On the Cognitive Impact of Endogenous and Exogenous Hormone Exposures Across the Lifespan

by

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ABSTRACT

Women are exposed to numerous endogenous and exogenous hormones across the lifespan. In the last several decades, the prescription of novel hormonal contraceptives and hormone therapies (HTs) have resulted in aging women that have a unique hormone exposure history; little is known about the impact of these hormone exposures on short- and long- term brain health. The goal of my dissertation was to understand how lifetime hormone exposures shape the female cognitive phenotype using several innovative approaches, including a new human spatial working memory task, the human radial arm maze (HRAM), and several rodent menopause models with variants of clinically used hormone treatments. Using the HRAM (chapter 2) and established human neuropsychological tests, I determined males outperformed females with high endogenous or exogenous estrogen levels on visuospatial tasks and the spatial working memory HRAM (chapter 3). Evaluating the synthetic estrogen in contraceptives, ethinyl estradiol (EE), I found a high EE dose impaired spatial working memory in ovariectomized (Ovx) rats, medium and high EE doses reduced choline-acetyltransferace-immunoreactive neuron population estimates in the basal forebrain following Ovx (chapter 4), and low EE impaired spatial cognition in ovary-intact rats (chapter 5). Assessing the impact of several clinically-used HTs, I identified a window of opportunity around ovarian follicular depletion outside of which the HT conjugated equine estrogens (CEE) was detrimental to spatial memory (chapter 6), as well as therapeutic potentials for synthetic contraceptive hormones (chapter 9) and bioidentical estradiol (chapter 7) during and after the transition to menopause. Chapter 6 and 7 findings, that estradiol and Ovx benefitted cognition after the menopause transition, but CEE did not, are perhaps due to the negative impact of ovarian-produced,

androstenedione-derived estrone; indeed, blocking androstenedione's conversion to estrone prevented its cognitive impairments (chapter 8). Finally, I determined that EE combined with the popular progestin levonorgestrel benefited spatial memory during the transition to menopause, a profile not seen with estradiol, levonorgestrel, or EE alone (chapter 9). This work identifies several cognitively safe, and enhancing, hormonal treatment options at different time points throughout female aging, revealing promising avenues toward optimizing female health.

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CHAPTER 1: GENERAL INTRODUCTION

Throughout the lifespan, women are exposed to constant shifts in hormones. In addition to constant exposure to endogenous hormones, which are hormones that are naturally produced by an organism, there is also the possibility of exposure to exogenous hormones, or hormones that originate from outside of the organism. Women's reproductive hormones, in particular, undergo many endogenously- and exogenously-triggered changes, including those that happen during perinatal development, puberty, with use of hormonal contraceptives, during pregnancy, throughout the transition to menopause, and with HT. Reproductive hormones such as estrogens, androgens, progesterone, and others, are responsible for the regulation of countless body functions in addition to their reproductive functions, including temperature, bone density, body fat composition and deposition, metabolism, and brain function. The breadth of hormone exposures women experience is impressive, and includes both natural and synthetic hormones. Moreover, several changes in hormone use trends across the past few decades contribute to an aging generation with a previously unrepresented hormonal history. The known pervasive and interactive effects each of these hormones has on brain and body function mean that these changing hormone use trends are likely to produce a unique generation of aging females.

Fortunately, scientists now have access to several important tools necessary to methodically investigate the effects that these hormone exposures have on brain function and cognition. Rodent behavioral research affords the opportunity to model many of the hormonal states that women experience throughout the lifespan. The rodent estrous cycle is remarkably similar to the human menstrual cycle, providing a model of the human

reproductive lifespan. Through surgical removal of the ovaries in rodents (ovariectomy; Ovx) we can model oophorectomy, a procedure that approximately 600,000 women undergo each year in the Unites States (ACOG, 2008; Rocca et al., 2009), as well as isolate the effects of individual ovarian hormones on the brain and body (Mennenga and Bimonte-Nelson, 2013). Treatment with the industrial chemical 4-vinylcyclohexene diepoxide (VCD) accelerates the process of atresia in ovarian follicles, producing a follicle-deplete, ovary-intact rodent with a hormone profile similar to that following human menopause (Mayer, et al., 2004, 2005; Acosta et al., 2009, 2010; Mennenga and Bimonte-Nelson, 2013). Both Ovx and VCD treatment can be manipulated independently of aging, allowing investigation into age-dependent and –independent effects of reproductive hormones.

17β-Estradiol (E2) is the most potent naturally circulating estrogen in women and rats, followed by estrone (E1) and estriol, in order of receptor affinity. Ethinyl estradiol (EE), a synthetic form of E2, is the most common estrogen in hormonal contraceptives (Shively, 1998), and is the only estrogen used in the contraceptive pill. National surveys estimate that 10.6 million women between 2006-2010 (Jones et al., 2012), and 17.3% of all women between 2006-2008 (Mosher and Jones, 2010), used oral contraceptives. Over 30 contraceptive formulations contain EE (Curtis et al., 2005; Hoffman et al., 2012) and EE is also found in hormone therapies (HTs) for menopausal women, such as Estinyl™ and Femhrt™ (Curtis et al., 2005; Hoffman et al., 2012). Understanding the cognitive impact of estrogens is critical, as exposure to exogenous estrogens occurs throughout the lifespan through both hormonal contraceptives and HTs. While EE is a synthetic analogue to E2, these estrogens have different pharmacological profiles (Coelingh

Bennink et al., 2004). Additionally, EE is more biologically active than E2 (Dickson and Eisenfeld, 1981) and cannot be converted to E1 or other weaker estrogens (Fotherby, 1996), whereas E2 can (Prokai-Tatrai, et al., 2005). These estrogens also exhibit different binding profiles across species (Paradiso et al., 2001).

Estrogen Receptors (ERs) are highly expressed in several cognitive brain regions, including the basal forebrain (BF) (Shughrue et al., 1999), which contains cholinergic cell bodies that project to the hippocampus (McEwen, 2001; Gibbs, 2010). These projections are known to be intimately involved in spatial learning and memory (Luine et al., 1986) and are required for E2 to benefit cognition in rodents (Gibbs, 2002, 2007); however, no studies have evaluated the impact of EE on this system. Although EE is among the most commonly prescribed estrogens for contraception, and is prescribed to women from puberty to post-menopause, most preclinical research on the cognitive impact of estrogens does not include EE (for reviews see: Bimonte-Nelson et al., 2010; Acosta et al., 2013).

Optimizing Hormonal Contraceptive Use During Young Adulthood

In human contraceptive users, no impact of EE-containing contraceptives was found on several tests of memory and concentration (Silber et al., 1987). Mordecai et al. (2008) found enhanced verbal memory during the active, compared to the inactive phase of oral contraceptives, although benefits were not seen on visuospatial measures. Although each of the contraceptive formulations used in these studies contained EE, other aspects of the formulations differed, including dose and which progestin was included. Thus, it is difficult to decipher whether or to what extent EE was responsible for these effects. In studies investigating EE as a HT, cognitive effects seem to depend on

memory domain as well. In aged ovariectomized (Ovx) rhesus monkeys, EE improved spatial working memory (Lacreuse et al., 2002), but impaired face recognition (Lacreuse and Herndon, 2003), and had no impact on executive function (Lacreuse et al., 2004). An fMRI study of menopausal women found EE-containing HTs increased frontal cortex activation during a working memory task (Smith, et al., 2006).

We performed an experiment in human participants to unite human and rodent cognitive research methodology and to determine whether a human analogue of a rodent testing paradigm produces a similar pattern of errors to that seen in rodents (see Table 1 for an overview of experiments). We also sought to determine whether established tasks that measure different domains of cognition in humans would account for unique portions of variance in HRAM scores. In experiment 1 (chapter 2), we tested whether a human radial arm maze (HRAM) could be a useful and valid measure of human spatial working memory. In experiment 2 (chapter 3), we then divided our participants according to hormonal status to determine whether sex, menstrual phase, or hormonal contraceptive use were associated with differences in performance on each of these tasks. Following the collection of these data, experiment 3 (chapter 4) was then performed to test the cognitive and neurobiological effects of daily administration of EE in Ovx rodents, to determine whether we could reproduce our human findings in rodents. Experiment 3 (chapter 4) utilized Ovx female rats with no circulating ovarian hormones in addition to the EE delivered exogenously by daily injections. While the Ovx model is a powerful tool that allows isolation of the cognitive effects of a single hormone, an animal model using ovary-intact female rats is necessary, given that most women retain their ovaries for the majority of their lives. Thus, experiment 4 (chapter 5) evaluated the effects of EE

administration in ovary-intact animals to determine how EE affects cognition when ovaries are present.

Optimizing the Cognitive Impact of Hormone Therapy in a Rodent Model of Natural Menopause

Around the fifth decade of life, women's eggs stop maturing, ovulation and menstruation become irregular, and eventually the menses cease; this natural irregularity and gradual cessation of the menses is known as menopause (NAMS; Curtis et al., 2005; Hoffman et al., 2012). With the halting of ovulation, ovarian production of estrogen and progesterone drastically decrease, resulting in several undesirable health consequences. Common issues faced by women undergoing menopause include hot flashes, bone density loss, cardiovascular changes, atrophy of vaginal tissue, and cognitive decline (Curtis et al., 2005; Hoffman et al., 2012). While human life expectancy is increasing, menopause onset has remained stable and can begin as early as age 40, meaning that women are living increasingly larger proportions of their lives in this hypo-estrogenic state (NAMS). Many women utilize estrogen-containing HT, which can alleviate several symptoms associated with menopause. Conjugated equine estrogens (CEE, tradename Premarin®, Prempro with the synthetic progestin Medroxyprogesterone acetate; MPA) were the most commonly prescribed estrogen component of HT in the US (Hersh et al., 2004). Fourteen million women in the US were estimated to use CEE in 2005, and CEE has been prescribed as HT since 1942 (Stefanick, 2005). CEE contains several estrogens, including many that are not naturally produced by women, trace amounts of E2, and over 50% E1 sulfate (E1S; Gleason et al., 2005), which is desulfated in the liver, converting it to E1. In many women, CEE HT is effective at attenuating or preventing symptoms of

menopause, including hot flashes, vaginal atrophy, and decreased bone density (Curtis et al., 2005); however, whether CEE reduces the cognitive decline associated with menopause remains unclear. The large Women's Health Initiative Memory Study (WHIMS) reported that CEE alone produced no change in risk of developing mild cognitive impairment (MCI) and marginally increased risk of probable dementia; CEE plus progestin treatment produced no change in MCI risk and increased the risk of probable dementia in menopausal women (Espeland et al., 2004; Shumaker et al., 2004), findings which prompted many women to discontinue their HT use altogether (ACOG, 2011).

Using the Ovx rodent as a model of surgical hormone loss, our and other laboratories have shown that E2 HT can benefit performance in multiple cognitive domains, including spatial reference memory, a form of long term memory for information that stays constant (Bimonte and Denenberg 1999; Gibbs, 2000; Bimonte-Nelson et al., 2006; Talboom et al., 2008), and spatial working memory, a form of short term memory for information that is updated (Bimonte and Denenberg, 1999). Our lab has also shown that CEE HT can benefit spatial working and reference memory in rats whose ovaries had been surgically removed via Ovx (Acosta et al., 2009). The Ovx model is an excellent tool to study the estimated 600,000 women per year who have undergone surgically induced menopause (ACOG, 2008; Rocca et al., 2009), and it is the gold standard for isolating the effects of exogenously administered ovarian hormones (Mennenga & Bimonte-Nelson, 2013); however, the Ovx model has limited generalizability to the majority of women who have undergone natural, transitional menopause and retained their follicle-deplete ovaries. Importantly, reproductive

senescence in women differs from reproductive senescence in female rats. The aging rat does not experience menopause; it experiences estropause, which includes several hormonal states indicative of irregular ovulation. Natural menopause can be more closely modeled in the rodent via the industrial chemical 4-vinylcyclohexene diepoxide (VCD). Treatment with VCD accelerates the natural process of atresia in the finite primary and primordial follicle pool, producing a gradual loss of follicles (Flaws et al., 1994; Springer, McAsey, et al., 1996; Springer, Tilly, et al., 1996; Borman, et al., 1999; Kao et al., 1999; Hu et al., 2002; Mayer, et al., 2002, 2004, 2005), leading to ovarian failure, and a drastic decrease in ovarian-derived E2 and progesterone (Hirshfield, 1991; Springer et al., 1996: Mayer, et al., 2004, 2005). Thus, treatment with VCD results in an ovary-intact, follicle-deplete rat with a hormone profile similar to that of a naturally menopausal woman.

Using the VCD and Ovx models, our lab was able to asses how CEE HT would impact cognition with transitional, versus abrupt, hormone loss. We showed that CEE improves performance on a spatial working and reference memory task following surgical menopause, but impairs performance on this task when administered following a VCD-induced transitional hormone loss and follicle-deplete ovaries were retained (Acosta et al., 2010). In surgically menopausal rats, CEE enhanced reference memory and two measures of working memory on the trial with the highest working memory load, similar to our previous findings. In contrast, VCD-treated rats showed the opposite effect, with errors increasing on these measures following CEE treatment. Further, CEE-treated Ovx rats showed better memory retention across an 8-hr delay relative to oil-

treated Ovx rats, while CEE exerted no retention benefit in transitionally menopausal rats.

There is accumulating evidence that a 'window of opportunity' for HT initiation following hormone loss exists. Clinical studies demonstrating a limited window of time during which HT can exert positive effects have given rise to the window of opportunity hypothesis (Resnick & Henderson, 2002; Zandi et al., 2002; MacLennan et al., 2006; Maki, 2006; Maki & Sundermann, 2009; Khoo et al., 2010). For example, recent reports have found that, in naturally menopausal women, HT initiated prior to menopause was beneficial to cognitive performance, while HT initiated post-menopause was detrimental (Greendale et al., 2009), and use of HT initiated during perimenopause has been shown to enhance memory and hippocampal activation, as detected by fMRI, in women (Maki et al., 2011). Several preclinical rodent studies also support the window of opportunity hypothesis for beneficial effects of HT on cognition (Gibbs, 2000; Daniel et al., 2006; Bohacek & Daniel, 2010) and brain health (Bohacek et al., 2008; Bohacek & Daniel, 2009). In middle-aged Ovx rats, E2 given immediately, but not 5 months after Ovx, enhanced spatial working memory on a land radial-arm maze (Daniel et al., 2006) and enhanced performance on the five-choice serial reaction time task (Bohacek & Daniel, 2010). Additionally, E2 given immediately or three months, but not 10 months, after Ovx enhanced delayed-match-to-position performance (Gibbs, 2000).

Thus, clinical and preclinical findings concur that the beneficial effects of estrogen HT may be dependent on early initiation. However, there have been no preclinical rodent studies evaluating this question utilizing a model of transitional menopause. In our previous study demonstrating detrimental effects of CEE in

transitionally menopausal ovary-intact rats (Acosta et al. 2010), CEE treatment initiation took place after follicular depletion had ensued. We then asked whether giving CEE at the onset of follicular depletion would still impair memory. The goals of experiment 5 (chapter 6) were to determine whether the cognitive impact of CEE HT is influenced by the timing of treatment initiation relative to the onset of follicular depletion, or the duration of treatment.

Our lab has collected an abundance of data that lead us to suspect that other estrogens may be capable of producing a more favorable cognitive outcome following follicular depletion. Androstenedione is an androgen that is produced by the ovaries and can be converted to E1 via the aromatase enzyme. In several studies, we have found an association between elevated circulating androstenedione levels and number of errors on the WRAM (Acosta et al., 2009; 2010; Camp et al. 2012). We have also shown that exogenous delivery of E1 to Ovx animals produces memory impairment (Engler-Chiurazzi et al., 2012), providing some insight into the possible hormonal mechanism underlying the negative cognitive effects of CEE following VCD treatment. These data suggest that androstenedione, the primary hormone released by follicle-deplete ovaries, may be impairing memory through its conversion to E1, via the aromatase enzyme. Administration of CEE, primarily composed of E1 sulfate, may exacerbate already impaired cognition by further increasing levels of E1. Mounting evidence suggests that CEE is not optimal for naturally menopausal women with an already imbalanced hormonal background. A HT that restores hormonal balance during and after the transition to menopause, such as E2, may provide cognitive benefits. Experiment 6 (chapter 7) was conducted to determine how E2 administration to follicle-deplete rats

would impact cognition compared to Ovx, which is the only treatment that has thus far been shown to improve memory following VCD-induced follicular depletion in rodents (Acosta et al., 2009b).

Hormonal Mechanisms Underlying the Cognitive Consequences of Natural Hormone Loss

With reproductive senescence, there is a drastic loss of ovarian-derived estrogen and progesterone, and the androgen androstenedione becomes the principal hormone released by the ovaries (Timaras et al., 1995). This androgen-rich hormone milieu is also seen in a rodent model of natural menopause via treatment with VCD. Accumulating evidence in the female rat suggests that androstenedione has a negative impact on cognition. Following a series of studies in which we found an association between higher levels of endogenously-produced androstenedione and poorer memory (Acosta et al., 2009b, 2010), we demonstrated that exogenous androstenedione administration to Ovx animals impaired spatial reference memory, working memory, and memory retention (Camp et al., 2012).

Understanding the effects of androstenedione on the brain and its function is critically important to understanding the cognitive impact of natural menopause; ovarian-derived androstenedione is present in menopausal women who maintain their ovaries, an effect observed for at least ten years after menopause ensues (Fogle et al., 2007). Drugs that block the activity of the aromatase enzyme (Santen et al., 2009), which catalyzes the conversion of androstenedione to E1, are some of the tools used to treat metastatic breast cancer prevalent in menopausal women (Glück et al., 2013), as well as manage estrogen-dependent endometrial carcinoma (Gao et al., 2014). In a subsequent study, we sought to

decipher the hormonal mechanisms underlying the negative cognitive impact of androstenedione using a rat model. Androstenedione could be exerting cognitive effects through a multitude of mechanistic pathways; it is a direct precursor to testosterone via the 17β-hydroxysteroid dehydrogenase (17β-HSD) enzyme, and to E1 via the aromatase enzyme, and it binds to androgen receptors (Horton & Tait, 1966; Jasuja et al., 2005). In the rodent model, testosterone administration has been shown to enhance spatial working memory (Bimonte-Nelson et al., 2003b), spatial reference memory (Benice & Raber, 2009), and performance on avoidance tasks (Flood et al., 1995; Edinger et al., 2004). Moreover, higher relative levels of testosterone are associated with better spatial ability in women, while lower relative levels of salivary testosterone were related to better spatial ability in men (Gouchie & Kimura, 1991). We have previously shown that E1 administration in Ovx rats produces cognitive impairments (Engler-Chiurazzi et al., 2012). Given these results, we hypothesized that androstenedione's conversion to E1, rather than its actions on the androgen receptor, underlies its negative cognitive impact.

In experiment 7 (chapter 8), we systematically evaluate whether androstenedione's conversion to E1, or its effects on the androgen receptor, are responsible for its negative cognitive effects in the surgically menopausal young adult rat. We utilize pharmacological manipulations that either block androstenedione's conversion to E1, or block androstenedione's androgenic effects by blocking activation of the androgen receptor. Anastrozole, a non-steroidal aromatase inhibitor, or flutamide, a non-steroidal anti-androgen, were co-administered with androstenedione to determine whether androstenedione impairs memory via its conversion to E1, or via its action on the androgen receptor, respectively. A secondary purpose of this study was to test the effects

of anastrozole given alone. Aromatase inhibitors such as anastrozole are used to treat and prevent recurrence of breast cancer (Santen et al., 2009). Elucidating the impact of aromatase and estrogen metabolism on the brain and its function is critical to our understanding of the systems-level alterations that occur with changes in both endogenous and exogenous steroid hormones.

Modeling Current Trends in Hormone Therapy

Since the early 2000's, HT prescription trends have shifted; the heavily publicized WHI and WHIMS results showing no cognitive benefits of CEE HT, and potential increased cognitive and health risks, prompted women to ask for alternative, safer HT regimens (ACOG, 2011; Endocrine Society, 2015). In response to this demand, many FDA-approved bioidentical E2-containing HTs are now available in the United States. In addition to these FDA-approved HT formulations, this demand has also opened a market for non-FDA-approved, custom compounded estrogen/estrogen+progestogen formulations. These formulations have gained popularity in the clinic; new estimates state that 28%-68% of HT prescriptions now fall under this category (NAMS, 2015a, 2015b), and these regimens have raised major health concerns amongst physicians, as they are not governmentally regulated, and may contain unsafe levels of various hormones (Endocrine Society, 2015; NAMS, 2015a, 2015b).

Another popular hormonal option for women beginning the transition to menopause is a hormonal contraceptive regimen, to regulate the menses (Curtis et al., 2005; Hoffman et al., 2012). It is still unknown how EE or any of the synthetic progestins utilized in hormonal contraceptives affect cognition in a rodent model of transitional menopause, although many physicians are now recommending these formulations for

prevention of unwanted pregnancies during the transition to menopause (Ikhena & Johnson, 2012). Other work from our lab has shown that treatment with MPA induces long-lasting cognitive impairments (Braden et al., 2010; 2011), and ongoing work in our laboratory suggests that a clinically relevant dose of another available progestin, levonorgestrel (levo), produces a favorable cognitive impact in young Ovx rats.

Findings from this dissertation suggest that EE-containing hormonal contraceptives may be a promising HT candidate for use during the menopause transition. Although low-dose EE negatively impacted memory in young-adult ovary-intact rodents in chapter 5, as well as in young adult women in chapter 3, we did not see an impact of low-dose EE in animals that had undergone Ovx in chapter 4. The results from this dissertation broadly suggest that the cognitive effects of estrogens are dependent on the hormonal profile of the user. EE's lack of conversion to E1 makes it a promising candidate for use by naturally menopausal women, and the necessary synthetic progestin may serve to replace the progestogenic stimulation lost with menopause. FDA-approved hormonal contraceptives may serve several functions, including alleviating non-cognitive symptoms of menopause, masking irregular hormone fluctuations, and preventing unwanted pregnancies during the transition to menopause. Hormonal contraceptives also do not incur the same cancer risks as traditional HTs, and may actually reduce the risk of ovarian and endometrial cancer (Hoffman et al., 2012). For experiment 8 (chapter 9) we tested the cognitive impact of the estrogens E2 and EE, as well as the synthetic progestin levo, and the combinations of each estrogen with levo during follicular depletion, to model clinically prescribed formulations of combined contraceptives and FDA-approved HTs that are currently prescribed (Curtis et al., 2005; Hoffman et al., 2012).

Overarching Aims of This Dissertation

The overarching purpose of my graduate work was to further scientific understanding of how various hormone exposures across the lifespan impact the trajectory of learning and memory throughout aging. I have addressed this goal by systematically modeling current hormone use trends in rodents via menopause induction models (both surgical and transitional) and by manipulating endogenous and exogenous estrogens, progestogens, and androgens. Through this dissertation research, I have discovered that endogenous and exogenous ovarian hormones impact the brain and its functions, work that, I hope, will translate to enriching brain health in women. Moreover, underscoring the translational approach of my work, I helped lead a team which created a human radial arm maze, thereby allowing gains in interdisciplinary understanding of hormonal effects on spatial learning and memory. Throughout the chapters of this dissertation, I aimed to model, as closely as possible, the current hormone use trends prevalent in women's healthcare today. The current aging generation is the first to have had exposure to hormonal contraceptives during young adulthood, and has also experienced several distinctive iterations of HT use trends during and after menopause; each of these major shifts in lifetime hormone exposures have produced an aging generation with a unique hormone history. It is my hope that the work in this dissertation allows insight into the cognitive impact of each of these various hormone exposures across the lifespan, therefore contributing to our understanding of the aging profile of current and future generations.

CHAPTER 2: NAVIGATING TO NEW FRONTIERS IN BEHAVIORAL NEUROSCIENCE: TRADITIONAL NEUROPSYCHOLOGICAL TESTS PREDICT HUMAN PERFORMANCE ON A RODENT-INSPIRED RADIAL ARM MAZE

Introduction

Spatial learning and memory, the ability to encode, store, and retrieve information about route navigation and object locations (Barnes et al., 1997), has been a major focus in the field of neuroscience since Tolman famously asserted that rodents utilize cognitive maps of their environments to navigate mazes (Tolman, 1948). Several decades and many landmark findings later, an abundance of rodent research probing the many facets of spatial navigation and numerous useful tools for measuring spatial learning and memory have been amassed (see Bimonte-Nelson et al., 2010 for review). In rodents, one of the most commonly used and widely recognized tests of spatial memory is the radial-arm maze (RAM) (Jarrard, 1993; Olton & Samuelson, 1976), which consists of a circular arena, from which multiple arms radiate outward. Rewards are typically located at the end of each arm, or a subset of the arms, depending on the specific task protocol, and the maze is surrounded by plentiful extra-maze environmental cues to aid in spatial navigation. The maze relies on positive and/or negative reinforcement to motivate animals to efficiently locate each reward using the fewest arm entries possible.

In the RAM task, rewards are typically not replaced once they have been located within each testing session, resulting in increasing task difficulty (i.e., the number of spatial locations the animal must avoid for successful performance) across trials, within each testing session. In the animal research literature, working memory is considered to be a form of short-term memory and is classically defined as information that is worked with,

kept ‘online’, and updated. In the RAM, working memory demand is elevated with each trial; once a reward is located at the end of an arm, the animal must then remember to avoid that arm on future trials for optimal task performance. This complexity makes the RAM a valuable instrument for evaluating the ability to handle a systematic increase in working memory load. It is well documented in both rats and mice that RAM errors increase within each day as trials progress and working memory demand escalates; however, errors decrease across multiple testing sessions as animals learn the task (Bimonte-Nelson et al., 2003; Bimonte & Denenberg, 1999; Camp et al., 2012; Hyde et al., 1998; Jarrard, 1993; Olton & Samuelson, 1976).

Evidence supports the assertion that, in humans, spatial learning and memory involves multiple complex cognitive processes similar to those measured in rodents. For example, in order to form a cognitive spatial map, humans also acquire knowledge about environmental cues (Shelton & McNamara, 2004; Taylor & Tversky, 1992). Additionally, human neuroimaging studies have discovered cell analogues to rodent place cells in the hippocampus, providing support for brain mechanisms similar to those of rats when mediating navigation through space (Ekstrom et al., 2003), further supporting the idea that humans, like rats, utilize a “cognitive map” of their environment. Many effective tasks have been developed to tap visuospatial ability, episodic memory, and working memory capacity in humans in both experimental and clinical settings. Tasks measuring general intelligence in humans, a domain that has yet to be defined or tested in rodents, have also been widely developed.

Rodent assessments of spatial memory are often also assessments of episodic memory, working memory capacity, as well as visuospatial ability. Rodent models have

been critical to our understanding of spatial learning and memory, the brain regions and mechanisms that confer navigational skills, and potential therapies and pharmacological treatments to improve quality of life in populations suffering from cognitive impairments. Rodent RAM research, specifically, has produced a wealth of translational knowledge by allowing for pharmacological, genetic, and environmental manipulations that are not ethically or logistically possible in human populations. Data collected with the rodent RAM have led researchers toward numerous discoveries and new directions with the potential to enrich and optimize cognitive function in humans; use of this paradigm is essential to decipher the infinitely complex neural mechanisms associated with learning and memory, as well as the influence of aging, disease, environmental changes, and countless other factors. It is generally thought that rodent performance on the RAM depends on visuospatial ability, working memory capacity, and an intact episodic memory, but not general intelligence. These same cognitive domains are readily evaluated in humans; however, it remains unclear whether working definitions of these cognitive domains in rodent and human research are functionally equivalent. The extent to which rodent RAM research is directly translational to human learning and memory persists as a key scientific question.

One approach to this immensely complex and dynamic issue is to create an intermediate testing instrument by adapting experimental paradigms from animals to humans. The aim of the present study was just that—to use a direct and literal translational approach to design a human task that measures the ability to remember and utilize information about spatial locations in a real world, walk-though environment, modeled after rodent RAMs. A complementary team of scientists with expertise in rodent maze

learning, human perception and memory, navigational behavior, and diagnostic clinical neuropsychology was assembled to construct an 11-arm, walk-through human RAM (HRAM), aiming to make the task as similar as possible to the rodent RAM. Performance on the HRAM was compared to performance on a battery of tests tapping cognitive domains that are hypothesized to underlie spatial learning and memory; namely, spatial reasoning ability, episodic memory, working memory, and general intelligence. The HRAM allowed me to translate and compare navigational error patterns, exactly as measured in rodent RAM studies, to performance on a battery of standard neuropsychological and cognitive tests in human participants.

My primary goal was to determine whether the HRAM produces a similar pattern of errors to that seen in rodents both within and across testing sessions. I expected to see HRAM errors change as a function of WM load and testing session. Specifically, I predicted that HRAM performance would decline as working memory demand became elevated within each testing session, but that performance would improve across testing sessions, similar to the pattern of performance seen in rodents. An additional goal of this study was to explore the relationship between HRAM performance and performance on commonly used neuropsychological and cognitive tests. In order to better understand the relationship between some of the most commonly used rodent and human methodology, I aimed to determine how much variance in HRAM performance could be predicted by scores on standard tests of visuospatial ability, working memory, episodic memory, and general intellectual ability. Because RAM performance relies on working memory and knowledge of spatial locations, I hypothesized that participants' scores on tests (defined in Methods section) of working memory capacity (OSpan, RSpan, RotSpan, SymSpan), and

visuospatial ability (MRT, JLAP) would predict performance on the HRAM. I also tested whether performance on a measure of episodic memory (RAVLT) would predict HRAM performance. I also wanted to investigate whether a measure of general intelligence would predict performance on the HRAM. The final goal of this project was to determine whether tasks that measure different domains of cognition in humans would account for unique portions of variance in HRAM scores, that is, whether each class of tests (i.e., working memory capacity tasks, visuospatial ability tasks, episodic memory tasks) contributed distinctly to overall prediction of HRAM performance. I aimed to assess the extent to which the addition of neuropsychological tests to a standard battery of cognitive tests would improve prediction of human ability to navigate and learn in a real-world environment. I predicted that performance on each group of tasks would account for unique variance in our HRAM task. The overarching goal of this study was to help expand knowledge of both human and rodent cognition, to allow broader interpretations of existing data in both species, and to facilitate translational connections between animal laboratory, human laboratory, and human clinical research domains.

Materials and Methods

Participants

A total of 157 participants (54 men and 103 women) were recruited from several psychology courses at Arizona State University. Mean age was 21.29 years (sd=3.75, range=18-47). Mean educational level in years was 14.43 (sd=1.29, 13-18 years range). There were no sex differences in age, education, or self-reported college GPA. Participation in the study was an option for extra credit in those courses. All procedures were approved by an Institutional Review Board for use of human participants in

research. Names were used only to assign course credit; all performance or questionnaire data were de-identified. All participants had normal or corrected to normal vision and no other obvious physical difficulties with the potential to affect their performance in the maze.

Human Radial Arm Maze

We developed and constructed a novel human radial-arm-maze (HRAM) to fit human proportions. A schematic and pictures of the HRAM are shown in figure 1. The maze frame consisted of a circular wooden center platform, 3.0 meters in diameter, 11 vertical pillars equally spaced around the center platform (standing 2.3 meters tall), and a circular ring around the top to stabilize the pillars. To create the walls of each arm, both ends of a solid black tarp were attached to sequential pillars at the edge of the center platform, and then wrapped around a heavy 2-meter tall cylinder forming the ends of each arm. The complete maze had 11 equally spaced arms extending from the center area, each 5 meters long by 1 meter wide, resulting in a total maze width of 13 meters. An 11-arm design was employed to create an asymmetrical arm pattern, thereby decreasing the chances for systematic strategies. This arrangement also allowed us to compare processing capacity of humans and rodents, which has classically been described by Miller as 7 ± 2 items of information in humans (Miller, 1956). More recent work has described working memory capacity limits of 3-5 pieces of information under certain circumstances (see: Cowan, 2010), however human working memory capacity for spatial locations in a radial-arm maze setting, specifically, has been estimated as 7 ± 2 items of information (Glassman et al., 1994; 1998). We built the maze with walls that extended above human height, and required participants to retrieve rewards from all 11 arms to

complete the maze. This provided a fully translational RAM task with actual locomotive motor movements, and full realism, as compared to virtual reality versions that can produce distortions due to computer lag, and lack a fully realistic array of location and depth cues. On the floor at the end of each arm was a 2' x 3' (0.6 meters x 0.9 meters) solid black floor mat, which served to conceal a reward. Each reward was a single bill of fake paper money; denominations varied across rewards. External visual cues on the room walls were present, including two basketball hoops on opposite ends of the room, solid black posters, and a clock.

Instructions were given to introduce the participants to the goals of the task and to prevent participants from simply sequentially proceeding down successive arms or every other arm. This was done to encourage participants to use utilize spatial strategies or to utilize more complex strategies than simple chaining to traverse the maze. Each participant was read the following instructions prior to maze testing:

"Money is under the mat at the end of each arm of this maze. Your goal is to find all of the money in the shortest amount of time. Once you find the money in an arm, it will not be replaced. Therefore, you should avoid going into any arm twice. Do not enter arms that are immediately next to each other or go in a pattern entering every other arm. Only travel into an arm immediately next to the one you previously entered if you absolutely must in order to obtain the remaining money, which means only do it when you are almost certain that you are on your last reward. If you do travel into an arm immediately next to the previous one you will be asked to stop and return to the center. Once you find money, please return it to the researcher located in the center of the maze. Then, wait until they tell you to go, and proceed to the rest of the arms to collect the

remaining money. During the course of testing please do not ask the researcher how many rewards remain or any other questions regarding your performance, as they are not permitted to respond."

Each participant started at the center of the maze; after receiving the instructions, the participant was told to begin collecting the rewards from each arm. For each trial, the researcher recorded the exact arm(s) the participant went down and recorded the time it took the participant to discover each reward. Upon locating a reward, the participant was instructed to return to the center of the maze, hand the reward to the researcher and then continue on to the next trial. This process was repeated until all 11 rewards were located, resulting in 11 total test trials (testing session A).

Following successful collection of all 11 rewards, participants were brought outside the maze and administered the WRAT-3, which served as a general measure of verbal intelligence. During this time (approximately 5 minutes), a second experimenter replaced all 11 rewards in the HRAM. After the WRAT-3, participants were tested on the HRAM a second time (testing session B), adhering to the same set of directions. Participants were scored based upon the number of total incorrect arm entries they made (HRAM Errors). Because all of the arms contain rewards at the beginning of each testing session, errors solely consist of repeat arm entries within each testing session and all errors are considered to be working memory errors. After completion of the HRAM and WRAT-3, participants were taken to a separate room and administered a general survey and the remaining cognitive tests.

Intelligence Measure

Between HRAM testing sessions, the WRAT-3 Reading subtest was administered, serving as a general measure of verbal intelligence. The WRAT-3 relies on the participants' ability to read aloud a list of increasingly less common irregularly spelled words, and is useful as an estimate of verbal intelligence (Lezak et al., 2004).

Episodic Memory

The Rey Auditory Verbal Learning Task (RAVLT) was used to assess episodic memory ability. In this task, participants must listen to and verbally recall words from a 15-item word list (List A) in 5 consecutive recall trials (Trials A1-A5; Total Words Learned). List A is then followed by recall of a distractor list (List B) in a single trial (Trial B1), and an immediate recall of List A (Trial A6; Retroactive Interference), which is often used as a measure of retroactive interference and short-term memory. After 20 minutes, delayed memory/long term memory recall is assessed in a single recall trial (Trial A7; Delayed Recall). Participants were scored on the number of words recalled correctly on each trial. Scores for Trials A1-A5 (Total Words Learned) were the total number of correctly recalled words across all five trials. Finally, participants complete a recognition trial discriminating words from List A from foils. We did not standardize scores by age or sex, but rather acknowledged age and sex as potential demographic variables that may influence scores on multiple tasks. Given that the rodent RAM has been reliably shown to be sensitive to both age and sex, this best facilitated our goal to examine the relationship between variance in our cognitive test scores and variance in our HRAM scores.

Visuospatial Ability

Two paper-and pencil measures of visuospatial ability were used in this study. The first was a version of the Vandenberg and Kuse Mental Rotation Task (MRT) (Vandenberg and Kuse, 1978), redesigned by Peters, et al., 1995. Version A of the MRT was used, which consists of 4 practice and 24 test questions. Each question is composed of five simple three-dimensional images made up of blocks. For each question, the objective is to match the target figure to a rotated version that is presented among a group of distractor items, which are either mirror images of the target figure or a different shape than the target figure. Participants were given 2 minutes to read the instructions and complete the practice items (not scored), 3 minutes to complete the first 12 items, and another 3 minutes to complete the remaining 12 items. Answers are considered correct only if the participant selects both correct images, with no partial credit for only one correct item (Peters et al., 1995). We also used the Judgment of Line Angle and Position-15 (JLAP) (Collaer, 2001) to measure visuospatial ability. The JLAP-15 consists of 20 test items and 5 practice items; each test item consists of two target line segments located directly above the comparison spectrum of 15 numbered lines in a 180° array. The target line segments were each 1 cm in length, whereas the comparison lines were 3 cm in length (Cherney & Collaer, 2005). Participants were given 2 minutes to read the instructions and complete the practice items (not scored), after which they were given 7 minutes to complete as many of the 20 test items as possible. Credit for correct answers is given only when both of the correct target lines are identified, with no partial credit for only one correct line. For the MRT and the JLAP, the score assigned was the total number of items answered correctly.

Working Memory Capacity

Working memory was assessed by a set of four computerized complex-span working memory tasks. These tests require participants to maintain mental memoranda (either verbal or spatial) in the face of completing a distracting task. These tests included verbal (Operation Span; OSpan and Reading Span; Rspan) and spatial (Symmetry Span; SymSpan and Rotation Span; RotSpan) working memory tasks (see Unsworth et al., 2009 for full task descriptions). In complex-span tasks, the participant is given verbal or spatial memoranda interspersed with distracting activity for a set of lists containing between 3 and 7 items. The participant's task is to remember the information in the order it was presented while simultaneously completing the distractor task. In all working memory tasks the dependent variable was the number of correct items recalled in the correct serial position.

Task Administration Overview

The HRAM (testing session A) was the first task participants completed as a measure of spatial working memory. The WRAT-3 was administered between the two HRAM testing sessions as a measure of verbal intelligence. The WRAT-3 was followed by a second HRAM testing session (testing session B), to determine whether participants improved performance across testing sessions. After completion of the second session of HRAM testing, participants completed a survey regarding health and demographic factors. Participants were then administered the RAVLT Trials A1-A6, MRT, JLAP, RAVLT Trial A7, and computer tasks. The testing battery was given in the same order for all participants. Upon completion of all tasks, participants were debriefed. The total time from beginning to completion was approximately 2 hours per participant.

Statistical Analyses

HRAM data were analyzed using repeated measures ANOVA, with HRAM Errors on Trials 1-11 and Sessions A and B as the repeated measures. Relations between performance on the MRT, JLAP-15, RAVLT, WRAT-3, RSpan, OSpan, SymSpan, and RotSpan with HRAM performance were examined with correlations and multiple regression analysis. In order to determine the extent to which each task predicts performance on the HRAM, a real-world, immersive task requiring spatial navigation, learning and memory, individual regressions were run with each task serving as the predictor and total errors made on both sessions of the HRAM combined (HRAM Total Errors) as the dependent (predicted) variable.

Additionally, hierarchical regression analysis was utilized to determine whether tasks measuring different domains of learning and memory offered unique predictive value to a regression equation predicting HRAM scores. Tasks that emerged as significant predictors of HRAM performance were entered into a regression equation in sequence, starting with the tasks that accounted for the largest proportion of variance in HRAM scores. Tasks were entered in clusters according to which cognitive domain they measure. The dependent variable for all equations was total errors made on both sessions of the HRAM combined (HRAM Total Errors). Four measures of working memory capacity, OSpan, RSpan, RotSpan, and SymSpan accounted for the largest proportion of variance in HRAM Total errors, and were entered as a first block of predictors to yield Equation 1:

HRAM Total Errors = b1OSpan+ b2RSpan+ b3RotSpan+ b4SymSpan+ b0

Two measures of visuospatial ability, MRT and JLAP were added to yield Equation 2:

HRAM Total Errors= b1OSpan+ b2RSpan+ b3RotSpan+ b4SymSpan+ b5MRT+ b6 JLAP+ b0

Gain in prediction from Equation 1 to Equation 2 assessed prediction from visuospatial ability measures over and above working memory capacity. Analyses were performed using SPSS 21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

Results

Human Radial Arm Maze Performance

There was a main effect of Trial [$F_{(10,1520)}$= 97.19; p< 0.0001] on HRAM Errors, with HRAM Errors increasing as trials progressed and working memory load increased (figure 2A). HRAM Errors increased from trial 8 to 9 (Trial 8: M= 0.28, SE= 0.04; Trial 9: M= 0.53, SE= 0.06; p< 0.05), from trial 9 to trial 10 (Trial 10: M=1.03; p<0.0001) and again from trial 10 to trial 11 (Trial 11: M=2.67, SE=0.11; p<0.0001). This increase in errors occurred when the number of arms participants needed to avoid exceeded roughly 8-9 items. HRAM Errors declined significantly across Testing Sessions (Both Sessions: M=0.436, SE=0.04, Session 1: M=0.51, SE=0.04, Session 2: M= 0.37, SE= 0.04; [$F_{(1,152)}$= 7.85; p< 0.01]). The pattern of performance across trials was the same across Testing Session, (Session x Trial interaction: $F_{(10,1520)}$= 1.80; p>0.05, NS]. Figure 2B shows error patterns observed in different versions of the rodent RAM for comparison.

Relationships Between General Intelligence and HRAM Performance

WRAT-3 scores did not correlate with HRAM Total Errors. Consistent with the lack of correlation, the WRAT-3 was not a significant predictor of HRAM Total Errors (figure 3).

Relationships Between Episodic Memory and HRAM Performance

RAVLT Total Words Learned, Retroactive Interference, and Delayed Recall did not correlate with HRAM Total Errors ($p>0.05$, NS). Regression analysis indicated that Total Words Learned, Retroactive Interference, and Delayed Recall trials of the RAVLT were not significant predictors of HRAM Total Errors (figure 4). Combining all measures of the RAVLT also did not predict HRAM Total Errors (Adjusted $R^2_{multiple}=0.00$, $F_{(3, 151)}= 0.88$, $p>0.05$, NS).

Relationships Between Visuospatial Tasks and HRAM Performance

Both visuospatial tasks, the MRT and JLAP, correlated negatively with HRAM Total Errors ($p<0.01$ and $p<0.0001$, respectively). For every additional question participants answered correctly on the MRT, HRAM errors decreased by 0.40 on average; errors decreased by 0.66 for each one point increase in JLAP (figure 5). The MRT and JLAP together predicted HRAM Total Errors (Adjusted $R^2_{multiple}=0.11$, $F_{(2, 152)}= 10.29$, $p<0.0001$).

Relationships Between Working Memory Capacity Tasks and HRAM Performance

Performance on the working memory capacity tasks, the OSpan, Rspan, RotSpan, and SymSpan, correlated negatively with HRAM Total Errors ($p<0.001$, $p<0.0001$, $p<0.05$, $p<0.05$, respectively). For every additional point earned on the Ospan or Rspan, HRAM Total Errors decreased by 0.17, on average; HRAM Total Errors decreased by

0.19 for each one-point increase in RotSpan or SymSpan scores (figure 6). The Ospan, Rspan, RotSpan, and SymSpan together predicted HRAM Total Errors (Adjusted $R^2_{multiple}$=0.09, $F_{(4,146)}$= 4.80, p<0.001).

Unique Predictive Value of Tasks Measuring Different Domains of Cognition

The baseline regression equation (Equation 1), including working memory capacity predictor variables, accounted for a significant proportion of variance in HRAM Total Errors (Adjusted $R^2_{multiple}$= 0.09, $F_{(4,146)}$=4.80, p<0.001). Only the Rspan predicted HRAM Total Errors when all other WM Span test scores were held constant (β=-0.12, 95% CI: [-0.24, 0.00], t=-1.99, p<0.05); none of the other WM Span tasks offered unique predictive value in a regression equation including all four tasks (Ospan: β=-0.10, 95% CI: [-0.22, 0.02], t=-1.59, p>0.05; RotSpan β=0.01, 95% CI: [-0.20, 0.23], t=0.13, p>0.05; SymSpan: β=0.01, 95% CI: [-0.20, 0.22], t=0.09, p>0.05).

The addition of two visuospatial tasks, MRT and JLAP, as predictor variables (MRT: b=-0.28; 95% CI [-0.55, -0.02]; t=-2.14; p<0.05; JLAP: b=-0.45; 95% CI [-0.78, -0.13]; t=-2.75; p<0.01) significantly increased the proportion of variance in HRAM Total Errors that was accounted for by our regression equation [Adjusted $R^2_{multiple}$= 0.18; $F_{change\ (2,144)}$=8.58; p<0.0001]. The Adjusted $R^2_{multiple}$ for Equation 2 indicated that adding MRT and JLAP as predictors roughly doubled the proportion of explained variance in HRAM Total Errors. JLAP scores offered predictive value over and above MRT scores (β=-0.55, 95% CI: [-0.88, -0.21], t=-3.22, p<0.01); however MRT scores were not predictive of HRAM Total Errors when JLAP scores were held constant (β=-0.23, 95% CI: [-0.50, 0.03], t=-1.73, p>0.05).

Discussion

The current study employed a human-sized, walk-through version of the RAM that was modeled after the rodent version used commonly in learning and memory research. The RAM has been used for decades to study spatial memory in the rodent. Notable landmark work includes that of Tolman in the 1940s utilizing the structurally-similar sunburst maze (Tolman, 1948), Olton utilizing the RAM in the 1970s (Olton & Samuelson, 1976; Olton, 1977; Olton & Feustle, 1981; Olton & Papas, 1979), and more recent work many decades later (e.g., Eckerman at al., 1980; Luine & Rodriguez, 1994; Bimonte & Denenberg, 1999, 2000; Bimonte et al., 2000; Bimonte-Nelson et al., 2003; Bimonte-Nelson et al., 2006; Daniel et al., 2006; Eckerman et al., 2008). Despite the many advantages of using animal models in research, there remain questions about the extent that findings in animals can truly be translated to humans, especially in the context of neurobehavioral assays. One approach to addressing this obviously complex issue is to create an intermediate testing instrument by adapting experimental paradigms from animals to humans. The present study did this through the development of the HRAM. Previous research teams have developed human versions of mazes, in particular the RAM, with their own unique set of parameters designed to answer their research questions (Astur et al., 2004; Bohbot et al., 2002; Glassman et al., 1998; Levy et al., 2005; O'Connor & Glassman, 1993; Scharine & McBeath, 2002). Our version of the HRAM was built with these prior studies in mind, and optimized the parameters to be as comparable as possible to the rodent version. I expected to see an increase in working memory errors as trials progressed. As predicted, participants began to make errors around trial 6, with the highest number of errors made on trial 11, when working memory demand was at its highest (figure 2A). The

increase in errors across trials in the HRAM is similar to that shown in the RAM with rat subjects (Bimonte & Denenberg, 1999, 2000; Bimonte et al., 2000; Bimonte-Nelson et al., 2003; Camp et al., 2012), as seen in figure 2B. Additionally, performance improved across testing sessions, indicating a learning effect, as seen in rodent RAMs (Bimonte & Denenberg, 1999, 2000; Bimonte et al., 2000; Bimonte-Nelson et al., 2003; Camp et al., 2012).

One major goal of this study was to explore the translational relationship between human performance on the HRAM and commonly used neuropsychological tests that tap spatial ability, episodic memory, working memory, and intelligence. Evaluating these relationships allowed us to determine which tests commonly used in clinical settings and cognitive psychology account for variance in performance on the HRAM, a commonly used rodent task adapted to humans. Of the battery of tests we administered in this study, the JLAP emerged as the strongest predictor of HRAM performance, accounting for 10% of HRAM Total Errors. The verbal working memory capacity tasks, the Ospan and Rspan, surfaced as the next strongest predictors, predicting 8% and 9% of the total variance in HRAM error scores, respectively. The MRT predicted 5% of variance on HRAM Total Errors, and the predictive value of the spatial working memory tasks (the RotSpan and SymSpan) was similar to the predictive value of the MRT, each predicting 4% of the total variance in HRAM error scores (Table 3). Total Words Learned, Retroactive Interference, and Delayed Recall measures of the RAVLT did not offer significant predictive value, nor did scores on the WRAT-3, an estimate of general intelligence.

When evaluating the nature of these tasks, plausible explanations for the observed relationships emerge. The predictive ability of the MRT and JLAP-15 may be attributable

to the proposed use of a mental visuospatial sketchpad (Baddeley, 2000). The visuospatial sketchpad has been theorized to be the temporary storage and manipulation of spatial and visual information, such as shapes, locations or speed of objects in space. The visuospatial sketchpad is theorized to contribute to performance in tasks that require planning of spatial movements, such as planning one's way through a complex environment like the HRAM and it is not surprising that better performance on visuospatial tasks predicts enhanced performance in a three-dimensional, immersive task that requires navigation through space. The working memory tasks used in this study assess the ability to 'hold on' to multiple pieces of information in the face of interference and an increase in working memory demand (e.g., Baddeley, 2003; Unsworth et al, 2009). Similarly, to perform well on the HRAM, participants must also maintain multiple pieces of information in the face of an increasing working memory load to successfully complete the task.

Performance on the HRAM did not correlate with the estimate of general verbal intelligence used in this study (reading subtest of the WRAT-3) or with new learning and long term delay measures of episodic memory, but did correlate with specific measures of visuospatial ability and working memory capacity, suggesting that the HRAM requires utilization of specific cognitive abilities of working memory and visuospatial skills rather than reliance on episodic memory or general verbal intelligence, as measured by the WRAT-3. Thus, our findings indicate that tasks measuring working memory (e.g., maintaining performance within the context of increased load or distracting stimuli) and visuospatial skills are correlated with performance on the RAM, a task used widely in rodent literature that we have fully adapted to human proportions.

Hierarchical regression analysis indicated that the proportion of HRAM error variance accounted for by each group of predictor variables (working memory capacity and visuospatial ability) was unique to that group of variables. Scores on the MRT and JLAP accounted for 9% of variance in HRAM scores, in addition to the 9% of variance accounted for by the four working memory capacity variables (Table 4). A regression equation including the working memory capacity tests and visuospatial ability tests, accounted for 18% of the total variance in HRAM error scores. These results suggest that including multiple measures in a cognitive battery increases the ability of the battery to predict how a participant would perform on tasks similar to the HRAM, which requires complex reasoning, such as recall of previous instances of navigating to spatial locations in a real-world setting. Additionally, these results support the hypothesis that successful performance on radial-arm maze tasks requires visuospatial abilities and sufficient working memory capacity.

In conclusion, our collaborative research group created a three-dimensional, fully immersive, walk-through version of the RAM designed specifically for human use, in order to create an intermediary translational instrument. The results indicate that human performance on the RAM is notably similar to rodent performance on the RAM, in that there is an exponential increase in errors as trials progress and task difficulty increases, but with the human error pattern revealing a larger processing capacity compared to rodents. The total number of errors per trial in humans remains low until the trial number exceeds a total similar to the classically defined human working memory capacity of 7 ±2 items (Miller 1956). Additionally, HRAM performance in our participants improved with repeated exposure to the task, indicating learning. We also demonstrated that performance

on the HRAM was related to performance on several tasks used in clinical neuropsychology and cognitive psychology, with a strong emphasis on tasks designed to measure spatial ability and working memory. The behavioral similarities seen in the rodent and human versions of the RAM, paired with the strong observed relationships between the HRAM and standard human working memory and visuospatial tasks, offer support to spatial working memory being the dominant construct common to rodents and humans that is reliably measured using existing testing procedures. Moreover, the HRAM has now been validated as a valuable instrument to translate, compare, and confirm models and findings in rodent research, cognitive neuroscience, navigational modeling, and neuropsychology. We took a collaborative and translational approach to bridge gaps between divergent, but closely related, fields of experimental and applied memory research. The successful implementation of the HRAM confirms our overarching goal to create a practical and useful basic– to applied- translational test instrument that can help us connect diverse behavioral domains to better understand learning, memory, and cognitive functioning processes.

CHAPTER 3: EFFECTS OF SEX, MENSTRUAL PHASE, AND HORMONAL CONTRACEPTIVES ON THE HUMAN RADIAL ARM MAZE AND A BATTERY OF NEUROPSYCHOLOGICAL AND COGNITIVE TASKS

Introduction

We previously performed a collaborative experiment in human participants with the goals to unite human and rodent cognitive research methodology and to validate a human analogue of a rodent testing paradigm as a viable measure of spatial working memory (Mennenga et al., 2014). Following substantiation of this task as a measure of multiple cognitive constructs, including both working memory capacity and visuospatial ability, I now wish to utilize the human radial arm maze (HRAM) to begin to directly translate preclinical rodent spatial learning and memory research to human participants, and vice versa. Numerous studies have reported sex differences in mental rotation and spatial perception abilities in humans, favoring men (Linn and Petersen, 1985), yet controversy remains concerning the extent that men outperform women in functional real-world spatial navigation tasks (Taylor and Tversky, 1996). My proficiency in translational cognitive endocrinology (Mennenga and Bimonte-Nelson, 2013), coupled with the newly validated HRAM as a human analogue to the rodent radial arm maze (Mennenga et al., 2014), now allow me to systematically address this question.

In the human literature, men typically excel on tasks that measure spatial ability, such as mental rotation of three-dimensional figures, spatial visualization, spatial perception, and targeting and intercepting objects (Linn and Petersen, 1985; Rahman and Wilson, 2003; Voyer et al., 1995), while women tend to perform better on tasks that measure non-spatial memory, such as episodic memory (Ruff et al., 1989; Trahan and

Quintana, 1990). There is also evidence in women that changes in estrogen levels across the menstrual cycle influence working memory (Hampson and Morley, 2013) and spatial ability (Mäntylä, 2013). In the rodent literature, males generally learn the land version of the RAM at a faster rate than females (Einon, 1980; Luine and Rodriguez, 1994; Roof, 1993; Williams et al., 1990), although sex differences are not always reported (Juraska et al., 1984; Kolb and Cioe, 1996; van Haaren, 1987), and females have been shown to encode more types of spatial information during learning than males (Williams et al., 1990).

Many endogenous ovarian hormone fluctuations as well as exogenous hormone exposures occur throughout the female lifespan. Relative levels of several hormones naturally change across the reproductive cycle, throughout aging, and with reproductive senescence. In addition to these natural changes across the lifespan, many women purposefully alter their ovarian hormone levels via regimens such as hormonal contraceptives and hormone therapy (HT). Explicating the cognitive effects of both endogenously produced and exogenously administered estrogens is necessary to optimize brain aging in women. Basic science and clinical research have progressed our understanding of how factors related to aging, menopause, and hormonal treatments impact cognitive function throughout aging, but a direct test of preclinical rodent research findings in human participants is lacking.

In the current study, I divided our participants according to hormonal status to determine whether sex, menstrual phase, or hormonal contraceptive use were associated with differences in performance on several tasks measuring different domains of cognitive function. The primary goal of this study was to determine whether differences

in visuospatial ability, working memory, and real-world spatial navigation would emerge between males and various groups of females with qualitatively different hormone profiles. I hypothesized that as working memory demand increases, a difference in spatial navigational ability will become apparent in the HRAM, such that males will outperform females experiencing high estrogen levels. This difference should manifest as an interaction between working memory load (trial) and errors on the HRAM, whereby all participants perform equally on the initial trials, but men outperform women with higher levels of estrogen on the final trials, when the fewest rewards remain and working memory load is highest. Further, I expected this difference in HRAM performance to correspond to differences in visuospatial task performance, rather than working memory capacity. I divided participants into four categories: men, women in the follicular phase of their menstrual cycle, which is characterized by relatively high levels of endogenous estrogens, women in the luteal phase of their menstrual cycle, which is characterized by relatively high levels of progesterone, and women actively taking hormonal contraceptives that include the synthetic estrogen, ethinyl estradiol (EE). These groups were chosen to broadly represent different hormone profiles that many women experience across their reproductive lifespan.

Materials and Methods

Participants

Participants were identical to those described in chapter 2 (experiment 1), with the exception of 26 participants that were excluded from analyses for one of more of the following reasons: no response on survey questions about current menstrual cycle phase or phase of hormonal contraceptive, potential pregnancy, contraceptive users in an

inactive pill phase, and use of non-EE containing hormonal contraceptives (progestin-only formulations).

Participants were grouped according to their sex and hormone status, and by whether they were utilizing EE-containing hormonal contraceptives. Women who were not taking hormonal contraceptives were divided according to the phase of their menstrual cycle at their time of testing, based on self-report of how many days had passed since their previous menses. Women that were on days 7-13 of their menstrual cycle were considered to be in the follicular phase and placed into the follicular group, and women who were on days 18-35 of the menstrual cycle were considered to be in the luteal phase and placed in the luteal group. These designations were chosen because women in the follicular phase of the menstrual cycle have high circulating levels of E2, whereas women in the luteal phase have high circulating levels of progesterone (Hoffman et al., 2012). We also collected information on the formulations of the various contraceptives that our participants were using; the oral combined contraceptives that our participants were taking contained between 20 and 35μg per day of EE as well as one of the following synthetic progestins: Desogestrel (0.15μg/day), Drosperinone (3.0μg/day), Ethynodiol Diacetate (1.0μg/day), Norethindrone (0.4μg/day), Norgestimate (0.18 or 0.25μg/day), Levonorgestrel (0.1μg/day), or Norethindrone Acetate (1.0 or 1.5μg/day). Groups were as follows: males (n=54), females tested while using combined oral contraceptives (n=23), females not on contraceptives tested during the follicular phase of their menstrual cycle (n=15), and females not on contraceptives tested during the luteal phase of the menstrual cycle (n=22).

Experimental Procedures

Experimental procedures were identical to those described in chapter 2 (experiment 1).

Statistical Analyses

Orthogonal planned comparisons were as follows: Male versus Females Follicular, Male versus Female Luteal, and Male versus Female Oral Contraceptives. Errors made on the HRAM were analyzed using repeated measures ANOVA, with Group (Males, Females Oral Contraceptives, Females Follicular, or Females Luteal) as the between variable, and Runs A and B, each containing Trials 1-11 as the repeated measure. Data were further broken up into low working memory load conditions (trials 2-6) and high working memory load conditions (trials 7-11) and analyzed using one-way ANOVAs with Group as the independent variable and average errors made under each working memory load condition as the dependent variable. Data from the WRAT-3, RAVLT, MRT, JLAP, OSpan, RSpan, RotSpan, and SymSpan were analyzed with one-way ANOVA with Group as the independent variable and total items correct as the dependent variable for each task.

Results

Human Radial Arm Maze Performance

There was a Trial x Hormone Group interaction ($F_{(30,1100)}= 1.47$, $p<0.05$) for Total Errors on the HRAM, such that as working memory load (trial) increased, group differences began to emerge (figure 7). When trials were grouped into low working memory demand and high working memory demand, there were no differences between the Male and Female Follicular groups ($F_{(1,67)}= 0.12$, $p>0.05$, NS; $\eta^2<0.01$), Male and

Female Luteal groups ($F_{(1,74)}$= 0.44, p>0.05, NS; η^2<0.01), or Male and Female Oral Contraceptive groups ($F_{(1,75)}$= 1.74, p>0.05, NS; η^2=0.02) for trials 2 through 6, which required participants to remember 1-5 previously visited spatial locations. There was a difference on Total Errors between the Male and Female Follicular groups ($F_{(1,67)}$= 4.38, p<0.05; η^2=0.06), and between the Male and Female Oral Contraceptive groups ($F_{(1,75)}$= 6.17, p<0.05; η^2=0.08), but not between the Male and Female Luteal groups ($F_{(1,74)}$= 0.14, p>0.05, NS; η^2<0.01), for trials 7 through 11, which corresponded to a demand of 6-10 previously visited spatial locations (figure 7).

Reading Proficiency

We found a difference in WRAT-3 scores between the Male and Female Follicular ($F_{(1,60)}$= 6.95, p<0.05; η^2=0.10), but not between the Male and Female Luteal ($F_{(1,67)}$= 1.54, p>0.05, NS; η^2=0.02) groups, or the Male and Female Oral Contraceptive groups ($F_{(1,71)}$= 2.26, p>0.05, NS; η^2=0.03). Mean scores on the WRAT-3 for each group were as follows: men (M=109.6; SD=7.2), contraceptive users (M=106.7; SD=9.0), follicular phase (M=103.3; SD=6.0), and luteal phase (M=103.7; 13.5). Although this difference in scores is statistically significant, it is not considered to be a clinically relevant difference, as these scores are all well within the range of normal reading ability.

Episodic Memory

There were no differences between Male and Female Follicular ($F_{(1,67)}$= 1.06, p>0.05, NS; η^2=0.02), Male and Female Luteal ($F_{(1,74)}$= 0.71, p>0.05, NS; η^2=0.01), or Male and Female Oral Contraceptive ($F_{(1,75)}$= 1.82, p>0.05, NS; η^2=0.02) on Total Words Learned (trials A1-A5). There were no differences between Male and Female Follicular ($F_{(1,66)}$= 1.08, p>0.05, NS; η^2=0.02), Male and Female Luteal ($F_{(1,73)}$= 0.38, p>0.05, NS;

η^2=0.01), or Male and Female Oral Contraceptive ($F_{(1,74)}$= 0.99, p>0.05, NS; η^2=0.01) on number of words recalled on the retroactive interference trial (trial A6). There were no differences between Male and Female Follicular ($F_{(1,67)}$= 2.29, p>0.05, NS; η^2=0.03), Male and Female Luteal ($F_{(1,74)}$= 0.85, p>0.05, NS; η^2=0.01), or Male and Female Oral Contraceptive ($F_{(1,75)}$= 2.05, p>0.05, NS; η^2=0.03) on number of words recalled following a 20-minute delay (trial A7; figure 9).

Visuospatial Tasks

There were differences in MRT scores between the Male and Female Follicular groups ($F_{(1,67)}$= 11.57, p<0.01; η^2=0.15), the Male and Female Luteal groups ($F_{(1,74)}$= 6.09, p<0.05; η^2=0.08), and the Male and Female Oral Contraceptive groups ($F_{(1,75)}$= 8.91, p<0.01; η^2=0.11). There was also a marginal difference in JLAP scores between the Male and Female Follicular groups ($F_{(1,67)}$= 3.54, p<0.10; η^2=0.05), and a significant difference between the Male and Female Oral Contraceptive groups ($F_{(1,75)}$= 5.73, p<0.05; η^2=0.07), but not between the Male and Female Luteal groups ($F_{(1,74)}$= 1.67, p>0.05, NS; η^2=0.02) (figure 10).

Working Memory Capacity Tasks

There were no differences between groups on the OSpan (Male versus Female Follicular: $F_{(1,65)}$= 0.28, p>0.05, NS; η^2<0.01; Male versus Female Luteal: $F_{(1,72)}$= 0.02, p>0.05, NS; η^2<0.01; Male versus Female Oral Contraceptive: $F_{(1,73)}$= 0.65, p>0.05, NS; η^2<0.01), RSpan (Male versus Female Follicular: $F_{(1,65)}$= 0.10, p>0.05, NS; η^2<0.01; Male versus Female Luteal: $F_{(1,72)}$= 2.31, p>0.05, NS; η^2=0.03; Male versus Female Oral Contraceptive: $F_{(1,73)}$= 0.06, p>0.05, NS; η^2<0.01), RotSpan (Male versus Female Follicular: $F_{(1,65)}$= 0.20, p>0.05, NS; η^2<0.01; Male versus Female Luteal: $F_{(1,72)}$= 0.57,

$p>0.05$, NS; $\eta^2<0.01$; Male versus Female Oral Contraceptive: $F_{(1,73)}= 0.43$, $p>0.05$, NS; $\eta^2<0.01$), or SymSpan (Male versus Female Follicular: $F_{(1,65)}= 0.03$, $p>0.05$, NS; $\eta^2<0.01$; Male versus Female Luteal: $F_{(1,72)}= 0.57$, $p>0.05$, NS; $\eta^2<0.01$; Male versus Female Oral Contraceptive: $F_{(1,73)}= 0.87$, $p>0.05$, NS; $\eta^2=0.01$) tasks (figure 11).

Discussion

A large sex difference in mental rotation and spatial perception abilities, favoring men, is typically found in humans (Linn and Petersen, 1985), yet there is controversy concerning the extent that men perform better in functional real-world spatial navigational tasks (Taylor and Tversky, 1996). Here, I evaluated the impact of hormone status on a battery of tasks measuring visuospatial ability, working memory, and episodic memory. I classified our female participants into three groups according to their hormone status: women in the estrogen-dominant follicular phase of the menstrual cycle, women in the progesterone-dominant luteal phase of the menstrual cycle, and women taking EE-containing hormonal contraceptives. I chose to evaluate performance separately in each of these groups in order to appraise how fluctuations in endogenously produced and exogenously administered estrogens impact several individual cognitive domains, as well as a real-world spatial navigational working memory task.

Utilizing the newly validated HRAM as a measure of spatial working memory (Mennenga et al., 2014), I found that sex differences were limited to comparisons between men and women experiencing high levels of endogenous or exogenous estrogens. Here, males outperformed women in the estrogen-dominant follicular phase of the menstrual cycle, and women on EE-containing hormonal contraceptives, but not women in the progesterone-dominant luteal phase of the menstrual cycle. Sex differences

have not been seen on other two-dimensional versions of HRAM tasks, including virtual reality versions (Astur et al., 2004; Levy et al., 2005), paper-pencil versions (O'Connor and Glassman, 1993), and a large outdoor version with arms painted on the grass (Glassman et al., 1998). The current results may be due to utilization of hormone status, rather than sex, as a variable of interest, or to the specific set of parameters utilized here that differed from prior versions of the maze. It appears that the implementation of an 11-arm paradigm was sufficient to tax the working memory capabilities of our participants (Mennenga et al., 2014). Because there was an interaction between Hormone Status and Trial, it is probable that sex differences would not be detectable in tasks that do not sufficiently tax the working memory system. It is also possible that the three-dimensional real-world nature of our maze is key to measuring spatial memory in humans.

I also investigated differences in visuospatial ability between groups, and found that men outperformed women on the MRT, a result in line with an abundance of prior research using this task (Astur et al., 2004; Cherney and Collaer, 2005; Linn and Petersen, 1985; Vandenberg and Kuse, 1978). Previous reports that have not accounted for female hormone status have reported sex differences on the JLAP, also favoring men (Cherney and Collaer, 2005). I now report that when women are characterized according to their hormone profile, sex differences on the JLAP are limited to women in the estrogen-dominant follicular phase of the menstrual cycle and women actively taking hormonal contraceptives. No difference was observed in JLAP performance between women in the progesterone-dominant luteal phase of the menstrual cycle and men, similar to our observations on HRAM performance. Following a meta-analysis, Linn and Petersen (1985) concluded that large sex differences in visuospatial ability were limited

to mental rotation tasks, and that other spatial perception tasks produced smaller male benefits. The findings support and expand on this notion; while males outperform females in the follicular and luteal phase of the menstrual cycle, as well as women taking EE-containing contraceptives on the MRT, the female disadvantage previously reported on the JLAP task only becomes apparent in women experiencing high levels of either endogenous or exogenous estrogens. Campbell and Collaer (2009) reported that sex differences on the JLAP are sensitive to the stereotype threat that men typically outperform women on this task. Participants in the current study were not told anything about expected performance on any of the tasks, and it is unknown how fluctuations in hormone levels might interact with environmental factors such as stereotype threat.

There were no differences in performance across groups on the OSpan, RSpan, RotSpan, or SymSpan, which are each measures of human working memory capacity. Thus, current findings signify that hormone status impacts visuospatial ability and HRAM performance, but not working memory capacity. In chapter 2, I reported that the working memory tasks (OSpan, RSpan, RotSpan, and SymSpan) and the visuospatial tasks (MRT and JLAP) utilized here each predict unique variance in performance on the HRAM. I now report that it is likely that successful navigation of the HRAM relies on working memory capacity as well as visuospatial ability, and that fluctuations in estrogen levels impact performance on the HRAM through disrupting visuospatial aptitude.

The HRAM task was created in order to allow direct translation between basic science and clinical research findings. Here, the HRAM, along with a battery of standard neuropsychology and cognitive psychology tasks tapping several domains of cognitive function, were utilized to probe the cognitive effects of fluctuations in female hormone

levels. The three groups of females utilized in this study (follicular phase, luteal phase, and hormonal contraceptive users) were chosen to broadly represent the various hormonal states that women experience across their reproductive lifespan. The current classifications were utilized only as a starting point; much heterogeneity remains within each of the three groups of women studied here. Variations in endogenous hormone production and in hormonal contraceptive formulations, including route of administration, dose of EE, and dose and type of progestin, are likely to produce unique cognitive effects. Additional hormonal states such as those seen during pregnancy, or following surgically induced or natural menopause are not represented in the current study, and are also likely to coincide with unique cognitive profiles. Further, a within-subjects study of the cognitive impact of hormonal fluctuations is necessary to determine whether the group differences observed here are reflective of permanent or transient changes in cognition. I hope that these findings will spur additional research into the contributions of ovarian hormones to individual cognitive profiles, and help to inform clinicians and researchers on the impact that various lifetime hormone exposures have on brain health and function across the entire lifespan.

CHAPTER 4: UNDERSTANDING THE COGNITIVE IMPACT OF THE CONTRACEPTIVE ESTROGEN ETHINYL ESTRADIOL: TONIC AND CYCLIC ADMINISTRATION IMPAIRS MEMORY, AND PERFORMANCE CORRELATES WITH BASAL FOREBRAIN CHOLINERGIC SYSTEM INTEGRITY

Introduction

Ethinyl estradiol (EE), a synthetic form of 17β-estradiol (E2), is the most common estrogen in hormonal contraceptives (Shively, 1998), and is the only estrogen used in the contraceptive pill. National surveys estimate that, from 2006-2010, 10.6 million women between 2006-2010 (Jones et al., 2012), and 17.3% of all women between 2006-2008 (Mosher and Jones, 2010), used oral contraceptives. Over 30 contraceptive formulations contain EE (Curtis et al., 2005), including both oral regimens and non-oral, tonic delivery regimens, such as the transdermal patch and vaginal ring. EE is also found in hormone therapies (HT) for menopausal women, such as Estinyl™ and Femhrt™ (Curtis et al., 2005). Understanding the cognitive impact of estrogens is critical, as exogenous exposure to estrogens occurs throughout the lifespan through contraceptives and HT. Of note, EE is a synthetic analogue to E2; however, these estrogens have different pharmacokinetic profiles (Coelingh Bennink et al., 2004). EE is more biologically active than E2 (Dickson and Eisenfeld, 1981) and cannot be converted to estrone or other weaker estrogens (Fotherby, 1996), whereas E2 can (Prokai-Tatrai, et al., 2005). These estrogens also have different binding profiles, which vary across species (Paradiso et al., 2001).

Although EE is among the most commonly prescribed estrogens for contraception, and is prescribed to women from puberty to post-menopause, most preclinical research on the cognitive impact of estrogens has focused on 17β-estradiol and other endogenous

estrogens, and does not include EE (for reviews see: Bimonte-Nelson et al., 2010; Acosta et al., 2013). Methodically evaluating EE in a rodent model allows the opportunity to systematically control for many variables that could impact cognitive scores, including mode of administration, dosing, endogenous hormone variations, age, and diet.

There have been a few studies investigating the cognitive effects of EE as a contraceptive or HT, with effects that vary across memory domains. In human contraceptive users, no impact of EE-containing contraceptives was found on several tests measuring memory and concentration (Silber et al., 1987). Another study found enhanced verbal memory during the active compared to the inactive phase of oral contraceptives, although benefits were not seen on visuospatial measures (Mordecai et al., 2008). Importantly, although each of the contraceptive formulations used in these studies contained EE, other aspects of the formulations differed, including dose and the progestin component. Thus, it is difficult to decipher whether or to what extent EE was responsible for these effects. In studies investigating EE as a HT, cognitive effects depend on the domain as well. In aged, ovariectomized (Ovx) rhesus monkeys, EE improved spatial working memory (Lacreuse et al., 2002), but impaired face recognition (Lacreuse and Herndon, 2003), and had no impact on executive function (Lacreuse et al., 2004). An fMRI study of menopausal women found EE-containing HTs increased frontal cortex activation during a working memory task (Smith, et al., 2006).

Estrogen Receptors (ERs) are highly expressed in several cognitive brain regions, including the basal forebrain (BF) (Shughrue et al., 1999), which contains cholinergic cell bodies that project to the hippocampus (McEwen, 2001; Gibbs, 2010). These projections are known to be intimately involved in spatial learning and memory (Luine et al., 1986)

and are required for E2 to benefit rodent performance on a spatial delay-match-to-position (Gibbs, 2002, 2007). E2 and EE may differentially affect the basal forebrain cholinergic system due to differing receptor binding abilities in the hippocampus (Paradiso et al., 2001); however, no studies have evaluated the impact of EE on these projections, or how this impact relates to spatial learning and memory.

The current study was performed to test the cognitive and neurobiological effects of cyclically administered EE, given via a daily injection, to model oral contraceptive use. In order to encompass the entire range of clinically used doses, an additional medium dose of EE was assessed, equivalent to the highest dose of EE currently available in contraceptives, (Curtis et al., 2005). Following behavioral evaluations, cholinergic cells in the BF were quantified using unbiased stereology and relations between cell populations and performance on cognitive tasks were evaluated. The current studies aim to isolate the cognitive and neurobiological effects of several clinically relevant administration regimens of EE, using the Ovx rodent model.

Materials and Methods

Subjects

Subjects were 36 female Fischer-344 rats raised at Harlan Laboratories (Indianapolis, IN). Animals were three months old at the beginning of the study, four months old at maze testing initiation, and five months old at euthanasia. After arrival, animals were pair-housed, had food and water ad-lib, and were maintained on a 12-h light/dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards.

Experimental Design and Hormone Treatments

At three months old, all animals received Ovx surgery. Rats were anesthetized via isoflurane inhalation, received bilateral dorsolateral incisions in the skin and peritoneum, and ovaries and tips of the uterine horn were ligatured and removed. Muscle and skin were then sutured closed. During surgery, rats received an injection of Rimadyl™ (5mg/ml/kg) for pain, and saline (2ml) to prevent dehydration.

Eighteen days after Ovx, animals started receiving daily, subcutaneous injections at a volume of 0.1ml, continuing until euthanasia. Rats were randomly assigned to one of four treatment groups (n=9 per group): vehicle (sesame oil), low EE (0.125μg per day), medium EE (0.18μg per day), or high EE (0.3μg per day). EE (Sigma, St. Louis, MO) was dissolved in sesame oil at the appropriate dose at the beginning of the study, then aliquoted into daily quantities and stored in the refrigerator (2-4°C) until needed. The medium EE dose was based on a 45-50μg per day regimen that an average woman weighing 60-70kg would be prescribed in an oral contraceptive (Curtis et al., 2005; Hoffman et al., 2012), adjusted to the weight of a rat (about 0.25kg).

Water Radial Arm Maze

Eighteen days later, subjects were tested for 13 days on the eight-arm, win-shift Water Radial-Arm Maze (WRAM) to evaluate spatial working and reference memory, as previously described (Bimonte and Denenberg, 1999; Bimonte et al., 2000, 2002, 2003; Bimonte-Nelson et al., 2003; Bimonte-Nelson et al., 2004). The maze was an eight-arm apparatus (each arm 38.1 x 12.7cm) filled with opaque, room temperature water. Water temperature was measured at the beginning of each day of testing, and was between 18-20°C for testing. Four of the eight maze arms contained hidden platforms (10cm

diameter) just beneath the surface of the water and spatial cues were present to aid the animals in spatial navigation. Each subject was assigned different platform locations that remained fixed across all days of testing. A subject was released from the start arm and given 3 minutes (min) to locate a platform. Once a platform was found, the animal remained on it for 15 seconds (s), then was returned to its heated testing cage for a 30s inter-trial interval (ITI). During the ITI, the just-found platform was removed from the maze and the water was cleaned to remove any debris and obscure olfactory cues. The animal was then placed back into the start arm and given another 3min to locate a platform. Each animal received four trials per day for 13 days, with the number of remaining platforms reduced by one on each subsequent trial. Thus, the working memory system was increasingly taxed as trials progressed within a day, allowing working memory load to be assessed. On the 13th day of testing, a six-hour delay was given between trials 2 and 3 to test delayed memory retention.

Morris Water Maze

One day after the WRAM, spatial reference memory was evaluated using the Morris water maze (MM). The apparatus was a round tub (188cm diameter) filled with opaque, room temperature water (18-20°C) containing a submerged platform (10cm diameter) in the northeast quadrant. The platform location remained fixed across all days and trials, with spatial cues available to aid the animals in spatial navigation, testing spatial reference memory (Morris et al., 1982). Animals received six trials per day for three days. At the beginning of each trial, animals were dropped off from one of four starting points (north, south, east or west), varying semi-randomly. Animals had 60s to locate the platform, where they remained for 15s before being placed back into a heated

cage for an ITI of 5-8min. To evaluate whether animals utilized a spatial strategy, a seventh probe trial was given on the third day of testing, during which the platform was removed and animals were given 60s to swim freely in the maze. A video camera and tracking system tracked and measured each rat's swim path (Ethovision; Noldus Instruments, Wageningen, The Netherlands).

Visible Platform Task

After completion of behavioral testing, motor and visual competences were evaluated using the visible platform task. This was a non-spatial adaptation of the spatial MM task, previously used to dissociate visual and motor acuity from place memory (Morris et al., 1982). This task is ideal for this purpose due to its similarity to other spatial water-maze tasks with respect to motor and visual requirements, differing only in that animals are not required to associate the location of the platform with distal cues. The apparatus was a rectangular tub (100 x 60cm) filled with clear room temperature water (18-20°C). A black platform (10cm wide) was positioned 4cm above the surface of the water, following previously published methods (Hunter et al., 2003). A ring of opaque curtains surrounded the maze, blocking all obvious spatial cues to prevent spatial navigation. Animals received six trials in one day. The drop off location remained the same across trials; however the platform location varied semi-randomly across three locations. Each rat had 90s to locate the platform, where it remained for 15s before being placed back into a heated cage for an ITI of 5-8min.

Markers of Peripheral Stimulation

To verify Ovx and subsequent hormone treatment, vaginal smears were taken at four months old for four days, after animals were given hormone treatment. Smears were

classified as proestrus, estrus, metestrus, or diestrus (Goldman et al., 2007; Engler-Chiurazzi et al., 2012). At euthanasia, uteri of all subjects were removed and trimmed of visible fat, and wet uterine weight (grams) was measured, as done previously (Westerlind et al., 1998; Engler-Chiurazzi et al., 2012). Osmotic pumps were visually inspected at euthanasia for visible cracks or tops that had come off of the pumps.

Euthanasia

Animals were euthanized one day after completion of the visible platform task by researchers blinded to treatment group. Animals were decapitated under isoflurane anesthesia; brains were rapidly removed and blocked just posterior to the BF. The anterior portion of each brain was fixed in 4% paraformaldehyde for 48 hours following removal, then transferred to 0.1 M phosphate-buffered solution (PB, pH 7.4). Brains were then soaked in 30% sucrose solution in PB for 72 hours, frozen, and sectioned at 40μm through the BF (plates 1–28 Paxinos and Watson, 2005) using a Microtome Cryostat (Microm HM 500 OM).

Serum Analyses

Serum levels of E2, E1 and EE were obtained from a subset of the vehicle and EE-medium groups to verify Ovx status and to determine whether experimental treatments resulted in circulating serum EE levels similar to those found in women taking EE-containing hormonal contraceptives. Blood was obtained via cardiocentesis at the time of euthanasia, and estrogen levels were determined using mass spectrometry with a lower detection limit of 10pg/ml.

Immunohistochemistry

ChAT-immunoreactive (ChAT-IR) cells in the BF were labeled using immunohistochemistry, following similar previously published protocols from our laboratory following treatment with estrogens (Acosta et al., 2009). Briefly, four animals from each group were selected, and a series of every third section through the BF was selected from each brain for immunohistochemistry processing, yielding six sections per animal 120μm apart, corresponding to plates 23-28 (roughly 1.2mm- 0.6mm Bregma) from Paxinos and Watson (2005), similar to prior publications (Gibbs, 2002). See supplemental materials for more detailed methods.

Stereology

Unbiased stereology was used to quantify ChAT-IR cells within the medial septum (MS) and vertical/diagonal bands (VDB), regions that contain neurons known to innervate the hippocampus (Lewis and Shute, 1967; Dutar et al., 1995; Banuelos et al., 2013). One researcher blind to treatment groups used the optical fractionator method, where the number of cells counted in a known, uniformly random sample of a region of interest is used to estimate the total cell population in that region (Gundersen, 1986; West, 1999; Banuelos et al., 2013).

Statistical Analyses

We planned a priori to assess the differences in maze performance between each EE group and the Vehicle group to compare effects of each dose to a "blank" ovarian hormone background. After completion of the study, we performed additional post-hoc comparisons between the EE-low and EE-high groups.

For WRAM analyses, orthogonal measures of working memory and reference memory errors were quantified as done previously in WRAM studies (Bimonte et al., 2000). Working memory correct (WMC) errors were the number of first and repeat entries into an arm that previously contained a platform within each session. Reference memory (RM) errors were the number of first entries into an arm that never contained a platform within each session. Working memory incorrect (WMI) errors were repeat entries into reference memory arms within each session.

WRAM testing was blocked into learning (days 2-7) and asymptotic (days 8-12) phases, based on prior studies (e.g., Bimonte and Denenberg, 1999; Bimonte et al., 2000; Hyde et al., 2000; Bimonte et al., 2003). Data were analyzed separately for each type of error using repeated measures ANOVA, with treatment as the between-groups variable and number of errors on each trial as the dependent variable. Steroid treatment induced differences on the lattermost portion of WRAM testing have been observed previously, with most pronounced effects on trial 4, the highest working memory load trial (Bimonte and Denenberg, 1999; Bimonte-Nelson et al., 2003, 2004; Braden et al., 2010); therefore, interactions between treatment and working memory load (trials) were analyzed. Fisher PLSD post-hoc tests were used, alpha level was set at 0.05.

MM data were analyzed using repeated measures ANOVA, with treatment as the between-groups variable and distance to the platform across days and trials as the dependent variable. Probe trial data were analyzed identically, except with percent distance in the northeast (platformed) and southwest (diagonally opposite of the platform) quadrants as the dependent variable.

Visible platform data were analyzed using repeated measures ANOVA, with treatment as the between-groups variable and latency to reach the platform on each trial as the dependent variable.

One-way ANOVA was used to analyze treatment group differences in the number weighted mean section thickness population estimate (ChAT-IR cell counts) in each region, and correlations between region population estimates and behavioral measures were examined. Accuracy of stereological estimates was evaluated using Gundersen's smoothness classification m=1 coefficients of error (CEs).

Results

Water Radial Arm Maze

When delivered via daily subcutaneous injection, there were no effects of EE treatment on WMC, WMI, or RM during the learning portion of testing. During the asymptotic phase of testing, similar to effects seen previously in our lab with tonic EE treatment (Mennenga et al., 2015), there was a Trial x Treatment interaction for WMC errors [$F_{(6,64)}$= 2.82; $p<0.05$] with a planned comparison showing that the high EE treated animals made more errors than vehicle treated animals as working memory load increased [$F_{(2,32)}$= 5.78; $p<0.01$] (figure 12). Post-hoc analyses also showed that the high EE group committed more WMC errors than the low EE (Fisher, $p<0.05$) and medium EE (Fisher, $p<0.05$) animals at the highest working memory load (figure 12). There were no differences in WMC errors between the vehicle group and the low EE or medium EE group, and there were no group differences for WMI or RM errors during the asymptotic phase of testing (figure 12). There were no group differences on the post-delay trials on day 13.

Morris Water Maze

There was a marginal Treatment x Day interaction for MM testing [$F_{(6,64)}$=2.21; p=0.05]. Further analyses revealed a main effect of Treatment [$F_{(3,32)}$= 3.22; p<0.05] for Day 1 of MM, whereby the vehicle group performed better than the low EE [$F_{(1,16)}$= 6.84; p<0.05], medium EE [$F_{(1,16)}$= 8.51; p<0.05], and high EE [$F_{(1,16)}$= 9.47; p<0.01] groups. There was no Treatment x Trial interaction for Day 1, indicating that this effect was present across all trials and was not carried by the initial exposure to the task on trial 1. There were no effects of Treatment for Days 2 or 3 of MM testing (Figure 13a). A higher percent distance was spent in the previously platformed quadrant versus the opposite quadrant [$F_{(1,32)}$ =374.33; p<0.0001] for the probe trial, with no quadrant by Treatment interaction, indicating that all groups spatially localized the platform quadrant by the end of testing (Figure 13b). Treatment did not impact number of crossings through the platform area during the probe trial (data not shown).

Visible Platform Task

The average escape time across all 6 trials was a rapid 7.71 seconds with a standard deviation of 7.47 seconds. There were no treatment effects for latency (data not shown), indicating that all animals possessed similar procedural capabilities to solve a water maze task (p>0.05).

Markers of Peripheral Stimulation

Fourteen days after the start of injections, all vehicle-treated rats exhibited diestrus smears indicating a lack of uterine stimulation, while animals treated with any dose of EE alternated between estrus and metestrus smears, with each smear showing numerous cornified cells, indicating uterine stimulation (Goldman et al., 2007). One

uterine weight score was lost due to experimental error and was not included in these analyses. For wet uterine weight, there was a significant effect of Treatment [$F_{(3,31)}$=29.88; p<0.0001], with uteri of vehicle-treated rats weighing less than low EE- [$F_{(1,15)}$= 62.17; p <0.0001], medium EE- [$F_{(1,16)}$= 117.36; p <0.0001], and high EE- [$F_{(1,16)}$= 109.10; p <0.0001] treated rats (figure 14).

Serum Analyses

Circulating serum E1 and E2 levels were below the lower limit for detection (10pg/ml) in all animals, verifying Ovx status. The mean circulating serum EE concentration in the medium EE treatment group was 23.17 pg/ml with a standard deviation of 12.50 pg/ml, which is remarkably similar to the range of serum levels found in women taking an oral contraceptive containing 35ug of EE near the beginning of their monthly cycle (Devineni et al., 2007).

ChAT Cell Counts

Mean measured tissue thickness was 27μm, CEs ranged from 0.05 to 0.10 and were less than half of the observed variation across subjects (coefficients of variation ranging from 0.21 to 0.34), indicating that the sampling and counting parameters utilized here were adequate to detect differences in cell populations among treatment groups (Gundersen and Osterby, 1981; Gundersen and Jensen, 1987; West, 1999; Dorph-Petersen et al., 2001).

There was a main effect of Treatment [$F_{(3,12)}$=3.66; p<0.05] in the VDB, whereby ChAT-IR cell counts were lower in the medium EE group (Fisher, p<0.05), and lower in the high EE group (Fisher, p=0.05), than those in the vehicle group (figure 15a). ChAT-IR cell counts in the low EE group did not differ from the vehicle group. Post-hoc tests

indicate that the ChAT-IR cell counts were lower in the medium EE group (Fisher, $p<0.05$) and marginally lower in the high EE group (Fisher, $p=0.09$), than the low EE group (figure 15a). There were no effects of Treatment on ChAT-IR cell counts in the MS (figure 15b).

The MS had a lower ChAT-IR cell count than the VDB ($F_{(1,12)}=96.49$, $p<0.001$) (figure 15c). There was also a positive correlation between ChAT-IR cell counts in the VDB and MS of the basal forebrain ($r=0.56$, $p<0.05$), indicating that animals with higher ChAT-IR cell counts in the MS tended to also have higher cell counts in the VDB (figure 15d).

Relationship Between Cholinergic Cell Population Estimates and Maze Performance

Correlations between behavioral data and BF cell counts were assessed to determine whether group differences in behavior data relate to changes in BF cholinergic cell populations. There was a negative correlation between ChAT-IR cell counts in the VDB and number of WMC errors on the highest load trial (trial 4) during the asymptotic portion of WRAM testing [$r= -0.55$; $p<0.05$], such that animals with lower ChAT-IR cell counts committed more WMC errors (figure 15e). Both intra- and inter- class correlations were assessed to ensure that the directionality of intra-class correlations agreed with that of the overall correlation across groups; all intra-class correlations were also negative, and thus in accordance with the overall correlation, but not significant (data not shown). VDB ChAT-IR cell counts did not correlate with any other types of errors and there were no correlations between MS ChAT-IR cell counts and behavior data.

Discussion

The present study was the first to investigate the effects of several doses of EE on cognition and the cholinergic system, in rodents. Overall, I found that: 1) EE impacted cognition in a dose- dependent manner, with high EE treatment impairing high demand spatial working memory, and low and medium EE treatment producing only modest transient impairments in a different memory domain, spatial reference memory, and 2) EE decreased the number of ChAT-positive neurons in the BF at medium and high doses. Analysis of brain and behavior measures revealed a relationship between ChAT-IR cell counts in the VDB and working memory performance on the WRAM. Specifically, animals with higher VDB ChAT-IR cell counts tended to make fewer working memory errors. Collectively, these results suggest that dose modifies the cognitive impact of EE; the high dose of EE produced working memory impairments, but low and medium doses of EE produced a transient impairment on only one task. These findings are clinically important, as the low EE treatment corresponds to the low end of available doses currently prescribed to women in contraceptive formulations. These doses were chosen to model the exact formulations currently prescribed to women, adjusted to the weight of a rat (Curtis et al., 2005).

While the cognitive profiles of the low and medium EE doses did not differ, the medium EE dose decreased the number of ChAT-positive cells in the VDB of the BF, while treatment with low EE did not alter this cell population, relative to vehicle treatment. Of note, while the present study did not detect overt maze learning or memory differences following treatment with the medium dose of EE, this dose was sufficient to alter our brain measure of cholinergic cell counts. Additionally, cell populations in the VDB of the BF

negatively correlated with maze errors. This effect size is large, and it can be alternately stated that as cell populations decreased, number of working memory errors in the maze increased. This information can be used to design and implement future studies investigating the mechanisms responsible for the cognitive impact of EE.

These findings, combined with results showing that our rat serum hormone levels correspond with serum levels in women using hormonal contraceptives, raise concerns about the impact EE has on the brain and its function, as clinically prescribed for women. It is still unknown how extended exposure to these hormones may modulate their impact or whether cessation of hormone treatment would attenuate these effects. It also remains to be determined whether exposure to EE early in life, such as for contraception, may impact the cognitive impact of hormone loss or estrogen-containing HT later in life.

The mechanisms by which EE modulates the BF cholinergic system are still unknown, although there are multiple points at which estrogens can influence this system. E2 is well known to interact with this system, however it has been reliably shown to produce an increase in BF cholinergic cell counts and there is strong evidence that E2 produces cognitive benefit through the BF cholinergic system (For review see Gibbs and Aggarwal, 1998, and Gibbs, 2010), the opposite of the impact of EE seen here. There is ample evidence that dose and duration of E2 administration alter its impact on the cholinergic system and, in fact, E2 delivered for a comparable duration, and at an equivalent dose, to the regimen used here has been shown to decrease cell populations in the MS (Gibbs, 2010). Although we do not see treatment group differences in the MS, the medium EE and high EE groups tended to have fewer ChAT-IR cells in the MS, relative to the vehicle group. This evidence, in conjunction with the clear behavioral deficits produced

by EE treatment here, seems to indicate that EE and E2 are working on similar targets, but in different manners.

Differences in the structure and function of EE and E2 may account for these opposing effects. For example, Paradiso et al., 2001 found that a small structural difference between human and rat α4β2 Nicotinic Acetylcholine Receptors (NAchRs) in the binding domain results in an inability of E2, but not EE, to potentiate this receptor in rats. Interestingly, the human α4β2 NAchR can be stimulated by both E2 and EE. These receptors, along with the α7 subtype, are the primary type of NAchRs present in the rodent brain (Flores et al., 1992), and are closely related to many cognitive processes, including hippocampal-dependent learning and memory (for review, see Hogg et al., 2002). Direct potentiation of hippocampal α4β2 NAchRs from exogenous EE may contribute to a downregulation in the production of endogenous acetylcholine. While this distinction has the potential to contribute to the opposing cognitive impacts of EE and E2, the cholinergic system is extensively complex and there are many factors yet to be investigated that will likely modulate the impact of different estrogens.

It is crucial to mention that clinically used EE-containing contraceptives and HTs require a progestin component to prevent the increased risk of endometrial cancer associated with unopposed estrogen. There are currently several clinically available progestins, each of which has a distinct pharmacological profile (Curtis et al., 2005). One commonly prescribed progestin, medroxyprogesterone acetate (MPA) (Curtis et al., 2005), when delivered alone, has been shown to impair spatial memory during treatment as well as several months later, when MPA levels are no longer detectable in serum (Braden et al., 2010, 2011). EE has yet to be methodically tested for cognition along with specific

progestins, and it is unknown how the inclusion of a progestin may influence the behavioral or brain impact of EE.

Results from the present study suggest that the contraceptive regimen that may produce the most favorable cognitive impact includes a low dose of EE (30-35μg EE/day or less). These findings also offer insight into how small differences in hormone structure and function can produce large differences in behavioral and brain profiles. Further studies are necessary to outline the many mechanisms by which estrogens can alter cognitive brain regions and how changes produced by exogenously delivered and endogenously circulating estrogens relate to cognitive function. The popularity that contraceptives have gained since their introduction in the 1960's has effectively changed the lifetime hormone profile of the average woman. The current aging generation is the first to have had long-term exposure to synthetic hormones and it is now crucial to understand how a lifetime of different endogenous and exogenous hormone exposures can influence cognitive aging. The broad goal of this research is to elucidate the impact that clinically prescribed hormones have on cognitive function and, ultimately, to optimize contraceptive and HT use for healthy cognitive aging, beginning in young adulthood. I hope the results of the current studies will set the stage for a series of future methodical investigations into the effects these clinically-prescribed hormones have on the brain and related memory processes.

CHAPTER 5: COGNITIVE IMPACT OF ETHINYL ESTRADIOL IN OVARY-INTACT YOUNG ADULT RATS

Introduction

Ethinyl estradiol (EE) is a synthetic analogue of the natural estrogen, 17β-estradiol (E2), and is the primary estrogen utilized in hormonal contraceptives (Shively, 1998). Numerous contraceptive formulations contain EE (Curtis et al., 2005), and EE is also found in hormone therapies (HTs) for menopausal women, such as Estinyl™ and Femhrt™ (Curtis et al., 2005). It has been estimated that 10.6 million women between 2006-2010 (Jones et al., 2012), and 17.3% of all women between 2006-2008 (Mosher and Jones, 2010), used EE-containing contraceptives. Elucidating the cognitive impact of EE is critical, as exogenous exposure to EE can occur during young adulthood and during the transition to menopause through contraceptives and HT. Of note, EE is a synthetic analogue to E2; however, these estrogens have different pharmacokinetic profiles (Bennink, 2004). EE is more biologically active than E2 (Dickson and Eisenfeld, 1981) and cannot be converted to estrone (E1) or other weaker estrogens (Fotherby, 1996), whereas E2 can (Prokai-Tatrai, et al., 2005). Each of these estrogens also exhibits a distinct binding profile, which varies across species (Paradiso et al., 2001).

Although EE is widely used by women, and many women take EE-containing hormonal formulations for a large portion of their lives, most preclinical research on the cognitive impact of estrogens has excluded EE (for review see: Acosta et al., 2013; Bimonte-Nelson et al., 2010). There have been a few studies investigating the cognitive effects of EE as both a contraceptive and HT, with effects that vary across memory domains (Silber et al., 1987; Mordecai et al., 2008). Although these studies each utilized

hormonal contraceptive formulations containing EE, the specific dose of EE and the included progestin differed. Because of these differences, it is unknown whether or to what extent EE produced these effects.

In chapter 4, I tested the cognitive and neurobiological effects of cyclically administered EE, given via a daily injection, to model oral contraceptive use. By methodically evaluating EE in the ovariectomized (Ovx) rodent model, I was able to investigate the effects of EE alone, without the effects of any other ovarian hormones. I found that EE in the Ovx model impacted cognition in a dose- dependent manner, with high EE treatments impairing high demand spatial working memory, and low treatment producing only modest transient impairments in a different memory domain, spatial reference memory. I also found that cyclic EE decreased the number of Choline Acetyltransferase (ChAT)-positive neurons in the basal forebrain at medium and high doses. Analysis of brain and behavior measures revealed a relationship between ChAT-positive cell counts in the VDB and working memory performance on the WRAM. Specifically, animals with higher VDB ChAT-positive cells tended to make fewer working memory errors. An animal model using ovary intact female rats is necessary, given that most women are ovary intact for the majority of their lives. In fact, only a small percentage of women have their ovaries removed via oopherectomy. The aim of the current study is to evaluate the effects of EE administration in ovary intact animals, to determine how the administration of EE affects cognition in ovary intact rats, as a model of hormonal contraceptive use.

Materials and Methods

Subjects

Thirty three-month old virgin F-344 female rats were raised at Harlan Laboratories (Indianapolis, IN). Similar to Study I, animals were three months old at the beginning of the study, four months old at maze testing initiation, and five months old at euthanasia. After arrival, animals were pair-housed, had food and water ad-lib, and were maintained on a 12-h light/dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards.

Experimental Design and Hormone Treatments

Rats were vaginally smeared and catalogued for 35 days to ensure that each animal's estrous cycle was regular, meaning that they consistently progressed through each phase of the estrous cycle within four or five days. Animals were then randomly assigned to one of three groups (n=10/group) and given either empty silastic tubing, or one of two lengths of silastic tubing containing EE (Dow Corning; 0.062in I.D. x 0.125in O.D.). Rats were anesthetized via isoflurane inhalation, a small incision was made, and a subcutaneous pocket was created in the dorsal scruff of the neck. A length of silastic tubing that was either empty (4mm long), or one of two lengths containing EE ((Sigma, St. Louis, MO; 2mm or 4mm long) was inserted below the skin, and the skin was closed with surgical staples. The 2mm length of silastic tubing was selected to produce circulating serum levels of EE similar to those that we have seen with our low EE dose, which is based on the 30-35μg daily regimen that an average 60-70kg woman would be prescribed in an oral contraceptive (Curtis et al., 2005; Hoffman et al., 2012), adjusted to the weight of a rat (about 0.25kg). The 4mm length of silastic tubing was chosen to produce circulating serum

levels similar to our previously used high dose of EE, which is one-tenth of a similar dose of E2 previously shown to enhance performance on spatial tasks (Talboom et al., 2008) and corresponds to a 75-80μg/day dose of EE (in a 60-70kg woman), within the range of previously available contraceptives for women in the 1960's, before the benefits of lower-dose formulations were known (Chadwick et al., 2012).

Eighteen plus or minus two days after silastic insertion surgeries, behavioral testing ensued. Behavioral testing consisted of: water radial arm maze, Morris water maze, open field, and object recognition. Figure 16 shows a timeline depicting the temporal relations among the various treatments.

Water Radial Arm Maze

Water radial arm maze testing was identical to the testing procedures described in chapter 4.

Morris Water Maze

Morris water maze testing was identical to the procedures described in chapter 4, with the exception that animals were tested for four trials per day across five days of testing, with an additional fifth probe trial on the final day of testing.

Markers of Peripheral Stimulation

To verify hormone treatment, vaginal smears were taken throughout the study. Smears were classified as proestrus, estrus, metestrus, or diestrus (Goldman et al., 2007; Engler-Chiurazzi et al., 2012; Mennenga and Bimonte-Nelson, 2013). At sacrifice, uteri of all subjects were removed and trimmed of visible fat, and wet uterine weight (grams) was measured, as done previously to confirm hormone status (Westerlind et al., 1998; Engler-Chiurazzi et al., 2012; Mennenga and Bimonte-Nelson, 2013). Serum was collected at

sacrifice via cardiocentesis and processed for levels of androstenedione, E2, E1, leutenizing hormone (LH), and follicle stimulating hormone (FSH). Levels were obtained using an iodinated radioimmunoassay (RIA) by the CORE Endocrine Laboratory at Pennsylvania State University, exactly as reported elsewhere (Acosta et al., 2009, 2010).

Statistical Analyses

WRAM testing was blocked into learning (days 2-7) and asymptotic (days 8-12) phases, based on prior studies (e.g., Bimonte and Denenberg, 1999; Bimonte et al., 2000; Hyde et al., 2000; Bimonte et al., 2003). Data were analyzed separately for each type of error using repeated measures ANOVA, with treatment as the between-groups variable and number of errors on each trial as the dependent variable. Steroid treatment induced differences on the lattermost portion of WRAM testing have been observed previously, with most pronounced effects on trial 4, the highest working memory load trial (Bimonte and Denenberg, 1999; Bimonte-Nelson et al., 2003, 2004; Braden et al., 2010); therefore, interactions between treatment and working memory load (trials) were analyzed. Fisher PLSD post-hoc tests were used, alpha level was set at 0.05.

MM data were analyzed using repeated measures ANOVA, with treatment as the between-groups variable and distance to the platform across days and trials as the dependent variable. Probe trial data were analyzed identically, except with percent distance in the northeast (platformed) and southwest (diagonally opposite of the platform) quadrants as the dependent variable.

Results

The high EE group was removed from the behavioral analyses due to extensive health issues that arose from the high EE treatment. By sacrifice, 5 out of the 10 animals in

this group had developed extremely enlarged uterine horns that appeared to be cancerous. The remaining 5 animals all also had very enlarged uterine horns, which are likely the result of this high dose of synthetic estrogen being given to animals with intact ovaries. Uterine stimulation was apparent in the low EE group as well, but it was not as extensive as seen in the high dose group. Behavioral data from the vehicle and low EE groups are presented.

Water Radial Arm Maze

There was a main effect of Treatment on Total Errors made on days 2-7 of WRAM testing, which is considered the learning phase of testing ($F_{(1,16)}= 4.80$, $p<0.05$), with the EE-treated group committing more errors across all trials than the vehicle-treated animals (figure 17). There was no interaction between Treatment and Trial ($F_{(3,48)}= 1.59$, $p=0.20$, NS), indicating that this difference was not specific to any trial. This difference was no longer apparent during the asymptotic portion of testing, days 8-12 ($F_{(1,16)}= 1.94$, $p=0.18$, NS) and there was no interaction between Treatment and Trial for this portion of testing ($F_{(3,48)}= 0.70$, $p=0.56$, NS) (figure 17).

Morris Water Maze

There were no main effects of Treatment on Total Swim Distance (cm) for the MM ($F_{(1,16)}= 1.08$, $p=0.31$, NS), but there was a Day x Treatment interaction ($F_{(4,64)}= 3.32$, $p<0.05$) (figure 18). Further analyses revealed that there was an effect of Treatment on Total Swim Distance on Day 1 of testing ($F_{(1,16)}= 5.25$, $p<0.05$), with the EE-treated animals swimming a shorter distance than the vehicle-treated animals across all four trials, suggesting that they covered a shorter distance during the allotted trial time (figure 18). Given that the majority of the animals did not locate the platform successfully during the

allotted trial time on the first day of testing with this testing protocol that only includes four trials per day, I analyzed only successful trials, in which the animal found the platform within the allotted trial time; there was no difference on any trial of Day 1 when only successful trials were included (Trial 1: no animals found the platform; Trial 2: $F_{(1,5)}$=0.05, p>0.05, NS; Trial 3: $F_{(1,7)}$=1.61, p>0.05, NS; Trial 4: $F_{(1,8)}$=0.47, p>0.05, NS; figure 19). Further, mean swim velocity (cm/s) was marginally decreased in the EE group ($F_{(1,16)}$=3.93, p<0.10; figure 19). By the end of the second day of testing, animals were able to locate the platform on their own on the majority of the trials given, and this difference was no longer apparent. For the probe trial, there was a main effect of Quadrant ($F_{(1,16)}$= 173.00, p<0.0001; figure 18), indicating that all animals preferred the previously-platformed quadrant over the diagonally opposite quadrant and were therefore likely employing a spatial strategy.

Markers of Peripheral Stimulation

There was a main effect of Treatment on androstenedione levels ($F_{(1,14)}$= 11.99, p<0.01), E2 levels ($F_{(1,16)}$= 5.54, p<0.05), and FSH levels ($F_{(1,17)}$= 8.32, p<0.05), such that EE treatment increases androstenedione levels, decreases E2 levels, and increased FSH levels, relative to vehicle treatment (figure 20). There was no effect of EE treatment on E1 levels ($F_{(1,17)}$= 0.05, p=0.83, NS) or LH levels ($F_{(1,17)}$= 0.92, p= 0.36, NS). There was an effect of Treatment on wet uterine weight ($F_{(1,17)}$= 14.85, p<0.01), such that EE treatment increased uterine weights (figure 20).

Discussion

I previously reported cognitive-impairing effects of high-dose EE in Ovx animals (Mennenga et al., 2015; chapter 4), and the current study now extends those findings to

ovary-intact animals. Here, EE treatment given to ovary-intact young adult rats produced impairments on the WRAM, and marginally decreased swim velocity on the MM. EE did not impact non-spatial object recognition. Silber et al. (1987) found no impact of EE-containing contraceptives on several tests measuring memory and concentration in women, and Mordecai et al. (2008) found enhanced verbal memory during the active compared to the inactive phase of oral contraceptives in women, although benefits were not seen on visuospatial measures. Thus, the results here are in keeping with the general finding that estrogenic effects are specific to memory domain. In chapter 3, I reported memory-domain-specific differences between males and females experiencing different hormonal states. It seems that visuospatial ability is particularly impacted by changes in estrogen status. Thus, the spatial memory tasks utilized in the current set of experiments may be ideal to detect the cognitive impact of EE and other estrogens.

EE also increased serum follicle stimulating hormone and androstenedione, decreased serum E2, and did not impact serum estrone or LH in these ovary-intact young adult rats. Our lab has shown in several studies that elevated androstenedione levels are associated with impaired performance on several maze tasks (Acosta et al., 2009, 2010; Camp et al., 2012; Mennenga et al., 2014), implicating this hormone in the cognitive impairments seen here. This study serves only as the starting point from which future investigations into the cognitive effects of natural and synthetic hormones can be derived; investigations into the many administration parameters that vary by formulation are needed. Follow-up studies utilizing popular available synthetic progestins will be necessary to determine how the inclusion of another hormone might offset or interact with the effects of EE alone. Also, the doses investigated in this dissertation cover a wide

range, and more refined studies of dose-response relationships will be valuable towards understanding how contraceptive formulations impact the brain and its function. Additionally, administration route is an important factor to consider when studying the behavioral pharmacology of any drug. Contraceptives are delivered via several different mechanisms, including tonic regimens that release a steady rate of hormone across time, such as transdermal patches or the vaginal ring, and cyclic regimens that are delivered in a daily pill, resulting in a steady rise and fall of hormone levels across time. Phasic formulations that deliver EE and progestins in a pattern more closely representative of the natural fluctuations in hormone levels that happen across the menstrual cycle are also available; these formulations are as-of-yet unexplored for effects on cognition.

There is an abundance of hormone formulations available to women, and these treatments are prescribed for many purposes, including for contraception, endometrial and ovarian regulation, and for relief from symptoms associated with menopause. This wide array of available formulations means that there is ample potential for cognitively detrimental treatments to unknowingly be given to women; however, this also means that we, as scientists, have a long list of potentially cognitively neutral, or even beneficial formulations to study. Since each of these formulations achieve similar clinical purposes, future women may have the opportunity to choose hormone treatments based on scientifically informed information on the whole-body impact of each available option.

CHAPTER 6: OPTIMIZING HORMONE THERAPY ACROSS THE MENOPAUSE TRANSITION I: WINDOW OF OPPORTUNITY

Introduction

Around the fifth decade of women's lives, their eggs stop maturing, ovulation and menstruation become irregular, and eventually menstruation stops; this natural cessation of the menses is known as menopause (NAMS; Curtis et al., 2005). With halting of ovulation, ovarian production of estrogens and progesterone drastically decrease, resulting in several undesirable health consequences. Common menopause-related issues include hot flashes, bone density loss, cardiovascular changes, vaginal atrophy, and cognitive decline (Curtis et al., 2005). Increasing life expectancy and stable age of natural menopause onset mean that women are now spending up to 40% of their lives post-menopause (ACOG, 2011). Many women utilize estrogen-containing hormone therapy (HT), which can alleviate several symptoms of menopause. Conjugated equine estrogens (CEE, tradename Premarin®, Prempro with the synthetic progestin Medroxyprogesterone acetate; MPA) were the most commonly prescribed estrogen component of HT in the US (Hersh et al., 2004); fourteen million women in the US were estimated to use CEE in 2005, and CEE has been prescribed as HT since 1942 (Stefanick, 2005). CEE contains several estrogens, including many that are not naturally produced by women, trace amounts of 17β-estradiol (E2), the most potent naturally circulating estrogen in women, and over 50% estrone sulfate (E1S; Gleason et al., 2005), which is desulfated in the liver, converting it to estrone (E1). In many women, CEE HT is effective at attenuating or preventing symptoms of menopause, including hot flashes, vaginal atrophy, and decreased bone density (Curtis et al., 2005); however, whether CEE reduces the cognitive decline associated with menopause remains unclear. The large

Women's Health Initiative Memory Study (WHIMS) reported that CEE alone produced no change in risk of developing mild cognitive impairment (MCI) and a trend for an increase in probable dementia incidence; CEE plus progestin treatment produced no change in MCI risk and increased the risk of probable dementia in menopausal women (Espeland et al., 2004; Shumaker et al., 2004), findings which prompted many women to discontinue their HT use altogether (ACOG, 2011).

There is accumulating evidence that a 'window of opportunity' for HT initiation following hormone loss exists (Singh et al., 2013). Clinical studies demonstrating a limited window of time during which HT can exert positive effects have given rise to the critical period, or window of opportunity, hypothesis (Resnick & Henderson, 2002; Zandi et al., 2002; MacLennan et al., 2006; Maki, 2006; Maki & Sundermann, 2009; Khoo et al., 2010). For example, recent reports have found that HT initiated prior to natural menopause was beneficial to cognitive performance; however, HT initiated post-menopause was detrimental (Greendale et al., 2009), and use of HT during the menopause transition has been shown to enhance memory and hippocampal function, as detected by fMRI, in women (Maki et al., 2011). Several preclinical rodent studies also support the window of opportunity hypothesis for beneficial effects of HT on cognition (Gibbs, 2000; Daniel et al., 2006; McLaughlin et al., 2008; Bohacek & Daniel, 2010) and brain health (Bohacek et al., 2008; Bohacek & Daniel, 2009), but this research has been limited to the Ovx model of hormone loss and to E2 as HT. In middle-aged Ovx rats, E2 given immediately, but not five months after Ovx, enhances spatial working memory (Daniel et al., 2006) and attentional processes on the five-choice serial reaction time task (Bohacek & Daniel, 2010).

Additionally, E2 given immediately or three months after Ovx, but not 10 months after Ovx, enhances delayed-match-to-position performance (Gibbs, 2000).

Using the ovariectomized (Ovx) rodent as an ovarian hormone 'blank slate', we showed that CEE HT exerted beneficial effects on spatial working and reference memory (Acosta et al., 2009; Engler-Chiurazzi et al., 2011). Importantly, reproductive senescence in women differs from reproductive senescence in female rats. The aging rat does not experience menopause; it experiences estropause, which includes several hormonal states indicative of irregular ovulation. Estropause can manifest as a persistent estrus state, associated with low to medium circulating levels of E2, E1, and the androgens testosterone and androstenedione, along with low levels of progesterone, or it can produce a persistent diestrus state with intermediate levels of E2, E1, and androstenedione, low testosterone, and high progesterone (Lu, 1979). Although these processes differ from menopause, there are notable similarities in the physiological triggers of these events. In both women and rodents, patterns of FSH and LH release from the pituitary gland change before changes in ovulation occur (Wise et al., 1999; Downs and Wise, 2009), and there is indication that changes in pituitary gonadotropin release, coupled with changes in ovarian function, lead to both estropause and menopause (Wise, 1999; Wise et al., 1999). These parallels are important, and lend support to the rodent as a reliable model of the human reproductive system; nonetheless, estropause is not the ideal model of human menopause. Likewise, the Ovx model is an excellent tool to study the estimated 600,000 women per year who have undergone surgically induced menopause (ACOG, 2008; Rocca et al., 2009), and it is the gold standard for isolating the effects of exogenously administered hormones (Mennenga & Bimonte-Nelson, 2013); however, the Ovx model has limited generalizability to the

majority of women who have undergone natural, transitional menopause and retained their follicle-deplete ovaries.

Natural menopause can be more closely modeled in the rodent via the industrial chemical 4-vinylcyclohexene diepoxide (VCD). Treatment with VCD accelerates the natural process of atresia in the finite primary and primordial follicle pool, producing a gradual loss of follicles (Flaws et al., 1994; Springer, McAsey, et al., 1996; Springer, Tilly, et al., 1996; Borman, et al., 1999; Kao et al., 1999; Hu et al., 2002; Mayer, et al., 2002, 2004, 2005), leading to ovarian failure, and a drastic decrease in ovarian-derived E2 and progesterone (Hirshfield, 1991; Springer et al., 1996: Mayer, et al., 2004, 2005). Thus, treatment with VCD results in an ovary-intact, follicle-deplete rat with a hormone profile similar to that of a naturally menopausal woman. Using the VCD model, our laboratory has previously shown that CEE administration can improve performance on a spatial working and reference memory task following surgical menopause, but can impair performance when administered following a VCD-induced transitional hormone loss (Acosta et al., 2010). We have repeatedly shown a positive relationship between circulating levels of androstenedione, the primary hormone released following follicular depletion, and maze errors (Acosta et al., 2009b, 2010; Camp et al., 2012), and we have also shown that exogenous androstenedione impairs spatial memory in Ovx rats (Camp et al., 2012), as does its estrogenic metabolite E1 (Engler-Chiurazzi et al., 2012). Moreover, pharmacological blockade of exogenous androstenedione's conversion to E1 prevented its negative cognitive impact (Mennenga et al., 2015). These results indicate that ovarian-produced androstenedione-derived E1 is contributing to the cognitive deficits associated

with transitional menopause. CEE is primarily composed of E1S that is converted to E1, making it unlikely to be beneficial for memory once follicular depletion has ensued.

Thus, clinical and preclinical findings concur that beneficial effects of estrogen HT may be dependent on early initiation; however, there have been no preclinical rodent studies on the window of opportunity utilizing a model of transitional menopause, or the popular HT CEE. In our previous study demonstrating detrimental effects of CEE in transitionally menopausal, ovary-intact rats (Acosta et al. 2010), CEE treatment initiation took place *after* follicular depletion had ensued, when androstenedione is the primary hormone released by the ovaries. I hypothesized that timing of treatment initiation relative to follicular depletion or duration of treatment alter the cognitive effects of CEE HT. I investigate whether giving CEE *at the onset* of accelerated follicular depletion changes its cognitive effects. The goals of the present study were to determine whether the cognitive impact of CEE HT is influenced by: the timing of treatment initiation relative to the onset of accelerated follicular depletion, or the duration of treatment. I evaluated cognition using tasks tapping spatial working and reference memory.

Materials and Methods

Subjects

Thirty-four eight month-old Fischer-344 female rats raised at the aging colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN) were used. After arrival, rats were pair-housed, had food and water ad-lib, and a 12-h light/dark cycle. Animals were 12 months old at the initiation of behavioral testing. All procedures were approved by the local Institutional Animal Care and Use Committee (IACUC) and adhered to National Institutes of Health (NIH) standards.

Experimental Design and Hormone Treatments

To make behavioral testing feasible, animals were run in two experimental waves, six weeks apart, with each treatment group represented in each wave. Rats were randomly divided into 4 groups (n in parentheses): Control (8), Post (9), Peri Long-Term (Peri-LT) (10), and Peri Short-Term (Peri-ST) (7). Figure 21 provides a comprehensive overview of the experimental design.

VCD Treatment

One week after arrival, animals in the Control, Post and Peri-LT groups received VCD treatment (160 mg/kg diluted in 50% DMSO/Saline at a volume of 2.5µl/g body weight; IP; Sigma-Aldrich, St. Louis, MO) while animals in the Peri-ST group received vehicle injections (50% DMSO/Saline at a volume of 2.5µl/g body weight; IP), for 15 days. VCD follicular depletion procedures were adapted from prior studies (Acosta et al., 2009, 2010; Mayer et al., 2002). Sixty-four days after the first VCD injection, a second set of VCD/Vehicle injections were administered. Peri-ST animals received VCD injection for 15 days (160 mg/kg diluted in 50% DMSO/Saline at a volume of 2.5µl/g body weight; IP; Sigma-Aldrich, St. Louis, MO), while Control, Post, and Peri-LT animals received vehicle injections (50% DMSO/Saline at a volume of 2.5µl/g body weight; IP).

CEE Treatment

Twenty-eight ±1 days after the beginning of VCD treatment for the Control, Post, and Peri-LT groups, CEE treatment was initiated for the Peri-LT group. CEE, in its unconstituted powder form, as prescribed to women (manufactured by Wyeth Pharmaceuticals Inc., Philadelphia, PA, obtained from a commercial pharmacy via veterinary prescription) was dissolved in sesame oil at a dose of 30µg/injection (injection

volume=0.1ml). One subcutaneous injection was given for two consecutive days followed by two days off, a pattern repeated throughout the study (exactly as done in Acosta et al., 2009). Using this injection regimen, we have previously shown CEE to alter cognition in middle-aged Ovx rats (Acosta et al., 2009), and we, and others, have shown E2 to impact hippocampal plasticity and memory (Korol and Kolo, 2002; McLaughlin et al., 2008; Woolley and McEwen, 1993). All other groups received sesame oil injections following the same administration regimen at a volume of 0.1ml.

Next, 90±1 days after the Control and Post groups' VCD treatment was initiated, CEE treatment began for the Post and Peri-ST groups. CEE administration for the Post and Peri-ST groups was identical to that of the Peri-LT group, with the exception of the treatment initiation time-point. The Control group continued to receive sesame oil injections following the same administration regimen at a volume of 0.1ml. These injection regimens were continued until sacrifice.

To confirm follicular depletion following VCD as well as CEE treatment, vaginal smears were performed during the 10 days prior to behavioral testing. Smears were classified as proestrus, estrous, metestrus or diestrus (Goldman et al., 2007). Behavioral testing began 107±1 days after the first VCD injection, 79±2 days after initiation of CEE treatment for the Peri-LT group, and 17 days after initiation of CEE treatment for the Post and Peri-ST groups Hormone/vehicle treatment was continued throughout behavioral testing, and animals received the last CEE/Sesame oil injection one day before sacrifice.

Water Radial Arm Maze

Water radial arm maze (WRAM) procedures were identical to those in chapter 4, except that on the 13th day of testing, a 6-hour delay was given between trials 2 and 3, and

on the 14th day of testing, an 8-hour delay was given between trials 2 and 3, to test delayed memory retention.

Morris Water Maze

Morris water maze (MM) procedures were identical to those in chapter 4.

Delay Match-to-Sample Asymmetrical Three-Choice Task

One day after MM testing concluded, spatial working memory and short-term memory retention were evaluated using a win-stay water-escape DMS asymmetrical place-learning task. The maze was an asymmetrical, four-arm apparatus (each arm 38.1 x 12.7cm) filled with opaque, room temperature water containing a submerged platform (10cm diameter) in one of the four arms. This task was identical to the win-stay DMS plus maze (Engler-Chiurazzi et al., 2011; Frick et al., 1995), with the exception of the asymmetrical arm configuration. Animals were released into a different start arm at the beginning of each trial, varying semi-randomly such that the animals were released from each of the three non-platformed arms twice within a day of testing. The platform remained in the same location within a day, but changed location across days. Animals received six trials/day for 8 days with 90 seconds to locate the platform, 15 seconds on the platform and a 30 second inter-trial-interval in a heated cage. On the 7th day of testing, a 6-hour delay was given between trials 1 and 2, and on the 8th day of DMS testing, an 8-hour delay was given between trials 1 and 2, to test delayed memory retention.

Visible Platform Task

Visible platform procedures were identical to those described in chapter 4.

Peripheral Markers of Treatment

To verify VCD and subsequent hormone treatment, vaginal smears were taken for ten days just prior to behavioral testing. Smears were classified as proestrus, estrus, metestrus, or diestrus, exactly as reported elsewhere (Goldman et al., 2007; Engler-Chiurazzi et al., 2012; Acosta et al., 2009, 2010). At sacrifice, uteri of all subjects were removed and trimmed of visible fat, ovaries were removed for histological processing, and wet uterine weight (grams) was measured, exactly as reported elsewhere (Westerlind et al., 1998; Engler-Chiurazzi et al., 2012; Acosta et al., 2009, 2010). Ovaries were removed, trimmed of fat, and preserved in 10% formalin. Sections were then processed for paraffin embedding and sectioned at 5μm, then stained using hematoxylin and eosin. Corpora lutea and follicle populations were counted.

Hormone Assays

Serum was collected at sacrifice via cardiocentesis and processed for levels of Androstenedione. Levels were obtained using an iodinated radioimmunoassay (RIA) by the CORE Endocrine Laboratory at Pennsylvania State University, exactly as reported elsewhere (Acosta et al., 2009, 2010).

Statistical Analyses

Orthogonal planned comparisons were set a priori. We first compared animals treated with CEE post-depletion (Post group), to animals given vehicle treatment (Control group). Next, we compared animals treated with CEE post-depletion (Post group) to each group of animals whose CEE treatment administration began during depletion (Peri-LT and Peri-ST groups). These comparisons were chosen 1) to replicate prior findings that CEE initiated post-depletion impairs memory relative to vehicle treatment and 2) to determine

whether previously observed CEE-induced cognitive impairments following transitional menopause could be reversed with early CEE initiation. We predicted that the animals treated with CEE post depletion (Post group) would make more WRAM errors than the animals that received vehicle treatment (Control group); this would be a direct replication of our prior findings using the VCD model (Acosta et al., 2010). Thus, alpha level was set at 0.05 (1-tail) for Post versus Control group WRAM and MM comparisons. We also anticipated that the Post group would make more errors than the Peri-ST and Peri-LT groups, based on prior findings from others evaluating a window of opportunity for cognitive benefits of E2 in the Ovx model (Gibbs, 2000; Daniel et al., 2006; Bohacek & Daniel, 2010). However, since early initiation of HT has never been evaluated in rodents using CEE or a transitional model of menopause, and the DMS 3-choice task has not been used to evaluate cognitive effects of CEE in the VCD model, alpha level for all remaining comparisons was set at 0.05 (2-tail).

WRAM errors were counted when the tip of a rat's snout crossed a mark on the outside of the arm (not visible from inside the maze; 11cm into the arm). Errors were initially quantified using previously established orthogonal measures of working and reference memory (Jarrard et al., 1984; Bimonte et al., 2000, 2002; Hyde et al., 2000; Braden et al., 2010, 2011). Working Memory Correct (WMC) errors included all entries into arms that previously contained a platform, Reference Memory (RM) errors included first entries into arms that never contained a platform and Working Memory Incorrect (WMI) errors included all subsequent entries into arms that never contained a platform. An initial analysis indicated that there was no interaction between Treatment and WRAM Error Type ($F_{(6,58)}$= 0.701; $p>0.05$, NS); therefore, total errors are presented. WRAM

testing was divided into 3 testing blocks of 4 days each (Block 1: Days 1-4, Block 2: Days 5-8, Block 3: Days 9-12). Data were analyzed using repeated measures ANOVA, with treatment as the between-groups variable and number of errors on each trial as the dependent variable. Estrogen-induced differences on the lattermost portion of WRAM testing have been observed previously, and the largest differences are typically seen on trial 4, the highest working memory load trial (Bimonte and Denenberg, 1999; Bimonte-Nelson et al., 2003, 2004; Braden et al., 2010; Acosta et al., 2010); therefore, data were analyzed separately within each testing block, and interactions between Treatment and working memory load (Trial) were investigated.

MM data were analyzed using repeated measures ANOVA, with treatment as the between-groups variable and distance to the platform as the dependent variable. Probe trial data were analyzed using repeated measures ANOVA with quadrant (Northeast vs. Southwest) as the between-groups variable and percent distance as the dependent variable, to determine the percent of each animal's swim distance spent in the previously platformed, versus the diagonally opposite quadrant.

For DMS testing, trial 1 was considered to be the information trial and was not included in analyses, trial 2 was the working memory trial and trials 3-6 were considered recent memory trials. Entry into any non-platformed arm was counted as an error. An arm entry was counted when the tip of a rat's snout reached a mark on the outside of the arm (not visible from the inside of the maze; 11 cm into the arm). DMS testing was divided into learning (days 1-3) and asymptotic (days 4-6) phases and analyzed separately for each phase of testing using repeated measures ANOVA, with Treatment at the between-groups variable and number of errors across days and trials as the repeated dependent measure.

Visible platform data were analyzed using repeated measures ANOVA, with treatment as the between-groups variable and latency to reach the platform on each trial as the dependent variable.

Wet uterine weights, serum hormone levels, ovarian follicles, and corpora lutea were all analyzed with one-way ANOVAs, with Treatment as the between-groups variable and wet uterine weights (g), circulating levels of Androstenedione (pg/ml), estimated number of follicles, and number of corpora lutea as the respective dependent variables. Subjects with serum hormone levels below the lower detectable limit of the assay were excluded from serum analyses.

Results

Water Radial Arm Maze

We did not observe an effect of Wave ($F_{(1,25)}$= 2.53; p>0.05, NS, η_G^2<0.01), nor a Wave x Treatment interaction ($F_{(3,25)}$= 1.19; p>0.05, NS, η_G^2<0.01) for WRAM errors, therefore analyses were collapsed across wave. An initial analysis indicated that there was no interaction between Treatment and WRAM Error Type (WMC, WMI, RM) for WRAM testing (days 1-12; $F_{(6,58)}$= 0.70; p>0.05, NS); therefore, total errors are presented. There were no effects of Treatment or Trial x Treatment interactions for Blocks 1 or 2. For Block 3, there was a main effect of Treatment, such that the Post group made more errors than the Control group ($F_{(1,15)}$=3.22, p≤0.05, η_G^2=0.01), as expected (Acosta et al., 2010) ($F_{(1,15)}$=3.22, p≤0.05, η_G^2=0.01). The Post group also made more error than the Peri-ST group ($F_{(1,14)}$=6.20, p≤0.05, η_G^2=0.03) across all trials, indicating a benefit of early treatment, but did not differ from the Peri-LT group ($F_{(1,17)}$=1.20, p>0.05, NS, η_G^2=0.01), indicating that the benefit of early initiation is restricted to short-term treatment (figure 22).

Morris Water Maze

We did not observe an effect of Wave ($F_{(1,26)}$= 1.22; p>0.05, NS, η_G^2=0.02), nor a Wave x Treatment interaction ($F_{(3,26)}$= 1.42; p>0.05, NS, η_G^2=0.04) for MM swim distance, therefore analyses were collapsed across wave. There were no differences in swim distance on Days 1-3 between the Post and Control groups ($F_{(1,15)}$=0.36, p>0.05, η_G^2=0.01; NS), the Post and Peri-LT groups ($F_{(1,17)}$=2.29, p>0.05, η_G^2=0.02; NS), nor the Post and Peri-ST groups ($F_{(1,14)}$=1.21, p>0.05, η_G^2=0.03; NS; Figure 23a). For the probe trial, there was a main effect of Quadrant ($F_{(1,30)}$=353.88, p≤0.0001, η_G^2=0.90; Figure 23b) in the absence of a Quadrant x Treatment interaction ($F_{(3,30)}$=1.38, p>0.05, η_G^2=0.10; NS). All treatment groups covered a higher percent distance in the previously platformed vs. the opposite quadrant, indicating that all groups spatially localized the platform by the end of testing.

Delay Match-to-Sample Asymmetrical Three-Choice Task

We did not observe an effect of Wave ($F_{(1,26)}$= 0.47; p>0.05, NS, η_G^2<0.01), nor a Wave x Treatment interaction ($F_{(3,26)}$= 2.35; p>0.05, NS, η_G^2=0.01) for DMS errors, therefore both waves are presented together. There was a Trial x Treatment interaction for the Control versus the Post group ($F_{(4, 60)}$=6.22; p≤0.001, η_G^2=0.08; Figure 24a), whereby the Post group made fewer errors on Trial 2, the working memory trial ($F_{(1,15)}$=16.26, p≤0.01, η_G^2=0.14; Figure 24a), during the learning phase of testing (Days 1-3). There were no differences between the Post and Peri-LT groups ($F_{(1,17)}$=0.66, p>0.05, η_G^2=0.01; NS) or Post and Peri-ST groups ($F_{(1,14)}$=0.03, p>0.05, η_G^2=0.04; NS) for the learning portion of testing (Figure 24a). There were no effects of Treatment for the asymptotic portion of DMS testing (Days 4-6; Figure 24b), and there were no effects of Treatment on errors following a 6- or 8-hour delay between trials 1 and 2.

We analyzed the number of perseverations into the previously-platformed arm (PPP Errors) on Days 2-3 of DMS testing to examine the animals' ability to switch from win-stay (return to the same locations) to win-shift (do not return to the same location) behavior across days (there is no previously platformed arm on Day 1). On trial 2, the working memory trial, the Post group made fewer PPP Errors than the Control group ($F_{(1,15)}$= 7.09, $p \leq 0.05$, η_G^2=0.14), indicating that the Control group continued to exhibit win-stay behavior on days 2 and 3 of DMS testing, while the Post animals were able to adapt to the new task rules more quickly. The Post group did not differ from the Peri-LT ($F_{(1,17)}$= 0.16, $p > 0.05$, η_G^2=0.01, NS) or Peri-ST ($F_{(1,14)}$= 2.85, $p > 0.05$, η_G^2=0.10, NS) groups on PPP Errors.

Peripheral Markers of Treatment

Animals in the Control group did not cycle through the four phases of the estrous cycle, but rather exhibited intermittent elongated estrus and diestrus phases, as expected in middle-aged female rats (Mennenga & Bimonte-Nelson, 2015). Animals treated with CEE exhibited consistent estrus smears containing primarily cornified cells, with some leukocytes, as expected (Acosta et al., 2009a, 2009b). We did not observe an effect of Wave, nor a Wave x Treatment interaction, for uterine weights (Wave: $F_{(1,26)}$= 1.90, $p > 0.05$, $\eta^2 < 0.07$, NS; Wave x Treatment: $F_{(3,26)}$= 2.82, $p > 0.05$, NS; η^2=0.24), corpora lutea (Wave: $F_{(1,26)}$= 0.001, $p > 0.05$, $\eta^2 < 0.01$, NS; Wave x Treatment: $F_{(3,26)}$= 0.82, $p > 0.05$, NS; η^2=0.08), or follicles (Wave: $F_{(1,26)}$= 0.57, $p > 0.05$, η^2=0.18, NS; Wave x Treatment: $F_{(3,26)}$= 0.43, $p > 0.05$, NS; η^2=0.05), therefore analyses were collapsed across wave. There was a main effect of Treatment on uterine weights ($F_{(3, 30)}$=4.44; $p \leq 0.05$, η^2=0.31; Figure 25a), such that the Control group had lower uterine weights than all CEE-treated groups (Post: Fisher, $p \leq 0.05$; Peri-LT: Fisher $p \leq 0.05$; Peri-ST: Fisher, $p \leq 0.01$). There was also a main

effect of Treatment on corpora lutea counts ($F_{(3, 30)}$=8.91; p≤0.001, η^2=0.47; Figure 25c), such that animals in the Peri-ST group had more corpora lutea than the Control (Fisher, p≤0.01), Post (Fisher, p≤0.001), and Peri-LT (Fisher, p≤0.0001) animals, indicating that animals in this group have recently ovulated (Haas et al., 2007), as expected, since this group had not completed follicular depletion at the time of sacrifice. There were no effects of Treatment on number of follicles in the ovaries ($F_{(3, 30)}$=1.59; p>0.05, η^2=0.14, NS; Figure 25b), and all treatment groups had fewer than 30 follicles remaining in their ovaries on average, indicating that the VCD treatment effectively depleted follicles in each treatment group.

Hormone Assays

We did not observe an effect of Wave ($F_{(1,25)}$= 0.14; p>0.05, NS, η^2<0.01), nor a Wave x Treatment interaction ($F_{(3,25)}$= 0.39; p>0.05, NS, η^2=0.05) for DMS errors, therefore analyses were collapsed across wave. There were no group differences in serum levels of androstenedione ($F_{(3,29)}$=0.13; p>0.05, η^2=0.02 NS; Figure 26). In all treatment groups, androstenedione levels positively correlated with total WRAM errors on Trials 1-4 across all days of testing (r=0.51, p≤0.05; figure 27), as well as on Trial 4 alone, the trial with the highest working memory load, (r=0.58, p≤0.01; figure 27), a replication of previous findings from our lab (Acosta et al., 2009, 2010; Camp et al., 2012).

Discussion

Here I evaluated whether the timing of treatment initiation relative to follicular depletion, or duration of treatment, alters the cognitive impact of CEE HT in the VCD rat model of transitional menopause. I replicated previous findings (Acosta et al., 2010), that CEE HT initiated after follicular depletion impairs spatial working memory. Relative to

vehicle treatment, CEE administered after follicular depletion produced impairments during the lattermost phase of WRAM testing, did not affect performance on the MM, and improved behavioral flexibility, allowing animals to more quickly adjust to the rules of DMS asymmetrical 3-choice task. Short-term CEE administration initiated at the beginning of follicular depletion improved performance relative to short-term CEE administration initiated after follicular depletion on the lattermost portion of WRAM testing (Peri ST versus Post groups). Short-term CEE administration initiated at the beginning of follicular depletion (Peri ST group) did not impact performance on the MM or the DMS asymmetrical 3-choice task, relative to CEE given after follicular depletion had ensued (Post group). CEE is known to confer several health benefits apart from its potential cognitive impact; therefore, while CEE does not appear to offer benefits for learning and memory outside of very specific treatment parameters, finding a delivery method that is cognitively neutral is nonetheless clinically promising. Our results indicate that current recommendations for HT during menopause, which include individualized decision-making, early initiation, and the shortest possible duration of treatment (NAMS, 2012), are optimal for CEE's cognitive impact.

A benefit of post-depletion CEE treatment did emerge; animals in the Post group made fewer errors than the control animals and the Peri ST group on the first three days of the DMS asymmetrical 3-choice task, as measured by Total Errors. Thus, the Post animals appear to be outperforming the Control group, so long as we operationally define performance as making the fewest number of Total Errors. However, if we pause to consider that this was the third maze in a cognitive battery, and it followed a fully win-stay version of the MM, we may wish to revise our operational definition of 'good' performance

on this new task; at least for the first few exposures, in which animals are not yet aware of the change in task rules. In fact, one may actually consider better performance on the second day of DMS testing, following MM learning, to be returning to the previously rewarded platform location. Prior to the MM, animals learned the WRAM, which also involved a win-stay across days rule; even though animals must not return to platforms within a day, the task starts with the same subset of baited arms at the beginning of each day. Additionally, the win-stay-within-a-day feature of the DMS task likely acts to further reinforce the behavior of returning to the previously rewarded location during the initial exposures to the task. Indeed, when we carefully examined the animals' performance during the first exposures to this task, we made an interesting discovery: the control animals made more errors into the arm of the maze that had contained the platform on the previous day.

The behavioral assessments utilized in this study bring to light the complexity that exists when measuring constructs in non-verbal animals. As behaviorists, we are forced to rely on operational definitions that we can be reluctant to deviate from for various reasons including consistency, replication and ease of interpretation. Because evaluation depends on the task at hand, these operational definitions are typically tied to particular tasks that are used to evaluate specific domains of learning and memory. However, consideration must be given to the greater structure into which each of these individual tasks is implemented. When utilizing multiple tasks in sequence, the way that performance should be defined may be different on the first exposure, depending on what set of rules the animals have previously learned and consistently been rewarded for following. It may seem, at first consideration, that three days of failure to update to this new rule is a

substantial amount of time. However, because the DMS platform location is constant across six trials within each day, it is not until the first trial of the second day of testing that the animals experience the first event contrary to the win-stay rule previously learned. Then, it is not until the first trial of the third day of testing that the second reinforcement opportunity for this new rule is presented. When viewed in this context, it seems reasonable for an animal to return to the previously rewarded location on each of these instances. Thus, we can conclude that the Control animals exhibited a deficit in adapting to the demands of a different spatial learning task, relative to Post animals. However, we do not believe that this is a deficit in spatial memory, as the Control animals exhibited more entries into the arm that they were rewarded for entering repeatedly on the previous day. Rather, this behavior is indicative of an intact spatial memory coupled with difficulty updating to the rules of a new task. Whether this effect is beneficial or detrimental depends on the situation and the way that a 'good' outcome is defined. If we are concerned with an organism's ability to quickly adjust to new situations with changing demands, then Post treatment appears to produce a benefit relative to the Control group. Likewise, if we are concerned with an organism's ability to recall a previously rewarded spatial location, the Post group no longer outperforms the Control group.

As the Peri-ST group had a delayed onset of VCD treatment, their VCD-induced ovarian depletion was not complete at the onset of testing or at sacrifice. While all other groups had completed their depletion process by the beginning of behavioral testing (107±1 days after the first VCD injection), animals in the Peri-ST group began behavioral testing 43±1 days after their first VCD injection, and were sacrificed 72±1 days after their first VCD injection, inside of the 90 days that VCD treatment requires to fully deplete ovarian

follicles. It is likely that the Peri-ST group continued ovulating intermittently throughout testing. Although I did not include a non-VCD control group, all groups exhibited very low follicle counts, relative to what would be expected in a normally cycling animal (Mayer et al., 2002). Elevated follicle and corpora lutea counts observed in the Peri-ST group lend further support to the likelihood that follicular depletion in this group was underway, but not complete.

Of interest, the only indication of a cognitive benefit of CEE HT was seen in the group of animals that was midway through the follicular depletion process. Moreover, the cognitive protection seen with CEE HT administered early during follicular depletion is not seen when the treatment is continued long-term. This is in line with recent findings from the Study of Women's Health Across the Nation (SWAN), indicating that the cognitive impairments seen with menopause, and the cognitive benefits of estrogen-containing HT, are limited to the menopausal transition. In fact, post-menopausal HT use impairs cognition, even when HT was initiated before the final menstrual period (Greendale et al., 2010). Further, the cognitive impairments seen during late perimenopause do not appear to be due to depressive, anxiety, sleep, or vasomotor symptoms (Greendale et al., 2009), implicating a direct effect of perimenopause on learning and memory. The effect sizes of the behavioral differences we report here are small, indicating that group membership explains only a small proportion of variability on these tasks; many other, as of yet undefined, factors are likely to contribute to the cognitive effects of CEE-containing HT during and after menopause. Animal models provide a promising avenue for the exploration of these questions, as they allow systematic manipulation of menopause

variants independently of aging, permitting experimental control not possible in human research.

Several studies offer insight into the putative mechanisms by which the transition to menopause impacts cognition. Degradation of functionality in the septo-hippocampal cholinergic system has been previously proposed as an underlying cause of the window of opportunity for HT following Ovx (Gibbs 2000; Gibbs, 2002; Gibbs 2010). Emerging work also indicates a widespread deregulation of brain metabolic function associated with loss of estrogen stimulation that likely underlies the cognitive impact of menopause and subsequent HT (Yin et al., 2015; Brinton et al., 2015). Our findings indicate that similar mechanisms likely exist in the VCD model of natural menopause, creating a window of opportunity for cognitively safe HT administration. We have previously reported that elevated circulating levels of androstenedione are associated with poorer memory (Acosta et al., 2009, 2010, Camp et al., 2012), and that exogenous androstenedione impairs memory (Camp et al., 2012), an effect that is blocked by preventing its conversion to E1 (Mennenga et al., 2015). Further, we have shown that exogenous E1 administration produces cognitive impairments (Engler-Chiurazzi et al., 2011), and higher circulating levels of E1 are related to poorer memory (Mennenga et al., 2015). These reports, coupled with the current results, lead us to propose that elevated levels of ovarian-produced androstenedione-derived E1, relative to other estrogens, serve to exacerbate the cognitive effects of hormone loss with menopause.

Collectively, these results suggest that there is a limited window of opportunity around the onset of natural menopause, during which benefits can be seen with CEE treatment, and outside of which cognitive detriments are seen with CEE administration,

even if initiation is early. Additionally, I conclude that the ovaries remain active following follicular depletion, and androstenedione-derived E1 from these follicle-deplete ovaries negatively impacts spatial memory. Accumulating research evaluating multiple systems collectively indicates that CEE is not the optimal solution to alleviate the hormonal imbalance brought on by menopause, likely due to its high E1S content. It is possible that an alternative estrogen, such as E2, may act to help restore the hormonal balance that changes with menopause, providing cognitive benefit where CEE produces impairments. These findings draw attention to the need for investigation into the cognitive effects of the transition to menopause, as well as of alternative HTs.

CHAPTER 7: OPTIMIZING HORMONE THERAPY ACROSS THE MENOPAUSE TRANSITION II: BIOIDENTICAL ESTROGEN

Introduction

Around the fifth decade of life, women will experience menstrual irregularity and eventual cessation of the menses known as menopause (NAMS; Curtis et al., 2005; Hoffman et al., 2012). With menopause comes a halting of ovulation, and thus a drastic decline in ovarian production of the hormones estrogen and progesterone. Loss of these hormones results in several undesirable health consequences, including hot flashes, bone density loss, cardiovascular changes, atrophy of vaginal tissue, and cognitive decline (Curtis et al., 2005; Hoffman et al., 2012). Although advances in health and medicine have resulted in an increasing average life expectancy, menopause onset has remained stable and typically begins in a woman's 40's. This means that women are living increasingly larger proportions of their lives (up to 40% of the expected lifespan in the United States) in a hypo-estrogenic menopausal state (NAMS; ACOG Women's Health 2011). Many women choose to utilize estrogen-containing hormone therapy (HT), to alleviate the health consequences of menopause. Conjugated Equine Estrogens (CEE) have been prescribed to menopausal women as HT since 1942, and CEE became the most commonly prescribed HT in the US in the early 2000's; approximately fourteen million women in the US were estimated to use CEE at this time (Hersh et al., 2004; Stefanick, 2005). CEE contains minute amounts of 17β- estradiol (E2), the most potent naturally circulating estrogen in women and rats, and is over 50% estrone sulfate (E1s; Gleason et al., 2005), which is converted to estrone (E1), another estrogen, by the liver.

CEE HT is effective at alleviating many of the non-cognitive symptoms of menopause in women; however, whether CEE prevents the cognitive decline associated with menopause remains uncertain. Several studies have examined the impact of CEE on health and cognition, including the large Women's Health Initiative (WHI). However, in 2002, the WHI announced that the estrogen plus progestin vs placebo trial would be halted due to an increased risk of breast cancer and cardiovascular disease associated with estrogen plus progestin treatment (Writing Group for the Women's Health Initiative Investigators, 2002). Two years later, the WHI memory study (WHIMS), an ancillary study to the WHI, reported that in menopausal women, CEE alone did not affect risk of developing mild cognitive impairment (MCI), and non-significantly increased the risk of probable dementia, while CEE plus progestin treatment did not impact MCI risk and increased the risk of probable dementia in (Espeland et al., 2004; Shumaker et al., 2004). Following the publication and heavy media coverage of the WHI and WHIMS results, many women halted their HT regimens altogether. In fact, according to the American Congress of Obstetricians and Gynecologists (ACOG), roughly 65% of women using HT stopped in response to the 2002 WHI study results. This negative public reaction led to just over 76 million HT prescriptions being dispensed in 2003, compared to almost 129 million prescriptions dispensed three years earlier, in 2000 (ACOG Women's Health, 2011). However, by 2004, the year the WHIMS study results were released, the ACOG reports that one in four of the women who previously discontinued HT re-initiated it (ACOG Women's Health, 2011). Since then, a demand for alternative, safer HTs has led to a huge shift in prescription rates. A 2015 report from the Endocrine Society states that almost half of the prescriptions filled for HT are now custom-compounded bioidentical hormones

(Endocrine Society, 2015). Bioidentical hormones have the exact same chemical and molecular structure as hormones found naturally circulating in the human body prior to menopause; these hormones have gained popularity and have also created a great deal of confusion among the general public about HT options (Sood et al., 2011).

Our lab has previously investigated the cognitive impact of both CEE and so-called 'bioidentical' E2 HT following surgical removal of the ovaries (ovariectomy; Ovx). We have previously shown that CEE HT benefits spatial working memory, a form of short term memory for information that is updated, and spatial reference memory, a form of long term memory for information that stays constant, and protects against scopolamine-induced amnesia (Acosta et al., 2009a). Our and others' laboratories have shown that E2 HT following Ovx can also benefit performance in multiple cognitive domains, including spatial working memory (Daniel et al., 1997; Fader et al., 1999; Bimonte & Denenberg, 1999; Bohacek & Daniel, 2007; Gibbs and Johnson, 2008; Rodgers et al., 2010), and spatial reference memory (Bimonte and Denenberg 1999; Gibbs, 2000; Bimonte-Nelson et al., 2006; Talboom et al., 2008).

Notably, the aging female rat experiences reproductive senescence known as estropause, which includes several hormonal states indicative of irregular ovulation, and differs from human menopause. Estropause involves either a persistent estrus state, or a persistent diestrus state, with hormone profiles dissimilar to those of naturally menopausal women (Lu, 1979). Although the aging rat does not experience menopause, both rodent estropause and human menopause appear to result from simultaneous changes in pituitary gonadotropin release and changes in ovarian function (Wise, 1999; Wise et al., 1999), rendering the rodent a reliable model of the human reproductive system and, to some

extent, of human reproductive aging. Nonetheless, estropause does not produce hormone profiles similar to those seen in natural human menopause. The Ovx model is a valuable model that affords the opportunity to isolate effects of individual ovarian hormones, and it is an appropriate model for the many hundreds of thousands of women who undergo surgically induced menopause (ACOG, 2008; Rocca et al., 2009). However, it has limited generalizability to the population of women who have undergone natural, transitional menopause. Treatment with the industrial chemical 4-vinylcyclohexene diepoxide (VCD) can be used as a rodent model of transitional human menopause. Treatment with VCD accelerates the natural process of atresia in the finite primary and primordial follicle pools, producing a gradual loss of follicles (Flaws et al., 1994; Springer et al., 1996; Springer, McAsey, et al., 1996; Springer, Tilly, et al., 1996; Borman, et al., 1999; Kao et al., 1999; Hu, et al., 2001; Hu et al., 2002; Mayer, et al., 2002; Mayer et al., 2004; Mayer et al., 2005), leading to halting of ovulation and a drastic decrease in ovarian production of estrogens and progesterone (Springer et al., 1996: Mayer, et al., 2004; Mayer et al., 2005), creating a rodent with a hormone profile more similar to that of a naturally menopausal woman than following estropause or Ovx.

Using this VCD model, our lab has demonstrated that transitional hormone loss impairs spatial working memory if the follicle-deplete ovaries are retained, but improves spatial working memory if the residual ovary is removed following follicular depletion, indicating that the follicle-deplete ovary itself is negatively impacting cognition (Acosta et al., 2009). Acosta et al., 2010 reported another important distinction, whereby CEE improves cognition following surgical menopause, but impairs cognition following VCD-induced menopause, showing that follicle-deplete ovaries alter the cognitive impact of HT.

As a whole, these studies imply that the ovaries remain active following follicular depletion, and the hormones produced by these follicle-deplete ovaries may be responsible for memory impairments associated with menopause. This series of results led us to investigate the cognitive impact of the androgen androstenedione. Androstenedione is the primary hormone produced by follicle-deplete ovaries, and it can be converted to E1 via the aromatase enzyme. In several studies, we have reported that high serum levels of androstenedione are associated with worse performance on the WRAM (Acosta et al., 2009b; 2010; Camp et al. 2012), and we have also shown that exogenous administration of androstenedione to Ovx rats impairs spatial memory (Camp et al., 2012). Our lab also demonstrated that exogenous delivery of E1, a metabolite of androstenedione, to Ovx animals produces memory impairment (Engler-Chiurazzi et al., 2012), and that a pharmacological blockade of androstenedione's conversion to E1 prevents its negative cognitive impact (Mennenga et al., 2015). Collectively, these data suggest that androstenedione, the primary hormone released following ovarian follicular depletion, impairs memory in the VCD model of menopause through its conversion to E1.

These experiments led to the development of the hypothesis that high levels of ovarian androstenedione-derived E1 relative to E2 are responsible for impaired memory following follicular depletion. Given this assumption, administration of CEE, composed primarily of E1 sulfate, is unlikely to benefit cognition following follicular depletion. I now suspect that bioidentical E2 may be capable of producing a more favorable cognitive outcome than CEE following ovarian follicular depletion. I predicted that E2 will benefit cognition in follicle-delete rats by bringing hormone ratios closer to those found prior to menopause. The current experiment was conducted to determine how E2 administration to

VCD-treated follicle-deplete rats would impact cognition. I also wished to compare E2 treatment to Ovx, which is the only treatment that has thus far been shown to improve memory following VCD-induced follicular depletion in rodents (Acosta et al., 2009b).

Materials and Methods

Subjects

Subjects were 33 eight month-old Fischer-344 female rats raised at the aging colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN) were used. After arrival, rats were pair-housed, had food and water ad-lib, and a 12-h light/dark cycle. Animals were 11 months old at the initiation of behavioral testing. All procedures were approved by the local Institutional Animal Care and Use Committee (IACUC) and adhered to National Institutes of Health (NIH) standards. Animals were randomly assigned to one of three treatments groups, as follows (n per group): VCD (11), VCD-E2 (11), VCD-Ovx (11).

VCD, Ovx, and E2 Treatments

One week after arrival, animals received VCD treatment (160 mg/kg diluted in 50% DMSO/Saline at a volume of 2.5µl/g body weight; IP; Sigma-Aldrich, St. Louis, MO) exactly as reported previously (Acosta et al., 2009b, 2010). Seventy-four days into the follicular depletion process, Ovx or sham surgeries were conducted. Rats were anesthetized via isoflurane inhalation, received bilateral dorsolateral incisions in the skin and peritoneum, and ovaries and tips of the uterine horn were ligatured and removed. Muscle and skin were then sutured closed. During surgery, rats received an injection of Rimadyl^{TM} (5 mg/ml/kg) for pain, and saline (2 ml) to prevent dehydration. Sham surgeries consisted of skin and muscle incisions and sutures only. Ninety days after VCD treatment began,

animals underwent a second surgery to have a subcutaneous osmotic Alzet pump placed into the scruff of their neck. The Alzet 2006 model was used, which held a total of 200μl of solution, released for 6 weeks, at a rate of 0.15μl per hour. Rats were anesthetized via isoflurane inhalation, a small incision was made, and a subcutaneous pocket was created in the dorsal scruff of the neck. A pump filled with vehicle (polyethylyene glycol) or E2 (3μg/day, released at a steady rate across time) dissolved in polyethylyene glycol was inserted below the skin, and the skin was closed with surgical staples.

One hundred seven days after the beginning of VCD treatment, water radial arm maze testing began, followed by Morris water maze testing. These animals were a subset of a larger group of animals, which also received an additional treatment; therefore, they also received daily subcutaneous injections of 0.5ml PEG, starting on the day of E2 or vehicle pump insertion and continuing to sacrifice. Figure 28 shows the treatment groups and a timeline for the study.

Water Radial Arm Maze

Water radial arm maze (WRAM) procedures were identical to those in chapter 4, except that on the 13th day of testing, a 8-hour delay was given between trials 2 and 3 to test delayed memory retention.

Morris Water Maze

Morris water maze (MM) procedures were identical to those in chapter 5.

Visible Platform Task

Visible platform procedures were identical to those in chapter 4.

Peripheral Markers of Treatment

At sacrifice, uteri of all subjects were removed and trimmed of visible fat, ovaries were removed for histological processing, and wet uterine weight (grams) was measured, exactly as reported elsewhere (Westerlind et al., 1998; Engler-Chiurazzi et al., 2012; Acosta et al., 2009, 2010). Ovaries were removed, trimmed of fat, and preserved in 10% formalin. Sections were then processed for paraffin embedding and sectioned at 5μm, then stained using hematoxylin and eosin. Corpora lutea and follicle populations were counted.

Hormone Assays

Serum was collected at sacrifice via cardiocentesis and processed for levels of androstenedione and Progesterone. An iodinated radioimmunoassay (RIA) was used by the CORE Endocrine Laboratory at Pennsylvania State University, exactly as reported elsewhere (Acosta et al., 2009, 2010) to determine serum hormone levels.

Statistical Analyses

Orthogonal planned comparisons were set a priori; we compared the group that underwent follicular depletion followed by sham surgery and vehicle treatment (VCD group) to the E2-treated animals (VCD-E2 group) and the Ovx animals (VCD-Ovx group). We predicted that the VCD-Ovx group would outperform the VCD group on the WRAM; this would be a replication of our prior findings using the VCD model (Acosta et al., 2009b). We predicted that E2 treatment would also improve performance on the WRAM task, however the cognitive impact of E2 has never been investigated following VCD treatment. Thus, alpha level was set at 0.05 (1-tail) for VCD vs VCD-Ovx comparisons, and alpha level for comparisons of VCD versus VCD-E2 was set at 0.05 (2-tail).

WRAM errors were divided into 3 testing blocks of 4 days each, except the first testing block, which only included three days because we excluded the first day of maze testing (Block 1: Days 2-4, Block 2: Days 5-8, Block 3: Days 9-12). Data were analyzed with repeated measures ANOVA. Treatment was used as the between-groups variable and number of errors on each trial was the dependent variable.

MM data were also analyzed with repeated measures ANOVA. Again, treatment served as the between-groups variable, however distance to the platform was the dependent variable for MM analyses. Probe trial data were analyzed with repeated measures ANOVA with quadrant (Northeast vs. Southwest) as the between-groups variable and percent swim distance as the dependent variable. MM probe analysis was necessary to determine whether any of the treatments impacted animals' use of a spatial navigation strategy.

Wet uterine weights, serum hormone levels, ovarian follicles, and corpora lutea were each analyzed using one-way ANOVA with Treatment as the between-groups variable in each analysis and wet uterine weights (g), circulating hormone levels, estimated number of follicles, and number of corpora lutea as the respective dependent variables for each analysis. Fisher's post-hoc tests were used following significant omnibus tests.).

Generalized eta squared (η_G^2) is reported for mixed designs that utilize both between- and within- subjects variables (WRAM, MM, DMS behavior data), and eta squared (η^2) is reported for analyses that include only a single between-subjects independent variable (Wet uterine weights, serum hormone levels, ovarian follicles, and corpora lutea), to allow comparability of effect sizes across variables (Olejnik & Algina, 2003; Bakeman, 2005). Effect sizes are interpreted by Cohen's guidelines for η^2 (0.02=small, 0.13=medium, 0.26=large; Cohen, 1988; Bakeman, 2005).

Results

Water Radial Arm Maze

On the first block of WRAM testing, days 2-4, there was a Trial x Treatment interaction for the VCD and VCD-Ovx groups ($F_{(3,60)}$= 2.52, $p<0.05$; η_G^2=0.04; figure 29), such that on trial 4, the trial with the highest working memory load, the VCD animals made more errors than the Ovx animals ($F_{(1,20)}$= 2.96, $p<0.05$; η_G^2=0.06; figure 29). There was also a marginal Trial x Treatment interaction for the VCD and VCD-E2 groups ($F_{(3,60)}$= 2.48, $p<0.10$; η_G^2=0.04; figure 29), such that on trial 4, the trial with the highest working memory load, the VCD animals made marginally more errors than the VCD-E2 animals ($F_{(1,20)}$= 2.94, $p<0.10$; η_G^2=0.06; figure 29). On the second testing block, days 5-8, there was a Trial x Treatment interaction for the VCD and VCD-E2 groups ($F_{(3,60)}$= 3.70, $p<0.05$; $\eta_G^2<0.01$; figure 29). On Trial 4, the trial with the highest working memory load, E2 treatment enhanced performance; the E2 animals made fewer errors than the VCD group ($F_{(1,20)}$= 4.68, $p<0.05$; η_G^2=0.04; figure 29). There were no effects of Treatment on Days 9-12 of testing (figure 29).

Morris Water Maze

There were no effects of Treatment on MM performance for the VCD versus Ovx ($F_{(1,20)}$= 0.86, p=0.37, NS; η_G^2=0.01) or for the VCD versus E2 ($F_{(1,20)}$= 0.46, p=0.51, NS; η_G^2=0.01) groups. On the probe trial, there was a main effect of Quadrant ($F_{(1,30)}$= 150.65, $p<0.0001$; η_G^2=0.80), with no effect of Treatment ($F_{(2,30)}$= 0.68, p= 0.52; η_G^2=0.01; NS), or Quadrant x Treatment interaction ($F_{(2,30)}$= 0.67, p= 0.52; η_G^2=0.03; NS).

Peripheral Markers of Treatment

There were no differences in total number of follicles present in the ovaries of the E2 and Vehicle groups at sacrifice ($F_{(1,20)}$= 0.93, p= 0.35; η^2=0.04; NS; figure 30), or the number of corpora lutea ($F_{(1,20)}$= 0.46, p= 0.51; η^2=0.02; NS; figure 30). There was a main effect of Treatment on wet uterine weights ($F_{(2,30)}$= 14.93, p<0.0001; η^2=0.50; figure 30), with E2-treated animals having heavier uterine horns than vehicle-treated or Ovx animals (Fisher, p<0.0001). There was no difference between the Vehicle and Ovx groups in uterine weight, indicating a lack of uterine stimulation in the Vehicle-treated VCD animals.

Serum Hormone Levels

There was a main effect of treatment on serum E2 levels ($F_{(2,30)}$= 17.85, p<0.0001; η^2=0.54; figure 31), with the E2-treated group showing higher circulating levels of E2 than both the Ovx and Vehicle groups (Fisher, p<0.0001). There was no difference in serum E2 levels between the Ovx and Vehicle groups at sacrifice (Fisher, p=0.76; NS; Vehicle M=5.42pg/ml, Ovx M=1.45pg/ml), suggesting there is a negligible amount of ovarian-derived circulating E2 in animals treated with the VCD regimen utilized here.

There was also a main effect of treatment on serum E1 levels ($F_{(2,27)}$= 21.55, p<0.0001; η^2=0.62; figure 31), with the E2-treated group showing higher circulating levels of E1 than both the Ovx and Vehicle groups (Fisher, p<0.0001). There was no difference in serum E1 levels between the Ovx and Vehicle groups (Fisher, p=0.64, NS; Vehicle M=39.42pg/ml, Ovx M=36.76pg/ml), again suggesting a very minor amount of ovarian-derived estrogen in our Vehicle group following VCD-induced follicular depletion.

Finally, there was a main effect of treatment on serum androstenedione levels ($F_{(2,27)}$= 8.86, p<0.01; η^2=0.40; figure 31), with the E2-treated group and the Ovx group

showing lower circulating levels of androstenedione than the Vehicle group (Fisher, $p<0.01$). There was no difference in serum androstenedione between the Ovx and E2 groups (Fisher= 0.22, NS; Ovx M= 0.09ng/ml, E2 M=0.21ng/ml), indicating that treatment with E2 reduces circulating androstenedione levels to the same extent as surgical removal of the follicle-deplete ovaries.

Discussion

Through a series of studies, I have investigated how various HT parameters, including timing of administration, length of exposure, and type of estrogen, modify the cognitive effects of HT following VCD-induced follicular depletion in rodents. Our lab has shown previously that CEE treatment initiated post-depletion produces memory detriments (Acosta et al., 2010), and that surgical removal of follicle-deplete ovaries produces a cognitive benefit (Acosta et al., 2009b). I now demonstrate that bioidentical E2 has a favorable impact on cognition following follicular depletion, possibly because of E2's ability to correct the hormonal imbalances created by disruption of ovulation. In the current study, E2 treatment produced marginal benefits on the earliest block of WRAM testing, and improved performance on the second testing block of WRAM. Benefits of E2 on radial arm maze performance have been previously reported numerous times in Ovx animals (Daniel et al., 1997; Fader et al., 1999; Bimonte & Denenberg, 1999; Bohacek & Daniel, 2007; Gibbs and Johnson, 2008; Rodgers et al., 2010), but the cognitive impact of E2 had previously never been investigated following VCD-induced follicular depletion. E2 is now the fist clinically-utilized HT to be shown to improve cognition in the VCD rodent model of menopause.

Ovx during follicular depletion also improved performance on the initial learning phase of WRAM testing. Acosta et al. (2009b) reported a benefit of Ovx treatment following a 4-hour delay between trials 2 and 3 of the WRAM in 14 month-old VCD-treated rats. Acosta et al., 2010 utilized slightly younger rats that were 11 months old at the initiation of maze testing, and did not find differences between vehicle- and CEE-treated groups following a 4-hour delay. In the present study, animals were 11 months old at the initiation of maze testing, identical to the age in Acosta et al., 2010, which prompted us to instill a more challenging, 8-hour delay at the end of WRAM testing. Our groups did not differ in performance following the delay, and all groups performed very well in spite of the challenge, making fewer than 3 total errors on average across the post-delay trials. The younger age of these animals likely produced a steeper learning curve than what a 14-month-old rodent would exhibit, resulting in Ovx benefits manifesting earlier during WRAM testing than what was observed in Acosta et al., 2009b.

Serum hormone levels of E1, E2 and androstenedione were measured to gain insight into the potential mechanism by which each of these treatments may be impacting brain function. Here, E2 treatment increased circulating E1 and E2, and decreased circulating androstenedione levels, compared to vehicle treatment. These effects were large and, together, amount to a substantial multi-hormone shift towards a hormone profile similar to pre-follicular-depletion (increased E1 and E2, decreased androstenedione), possibly resulting in the observed cognitive benefit of E2 treatment. Ovx during follicular depletion resulted in decreased androstenedione levels, but did not alter E1 or E2 levels relative to sham surgery with vehicle treatment. Interestingly, this set of hormonal changes represents only a partial shift towards the pre-follicular-depletion hormone profile, slightly

less than what is produced by E2 treatment. This partial shift is in line with the less robust cognitive benefits observed with Ovx, relative to E2. Exactly how these hormone levels impact brain function and spatial memory remains to be determined. Recent work indicates loss of estrogen signaling as a trigger for extensive metabolic dysfunction in brain cells (Yin et al., 2015; Brinton et al., 2015); this metabolic deregulation is a potential downstream result of ovarian follicular depletion that would likely be offset by exogenous E2 treatment.

Importantly, the timing of the E2 administration regimen here, where we show that post-depletion administration of E2 improves memory, was identical to that of the CEE administration in Acosta et al. (2010), where we showed that post-depletion administration of CEE impairs memory in the VCD model of menopause. It remains to be determined whether the same cognitive benefits would be seen with E2 administered during follicular depletion. Of note, the E2 administration regimen utilized here, as well in as clinical E2-containing HTs, is insufficient to prevent pregnancy. Gynecologists now recommend that sexually active women utilize some form of contraception during perimenopause, due to the risks that unintended pregnancies during this time pose to the mother and fetus (Ikhena and Johnson, 2012). Notably, the hormones utilized in contraceptive formulations are different than those marketed as HTs. Ethinyl estradiol (EE), a synthetic form of natural E2, is the primary estrogen utilized in contraceptive formulations (Curtis et al., 2004; Hoffman et al., 2012). Many women choose to take EE-containing contraceptives over E2-containing HT in order to prevent unintended pregnancy during the transition to menopause. Future studies investigating the cognitive and whole-body impact of each of these estrogens during perimenopause are necessary.

The results presented here lend support to the hypothesis that androstenedione-derived E1 produces cognitive impairments in follicle-deplete rodents. It follows that, although both E1 and E2 are bioidentical hormones, administration of E1-containing compounds is unlikely to benefit cognition following follicular depletion, whereas administration of E2 should provide cognitive protection. The findings reported here have considerable clinical implications; beginning with the broad message that HT will likely produce optimal benefits with minimal risks only if it is tailored to each woman's personal hormonal makeup. Further, as a woman's hormone profile changes across time, different HT regimens may offer cognitive benefit or detriment. A recent publication from the Study of Women's Health Across the Nation (SWAN) identifies four distinct clusters of E2 change patterns across the menopausal transition, as well as 3 distinct patterns of follicle-stimulating hormone change (Tepper et al., 2012). Understanding and predicting these hormone change trajectories at an individualized level may prove to be crucial for optimization of HT during the menopausal transition. Further individualization along with modeling of the natural hormone cycle will likely result in a more comprehensive HT that confers even greater cognitive and general health benefits.

CHAPTER 8: PHARMACOLOGICAL BLOCKADE OF THE AROMATASE ENZYME, BUT NOT THE ANDROGEN RECEPTOR, REVERSES ANDROSTENEDIONE-INDUCED COGNITIVE IMPAIRMENTS IN YOUNG SURGICALLY MENOPAUSAL RATS

Introduction

By the year 2050, the population over the age of 65 in the U.S. is projected to reach 88.5 million people, more than double what it was in the year 2010, and more than half of the population will be female (US Census, 2010). Around the fifth decade of life, most females experience menopause, whereby eggs stop maturing, and eventually ovulation and menstruation cease. With this reproductive senescence, there is a drastic loss of ovarian-derived estrogen and progesterone, and the androgen androstenedione becomes the principal hormone released by the ovaries (Timaras et al., 1995). This androgen-rich hormone milieu is also seen in a rodent model of natural menopause via treatment with 4-vinylcyclohexene diepoxide (VCD), an industrial chemical that induces gradual depletion of primary and primordial follicles in the female rat (Mayer at al., 2002, 2004; Acosta et al., 2009b, 2010).

Accumulating evidence in the female rat suggests that androstenedione has a negative impact on cognition. Our laboratory previously demonstrated that VCD-induced, transitional menopause in middle-aged, female rats elicits inferior cognitive performance across multiple domains, compared to rats that had undergone surgical menopause via ovariectomy (Ovx). Of note, this finding is not apparent in animals that have undergone Ovx following their VCD treatment; such that the follicle-deplete ovaries were removed after follicular depletion had ensued (Acosta et al., 2009b). An unexpected finding from

this study was that higher serum levels of androstenedione, which is released from the follicle-deplete menopausal ovary (Timaras et al., 1995), correlated with poorer memory scores in follicle-deplete, VCD treated rats (Acosta et al., 2009b). In a follow-up study, we again found that higher androstenedione levels correlated with impaired performance in transitionally menopausal rats demonstrating an androgen-rich serum profile (Acosta et al., 2010). This correlation was evident for multiple types of errors representing several domains of memory, including reference memory, a form of long-term memory that remains constant across all days and trials, as well as two orthogonal measures of working memory, a form of short-term memory that requires updating of information (Acosta et al., 2010). If androstenedione is truly related to poorer memory, impairments should be revealed after administration of androstenedione to a "blank" ovarian hormone template. To test this hypothesis, I performed a study in which middle-aged (14 month old) Ovx rats were administered either vehicle or one of two doses of androstenedione, and then tested with a battery of mazes that assess learning and memory. Relative to vehicle treatment, androstenedione administration impaired spatial reference memory on the Morris water maze, was detrimental to performance on the water radial-arm maze (WRAM) when the working memory load was most demanding, and impaired memory retention on a win-stay delay match to sample (DMS) task (Camp et al., 2012). Thus, in several different studies we have shown that androstenedione, released from the follicle-deplete ovary in both women and rats, markedly impairs memory.

Understanding the effects of androstenedione on the brain and its function is critically important to understanding the cognitive impact of natural menopause; ovarian-derived androstenedione is present in menopausal women who maintain their ovaries, an

effect observed for at least ten years after menopause ensues (Fogle et al., 2007). Drugs that block the activity of the aromatase enzyme (Santen et al., 2009), which catalyzes the conversion of androstenedione to the estrogen estrone, are some of the tools used to treat metastatic breast cancer prevalent in menopausal women (Glück et al., 2013), as well as manage estrogen-dependent endometrial carcinoma (Gao et al., 2014). Here, we seek to decipher the hormone mechanism(s) underlying the negative cognitive impact of androstenedione using a rat model. Androstenedione could be exerting cognitive effects through a multitude of mechanistic pathways; it is a direct precursor to testosterone via the 17β-hydroxysteroid dehydrogenase (17β-HSD) enzyme, and to estrone via the aromatase enzyme, and, further, it binds to androgen receptors (Horton & Tait, 1966; Jasuja et al., 2005). In the rodent model, testosterone administration has been shown to enhance spatial working memory (Bimonte-Nelson et al., 2003b), spatial reference memory (Benice & Raber, 2009), and performance on avoidance tasks (Flood et al., 1995; Edinger et al., 2004). There is also evidence that higher relative levels of testosterone are associated with better spatial ability performance in women, while lower relative levels of salivary testosterone were related to better spatial ability performance in men (Gouchie & Kimura, 1991). We have previously shown that estrone administration in Ovx rats produces cognitive impairments (Engler-Chiurazzi et al., 2012). Given these results, we now hypothesize that androstenedione's conversion to estrone underlies its negative cognitive impact, rather than its actions on the androgen receptor.

The primary purpose of the current study was to systematically evaluate whether androstenedione's conversion to estrone, or its effects on the androgen receptor, are responsible for the negative cognitive effects of androstenedione administration in the

surgically menopausal young adult rat. Herein, I tested the hormonal mechanism underlying the previously observed androstenedione-induced cognitive impairments using pharmacological manipulations that either block androstenedione's conversion to estrone, or block androstenedione's androgenic effects by blocking activation of the androgen receptor. Anastrozole, a non-steroidal aromatase inhibitor, or flutamide, a non-steroidal anti-androgen, were co-administered with androstenedione to determine whether androstenedione impairs memory via its conversion to estrone, or via its action on the androgen receptor, respectively. A secondary purpose of this study was to test the effects of anastrozole given alone. Indeed, aromatase inhibitors such as anastrozole are currently used to treat breast cancer and prevent breast cancer recurrence (Santen et al., 2009). Elucidating the impact of aromatase and estrogen metabolism on the brain and its function is critical to our understanding of the systems-level alterations that occur with changes in both endogenous and exogenous steroid hormones.

Materials and Methods

Subjects

Forty-eight four-month-old Fischer-344 virgin female rats born and raised at the National Institute on Aging colony at Harlan Laboratories (Indianapolis, IN) were used. Upon arrival, rats were pair housed, had access to food and water ad-lib, and were maintained on a 12-hour light/dark cycle at the Arizona State University animal facility. All procedures were approved by the local IACUC committee and adhered to NIH standards. Rats arrived two weeks before experiment initiation.

Experimental Design and Hormone Treatments

All rats received Ovx 13-14 days before the start of behavioral testing. Animals received bilateral dorsolateral incisions in the skin and peritoneum, the ovaries and tips of the uterine horns were ligatured and removed, and the muscle and skin were then sutured closed. During surgery, rats received an injection of Rimadyl (5mg/ml/kg) for pain and saline (2ml) to prevent dehydration. Hormone or vehicle treatment began 2-3 days after surgery (11 days before behavioral testing ensued) and continued until sacrifice. All assigned treatments were administered daily via subcutaneous injection into the scruff of the neck at an injection volume of 0.5ml. Rats were randomly assigned to one of five treatment groups: Vehicle (n=10), Androstenedione (n=10), Androstenedione+Anastrozole (n=10), Androstenedione+Flutamide (n=10), and Anastrozole (n=10). Vehicle-treated animals received 0.5ml of polyethylene glycol (PEG) (Sigma-Aldrich, St. Louis, MO, USA) only. All rats receiving androstenedione (Steraloids, Newport, RI, USA) were given 2mg daily dissolved in PEG; this dose of androstenedione was based on previous literature (Lea & Flanagan, 1998; Sprando et al., 2004; Camp et al., 2012) and has been shown to produce working memory impairments in middle-aged Ovx rats (Camp et al., 2012). Animals in the Androstenedione+Anastrozole group received 0.025mg/day anastrozole (Tocris, Minneapolis, MN, USA) co-administered with 2mg androstenedione treatment, in order to block activity of the aromatase enzyme, preventing the conversion of androstenedione to estrone. Animals in the Androstenedione+Flutamide treatment group received 27.5mg of flutamide (Sigma-Aldrich, St. Louis, MO, USA) co-administered with 2mg androstenedione treatment, to block the action of testosterone on androgen receptors. The Anastrozole treatment group received 0.025mg/day anastrozole dissolved in PEG.

Twelve days after the initiation of hormone treatment administration, behavioral testing began. Behavioral testing commenced approximately one hour after injections each day, and all treatment groups were counterbalanced across testing squads. All rats were subjected to the complete battery of behavioral evaluations. The order of behavior tests is concordant with our prior studies showing correlations between serum androstenedione levels and memory (Acosta et al., 2009b; Acosta et al., 2010; Camp et al., 2012). Figure 32 contains a timeline with depictions of each behavioral task used.

Water Radial Arm Maze

WRAM procedures were identical to those in chapter 4.

Delay Match-to-Sample Asymmetrical Three-Choice Task

Delay-match-to-sample (DMS) procedures were identical to those described in chapter 6, except that animals were given 7 days to learn the task.

Morris Water Maze

Morris water maze procedures were identical to those in chapter 4.

Visible Platform Task

Visible platform procedures were identical to those in chapter 4.

Uterine Weights

Prior studies have shown that androgens can stimulate the uterus and lead to increased uterine weight (Ruizeveld de Winter et al., 1991; Horie et al., 1992). To further validate androstenedione's effects on uterine tissues, at sacrifice the uteri of all subjects were removed, trimmed of visible fat, and immediately weighed (wet weight; g).

Serum Hormone Levels

At the time of sacrifice, blood was collected via cardiocentesis. Blood was allowed to clot at 4°C (Vacutainer 367986, Becton Dickinson and Company, Franklin Lakes, NJ, USA), serum was collected after centrifugation for 20min at 4°C, and serum was stored at -20°C until assays were performed. Serum hormone levels were determined by radioimmunoassay using previously described methods (Acosta et al., 2010; Camp et al., 2012). Androstenedione was measured in serum using a solid-phase radioimmunoassay (Beckman-Coulter, Webster, TX), based on androstenedione-specific antibodies immobilized to the wall of polypropylene tubes and a ^{125}I-labeled androstenedione tracer. Interassay Precision: CV of 7% at mean of 1.1ng/ml (3.8nmol/L), CV of 5% at mean of 3.8 ng/ml (13.3nmol/L). Functional Sensitivity: 1ng/ml.

Testosterone was determined in serum using a competitive solid-phase radioimmunoassay (Beckman-Coulter, Webster, TX) that relies on testosterone-specific antibodies that are immobilized to the wall of polypropylene tubes and compete for testosterone in the sample or purified testosterone standards with ^{125}I-labeled testosterone added to the tube as the tracer. Interassay Precision for the assay averages 7% at a mean value of 84ng/dl (2.9nmol/L) and less than 5 % at a mean value of 403ng/dl (13.9nmol/L). Functional sensitivity of the assay is 15ng/dl (0.5nmol/L).

Estrone was determined in serum using a competitive radioimmunoassay (Beckman-Coulter, Webster, TX) with ^{125}I-labeled estrone and a highly specific primary antibody. Separation of bound and free antigen was achieved using a double antibody system. Interassay Precision for the assay averages 11% at a mean value of 35pg/ml. Functional sensitivity of the assay is 5pg/ml.

Statistical Analyses

Statistical analyses were identical to those in chapter 4, with the exceptions that WRAM testing was divided into three four-day blocks (Block 1=Days 1-4, Block 2=Days 5-8, Block 3=Days 9-12), and we did not choose comparisons a priori. For DMS testing, data were analyzed using repeated measures ANOVA with Treatment as the independent variable and number of total errors across Days and Trials as the repeated measure. Morris water maze testing was blocked into six three-trial blocks (two Blocks per Day) and analyzed using repeated measures ANOVA with Treatment as the independent variable and swim distance across Blocks and Trials as the repeated measure. Probe trial data were analyzed identically to the analysis in chapter 4. Visible platform data were analyzed identically to the analysis in chapter 4.

Two-tailed tests were used throughout, and alpha was set at 0.05. Uterine weights (g), serum androstenedione levels (ng/ml), serum testosterone levels (ng/dl), and serum estrone levels (pg/ml) were analyzed separately using one-way ANOVA, with each respective measure as the dependent variable and Treatment as the independent variable.

Results

Water Radial Arm Maze

Errors decreased across block for all three memory measures on the WRAM, indicating learning (main effect of Block for WMC [$F_{(2,88)} = 52.13$, $p < 0.0001$], WMI [$F_{(2,88)} = 55.39$, $p < 0.0001$], and RM [$F_{(2,88)} = 69.25$, $p < 0.0001$] errors. There were no Treatment effects for WMC, WMI, and RM for Block 1 (Days 1-4) or Block 2 (Days 5-8) of WRAM testing. We have previously observed effects of exogenous treatment with both androgens and estrogens during the latter portion of testing, so we were particularly interested at

effects at the latter testing block (Acosta et al., 2010; Bimonte & Denenberg, 1999; Bimonte-Nelson et al., 2003b; Camp et al., 2012). On Block 3, as predicted, a general pattern emerged, revealing that androstenedione-induced impairments were negated by the addition of the aromatase inhibitor anastrozole, but not by blockade of the androgen receptor through the addition of flutamide. This pattern was observed for all three types of errors evaluated on the WRAM.

For Block 3 of WRAM testing, there was a Treatment x Trial interaction for WMC errors [$F_{(8,88)}$ =3.05, $p<0.01$; figure 33a]. For Trial 4, the trial with the highest working memory load, there was a main effect of Treatment for WMC errors [$F_{(4,44)}$ =4.31, $p<0.01$; figure 33a]; post hoc analysis revealed that, on Trial 4, the Androstenedione group committed more WMC errors compared to the Vehicle group (Fisher, $p<0.001$); the addition of aromatase inhibition via anastrozole treatment reversed this androstenedione-induced impairment [Androstenedione vs. Androstenedione+Anastrozole, Fisher, $p<0.01$]. At the highest memory load for WMC, the Androstenedione group also made more errors than the Anastrozole group (Fisher, $p<0.01$) group, and the Androstenedione+Flutamide group committed more errors than the Vehicle group (Fisher, $p<0.05$).

Similar to the effect on Block 3 for WMC, there was also an effect on Block 3 for WMI, with a Treatment x Trial interaction for WMI errors [$F_{(12,132)}$ =5.36, $p<0.0001$; figure 33b]. For Trial 4, there was a main effect of Treatment for WMI errors [$F_{(4,44)}$ =5.90, $p<0.001$; figure 33b]; post hoc analysis revealed that, on this trial requiring the highest working memory demand, the Androstenedione group committed more WMI errors compared to Vehicle (Fisher, $p<0.01$). Again, the addition of anastrozole reversed the impairing effect of andostenedione at the highest working memory load [Androstenedione

vs Androstenedione+Anastrozole (Fisher, $p<0.01$)], and the Androstenedione group made more errors than the Anastrozole group (Fisher, $p<0.01$). Post hoc analysis also demonstrated that the Androstenedione+Flutamide group committed more WMI errors on Trial 4 than the Vehicle (Fisher, $p<0.01$), Androstenedione+Anastrozole (Fisher, $p<0.01$), and Anastrozole (Fisher, $p<0.01$) groups.

A main effect of Treatment for RM errors was also revealed [$F_{(4,44)} =6.30$, $p<0.001$; figure 33c] for Block 3 of WRAM testing. Post hoc analysis demonstrated that the Androstenedione group committed more RM errors than the Vehicle group (Fisher, $p<0.001$), and, in accordance with effects for both orthogonal working memory error types for the WRAM, the addition of anastrozole reversed reference memory impairments induced by androstenedione [Androstenedione vs. Androstenedione+Anastrozole, Fisher, $p<0.05$]. The Androstenedione group also made more RM errors than the Anastrozole group (Fisher, $p<0.05$), and the Androstenedione+Flutamide group committed more RM errors compared to Vehicle (Fisher, $p<0.001$), Androstenedione+Anastrozole (Fisher, $p<0.01$), and Anastrozole (Fisher, $p<0.05$) groups.

Hormone treatment did not impact performance on the delayed memory retention of multiple platform locations, as there were no treatment effects on the post-delay trials on Day 13 for WMC, WMI, or RM errors on the WRAM.

Delay Match-to-Sample Asymmetrical Three-Choice Task

There was a main effect of Day [$F_{(6,264)} =17.17$, $p<0.0001$] with Total Errors decreasing as days progressed. There were no Treatment effects for Total Errors (Days 1-7; figure 34), nor was there a Treatment x Day interaction.

Morris Water Maze

Analyses revealed a main effect of Block [$F_{(5,220)}$ =150.06, p<0.0001], with swim distance decreasing across blocks showing learning. There was a Treatment x Block interaction for Morris water maze testing [$F_{(20,220)}$=1.84; p<0.05; figure 35a]. For Block 1, there was a main effect of Treatment [$F_{(4,44)}$=2.96; p<0.05; figure 35b]; post hoc analyses revealed that the Androstenedione+Anastrozole group swam a shorter distance to the platform than the Vehicle (Fisher, p<0.05), Androstenedione (Fisher, p<0.05), and the Androstenedione+Flutamide group (Fisher, p<0.01). For the probe trial, there was a main effect of Quadrant [$F_{(1,44)}$=982.20; p<0.0001; figure 35c] in the absence of a Quadrant x Treatment interaction [$F_{(4,44)}$=2.30; p>0.05, NS; figure 35c], indicating that all groups equally localized the platform using spatial navigation by the end of Morris water maze testing.

Visible Platform Task

Figure 36 shows the mean+SEM latency to escape value for each group across all trials for the one day of visible platform testing. There was a main effect of Trial [$F_{(5,220)}$ =9.32, p<0.0001], with latency decreasing as trials progressed within the day of visible platform testing (figure 36). There were no Treatment main effects [$F_{(4,44)}$=1.49, p<0.05, NS] on latency to escape for the visible platform task. However, there was a Treatment x Trial interaction [$F_{(20,220)}$ =2.35, p<0.01], such that there was a main effect of Treatment on Trial 1 [$F_{(4,44)}$ =3.65, p<0.05]. Further analyses indicated that the Vehicle group took a longer time to reach the platform than the Androstenedione (Fisher, p<0.05), Androstenedione+Anastrozole (Fisher, p<0.01), and Anastrozole (Fisher, p<0.001) groups; no hormone treated group differed from any other hormone treated group. Most

importantly, there were no effects of Treatment on any of the remaining trials (Trials 2-6), each animal successfully located the platform on every trial, and by the last trial, all groups found the platform within 16s, thereby allowing interpretation that animals demonstrated the procedural skills necessary to complete a water maze task.

Uterine Weights

There was a main effect of Treatment for uterine weights [$F_{(4,43)}$=13.03; $p<0.0001$; figure 37]. The Androstenedione group had higher uterine weights than the Vehicle (Fisher, $p<0.0001$), Androstenedione+Anastrozole (Fisher, $p<0.001$), Androstenedione+Flutamide (Fisher, $p<0.0001$), and Anastrozole (Fisher, $p<0.0001$) groups. The Androstenedione+Anastrozole group also had higher uterine weights than the Anastrozole group (Fisher, $p<0.05$).

Serum Hormone Levels

There was a main effect of Treatment for serum androstenedione [$F_{(4,31)}$=6.04; $p<0.01$; figure 38a]. Androstenedione treatment increased serum androstenedione levels in all groups receiving this androgen, relative to vehicle treatment [Vehicle vs. Androstenedione (Fisher, $p<0.01$), Vehicle vs. Androstenedione+Flutamide (Fisher, $p<0.05$), Vehicle vs. Androstenedione+Anastrozole (Fisher, $p<0.01$)], and relative to treatment with anastrozole alone [Anastrozole group vs. Androstenedione group (Fisher, $p<0.001$), Anastrozole vs. Androstenedione+Flutamide group (Fisher, $p<0.05$), Anastrozole vs. Androstenedione+Anastrozole group (Fisher, $p<0.01$)].

A main effect of Treatment for serum testosterone was also demonstrated [$F_{(4,28)}$=4.60; $p<0.01$; figure 38b]. The Androstenedione group had higher testosterone serum levels than the Vehicle (Fisher, $p<0.01$) and Anastrozole (Fisher, $p<0.01$) groups,

and the Androstenedione+Anastrozole group also had higher serum testosterone levels than the Vehicle (Fisher, $p<0.05$) and Anastrozole (Fisher, $p<0.05$) groups.

The analysis of serum estrone revealed a main effect of Treatment as well [$F_{(4,26)}=96.67$; $p<0.0001$; figure 38c]. The Androstenedione group had higher serum estrone levels than the Vehicle group (Fisher, $p<0.001$), and the addition of anastrozole decreased estrone levels (Androstenedione vs. Androstenedione+Anastrozole Fisher, $p<0.05$), confirming that the anastrozole treatment used herein effectively reduced androstenedione's conversion to estrone. The Androstenedione group also had higher serum levels than the Anastrozole group (Fisher, $p<0.001$), and the Androstenedione+Flutamide group had higher serum estrone levels than the Vehicle (Fisher, $p<0.0001$), Anastrozole (Fisher, $p<0.0001$), Androstenedione (Fisher, $p<0.0001$), and Androstenedione+Anastrozole groups (Fisher, $p<0.0001$). Additionally, the Androstenedione+Anastrozole group tended to have higher serum estrone levels than both the Vehicle ($p=0.05$), and Anastrozole ($p=0.05$) groups, suggesting that the addition of anastrozole did not completely block aromatase activity in this model.

Correlations Between Serum Hormone Levels and Behavioral Tests

Serum estrone levels correlated with average Total Errors on Block 3 of WRAM testing across all four trials ($r=0.39$; $p<0.05$; figure 39a), as well as on Trial 4, the trial with the highest working memory load ($r=0.36$; $p<0.05$; figure 39b). Because we found a clear bimodal distribution in estrone levels, whereby the Androstenedione+Flutamide group had higher estrone levels than all other groups and therefore held the potential to exert a large amount of influence over these analyses, we also assessed each of these correlations excluding the Androstenedione+Flutamide group. With the Androstenedione+Flutamide

group excluded, we found that serum estrone levels still correlated with average Total Errors on Block 3 of WRAM testing across all four trials ($r=0.58$; $p<0.01$; figure 39a), as well as on Trial 4, the trial with the highest working memory load ($r=0.62$; $p<0.01$; figure 39b).

Discussion

Our laboratory has recently reported that androstenedione produces spatial memory impairments in the female rat. Specifically, we have found positive correlations between endogenous androstenedione levels and maze error scores, and subsequently confirmed these relationships by methodically manipulating androstenedione levels in older Ovx rats and showing that exogenous androstenedione treatment impairs memory across multiple domains (Acosta et al., 2009b, 2010; Camp et al., 2012). The present goals were to extend our previous findings and demonstrate that exogenous androstenedione administration produces memory impairment in young adult animals, and to evaluate the hormonal mechanism(s) underlying these androstenedione-induced cognitive impairments. Because we have previously observed spatial memory impairments following tonic administration of estrone (Engler-Chiurazzi et al., 2012), I hypothesized that the conversion of androstenedione to estrone was, at least in part, responsible for the memory impairments observed when androstenedione is administered to otherwise ovarian-hormone blank (Ovx) animals.

Replicating our previous findings in middle-aged animals, in the current study androstenedione impaired several dimensions of cognition including spatial reference and working memory in young adult Ovx rats. Offering support to our hypothesis regarding the mechanism underlying these effects, androstenedione administration did not induce

memory impairments on any measure evaluated here when it was paired with an aromatase inhibitor, anastrozole. Anastrozole blocks the activity of the aromatase enzyme, which is responsible for the conversion of androstenedione to estrone. This treatment still allows the exogenously delivered androstenedione to act both directly as well as indirectly, through its conversion to testosterone, on the androgen receptor. Pharmacological blockade of androgen receptor activation did not block the cognitive impairing effects of androstenedione. Together, these findings offer support to the tenet that androstenedione produces robust memory impairments due to its conversion to estrone, rather than due to its androgenic effects.

Androgens are typically thought of as masculine hormones and are rarely associated with menopause. However, increasing evidence indicates that studying the impact of androgens on cognition is crucial to our understanding of natural transitional menopause and associated cognitive changes. Female rats have been shown to express high concentrations of androgen receptors in cognitive brain areas such as the hippocampus and cerebral cortex (Simerly et al., 1990), which have been shown to be sensitive to both Ovx and androgen administration (Lu et al., 1998), and activation of which could impact cognitive function through gene transcription (McPhaul & Young, 2001). There has been a paucity of research evaluating the learning and memory effects of endogenous or exogenously administered androstenedione. In fact, as far as we are aware, the current experiment and our prior research findings (Camp et al., 2012) are the only studies testing the effects of androstenedione administration on learning and memory in the rat.

Much of the prior research testing the effects of androgens on rodent cognition has focused on dihydrotestosterone, testosterone, and dehydroepiandrosterone. Interestingly, while

reports indicate that dihydrotestosterone has no impact on spatial working or reference memory (Raber et al., 2002; Bimonte-Nelson et al., 2003b; Benice & Raber, 2009), we and others have shown that testosterone administration enhances working memory (Bimonte-Nelson et al., 2003b), spatial reference memory (Benice & Raber, 2009), and performance on avoidance tasks (Flood et al., 1995; Edinger et al., 2004).

The metabolism of androstenedione versus testosterone is likely related to the divergence in their respective cognitive impacts; testosterone is directly aromatized to 17β-estradiol, whereas androstenedione is directly aromatized to estrone. Many studies have demonstrated that estradiol can enhance cognition in female rats (e.g. Bimonte & Denenberg 1999; Gibbs, 1999, 2005; Gibbs et al., 2004; Daniel et al., 2006; Talboom, 2008; Rodgers et al., 2010; for review see Acosta et al., 2013). Thus far, the only two studies investigating the cognitive impact of estrone have found that estrone treatment was detrimental to contextual fear conditioning in young adult female rats (Barha et al., 2010), as well as working memory in middle-aged female rats (Engler-Chiurazzi et al., 2012).

The potential clinical implications of the current findings are far-reaching. Indeed, this work could generate new insight into the already immensely complex relationship between the loss of ovarian hormones in menopause and memory changes (Weber & Mapstone, 2009; Weber et al., 2013; Fischer et al., 2014). Cognitive effects likely depend on an individual's menopause status, including whether they have intact ovaries (Nappi et al., 1999), what phase of the menopause transition they are in (Weber et al., 2013), circulating levels of androstenedione, as well as other steroid hormones and gonadotropins (Acosta et al., 2009b), and prior hormone exposure history (Bimonte-Nelson et al., 2010). Knowledge of how these factors interact is particularly salient towards our goal of

optimizing hormone therapy for relief of menopausal symptoms. For example, we have demonstrated that conjugated equine estrogen (CEE) hormone therapy benefits cognition following surgical hormone loss, but impairs cognition following transitional menopause in which the residual, androstenedione-producing ovaries remain intact (Acosta et al., 2010). The current results underscore the tenet that CEE is not the optimal hormone therapy for menopausal women. Support for this assertion comes from several intersecting lines of evidence, including the current data indicating that this may be especially relevant for women who retain their ovaries; indeed, CEE is over 50% estrone sulfate (Kuhl, 2005; Gleason et al., 2005). Estrone sulfate is converted to estrone by the liver, further adding to the estrone load derived from ovarian-produced androstenedione. It is possible that a bioidentical estradiol hormone therapy approach may produce more favorable cognitive outcomes, as it would act to bring the hormonal milieu closer to ratios seen in pre-menopausal women (Kuhl, 2005; Gleason et al., 2005).

The study of aromatase and estrogen metabolism is critical to moving the endocrine field forward, and to our understanding of systems-level changes occurring with hormone loss and replacement during menopause. Highlighting the need for a non-estrogenic compound that could safely relieve some of the symptoms of menopause, many women are unable to utilize estrogen-inclusive hormone therapy due to an increased risk of, or history of, breast cancer. The aromatase enzyme is found in breast tissue, and aromatase inhibitors are currently used to treat breast cancer and prevent breast cancer recurrence (Santen et al., 2009). Furthermore, there is a greater degree of androstenedione aromatization to estrogen as the body mass index and obesity increase in postmenopausal women, suggesting that conversion of androstenedione to estrogens can vary across the menopausal population

(Santen et al., 2009). Should aromatase inhibitors prove to offset some of the negative cognitive consequences of menopause, this would further add to their value. In fact, it is noteworthy that, in the current study, anastrozole alone did not impair any of our many measures of cognition; indeed, anastrozole is one of the currently prescribed aromatase inhibitors used for breast cancer. Important future directions include developing a better understanding of the downstream hormone and brain mechanism(s) through which androstenedione and estrone produce cognitive impairments. A primary goal of this research is to evaluate alternative hormone therapy options that produce favorable outcomes for improved cognition in the menopausal female, utilizing a systematic approach that acknowledges and accounts for contributions of the many interacting variables that produce cognitive changes throughout aging.

CHAPTER 9: EVALUATION OF THE COGNITIVE IMPACT OF HORMONAL CONTRACEPTIVES DURING THE MENOPAUSAL TRANSITION

Introduction

Through the set of experiments performed for this dissertation, I have shown that several endogenous and exogenous hormone exposures across the lifespan have the potential to impact cognition. Chapter 3 demonstrated that use of EE-containing contraceptives is associated with worse performance in women tested on the human analogue of the rat radial-arm maze task (the HRAM), as well as poorer performance on tasks that measure visuospatial ability, compared to men. Chapter 4 reported similar impairments in spatial working memory following administration of EE in young adult Ovx rats, and chapter 5 extended those findings to ovary-intact rats. Chapter 6 showed that there is a narrow window of opportunity around VCD-induced follicular depletion during which CEE does not produce cognitive impairments, and chapter 7 demonstrated that exogenous administration of E2 following VCD-induced follicular depletion produces spatial memory enhancements. Finally, in chapter 8, I collected data to support our hypothesis, that follicle-deplete ovarian-derived androstenedione's conversion to E1 via the aromatase enzyme underlies the working memory impairments associated with elevated androstenedione levels. Generally, the work so far in this dissertation suggests that alterations in estrogen levels impact working memory, while leaving reference memory comparatively unaffected, and that background hormone profile is important to consider when designing or choosing hormone treatments. This work also indicates that animals that have undergone follicular depletion and retained their follicle-deplete ovaries respond more strongly to exogenous E2 treatment behaviorally than to CEE treatment, or removal of the

ovaries (Ovx), perhaps because E2 produces a comprehensive hormonal shift towards higher E1 and E2 levels, and lower androstenedione levels than what is seen with CEE or Ovx.

Through the last decade, there has been a massive shift in HT prescription trends; the heavily publicized WHI and WHIMS results showing no cognitive benefits of CEE HT, coupled with increased cognitive and health risks in some cases, spurred a high demand for alternative, safer prescription HT regimens (ACOG, 2011; Endocrine Society, 2015). Several FDA-approved bioidentical E2-containing HTs are now available in the United States, including numerous transdermal, systemic, tonic-E2-releasing patches (Alora, Climara, Estraderm, Minivelle®, Vivelle-Dot, and other generic versions), gels (Divigel®, EstroGel, Elestrin), and a spray (Evamist®; NAMS). The Estrace vaginal cream, Estring® vaginal ring, and Vagifem® vaginal mist are also available for local vaginal, non-systemic use. The majority of these products have counterparts that also contain progestogens, for use by women that have not undergone hysterectomy (surgical removal of the uterus). Activella is an oral formulation that includes the progestin Norethindrone Acetate (NETA), Prefest® is another oral formulation that utilizes Norgestimate, another synthetic progestin, CombiPatch is a transdermal patch with NETA, and Climara Pro is a transdermal patch that releases yet another synthetic progestin, levonorgestrel (levo; NAMS).

In addition to this long list of bioidentical FDA-approved HT formulations, a host of new, non-FDA-approved, custom compounded estrogen/estrogen+progestogen formulations have gained massive popularity in the clinic. This year, the Endocrine Society and the North American Menopause Society (NAMS) have published several reports expressing concern about rampant off-label prescribing of these new custom compounded

estrogen formulations as HT. These formulations are marketed as bioidentical customized estrogen cocktails, and now may be the dominant HT prescribed by physicians, accounting for 28% to 68% of currently used HTs (NAMS, 2015a, 2015b). Because these formulations are not FDA-approved, and therefore not governmentally regulated, it is difficult to track their use, and their safety is not guaranteed. Indeed, there are now major concerns that these formulations do not contain hormones in the proper ratios to render them safe; in many cases the amount of included progestogen was insufficient to protect the uterus from estrogenic stimulation, an oversight that can be deadly (Endocrine Society, 2015; NAMS, 2015a, 2015b).

In lieu of the above-described HT options, many physicians now also recommend an FDA-approved contraceptive regimen to women in the menopause transition to prevent unwanted pregnancies and to regulate the menstrual cycle, which would otherwise become increasingly irregular and unpredictable across the transition to menopause (Curtis et al., 2005; Hoffman et al., 2012; Ikhena & Johnson, 2012). These contraceptive formulations, while not FDA-approved for use as HT, are FDA-approved and regulated for contraceptive use, providing a general safety guarantee over that of custom-compounded HTs. The cognitive effects of the synthetic estrogen EE utilized in hormonal contraceptives, and the many available synthetic progestins, have never been evaluated in a rodent model of transitional menopause. Other work from our laboratory has shown that treatment with medroxyprogesterone acetate (MPA), a synthetic progestin, induces cognitive impairments in ovary-intact, as well as Ovx rats (Braden et al., 2010; 2011), and ongoing work in our laboratory suggests that a clinically relevant dose of levo, a structurally and functionally different progestin, may have a favorable cognitive impact in young Ovx rats.

Findings from this dissertation suggest that EE-containing hormonal contraceptives may serve as a more optimal HT during the menopause transition than the current FDA-approved regimens containing E2 or CEE, and the now-popular non-FDA-approved custom estrogen formulations. Although I saw a clear negative impact of low-dose EE in young-adult ovary-intact rodents in chapter 5, as well as in young adult women in chapter 3, I did not see any impact of low-dose EE in Ovx animals in chapter 4, and collectively, the results from this dissertation suggest that the cognitive effects of estrogens depend heavily on the hormonal profile they are delivered to. EE's lack of conversion to E1 makes it an especially promising candidate to supplement the hormonal profile of naturally menopausal women. The addition of a synthetic progestin may further serve to replace the progestogenic stimulation lost with menopause. It is also important to consider that the current FDA-approved E2 and CEE-containing HT regimens are not sufficient to halt ovulation, and therefore are not useful as contraceptives. FDA-approved hormonal contraceptives may serve the additional function of disguising the irregular hormone fluctuations that occur during the transition to menopause, which current FDA-approved HT prescriptions are not capable of. Moreover, hormonal contraceptives also provide the added benefit of preventing unwanted pregnancies during the transition to menopause, and do not incur the same cancer risks as traditional HTs. In fact, combined contraceptive use is associated with a reduced risk of ovarian and endometrial cancer (Hoffman et al., 2012).

For my final experiment, I tested the cognitive impact of the estrogens E2 and EE, as well as the synthetic progestin levo, and the combinations of each estrogen with levo during follicular depletion. These hormone regimens were specifically chosen to model clinically prescribed formulations of combined contraceptives and FDA-approved HTs that

are currently prescribed as closely as possible (Curtis et al., 2005; Hoffman et al., 2012). I predicted that E2- and EE-containing treatments would improve performance on a spatial working memory task, and that EE+levo would produce the most robust cognitive benefits, relative to no hormone treatment during the transition to menopause.

Materials and Methods

Subjects

Subjects were 59 eight-month-old Fisher-344 rats raised at the National Institute on Aging colony at Harlan Laboratories (Indianapolis, IN). After arrival at the ASU Tempe campus facilities, rats were pair-housed, had access to food and water ad-lib, and were housed on a 12-h light/dark cycle. All procedures were approved by the local Institutional Animal Care and Use Committee (IACUC) and adhered to National Institutes of Health (NIH) standards.

Experimental Design and Hormone Treatments

Animals received VCD treatment exactly as described in chapters 7 and 8. Thirty days after VCD treatment was initiated, administration of Vehicle (n=10), E2 (n=10), EE (n=10), levo (n=10), E2+levo (n=9), or EE+levo (n=10) began. Treatment was given via subcutaneous Alzet osmotic pump, releasing a tonic dose of hormone treatment for the remainder of the study.

EE in combination with levo exists in over twenty different contraceptive formulations, including Alesse, Aviane, Lutera, Nordette, Altavera, and several other generic variations. Doses of EE in these formulations range from 10-30μg/day, while EE as a HT is given at a lower dose (2.5 or 5.0μg/day), and clinically used doses of levo range from 0.05-0.15mg/day. Here, we utilized the low EE dose from chapter 4 (0.125μg/day),

which is equivalent to a roughly 30-35μg/day dose of EE in women, when corrected for differences in body weight, and the dose of E2 that was used in chapter 7 (0.3μg/day). We chose a dose of 0.6μg/day of levo because ongoing work in our laboratory suggests this regimen may have a favorable impact on cognition. This dose in a rat is equivalent to roughly 1.4-1.7mg/day dose in women, when corrected for body weight. Figure 40 shows a detailed experimental timeline.

Water Radial Arm Maze

Water radial arm maze (WRAM) procedures were identical to those in chapter 4.

Morris Water Maze

Morris water maze (MM) procedures were identical to those in chapter 5.

Visible Platform Maze

Visible platform procedures were identical to those described in chapter 4.

Peripheral Markers of Treatment

Wet uterine weights (g) were measured at sacrifice to verify hormone treatment. Ovaries were collected and preserved in 10% formalin for future evaluation of follicle and corpora lutea counts.

Serum Hormone Levels

Animals were given two days off from behavioral testing before sacrifice, at which time serum was collected for hormone assays, exactly as described in chapter 4.

Statistical Analyses

WRAM, MM, and visible platform data were analyzed exactly as described in chapters 7 and 8, with the exception that two independent between-subjects variables will

be used: Estrogen (three levels: Vehicle, E2, EE) and Progestin (two levels: Vehicle, Levo), rather than one between subjects treatment variable.

Results

Water Radial Arm Maze

For Block 1 (days 2-3) of WRAM testing, there was a Trial x Estrogen x Progestin interaction ($F_{(6,159)}$=2.33, $p<0.05$; η_G^2=0.04; figure 41a), with group differences appearing on Trial 4, the trial with the highest working memory load. On Trial 4, there was a Estrogen x Progestin interaction ($F_{(2,53)}$=3.39, $p<0.05$; η_G^2=0.05; figure 41b), whereby levo treatment produced impairments relative to no levo treatment in animals treated with no estrogen (Fisher, $p<0.05$), and in animals treated with E2 (Fisher, $p<0.05$), but not in animals treated with EE (Fisher, $p>0.05$).

On Block 2 of WRAM testing (days 4-6), there was a Trial x Estrogen interaction ($F_{(6,159)}$=4.47, $p<0.001$; η_G^2=0.04; figure 41c) and a Trial by Progestin interaction ($F_{(3,159)}$=3.93, $p<0.01$; η_G^2=0.02figure 41e), with treatment differences emerging on Trial 4, the trial with the highest working memory load. On Trial 4, there was a main effect of estrogen ($F_{(2,53)}$=5.23, $p<0.01$; η_G^2=0.06; figure 41d), with E2-treated animals performing better than those treated with vehicle (Fisher, $p<0.05$) or EE (Fisher, $p<0.05$), regardless of Progestin treatment. There was also a main effect of Progestin on Trial 4 ($F_{(1,53)}$=5.40, $p<0.05$; η_G^2=0.03; figure 41f), with levo-treated animals outperforming animals that did not receive levo (Fisher, $p<0.05$), regardless of Estrogen treatment.

There was a Trial x Estrogen x Progestin interaction on Block 3 of WRAM testing (days 7-9; $F_{(6,159)}$=3.66, $p<0.01$; η_G^2=0.04; figure 41g), with group differences on Trial 4, the highest working memory load trial. On Trial 4, there was an Estrogen x Progestin

interaction ($F_{(2,53)}$=4.73, $p<0.05$; η_G^2=0.07; figure 41h), whereby treatment with E2 produced spatial memory impairment in animals that did not receive levo, relative to treatment with EE (Fisher, $p<0.05$) or no estrogen (Fisher, $p<0.05$). There were no effects of Estrogen within the levo-treated groups (Fisher, $p>0.05$).

There were no effects of Estrogen ($F_{(2,53)}$= 0.46, $p>0.05$, NS; $\eta_G^2<0.01$) or Progestin ($F_{(1,53)}$= 2.99, $p>0.05$, NS; $\eta_G^2<0.01$) on Block 4 of WRAM testing (days 10-12), and there were no effects of Estrogen or Progestin on delayed memory retention (day 13).

Morris Water Maze

There were no effects of Estrogen ($F_{(2,53)}$= 0.80, $p>0.05$, NS; $\eta_G^2<0.01$; figure 42a) or Progestin on MM performance ($F_{(1,53)}$= 0.03, $p>0.05$, NS; $\eta_G^2<0.01$; figure 42a). On the probe trial, there was a main effect of Quadrant ($F_{(1,53)}$= 430.58, $p\leq0.0001$; η_G^2=0.86), with no Estrogen x Quadrant interaction ($F_{(2,53)}$= 0.20, $p>0.05$; $\eta_G^2<0.01$; NS), but there was a Progestin x Quadrant interaction ($F_{(1,53)}$= 3.81, $p<0.05$; η_G^2=0.03). This prompted us to analyze the first half of the Probe trial, to determine whether animals began searching in other quadrants after a lack of reward in the NE quadrant for the first 30 seconds. Indeed, during the first 30 seconds of the probe trial, there was a main effect of Quadrant ($F_{(1,53)}$= 261.96, $p\leq0.0001$; η_G^2=0.93; figure 42b), with no Estrogen x Quadrant interaction ($F_{(2,53)}$= 0.10, $p>0.05$; $\eta_G^2<0.01$; NS; figure 42b), and no Progestin x Quadrant interaction ($F_{(1,53)}$= 2.44, $p>0.05$; η_G^2=0.03; NS; figure 42b), demonstrating that all treatment groups localized the platform by the end of MM testing.

Visible Platform Task

There was no effect of Estrogen on escape latency in the visible platform task ($F_{(2,53)}$= 0.03, $p>0.05$; $\eta_G^2<0.01$; NS), but there was a marginal effect of Progestin on

escape latency ($F_{(1,53)}$= 3.19, $p<0.10$; η_G^2=0.01). Animals treated with levo tended to escape from the maze faster than those that did not receive Levo (Fisher, $p<0.10$), indicating that this treatment enhanced some procedural aspect of water-maze performance. Although this effect was present, all groups exhibited mean escape times of less than 10 seconds across all six trials, and the levo-induced benefit on this task amounted to an average escape time of less than two seconds faster than that of non-levo treated animals (no levo: M=9.32s, SD=7.47s; levo: M=7.95s, SD=7.16s).

Peripheral Markers of Treatment

There was a main effect of Estrogen on Uterine Weights ($F_{(2,53)}$=8.51, $p<0.001$; η_G^2=0.24; figure 43a), with E2-treated animals exhibiting heavier uterine horns at sacrifice than animals that received EE or no estrogen, regardless of Progestin treatment. There was no impact of Progestin treatment on Uterine Weights ($F_{(1,53)}$=1.20, $p>0.05$; η_G^2=0.02; figure 43a). and no Estrogen x Progestin interaction ($F_{(1,53)}$=0.10, $p>0.05$; NS; $\eta_G^2<0.01$; figure 43a). Relationships between E2 levels and uterine weights by group are shown in figure 44.

Serum Hormone Levels

There was a main effect of Estrogen on circulating serum levels of E2 ($F_{(2,53)}$=93.77, $p<0.0001$; η_G^2=0.78; figure 43b), with higher E2 levels in groups given E2 compared to those given EE or no estrogen, regardless of Progestin treatment. There was no impact of Progestin treatment on serum E2 levels ($F_{(1,53)}$=1.17, $p>0.05$; η_G^2=0.02; figure 43b).

Discussion

The aim of the current study was to determine whether EE-containing contraceptive formulations might serve as cognitively protective HTs during the transition to menopause.

I compared tonic EE and E2 treatment, with and without the popular progestin levo, to vehicle treatment in animals undergoing the end stages of ovarian follicular depletion, as a model of the transition to menopause. I observed a benefit of E2 treatment during the middle of WRAM testing, similar to the effects of E2 in post-depletion animals that we reported in chapter 7. However, I also observed a negative impact of E2 treatment later in testing, which is unique to E2 given during follicular depletion; this negative effect was not seen in post-depletion animals with an identical E2 treatment in chapter 7. Of note, the only treatment regimens utilized here that are not sufficient to halt ovulation are those for the Control and E2 alone groups: the inconsistency in E2's effects on cognition may reflect the inconsistent endogenous hormone levels that this exogenous treatment is added to. In support of this hypothesis, the addition of levo, which alone is sufficient to halt ovulation and mask the irregular endogenous hormone levels associated with the transition to menopause, negates the impairment seen with E2 alone, without negating the benefit of E2 seen earlier in testing. The addition of levo treatment to E2 treatment does, however, induce a deficit on the initial testing block that is not seen with E2 alone. Thus, while it seems that treatment with E2 produces a favorable cognitive impact after follicular depletion is complete, modeling post-menopause, as we reported in chapter 7, we now report that E2 alone produces both cognitive enhancement and impairment when administered during the transition to menopause.

I saw no cognitive impact of EE alone, relative to no hormone treatment. These results are identical to the cognitive profile of the corresponding dose of EE that I reported in Ovx animals in chapter 4, and differs from the cognitive impact of a similar dose of EE that I observed in regularly cycling, ovary-intact young adult rats in chapter 5. While I did

not observe any overt cognitive benefits of EE treatment, I still consider the observed neutral cognitive impact to be a positive result; EE produces the same estrogenic stimulation that FDA-approved E2 or CEE HTs yield, without the negative cognitive impact that we observed with CEE in chapter 6, or with E2 or levo in the current experiment.

I also conclude that the cognitive impact of levo depends on the estrogen treatment that it is administered with. In animals that received no estrogen, and in animals that received E2, levo impaired WRAM performance on the first block of testing, but improved performance in later testing blocks, relative to animals that did not receive levo. In animals that received EE treatment, levo produced a benefit on the second block of WRAM testing, with no negative impact on any other blocks. Thus, the treatment utilized here that models over 20 different currently available FDA-approved hormonal contraceptives (EE+levo) produced the most favorable cognitive profile, relative to no hormone treatment at all, E2 treatment alone, EE treatment alone, levo treatment alone, or E2 plus levo treatment.

Altogether, I report here that treatment with E2 or levo alone, or a combination of E2 and levo during the transition to menopause results in mixed cognitive effects, including both improved and worsened performance on our WRAM task. Treatment with EE alone did not impact performance on any of the tasks utilized here, and treatment with EE plus levo produced a modest benefit on our WRAM task. This EE+levo-induced memory benefit during the transition to menopause is an incredibly exciting finding, as this treatment regimen is widely available, free to most women in the United States, and produces all of the non-cognitive benefits of currently approved HTs, in addition to pregnancy prevention not offered by traditional HTs. More investigation into the many

different variations of contraceptive formulations currently available is sorely needed. The results here are specific to tonic treatment regimens, while many of the available formulations (and the most popular) are administered in a daily cycle. Previous and ongoing work from our lab suggests that the cognitive impact of EE (Mennenga et al., 2015a) and of levo may vary between tonic and cyclic regimens.

Also, the cognitive impacts of each of the numerous available synthetic progestins have only just started to be explored. There has been no exploration into the cognitive impact of phasic contraceptive formulations, which even more closely model the reproductive hormone cycle and may produce an even better cognitive outcome. To my knowledge, along with two other reports from our laboratory on the memory effects of MPA (Braden et al., 2010, 2011), and ongoing unpublished work from our lab, this is the only basic science evaluation of the cognitive impact of any synthetic progestin to date. This is also, to my knowledge, the only basic science investigation into various available hormone treatments in a model of the transition to menopause. Thus, I am very pleased to report that I have discovered a promising new avenue for investigation into cognitively protective HTs to be utilized during the transition to menopause. I hope that this work opens a new road to safe, affordable, easy-to-access HT options during the transition to menopause.

CHAPTER 10: GENERAL DISCUSSION

Women are exposed to constant shifts in endogenous and exogenous hormones throughout life. These exposures have pervasive and interactive impacts on many physiological functions, spanning multiple systems. Women's reproductive hormones, in particular, undergo both endogenously- and exogenously- triggered changes, including those that happen during perinatal development, puberty, with use of hormonal contraceptives, during pregnancy, throughout the transition to menopause, and with hormone therapy (HT). Reproductive hormones such as estrogens, androgens, progesterone, and others, are responsible for the regulation of many body functions in addition to their influence on reproductive functions. Internal hormone secretions serve to regulate body temperature, bone density, body fat composition and deposition, metabolism, brain function, and much more (Mennenga and Bimonte-Nelson, 2015). Moreover, the breadth of the types of hormone exposures women experience is impressive, including both natural and synthetic hormones, and several changes in hormone use trends across the last few decades may produce a unique generation of aging females.

I have studied four distinct types of hormone profiles in this dissertation: the ovary-intact, reproductively viable hormone profile, which includes several distinct hormonal states in a cyclic pattern, the ovariectomy (Ovx) hormone profile, which is void of ovarian hormones, the mid-follicular depletion hormone profile, which includes several hormonal states that occur in an irregular pattern, and the post-follicular depletion hormone profile, which involves low levels of endogenous estrogens and progesterone and moderate levels of the androgen androstenedione. Further, I have evaluated individual contributions to cognitive ability from several hormones, including androstenedione, several estrogens

(ethinyl estradiol, EE; conjugated equine estrogens, CEE; estradiol, E2), and a synthetic progestin (levonorgestrel, levo). Results largely indicate that which hormonal treatments produce an optimal cognitive impact depends on which of the above hormone profiles is in question. Altogether, the data that I have collected for my dissertation indicate that both endogenous and exogenous hormonal fluctuations across the lifespan impact specific domains of cognition, and are likely to play an important role in shaping the cognitive phenotype throughout aging.

Optimizing Hormonal Contraceptive Use During Young Adulthood

I have shown in both humans and rodents that treatment with the estrogen found in combined contraceptives (EE) is associated with poorer spatial memory; however, in both species, differences only became apparent when working memory demand was highest (chapters 3 and 4). In the human radial arm maze (HRAM), which I have demonstrated to be a reliable measure of human spatial working memory (chapter 2), I was able to detect detriments in performance on the highest working memory trials in women with relatively high circulating estrogens (those taking combined contraceptives and those in the follicular phase of the menstrual cycle), compared to males (chapter 3).

In several follow-up studies, I investigated how the estrogenic component of combined oral contraceptives, EE, affects spatial learning and memory in both Ovx and ovary-intact young-adult female rats (chapters 4 and 5). In this series of studies, I showed that a high dose of EE impaired spatial working memory in Ovx rodents, and both medium and high doses of EE reduced the number of ChAT-IR neurons in the basal forebrain (chapter 4). Further, the observed behavioral effects correlated with the observed changes in the cholinergic system, suggesting that EE may be affecting spatial learning and memory

through its impact on this system (chapter 4). I noted a similar pattern of effects in another study, in which I administered EE to ovary-intact rats that were actively cycling through the estrous cycle. In this study, a low dose of EE, comparable to the doses clinically prescribed to women, produced deficits in spatial learning and memory (chapter 5).

The findings that I report in chapters 2 through 5 answer many questions about the cognitive impact of hormonal contraceptives, but they also raise many new questions. Due to the between-subjects nature of the studies utilizing the HRAM in chapters 2 and 3, I cannot be sure whether the group differences in visuospatial and spatial working memory tasks are due to differences in hormonal profiles, or other subject variables that were not accounted for. A within-subjects study is necessary to know whether the differences we report here change across time within women. Similarly, the data presented here does not allow insight into the permanency of EE's impact on cognition. Past research from our lab found long lasting cognitive impairments with a different synthetic progestin, MPA (Braden et al., 2011), raising a possibility that the observed effects might be permanent, or long lasting. Finally, while findings from chapter 4 suggest that EE may be exerting its cognitive effects through the cholinergic system, we do not know precisely how. Additional work is necessary to elucidate the exact mechanisms by which EE impacts cognition.

Optimizing the Cognitive Impact of Hormone Therapy in a Rodent Model of Natural Menopause

My next series of studies was aimed at optimizing the cognitive impact of HT across the transition to menopause. I investigated how various parameters, including timing of hormone administration initiation, length of hormone exposure time, and type of

estrogen, impacted the cognitive effects of HT during and after VCD-induced follicular depletion (chapters 6 and 7). I replicated a previous lab finding, that CEE treatment initiated post-depletion produces memory detriments (chapter 6; Acosta et al., 2010). Additionally, I extended the previous findings to incorporate multiple administration parameters, and discovered that timing of treatment initiation alters the cognitive impact of CEE in a model of human menopause. I also reported that post-depletion treatment with bioidentical E2 provides a cognitive benefit, akin to that seen with surgical removal of the ovaries (chapter 7).

These findings have massive clinical implications; beginning with the broad message that HT will likely produce optimal benefits with minimal risks only if it is tailored to each woman's personal hormonal makeup. More specifically, I have demonstrated that bioidentical E2 as a HT appears to be a more viable option for cognition than CEE post-menopause, possibly because of the existing hormonal imbalances created by the disruption of the menstrual cycle. Clinical work from the large study of women's health across the nation (SWAN) has detected several distinct hormonal profiles in women across the transition to menopause (Tepper et al., 2012). Future research into the individual differences in the menopause transition experience is likely to result in further optimization of HTs for cognition. Whether the same clusters of hormone profiles are seen across time in the VCD model is unknown; collection of circulating hormone levels across time following VCD treatment may allow even more refined modeling of natural human menopause.

Hormonal Mechanisms Underlying the Cognitive Consequences of Natural Hormone Loss

I utilized pharmacological blockades to investigate the hormonal mechanism by which androstenedione may be impairing memory following follicular depletion. The results supported our hypothesis, that androstenedione is producing cognitive impairments through its conversion to E1, rather than through its actions on the androgen receptor (chapter 8). This finding helps to identify exactly which parts of the endogenous hormone milieu are negative for cognition. It seems that elevated levels of E1 relative to E2 negatively impact cognition during and after the transition to menopause.

At this point, our lab has identified three different manipulations that each serve to alter this hormonal ratio, and that each benefit spatial learning and memory. Surgical removal of the follicle-deplete ovaries has now been shown in two separate studies to improve aspects of cognition (Acosta et al., 2009b; chapter 7), administration of exogenous E2 has also been shown to enhance cognition (experiment 7), and blocking conversion of androgens to estrogens via pharmacological inhibition of the aromatase enzyme has also now been shown to negate androstenedione-induced cognitive impairments. Thus, it seems likely that there will be multiple ways in which hormone profiles can be altered to benefit cognition throughout the transition to menopause, aside from the administration of exogenous hormone treatments.

Modeling Current Trends in Hormone Therapy

Finally, I sought to determine whether EE-containing contraceptives might be cognitively protective during the transition to menopause. I did this by comparing tonic EE and E2 treatment, both with and without the popular progestin levo, to vehicle treatment in

animals undergoing the end stages of ovarian follicular depletion, as a model of the transition to menopause. I found that E2, levo, and E2 combined with levo each produce mixed cognitive effects, which include both benefits and detriments to spatial memory (chapter 9). EE alone did not impact cognition, as measured by the WRAM and MM, an effect that is promising, given that EE is capable of producing all of the non-cognitive benefits of E2 and CEE. Even more exciting was that EE in combination with levo, modeling currently available FDA-approved hormonal contraceptive formulations, produced a transient benefit on the WRAM (chapter 9). Together, these results indicate that currently available, FDA-approved combined contraceptives (containing both EE and a synthetic progestin) may serve as a more optimal HT for women that are experiencing the transition to menopause than either bioidentical E2 or CEE HT.

There are several possible reasons that our EE plus levo treatment produced different cognitive effects than either CEE or E2 during the transition to menopause. First, the estrogenic component of hormonal contraceptives is unique in several respects: it is not converted to other estrogens, and is more resistant to enzymatic degradation in general than natural estrogens, conferring a distinct pharmacokinetic profile, and its pharmacodynamic profile differs from that of natural E2 and CEE as well. Combined contraceptives are also unique candidates for HT due to their ability to halt ovulation, therefore altering endogenous production of estrogens and other hormones. Traditional HTs, such as E2 and CEE, are not sufficient to halt ovulation, meaning that irregular production of ovarian hormones continues in addition to the exogenous administration of these hormone treatments. Not only does this distinction mean that traditional HTs do not offer protection against unwanted pregnancies, it also means that the endogenous hormone profile likely

differs, depending on which exogenous hormone formulation is administered. Thus, a treatment that masks irregular endogenous hormone fluctuations in addition to providing estrogenic and progestogenic stimulation, such as a combined hormonal contraceptive, may be the optimal HT choice for women undergoing the transition to menopause. This masking of endogenous hormone fluctuations may not be necessary once follicular depletion is complete, and ovulation has ceased.

General Conclusions

Broadly, the goal of my graduate work was to elucidate the parameters that determine how endogenous and exogenous hormones impact cognition, particularly spatial learning and memory. My conclusions from the completed experiments are that endogenous and exogenous hormone treatments have the potential to impact spatial memory, and that the direction of these effects largely depends on many individual factors. First and foremost, the existing hormone profile of the user determines the cognitive impact of hormone exposures. In young adult, naturally-cycling and Ovx organisms, I conclude that, although the presence of the ovaries alters the cognitive effects of EE treatment, generally, lower doses of EE delivered via tonic regimens (as opposed to daily cyclic regimens) produce the best cognitive outcomes. In our model of transitional menopause, I established that optimal treatments may vary, depending on which stage of the menopause transition the user is in. During follicular depletion, combined contraceptives, specifically those containing EE and levo and delivered in a tonic regimen, produce a more favorable cognitive profile than bioidentical E2, levo, or E2 combined with levo. Natural, bioidentical E2, while producing mixed cognitive results when delivered during follicular

depletion, appears to be a viable HT option when delivered after follicular depletion is complete.

Although the work performed for this dissertation furthered our knowledge of how to optimize hormone exposures across the lifespan, several questions remain unanswered. First, we do not know whether the cognitive effects of most of the treatments examined here are transient or permanent. Major shifts in HT use trends and the fairly recent availability of hormonal contraceptives mean that current and future generations have been exposed to entirely different hormones throughout their lifespan than any previous generation. Work from our laboratory utilizing the synthetic progestin MPA suggests that these effects may be long lasting, therefore contributing permanently to the cognitive aging profile (Braden et al., 2011). If the treatments investigated in this dissertation do have long-lasting effects on the brain or body, then current and future generations are likely to experience cognitive aging differently than any previous generation. Administration parameters, variations in dose and type of hormone, variations in endogenous hormone levels, and other factors, such as verbal intelligence, may serve to further impact the cognitive effects of any hormone treatment.

Thus, much more investigation into the numerous parameters surrounding hormone treatment administration is sorely needed. It is my sincere hope that work from this dissertation will serve as a starting point from which future studies can be designed to continue to elucidate how each of these parameters impact learning and memory throughout the entire lifespan. I purposely investigated multiple treatment regimens that are already available to women in an effort to minimize the amount of time that the translation of my findings to the clinic would take. Contraceptive hormones were initially designed to

regulate the menses, providing relief from irregular menstrual cycles. This feature makes them an especially interesting candidate for HT. Future studies may allow clinicians to develop the optimal transition from the reproductive cycle to reproductive senescence; through utilization of cyclic hormonal contraceptive regimens, the menstrual cycle could even be simulated through the end of life. We do not yet know enough of the impact that such treatments may have on the body as a whole to assert this as a definite possibility, however it is possible that the findings from this dissertation lead to revision of hormone treatment recommendations, and potentially even re-thinking the type of hormone treatments that are delivered. Should the effects reported in this dissertation prove to be permanent or long lasting, that means that permanent or long lasting detriments, but also benefits, to cognition may be possible to achieve through short-term treatments. Such findings could be utilized to improve the way that current and future generations experience cognitive aging.

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APPENDIX A

PUBLISHED RESEARCH AUTHORIZATION

I have secured permission from all authors to include published research in the current dissertation.

APPENDIX B

PERFECT MARGARITA

PERFECT MARGARITA

Ingredients

2 ounces silver/blanco tequila

1 tablespoon kosher salt

4 limes

½ an orange

2 tablespoons light agave nectar

¾ cup ice cubes

Instructions

Pour 1/2-ounce of tequila into a small saucer. Spread kosher salt in a separate small saucer. Wet the rim of a glass in the tequila. Lift out of the tequila and hold upside down for 10 seconds to dry slightly, and then dip the rim of the glass into the salt. Juice two limes into the bottom of a cocktail shaker. Cut the remaining two limes and the orange into quarters and add them to the shaker. Add agave nectar and muddle. Add the remaining 1 1/2 ounces of tequila and any remaining on the saucer. Add ice to the shaker, cover and shake for 30 seconds. Strain the mixture through a cocktail strainer into the salt-rimmed glass, garnish with lime slice, and serve immediately.

Figure 1. Experiment 1 schematics and pictures of the human radial arm maze. Depiction of the HRAM and surrounding space.

Figure 1

Diagram and Pictures of the Human Radial Arm Maze and Surrounding Environment

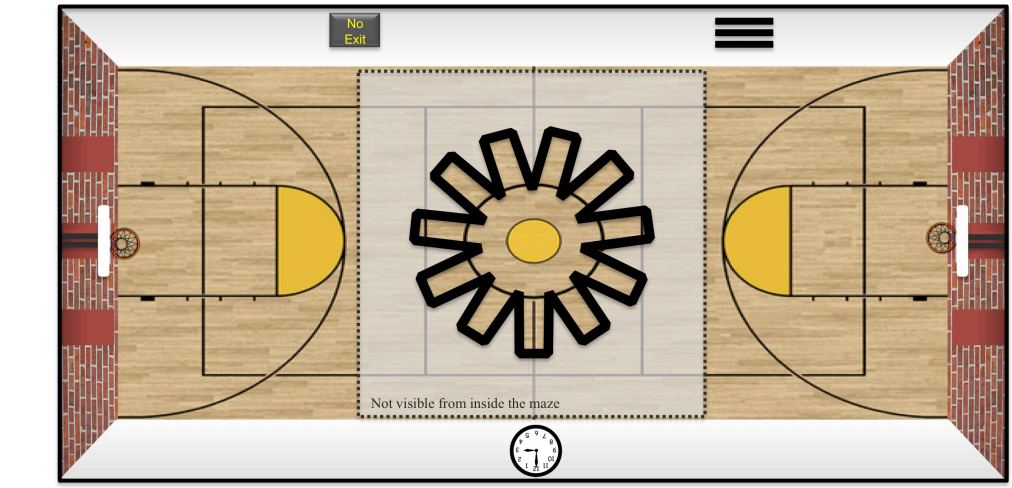


Figure 2. Experiment 1 Human and Rodent Radial Arm Maze Performance. There was a main effect of Trial [$F_{(10,1520)}$= 97.19; p< 0.0001] on HRAM Errors, with HRAM Errors increasing as trials progressed and working memory load increased. HRAM Errors increased from trial 8 to 9 (Trial 8: M= 0.28, SE= 0.04; Trial 9: M= 0.53, SE= 0.06; p< 0.05), from trial 9 to trial 10 (Trial 10: M=1.03; p<0.0001) and again from trial 10 to trial 11 (Trial 11: M=2.67, SE=0.11; p<0.0001). This increase in errors occurred when the number of arms participants needed to avoid exceeded roughly 8-9 items. HRAM Errors declined significantly across Testing Sessions (Both Sessions: M=0.436, SE=0.04, Session 1: M=0.51, SE=0.04, Session 2: M= 0.37, SE= 0.04; [$F_{(1,152)}$= 7.85; p< 0.01]). The pattern of performance across trials was the same across Testing Session, (Session x Trial interaction: $F_{(10,1520)}$= 1.80; p>0.05, NS]. Figure 2B shows error patterns observed in different versions of the rodent RAM for comparison.

Figure 2

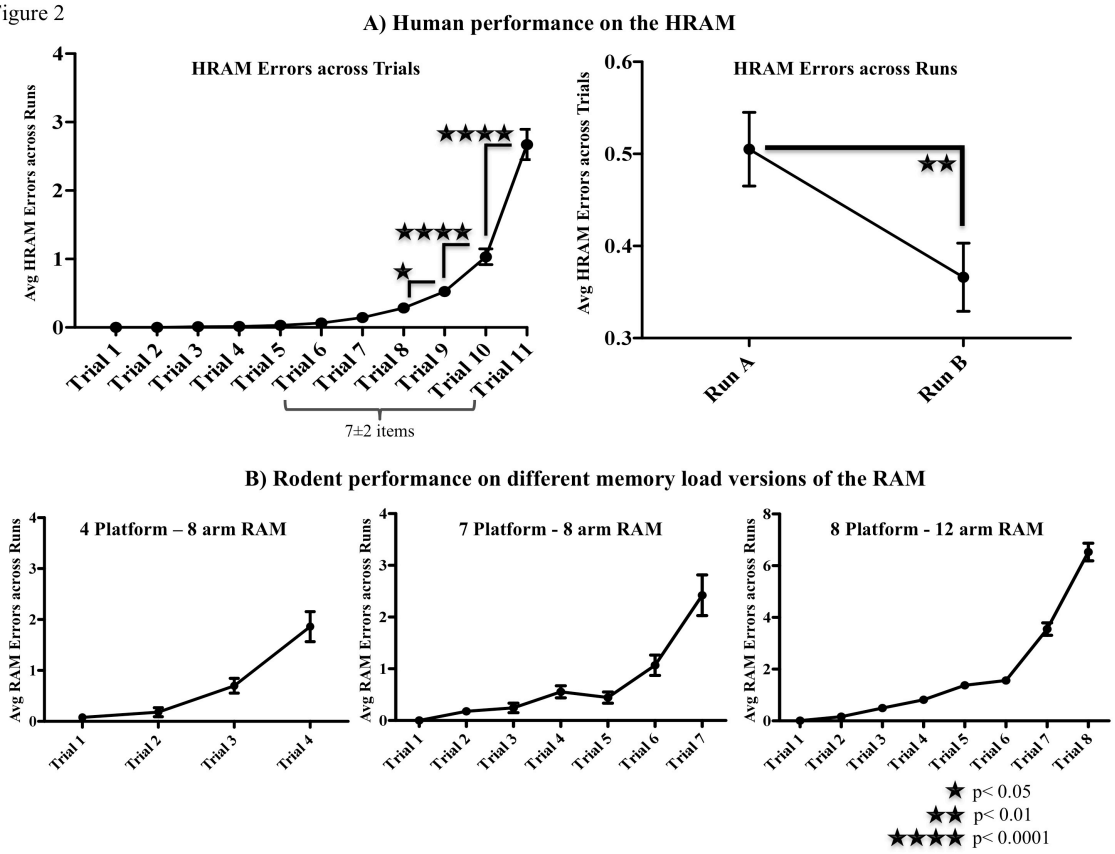
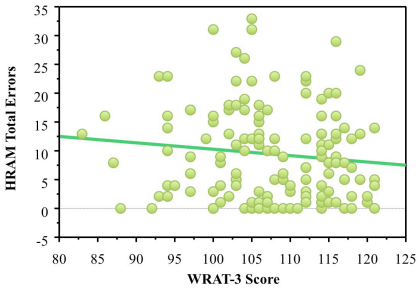

A) Human performance on the HRAM
HRAM Errors across Trials
Avg HRAM Errors across Runs
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
Trial 6
Trial 7
Trial 8
Trial 9
Trial 10
Trial 11
7±2 items
HRAM Errors across Runs
Avg HRAM Errors across Trials
Run A
Run B
B) Rodent performance on different memory load versions of the RAM
4 Platform – 8 arm RAM
7 Platform - 8 arm RAM
8 Platform - 12 arm RAM
Avg RAM Errors across Runs
p< 0.05
p< 0.01
p< 0.0001

Figure 3. Experiment 1 Human Radial Arm Maze Scores as Predicted by Verbal Intelligence Measure. WRAT-3 scores did not correlate with HRAM Total Errors.

Figure 3

Verbal intelligence predicting HRAM errors



HRAM Total Errors= 21.56 - 0.11*WRAT-3; R^2=0.01

Figure 4. Experiment 1 Human Radial Arm Maze Scores as Predicted by Episodic Memory Measures. RAVLT Total Words Learned, Retroactive Interference, and Delayed Recall did not correlate with HRAM Total Errors ($p>0.05$, NS). Regression analysis indicated that Total Words Learned, Retroactive Interference, and Delayed Recall trials of the RAVLT were not significant predictors of HRAM Total Errors. Combining all measures of the RAVLT also did not predict HRAM Total Errors (Adjusted $R^2_{multiple}=0.00$, $F_{(3, 151)}= 0.88$, $p>0.05$, NS).

Figure 4

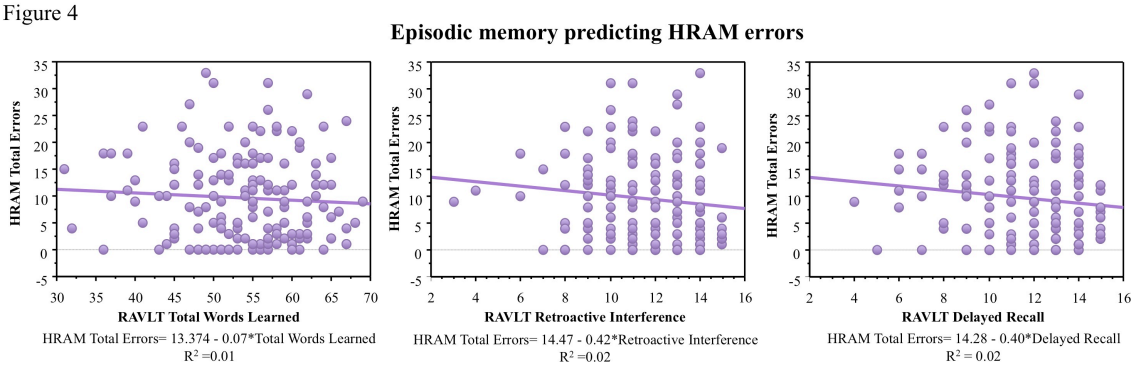

Episodic memory predicting HRAM errors
HRAM Total Errors
RAVLT Total Words Learned
HRAM Total Errors= 13.374 - 0.07*Total Words Learned
R² =0.01
HRAM Total Errors
RAVLT Retroactive Interference
HRAM Total Errors= 14.47 - 0.42*Retroactive Interference
R² =0.02
HRAM Total Errors
RAVLT Delayed Recall
HRAM Total Errors= 14.28 - 0.40*Delayed Recall
R² = 0.02

Figure 5. Experiment 1 Human Radial Arm Maze Scores as Predicted by Visuospatial Ability Measures. Both visuospatial tasks, the MRT and JLAP, correlated negatively with HRAM Total Errors ($p<0.01$ and $p<0.0001$, respectively). For every additional question participants answered correctly on the MRT, HRAM errors decreased by 0.40 on average; errors decreased by 0.66 for each one point increase in JLAP. The MRT and JLAP together predicted HRAM Total Errors (Adjusted $R^2_{multiple}=0.11$, $F_{(2, 152)}= 10.29$, $p<0.0001$).

Figure 5

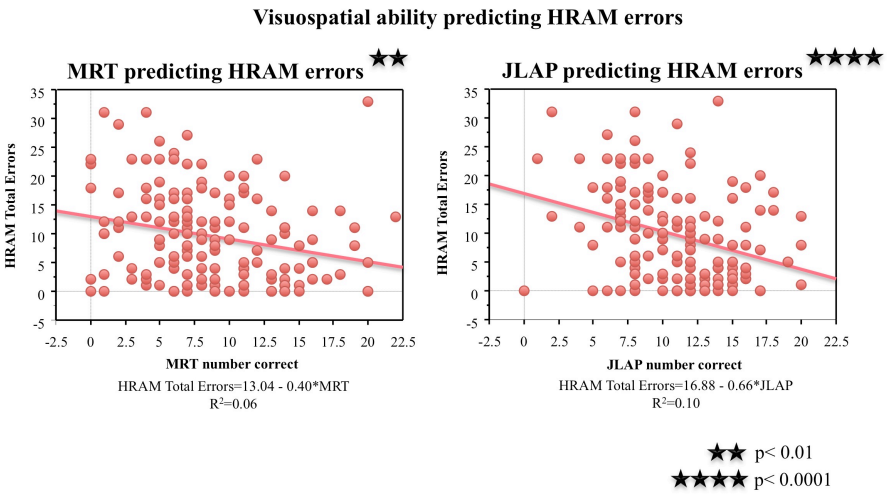

Visuospatial ability predicting HRAM errors
MRT predicting HRAM errors
HRAM Total Errors
MRT number correct
HRAM Total Errors=13.04 - 0.40*MRT
R²=0.06
JLAP predicting HRAM errors
HRAM Total Errors
JLAP number correct
HRAM Total Errors=16.88 - 0.66*JLAP
R²=0.10
★★ p< 0.01
★★★★ p< 0.0001

Figure 6. Experiment 1 Human Radial Arm Maze Scores as Predicted by Working Memory Measures. Performance on the working memory capacity tasks, the OSpan, Rspan, RotSpan, and SymSpan, correlated negatively with HRAM Total Errors ($p<0.001$, $p<0.0001$, $p<0.05$, $p<0.05$, respectively). For every additional point earned on the Ospan or Rspan, HRAM Total Errors decreased by 0.17, on average; HRAM Total Errors decreased by 0.19 for each one-point increase in RotSpan or SymSpan scores. The Ospan, Rspan, RotSpan, and SymSpan together predicted HRAM Total Errors (Adjusted $R^2_{multiple}=0.09$, $F_{(4,146)}= 4.80$, $p<0.001$).

Figure 6

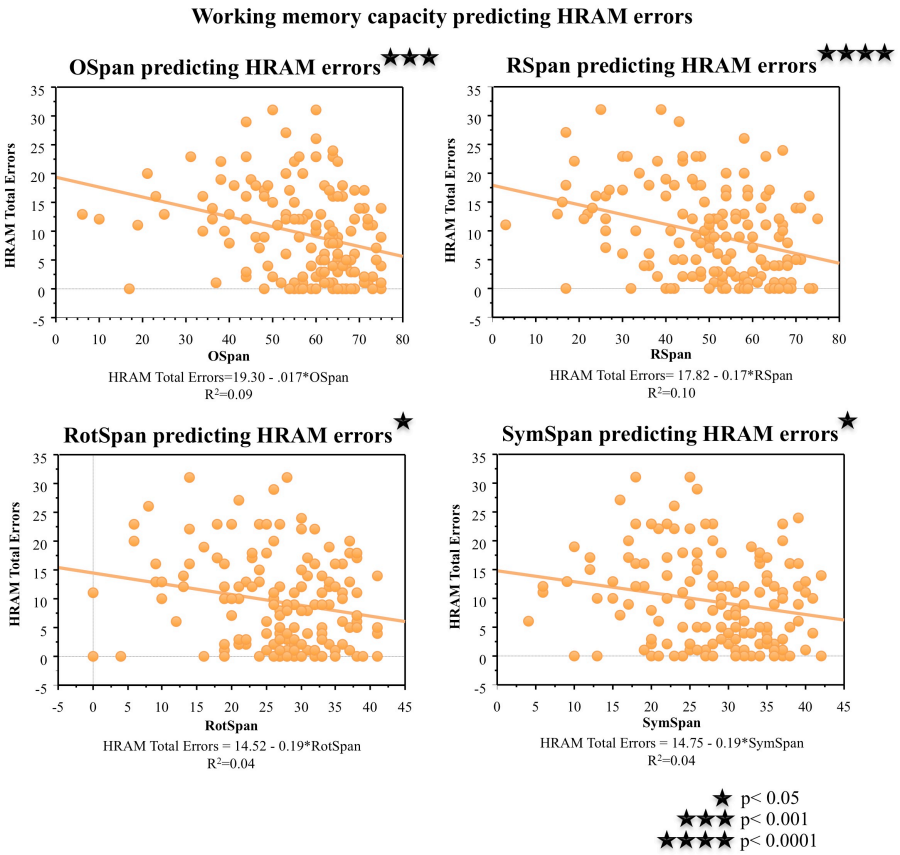

Working memory capacity predicting HRAM errors
OSpan predicting HRAM errors★★★
HRAM Total Errors
OSpan
HRAM Total Errors=19.30 - .017*OSpan
R²=0.09
RSpan predicting HRAM errors★★★★
HRAM Total Errors
RSpan
HRAM Total Errors= 17.82 - 0.17*RSpan
R²=0.10
RotSpan predicting HRAM errors★
HRAM Total Errors
RotSpan
HRAM Total Errors = 14.52 - 0.19*RotSpan
R²=0.04
SymSpan predicting HRAM errors★
HRAM Total Errors
SymSpan
HRAM Total Errors = 14.75 - 0.19*SymSpan
R²=0.04
★ p< 0.05
★★★ p< 0.001
★★★★ p< 0.0001

Figure 7. Experiment 2 Human Radial Arm Maze Performance Across Trials. There was a Trial x Hormone Group interaction ($F_{(30,1100)}$= 1.47, $p<0.05$) for Total Errors on the HRAM, such that as working memory load (trial) increased, group differences began to emerge. When trials were grouped into low working memory demand and high working memory demand, there were no differences between the Male and Female Follicular groups ($F_{(1,67)}$= 0.12, $p>0.05$, NS; $\eta^2<0.01$), Male and Female Luteal groups ($F_{(1,74)}$= 0.44, $p>0.05$, NS; $\eta^2<0.01$), or Male and Female Oral Contraceptive groups ($F_{(1,75)}$= 1.74, $p>0.05$, NS; $\eta^2=0.02$) for trials 2 through 6, which required participants to remember 1-5 previously visited spatial locations. There was a difference on Total Errors between the Male and Female Follicular groups ($F_{(1,67)}$= 4.38, $p<0.05$; $\eta^2=0.06$), and between the Male and Female Oral Contraceptive groups ($F_{(1,75)}$= 6.17, $p<0.05$; $\eta^2=0.08$), but not between the Male and Female Luteal groups ($F_{(1,74)}$= 0.14, $p>0.05$, NS; $\eta^2<0.01$), for trials 7 through 11, which corresponded to a demand of 6-10 previously visited spatial locations.

Figure 7

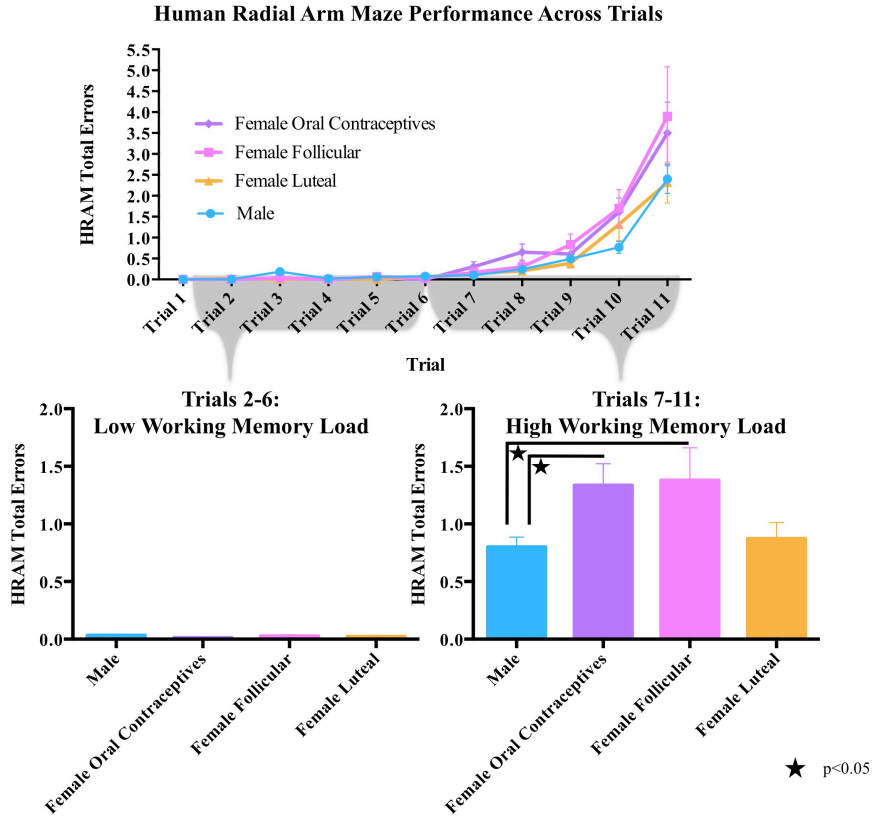

Human Radial Arm Maze Performance Across Trials
HRAM Total Errors
Female Oral Contraceptives
Female Follicular
Female Luteal
Male
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
Trial 6
Trial 7
Trial 8
Trial 9
Trial 10
Trial 11
Trial
Trials 2-6:
Low Working Memory Load
Trials 7-11:
High Working Memory Load
Male
Female Oral Contraceptives
Female Follicular
Female Luteal
p<0.05

Figure 8. Experiment 2 Reading Proficiency Task. We found a difference in WRAT-3 scores between the Male and Female Follicular ($F_{(1,60)}$= 6.95, $p<0.05$; η^2=0.10), but not between the Male and Female Luteal ($F_{(1,67)}$= 1.54, $p>0.05$, NS; η^2=0.02) groups, or the Male and Female Oral Contraceptive groups ($F_{(1,71)}$= 2.26, $p>0.05$, NS; η^2=0.03). Mean scores on the WRAT-3 for each group were as follows: men (M=109.6; SD=7.2), contraceptive users (M=106.7; SD=9.0), follicular phase (M=103.3; SD=6.0), and luteal phase (M=103.7; 13.5).

Figure 8

Reading Proficiency

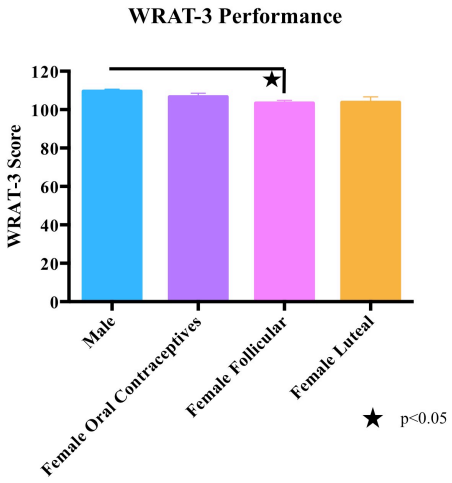


Figure 9. Experiment 2 Episodic Memory Tasks. There were no differences between Male and Female Follicular ($F_{(1,67)}$= 1.06, p>0.05, NS; η^2=0.02), Male and Female Luteal ($F_{(1,74)}$= 0.71, p>0.05, NS; η^2=0.01), or Male and Female Oral Contraceptive ($F_{(1,75)}$= 1.82, p>0.05, NS; η^2=0.02) on Total Words Learned (trials A1-A5). There were no differences between Male and Female Follicular ($F_{(1,66)}$= 1.08, p>0.05, NS; η^2=0.02), Male and Female Luteal ($F_{(1,73)}$= 0.38, p>0.05, NS; η^2=0.01), or Male and Female Oral Contraceptive ($F_{(1,74)}$= 0.99, p>0.05, NS; η^2=0.01) on number of words recalled on the retroactive interference trial (trial A6). There were no differences between Male and Female Follicular ($F_{(1,67)}$= 2.29, p>0.05, NS; η^2=0.03), Male and Female Luteal ($F_{(1,74)}$= 0.85, p>0.05, NS; η^2=0.01), or Male and Female Oral Contraceptive ($F_{(1,75)}$= 2.05, p>0.05, NS; η^2=0.03) on number of words recalled following a 20-minute delay (trial A7).

Figure 9

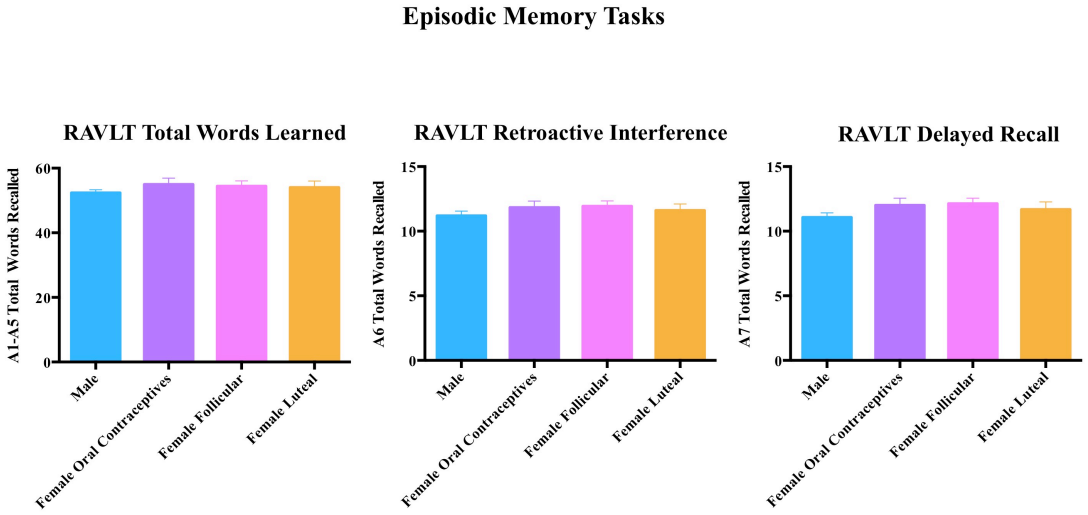

Episodic Memory Tasks
RAVLT Total Words Learned
A1-A5 Total Words Recalled
60
40
20
0
Male
Female Oral Contraceptives
Female Follicular
Female Luteal
RAVLT Retroactive Interference
A6 Total Words Recalled
15
10
5
0
Male
Female Oral Contraceptives
Female Follicular
Female Luteal
RAVLT Delayed Recall
A7 Total Words Recalled
15
10
5
0
Male
Female Oral Contraceptives
Female Follicular
Female Luteal

Figure 10. Experiment 2 Visuospatial Ability Tasks. There were differences in MRT scores between the Male and Female Follicular groups ($F_{(1,67)}$= 11.57, $p<0.01$; η^2=0.15), the Male and Female Luteal groups ($F_{(1,74)}$= 6.09, $p<0.05$; η^2=0.08), and the Male and Female Oral Contraceptive groups ($F_{(1,75)}$= 8.91, $p<0.01$; η^2=0.11). There was also a marginal difference in JLAP scores between the Male and Female Follicular groups ($F_{(1,67)}$= 3.54, $p<0.10$; η^2=0.05), and a significant difference between the Male and Female Oral Contraceptive groups ($F_{(1,75)}$= 5.73, $p<0.05$; η^2=0.07), but not between the Male and Female Luteal groups ($F_{(1,74)}$= 1.67, $p>0.05$, NS; η^2=0.02).

Figure 10

Visuospatial Ability Tasks

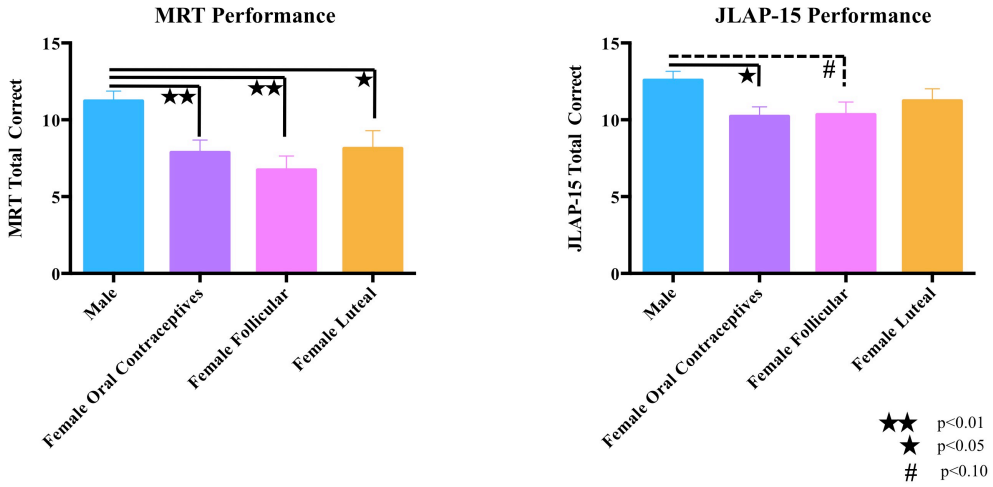


Figure 11. Experiment 2 Working Memory Capacity Tasks. There were no differences between groups on the OSpan (Male versus Female Follicular: $F_{(1,65)}= 0.28$, $p>0.05$, NS; $\eta^2<0.01$; Male versus Female Luteal: $F_{(1,72)}= 0.02$, $p>0.05$, NS; $\eta^2<0.01$; Male versus Female Oral Contraceptive: $F_{(1,73)}= 0.65$, $p>0.05$, NS; $\eta^2<0.01$), RSpan (Male versus Female Follicular: $F_{(1,65)}= 0.10$, $p>0.05$, NS; $\eta^2<0.01$; Male versus Female Luteal: $F_{(1,72)}= 2.31$, $p>0.05$, NS; $\eta^2=0.03$; Male versus Female Oral Contraceptive: $F_{(1,73)}= 0.06$, $p>0.05$, NS; $\eta^2<0.01$), RotSpan (Male versus Female Follicular: $F_{(1,65)}= 0.20$, $p>0.05$, NS; $\eta^2<0.01$; Male versus Female Luteal: $F_{(1,72)}= 0.57$, $p>0.05$, NS; $\eta^2<0.01$; Male versus Female Oral Contraceptive: $F_{(1,73)}= 0.43$, $p>0.05$, NS; $\eta^2<0.01$), or SymSpan (Male versus Female Follicular: $F_{(1,65)}= 0.03$, $p>0.05$, NS; $\eta^2<0.01$; Male versus Female Luteal: $F_{(1,72)}= 0.57$, $p>0.05$, NS; $\eta^2<0.01$; Male versus Female Oral Contraceptive: $F_{(1,73)}= 0.87$, $p>0.05$, NS; $\eta^2=0.01$) tasks.

Figure 11

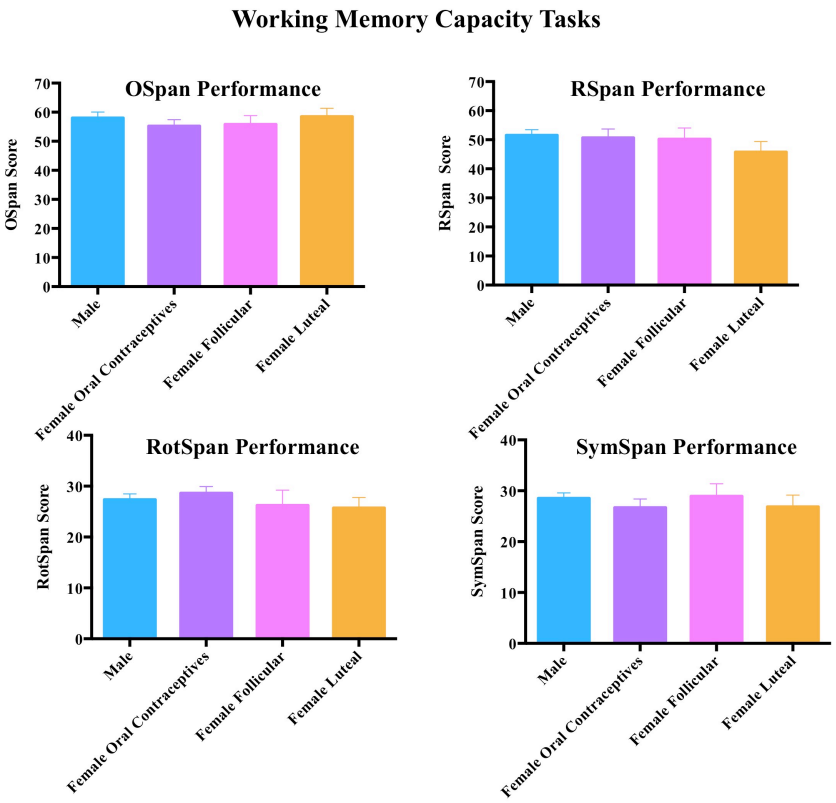

Working Memory Capacity Tasks
OSpan Performance
OSpan Score
RSpan Performance
RSpan Score
RotSpan Performance
RotSpan Score
SymSpan Performance
SymSpan Score
Male
Female Oral Contraceptives
Female Follicular
Female Luteal

Figure 12. Experiment 3 Water Radial Arm Maze Performance. When delivered via daily subcutaneous injection, there were no effects of EE treatment on WMC, WMI, or RM during the learning portion of testing. During the asymptotic phase of testing, similar to effects seen previously in our lab with tonic EE treatment (Mennenga et al., 2015), there was a Trial x Treatment interaction for WMC errors [$F_{(6,64)}$= 2.82; $p<0.05$] with a planned comparison showing that the high EE treated animals made more errors than vehicle treated animals as working memory load increased [$F_{(2,32)}$= 5.78; $p<0.01$]. Post-hoc analyses also showed that the high EE group committed more WMC errors than the low EE (Fisher, $p<0.05$) and medium EE (Fisher, $p<0.05$) animals at the highest working memory load.

Figure 12

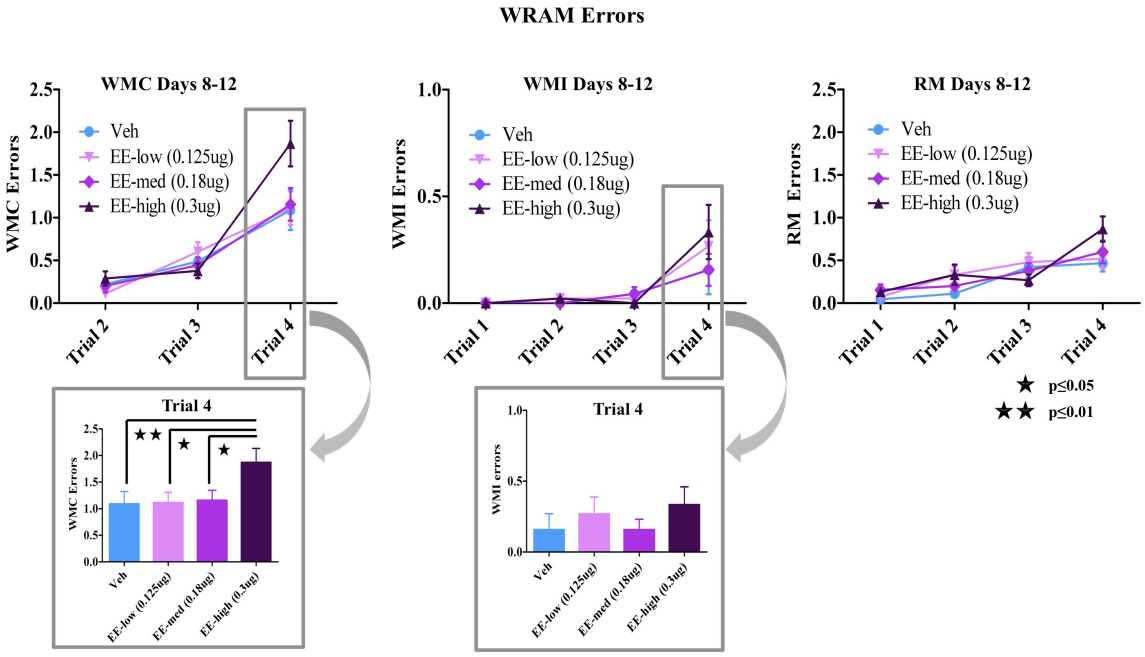

WRAM Errors
WMC Days 8-12
WMC Errors
Veh
EE-low (0.125ug)
EE-med (0.18ug)
EE-high (0.3ug)
Trial 2
Trial 3
Trial 4
Trial 4
WMC Errors
Veh
EE-low (0.125ug)
EE-med (0.18ug)
EE-high (0.3ug)
WMI Days 8-12
WMI Errors
Trial 1
Trial 2
Trial 3
Trial 4
Trial 4
WMI errors
RM Days 8-12
RM Errors
p≤0.05
p≤0.01

Figure 13. Experiment 3 Morris Water Maze Performance. There was a marginal Treatment x Day interaction for MM testing [$F_{(6,64)}$=2.21; p=0.05]. Further analyses revealed a main effect of Treatment [$F_{(3,32)}$= 3.22; p<0.05] for Day 1 of MM, whereby the vehicle group performed better than the low EE [$F_{(1,16)}$= 6.84; p<0.05], medium EE [$F_{(1,16)}$= 8.51; p<0.05], and high EE [$F_{(1,16)}$= 9.47; p<0.01] groups. There was no Treatment x Trial interaction for Day 1, indicating that this effect was present across all trials and was not carried by the initial exposure to the task on trial 1. There were no effects of Treatment for Days 2 or 3 of MM testing. A higher percent distance was spent in the previously platformed quadrant versus the opposite quadrant [$F_{(1,32)}$ =374.33; p<0.0001] for the probe trial, with no quadrant by Treatment interaction, indicating that all groups spatially localized the platform quadrant by the end of testing.

Figure 13

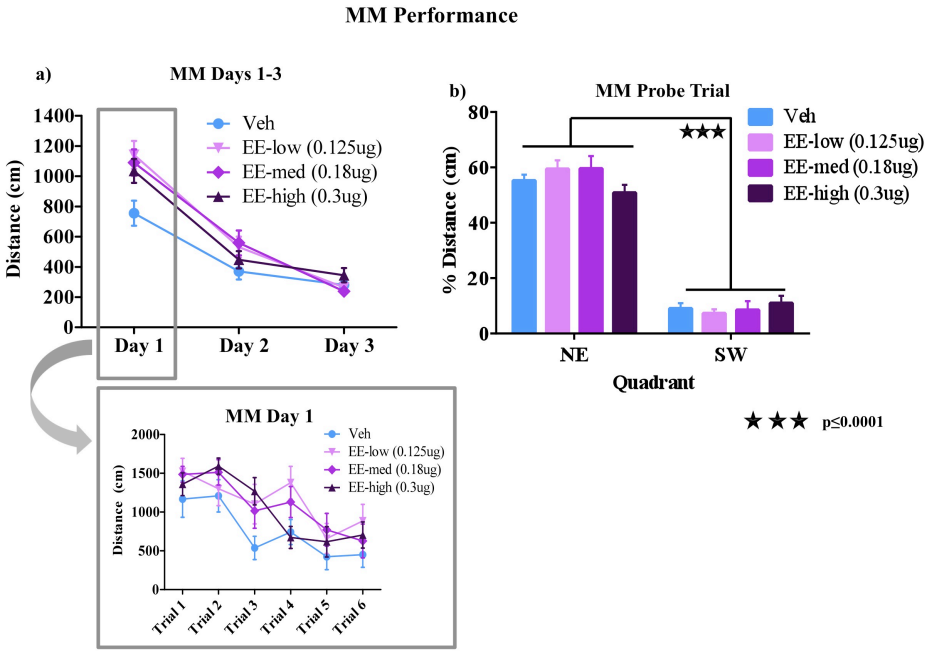

MM Performance
a)
MM Days 1-3
Distance (cm)
1400
1200
1000
800
600
400
200
0
Day 1
Day 2
Day 3
Veh
EE-low (0.125ug)
EE-med (0.18ug)
EE-high (0.3ug)
MM Day 1
2000
1500
1000
500
0
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
Trial 6
b)
MM Probe Trial
% Distance (cm)
80
60
40
20
0
★★★
NE
SW
Quadrant
★★★ p≤0.0001

Figure 14. Experiment 3 ChAT Cell Population Estimates. For wet uterine weight, there was a significant effect of Treatment [$F_{(3,31)}$=29.88; p<0.0001], with uteri of vehicle-treated rats weighing less than low EE- [$F_{(1,15)}$= 62.17; p <0.0001], medium EE- [$F_{(1,16)}$= 117.36; p <0.0001], and high EE- [$F_{(1,16)}$= 109.10; p <0.0001] treated rats.

Figure 14

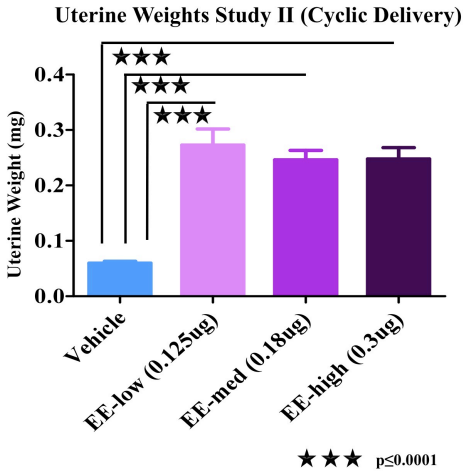

Uterine Weights Study II (Cyclic Delivery)
Uterine Weight (mg)
0.4
0.3
0.2
0.1
0.0
★★★
★★★
★★★
Vehicle
EE-low (0.125ug)
EE-med (0.18ug)
EE-high (0.3ug)
★★★ p≤0.0001

Figure 15. Experiment 3 Uterine Weights. There was a main effect of Treatment [$F_{(3,12)}$=3.66; $p<0.05$] in the VDB, whereby ChAT-IR cell counts were lower in the medium EE group (Fisher, $p<0.05$), and lower in the high EE group (Fisher, $p=0.05$), than those in the vehicle group. ChAT-IR cell counts in the low EE group did not differ from the vehicle group. Post-hoc tests indicate that the ChAT-IR cell counts were lower in the medium EE group (Fisher, $p<0.05$) and marginally lower in the high EE group (Fisher, $p=0.09$), than the low EE group. There were no effects of Treatment on ChAT-IR cell counts in the MS. The MS had a lower ChAT-IR cell count than the VDB ($F_{(1,12)}$=96.49, $p<0.001$). There was also a positive correlation between ChAT-IR cell counts in the VDB and MS of the basal forebrain ($r=0.56$, $p<0.05$), indicating that animals with higher ChAT-IR cell counts in the MS tended to also have higher cell counts in the VDB. There was a negative correlation between ChAT-IR cell counts in the VDB and number of WMC errors on the highest load trial (trial 4) during the asymptotic portion of WRAM testing [$r= -0.55$; $p<0.05$], such that animals with lower ChAT-IR cell counts committed more WMC errors.

Figure 15

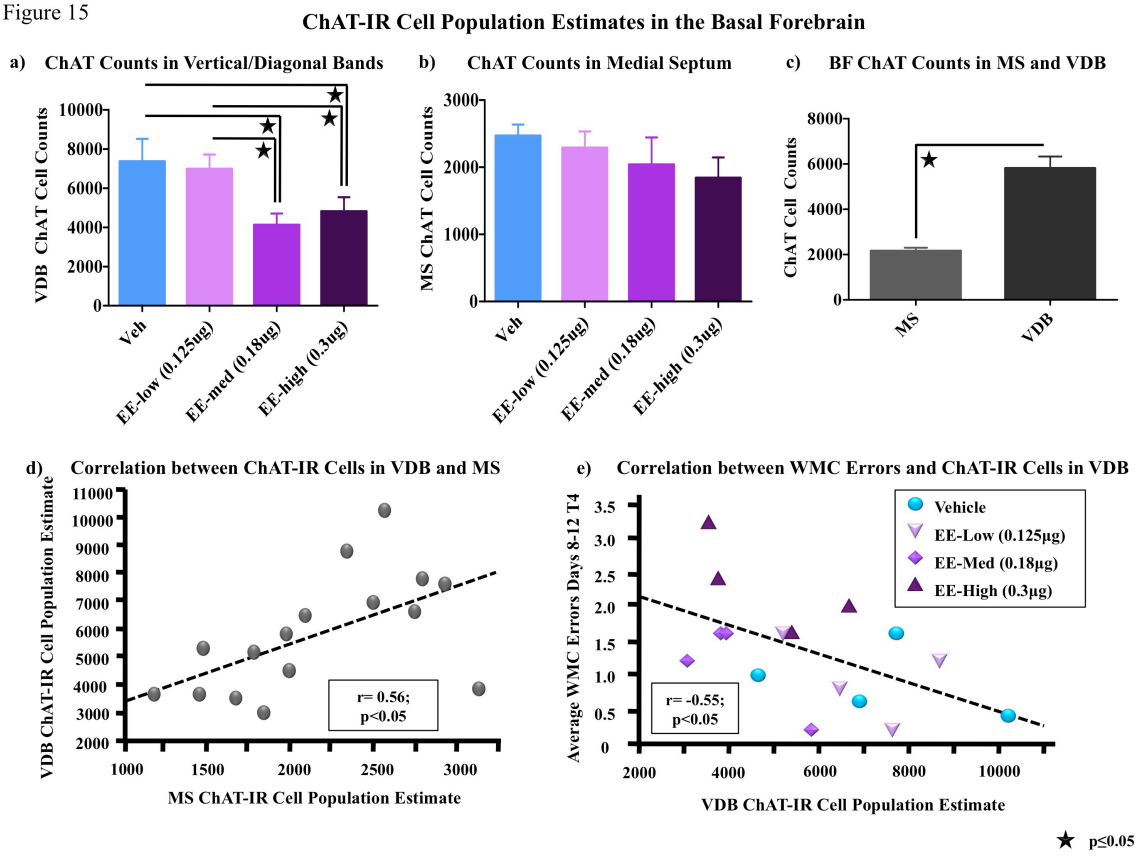

ChAT-IR Cell Population Estimates in the Basal Forebrain
a) ChAT Counts in Vertical/Diagonal Bands
VDB ChAT Cell Counts
Veh
EE-low (0.125ug)
EE-med (0.18ug)
EE-high (0.3ug)
b) ChAT Counts in Medial Septum
MS ChAT Cell Counts
c) BF ChAT Counts in MS and VDB
ChAT Cell Counts
MS
VDB
d) Correlation between ChAT-IR Cells in VDB and MS
VDB ChAT-IR Cell Population Estimate
MS ChAT-IR Cell Population Estimate
r= 0.56;
p<0.05
e) Correlation between WMC Errors and ChAT-IR Cells in VDB
Average WMC Errors Days 8-12 T4
VDB ChAT-IR Cell Population Estimate
Vehicle
EE-Low (0.125µg)
EE-Med (0.18µg)
EE-High (0.3µg)
r= -0.55;
p<0.05
★ p≤0.05

Figure 16. Experiment 4 Timeline and Depiction of Behavioral Tasks. Depiction of temporal relations between experimental manipulations.

Figure 16

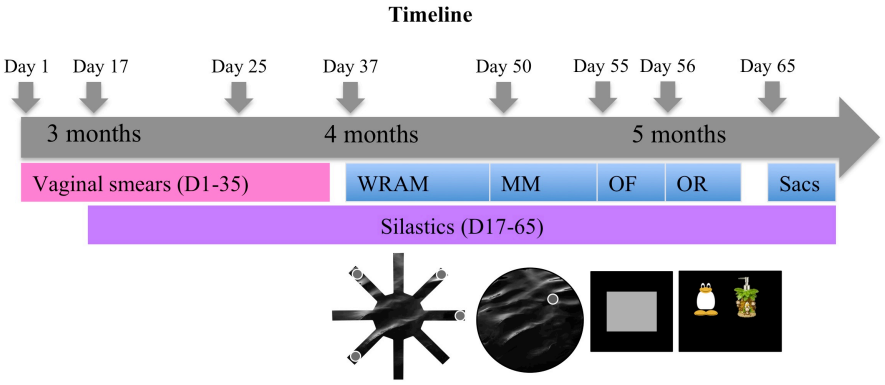

Timeline
Day 1
Day 17
Day 25
Day 37
Day 50
Day 55
Day 56
Day 65
3 months
4 months
5 months
Vaginal smears (D1-35)
WRAM
MM
OF
OR
Sacs
Silastics (D17-65)

Figure 17. Experiment 5 Water Radial Arm Maze Performance. There was a main effect of Treatment on Total Errors made on days 2-7 of WRAM testing, which is considered the learning phase of testing ($F_{(1,16)}$= 4.80, $p<0.05$), with the EE-treated group committing more errors across all trials than the vehicle-treated animals. There was no interaction between Treatment and Trial ($F_{(3,48)}$= 1.59, p=0.20, NS), indicating that this difference was not specific to any trial. This difference was no longer apparent during the asymptotic portion of testing, days 8-12 ($F_{(1,16)}$= 1.94, p=0.18, NS) and there was no interaction between Treatment and Trial for this portion of testing ($F_{(3,48)}$= 0.70, p=0.56, NS).

Figure 17

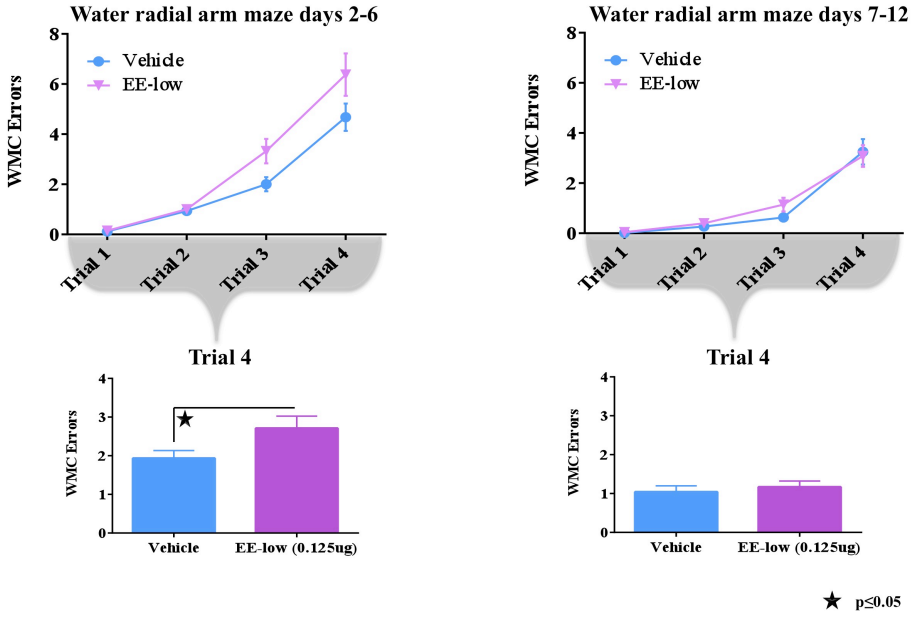

Water radial arm maze days 2-6
WMC Errors
Vehicle
EE-low
Trial 1
Trial 2
Trial 3
Trial 4
Trial 4
WMC Errors
Vehicle
EE-low (0.125ug)
Water radial arm maze days 7-12
WMC Errors
Vehicle
EE-low
Trial 1
Trial 2
Trial 3
Trial 4
Trial 4
WMC Errors
Vehicle
EE-low (0.125ug)
★ p≤0.05

Figure 18. Experiment 4 Morris Water Maze Performance. There were no main effects of Treatment on Total Swim Distance (cm) for the MM ($F_{(1,16)}$= 1.08, p=0.31, NS), but there was a Day x Treatment interaction ($F_{(4,64)}$= 3.32, p<0.05). Further analyses revealed that there was an effect of Treatment on Total Swim Distance on Day 1 of testing ($F_{(1,16)}$= 5.25, p<0.05), with the EE-treated animals swimming a shorter distance than the vehicle-treated animals across all four trials, suggesting that they covered a shorter distance during the allotted trial time. For the probe trial, there was a main effect of Quadrant ($F_{(1,16)}$= 173.00, p<0.0001), indicating that all animals preferred the previously-platformed quadrant over the diagonally opposite quadrant and were therefore likely employing a spatial strategy.

Figure 18

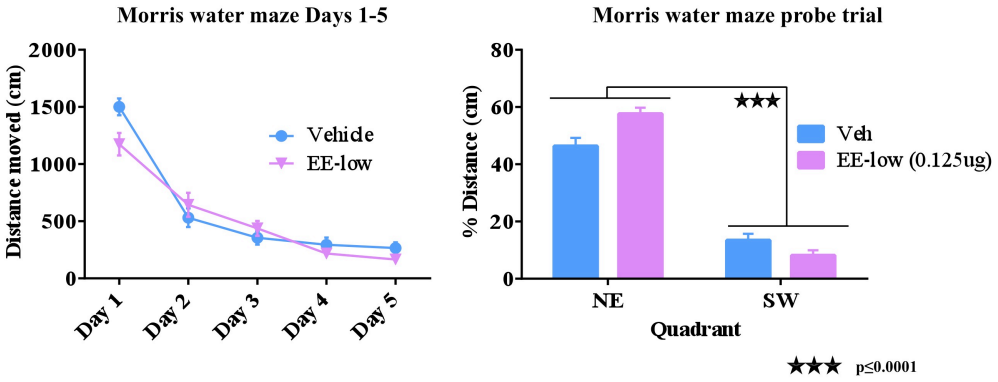

Morris water maze Days 1-5
Distance moved (cm)
2000
1500
1000
500
0
Vehicle
EE-low
Day 1
Day 2
Day 3
Day 4
Day 5
Morris water maze probe trial
% Distance (cm)
80
60
40
20
0
★★★
Veh
EE-low (0.125ug)
NE
SW
Quadrant
★★★ p≤0.0001

Figure 19. Experiment 4 Morris Water Maze Day 1 Performance. There was no difference on any trial of Day 1 when only successful trials were included (Trial 1: no animals found the platform; Trial 2: $F_{(1,5)}=0.05$, $p>0.05$, NS; Trial 3: $F_{(1,7)}=1.61$, $p>0.05$, NS; Trial 4: $F_{(1,8)}=0.47$, $p>0.05$, NS). Further, mean swim velocity (cm/s) was marginally decreased in the EE group ($F_{(1,16)}=3.93$, $p<0.10$).

Figure 19

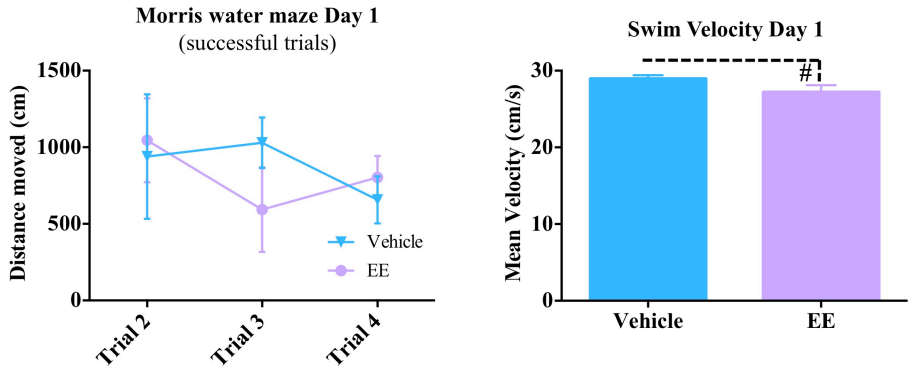

Morris water maze Day 1
(successful trials)
Distance moved (cm)
1500
1000
500
0
Trial 2
Trial 3
Trial 4
Vehicle
EE
Swim Velocity Day 1
Mean Velocity (cm/s)
30
20
10
0
#
Vehicle
EE

Figure 20. Experiment 4 Markers of Peripheral Stimulation. There was a main effect of Treatment on androstenedione levels ($F_{(1,14)}$= 11.99, $p<0.01$), E2 levels ($F_{(1,16)}$= 5.54, $p<0.05$), and FSH levels ($F_{(1,17)}$= 8.32, $p<0.05$), such that EE treatment increases androstenedione levels, decreases E2 levels, and increased FSH levels, relative to vehicle treatment.

Figure 20

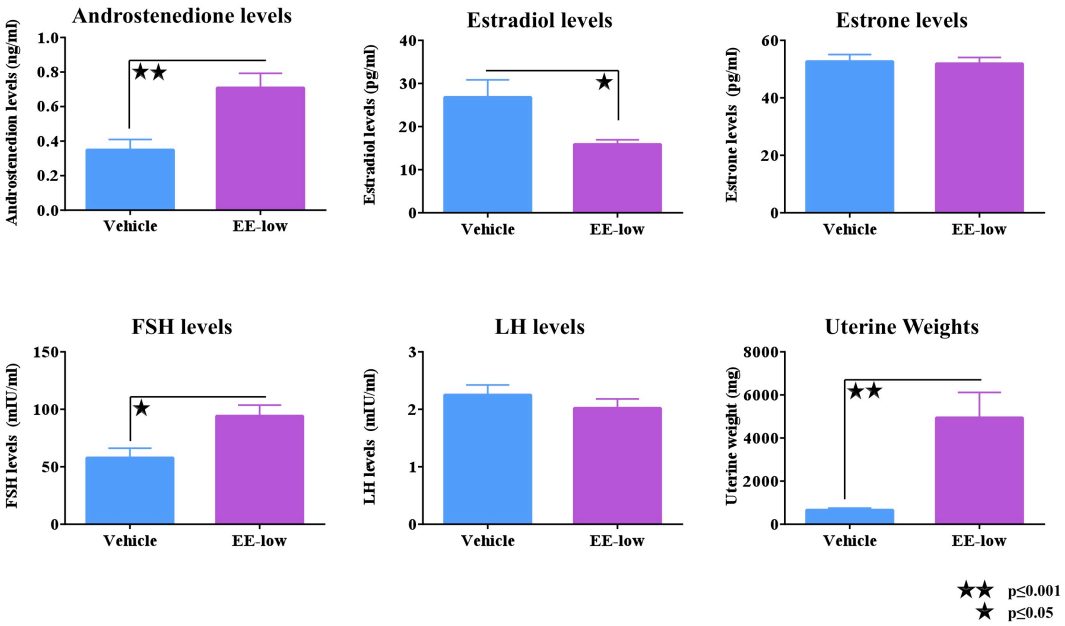

Androstenedione levels
Androstenedion levels (ng/ml)
1.0
0.8
0.6
0.4
0.2
0.0
Vehicle
EE-low
Estradiol levels
Estradiol levels (pg/ml)
40
30
20
10
0
Vehicle
EE-low
Estrone levels
Estrone levels (pg/ml)
60
40
20
0
Vehicle
EE-low
FSH levels
FSH levels (mIU/ml)
150
100
50
0
Vehicle
EE-low
LH levels
LH levels (mIU/ml)
3
2
1
0
Vehicle
EE-low
Uterine Weights
Uterine weight (mg)
8000
6000
4000
2000
0
Vehicle
EE-low
★★ p≤0.001
★ p≤0.05

Figure 21. Experiment 5 Timeline and Depiction of Behavioral Tasks Used. Depiction of temporal relations between experimental manipulations.

Figure 21

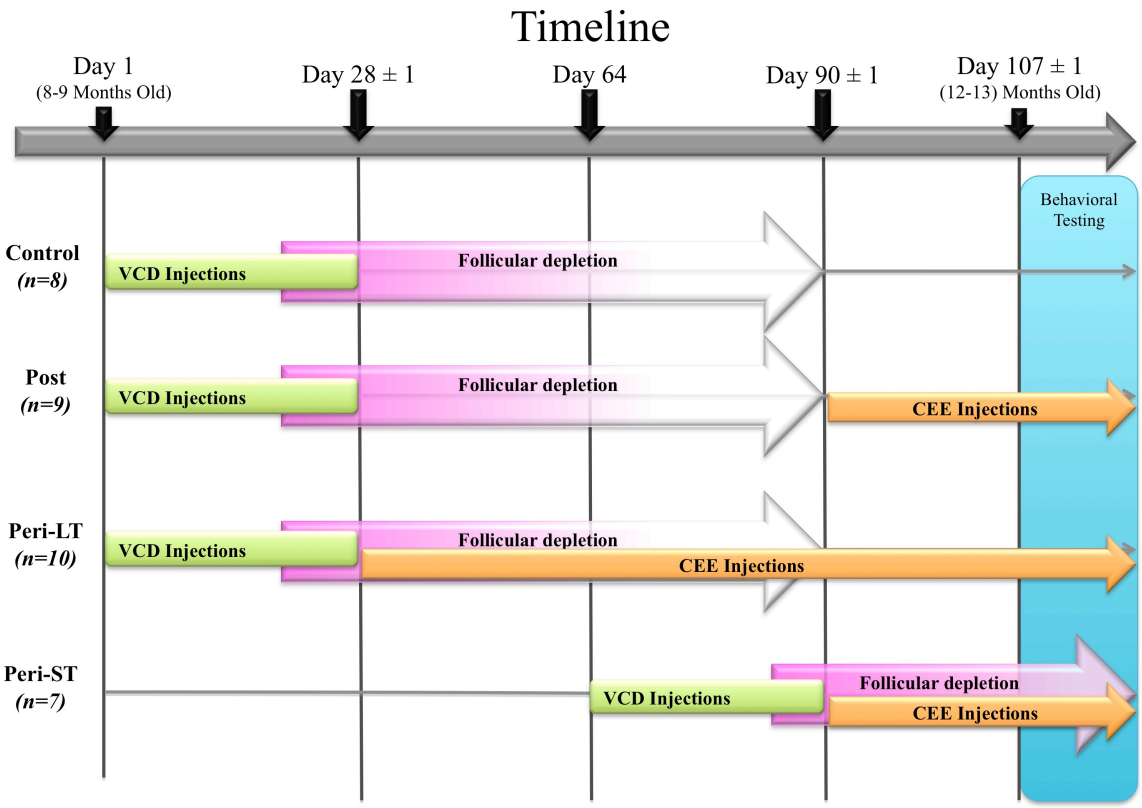

Timeline
Day 1
(8-9 Months Old)
Day 28 ± 1
Day 64
Day 90 ± 1
Day 107 ± 1
(12-13) Months Old)
Behavioral Testing
Control
(n=8)
VCD Injections
Follicular depletion
Post
(n=9)
VCD Injections
Follicular depletion
CEE Injections
Peri-LT
(n=10)
VCD Injections
Follicular depletion
CEE Injections
Peri-ST
(n=7)
VCD Injections
Follicular depletion
CEE Injections

Figure 22. Experiment 5 Water Radial Arm Maze Performance. For Block 3, there was a main effect of Treatment, such that the Post group made more errors than the Control group ($F_{(1,15)}$=3.22, p≤0.05, η_G^2=0.01), as expected (Acosta et al., 2010) ($F_{(1,15)}$=3.22, p≤0.05, η_G^2=0.01). The Post group also made more error than the Peri-ST group ($F_{(1,14)}$=6.20, p≤0.05, η_G^2=0.03) across all trials, indicating a benefit of early treatment, but did not differ from the Peri-LT group ($F_{(1,17)}$=1.20, p>0.05, NS, η_G^2=0.01), indicating that the benefit of early initiation is restricted to short-term treatment.

Figure 22

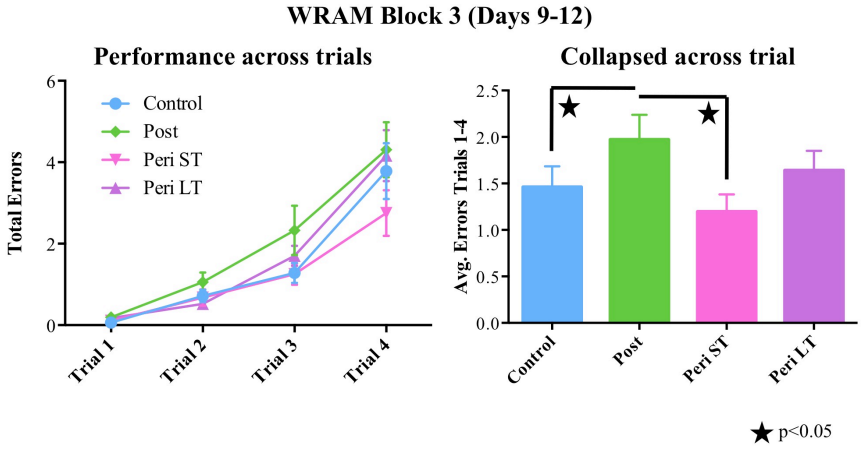

WRAM Block 3 (Days 9-12)
Performance across trials
Collapsed across trial
Total Errors
Avg. Errors Trials 1-4
Control
Post
Peri ST
Peri LT
Trial 1
Trial 2
Trial 3
Trial 4
★ p<0.05

Figure 23. Experiment 5 Morris Water Maze Performance. We did not observe an effect of Wave ($F_{(1,26)}$= 1.22; p>0.05, NS, η_G^2=0.02), nor a Wave x Treatment interaction ($F_{(3,26)}$= 1.42; p>0.05, NS, η_G^2=0.04) for MM swim distance, therefore analyses were collapsed across wave. There were no differences in swim distance on Days 1-3 between the Post and Control groups ($F_{(1,15)}$=0.36, p>0.05, η_G^2=0.01; NS), the Post and Peri-LT groups ($F_{(1,17)}$=2.29, p>0.05, η_G^2=0.02; NS), nor the Post and Peri-ST groups ($F_{(1,14)}$=1.21, p>0.05, η_G^2=0.03; NS). For the probe trial, there was a main effect of Quadrant ($F_{(1,30)}$=353.88, p≤0.0001, η_G^2=0.90) in the absence of a Quadrant x Treatment interaction ($F_{(3,30)}$=1.38, p>0.05, η_G^2=0.10; NS).

Figure 23

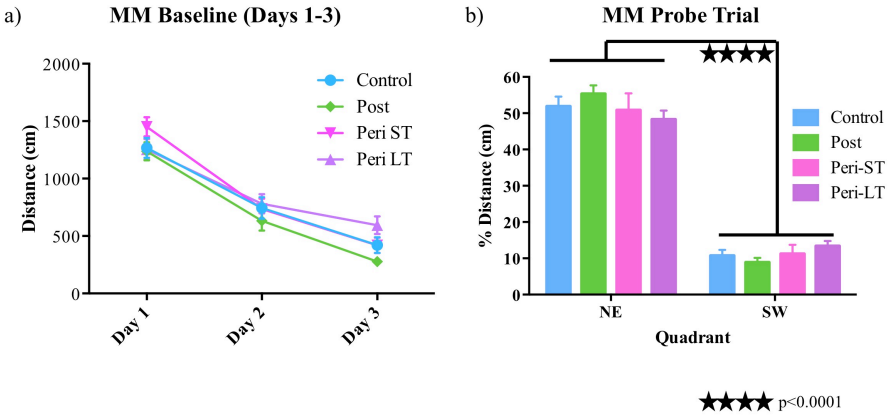

a)
MM Baseline (Days 1-3)
Distance (cm)
2000
1500
1000
500
0
Day 1
Day 2
Day 3
Control
Post
Peri ST
Peri LT
b)
MM Probe Trial
% Distance (cm)
60
50
40
30
20
10
0
NE
SW
Quadrant
Control
Post
Peri-ST
Peri-LT
★★★★
★★★★ p<0.0001

Figure 24. Experiment 5 Delay-Match-to-Sample Asymmetrical Three-Choice Task Performance. We did not observe an effect of Wave ($F_{(1,26)}$= 0.47; p>0.05, NS, η_G^2<0.01), nor a Wave x Treatment interaction ($F_{(3,26)}$= 2.35; p>0.05, NS, η_G^2=0.01) for DMS errors, therefore both waves are presented together. There was a Trial x Treatment interaction for the Control versus the Post group ($F_{(4, 60)}$=6.22; p≤0.001, η_G^2=0.08), whereby the Post group made fewer errors on Trial 2, the working memory trial ($F_{(1,15)}$=16.26, p≤0.01, η_G^2=0.14), during the learning phase of testing (Days 1-3). There were no differences between the Post and Peri-LT groups ($F_{(1,17)}$=0.66, p>0.05, η_G^2=0.01; NS) or Post and Peri-ST groups ($F_{(1,14)}$=0.03, p>0.05, η_G^2=0.04; NS) for the learning portion of testing (Figure 24a). There were no effects of Treatment for the asymptotic portion of DMS testing (Days 4-6), and there were no effects of Treatment on errors following a 6- or 8-hour delay between trials 1 and 2.

Figure 24

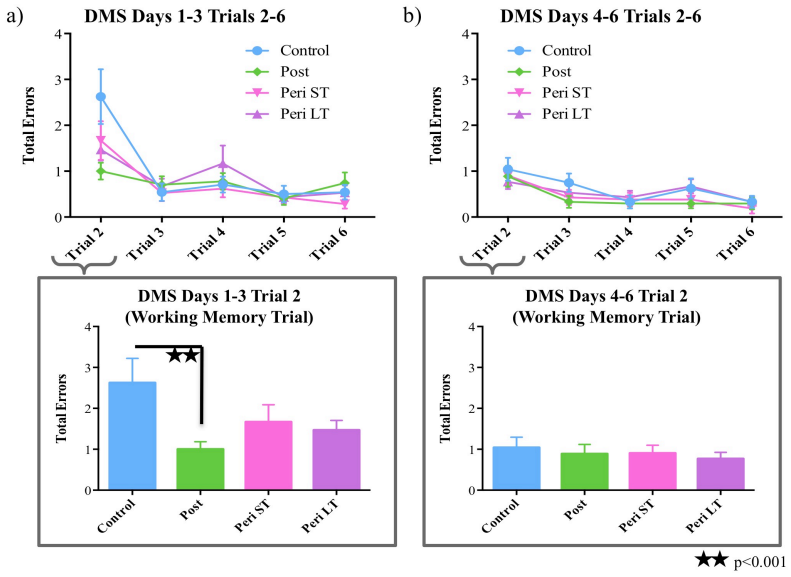

a)
DMS Days 1-3 Trials 2-6
Total Errors
Control
Post
Peri ST
Peri LT
Trial 2
Trial 3
Trial 4
Trial 5
Trial 6
DMS Days 1-3 Trial 2
(Working Memory Trial)
Total Errors
Control
Post
Peri ST
Peri LT
b)
DMS Days 4-6 Trials 2-6
Total Errors
Control
Post
Peri ST
Peri LT
Trial 2
Trial 3
Trial 4
Trial 5
Trial 6
DMS Days 4-6 Trial 2
(Working Memory Trial)
Total Errors
Control
Post
Peri ST
Peri LT
★★ p<0.001

Figure 25. Experiment 5 Peripheral Markers of Treatment Verification. There was a main effect of Treatment on uterine weights ($F_{(3, 30)}$=4.44; p≤0.05, η^2=0.31), such that the Control group had lower uterine weights than all CEE-treated groups (Post: Fisher, p≤0.05; Peri-LT: Fisher p≤0.05; Peri-ST: Fisher, p≤0.01). There was also a main effect of Treatment on corpora lutea counts ($F_{(3, 30)}$=8.91; p≤0.001, η^2=0.47), such that animals in the Peri-ST group had more corpora lutea than the Control (Fisher, p≤0.01), Post (Fisher, p≤0.001), and Peri-LT (Fisher, p≤0.0001) animals.

Figure 25

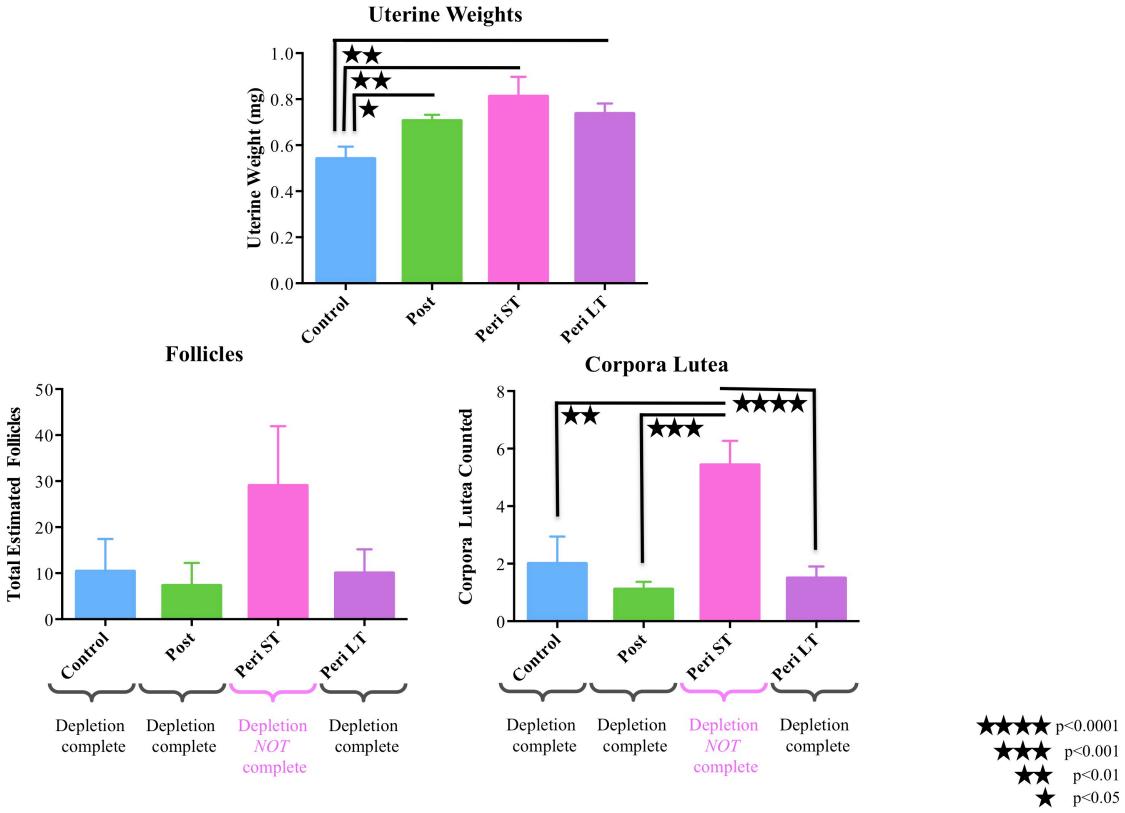

Uterine Weights
Uterine Weight (mg)
1.0
0.8
0.6
0.4
0.2
0.0
Control
Post
Peri ST
Peri LT
Follicles
Total Estimated Follicles
50
40
30
20
10
0
Depletion complete
Depletion complete
Depletion NOT complete
Depletion complete
Corpora Lutea
Corpora Lutea Counted
8
6
4
2
0
Depletion complete
Depletion complete
Depletion NOT complete
Depletion complete
p<0.0001
p<0.001
p<0.01
p<0.05

Figure 26. Experiment 5 Serum Levels of Androstenedione. There were no group differences in serum levels of androstenedione ($F_{(3,29)}$=0.13; p>0.05, η^2=0.02 NS).

Figure 26

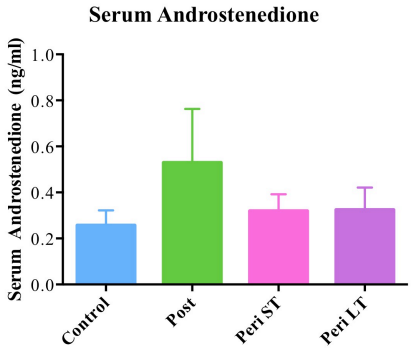
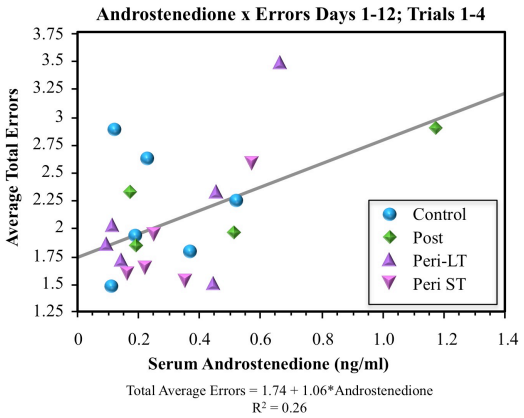

Serum Androstenedione
Serum Androstenedione (ng/ml)
1.0
0.8
0.6
0.4
0.2
0.0
Control
Post
Peri ST
Peri LT

Figure 27. Experiment 5 Correlations Between Behavioral Scores and Serum Androstenedione Levels. In all treatment groups, androstenedione levels positively correlated with total WRAM errors on Trials 1-4 across all days of testing ($r=0.51$, $p\leq0.05$), as well as on Trial 4 alone, the trial with the highest working memory load, ($r=0.58$, $p\leq0.01$).

Figure 27


Androstenedione x Errors Days 1-12; Trials 1-4
Average Total Errors
Serum Androstenedione (ng/ml)
Control
Post
Peri-LT
Peri ST
Total Average Errors = 1.74 + 1.06*Androstenedione
R² = 0.26

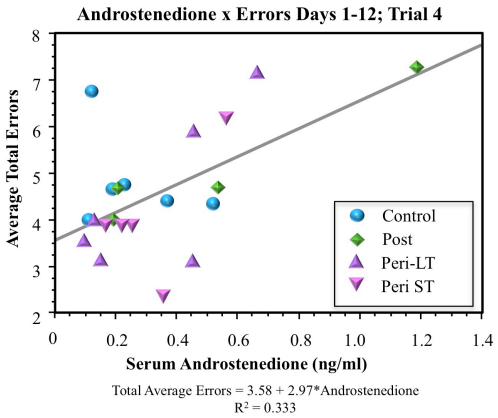

Androstenedione x Errors Days 1-12; Trial 4
Average Total Errors
Serum Androstenedione (ng/ml)
Control
Post
Peri-LT
Peri ST
Total Average Errors = 3.58 + 2.97*Androstenedione
R² = 0.333

Figure 28. Experiment 6 Timeline and Depiction of Behavioral Tasks Used. Depiction of temporal relations between experimental manipulations.

Figure 28

Group	VCD	Sx	Injection	Alzet Pump
Control	VCD	Sham	PEG	PEG
E2	VCD	Sham	PEG	3ug/day
Ovx	VCD	Ovx	PEG	PEG

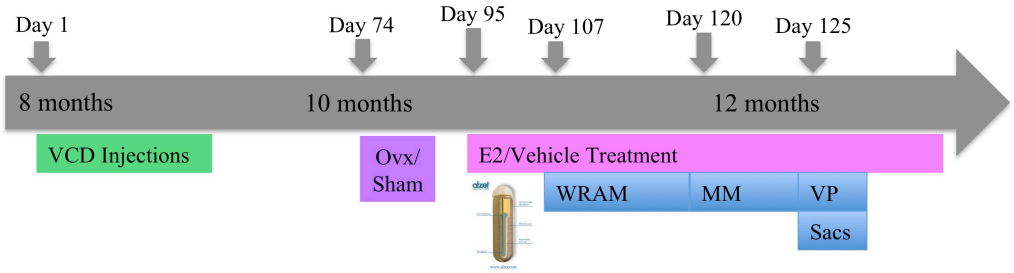


Figure 29. Experiment 6 Water Radial Arm Maze Performance. On the first block of WRAM testing, days 2-4, there was a Trial x Treatment interaction for the VCD and VCD-Ovx groups ($F_{(3,60)}$= 2.52, $p<0.05$; η_G^2=0.04), such that on trial 4, the trial with the highest working memory load, the VCD animals made more errors than the Ovx animals ($F_{(1,20)}$= 2.96, $p<0.05$; η_G^2=0.06). There was also a marginal Trial x Treatment interaction for the VCD and VCD-E2 groups ($F_{(3,60)}$= 2.48, $p<0.10$; η_G^2=0.04), such that on trial 4, the trial with the highest working memory load, the VCD animals made marginally more errors than the VCD-E2 animals ($F_{(1,20)}$= 2.94, $p<0.10$; η_G^2=0.06). On the second testing block, days 5-8, there was a Trial x Treatment interaction for the VCD and VCD-E2 groups ($F_{(3,60)}$= 3.70, $p<0.05$; $\eta_G^2<0.01$). On Trial 4, the trial with the highest working memory load, E2 treatment enhanced performance; the E2 animals made fewer errors than the VCD group ($F_{(1,20)}$= 4.68, $p<0.05$; η_G^2=0.04). There were no effects of Treatment on Days 9-12 of testing.

Figure 3

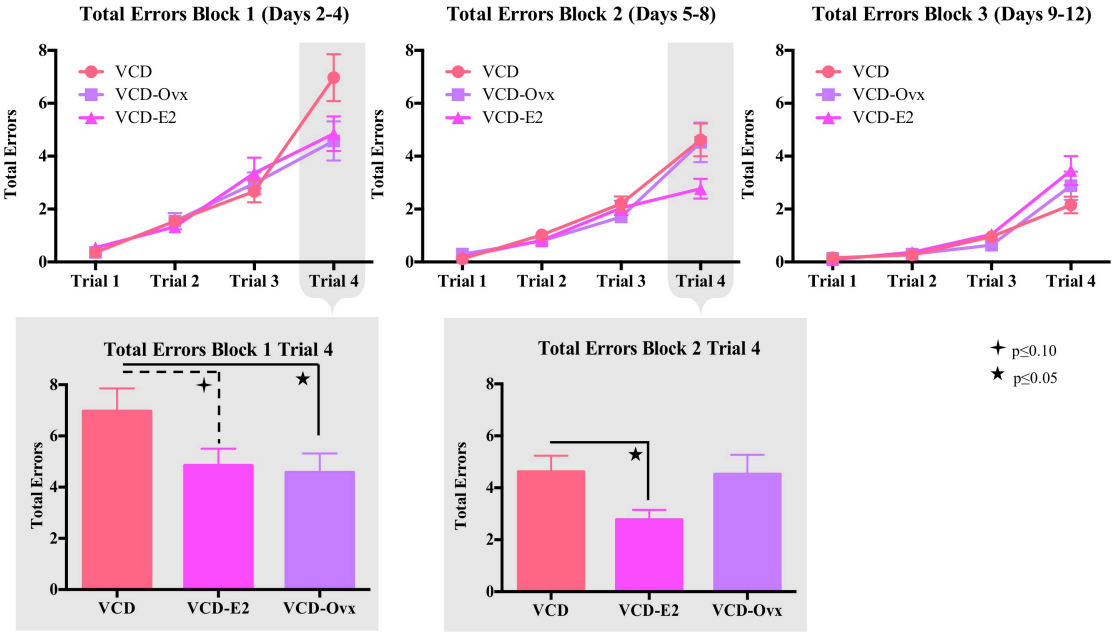

Total Errors Block 1 (Days 2-4)
Total Errors Block 2 (Days 5-8)
Total Errors Block 3 (Days 9-12)
VCD
VCD-Ovx
VCD-E2
Total Errors
Trial 1
Trial 2
Trial 3
Trial 4
Total Errors Block 1 Trial 4
Total Errors Block 2 Trial 4
VCD
VCD-E2
VCD-Ovx
✦ p≤0.10
★ p≤0.05

Figure 30. Experiment 6 Follicle Counts, Corpora Lutea Counts and Uterine Weights. There were no differences in total number of follicles present in the ovaries of the E2 and Vehicle groups at sacrifice ($F_{(1,20)}$= 0.93, p= 0.35; η^2=0.04; NS), or the number of corpora lutea ($F_{(1,20)}$= 0.46, p= 0.51; η^2=0.02; NS). There was a main effect of Treatment on wet uterine weights ($F_{(2,30)}$= 14.93, p<0.0001; η^2=0.50), with E2-treated animals having heavier uterine horns than vehicle-treated or Ovx animals (Fisher, p<0.0001). There was no difference between the Vehicle and Ovx groups in uterine weight, indicating a lack of uterine stimulation in the Vehicle-treated VCD animals.

Figure 30

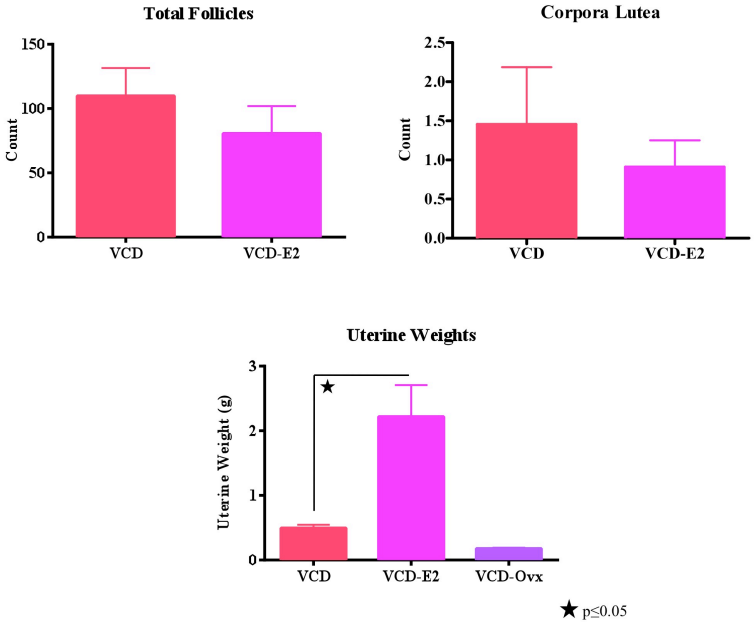

Total Follicles
Count
150
100
50
0
VCD
VCD-E2
Corpora Lutea
Count
2.5
2.0
1.5
1.0
0.5
0.0
VCD
VCD-E2
Uterine Weights
Uterine Weight (g)
3
2
1
0
VCD
VCD-E2
VCD-Ovx
★ p≤0.05

Figure 31. Experiment 6 Serum Levels of E2, Estrone, and Androstenedione. There was a main effect of treatment on serum E2 levels ($F_{(2,30)}$= 17.85, $p<0.0001$; η^2=0.54), with the E2-treated group showing higher circulating levels of E2 than both the Ovx and Vehicle groups (Fisher, $p<0.0001$). There was no difference in serum E2 levels between the Ovx and Vehicle groups at sacrifice (Fisher, p=0.76; NS; Vehicle M=5.42pg/ml, Ovx M=1.45pg/ml).

Figure 31

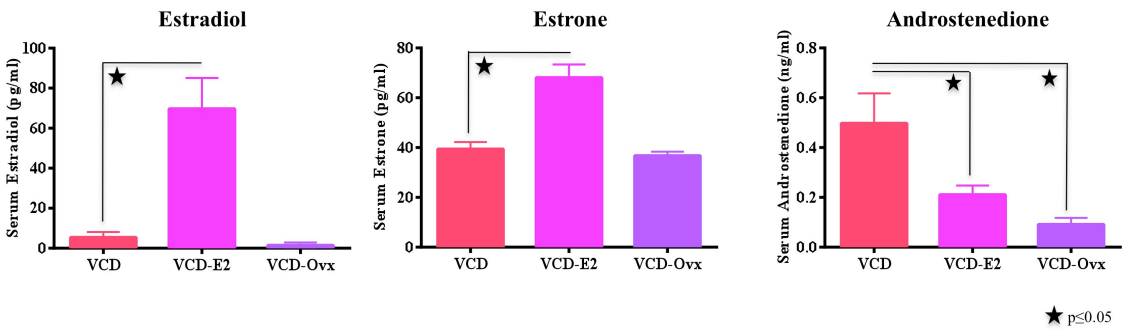

Estradiol
Serum Estradiol (pg/ml)
100
80
60
40
20
0
VCD
VCD-E2
VCD-Ovx
Estrone
Serum Estrone (pg/ml)
80
60
40
20
0
VCD
VCD-E2
VCD-Ovx
Androstenedione
Serum Androstenedione (ng/ml)
0.8
0.6
0.4
0.2
0.0
VCD
VCD-E2
VCD-Ovx
★ p≤0.05

Figure 32. Experiment 7 Timeline and Depiction of Behavioral Tasks Used. Depiction of temporal relations between experimental manipulations.

Figure 32

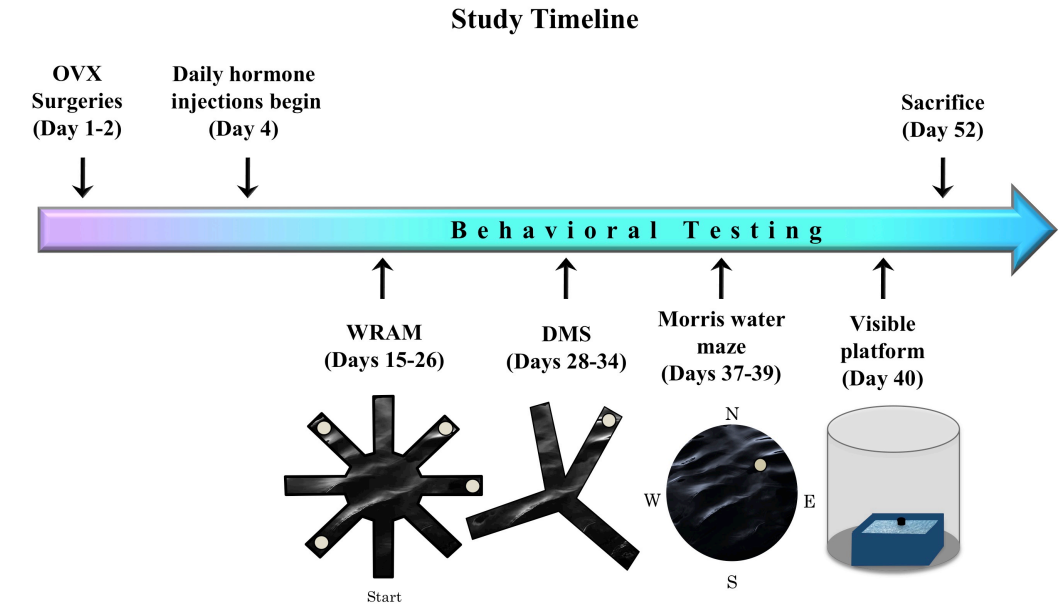

Study Timeline
OVX Surgeries (Day 1-2)
Daily hormone injections begin (Day 4)
Sacrifice (Day 52)
Behavioral Testing
WRAM (Days 15-26)
DMS (Days 28-34)
Morris water maze (Days 37-39)
Visible platform (Day 40)
Start
N
W
E
S

Figure 33. Experiment 7 Water Radial Arm Maze Performance. For Block 3 of WRAM, there was a Treatment x Trial interaction for WMC errors [$F_{(8,88)}$ =3.05, $p<0.01$]. For Trial 4, there was a main effect of Treatment [$F_{(4,44)}$ =4.31, $p<0.01$]; the Androstenedione group did worse than the Vehicle group (Fisher, $p<0.001$); the addition of anastrozole treatment reversed this androstenedione-induced impairment (Fisher, $p<0.01$). On Trial 4, the Androstenedione group also did worse than the Anastrozole group (Fisher, $p<0.01$) group, and the Androstenedione+Flutamide group did worse than the Vehicle group (Fisher, $p<0.05$). There was also an effect on Block 3 for WMI, with a Treatment x Trial interaction for WMI errors [$F_{(12,132)}$ =5.36, $p<0.0001$]. For Trial 4, there was a main effect of Treatment [$F_{(4,44)}$ =5.90, $p<0.001$]; the Androstenedione group committed more WMI errors than Vehicle (Fisher, $p<0.01$). The addition of anastrozole reversed the impairing effect of andostenedione on Trial 4 (Fisher, $p<0.01$), and the Androstenedione group made more errors than the Anastrozole group (Fisher, $p<0.01$). The Androstenedione+Flutamide group committed more WMI errors on Trial 4 than the Vehicle (Fisher, $p<0.01$), Androstenedione+Anastrozole (Fisher, $p<0.01$), and Anastrozole (Fisher, $p<0.01$) groups. There was a main effect of Treatment for RM errors [$F_{(4,44)}$ =6.30, $p<0.001$] for Block 3 of WRAM testing. The Androstenedione group committed more RM errors than the Vehicle group (Fisher, $p<0.001$), and anastrozole reversed reference memory impairments induced by androstenedione (Fisher, $p<0.05$). The Androstenedione group also made more RM errors than the Anastrozole group (Fisher, $p<0.05$), and the Androstenedione+Flutamide group made more RM errors than Vehicle (Fisher, $p<0.001$), Androstenedione+Anastrozole (Fisher, $p<0.01$), and Anastrozole (Fisher, $p<0.05$) groups.

Figure 33

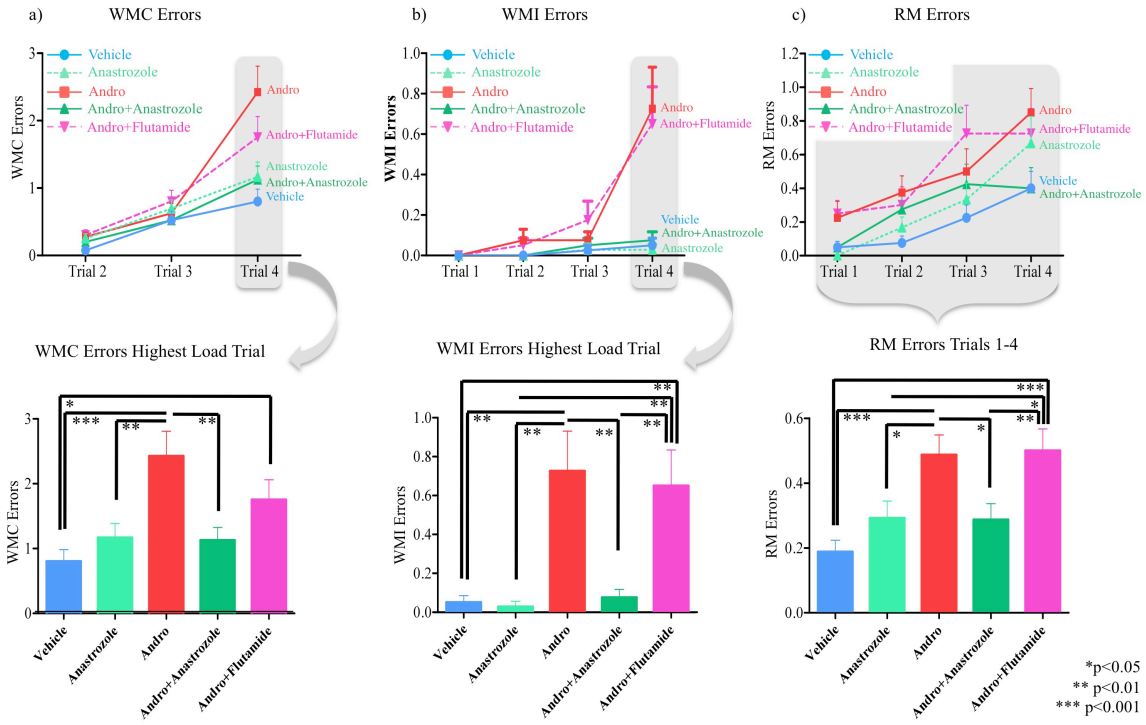

a)
WMC Errors
Vehicle
Anastrozole
Andro
Andro+Anastrozole
Andro+Flutamide
WMC Errors
Trial 2
Trial 3
Trial 4
b)
WMI Errors
WMI Errors
Trial 1
Trial 2
Trial 3
Trial 4
c)
RM Errors
RM Errors
Trial 1
Trial 2
Trial 3
Trial 4
WMC Errors Highest Load Trial
WMI Errors Highest Load Trial
RM Errors Trials 1-4
Vehicle
Anastrozole
Andro
Andro+Anastrozole
Andro+Flutamide
*p<0.05
** p<0.01
*** p<0.001

Figure 34. Experiment 7 Delay Match-to-Sample Performance. There was a main effect of Day [$F_{(6,264)} = 17.17$, $p < 0.0001$] with Total Errors decreasing as days progressed. There were no Treatment effects for Total Errors (Days 1-7), nor was there a Treatment x Day interaction.

Figure 34

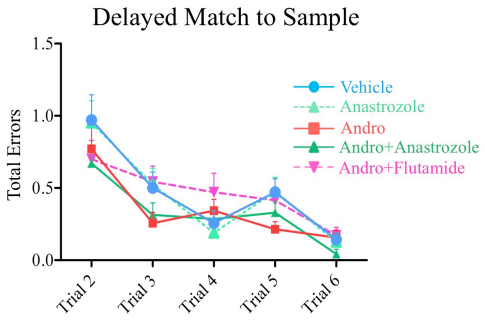

Delayed Match to Sample
Total Errors
1.5
1.0
0.5
0.0
Trial 2
Trial 3
Trial 4
Trial 5
Trial 6
Vehicle
Anastrozole
Andro
Andro+Anastrozole
Andro+Flutamide

Figure 35. Experiment 7 Morris Water Maze Performance. Analyses revealed a main effect of Block [$F_{(5,220)}$ =150.06, $p<0.0001$], with swim distance decreasing across blocks showing learning. There was a Treatment x Block interaction for Morris water maze testing [$F_{(20,220)}$=1.84; $p<0.05$]. For Block 1, there was a main effect of Treatment [$F_{(4,44)}$=2.96; $p<0.05$]; post hoc analyses revealed that the Androstenedione+Anastrozole group swam a shorter distance to the platform than the Vehicle (Fisher, $p<0.05$), Androstenedione (Fisher, $p<0.05$), and the Androstenedione+Flutamide group (Fisher, $p<0.01$). For the probe trial, there was a main effect of Quadrant [$F_{(1,44)}$=982.20; $p<0.0001$] in the absence of a Quadrant x Treatment interaction [$F_{(4,44)}$=2.30; $p>0.05$, NS], indicating that all groups equally localized the platform using spatial navigation by the end of Morris water maze testing.

Figure 35

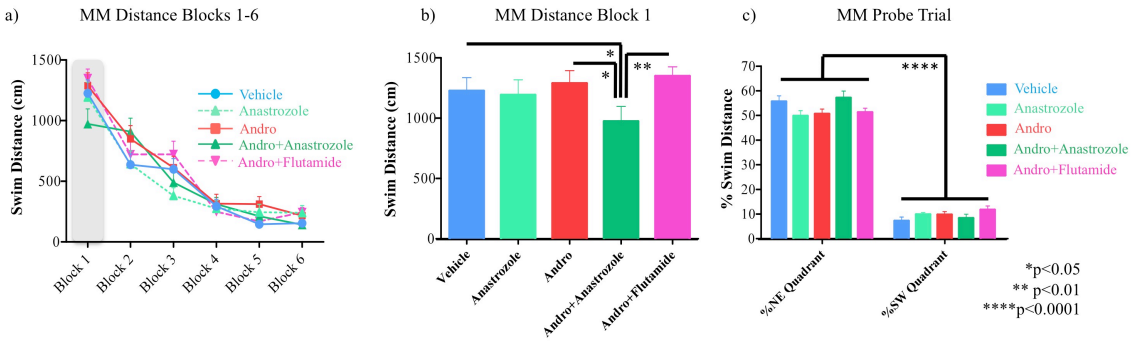

a)
MM Distance Blocks 1-6
Swim Distance (cm)
1500
1000
500
0
Block 1
Block 2
Block 3
Block 4
Block 5
Block 6
Vehicle
Anastrozole
Andro
Andro+Anastrozole
Andro+Flutamide
b)
MM Distance Block 1
Swim Distance (cm)
1500
1000
500
0
*
*
**
Vehicle
Anastrozole
Andro
Andro+Anastrozole
Andro+Flutamide
c)
MM Probe Trial
% Swim Distance
70
60
50
40
30
20
10
0
****
%NE Quadrant
%SW Quadrant
Vehicle
Anastrozole
Andro
Andro+Anastrozole
Andro+Flutamide
*p<0.05
** p<0.01
****p<0.0001

Figure 36. Experiment 7 Visible Platform Performance. There was a main effect of Trial [$F_{(5,220)}$ =9.32, $p<0.0001$], with latency decreasing as trials progressed within the day of visible platform testing. There were no Treatment main effects [$F_{(4,44)}$=1.49, $p<0.05$, NS] on latency to escape for the visible platform task. However, there was a Treatment x Trial interaction [$F_{(20,220)}$ =2.35, $p<0.01$], such that there was a main effect of Treatment on Trial 1 [$F_{(4,44)}$ =3.65, $p<0.05$]. Further analyses indicated that the Vehicle group took a longer time to reach the platform than the Androstenedione (Fisher, $p<0.05$), Androstenedione+Anastrozole (Fisher, $p<0.01$), and Anastrozole (Fisher, $p<0.001$) groups; no hormone treated group differed from any other hormone treated group. There were no effects of Treatment on any of the remaining trials (Trials 2-6).

Figure 36

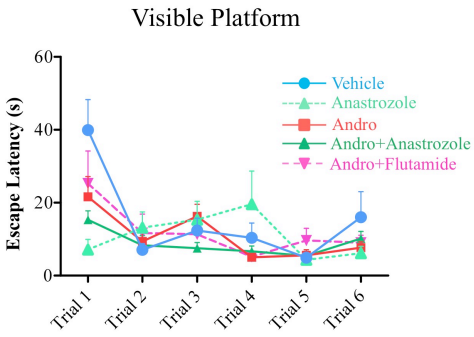

Visible Platform
Escape Latency (s)
60
40
20
0
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
Trial 6
Vehicle
Anastrozole
Andro
Andro+Anastrozole
Andro+Flutamide

Figure 37. Experiment 7 Uterine Weights. There was a main effect of Treatment for uterine weights [$F_{(4,43)}$=13.03; $p<0.0001$]. The Androstenedione group had higher uterine weights than the Vehicle (Fisher, $p<0.0001$), Androstenedione+Anastrozole (Fisher, $p<0.001$), Androstenedione+Flutamide (Fisher, $p<0.0001$), and Anastrozole (Fisher, $p<0.0001$) groups. The Androstenedione+Anastrozole group also had higher uterine weights than the Anastrozole group (Fisher, $p<0.05$).

Figure 37

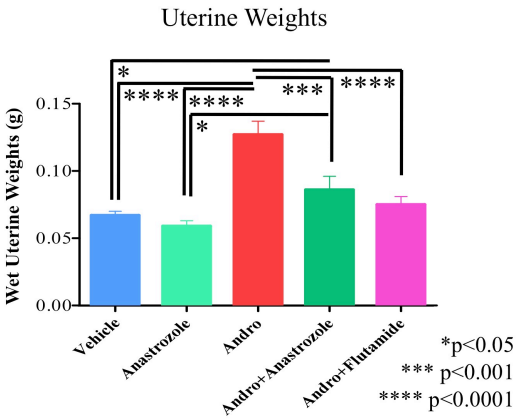

Uterine Weights
Wet Uterine Weights (g)
0.15
0.10
0.05
0.00
*
****
****
*
***
****
Vehicle
Anastrozole
Andro
Andro+Anastrozole
Andro+Flutamide
*p<0.05
*** p<0.001
**** p<0.0001

Figure 38. Experiment 7 Serum Hormone Levels. There was a main effect of Treatment for serum androstenedione [$F_{(4,31)}$=6.04; p<0.01]. Androstenedione increased serum androstenedione levels in all groups receiving this androgen, relative to vehicle treatment [Vehicle vs. Androstenedione (Fisher, p<0.01), Vehicle vs. Androstenedione+Flutamide (Fisher, p<0.05), Vehicle vs. Androstenedione+ Anastrozole (Fisher, p<0.01)], and relative to treatment with anastrozole alone [Anastrozole group vs. Androstenedione group (Fisher, p<0.001), Anastrozole vs. Androstenedione+Flutamide group (Fisher, p<0.05), Anastrozole vs. Androstenedione+ Anastrozole group (Fisher, p<0.01)]. There was a main effect of Treatment for serum testosterone [$F_{(4,28)}$=4.60; p<0.01]. The Androstenedione group had higher testosterone serum levels than Vehicle (Fisher, p<0.01) and Anastrozole (Fisher, p<0.01) groups, and the Androstenedione+Anastrozole group also had higher serum testosterone levels than Vehicle (Fisher, p<0.05) and Anastrozole (Fisher, p<0.05) groups. The analysis of serum estrone revealed a main effect of Treatment as well [$F_{(4,26)}$=96.67; p<0.0001]. The Androstenedione group had higher serum estrone levels than the Vehicle group (Fisher, p<0.001), and the addition of anastrozole decreased estrone levels (Androstenedione vs. Androstenedione+Anastrozole Fisher, p<0.05). The Androstenedione group had higher serum levels than the Anastrozole group (Fisher, p<0.001), and the Androstenedione+ Flutamide group had higher serum estrone levels than the Vehicle (Fisher, p<0.0001), Anastrozole (Fisher, p<0.0001), Androstenedione (Fisher, p<0.0001), and Androstenedione+Anastrozole groups (Fisher, p<0.0001). The Androstenedione+Anastrozole group tended to have higher serum estrone levels than the Vehicle (p=0.05), and Anastrozole (p=0.05) groups.

Figure 38

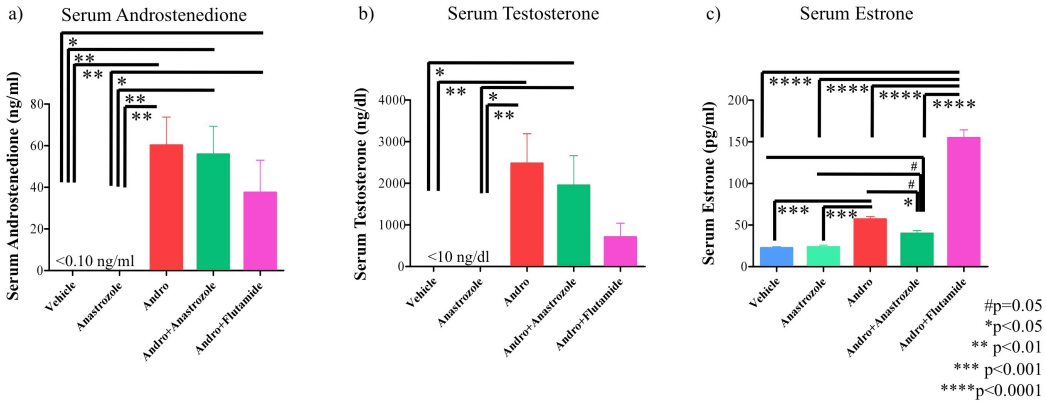

a)
Serum Androstenedione
Serum Androstenedione (ng/ml)
<0.10 ng/ml
b)
Serum Testosterone
Serum Testosterone (ng/dl)
<10 ng/dl
c)
Serum Estrone
Serum Estrone (pg/ml)
Vehicle
Anastrozole
Andro
Andro+Anastrozole
Andro+Flutamide
#p=0.05
*p<0.05
** p<0.01
*** p<0.001
****p<0.0001

Figure 39. Experiment 7 Correlations Between Serum Estrone Levels and Cognitive Performance. Serum estrone levels correlated with average Total Errors on Block 3 of WRAM testing across all four trials ($r=0.39$; $p<0.05$), as well as on Trial 4, the trial with the highest working memory load ($r=0.36$; $p<0.05$). Because we found a clear bimodal distribution in estrone levels, whereby the Androstenedione+Flutamide group had higher estrone levels than all other groups and therefore held the potential to exert a large amount of influence over these analyses, we also assessed each of these correlations excluding the Androstenedione+Flutamide group. With the Androstenedione+Flutamide group excluded, we found that serum estrone levels still correlated with average Total Errors on Block 3 of WRAM testing across all four trials ($r=0.58$; $p<0.01$), as well as on Trial 4, the trial with the highest working memory load ($r=0.62$; $p<0.01$).

Figure 39

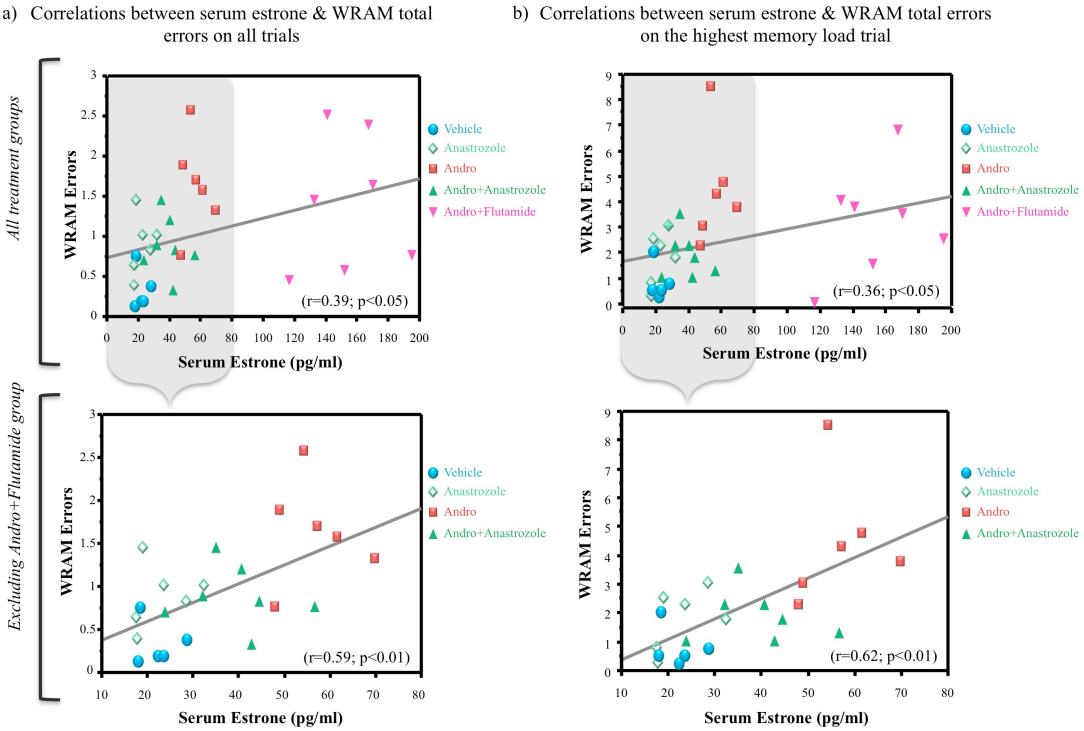

a) Correlations between serum estrone & WRAM total errors on all trials
b) Correlations between serum estrone & WRAM total errors on the highest memory load trial
All treatment groups
Excluding Andro+Flutamide group
WRAM Errors
Serum Estrone (pg/ml)
(r=0.39; p<0.05)
(r=0.36; p<0.05)
(r=0.59; p<0.01)
(r=0.62; p<0.01)
Vehicle
Anastrozole
Andro
Andro+Anastrozole
Andro+Flutamide

Figure 40. Experiment 8 Timeline and Depiction of Behavioral Tasks Used. Depiction of temporal relations between experimental manipulations.

Figure 40

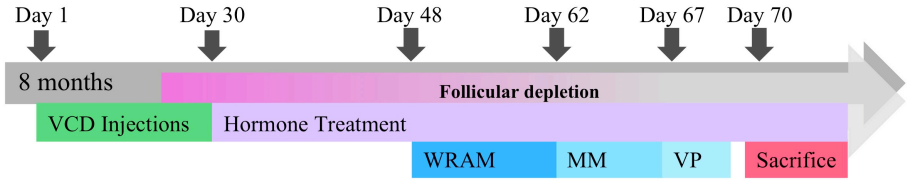

Day 1
Day 30
Day 48
Day 62
Day 67
Day 70
8 months
Follicular depletion
VCD Injections
Hormone Treatment
WRAM
MM
VP
Sacrifice

Figure 41. Experiment 8 Water Radial Arm Maze Performance. For Block 1 (days 2-3) of WRAM testing, there was a Trial x Estrogen x Progestin interaction ($F_{(6,159)}$=2.33, $p<0.05$; η_G^2=0.04), with group differences appearing on Trial 4, the trial with the highest working memory load. On Trial 4, there was a Estrogen x Progestin interaction ($F_{(2,53)}$=3.39, $p<0.05$; η_G^2=0.05), whereby levo treatment produced impairments relative to no levo treatment in animals treated with no estrogen (Fisher, $p<0.05$), and in animals treated with E2 (Fisher, $p<0.05$), but not in animals treated with EE (Fisher, $p>0.05$). On Block 2 of WRAM testing (days 4-6), there was a Trial x Estrogen interaction ($F_{(6,159)}$=4.47, $p<0.001$; η_G^2=0.04) and a Trial by Progestin interaction ($F_{(3,159)}$=3.93, $p<0.01$; η_G^2=0.02), with treatment differences emerging on Trial 4, the trial with the highest working memory load. On Trial 4, there was a main effect of estrogen ($F_{(2,53)}$=5.23, $p<0.01$; η_G^2=0.06), with E2-treated animals performing better than those treated with vehicle (Fisher, $p<0.05$) or EE (Fisher, $p<0.05$), regardless of Progestin treatment. There was also a main effect of Progestin on Trial 4 ($F_{(1,53)}$=5.40, $p<0.05$; η_G^2=0.03), with levo-treated animals outperforming animals that did not receive levo (Fisher, $p<0.05$), regardless of Estrogen treatment. There was a Trial x Estrogen x Progestin interaction on Block 3 of WRAM testing (days 7-9; $F_{(6,159)}$=3.66, $p<0.01$; η_G^2=0.04), with group differences on Trial 4, the highest working memory load trial. On Trial 4, there was an Estrogen x Progestin interaction ($F_{(2,53)}$=4.73, $p<0.05$; η_G^2=0.07), whereby treatment with E2 produced spatial memory impairment in animals that did not receive levo, relative to treatment with EE (Fisher, $p<0.05$) or no estrogen (Fisher, $p<0.05$). There were no effects of Estrogen within the levo-treated groups (Fisher, $p>0.05$).

Figure 41

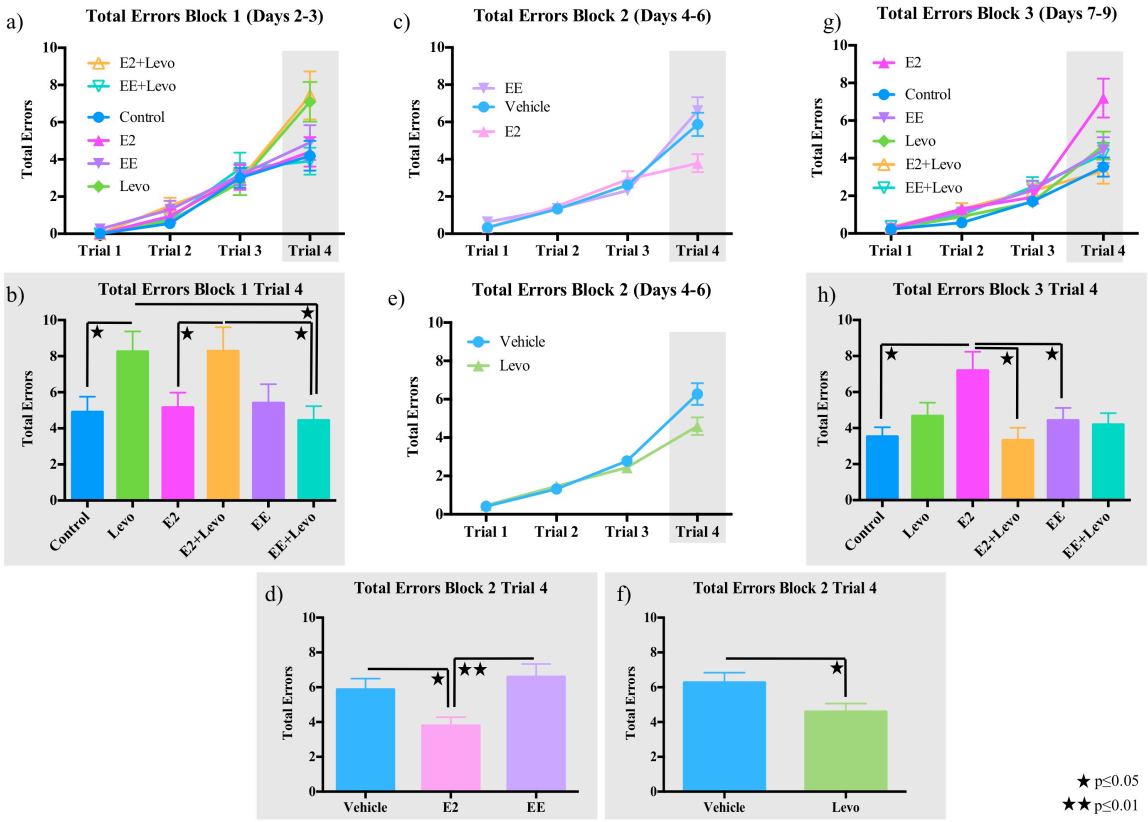

a)
Total Errors Block 1 (Days 2-3)
E2+Levo
EE+Levo
Control
E2
EE
Levo
Total Errors
Trial 1
Trial 2
Trial 3
Trial 4
c)
Total Errors Block 2 (Days 4-6)
EE
Vehicle
E2
g)
Total Errors Block 3 (Days 7-9)
E2
Control
EE
Levo
E2+Levo
EE+Levo
b)
Total Errors Block 1 Trial 4
Control
Levo
E2
E2+Levo
EE
EE+Levo
e)
Total Errors Block 2 (Days 4-6)
Vehicle
Levo
h)
Total Errors Block 3 Trial 4
d)
Total Errors Block 2 Trial 4
Vehicle
E2
EE
f)
Total Errors Block 2 Trial 4
Vehicle
Levo
★ p≤0.05
★★ p≤0.01

Figure 42. Experiment 8 Morris Water Maze Performance. There were no effects of Estrogen ($F_{(2,53)}$= 0.80, p>0.05, NS; η_G^2<0.01) or Progestin on MM performance ($F_{(1,53)}$= 0.03, p>0.05, NS; η_G^2<0.01). On the probe trial, there was a main effect of Quadrant ($F_{(1,53)}$= 430.58, p≤0.0001; η_G^2=0.86), with no Estrogen x Quadrant interaction ($F_{(2,53)}$= 0.20, p>0.05; η_G^2<0.01; NS), but there was a Progestin x Quadrant interaction ($F_{(1,53)}$= 3.81, p<0.05; η_G^2=0.03). This prompted us to analyze the first half of the Probe trial, to determine whether animals began searching in other quadrants after a lack of reward in the NE quadrant for the first 30 seconds. Indeed, during the first 30 seconds of the probe trial, there was a main effect of Quadrant ($F_{(1,53)}$= 261.96, p≤0.0001; η_G^2=0.93), with no Estrogen x Quadrant interaction ($F_{(2,53)}$= 0.10, p>0.05; η_G^2<0.01; NS), and no Progestin x Quadrant interaction ($F_{(1,53)}$= 2.44, p>0.05; η_G^2=0.03; NS), demonstrating that all treatment groups localized the platform by the end of MM testing.

Figure 42

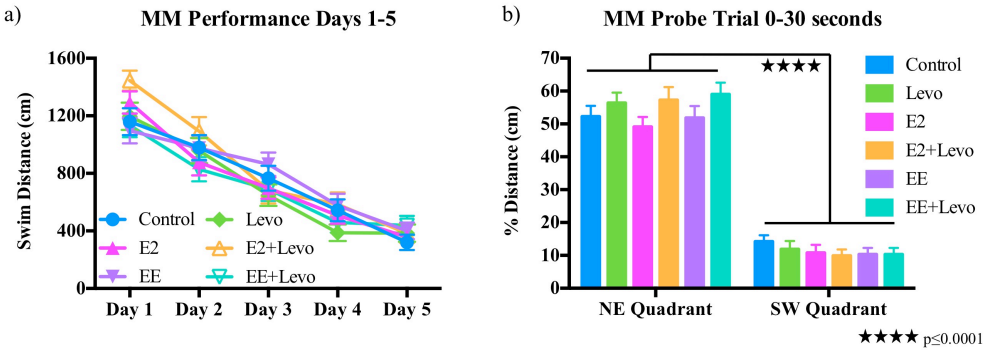

a)
MM Performance Days 1-5
Swim Distance (cm)
1600
1200
800
400
0
Day 1
Day 2
Day 3
Day 4
Day 5
Control
Levo
E2
E2+Levo
EE
EE+Levo
b)
MM Probe Trial 0-30 seconds
% Distance (cm)
70
60
50
40
30
20
10
0
NE Quadrant
SW Quadrant
★★★★
★★★★ p≤0.0001

Figure 43. Experiment 8 Uterine Weights and Serum Hormone Levels. There was a main effect of Estrogen on Uterine Weights ($F_{(2,53)}$=8.51, $p<0.001$; η_G^2=0.24), with E2-treated animals exhibiting heavier uterine horns at sacrifice than animals that received EE or no estrogen, regardless of Progestin treatment. There was no impact of Progestin treatment on Uterine Weights ($F_{(1,53)}$=1.20, $p>0.05$; η_G^2=0.02). There was no Estrogen x Progestin interaction ($F_{(1,53)}$=0.10, $p>0.05$; NS; $\eta_G^2<0.01$).

Figure 43

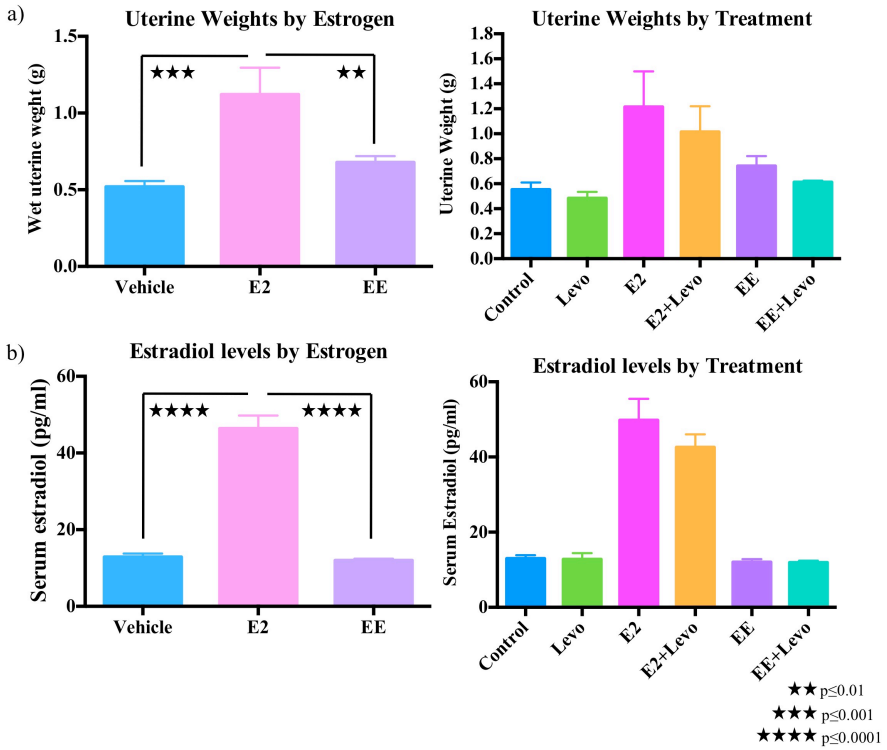
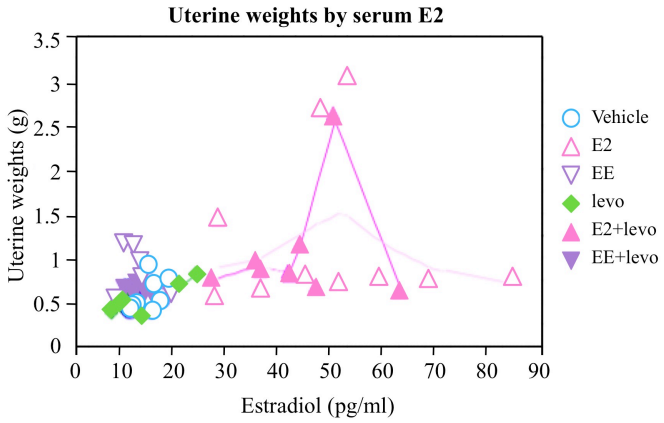

a)
Uterine Weights by Estrogen
Wet uterine weght (g)
1.5
1.0
0.5
0.0
★★★
★★
Vehicle
E2
EE
Uterine Weights by Treatment
Uterine Weight (g)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Levo
E2
E2+Levo
EE
EE+Levo
b)
Estradiol levels by Estrogen
Serum estradiol (pg/ml)
60
40
20
0
★★★★
★★★★
Vehicle
E2
EE
Estradiol levels by Treatment
Serum Estradiol (pg/ml)
60
40
20
0
Control
Levo
E2
E2+Levo
EE
EE+Levo
★★ p≤0.01
★★★ p≤0.001
★★★★ p≤0.0001

Figure 44. Experiment 8 Relations Between Uterine Weights and Serum Hormone Levels. Scatterplot of serum E2 levels (pg/ml) by uterine weights (g), split by group. Lowess smooth line fitting 66% of the points is shown by group.

Figure 44


Uterine weights by serum E2
Uterine weights (g)
3.5
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
80
90
Estradiol (pg/ml)
Vehicle
E2
EE
levo
E2+levo
EE+levo