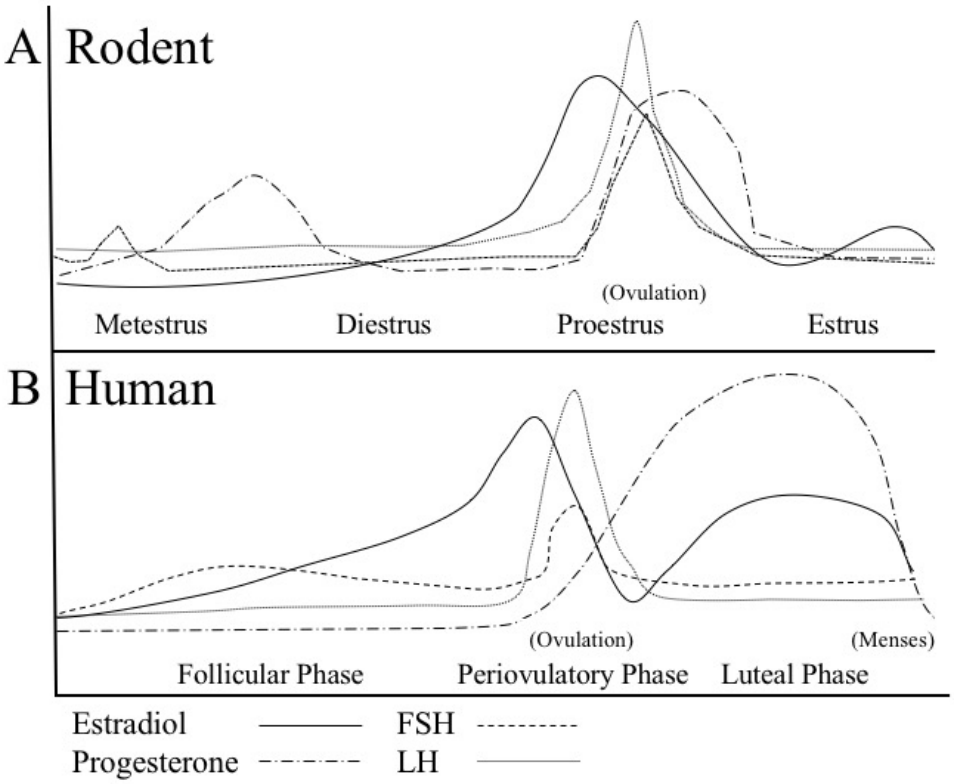

Figure 5: The Ovarian Hormone Cycle During the Reproductive Stage. A) The rodent estrous cycle is characterized by four phases. Proestrus is the shortest phase, lasting less than a day, wherein a critical level of increasing 17β-estradiol is thought to trigger the LH surge, inducing ovulation. FSH and progesterone also peak during proestrus. The proestrus phase is followed by the estrus phase. In estrus, LH, FSH, and progesterone decline to baseline levels, while 17β-estradiol levels are moderately low. In metestrus, 17β-estradiol is low, and a transient increase in FSH occurs. Some 17β-estradiol and high amounts of progesterone are released from the corpora lutea following ovulation, increasing circulating progesterone levels between the metestrus and diestrus phases. Gonadal hormones return to baseline as the estrous cycle begins again. B) The human menstrual cycle has three distinct phases. The follicular phase is characterized by steadily increasing 17β-estradiol levels. At a critical level of 17β-estradiol, LH surges, with the trigger for ovulation occurring in the periovulatory phase. Ovulation is followed by the luteal phase, wherein progesterone levels increase and 17β-estradiol is present at a moderate level.

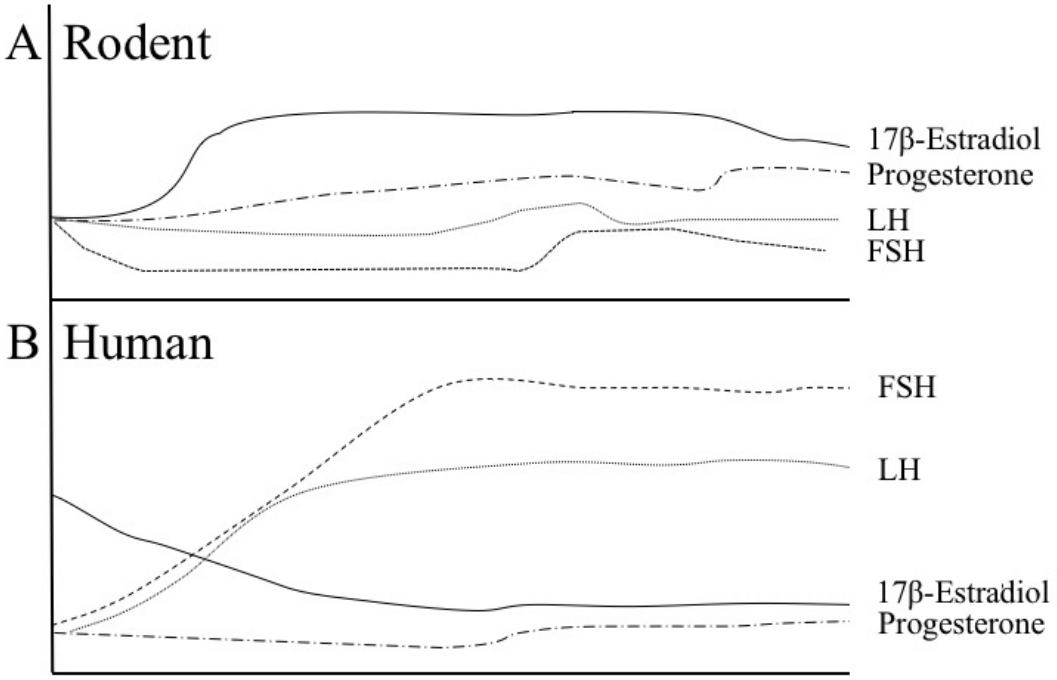

Figure 6: Ovarian Hormone Levels in Reproductive Senescence. A) Rodents commonly enter a persistent estrus state during reproductive senescence (estropause), characterized by moderate to high 17β-estradiol levels and moderate progesterone, LH, and FSH levels as a result of disrupted feedback between the ovaries and hypothalamus/pituitary. b) Human reproductive senescence (menopause) is characterized by low, sometimes undetectable, levels of 17β-estradiol and progesterone, but increased levels of the gonadotropins, FSH and LH.

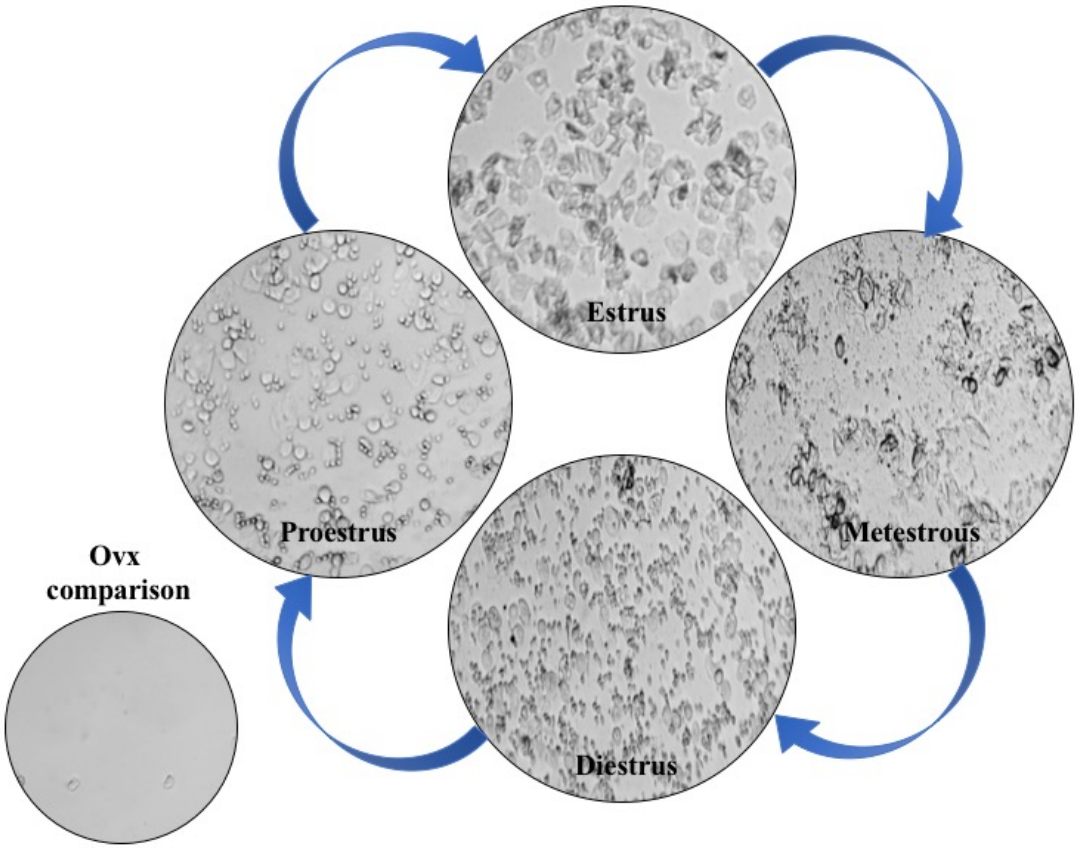

Figure 7: The Estrous Cycle. Representative images of the rat estrous cycle with vaginal cytology. Animals in estrus exhibit cornified cells in their vaginal smears. Metestrus smears contain a combination of cornified cells, leukocytes, needle-like, and round epithelial cells. Diestrus smears are characterized by a large number of leukocytes, and proestrus smears contain round epithelial cells and some cornified cells, sometimes presenting in clustered groups.

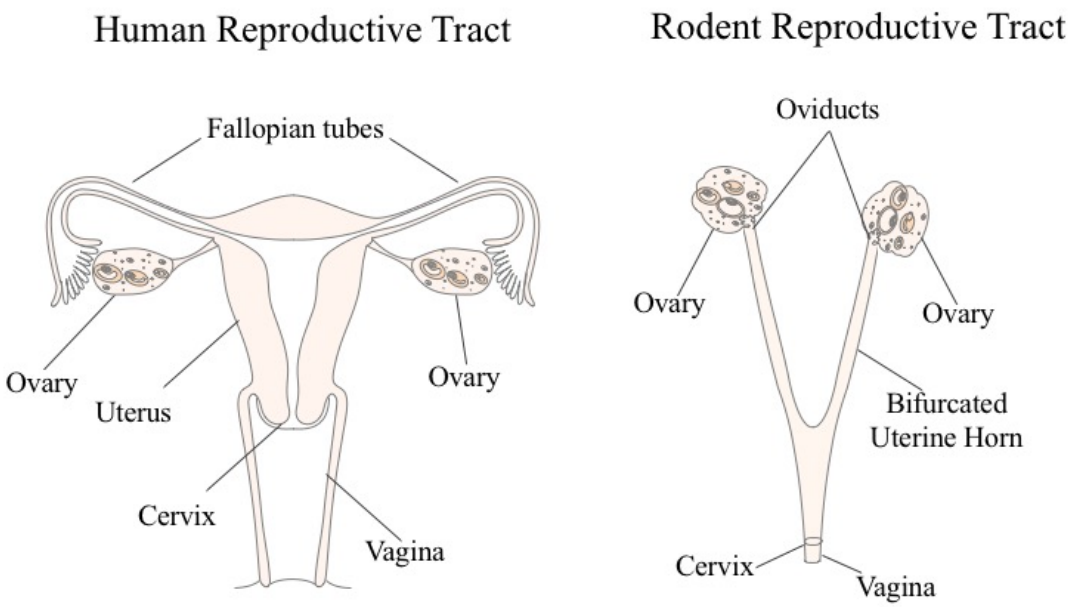

Figure 8: Human and Rodent Reproductive Tracts. A comparison of the human and rodent reproductive tracts, with analogous structures labeled.

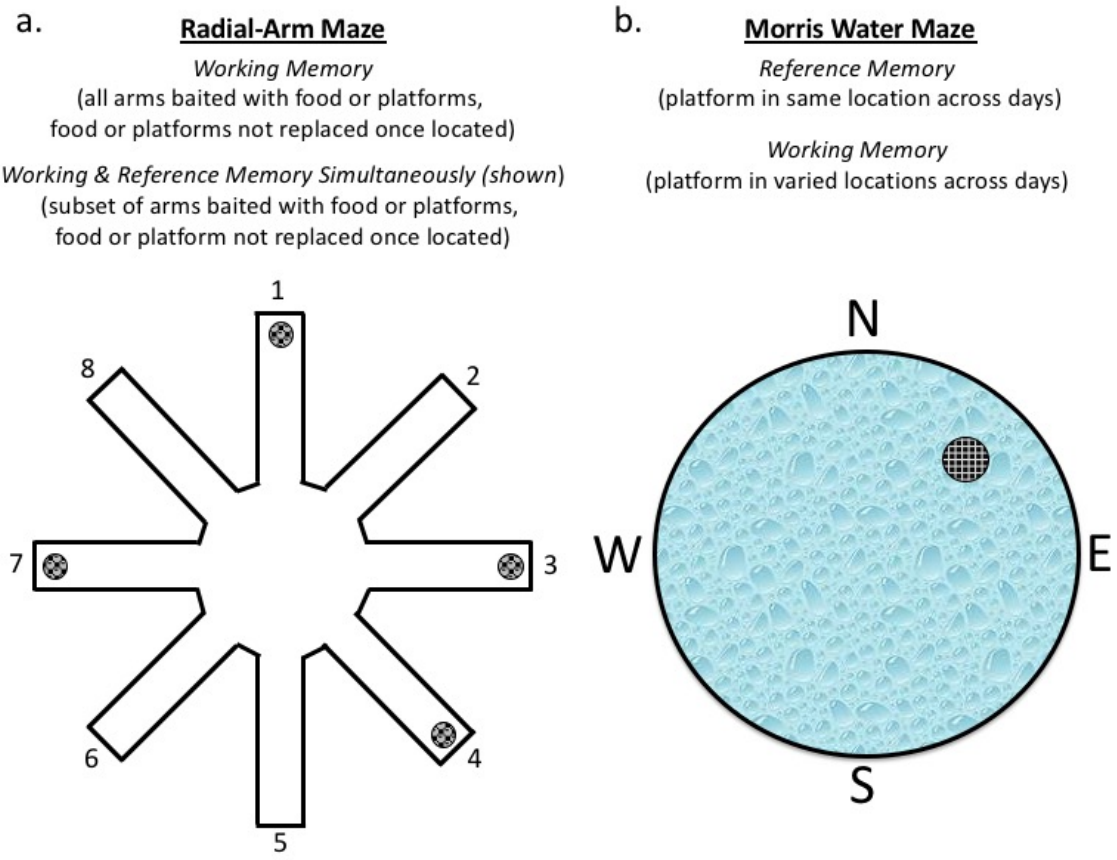

Figure 9: a) A schematic of an eight-arm radial-arm maze with a subset of arms baited to represent a working and reference memory task. The arms are baited with a food reward in the land version of the maze, and a hidden platform just beneath the surface of the water in the water escape version of the maze. B) A schematic of the Morris water maze, a reference memory task, with the hidden platform positioned in the northeast quadrant of the maze.

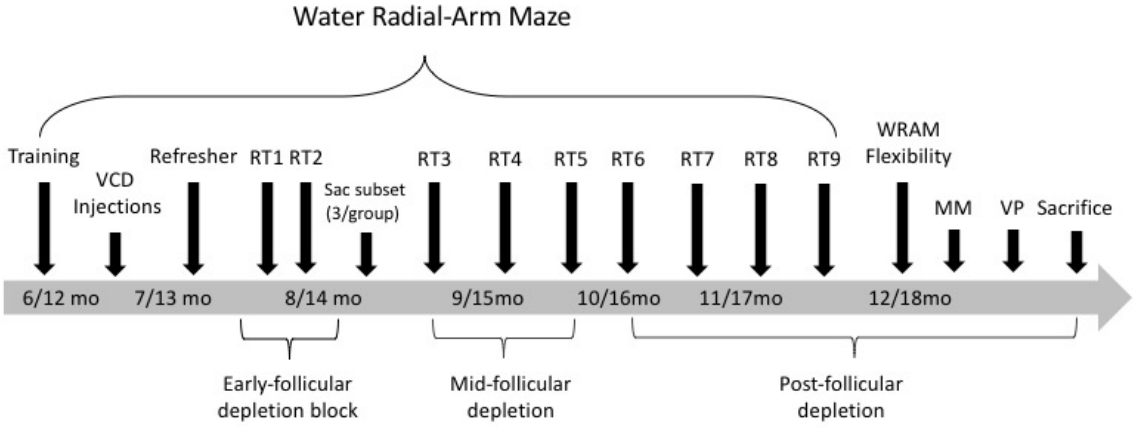

Figure 10: Study Timeline. Age of the young and middle-aged animals in months (mo) at time of assessment is indicated in the arrow. Animals received training for 12 days on the water radial-arm maze (WRAM). A one-month interim occurred during which animals received Vehicle or VCD injections. After a two-day refresher, subjects were tested for two-day retests (RT) every other week for four months, capturing the VCD-treated animals' transition from early follicular depletion to a post-follicle-deplete state. A subset of animals was sacrificed after RT2 to obtain a snapshot of serum hormone levels and ovarian follicular depletion early in the transition. Following WRAM RTs, remaining subjects were tested on a WRAM flexibility task, Morris water maze, and Visible Platform tasks prior to sacrifice.

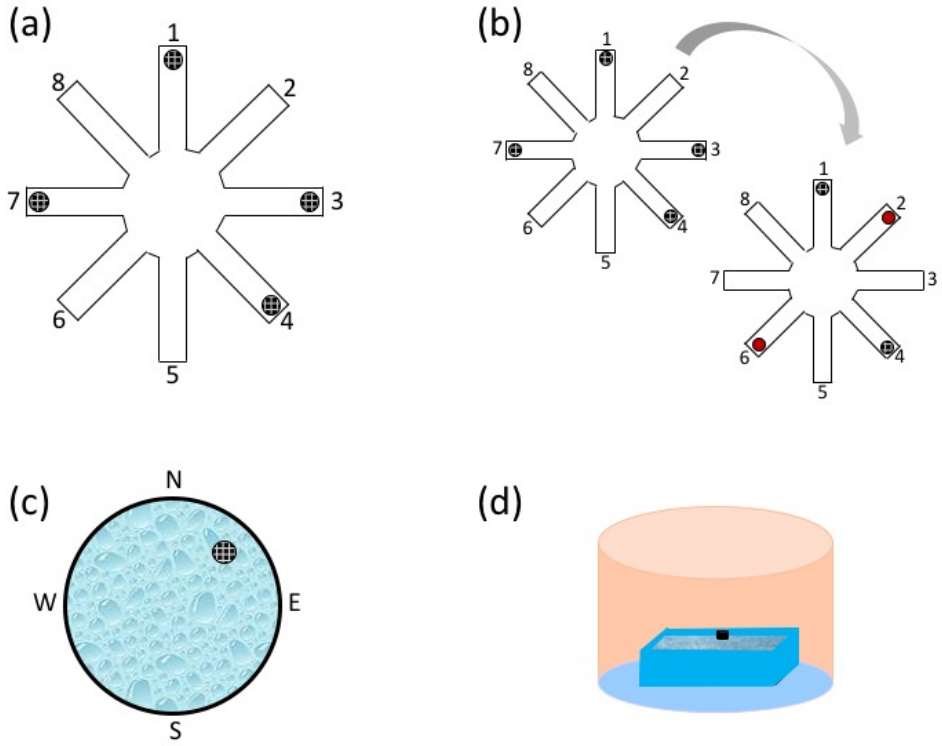

Figure 11: Schematics of the behavioral battery used throughout the experiment. (a) The water radial-arm maze (WRAM) contains four hidden platforms beneath the surface of the water, represented by black circles. Subjects were each assigned a set of platform locations that remained constant throughout retests. Platform locations varied across animals and were counterbalanced for age and treatment group. After one platform was located on each trial, the just-found platform was removed from the maze for the remainder of the day. (b) The WRAM Flexibility task occurred after RT9. Each subject was given two familiar platform locations from their initial platform location assignment (e.g. arms 1,4), and two platform locations were flipped to be located in a novel arm (e.g, platforms in arms 3 and 7 were moved to arms 2 and 6, indicated by the red circles), resulting in two novel spatial locations that require updating. (c) The Morris water maze was a large round tub with a hidden platform submerged beneath the water's surface in the northeast quadrant. The platform location remained constant across all baseline days and trials. Subjects were dropped off from each cardinal direction (north, south, east, and west) once per day. The order in which the drop off locations occurred was the same for all animals within a day, but varied across days. After the fourth trial on Day 5, the platform as completely removed from the maze to conduct the probe trial; animals were dropped off from the west for the probe trial. (d) The visible platform was a rectangular tub filled with clear water, with a black platform placed approximately 4 cm above the surface of the water. Opaque curtains were hung in a circular fashion around the room to block any spatial and geometric cues. Animals were dropped off from the south wall and were given 90s to reach the platform, which was located on the north wall. The platform location was the same for all animals within a trial, but varied across trials (left, center, and right of the drop off location).

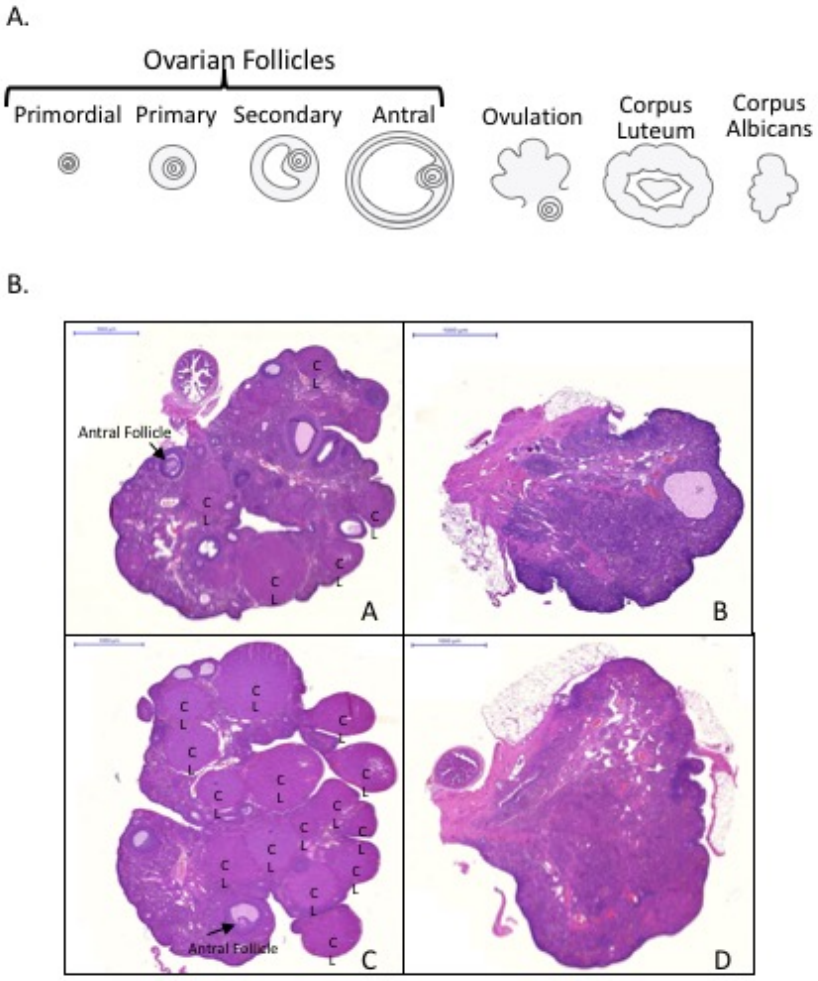

Figure 12: (a) A schematic of the different phases of ovarian follicle growth, beginning with the primordial, resting follicle pool, and progressing from primary to secondary to antral (pre-ovulatory) stages of growth. Following ovulation of the mature egg, the remaining follicle becomes the corpus luteum, a temporary endocrine structure that secretes progesterone and low levels of estrogens. The corpus luteum eventually regresses into the corpus albicans, which no longer secretes ovarian hormones. Of these ovarian follicle stages, VCD accelerates atresia (programmed cell death) for primordial and primary ovarian follicles. (b) Representative ovary micrographs from each group. (i) Rat ovary from the Young-Vehicle group (ii) Rat ovary from the Young-VCD group (iii) Rat ovary from the Middle-Aged-Vehicle group (iv) Rat ovary from the Middle-Aged-VCD group. Note that long-term exposure to VCD is indicated by the loss of corpora lutea structures due to ovarian failure. All micrographs are depicted at 2x and the scale bar is 1000 μm.

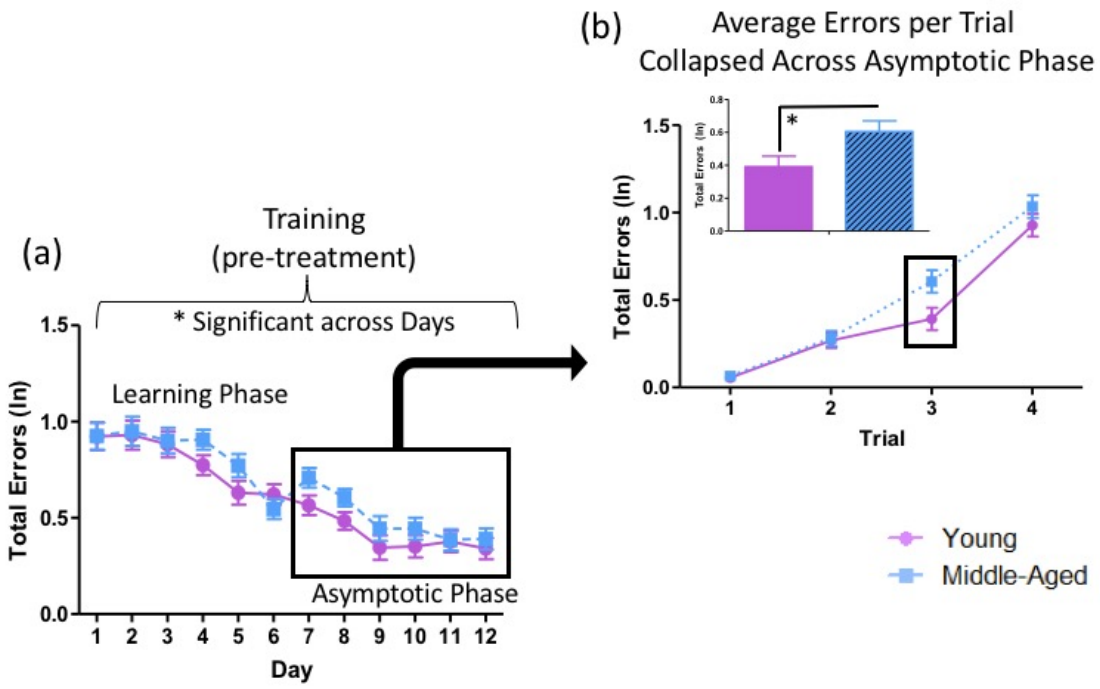

Figure 13: WRAM performance for Training (pre-treatment). (a) Across the 12 days of baseline testing, there was a main effect of Age, where Middle-Aged animals made more total errors than Young animals collapsed across all days of testing. (b) Average total errors (ln) per trial were evaluated for the asymptotic phase of testing (D7-12). Trial 3 alone, a higher working memory load trial, revealed a main effect of Age, where Middle-Aged animals made more errors than Young animals (* = $p<0.05$).

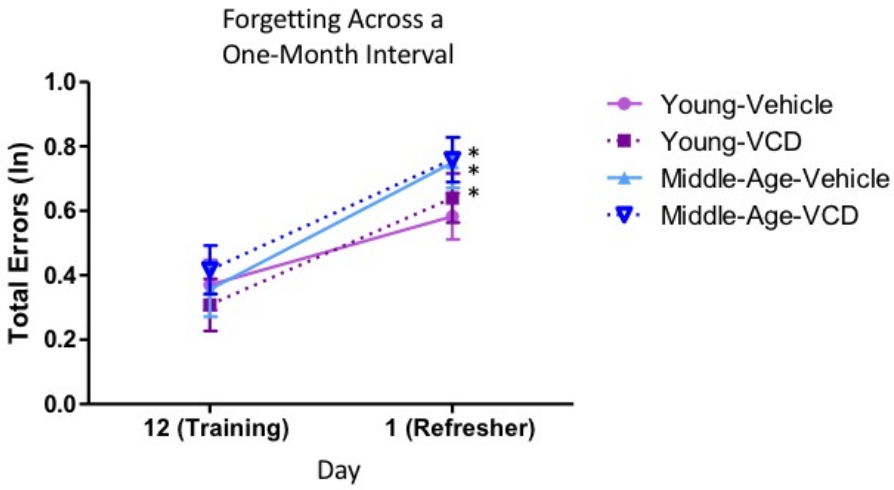

Figure 14: Forgetting for WRAM performance across a one-month interval. Between the last day of training (prior to treatment) and the first day of the refresher one month later (after treatment), Young-Vehicle treated animals did not exhibit significant forgetting, but the Young-VCD, Middle-Age-Vehicle, and Middle-Aged-VCD showed forgetting (* = $p<0.05$).

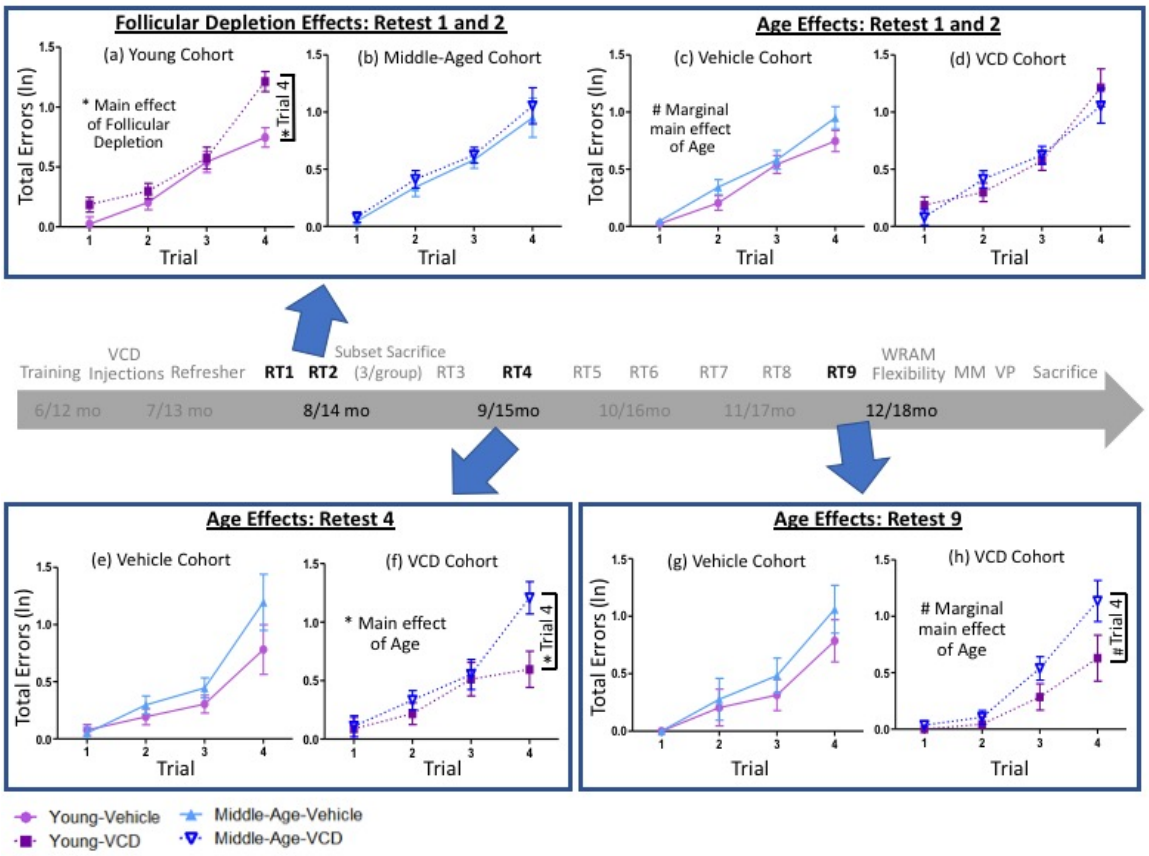

Figure 15: WRAM performance across the transition to follicular depletion. (a) Within the Young cohort, a main effect of Follicular Depletion was observed, where VCD-treated animals made more errors than Vehicle-treated animals, and this was particularly evident on Trial 4 early in follicular depletion. (c) Within the Vehicle cohort, there was a marginal main effect of Age where Middle-Aged animals tended to make more errors than Young animals. (b,d) There were no significant differences within the Middle-Aged cohort or the VCD cohort early in follicular depletion. (e) There were no differences in performance in the Vehicle cohort in mid-follicular depletion. (f) Within the VCD cohort, there was a main effect of Age where Middle-Aged animals made more errors than Young animals collapsed across trial, and this was particularly evident on Trial 4 in mid-follicular depletion. (g) There were no differences in performance in the Vehicle-treated cohort in post-follicular depletion. (h) For the VCD cohort, there was a marginal main effect of Age collapsed across trials, and a marginal Age effect on Trial 4, where Middle-Aged animals made more errors than Young animals in post-follicular depletion (* = $p<0.05$, # = $p<0.10$).

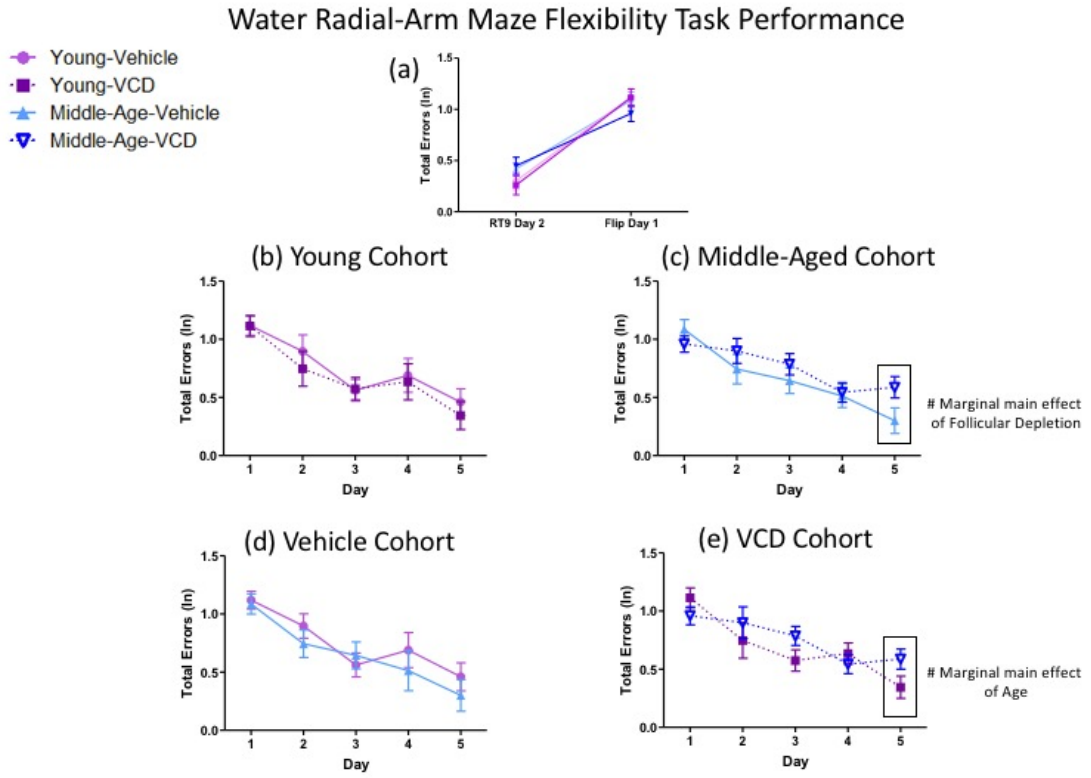

Figure 16: Water radial-arm maze flexibility task performance. (a) From the last day of RT9 to the first day of the flexibility task with two flipped platform locations, all subjects had impaired performance on the first day of exposure to the two flipped spatial locations, regardless of age or follicular depletion status. (b) No differences in performance were noted within the Young cohort across days, regardless of follicular depletion status. (c) Within the Middle-Aged cohort, on the final day of the flexibility task, there was a marginal main effect of Follicular Depletion, where VCD-treated animals made more errors than Vehicle-treated counterparts. (d) No differences in performance were noted within the Vehicle cohort across days, regardless of age. (e) Within the VCD cohort, on the final day of the flexibility task, there was a marginal main effect of Age, where older animals made more errors than younger animals. (# = $p<0.10$).

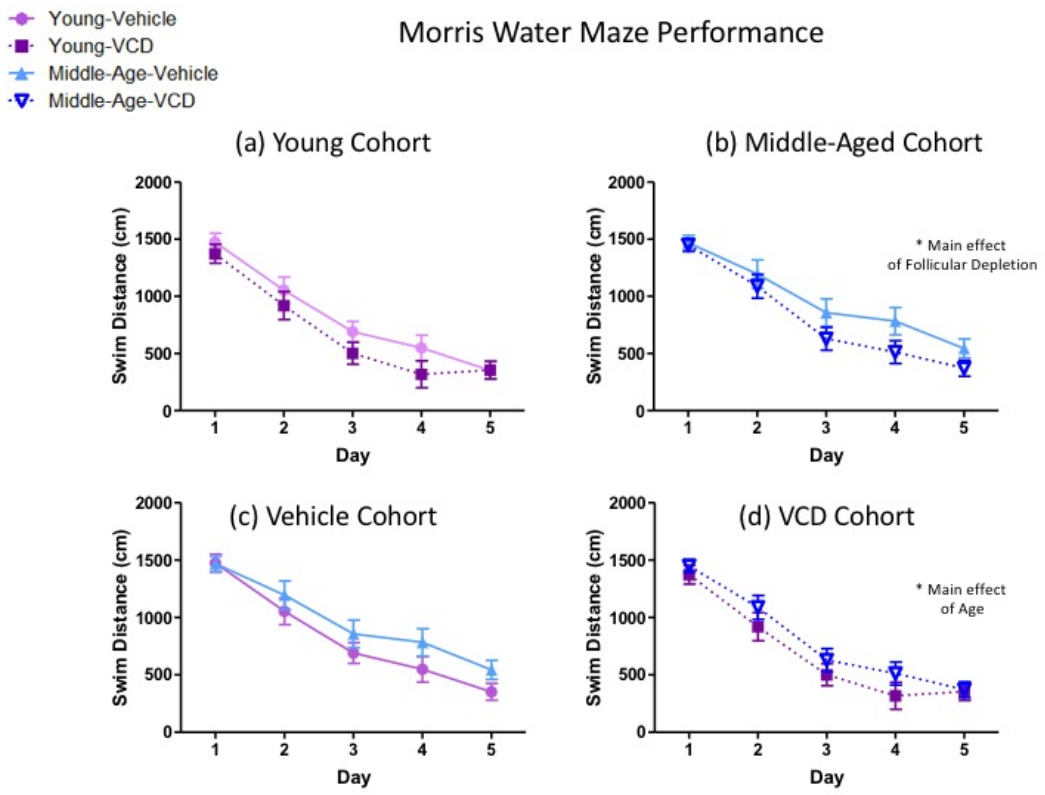

Figure 17: Morris water maze performance, average swim distance across four trials per day. (a) Swim distance did not vary in the Young cohort, regardless of follicular depletion status. (b) Within the Middle-Aged cohort, there was a main effect of Follicular Depletion, where VCD-treated animals swam less distance than Vehicle-treated animals. (c) Swim distance did not vary in the Vehicle cohort, regardless of age. (d) Within the VCD cohort, there was a main effect of Age, where Young animals swam less distance than Middle-Aged animals across days. (* = $p<0.05$).

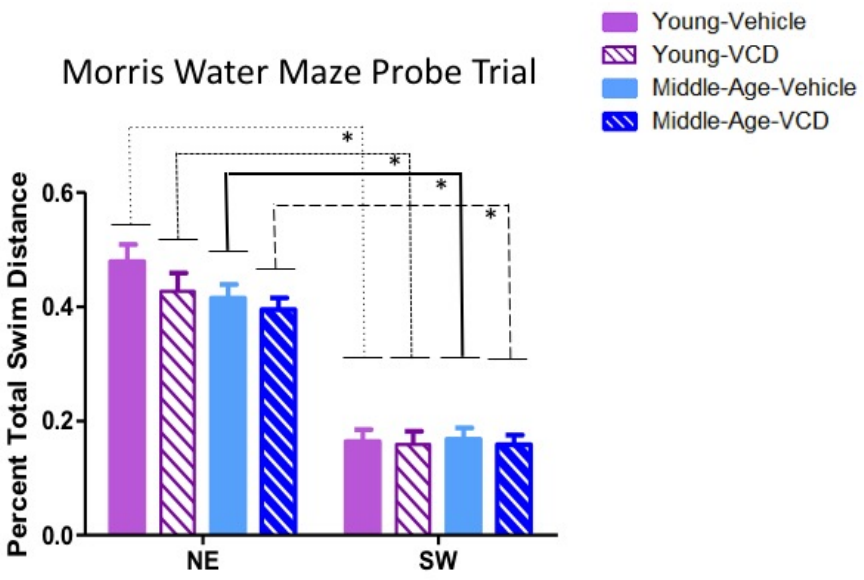

Figure 18: Morris water maze probe trial performance. Each treatment group, assessed separately, had a greater percent of total swim distance in the northeast (target) quadrant compared to the southwest (opposite) quadrant during the probe trial, indicating all animals spatially localized to the platform location, regardless of age or follicular depletion status. (* = $p<0.05$).

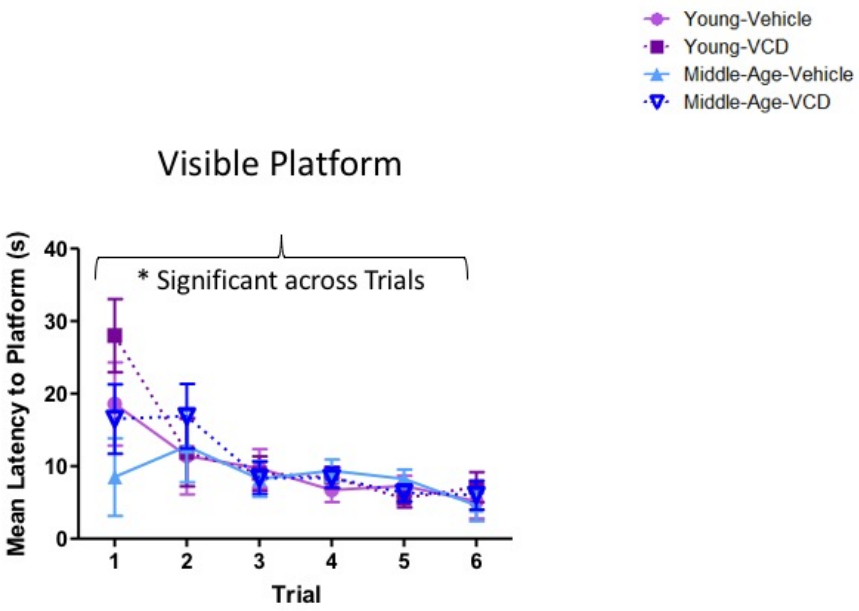

Figure 19: Visible platform performance. All animals, regardless of age or follicular depletion status, decreased latency (s) to the visible platform across six trials, confirming that all subjects could perform the motor and visual components of water maze tasks. (* = $p<0.05$).

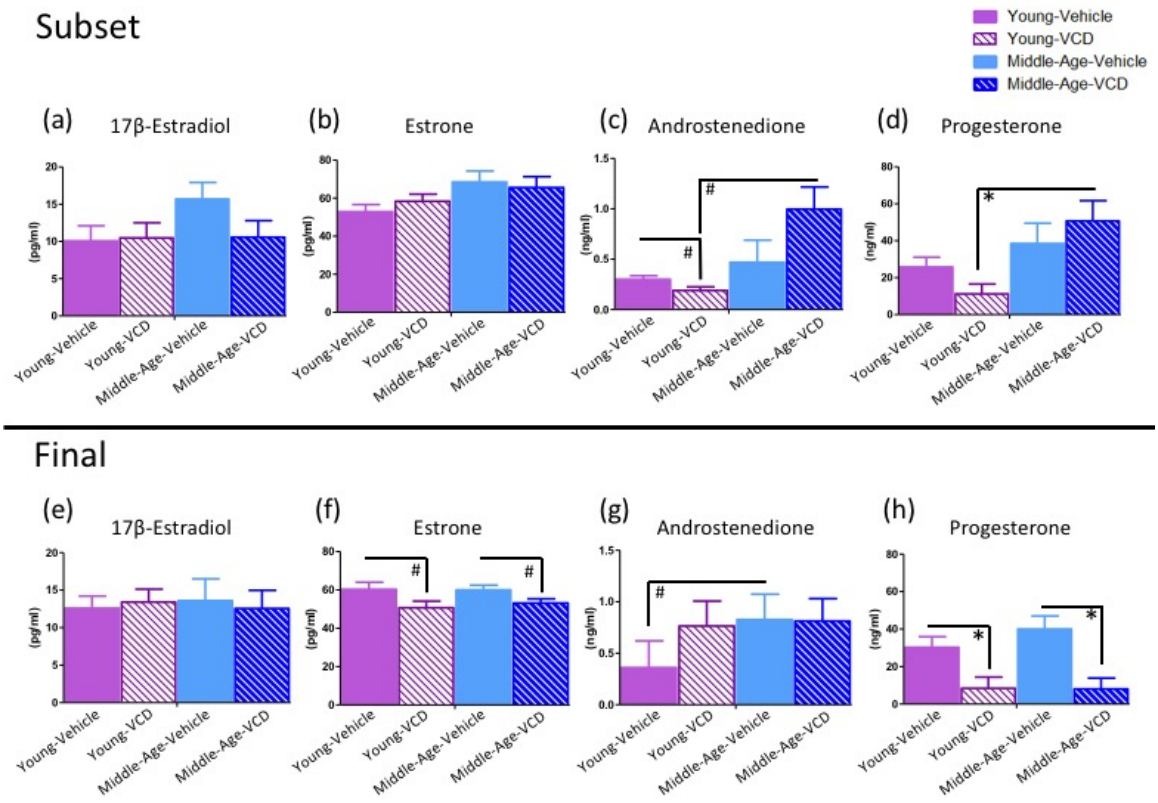

Figure 20: Circulating serum hormone levels for the subset (a-d) and end sacrifice (e-h) time points. (a) Mean ± SEM for 17β-estradiol serum levels (pg/ml) for the subset sacrifice. No differences were observed among treatment groups at the early follicular depletion time point. (b) Mean ± SEM for estrone serum levels (pg/ml) for the subset sacrifice. No differences were observed among treatment groups at the early follicular depletion time point. (c) Mean ± SEM for androstenedione serum levels (ng/ml) for the subset sacrifice. Young-VCD animals had marginally less androstenedione compared to age-matched controls, and to Middle-Aged-VCD animals. (d) Mean ± SEM for progesterone serum levels (ng/ml) for the subset sacrifice. Young-VCD animals had less progesterone than Middle-Aged-VCD animals at the early follicular depletion time point. (e) Mean ± SEM for 17β-estradiol serum levels (pg/ml) for the end sacrifice. No differences were observed among treatment groups at the post-follicular depletion time point. (f) Mean ± SEM for estrone serum levels (pg/ml) for the end sacrifice. Within the Young cohort and within the Middle-Aged cohort, there were marginal main effects of Follicular Depletion where VCD-treated animals had marginally less estrone compared to Vehicle-treated animals within both age groups. (g) Mean ± SEM for androstenedione serum levels (ng/ml) for the end sacrifice. Middle-Aged Vehicle animals had marginally more androstenedione than Young Vehicle animals. (h) Mean ± SEM for progesterone serum levels (ng/ml) for the end sacrifice. Within the Young cohort and within the Middle-Aged cohort, there was a main effect of Follicular Depletion wherein VCD-treated animals had less progesterone than Vehicle-treated animals within both age groups. (* = $p<0.05$; # = $p<0.10$).

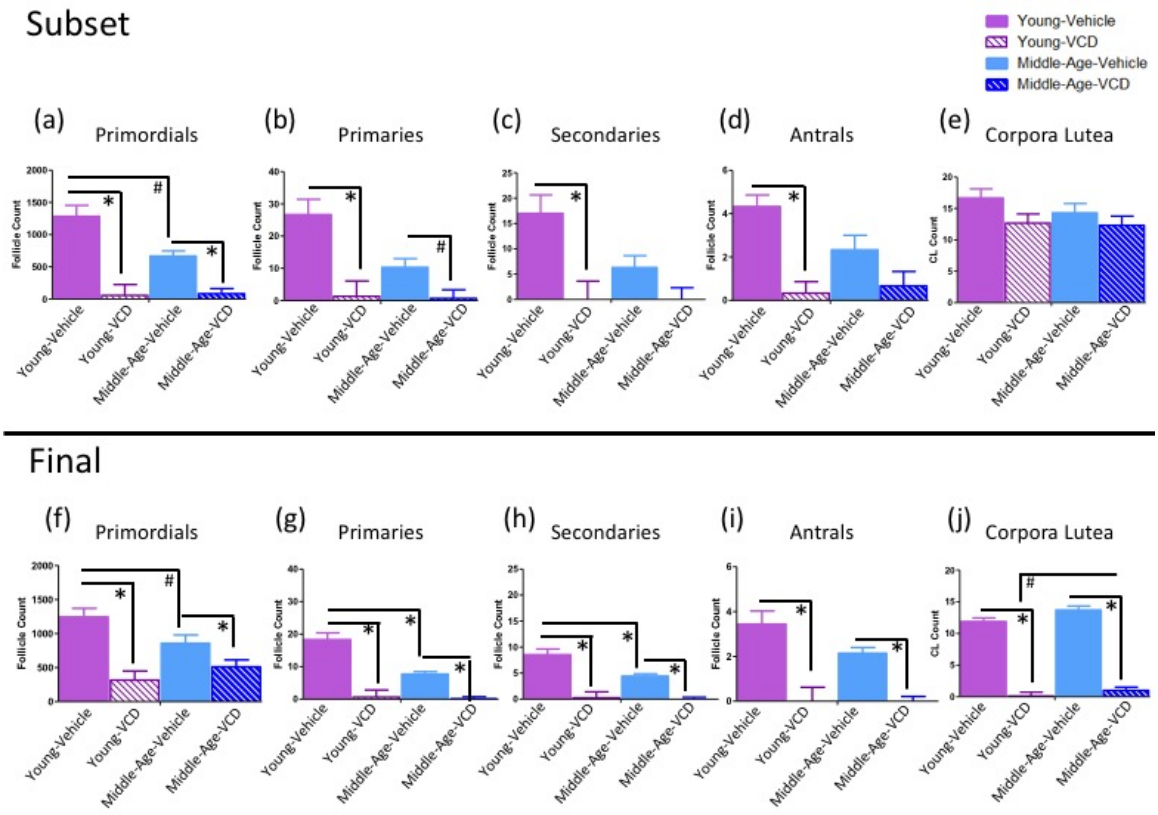

Figure 21: Ovarian follicle and corpora lutea counts for the subset (a-e) and end (f-j) sacrifice. (a-e) Young-VCD animals had fewer primordial, primary, secondary, and antral follicles compared to their Young-Vehicle controls at the subset time point. Middle-Aged-VCD animals had fewer primordial cells and marginally fewer primary cells compared to Middle-Aged-Vehicles, but did not differ from their Vehicle-treated counterparts for secondary or antral follicle counts at the subset time point. Animals did not differ in corpora lutea count, regardless of age or follicular depletion status, at the early follicular depletion time point. (f-j) Young and Middle-Aged VCD-treated animals had fewer primordial, primary, secondary, and antral follicle counts and fewer corpora lutea compared to their respective age-matched, Vehicle-treated controls, indicating that VCD treatment effectively depleted ovarian follicles. Middle-Aged Vehicle animals had marginally fewer primordial, and significantly fewer primary and secondary ovarian follicles than younger Vehicle-treated animals, but did not differ for antral follicle count or corpora lutea counts, at the end sacrifice time point, suggesting that rodents experience some follicular depletion with normal aging. (* = $p<0.05$; # = $p<0.10$).

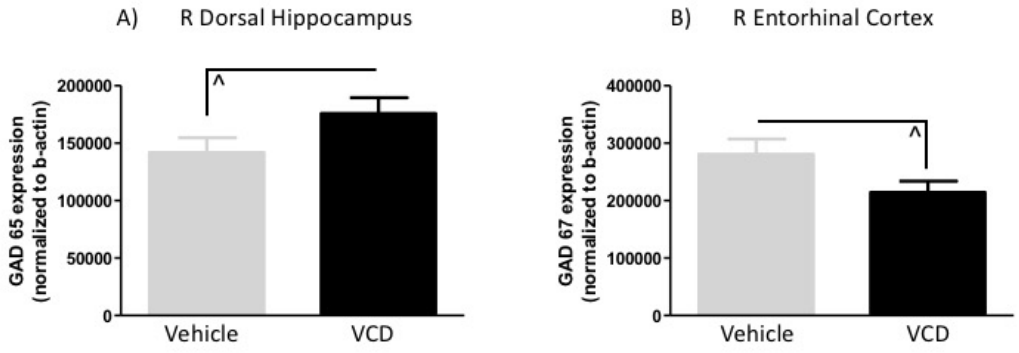

Figure 22: Western blot protein analysis. VCD-induced follicular depletion tended to increase GAD65 expression in the Dorsal Hippocampus (p=0.08), and tended to decrease GAD67 expression in the Entorhinal Cortex (p=0.06) compared to Vehicle controls.

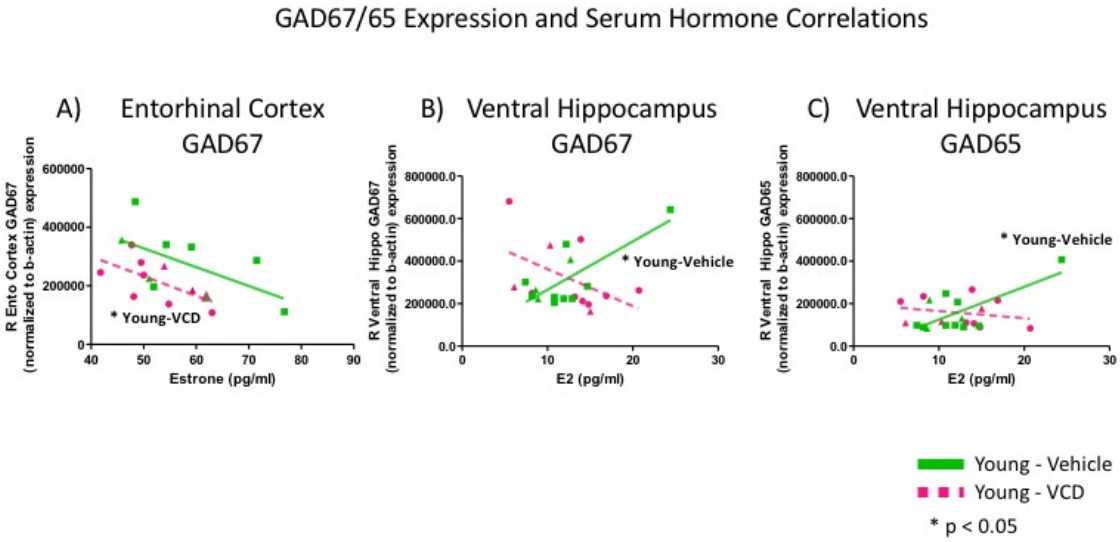

Figure 23: Region-Specific Correlations Between Serum Hormone Levels and GAD65 and GAD67. For Young-VCD rats, higher serum estrone (pg/mL) was associated with lower GAD67 expression in the Entorhinal Cortex. For Young-Vehicle rats, higher serum E2 (pg/mL) was associated with increased GAD67 and GAD65 expression in the Ventral Hippocampus (*$p < 0.05$).

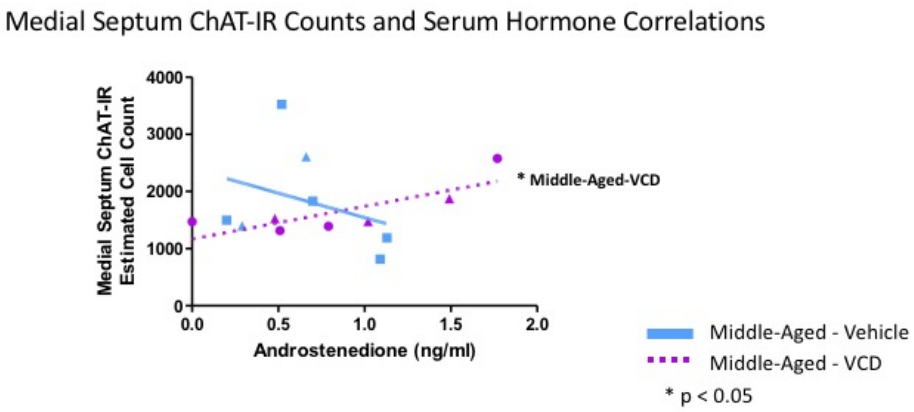

Figure 24: Region-Specific Correlations Between Serum Hormone Levels and ChAT-IR cell counts. Increased androstenedione serum (ng/mL) were associated with increased ChAT-IR estimated cell counts in the medial septum of the basal forebrain for the Middle-Aged-VCD group ($p < 0.05$).

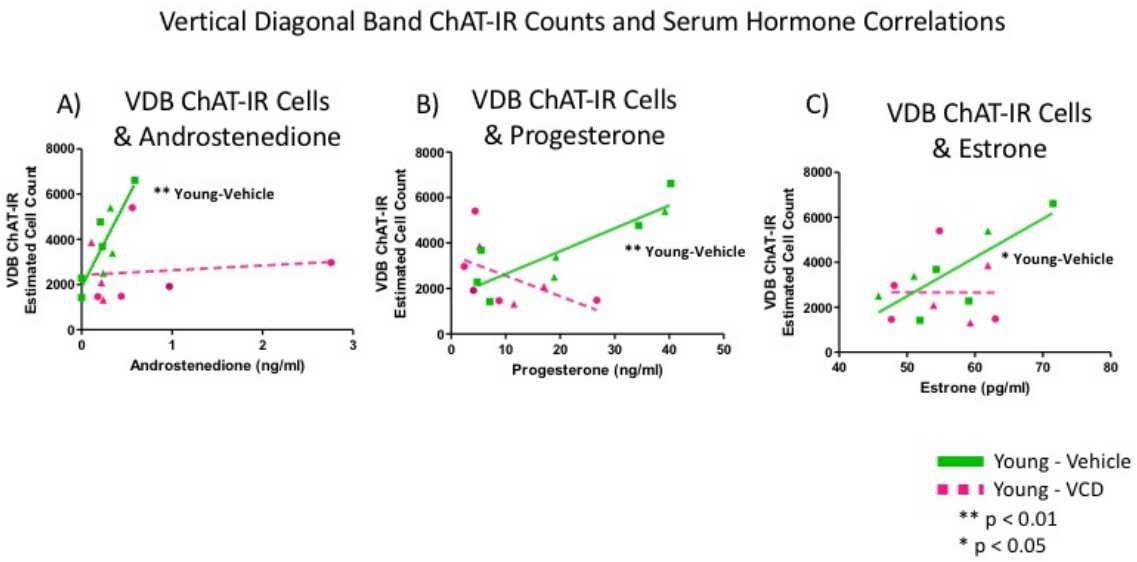

Figure 25: Region-Specific Correlations Between Serum Hormone Levels and ChAT-IR cell counts. For Young-Vehicle rats, higher serum androstenedione (ng/mL), progesterone (ng/mL), and estrone levels (pg/mL) were associated with more ChAT-IR estimated cell counts within the VDB of the basal forebrain. These associations were not present in age-matched counterparts that underwent follicular depletion.

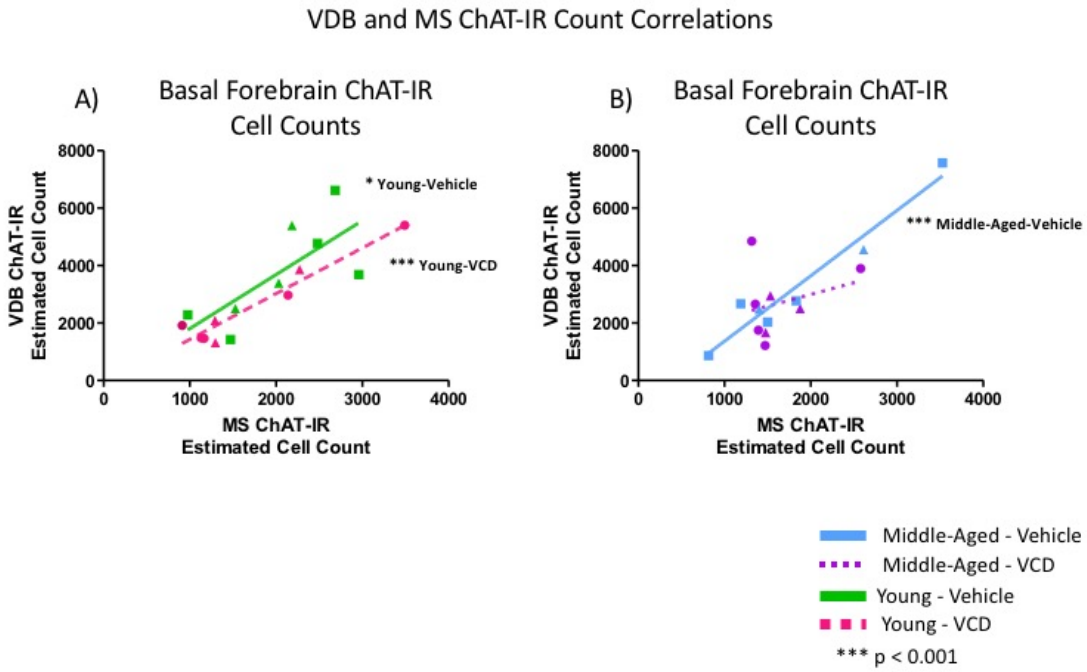

Figure 26: ChAT-IR correlations within the basal forebrain. The number of ChAT-IR cells in the medial septum within the basal forebrain correlated with estimated counts in the vertical/diagonal bands for both young groups and for Middle-Aged Vehicle rats. The association between the number of ChAT-IR cells in these regions of interest did not reach significant for the Middle-Aged-VCD group.

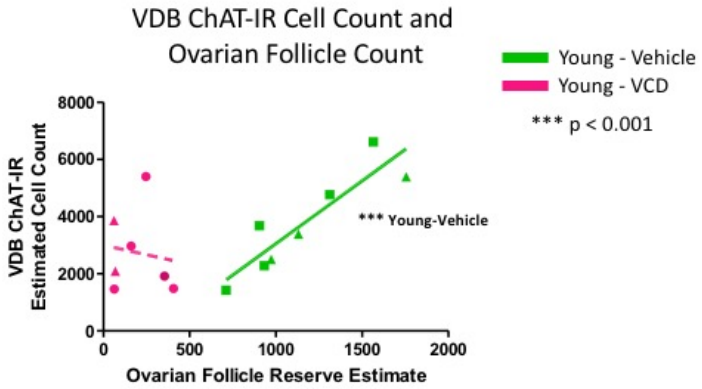

Figure 27: ChAT-IR correlations with ovarian follicle reserve. Primordial follicle counts, a putative marker of ovarian reserve, positively correlated with ChAT-IR cell estimates within the VDB for Young-Vehicle rats, but not for age-matched VCD-treated counterparts.

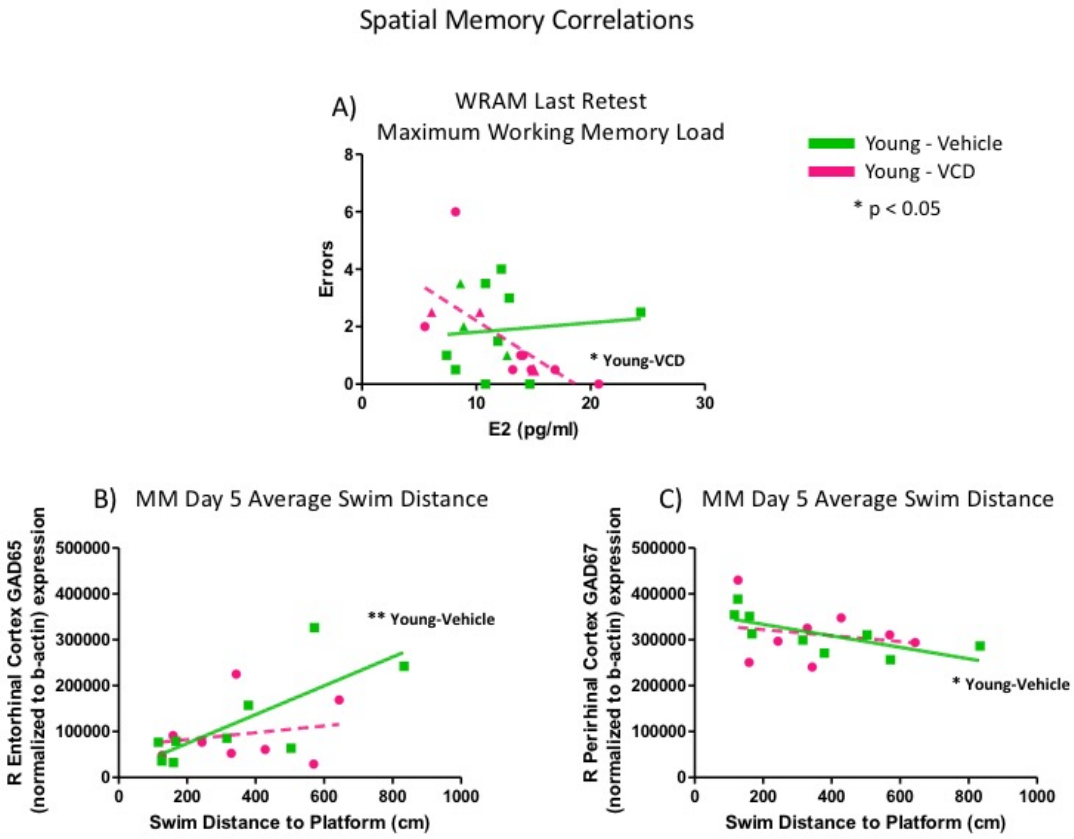

Figure 28: Correlations for Spatial Memory Performance, Ovarian Hormone Levels, and Brain Measures. Higher endogenous levels of E2 (pg/mL) were associated with fewer errors on the maximum working memory load trial of the WRAM for Young-VCD-treated rats on their last retest prior to sacrifice. For MM performance, increased GAD65 expression in the Entorhinal Cortex, and lower GAD67 expression in the Perirhinal Cortex were associated with poorer performance on the last day of testing for Young-Vehicle rats.

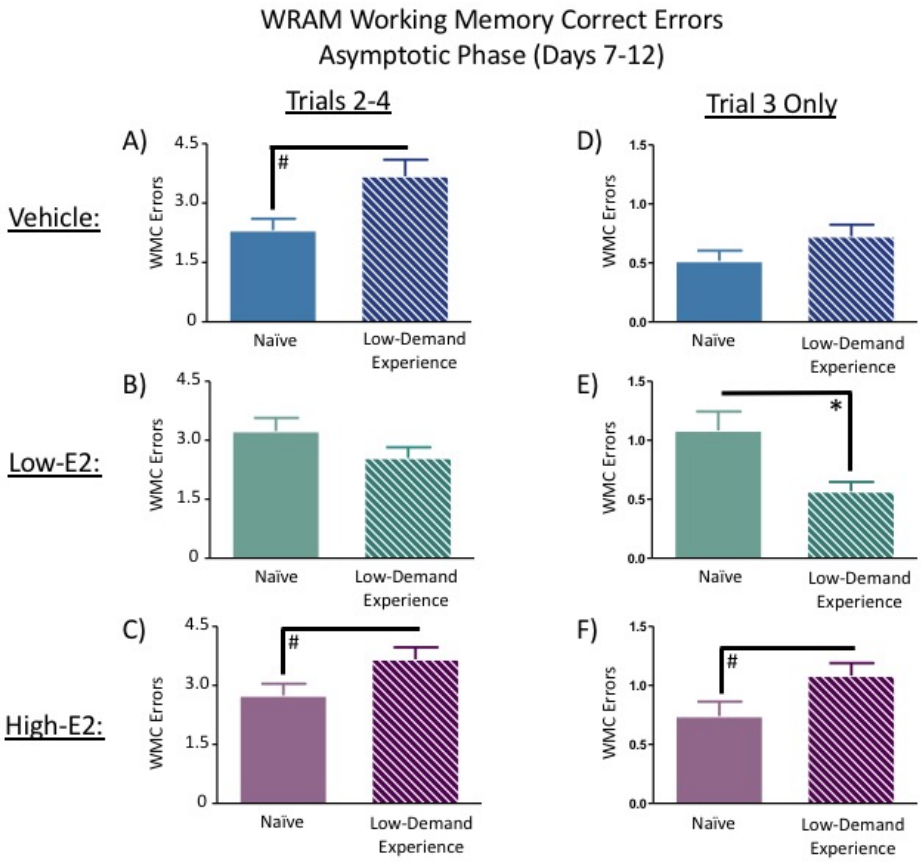

Figure 29: Working memory performance during the asymptotic phase of the WRAM. (A-C) Prior low-demand experience tended to impair performance for the Vehicle treated rats and the High-E2 treated rats compared to their naïve counterparts across all trials, although this was marginal and did not reach statistical significance. (D-F) When the moderate working memory load trial (Trial 3) was assessed alone, the Low-E2 treated rats with prior low-demand experience exhibited enhanced performance compared to Naïve Low-E2-treated rats. High-E2 treated rats with prior low-demand experience tended to make more errors on the moderate working memory load trial than High-E2 treated rats naïve to the task, but this effect was marginal and did not reach significance. Data are presented as the mean ± SEM. * $p < 0.05$, # $p < 0.10$.

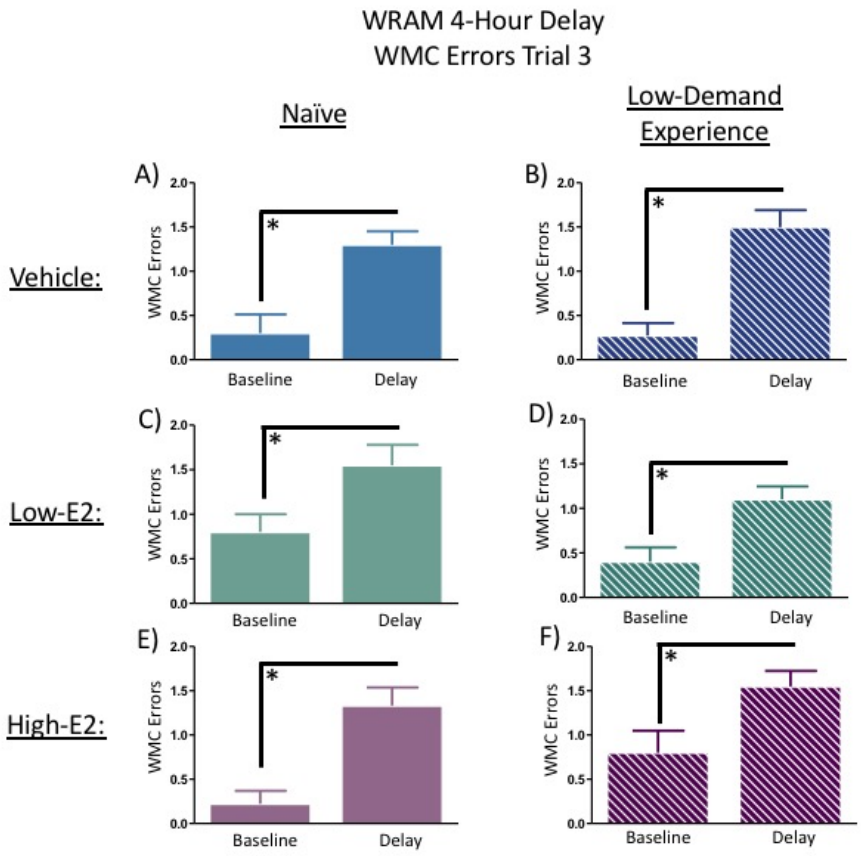

Figure 30: Delayed memory retention on the WRAM. (A-F) Following a four-hour delay between trials 2 and 3, all subjects, regardless of treatment group and prior experience, made more WMC errors on Trial 3 after the delay compared to Trial 3 WMC performance on the last day of baseline testing (Day 12). Data are presented as the mean ± SEM. * $p < 0.05$, # $p < 0.10$.

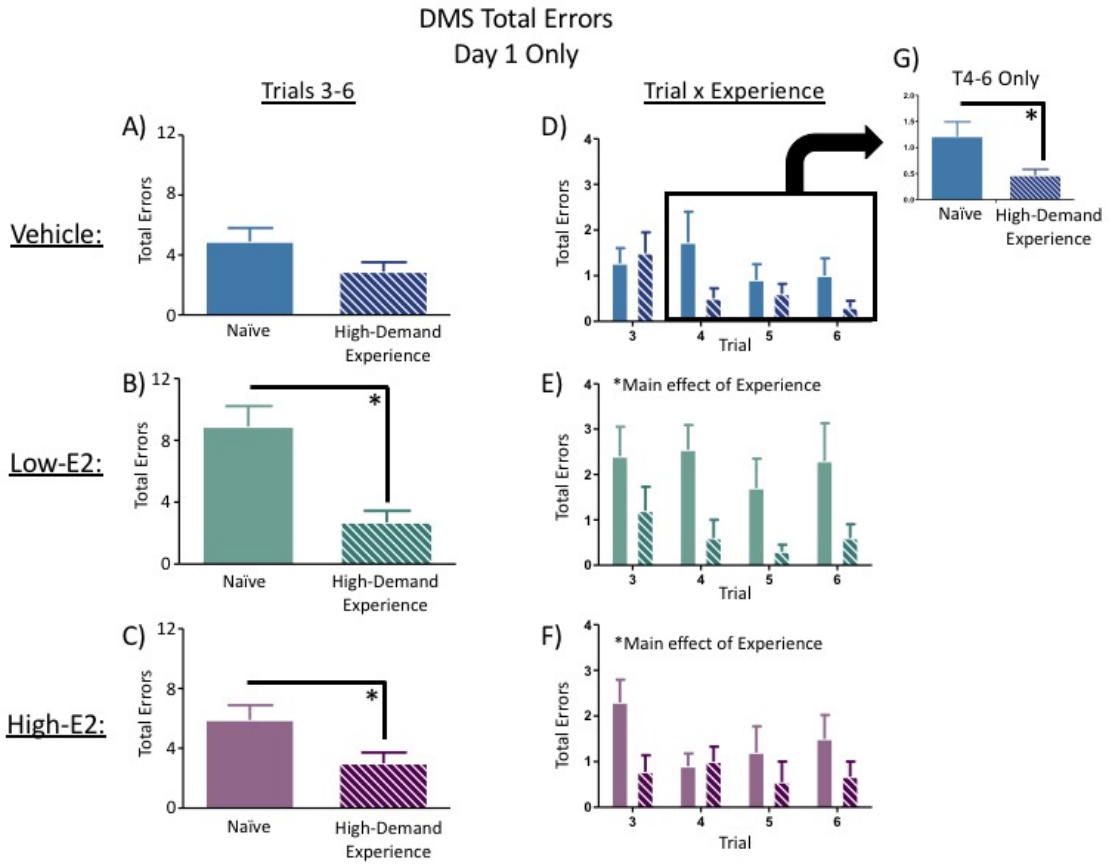

Figure 31: Total errors committed on the first day of DMS testing. (A-C, E-F) Low-E2 and High-E2 treated rats with prior high-demand experience made fewer errors than their naïve counterparts on trials 3-6 of DMS. (D,G) The Vehicle treated rats with high-demand experience made fewer errors than naïve Vehicle treated rats only on trials 4-6, indicating that even with high-demand experience, Ovx rats without exogenous E2 treatment require more exposures to the task before exhibiting enhanced performance compared to rats naïve to the task. Data are presented as the mean ± SEM. * $p < 0.05$, # $p < 0.10$.

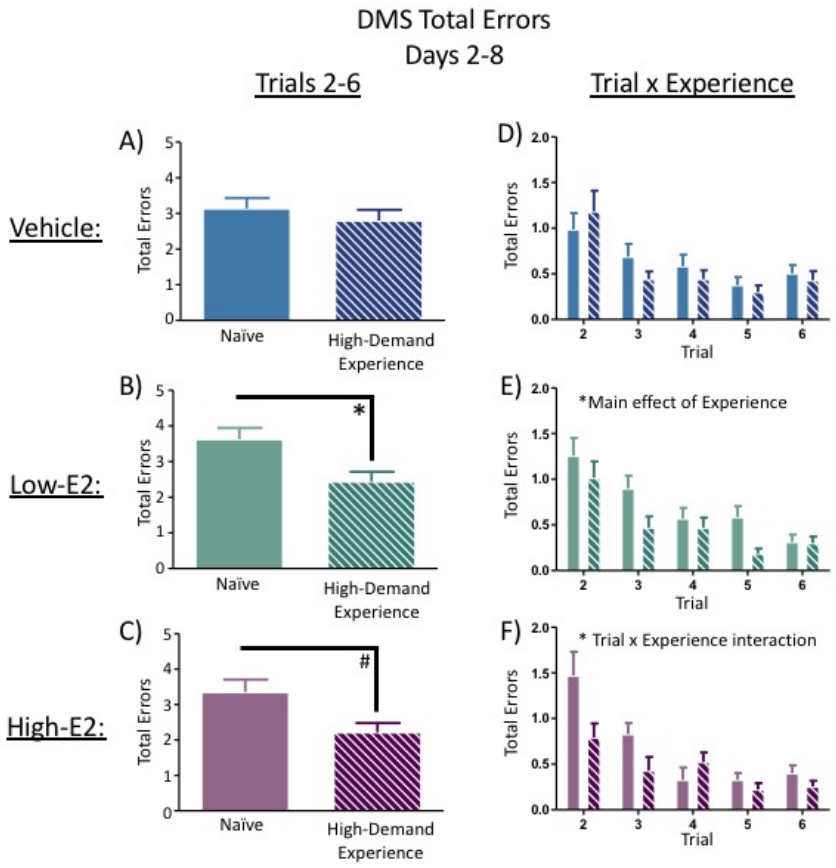

Figure 32: Total errors committed on Days 2-8 of DMS testing. (A-F) Low-E2 treated rats with prior high demand experience exhibited enhanced performance on trials 2-6 of the DMS across baseline testing compared to Low-E2 treated rats naïve to the task. The High-E2 treated rats with prior experience tended to make fewer errors than the High-E2 treated rats naïve to the task. Data are presented as the mean ± SEM. * $p < 0.05$, # $p < 0.10$.

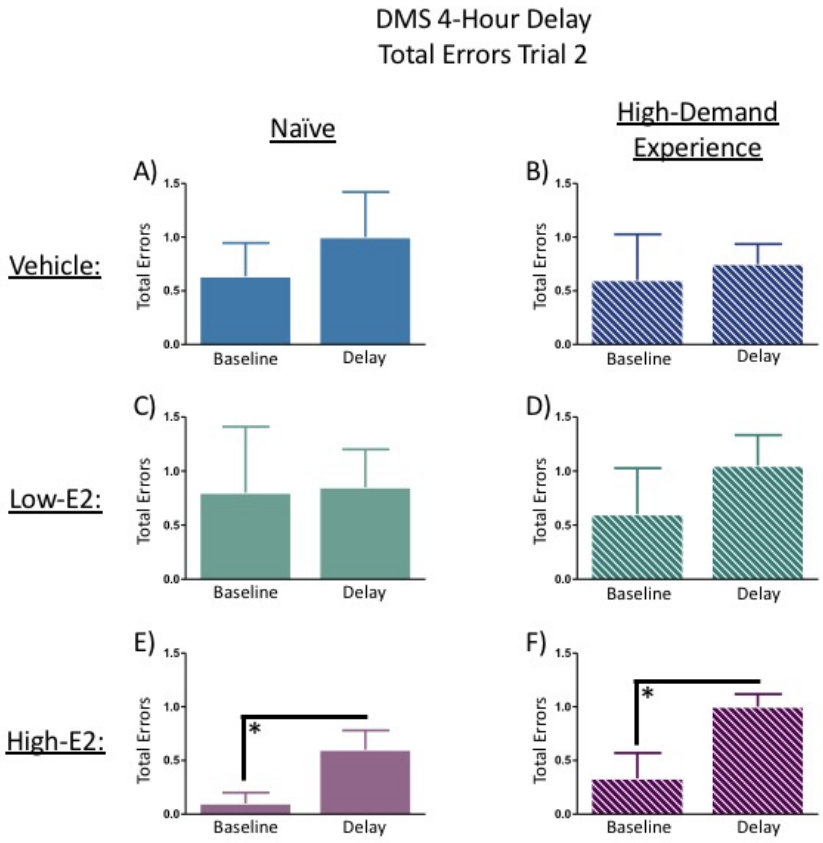

Figure 33: Delayed memory retention on the DMS. (A-D) Following a four-hour delay between trials 1 and 2, Vehicle treated rats and Low-E2 treated rats did not exhibit an impairment following the delay, regardless of prior testing experience. (E-F) Both High-E2 treated rats naïve to the DMS and High-E2 treated rats with prior high-demand experience showed a significant increase in total errors committed prior to finding the platform on the post-delay trial. Data are presented as the mean ± SEM. * $p < 0.05$, # $p < 0.10$.

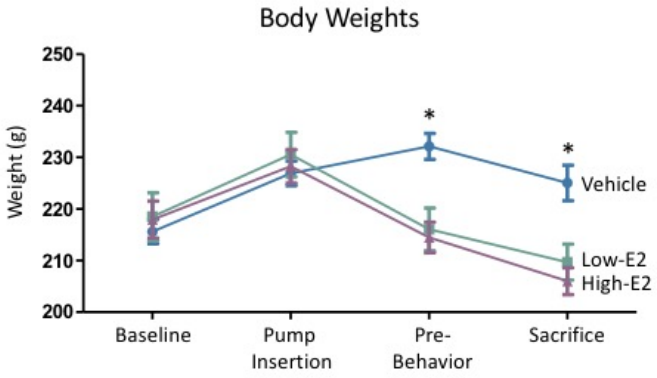

Figure 34: Body weights. Body weight (grams) increased for all subjects between Ovx and Pump Insertion time points. Following hormone treatment initiation, Ovx rats treated with Low-E2 or High-E2 decreased body weights compared to Ovx-Vehicle treated rats. This difference between Vehicle treated rats and E2 treated rats persisted through the end of the experiment. Data are presented as the mean ± SEM. * $p < 0.05$, # $p < 0.10$.

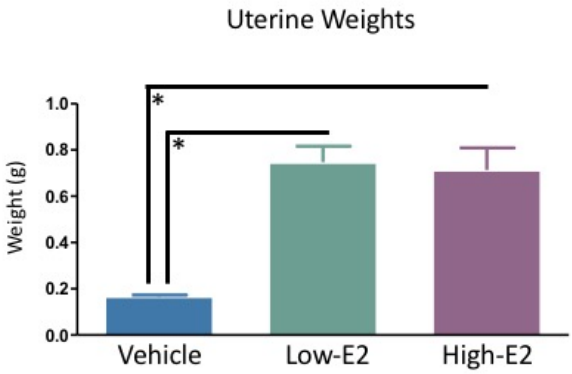

Figure 35: Uterine wet weights at euthanization. All groups were Ovx. The Low-E2 and High-E2 treated rats exhibited significantly heavier uterine wet weights at sacrifice compared to the Vehicle treated rats. Data are presented as the mean ± SEM. * $p < 0.05$, # $p < 0.10$.

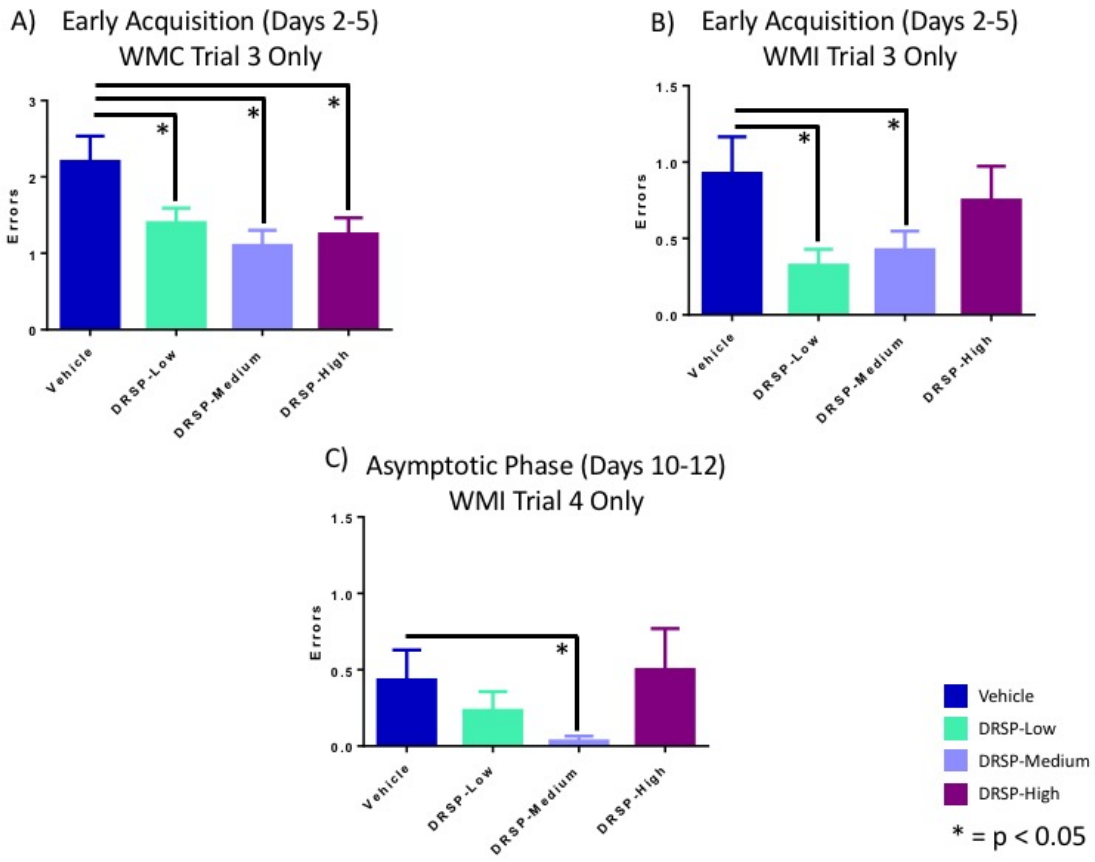

Figure 36: Study 1 Water radial-arm maze. A) During Early Acquisition, each DRSP dose enhanced WMC performance compared to Vehicle on a high working memory load trial. B) This benefit was extended to WMI errors for DRSP-Low and DRSP-Medium doses compared to Vehicle. C) During the Asymptotic Phase, DRSP-Medium continued to enhance performance compared to Vehicle treatment for WMI errors on the maximum working memory load trial.

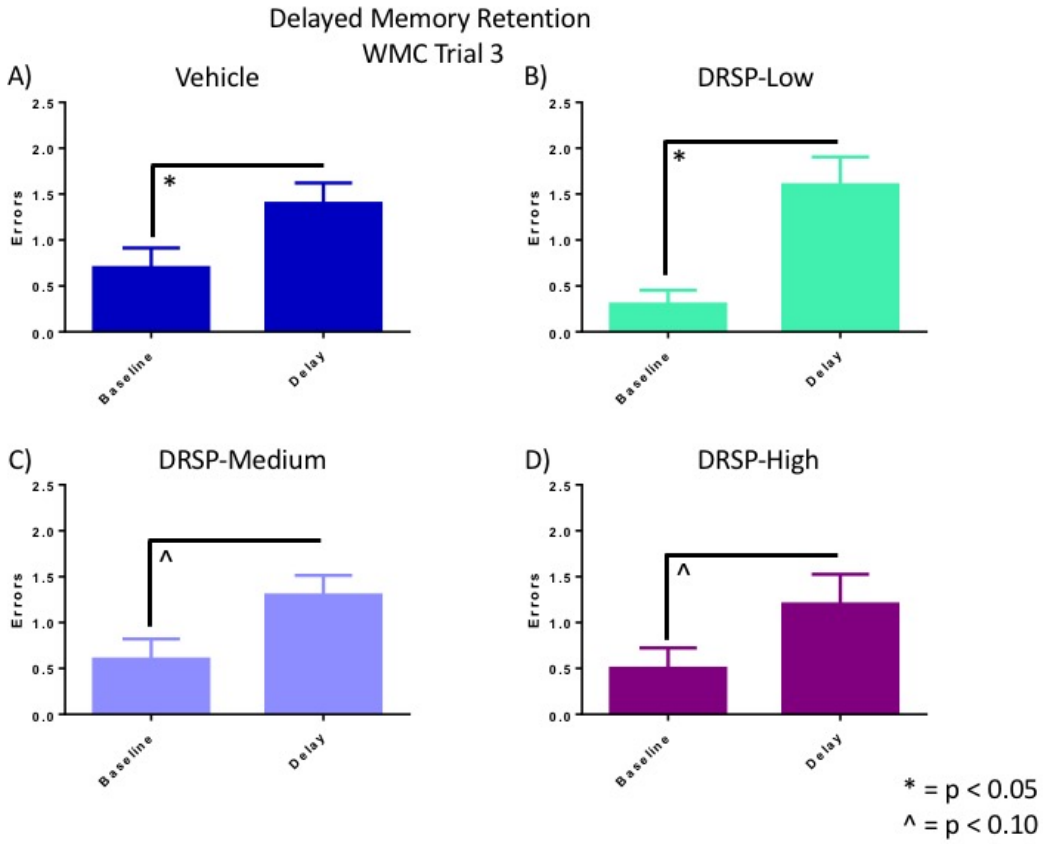

Figure 37: Study 1 Water radial-arm maze delay. Vehicle (A) and DRSP-Low (B) treated rats showed impaired delayed memory retention following a six hour delay between trials 2 and 3. DRSP-Medium (C) and DRSP-High (D) groups trended toward a delay-related impairment, but this effect did not reach significance in these groups.

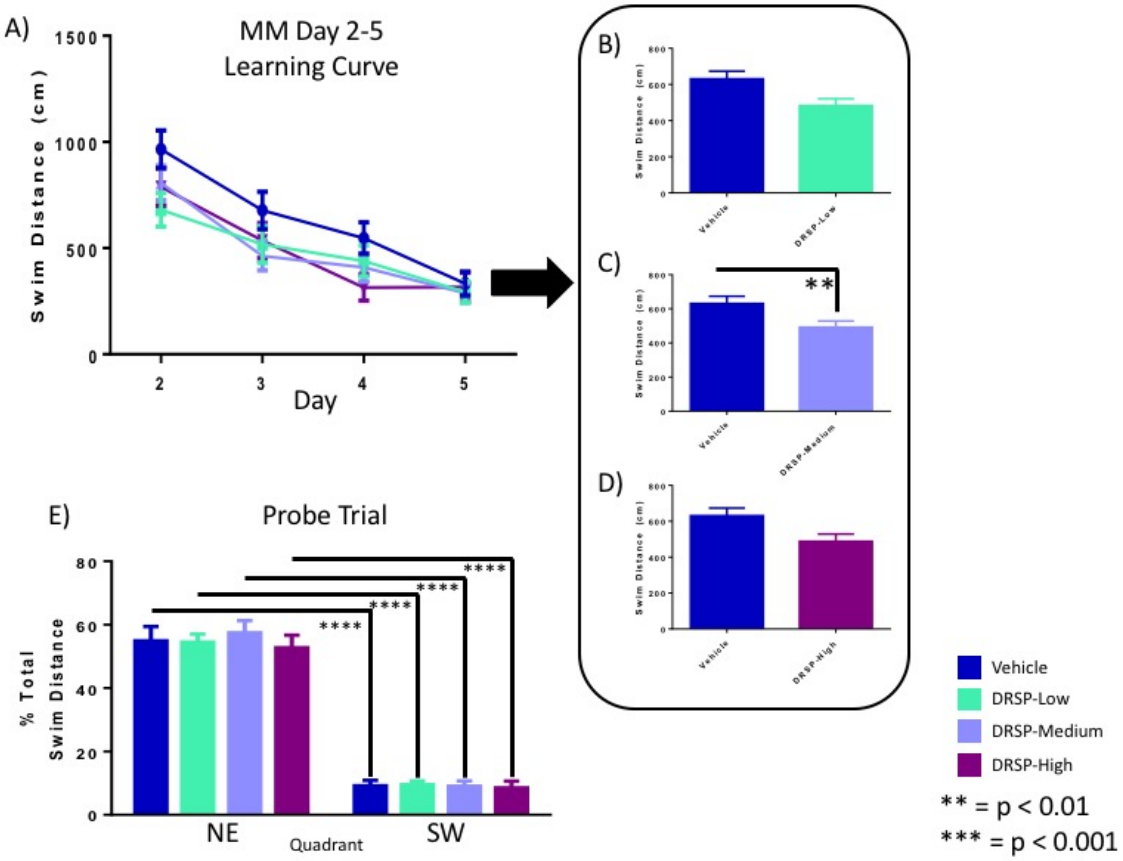

Figure 38: Study 1 Morris water maze. Across Days 2-5 (A), the DRSP-Medium group showed enhanced performance compared to the Vehicle group (C). All groups localized to the target quadrant during the probe trial (E).

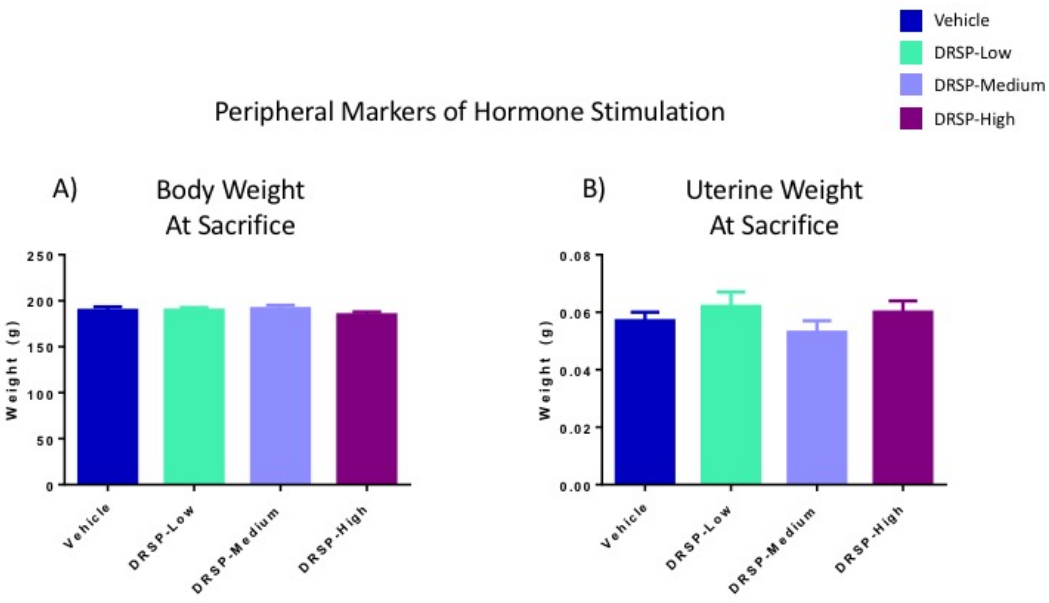

Figure 39: Study 1 Body weight and uterine weights. There were no treatment differences in body weight or uterine horn weights at sacrifice.

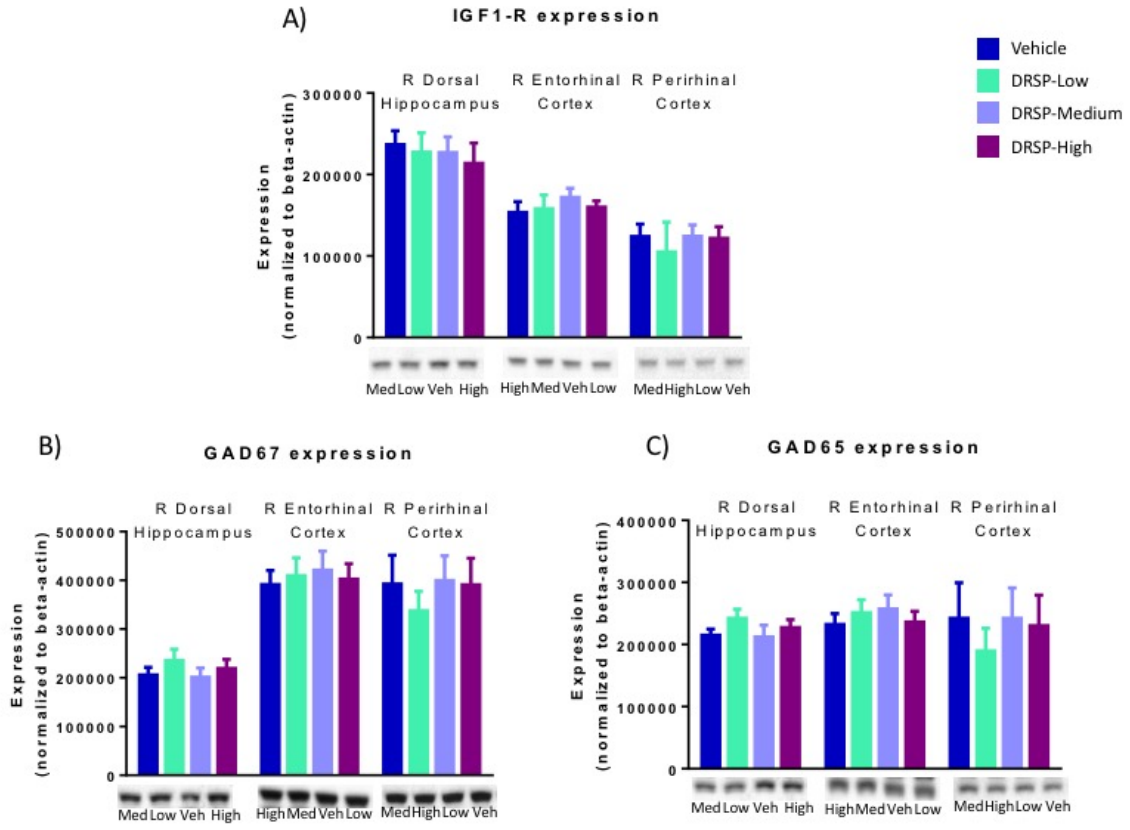

Figure 40: Study 1 Western blot protein analysis. There were no Treatment differences in IGF1-R expression, GAD65 expression, or GAD67 expression for any brain region evaluated.

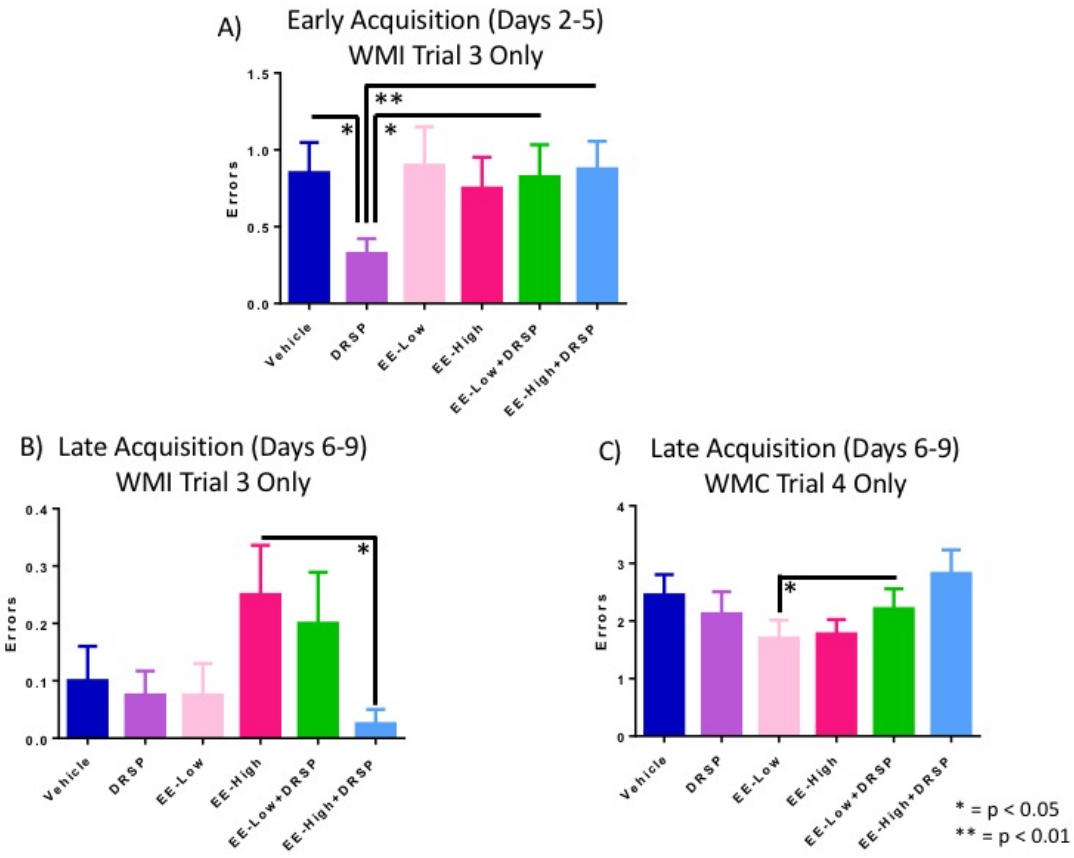

Figure 41: Study 2 Water radial-arm maze. A) The DRSP group showed enhanced WMI performance on the high working memory load trial compared to Vehicle, EE-Low+DRSP, and EE-High+DRSP groups. B) EE-High+DRSP treatment attenuated EE-High impairment on the high working memory load trial during Late Acquisition. C) EE-Low+DRSP impaired memory on the maximum working memory load trial compared to EE-alone treatment during Late Acquisition.

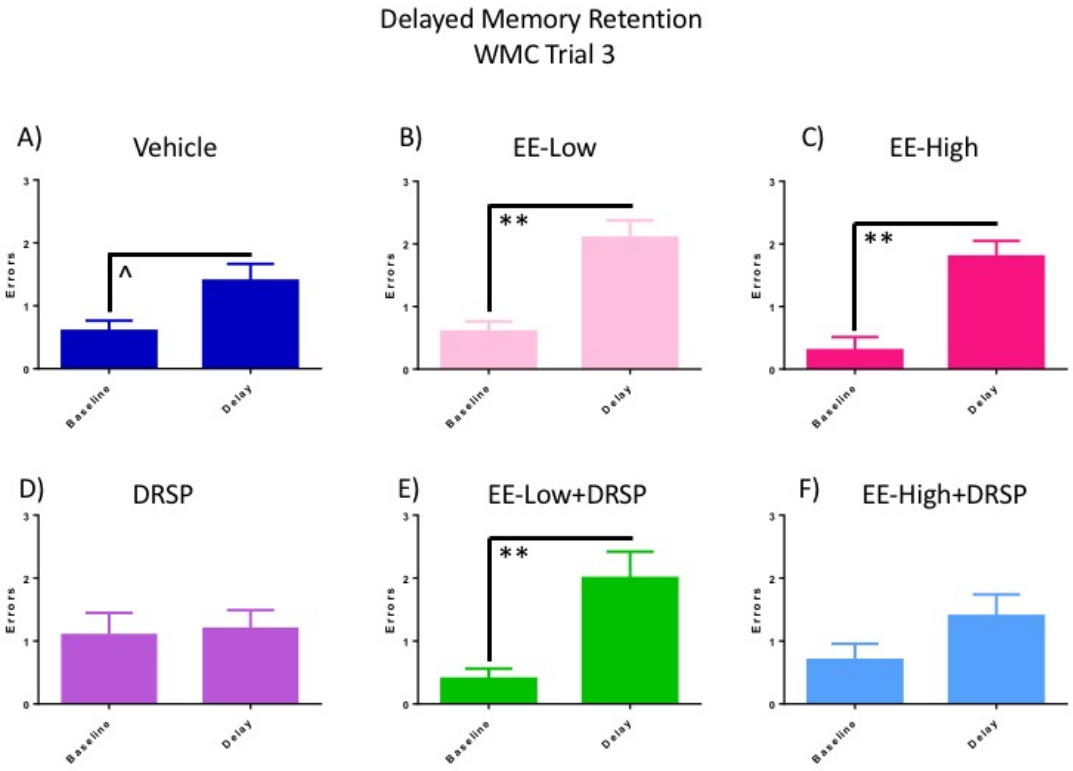

Figure 42: Study 2 Water radial-arm maze delay. A) The Vehicle group tended to show impairments following a six-hour delay B,C, E) EE-Low, EE-High, and EE-Low+DRSP groups showed significantly impaired working memory performance following a six-hour delay. D,F) The DRSP group and the EE-High+DRSP group did not significantly increase errors committed following a six-hour delay.

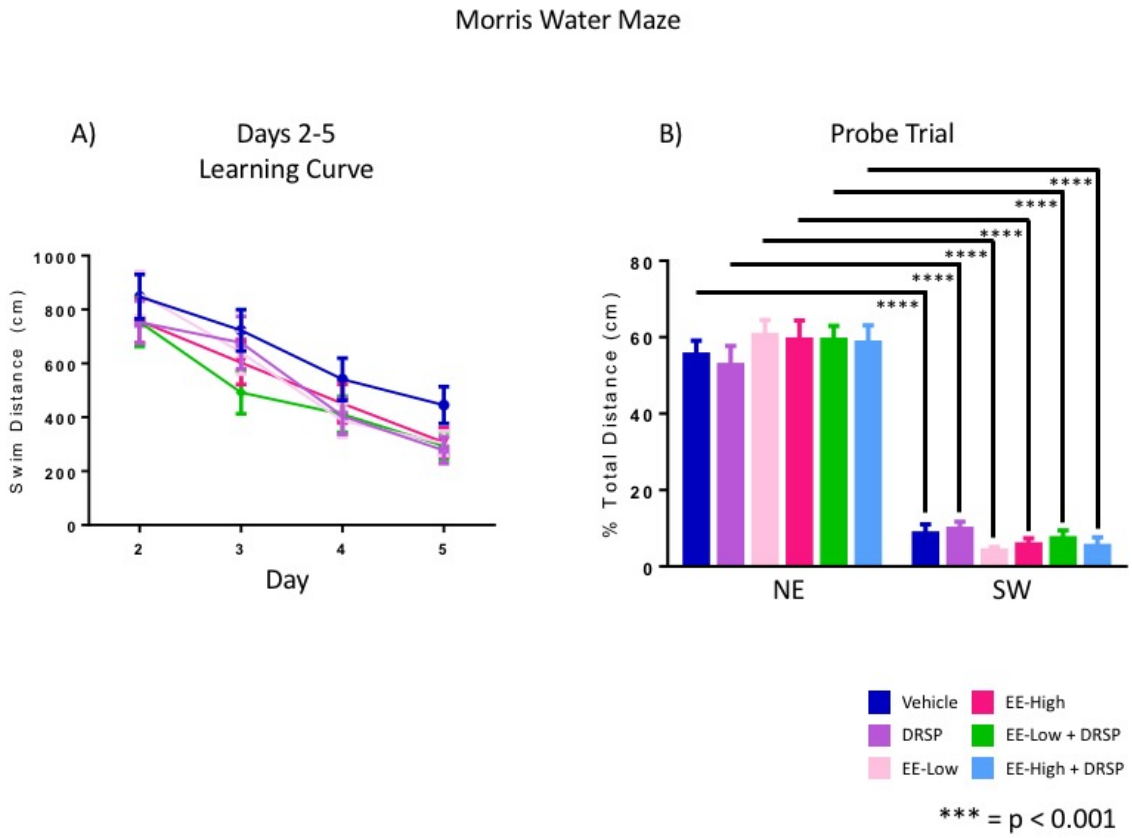

Figure 43: Study 2 Morris water maze. A) There were no Treatment differences across MM Days 2-5. B) All groups localized to the target quadrant during the probe trial.

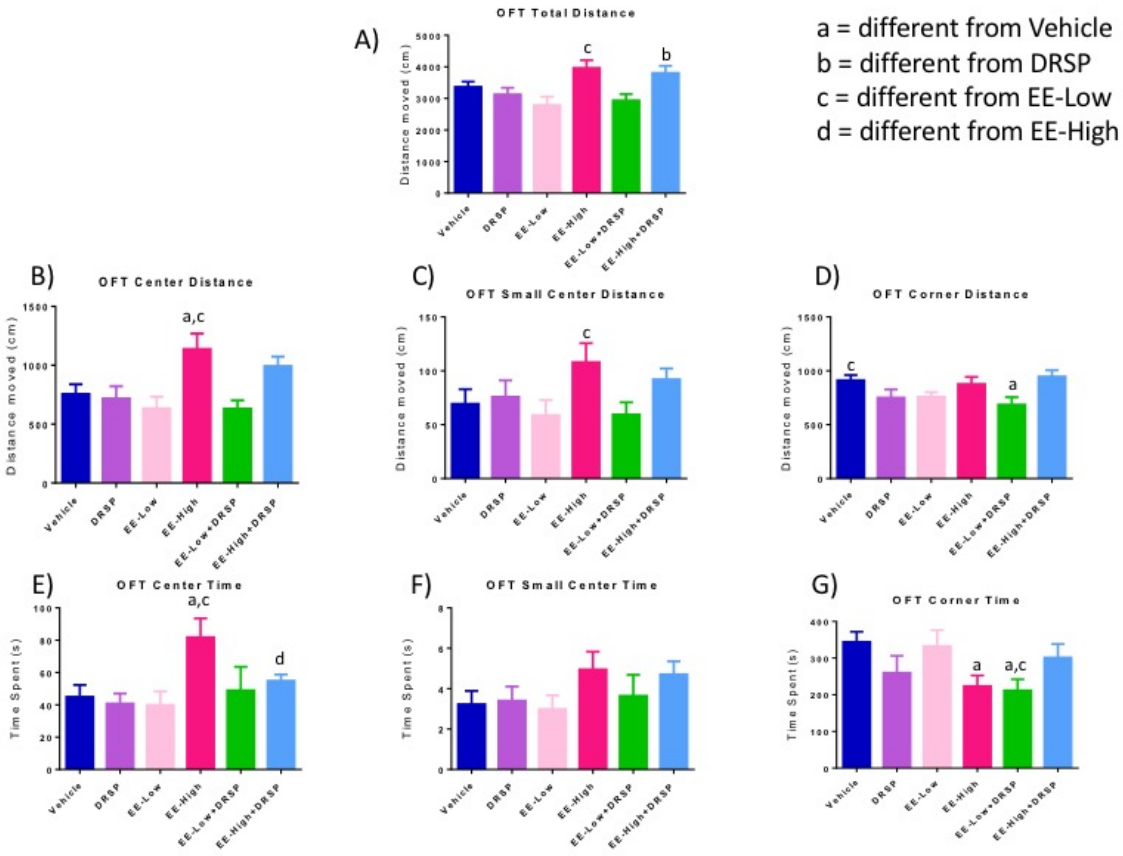

Figure 44: Open field task performance. The EE-High group showed increased locomotor activity compared to the Vehicle group and the EE-High+DRSP group showed increased locomotor activity compared to the DRSP group. EE-treated groups showed evidence of anxiolytic behavior.

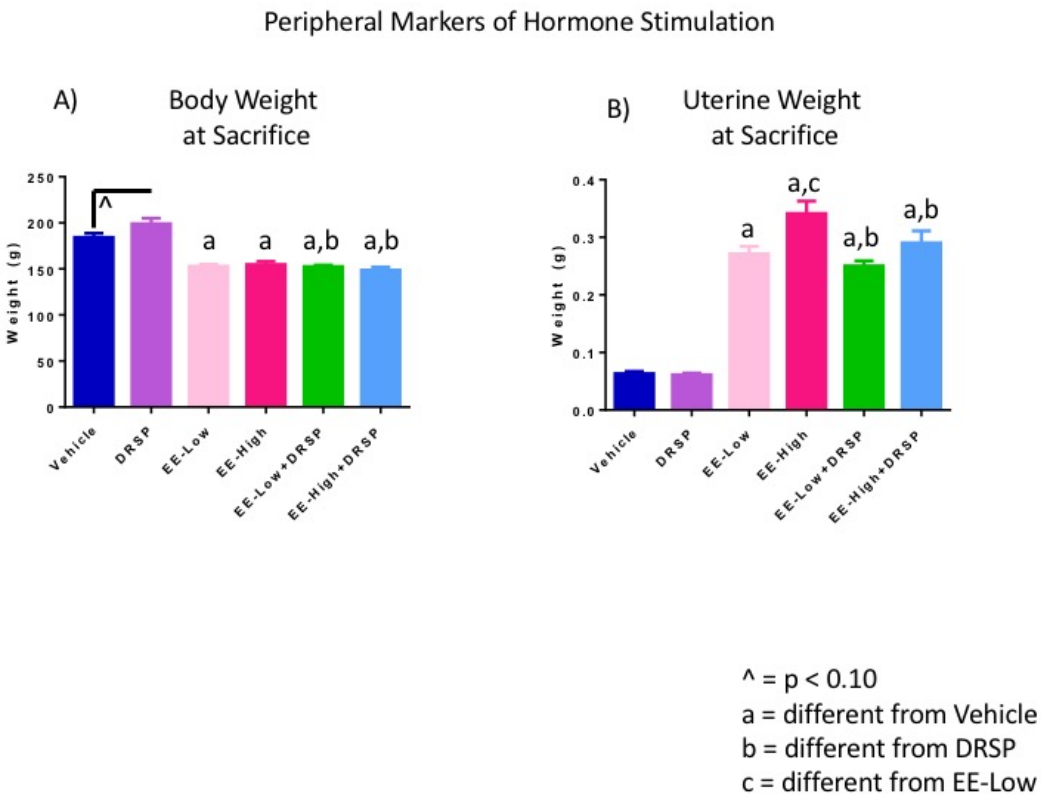

Figure 45: Study 2 Body weight and uterine weight. A) EE-treated groups weighed significantly less than Vehicle groups. The DRSP group weighed more than EE-Low+DRSP and EE-High+DRSP groups, and tended to weigh more than the Vehicle group. Of note, two-group comparisons were not made between DRSP and EE-Low or EE-High groups. B) Uterine weights from rats treated with EE weighed significantly more at euthanasia. Uterine weights from the DRSP group weighed less than EE-Low+DRSP and EE-High+DRSP groups, but did not differ from Vehicle. Of note, two-group comparisons were not made between DRSP and EE-Low or EE-High groups.

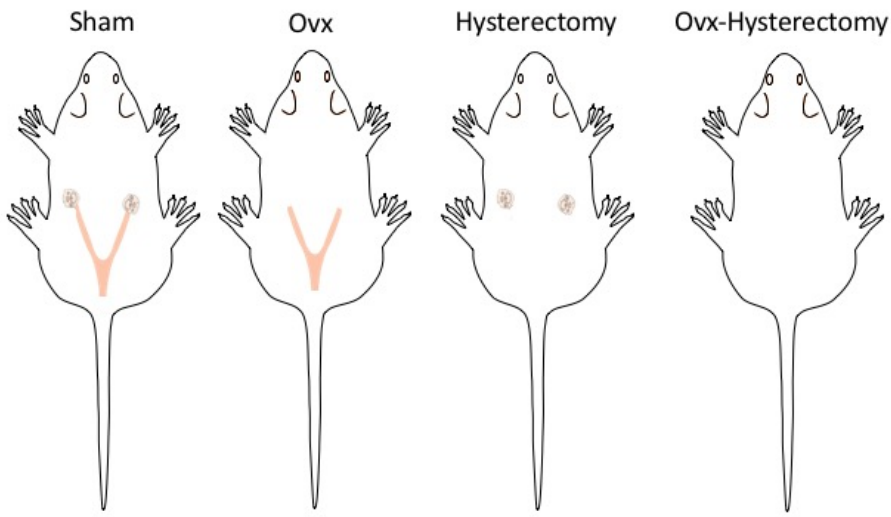

Figure 46: Diagram of the surgical manipulations performed. Sham-treated rats had intact an uterus and ovaries, Ovx-treated rats had an intact uterus, Hysterectomy-treated rats had intact ovaries, and Ovx-Hysterectomy-treated rats had both the uterus and ovaries removed.

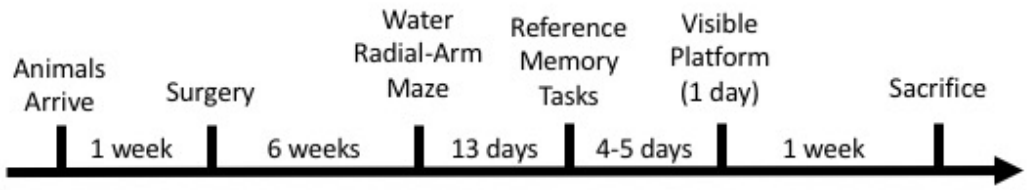

Figure 47: Study Timeline. Rats were tested on a battery of spatial working and reference memory tasks six weeks following surgical manipulations. One week following the last behavioral assay, subjects were euthanized.

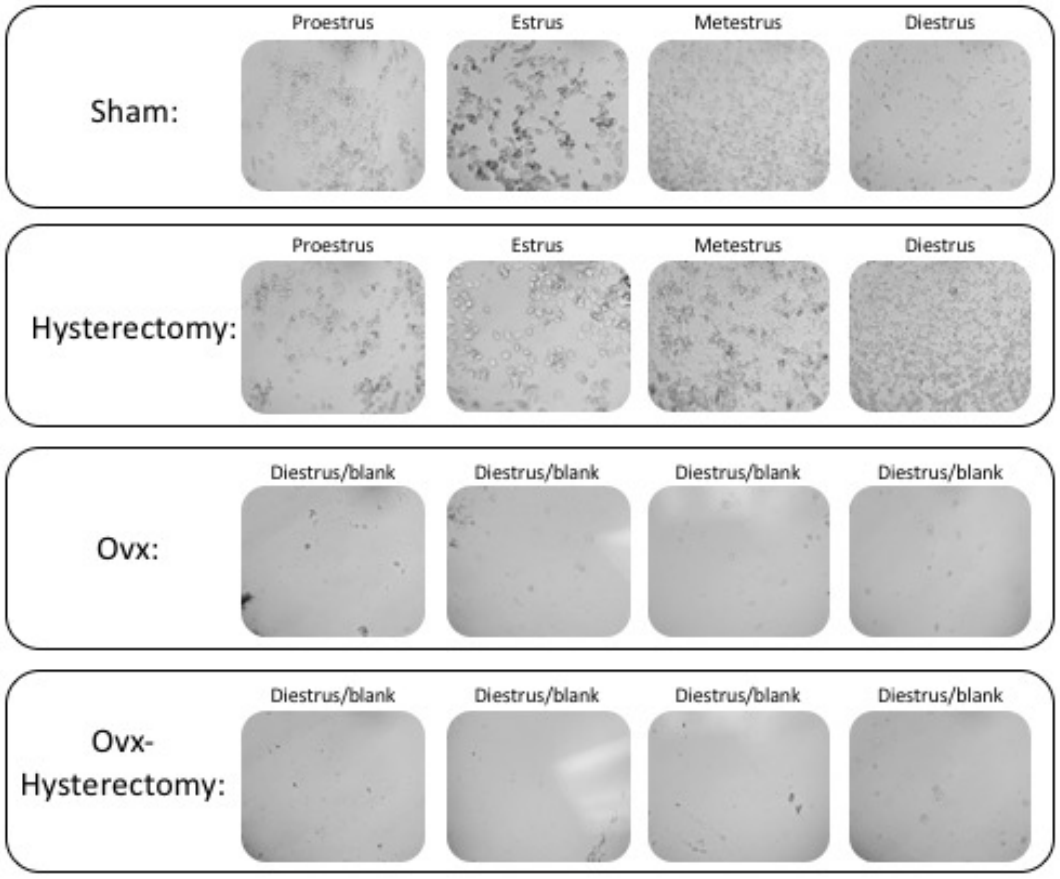

Figure 48: Representative images from consecutive days of vaginal cytology monitoring. Rats in the Sham and Hysterectomy groups (i.e., rats with intact ovaries) showed normal 4-5 day estrous cycling when examined 2-3 weeks after surgery. Ovx and Ovx-Hysterectomy rats (i.e., rats that experienced surgical removal of the ovaries) showed mostly blank vaginal smear cytology two weeks after surgery, indicating successful removal of the ovaries.

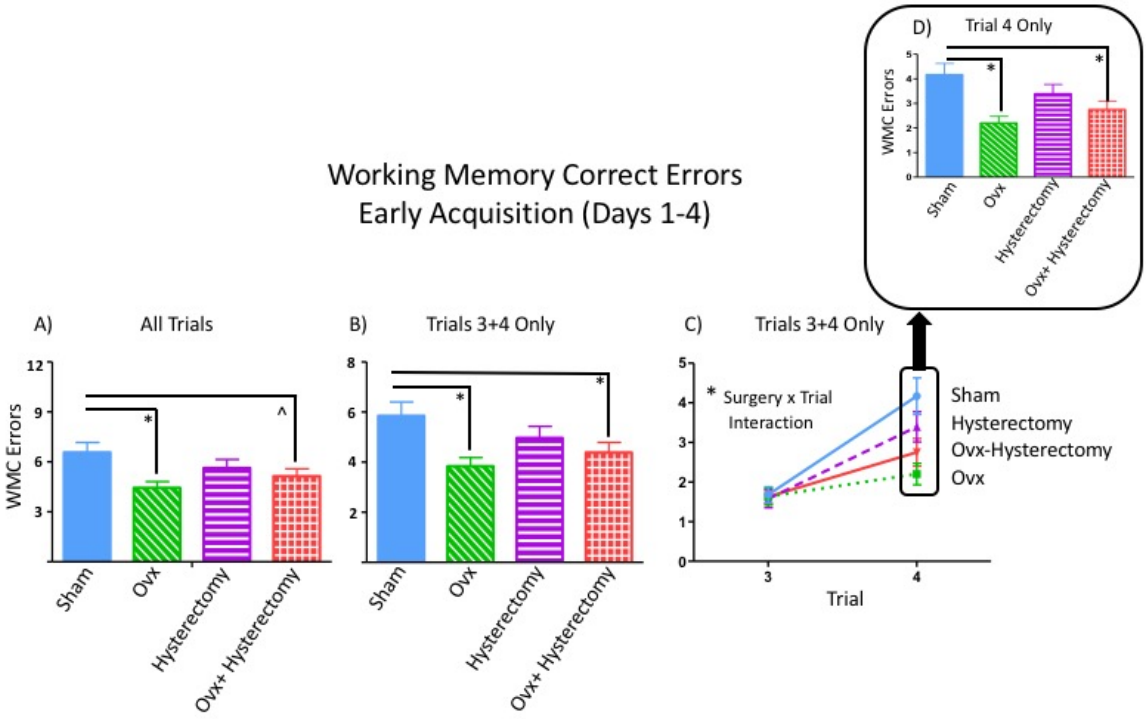

Figure 49: Working memory correct errors during the early acquisition phase of the water radial-arm maze (days 1-4). A) There was a main effect of Surgery across all days and trials within the early acquisition block. B) There was a main effect of Surgery across Days 1-4 on the high working memory load trials (Trials 3+4). C) There was a Surgery x Trial interaction on high working memory load trials (Trials 3+4), with D) a main effect of Surgery evident on Trial 4 alone across Days 1-4. * = $p < 0.05$ ^ = $p < 0.10$

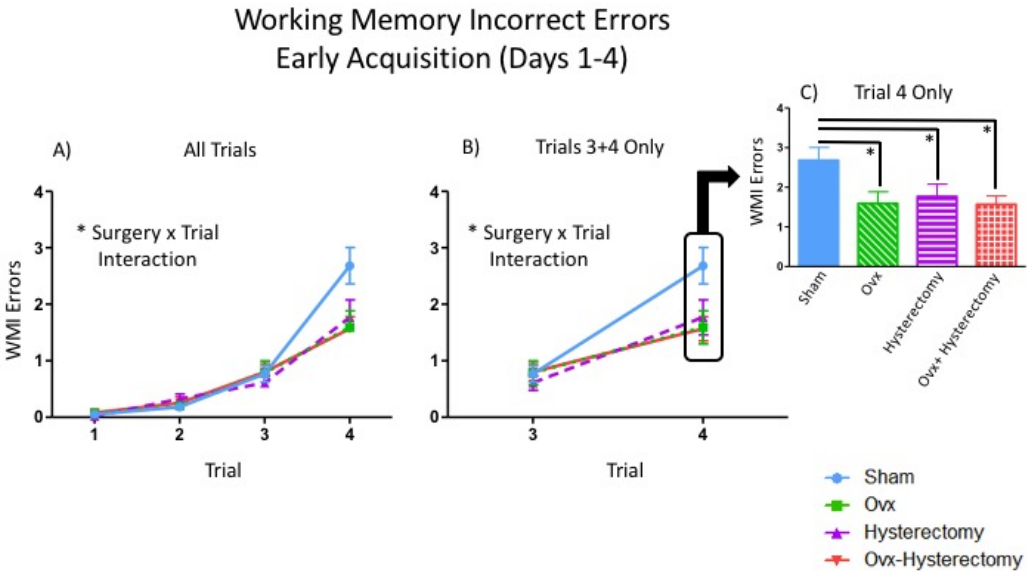

Figure 50: Working memory incorrect errors during the early acquisition phase of the water radial-arm maze (days 1-4). A) There was a Surgery x Trial interaction was present across Days 1-4 for WMI errors. B) There was a Surgery x Trial interaction on the high working memory load trials (Trials 3+4), with C) a main effect of Surgery on Trial 4 alone. * = $p < 0.05$

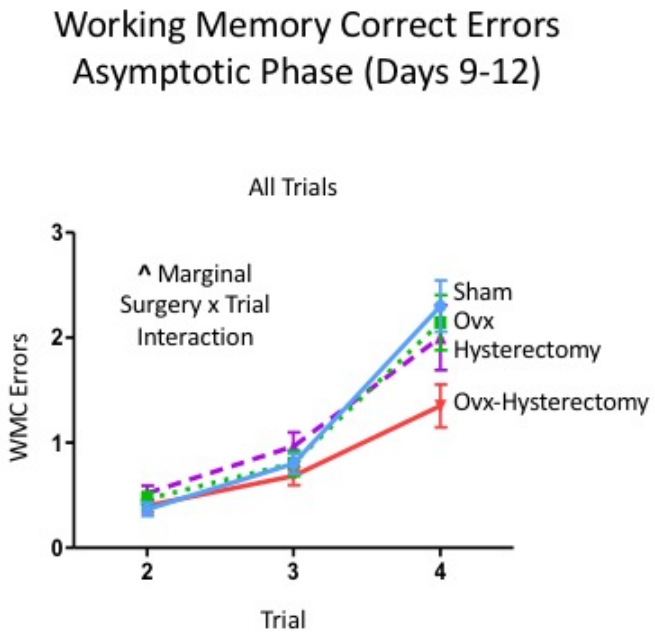

Figure 51: Working memory correct errors during the asymptotic phase of the water radial-arm maze (days 9-12). A marginal Surgery x Trial interaction was present for WMC errors. ^ = $p < 0.10$

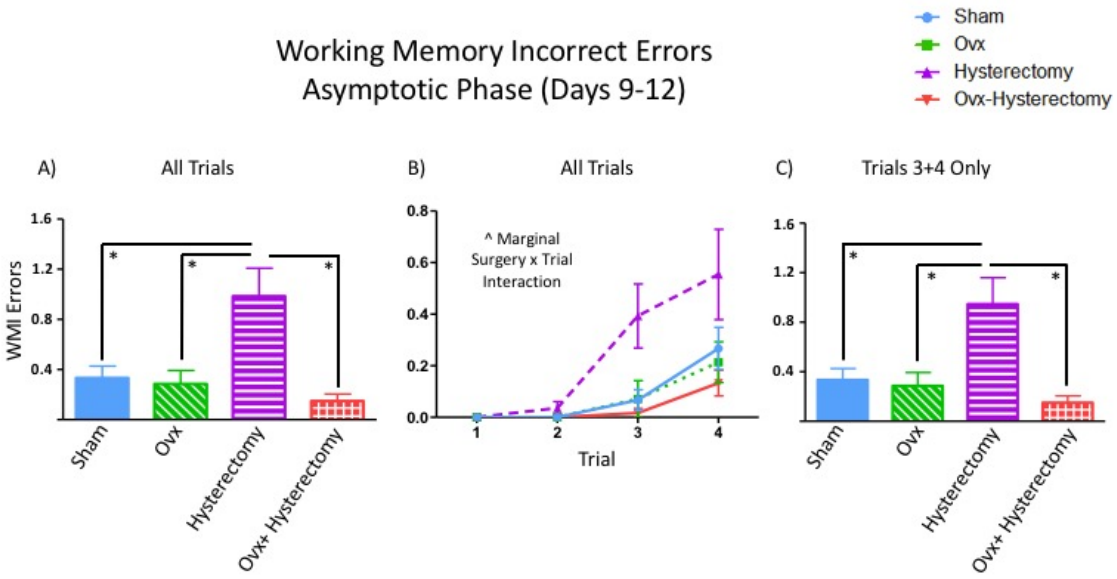

Figure 52: Working memory incorrect errors during the asymptotic phase of the water radial-arm maze (days 9-12). A) A main effect of Surgery was evident across all days and trials within the asymptotic phase block. B) A marginal Surgery x Trial interaction was found across all days and trials in the asymptotic phase. C) A main effect of Surgery was shown for the high working memory load trials (Trials 3+4) for the asymptotic phase block. * = $p < 0.05$ ^ = $p < 0.10$

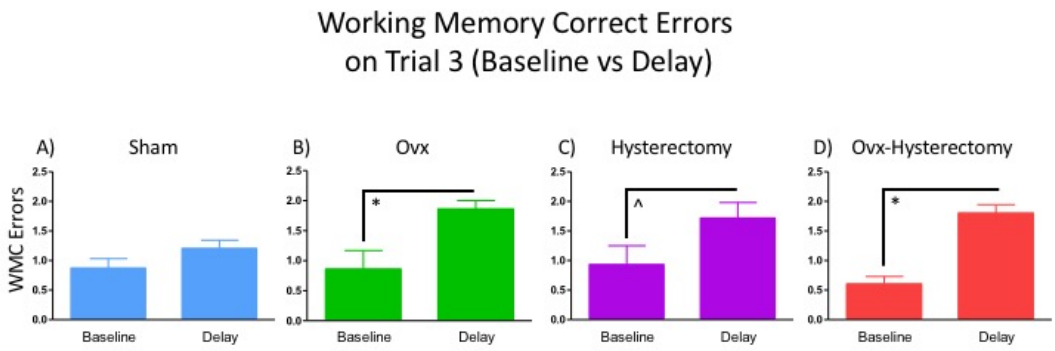

Figure 53: Water radial-arm maze delay data. A-D demonstrate working memory correct errors of each surgery group on Trial 3 of the last day of baseline testing (Day 12) compared to the number of working memory correct errors committed on Trial 3 of the delay day (first post-delay trial on Day 13). * = $p < 0.05$ ^ = $p < 0.10$

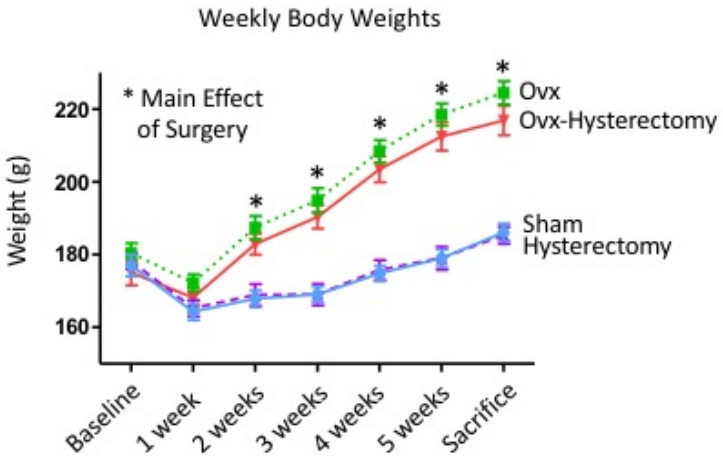

Figure 54: Weekly body weights were collected beginning on the date of surgery (baseline) and continued through euthanization. Body weights began to significantly diverge between rats with ovaries (i.e., Sham and Hysterectomy groups) versus rats without intact ovaries (i.e., Ovx and Ovx-Hysterectomy groups) beginning two weeks after surgical manipulation. * = $p < 0.05$

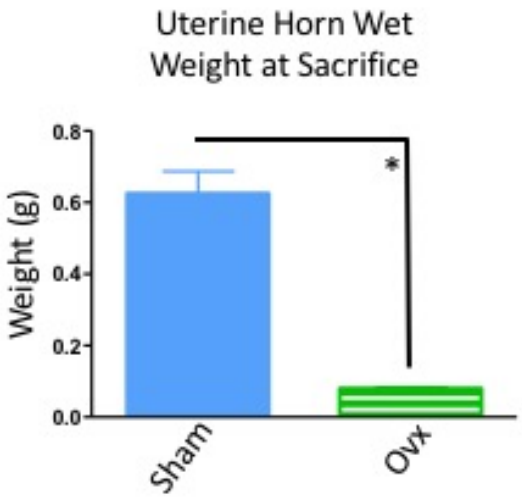

Figure 55: Uterine wet weights from rats that had an intact uterus throughout the experiment (Sham and Ovx groups). Uteri from Sham-operated rats weighed significantly more than those of the Ovx group, indicating successful removal of the ovaries in the Ovx group and a lack of ovarian hormone stimulation of the uterus. * = $p < 0.05$

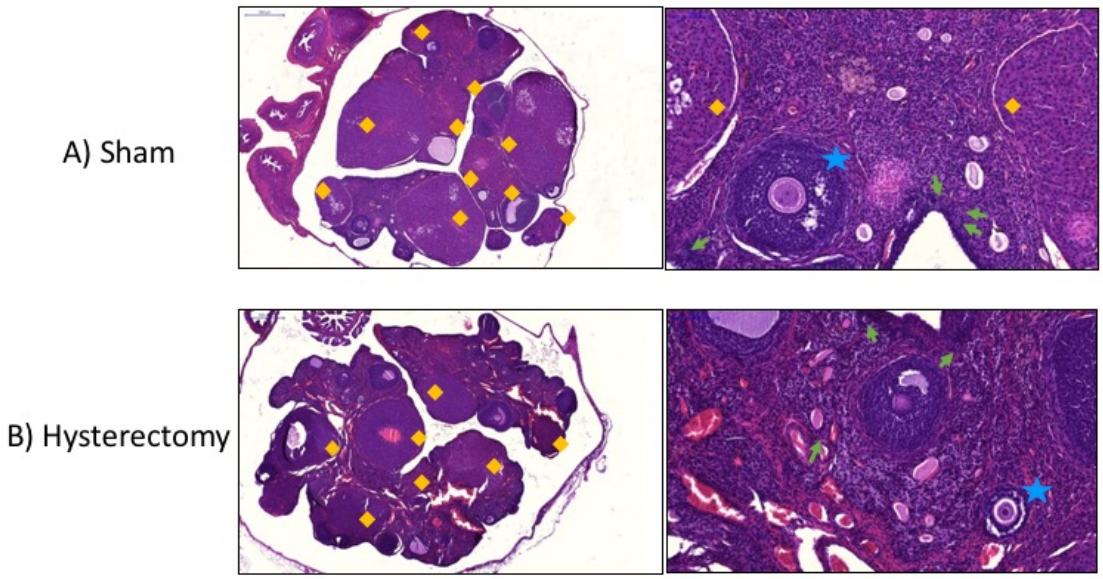

Figure 56: Representative ovarian micrographs stained with hematoxylin and eosin Y-phloxine B from A) a sham-operated rat and B) a Hysterectomy-operated rat. Low magnification images are 3.45x magnification with a scale bar of 500 um. High magnification images are at 20x magnification with a scale bar of 100 um. Corpora lutea are designated by orange diamonds. Primordial follicles are denoted with a green arrow. Secondary follicles are marked with blue stars.

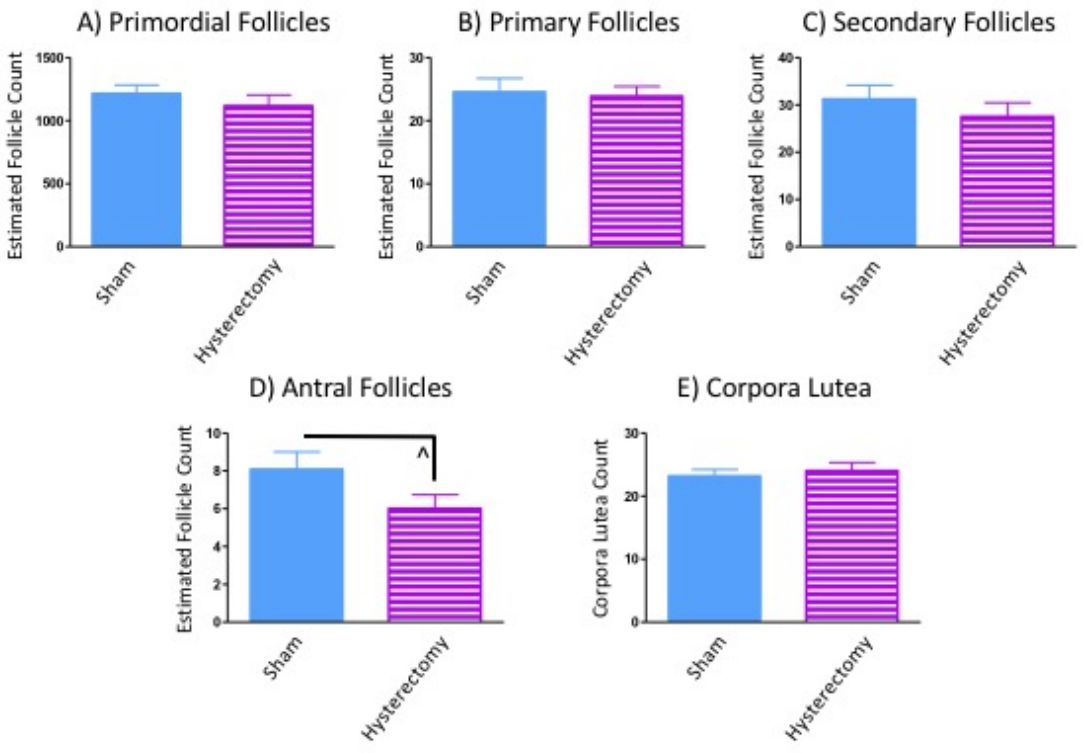

Figure 57: Healthy ovarian follicle count estimates from groups that had intact ovaries throughout the experiment (Sham and Hysterectomy groups). Primordial, primary, and secondary follicles did not differ between groups. Hysterectomy-operated rats had marginally fewer antral follicles compared to Sham-operated rats. Corpora lutea counts did not differ between groups. ^ = $p < 0.10$

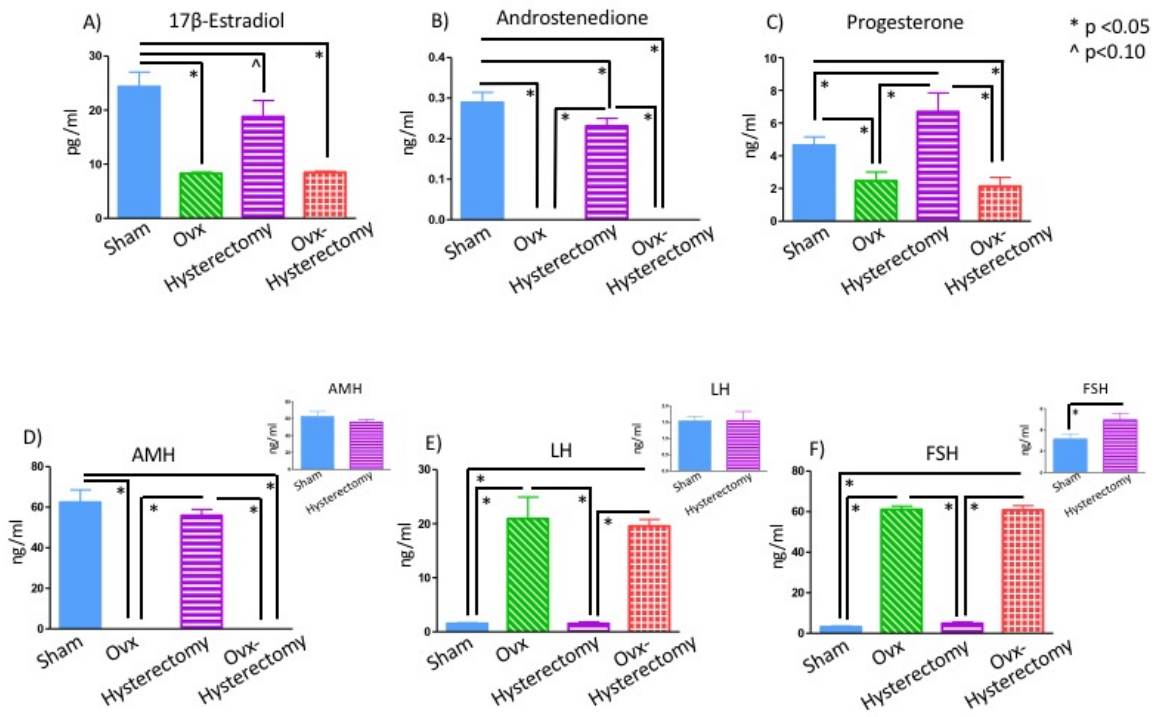

Figure 58: Circulating serum ovarian hormone levels from blood collected at euthanization. A) Sham-operated rats had more 17β-estradiol compared to Ovx and Ovx-Hysterectomy groups, and marginally more 17β-estradiol compared to the Hysterectomy group. B) Ovx and Ovx-Hysterectomy groups had non-detectable levels of androstenedione at euthanization. Hysterectomy-operated rats had less circulating androstenedione compared to Sham-operated rats. C) Ovx and Ovx-Hysterectomy groups had less progesterone than Sham and Hysterectomy groups. Hysterectomy-operated rats had more progesterone present than Sham-operated rats. D) Ovx and Ovx-Hysterectomy groups had undetectable levels of AMH at euthanization. AMH levels in Hysterectomy and Sham rats did not differ from each other. E) Both groups without ovaries had increased LH levels compared to Sham and Hysterectomy groups, indicating successful ovary removal. LH levels did not differ between Sham and Hysterectomy groups. F) Both groups without ovaries had increased FSH levels compared to Sham and Hysterectomy groups, indicating successful ovary removal. The Hysterectomy group had increased FSH levels compared to the Sham group.

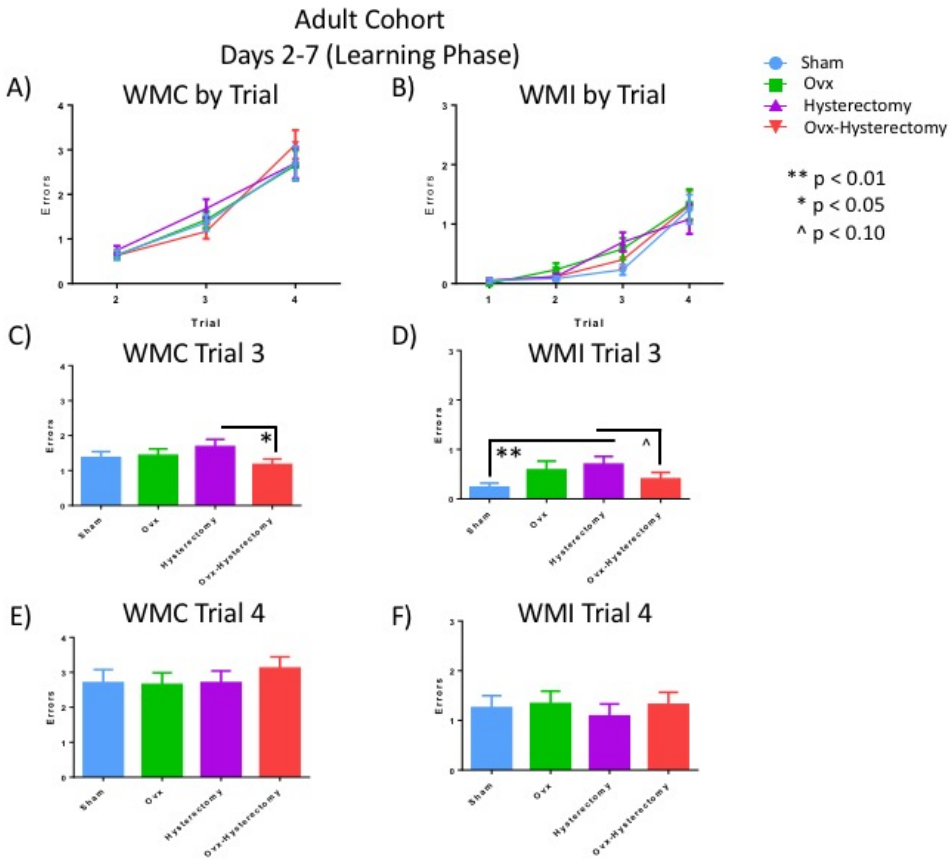

Figure 59: WRAM Learning Phase (Adult Cohort; 7 mo old, tested 6 weeks after surgery). A) WMC errors across trials B) WMI errors across trials C) Hysterectomy rats made more WMC errors on the moderate working memory load trial, Trial 3, compared to Ovx-Hysterectomy rats. D) Hysterectomy rats made more WMI errors on the maximum working memory load trial, Trial 4, compared to Sham rats. Ovx-Hysterectomy rats tended to make fewer errors than Hysterectomy rats. E) There were no group differences for WMC errors on Trial 4. F) There were no group differences for WMI errors on Trial 4. ** $p < 0.01$ * $p < 0.05$ ^ $p < 0.10$

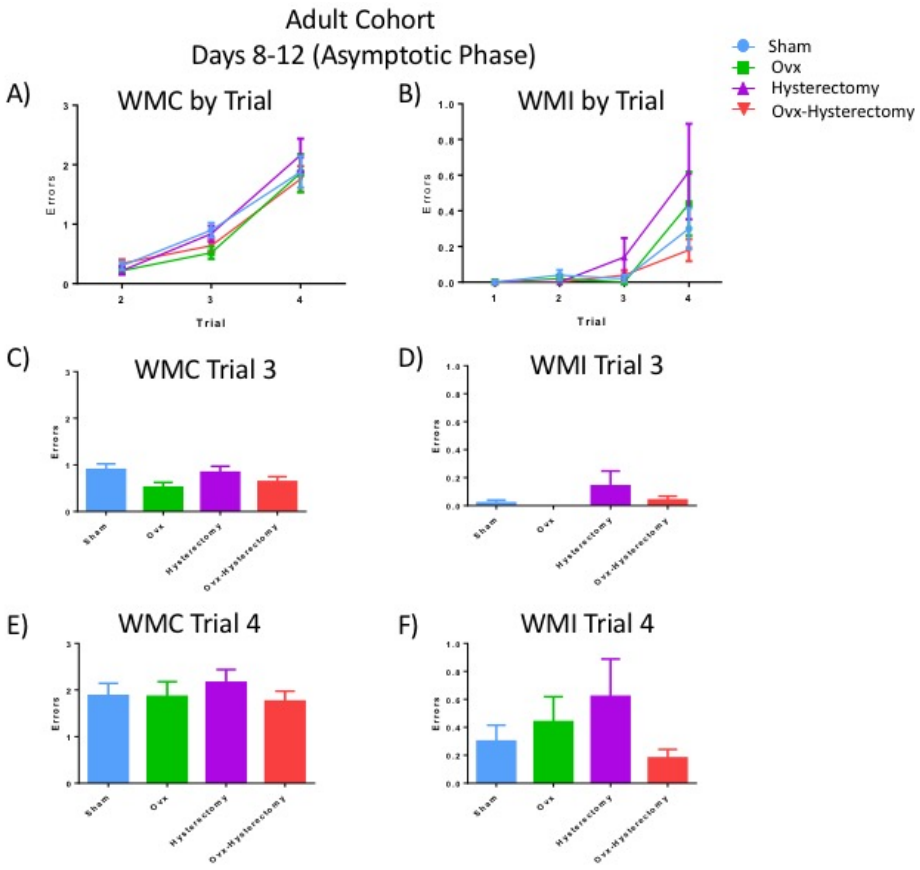

Figure 60: WRAM Asymptotic Phase (Adult Cohort; 7 mo old, tested 6 weeks after surgery). There were no group differences for any planned comparison during the Asymptotic phase of testing. A) WMC errors across trials B) WMI errors across trials C) Trial 3 WMC errors D) Trial 3 WMI errors E) Trial 4 WMC errors F) Trial 4 WMI errors

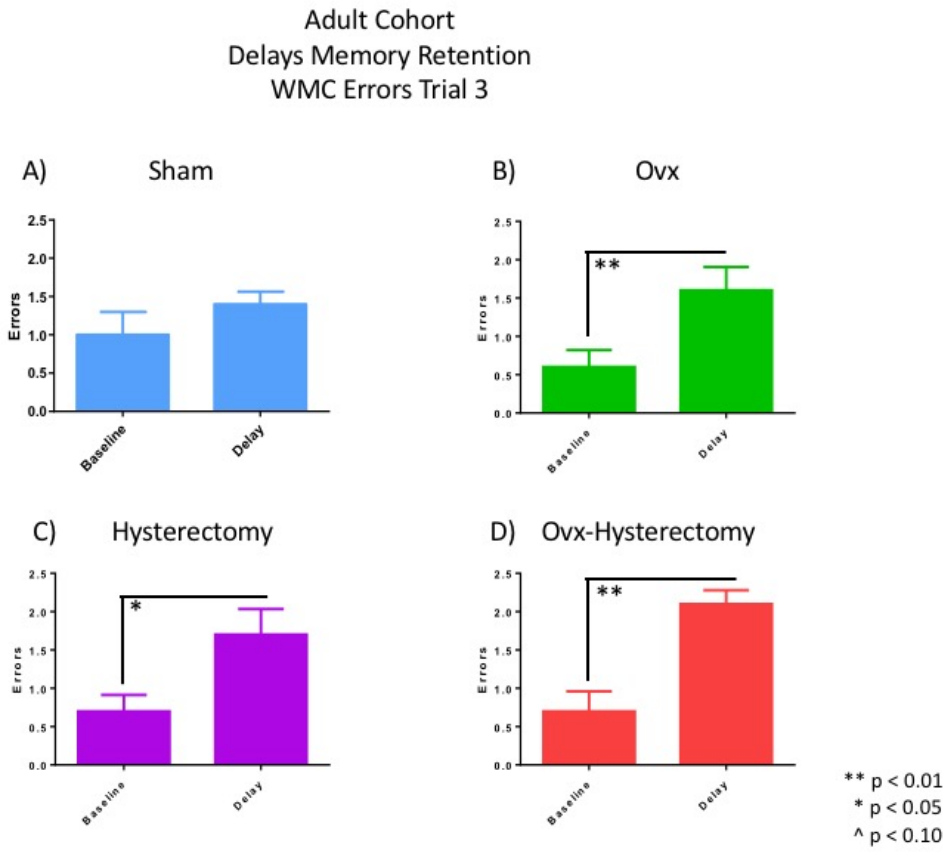

Figure 61: WRAM Delayed Memory Retention (Adult Cohort; 7 mo old, tested 6 weeks after surgery). Sham rats did not show a significant delay-induced working memory impairment (A). Ovx rats (B), Hysterectomy rats (C), and Ovx-Hysterectomy rats (D) committed more WMC errors on Trial 3 following a six-hour delay compared to the last day of baseline performance on Trial 3. ** $p < 0.01$ * $p < 0.05$ ^ $p < 0.10$

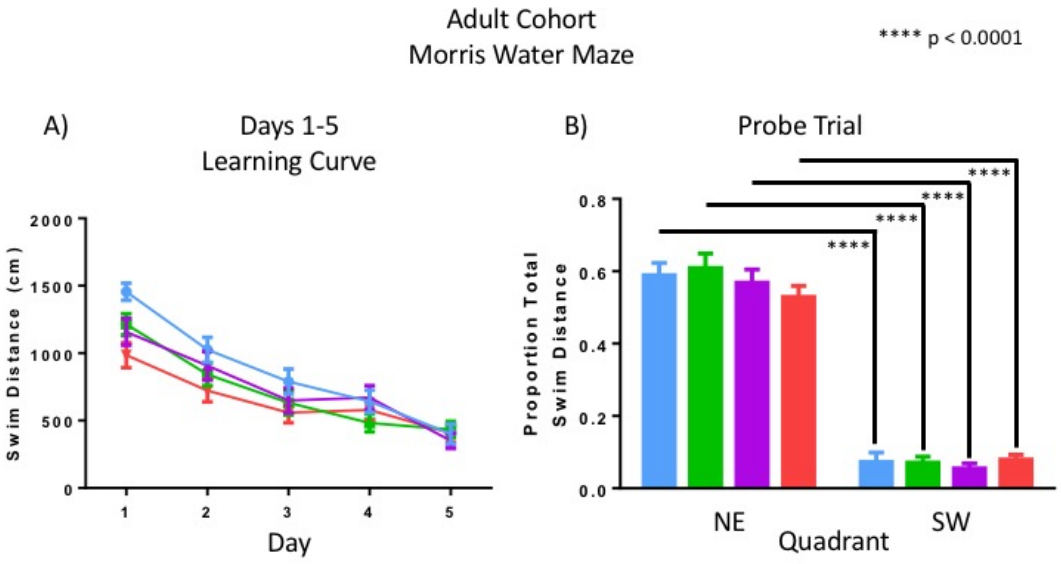

Figure 62: Morris Water Maze (Adult Cohort; 7 mo old, tested 6 weeks after surgery). A) There were no group differences in any planned comparison across days. B) Each Surgery group spent a greater proportion of total distance swimming in the Target (NE) Quadrant compared to the Opposite (SW) Quadrant during the probe trial, indicating spatial localization to the platform location. **** $p < 0.0001$

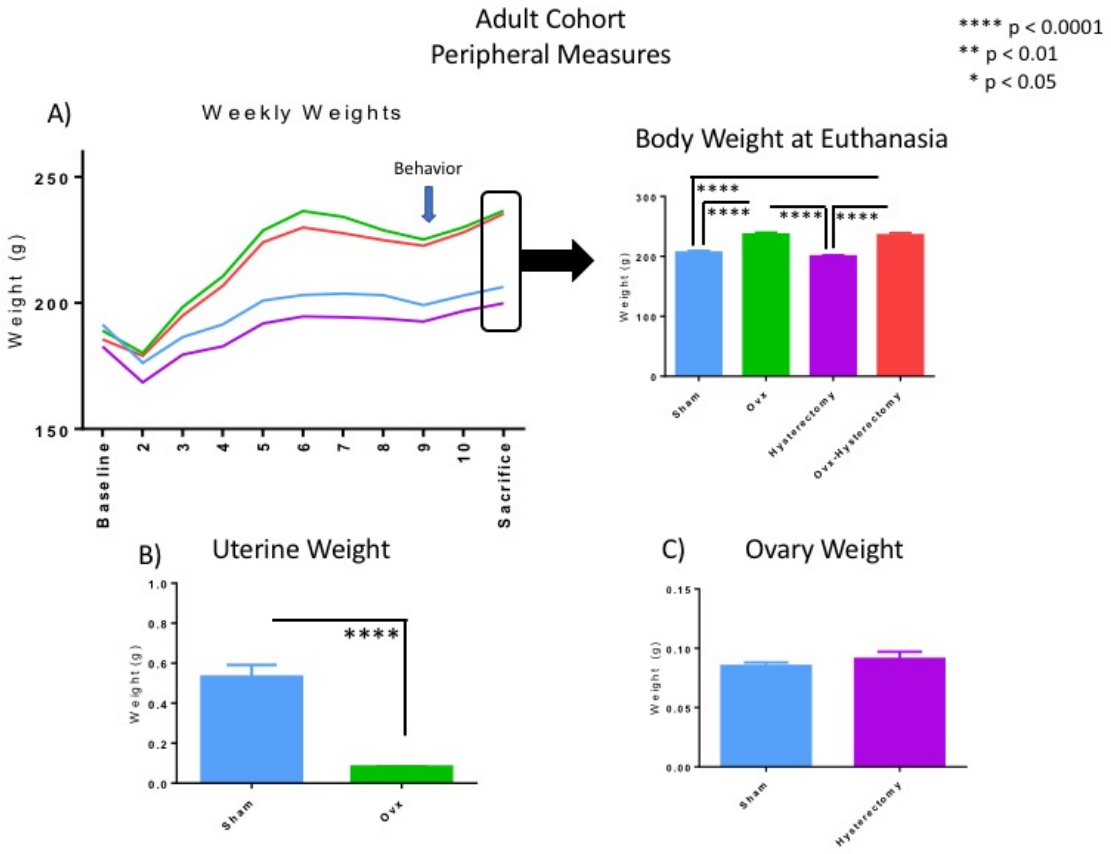

Figure 63: Peripheral Measures (Adult Cohort; 7 mo old, tested 6 weeks after surgery). A) Body weights were tracked weekly across the experiment. At euthanasia, ovary-intact groups (Sham, Hysterectomy) weighed less than Ovx groups (Ovx, Ovx-Hysterectomy). B) Uterine wet weight was obtained at euthanasia; Sham rats had increased uterine weights compared to Ovx rats. C) Ovary wet weights obtained at euthanasia did not differ between Sham and Hysterectomy rats at this time point. **** $p < 0.0001$ ** $p < 0.01$ * $p < 0.05$

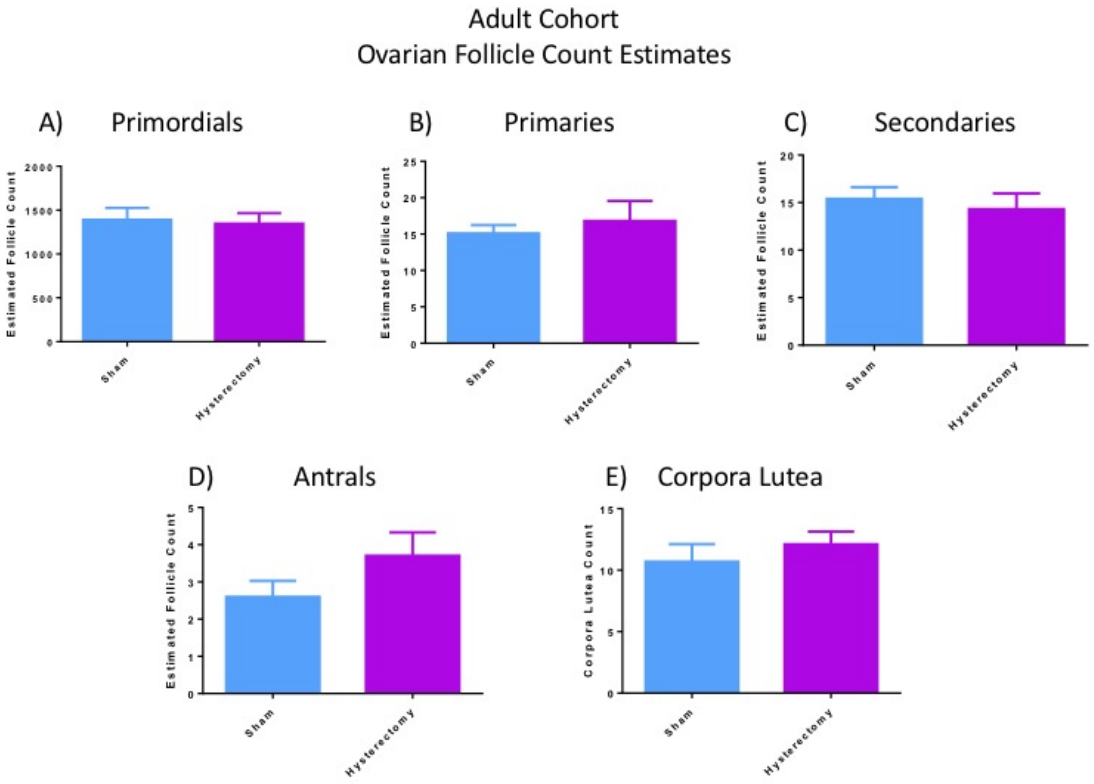

Figure 64: Ovarian Follicle Counts Estimates (Adult Cohort; 7 mo old, tested 6 weeks after surgery). There were no differences in Primordial Follicles (A), Primary Follicles (B), Secondary Follicles (C), Antral Follicles (D), or Corpora Lutea Counts (E) at this time point.

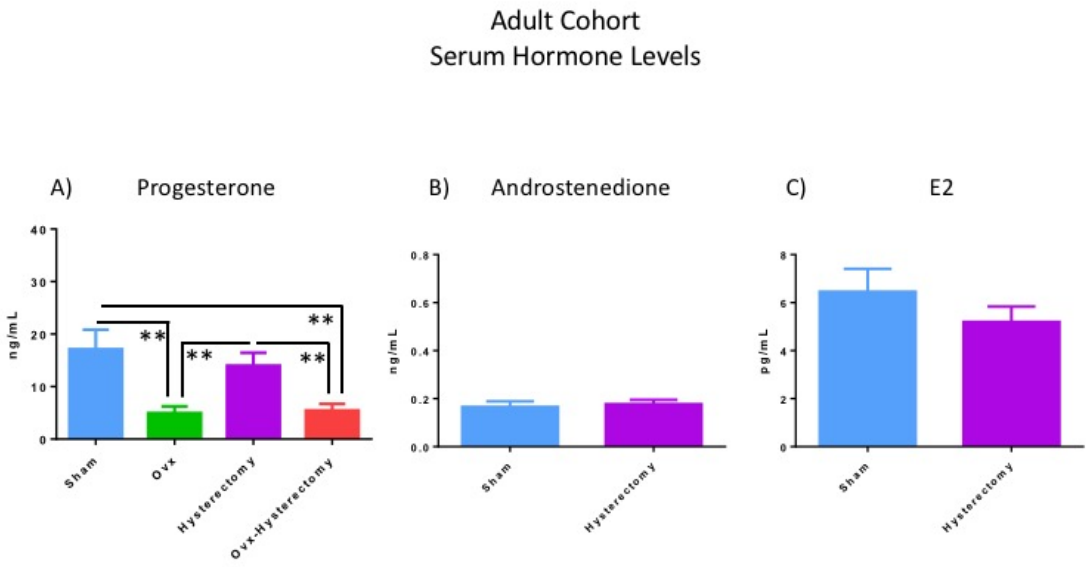

Figure 65: Serum Hormone Levels (Adult Cohort; 7 mo old, tested 6 weeks after surgery). A) Progesterone levels were significantly decreased in Ovx groups (Ovx, Ovx-Hysterectomy) when compared to ovary-intact groups (Sham, Hysterectomy). B) Androstenedione levels did not differ at this time point between Sham and Hysterectomy groups. C) E2 levels did not differ at this time point between Sham and Hysterectomy groups. ** $p < 0.01$.

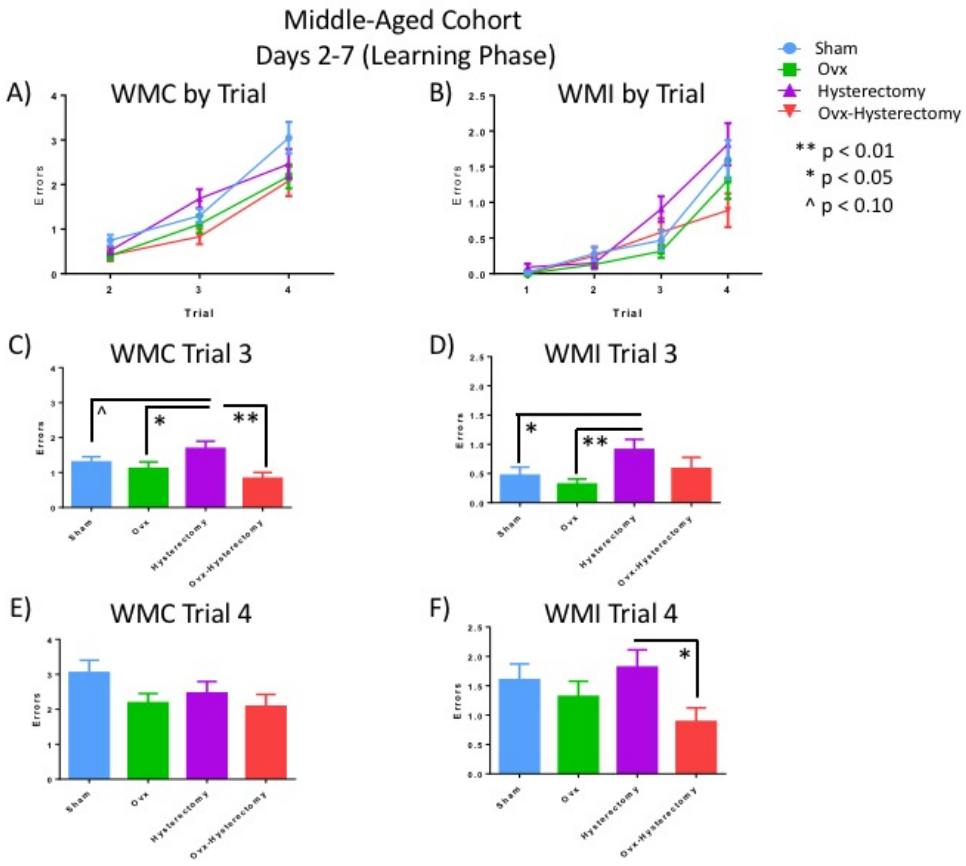

Figure 66: WRAM Learning Phase (Middle-Aged Cohort; 12 mo old, tested 7 months after surgery). A) WMC errors across trials B) WMI errors across trials C) Hysterectomy rats made more WMC errors on the moderate working memory load trial, Trial 3, compared to Ovx and Ovx-Hysterectomy rats. Sham rats tended to make fewer errors than Hysterectomy rats. D) Hysterectomy rats made more WMI errors on the maximum working memory load trial, Trial 4, compared to Sham rats and Ovx rats. E) There were no group differences for WMC errors on Trial 4. F) Hysterectomy rats made more WMI errors on Trial 4 compared to Ovx-Hysterectomy rats. ** $p < 0.01$ * $p < 0.05$ ^ $p < 0.10$

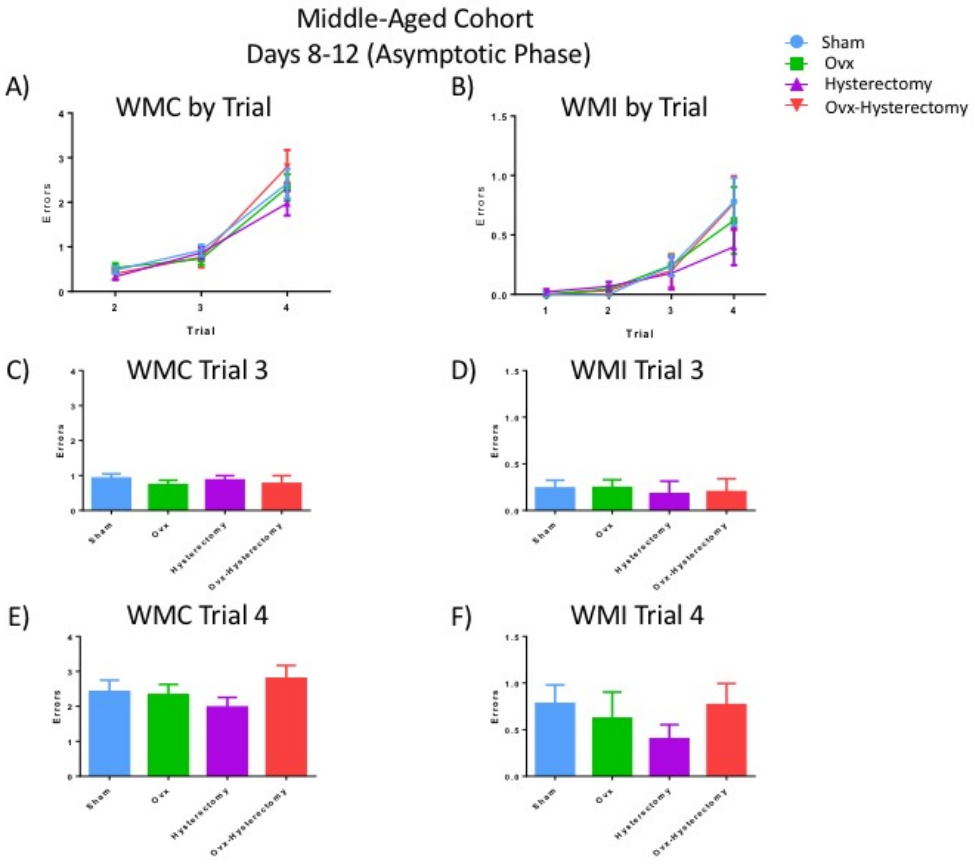

Figure 67: WRAM Asymptotic Phase (Middle-Aged Cohort; 12 mo old, tested 7 months after surgery). There were no group differences for any planned comparison during the Asymptotic phase of testing. A) WMC errors across trials B) WMI errors across trials C) Trial 3 WMC errors D) Trial 3 WMI errors E) Trial 4 WMC errors F) Trial 4 WMI errors

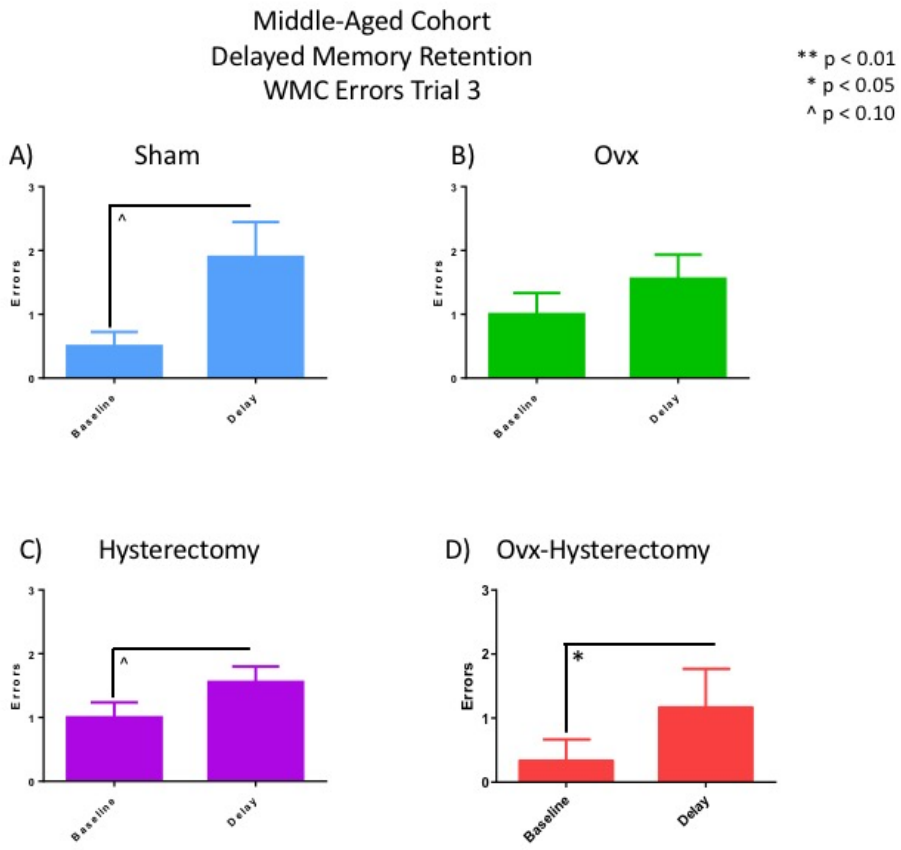

Figure 68: WRAM Delayed Memory Retention (Middle-Aged Cohort; 12 mo old, tested 7 months after surgery). A) Sham rats tended to make more WMC errors on Trial 3 following a six-hour delay compared to performance on the last day of baseline testing. B) Ovx rats did not significantly increase in errors following a six-hour delay. C) Hysterectomy rats tended to make more WMC errors on Trial 3 following a six-hour delay compared to performance on the last day of baseline testing. D) Ovx-Hysterectomy rats made more WMC errors on Trial 3 following a six-hour delay compared to performance on the last day of baseline testing. * $p < 0.05$ ^ $p < 0.10$

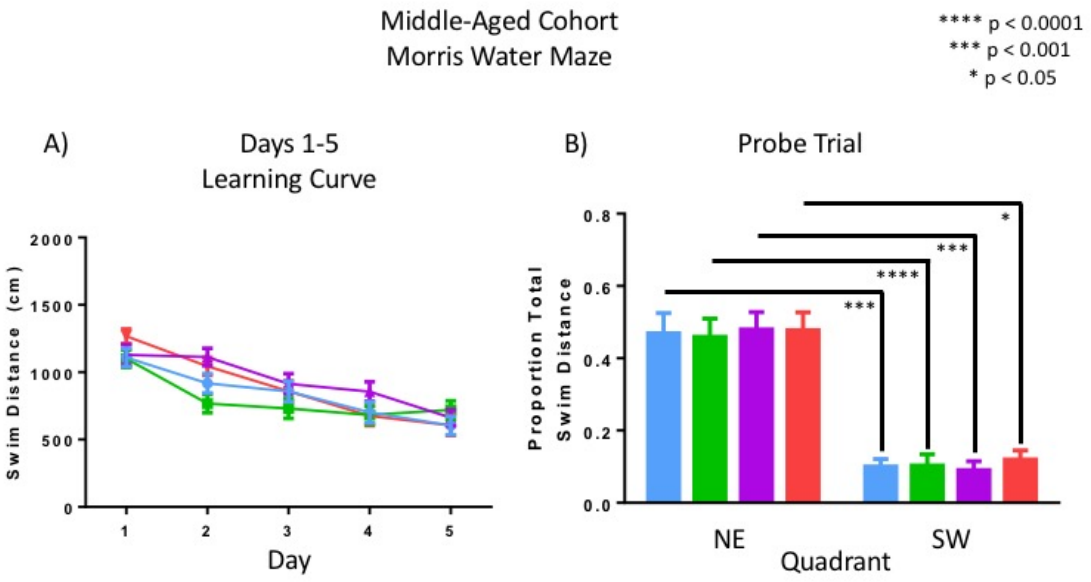

Figure 69: Morris Water Maze (Middle-Aged Cohort; 12 mo old, tested 7 months after surgery). A) There were no group differences in any planned comparison across days. B) Each Surgery group spent a greater proportion of total distance swimming in the Target (NE) Quadrant compared to the Opposite (SW) Quadrant during the probe trial, indicating spatial localization to the platform location. **** $p < 0.0001$

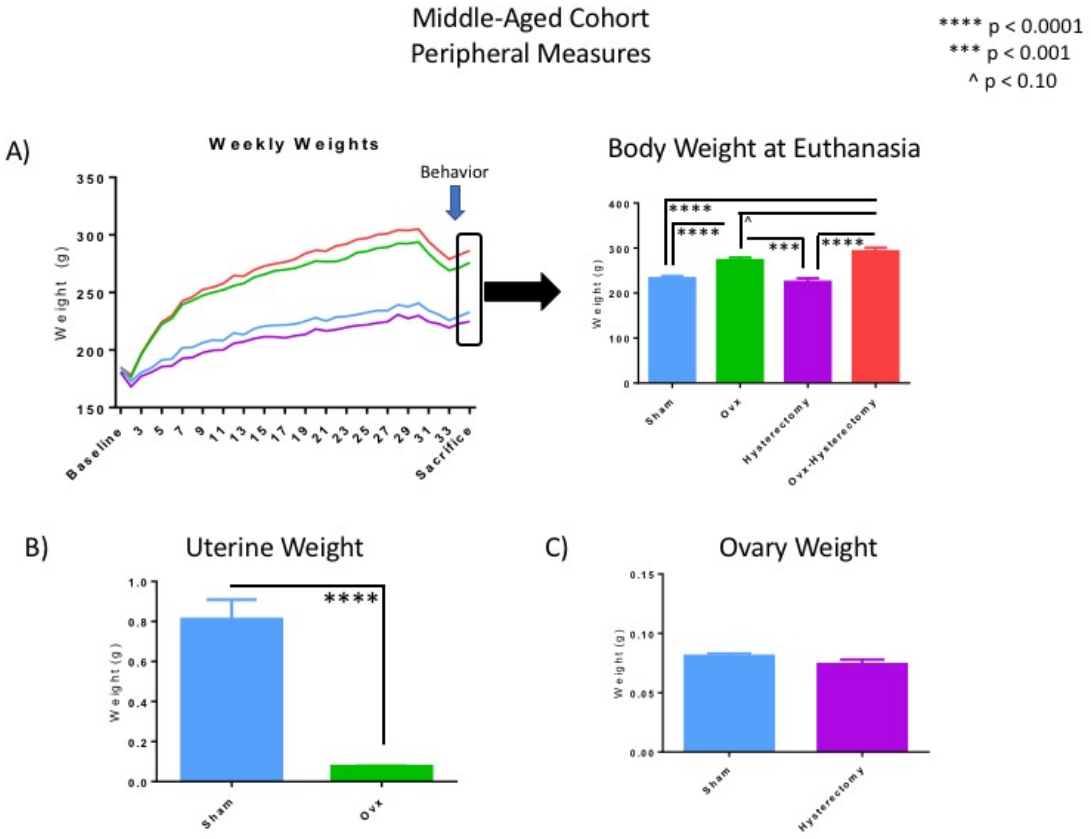

Figure 70: Peripheral Measures (Middle-Aged Cohort; 12 mo old, tested 7 months after surgery). A) Body weights were tracked weekly across the experiment. At euthanasia, ovary-intact groups (Sham, Hysterectomy) weighed less than Ovx groups (Ovx, Ovx-Hysterectomy). The Ovx group tended to weigh less than the Ovx-Hysterectomy group. B) Uterine wet weight was obtained at euthanasia; Sham rats had increased uterine weights compared to Ovx rats. C) Ovary wet weights obtained at euthanasia did not differ between Sham and Hysterectomy rats at this time point. **** $p < 0.0001$ *** $p < 0.001$ ^ $p < 0.10$

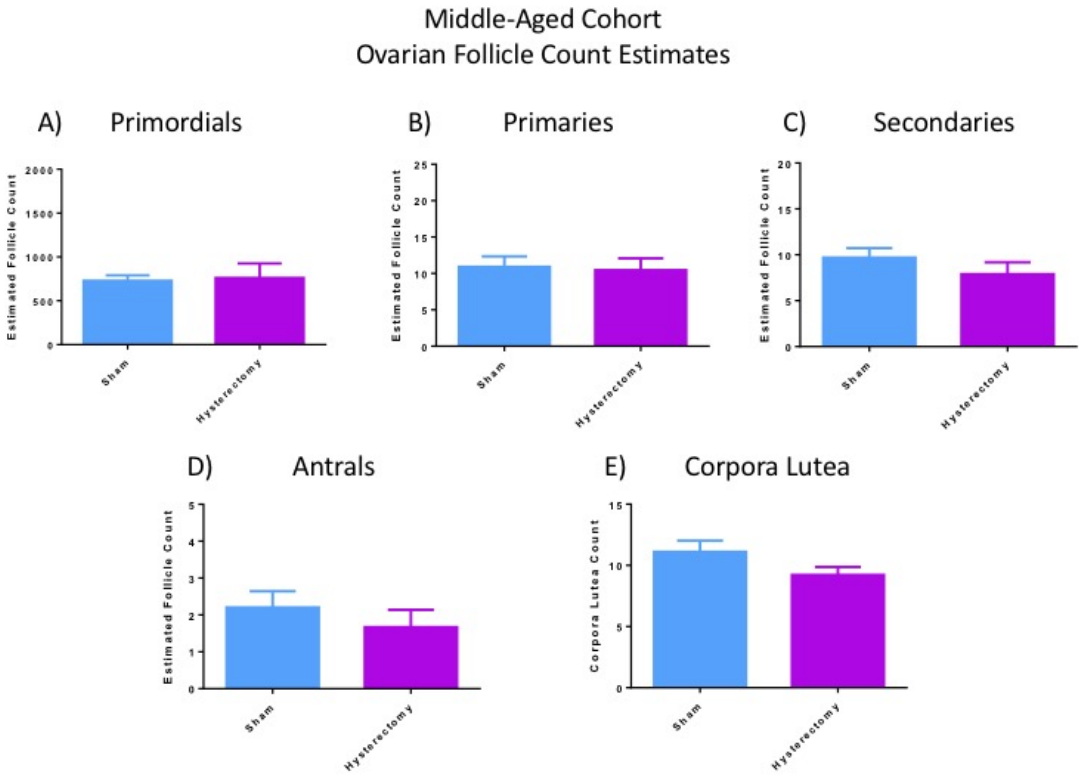

Figure 71: Ovarian Follicle Counts (Middle-Aged Cohort; 12 mo old, tested 7 months after surgery). There were no differences in Primordial Follicles (A), Primary Follicles (B), Secondary Follicles (C), Antral Follicles (D), or Corpora Lutea Counts (E) at this time point.

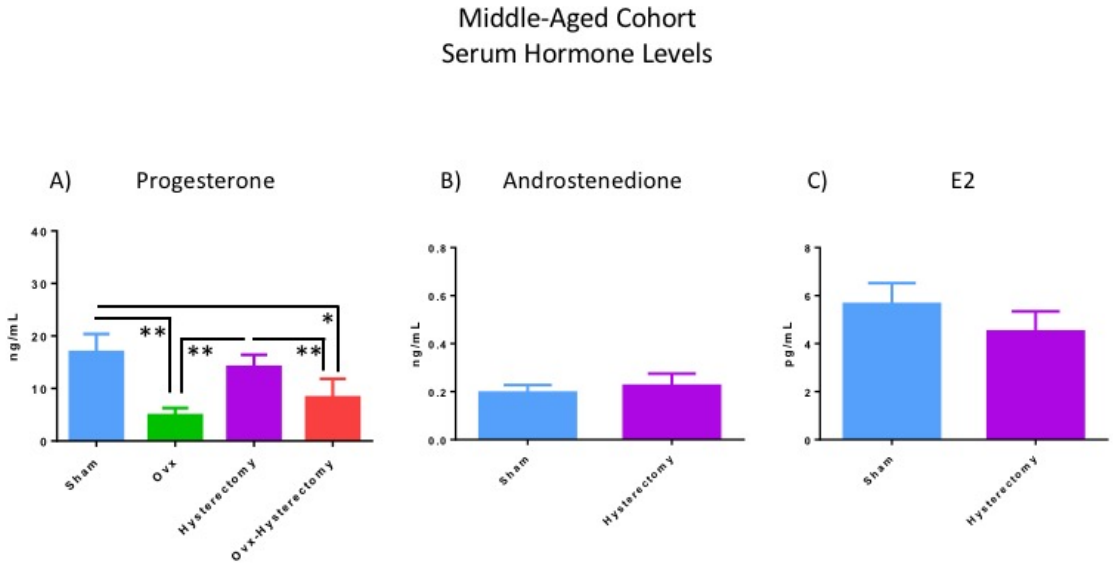

Figure 72: Serum Hormone Levels (Middle-Aged Cohort; 12 mo old, tested 7 months after surgery). A) Progesterone levels were significantly decreased in Ovx groups (Ovx, Ovx-Hysterectomy) when compared to ovary-intact groups (Sham, Hysterectomy). B) Androstenedione levels did not differ at this time point between Sham and Hysterectomy groups. C) E2 levels did not differ at this time point between Sham and Hysterectomy groups. ** $p < 0.01$ * $p < 0.05$.

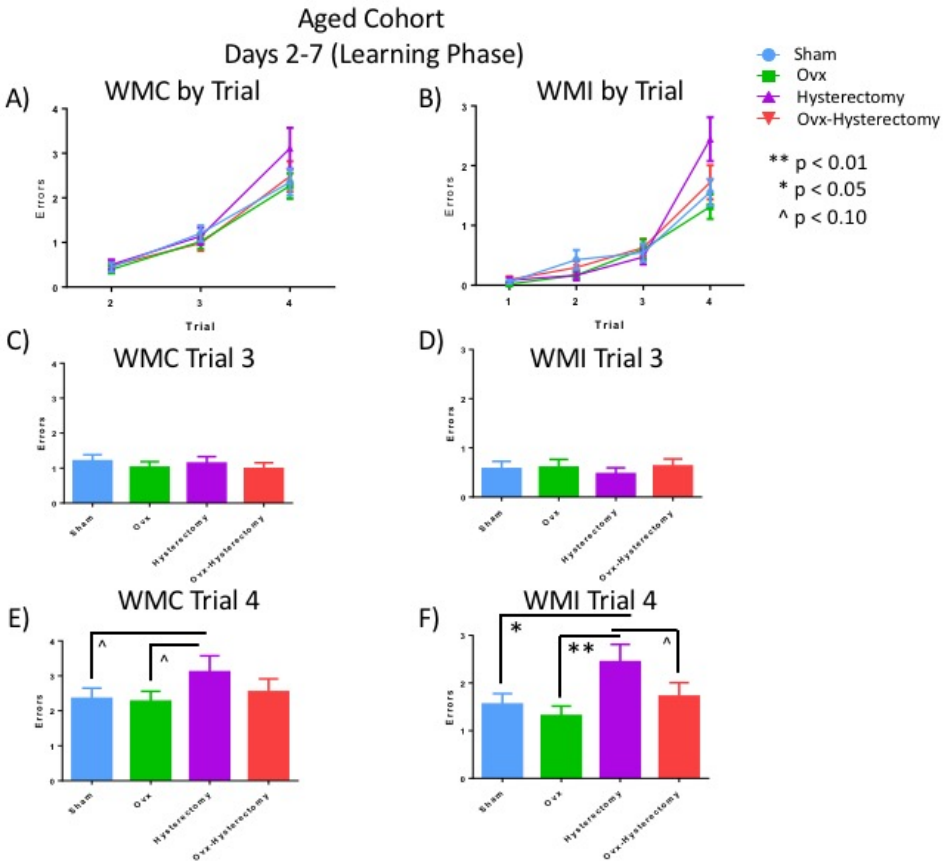

Figure 73: WRAM Learning Phase (Aged Cohort; 18 mo old, tested 12 months after surgery). A) WMC errors across trials B) WMI errors across trials C) There were no group differences for WMC errors on Trial 3 for any planned comparison. D) There were no group differences for WMI errors on Trial 3 for any planned comparison. E) Hysterectomy rats tended to make more WMC errors on Trial 4 when compared to Sham rats and compared to Ovx rats. F) Hysterectomy rats made more WMI errors on Trial 4 compared to Sham rats and compared to Ovx rats; Ovx-Hysterectomy rats tended to make fewer WMI errors than Hysterectomy rats on Trial 4 at this time point. ** $p < 0.01$ * $p < 0.05$ ^ $p < 0.10$

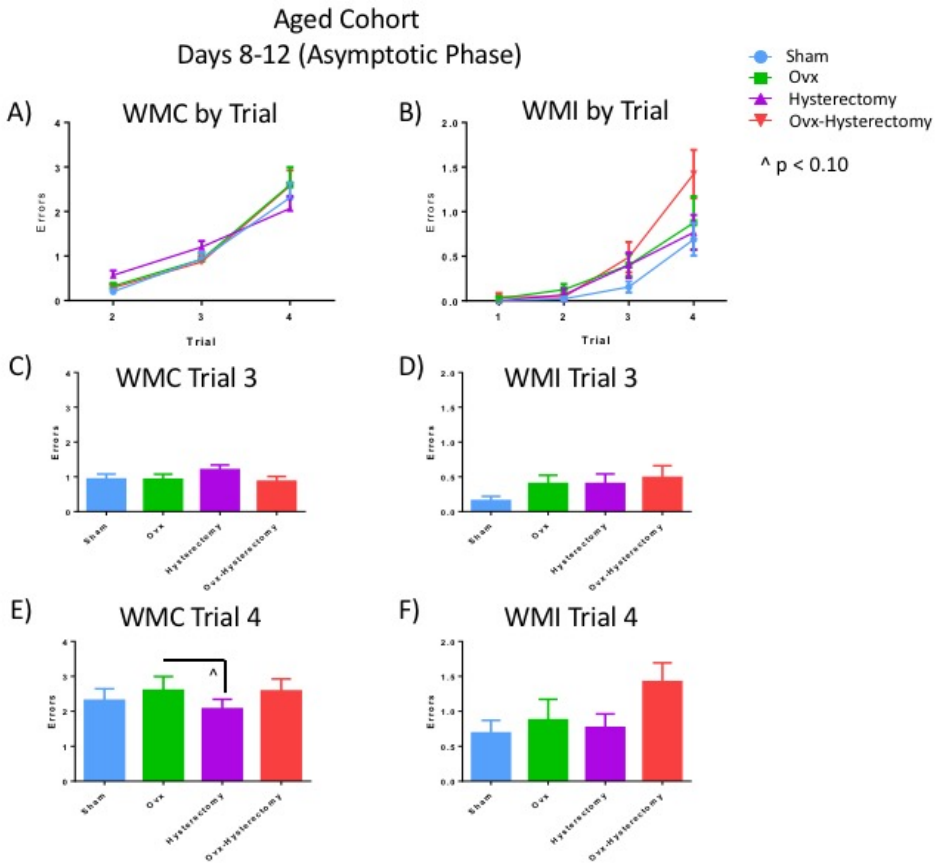

Figure 74: WRAM Asymptotic Phase (Aged Cohort; 18 mo old, tested 12 months after surgery). A) WMC errors across trials B) WMI errors across trials C) There were no group differences for WMC errors on Trial 3 for any planned comparison. D) There were no group differences for WMI errors on Trial 3 for any planned comparison. E) Hysterectomy rats tended to make fewer WMC errors on Trial 4 compared to Ovx rats, although this effect did not reach statistical significance. F) There were no group differences for WMI errors on Trial 4 for any planned comparison. ^ $p < 0.10$

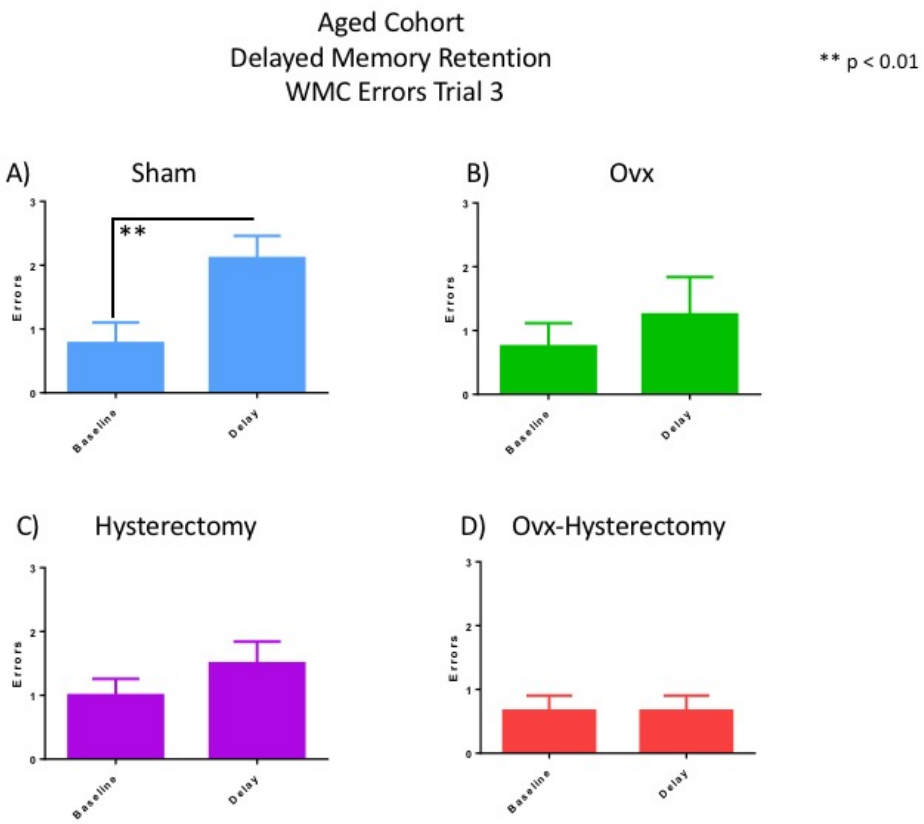

Figure 75: WRAM Delayed Memory Retention (Aged Cohort; 18 mo old, tested 12 months after surgery). A) Sham rats made more WMC errors on Trial 3 following a six-hour delay compared to performance on the last day of baseline testing. Ovx rats (B), Hysterectomy rats (C), and Ovx-Hysterectomy rats (D) did not exhibit a significant increase in WMC errors following a six-hour delay compared to performance on the last day of baseline testing within each group. ** $p < 0.01$

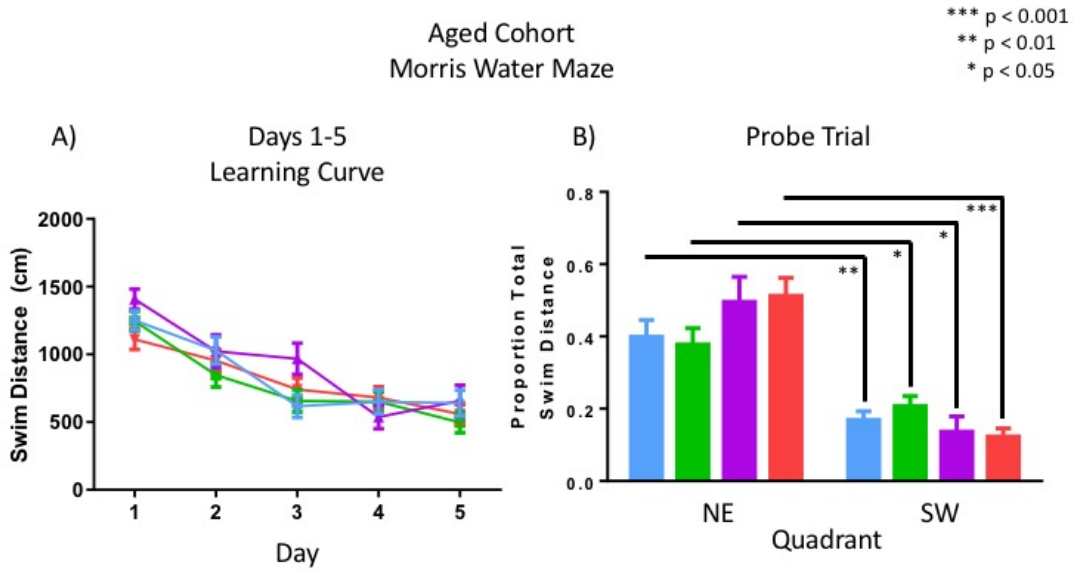

Figure 76: Morris Water Maze (Aged Cohort; 18 mo old, tested 12 months after surgery). A) There were no group differences in any planned comparison across days. B) Each Surgery group spent a greater proportion of total distance swimming in the Target (NE) Quadrant compared to the Opposite (SW) Quadrant during the probe trial, indicating spatial localization to the platform location. *** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$

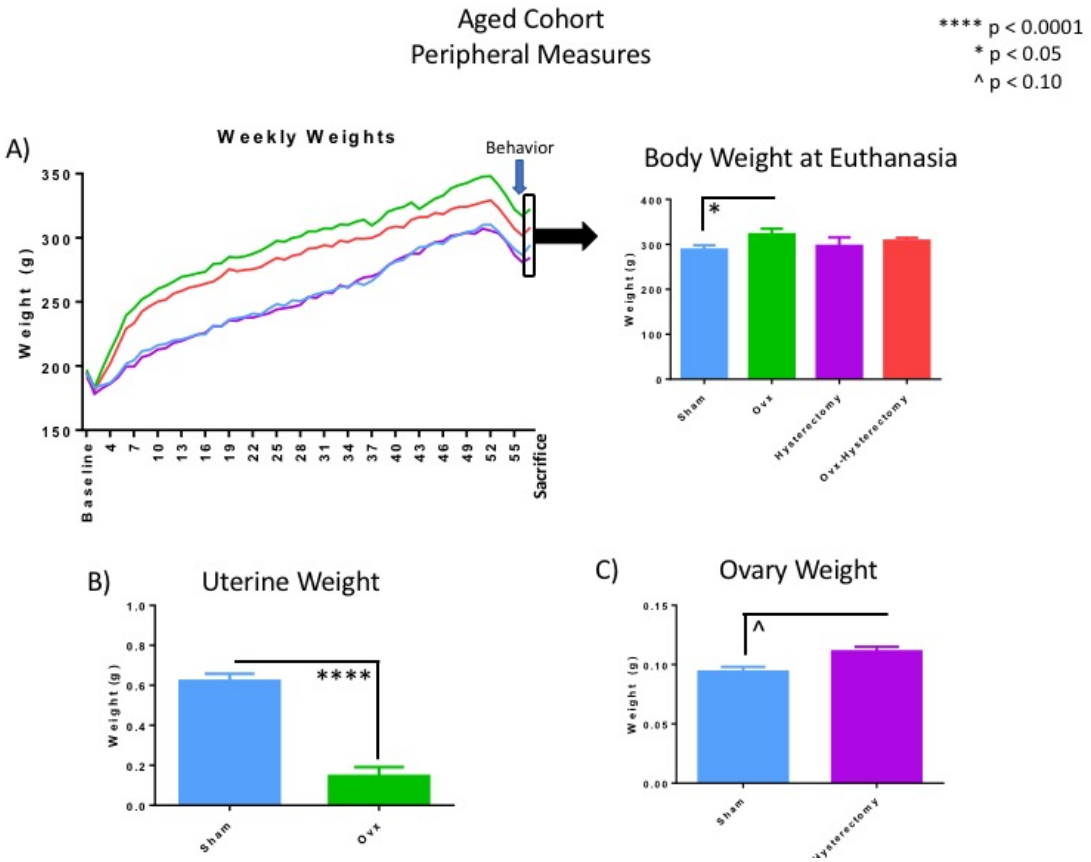

Figure 77: Peripheral Measures (Aged Cohort; 18 mo old, tested 12 months after surgery). A) Body weights were tracked weekly across the experiment. At euthanasia, Sham rats weighed less than Ovx rats. B) Uterine wet weight was obtained at euthanasia; Sham rats had increased uterine weights compared to Ovx rats. C) Ovary wet weights obtained at euthanasia tended to be increased in Hysterectomy rats compared to Sham rats, after correcting for body weight. **** $p < 0.0001$ * $p < 0.05$ ^ $p < 0.10$

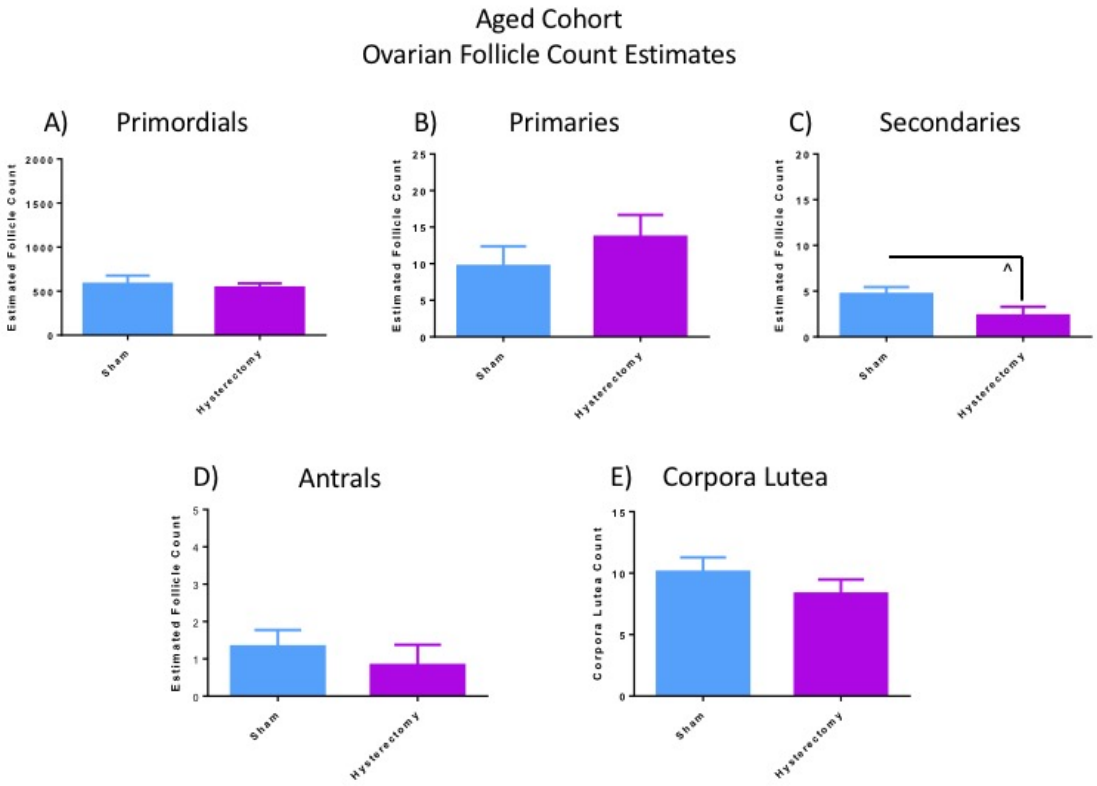

Figure 78: Ovarian Follicle Counts (Aged Cohort; 18 mo old, tested 12 months after surgery). There were no differences in Primordial Follicles (A), Primary Follicles (B), Antral Follicles (D), or Corpora Lutea Counts (E) at this time point. Hysterectomy rats tended to have fewer Secondary Follicles compared to Sham rats, although this effect did not reach statistical significance (C). ^ $p < 0.10$

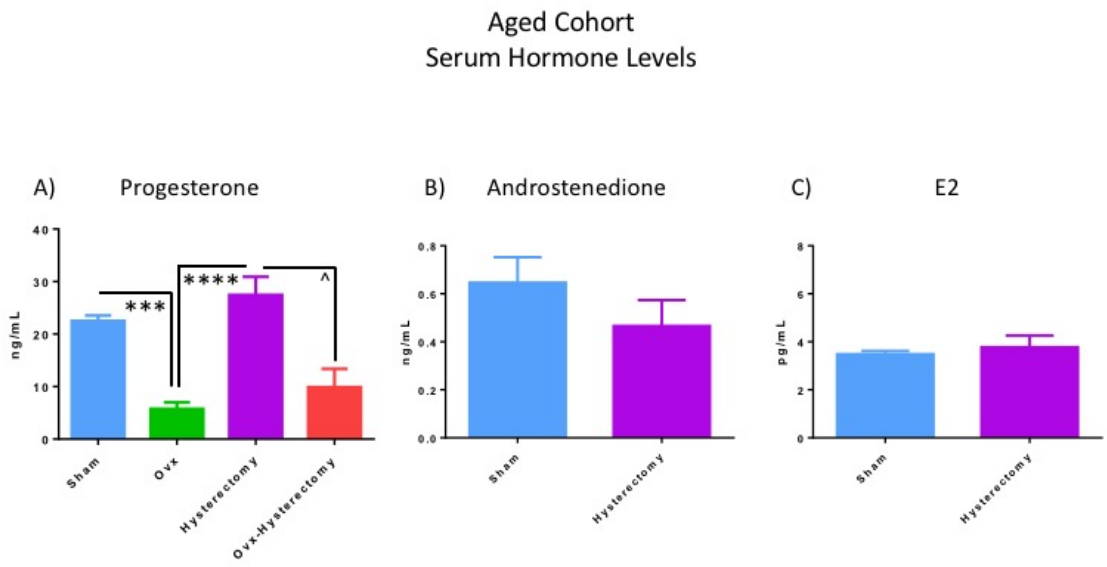

Figure 79: Serum Hormone Levels (Aged Cohort; 18 mo old, tested 12 months after surgery). A) Progesterone levels were significantly decreased Ovx rats when compared to Sham rats and compared to Hysterectomy rats. Ovx-Hysterectomy rats tended to have lower Progesterone levels compared to Hysterectomy rats. B) Androstenedione levels did not differ at this time point between Sham and Hysterectomy groups. C) E2 levels did not differ at this time point between Sham and Hysterectomy groups. **** $p < 0.0001$ *** $p < 0.001$ ^ $p < 0.10$.

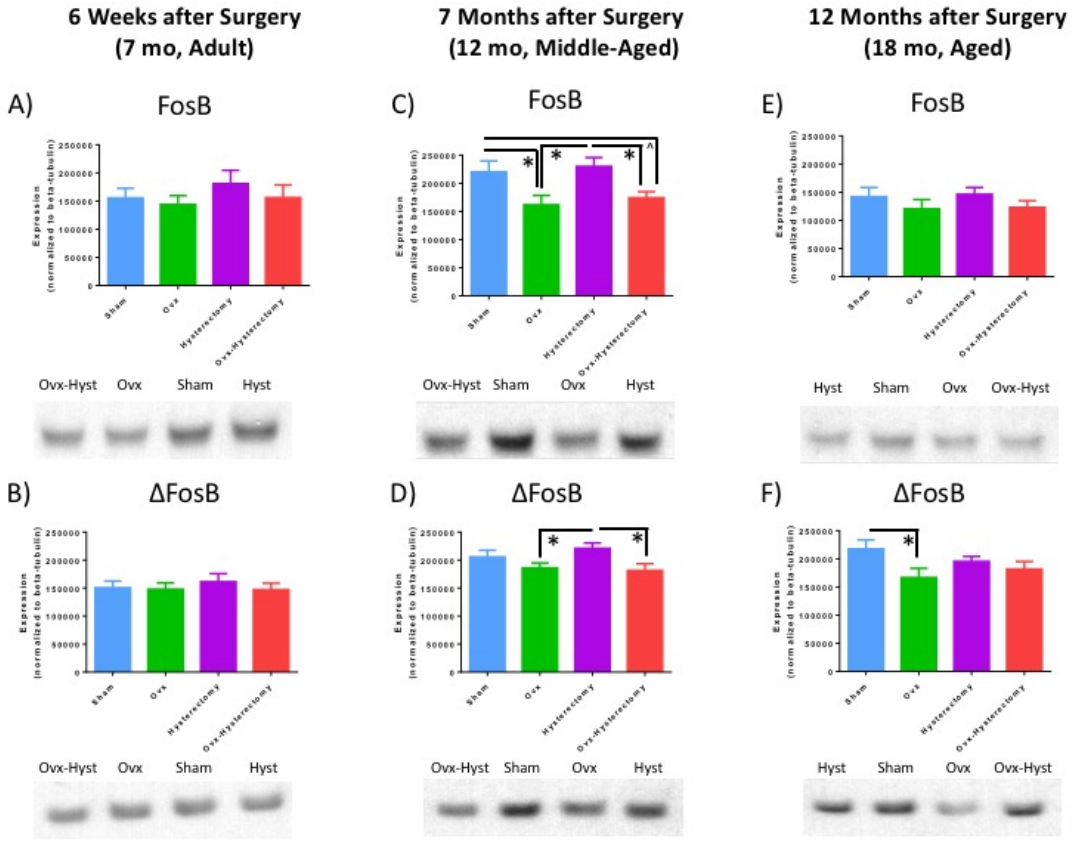

Figure 80: FosB and ΔFosB Expression in the Entorhinal Cortex. A) The Adult cohort did not exhibit changes in FosB or B) ΔFosB expression in the Entorhinal Cortex. C) Middle-Aged Ovx rats had less FosB expression compared to Sham rats and compared to Hysterectomy rats. Ovx-Hysterectomy rats had less FosB expression compared to Hysterectomy rats and marginally less FosB expression compared to Sham rats. D) Middle-Aged Hysterectomy rats tended to exhibit greater ΔFosB expression compared to Ovx rats and Ovx-Hysterectomy rats. E) Aged rats did not exhibit altered FosB expression. F) Aged Ovx rats had less ΔFosB expression than Sham rats.

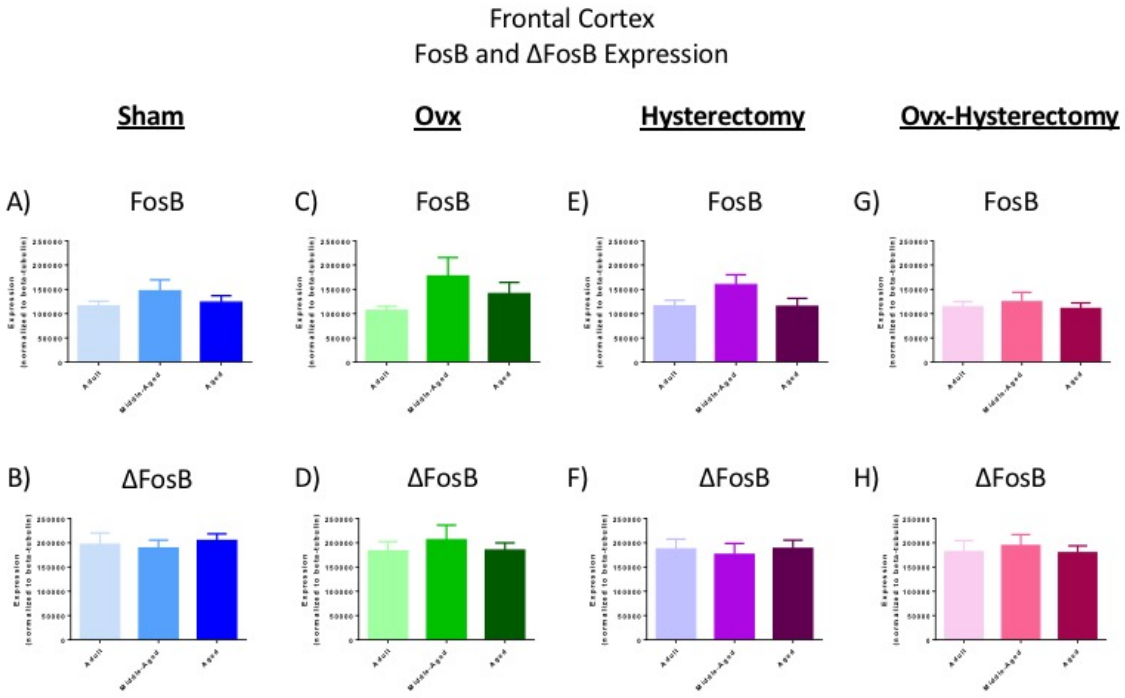

Figure 81: Age Effects on FosB and ΔFosB Expression in the Frontal Cortex. There was no significant age-related change in FosB expression (A, C, E, G) or ΔFosB expression for any surgery group (B, D, F, H).

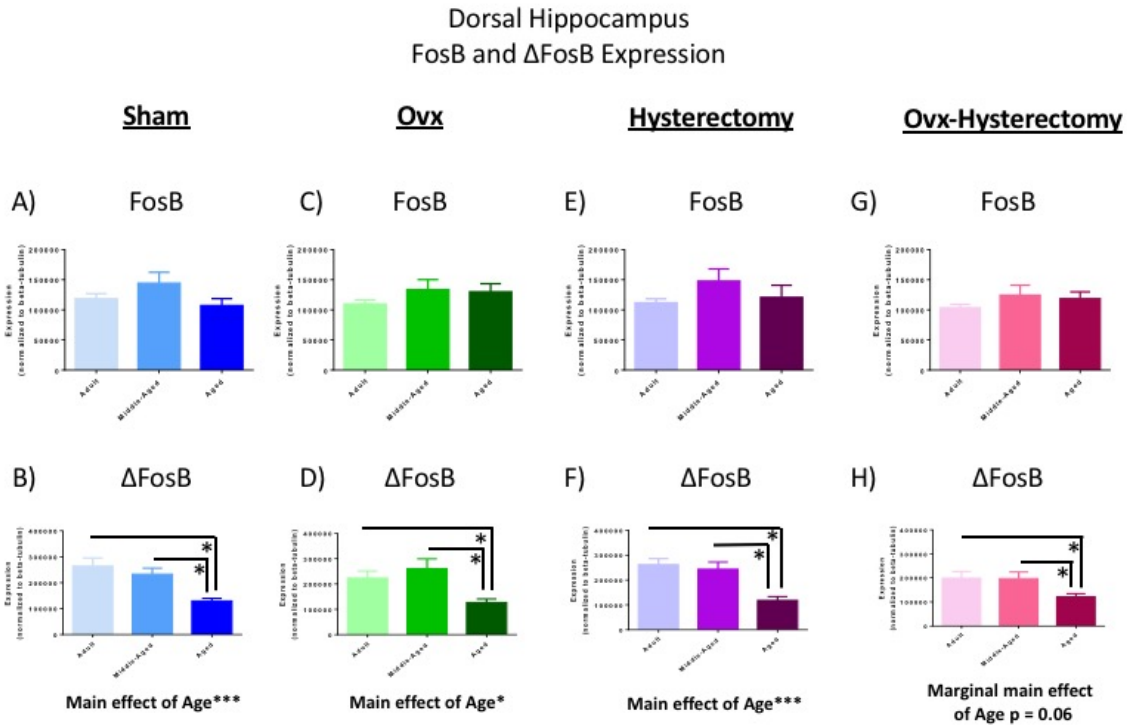

Figure 82: Age Effects on FosB and ΔFosB Expression in the Dorsal Hippocampus. There were no age-related changes in FosB expression with age in any surgery group (A, C, E, G). However, all subjects, regardless of surgery type, exhibited an decrease in ΔFosB expression in the Aged time point, when rats were 18 months old (B, D, F, H).

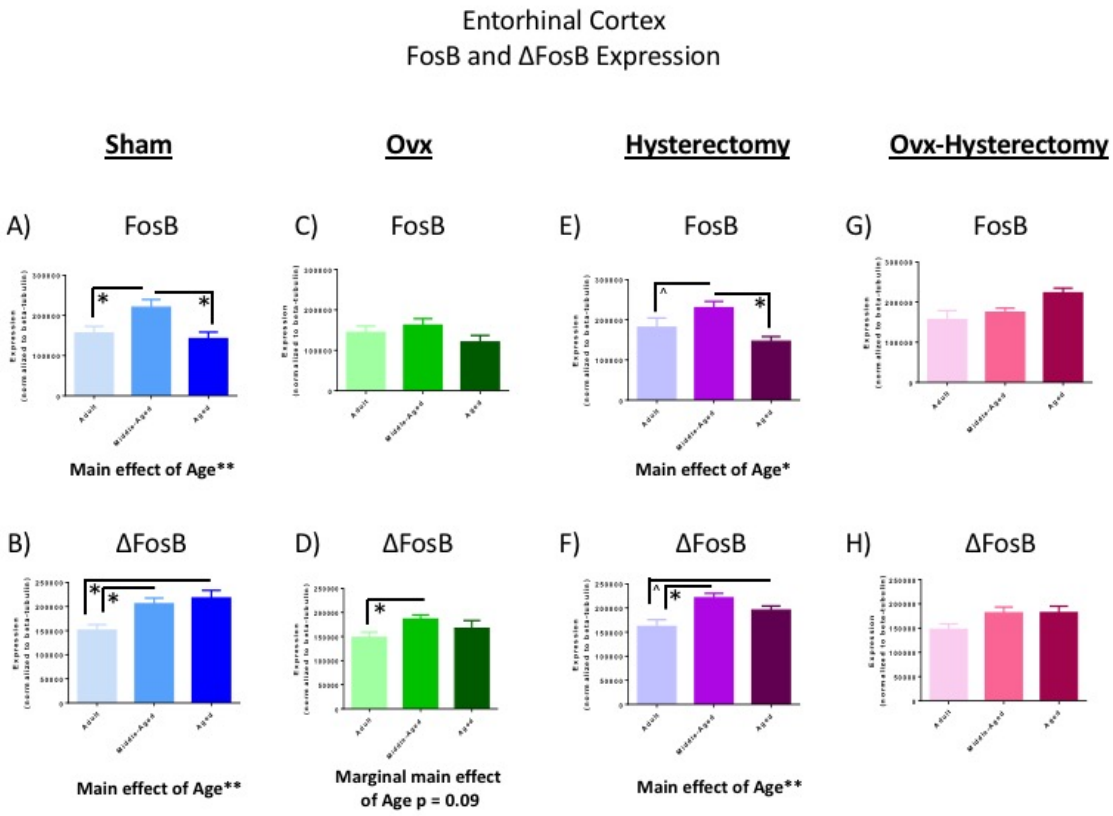

Figure 83: Age Effects on FosB and ΔFosB Expression in the Entorhinal Cortex. A) Sham rats showed a transient increase in FosB expression in Middle Age. B) Sham rats had more ΔFosB expression in the Middle-Aged cohort and the Aged cohort compared to the Adult cohort. C) Ovx rats did not exhibit changes in FosB expression across the three cohorts in the Entorhinal Cortex. D) ΔFosB expression tended to increase in Middle-Aged Ovx rats compared to Adult Ovx rats. E) Hysterectomy rats tended to exhibit greater FosB expression in the Middle-Aged cohort compared to the Adult cohort. However, FosB expression was lower in the Aged cohort than the Middle-Aged cohort. F) Adult Hysterectomy rats had lower ΔFosB expression compared to the Middle-Aged cohort and tended to have lower ΔFosB expression compared to the Aged cohort. G) Ovx-Hysterectomy rats did not exhibit changes in FosB expression across the three cohorts. H) Ovx-Hysterectomy rats did not exhibit changes in ΔFosB expression across the three cohorts.

Hormone Correlation Summary

Young-Vehicle	ChAT-IR VDB	ChAT-IR MS	Entorhinal Cortex GAD67	Ventral Hippocampus GAD65	Ventral Hippocampus GAD67
Estradiol	x	x	x	Positive	Positive
Estrone	Positive	x	x	x	x
Androstenedione	Positive	x	x	x	x
Progesterone	Positive	x	x	x	x
Ovarian Follicle Reserve	Positive	x	x	x	x

Middle Aged-Vehicle	ChAT-IR VDB	ChAT-IR MS	Entorhinal Cortex GAD67	Ventral Hippocampus GAD65	Ventral Hippocampus GAD67
Estradiol	x	x	x	x	x
Estrone	x	x	x	x	x
Androstenedione	x	x	x	x	x
Progesterone	x	x	x	x	x
Ovarian Follicle Reserve	x	x	x	x	x

Young-VCD	ChAT-IR VDB	ChAT-IR MS	Entorhinal Cortex GAD67	Ventral Hippocampus GAD65	Ventral Hippocampus GAD67
Estradiol	x	x	x	x	x
Estrone	x	x	Negative	x	x
Androstenedione	x	x	x	x	x
Progesterone	x	x	x	x	x
Ovarian Follicle Reserve	x	x	x	x	x

Middle Aged-VCD	ChAT-IR VDB	ChAT-IR MS	Entorhinal Cortex GAD67	Ventral Hippocampus GAD65	Ventral Hippocampus GAD67
Estradiol	x	x	x	x	x
Estrone	x	x	x	x	x
Androstenedione	x	Positive	x	x	x
Progesterone	x	x	x	x	x
Ovarian Follicle Reserve	x	x	x	x	x

Figure 84: Age and Follicular Depletion Hormone Correlation Summary. Young-Vehicle rats (8-12 months of age) exhibited positive correlations between serum estrone, androstenedione, and progesterone levels, as well as ovarian follicle reserve estimates with ChAT-IR neurons in the vertical/diagonal bands within the basal forebrain, such that higher circulating levels of these hormones were associated more ChAT-IR cells in this brain region. Furthermore, higher 17β-estradiol levels were associated with more GAD65 and GAD67 expression in the Ventral Hippocampus. Age-matched counterparts that underwent VCD-induced follicular depletion at 6 months of age did not exhibit these correlations; however, there was a negative association between estrone levels and Entorhinal Cortex GAD67 expression, where higher estrone levels were associated with less GAD67 expression. Middle-Aged-VCD rats (14-18 months of age) that underwent VCD-induced follicular depletion at 12 months of age exhibited a positive correlation between androstenedione levels and ChAT-IR neurons in the medial septum, but Vehicle-treated, age-matched counterparts did not exhibit any associations between circulating hormone levels and brain measures.

Behavioral Correlation Summary

Young-Vehicle	Working Memory Errors	MM Swim Distance to Platform
Estradiol	x	x
Entorhinal Cortex GAD65	x	Positive
Perirhinal Cortex GAD67	x	Negative

Middle Aged-Vehicle	Working Memory Errors	MM Swim Distance to Platform
Estradiol	x	x
Entorhinal Cortex GAD65	x	x
Perirhinal Cortex GAD67	x	x

Young-VCD	Working Memory Errors	MM Swim Distance to Platform
Estradiol	Negative	x
Entorhinal Cortex GAD65	x	x
Perirhinal Cortex GAD67	x	x

Middle Aged-VCD	Working Memory Errors	MM Swim Distance to Platform
Estradiol	x	x
Entorhinal Cortex GAD65	x	x
Perirhinal Cortex GAD67	x	x

Figure 85: Age and Follicular Depletion Behavior Correlation Summary. Young-Vehicle rats (8-12 months old) exhibited a positive correlation between Entorhinal Cortex GAD65 expression and MM Swim Distance, such that higher GAD65 expression was associated with a longer swim distance to platform, indicating poorer performance. Conversely, Young-VCD rats showed a negative association between serum 17β-estradiol levels and working memory errors on the WRAM, where higher 17β-estradiol was associated with fewer working memory errors on the maximum working memory load trial, an indication of better performance. There were no associations between behavior measures and physiological measures in Middle-Aged rats (14-18 months old), regardless of follicular depletion status.

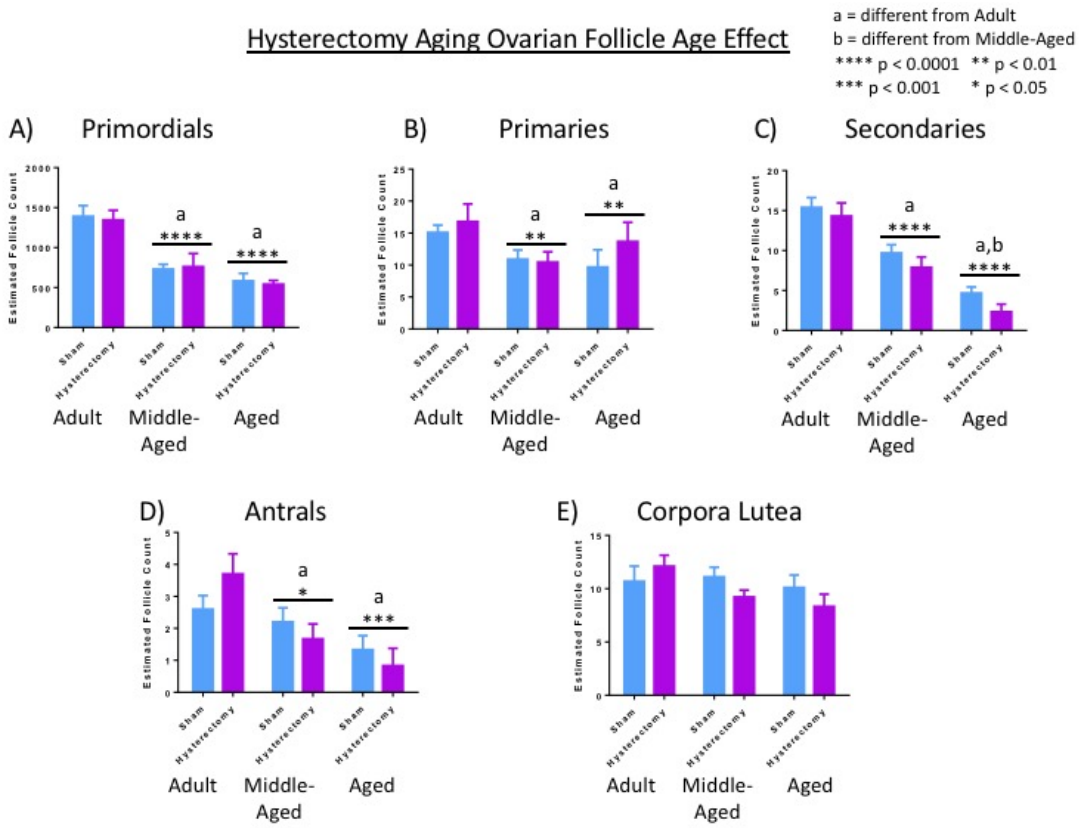

Figure 86: Hysterectomy Aging Ovarian Follicle Age Effect. Adult rats had more primordial (A), primary (B), secondary (C), and antral (D) follicles compared to Middle-Aged and Aged rats. Secondary follicles also significantly decreased from Middle-Age to Aged time points (C). Corpora lutea counts did not significantly differ with age.

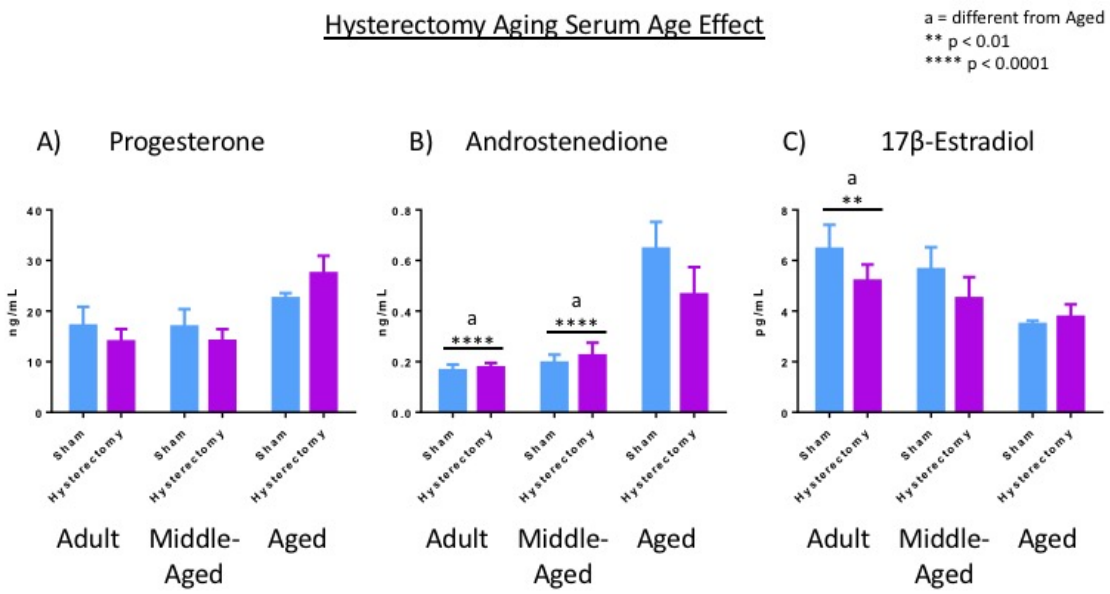

Figure 87: Hysterectomy Aging Serum Hormone Age Effect. A) Progesterone levels did not significantly change with age B) Androstenedione levels were increased in Aged rats compared to Adult and Middle-Aged time points C) Adult rats had higher serum 17β-estradiol levels compared to Aged rats.

APPENDIX A

TINTO DE VERANO

TINTO DE VERANO

Ingredients

1 bottle red wine

1 bottle lemon Fanta or Squirt soda

Garnish: Cinnamon and lemon twist

Instructions

Pour 8 oz of red wine into a glass. Combine with citrus-flavored soda to taste. Sprinkle cinnamon on top. Add a lemon twist for garnish, if desired. Serve chilled or with ice. Enjoy!

Ingredientes

1 botella de vino tinto

1 botella de refresco con sabor cítrico

Adorno: Canela y cáscara de limón

Instrucciones

Sírvase una copa de vino tinto (250 mL). Combine con el refresco cítrico a su gusto. Espolvoree la canela encima. Añada una cáscara de limón si desea. Sírvase frío o con hielo. ¡Disfrute!