Optimizing the Delivery of 17β-estradiol: Maximizing Beneficial Cognitive Effects While Minimizing Undesired Peripheral Stimulation in a Rat Model of Surgical Menopause

by

Alesia V. Prakapenka

A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved October 2018 by the
Graduate Supervisory Committee:

Heather Bimonte-Nelson, Chair
Rachael Sirianni
Cheryl Conrad
Sarah Stabenfeldt

ARIZONA STATE UNIVERSITY

December 2018

ABSTRACT

Estrogen-containing hormone therapy (HT) is approved for treatment of symptoms associated with menopause by the Food and Drug Administration. A common estrogen used in HT is 17β-estradiol (E2). Rodent models of menopause, and some clinical work as well, suggest a cognitively-beneficial role of E2. However, as of the 2017 statement released by the North American Menopause Society, HT is not currently advised for use as cognitive therapy in healthy, menopausal women, given that the data so far from existing clinical studies are not yet definitive. Indeed, the delivery of E2 treatment can be optimized to yield more consistent results on cognitive function, particularly considering that exogenously administered E2 gets rapidly metabolized and cleared from the body. Further, E2-containing HT must include a progestogen if prescribed to women with a uterus to oppose its undesired uterine stimulating effects, such as increased endometrial hyperplasia and cancer risks. Studies have shown that the addition of a progestogen to E2 treatment can attenuate the effects of E2 on cognition and brain variables associated with cognitive function. Thus, a brain-specific delivery platform of E2 treatment that would minimize the hormone's effects in the periphery while maintaining the beneficial cognitive effects is desirable. To achieve this goal, my dissertation work bridged two distinct scientific fields – behavioral neuroendocrinology and polymeric drug delivery – with the overarching aim of targeting the delivery of E2 to the brain to achieve maximal cognitively-beneficial effects with minimal undesired uterine stimulation. This aim was addressed via three distinct delivery strategies: 1) combining E2 with a cognitively-beneficial progestogen, 2) encapsulating E2 in polymeric nanoparticles, and 3) solubilizing E2 using cyclodextrins for intranasal

administration. Findings revealed that although all E2-containing treatments increased uterine horn weights, a marker of uterine stimulation, in middle-aged ovariectomized rats, some E2 treatment formulations yielded memory improvements, others were neutral in their effects on memory, and some impaired memory. Together, data from this dissertation set the stage for targeted E2 delivery research to optimize the cognitive therapeutic effects of E2 in the context of menopause while minimizing peripheral burden, leading to translationally relevant clinical implications for women's health.

ACKNOWLEDGMENTS

Over the last five and a half years, I have grown tremendously as a scientist, a friend, and overall as a person. I have been fortunate to be surrounded and mentored by an amazing group of scientists, and my wonderful family, as I ventured out on my Ph.D. endeavor. I want to thank and acknowledge you all from the bottom of my heart for your constant support throughout this adventure.

Heather and Rachael, thank you. Thank you for allowing me to pursue this line of research, and for guiding me through it. I am truly a better scientist because of you, and my love and passion for science has only been strengthened over the last several years. You have been there for me through the ups and downs of graduate school, and have generously shared your knowledge, experience, and valuable time with me. I value your mentorship, and I can honestly say I would not be where I am without the lessons and skills you have taught me. And to all of my graduate committee members, Heather, Rachael, Cheryl, and Sarah - it has been a pleasure to be mentored by each one of you. Thank you for your support, and for always being there for me. You have inspired me and motivated my work during graduate school, as well as my future career goals.

To my lab mates, both past and present, and to the graduate students in the Neuroscience and Behavioral Neuroscience programs, you were my rocks throughout it all, and many of you have become life-long friends. Thank you for your tremendous help and support, from being there at 7:00 am every day for injections to joining me in getting one more cup of coffee. You have helped make even the most stressful of days easier and enjoyable, and I am grateful for that.

And of course, a huge thank you to my family, which has grown even bigger over the last year! Mamik, Papik, and Deshik, you have been my cheerleaders from the start, and were always there if I ever needed advice, support, or just someone to talk to. Thank you for your advice, both in science and in life, and thank you for always believing in me. To my husband, Jon, thank you for supporting this stress ball, especially over the last two months as I finalized my dissertation. I am lucky to have met you, and to have had you by my side during the toughest milestones so far of my research career. And to my in-law family, especially Marty and Sherry, I want to thank you for always being there and offering your help in any way that you can. I thank you for your unwavering support, and I truly appreciate your eagerness to learn about my research.

And finally, the research presented in this dissertation would not have been possible without the funding support that I have been fortunate to receive, including the graduate research fellowship from the National Science Foundation, the ASU-BNI Fellowship, the ARCS award, the many travel awards for conferences, as well as the funding awarded to my co-mentors, Heather Bimonte-Nelson and Rachael Sirianni.

TABLE OF CONTENTS

CHAPTER Page

CHAPTER Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

Figure Page

CHAPTER 1

GENERAL INTRODUCTION

This chapter was submitted, and has undergone peer-review, as a chapter for the book entitled "Estrogens and Memory: Basic Research and Clinical Implications" for Oxford University Press, in May, 2018 titled:

Ovarian hormones, cognition, and reproductive aging: applications and implications for translating preclinical endocrine brain research to the clinic

A.V. Prakapenka, V.L. Peña, H.A. Bimonte-Nelson

Contribution: I was the first author for the submitted book chapter, and the two co-authors were Veronica L. Peña and Heather A. Bimonte-Nelson. I played a heavy role both in the writing as well as the editing stages of this chapter. Sections of the chapter were edited to fit the context of this dissertation.

Introduction

Hormones matter. In the last two decades, there has been an increasing number of studies addressing the impact of sex hormones on behaviors other than those related to reproduction. Indeed, the field of behavioral endocrinology has clearly demonstrated that hormones impact more than sex behaviors. Sex steroids such as estrogens, progesterone, and androgens have extensive and powerful effects on the body and exert both permanent and transient effects on innumerable systems, including the central nervous system. Animal scientific studies from the mid-20th century showing that sex steroids play an integral role in development and regulation of the brain and behavior (e.g. Beach and

Holz-Tucker, 1948; Phoenix et al., 1959; Davis et al., 1979) set the stage for detailed evaluation of the wide-reaching effects of sex steroid hormones on a multitude of systems and functions. Scientists have built upon this classic framework using clever, creative, and innovative approaches and tools to systematically investigate independent and interactive brain-behavioral effects of sex steroids (Bimonte, Mack, Stavnezer, & Denenberg, 2000; Fitch, Cowell, Schrott, & Denenberg, 1991; Williams, Barnett, & Meck, 1990). Researchers studying hormone effects on the brain and its functions have utilized animal models to methodically assess and control for biological and behavioral functions that are difficult to directly evaluate in human research studies. Systematic experiments in animal models have provided critical information regarding hormone-brain-behavior interactions. This interdisciplinary, systems work has spanned molecular to behavioral foci, with knowledge increasing in breadth and depth with every passing year.

Growing evidence now shows that there is an impressive array of cognitive behaviors and neurobiological mechanisms impacted by sex steroids, and this information has important real-world, translational applications and implications. For example, it is now well established that both males and females undergo endocrine changes as they age. In women, the 40's are a critical time in which age-related endocrine changes begin, as this period is marked by perimenopause-related irregular fluctuations of ovarian hormones and a transition to the postmenopause-related hormone nadir. These mid-life menopause-related changes coalesce with symptoms that can be broad and multi-system related, which greatly impacts health and quality of life. Women often opt to take hormone therapy to combat menopause-associated symptoms such as hot flashes,

osteoporosis, vaginal dryness, sleep disturbances, and changes in mood and cognition (NAMS, 2014). Thus, a considerable focus of behavioral endocrinology research has been to address questions related to hormone therapy, such as whether to take hormone therapy, and if so, what doses should be taken and when, and whether estrogen should be taken alone or with a progestogen. The more information researchers glean regarding the wide-reaching effects of menopause and hormone therapy on cognition, the more researchers have recognized that there is so much more to learn. It can be argued that every animal and human study involving ovarian hormone loss and hormone therapy has resulted in more questions, and has taught scientists that they must respect, value, and embrace the massive and diverse impact that sex hormones have on the brain and its functioning. Specifically, estrogens are thought to play a large role in hippocampal-dependent memory, and this role is impacted by multiple factors, including age at treatment initiation, treatment duration, type of hormone, dose of hormone, route of administration, and period of time between the loss of circulating ovarian hormones and hormone treatment initiation. This chapter aims to summarize key discoveries in the field of translational behavioral endocrinology, focusing on work using animal models to understand menopause and estrogen-including hormone therapies, and intends to provide vital background for developing delivery strategies to optimize estrogen's role on cognitive function while minimizing estrogen's peripheral burden.

Estrogens

In mammals, all naturally occurring steroid hormones are derived from the parent molecule, cholesterol. *Estrogens* are a class of steroid hormones that include

endogenously occurring hormones such as estrone (E1), 17β-estradiol (E2), and estriol (E3), their hydroxylated metabolites (e.g., 2-methoxyestradiol) and glucuronide and sulfate conjugates (e.g., estrone sulfate), and synthetic estrogens (e.g., ethinyl estradiol) (Kuhl, 2005; Lobo, 2007; Mattison, Karyakina, Goodman, & LaKind, 2014). Throughout this chapter, E2 will always refer to 17β-estradiol if specified within the cited paper, otherwise the estrogen will be referred to using the term provided in the cited paper. Table 1 summarizes the current U.S. Food and Drug Administration (FDA) approved estrogens for menopausal hormone therapy, along with their corresponding brand name and route of administration (U.S. FDA's Office of Women's Health, 2015). As seen in the table, the most common routes of administration for FDA-approved menopausal estrogen-based hormone therapy are oral (pill), transdermal (gel, spray, and patch), and local vaginal administration (vaginal ring, cream, and tablet). When assessing total circulating estrogen concentrations, using E2 as an example, there is typically only ~1-2% of free E2 available for activity at target tissue sites (D. C. Anderson, 1974; Kuhl, 2005). The rest of the total circulating E2 is bound to either sex hormone binding globulin or to albumin, decreasing the hormone's bioavailability (Lobo, 2007). Local application of estrogens via the *vaginal route* is considered to have little to no systemic effects, minimizing undesired estrogen exposure to peripheral organs as compared to alternate routes; however, following vaginal administration, there is evidence that estrogens can indeed enter systemic circulation (Labrie et al., 2009). For instance, E2 blood serum levels, measured via mass spectrometry assays 24 hours after one week of daily vaginal cream application (25 μg Vagifem or 1 g Premarin), were elevated by about 5-fold during the 24 hours following administration (Labrie et al., 2009). These data,

showing an increase from 3 pg/ml to 17 pg/ml in E2 concentration, demonstrate that local vaginal administration can increase systemic E2 (Labrie et al., 2009). Typical E2 circulating levels for a postmenopausal woman are < 20 pg/ml (Kuhl, 2005). Thus, these findings raise the question of whether the observed increase in E2 concentration following local administration is enough to produce undesired systemic side effects. Both oral and transdermal routes of administration produce levels of serum E2 that are over 20 pg/ml, which is higher than the levels found in postmenopausal women (Kuhl, 2005). The *oral route* of administration achieves a cyclic pattern of circulating E2 levels in women, as it is typically administered on a daily basis, and the *transdermal route* of administration produces more tonic circulating levels of E2 in women, as the hormone slowly enters circulation through absorption throughout the day for several days. With oral administration, E2 is immediately susceptible to first-pass metabolism, whereby E2 undergoes intestinal and hepatic conversion into estrogen metabolites and conjugates that are less potent compared to E2. E2 and the estrogen metabolites and conjugates then either enter the bile pool for enterohepatic circulation or enter the bloodstream (Kuhl, 2005; Ruoff & Dziuk, 1994). The transdermal route of administration circumvents first-pass metabolism of E2. With transdermal administration, E2 diffuses through the skin layers (stratum corneum, epidermis, and dermis) and then enters the bloodstream (Kuhl, 2005). Due to individual differences in metabolism (for oral) and rate of absorption (for transdermal), there are large inter- and intra- patient differences in circulating E2 concentrations and therapeutic effects of E2-based hormone therapy for menopause (Kuhl, 2005; Kuhnz, Gansau, & Mahler, 1993; Lobo, 2007). Additionally, high doses of E2, as high as ~1-2 mg E2/day for oral delivery and ~2-10 mg E2 for a four-day

transdermal patch (reportedly achieving ~50 μg circulating E2/day concentration), are required to overcome the barriers of oral and transdermal delivery and achieve desired therapeutic effects (Kuhl, 2005; Lees & Stevenson, 2001; Reginster et al., 1997). Thus, the route of administration can heavily dictate systemic circulation and activity of E2, and therefore, the hormone's potential for therapeutic effects.

Additionally, estrogens vary in efficacy as well as potency, as measured by each hormone's binding affinity to estrogen receptor α and estrogen receptor β. These two estrogen receptors are nuclear receptors that vary greatly in individual distribution and expression throughout the body (Kuhl, 2005; Kuiper et al., 1997). Among the naturally occurring estrogens, E2 has the strongest affinity to both estrogen receptors α and β, followed by E1 and then E3 (Kuhl, 2005; Kuiper et al., 1997). Some synthetic estrogens have markedly stronger affinity to the estrogen receptors than E2, such as ethinyl estradiol, which has a relative binding affinity of 190% to estrogen receptors compared to the 100% relative binding affinity of E2 (Blair et al., 2000). Estrogens that are derived from the urine of pregnant mares, called conjugated equine estrogens (CEE), are the estrogens present in Premarin (PREgnant MARe urINe), used primarily for menopausal hormone therapy (Lobo, 2007). CEE includes at least 10 different known estrogens, with the highest content as estrone sulfate (50%) and equilin sulfate (20-25%), and to a lesser amount the sulfate esters of 17α-estradiol, 17β-dihydroequilin, 17α-dihydroequilin, equilenin, 17β-dihydroequilenin, 17α-dihydroequilenin, Δ8,9-dehydroestrone, and E2 (Bhavnani, 2003; Bhavnani & Stanczyk, 2014; Kuhl, 2005; Lobo, 2007). Studies suggest that the relative binding affinity of each estrogen component in CEE to estrogen receptors in order from highest to lowest is: 17β-dihydroequilin, E2, 17β-dihydroequilenin, 17α-

dihydroequilin, E1, 17α-dihydroequilenin, 17α-estradiol, Δ8,9-dehydroestrone, equilenin, and equilin (Bhavnani, 2003; Lobo, 2007). Since all of the known estrogens in CEE are derived from equine sources, the majority of these estrogens are not naturally endogenous to women. Indeed, of the estrogens present in CEE, only E1 sulfate, E2 sulfate, and 17α-estradiol sulfate are endogenous to women (Kuhl, 2005; Lobo, 2007). Because estrogen receptors are found widely throughout the body *and* the brain, including brain areas important for cognition, such as the hippocampus, basal forebrain, and the cortex (Eyster, 2016; Kuhl, 2005; Mitra et al., 2003; Pérez, Chen, & Mufson, 2003), different clinically-prescribed estrogens may have distinct effects on various systems and their functions; the scope of this understanding includes not only reproductive domains, but also cognition and other aspects of brain functioning. This knowledge can aid in developing targeted estrogen-based formulations for the treatment of symptoms associated with menopause, and is important when considering other estrogen-containing prescriptions such as hormone-based contraceptives.

Female reproductive aging: Hormonal changes across lifespan

The *hypothalamic-pituitary-gonadal (HPG) axis* refers to the interplay between the hypothalamus, the pituitary, and the gonads to aid in development as well as regulation of multiple systems in the body, including the reproductive system. Increases in specific hormones released from each of these structures sends feedback information to each other to either increase (*positive feedback*) or decrease (*negative feedback*) the production and release of other hormones, as nicely evidenced in the role of the HPG in the reproductive cycle. Prior to discussing the effects of estrogens on spatial memory in

rodent models of menopause, it is important to understand the reproductive hormone changes across the lifespan of women and female rodents. Although the majority of observed changes in the hormonal milieu across a normal reproductive cycle in women and female rodents are similar (Figure 1), there are distinct species differences in the way the cycles change with age that must be considered when interpreting results related to reproductive senescence and estrogen-based hormone therapy in rodent models of reproductive aging. In this section, the changes in HPG axis signaling across a reproductive cycle and with aging in both humans and rodents are addressed.

Women: Menstrual cycle

The reproductive cycle in women is termed the *menstrual cycle* and lasts about 28 days. The average age of *menarche*, defined as the first *menstruation* or shedding of the uterine lining, for women in the U.S. is 12.8 years, which marks her first complete menstrual cycle (Kronenberg et al., 2008). Throughout the duration of the menstrual cycle, changes in circulating levels of ovarian hormones occur that reflect the maturation and release of an egg from the ovaries, as well as preparation of the uterus for fertilization. In the ovaries, immature eggs are contained within ovarian follicles. At the start of the menstrual cycle, during the *follicular phase*, gonadotropin releasing hormone (GnRH) is synthesized and released from the hypothalamus, which signals the anterior pituitary gland to produce and release follicle stimulating hormone (FSH). FSH stimulates growth of ovarian follicles, resulting in maturation of the egg contained within a dominant follicle, as well as increased proliferation of granulosa cells. As the egg matures, these granulosa cells start releasing estrogens in gradually increasing amounts

until a threshold is reached. Increasing estrogen levels prepare the uterus for pregnancy by stimulating growth of the endometrial lining. Additionally, androgen-producing theca cells surround the developing follicle and granulosa cells, and contribute to the estrogen surge by aromatizing androgens to estrogens (Magoffin, 2005). Once the estrogen threshold is reached, positive feedback mechanisms signal GnRH from the hypothalamus to initiate a burst release of luteinizing hormone (LH) from the anterior pituitary gland, and the consequent induction of *ovulation (ovulatory phase)*. Typically, one egg is released from one mature follicle within one menstrual cycle. After ovulation, the menstrual cycle enters the *luteal phase*, where a corpus luteum is formed from the follicle of the just-released egg. The corpus luteum releases progesterone, and to a lesser extent estrogens, to prime the uterus for fertilization. If the egg becomes fertilized, then the outer part of the zygote starts developing into the placenta and the inner part of the zygote starts developing into the fetus; the placenta produces progesterone in addition to multiple other hormones, peptides, and growth factors to aid in fetal development (Kronenberg et al., 2008). If the egg is not fertilized, then the negative feedback from increasing levels of progesterone and estrogen results in a decline in GnRH, LH, and FSH levels, in turn resulting in atrophy of the corpus luteum. As the corpus luteum degenerates, it becomes the non-hormone producing corpus albicans, leading to a decrease in progesterone and estrogen levels. The uterine lining is then shed via menstruation *(menstrual phase)*. Negative feedback from low levels of progesterone and estrogen to the hypothalamus and anterior pituitary re-starts the cycle, yielding increasing levels of GnRH released from the hypothalamus and increasing levels of FSH released from the anterior pituitary.

Rodent: Estrous cycle

The reproductive cycle of the female rodent, the *estrous cycle*, is similar to that of a woman's menstrual cycle in many ways, although it is much shorter, lasting only 4-5 days in standard laboratory rats and mice (Freeman, 2006; Haim, Shakhar, Rossene, Taylor, & Ben-Eliyahu, 2003). Comparable to the follicular, ovulatory, luteal, and menstrual phases of the menstrual cycle, there are four phases in the estrous cycle that signify ovarian follicle maturation and, thus, changes in circulating hormone levels: diestrus, proestrus, estrus, metestrus (Goldman, Murr, & Cooper, 2007; Koebele & Bimonte-Nelson, 2016; S. E. Mennenga & Bimonte-Nelson, 2015; Westwood, 2008). For ease of discussion here, the *diestrus* phase will be considered as marking the start of the estrous cycle. During diestrus, GnRH is released from the hypothalamus, signaling FSH release from the anterior pituitary gland to subsequently stimulate ovarian follicle growth (Freeman, 2006). As the eggs mature within the growing follicles, granulosa cells proliferate and release estrogens, which start preparing the uterine horns for pregnancy. The theca cell layer forms around the developing follicles and proliferating granulosa cells, releasing androgens that can then get metabolized into estrogens. Next, the animal enters the *proestrus* phase, where a high estrogen level threshold is reached and GnRH induces a burst release of LH from the anterior pituitary gland, prompting ovulation (Freeman, 2006; Haim et al., 2003). In the proestrus phase, development of the endometrial lining within the uterine horns progresses in preparation for fertilization. The proestrus phase is typically a short phase, lasting less than 24 hours. To produce litters of, on average, 8 pups in mice and 13 pups in rats, multiple eggs are released from mature follicles in one cycle (e.g.; Pritchett-Corning et al., 2009). The completion of ovulation

starts the *estrus* phase, which is marked by comparatively high, but decreasing, estrogen levels. The final phase, *metestrus*, is characterized by high progesterone and low estrogen levels released from the corpora lutea formed from the remaining follicles of the just-released eggs (Freeman, 2006). If the eggs are fertilized, then placentas will form and continue to produce progesterone as the zygotes develop. If the eggs are not fertilized, then the increasing levels of progesterone and estrogen will lead to negative feedback to the anterior pituitary and hypothalamus, triggering a decline in GnRH as well as LH and FSH levels, along with atrophy of the corpora lutea. As the estrous cycle starts over, the corpora lutea degenerates into corpora albicans and the uterine lining is reabsorbed, rather than shed as in women. Subsequent low levels of progesterone and estrogens will negatively feed back to the anterior pituitary and the hypothalamus, and the estrous cycle will start again with the release of increasing GnRH and FSH levels from the hypothalamus and anterior pituitary, respectively. Unlike in women, it is possible for female rodents to enter a state of pseudopregnancy after receiving sufficient vaginal stimulation. A pseudopregnant rodent will not have fertilized eggs, but the presence of the corpora lutea will be prolonged due to prolactin release from the pituitary, resulting in sustained levels of high progesterone and low estrogens (Stocco, Telleria, & Gibori, 2007; Westwood, 2008).

Women: Menopause

The transition to *menopause*, the point at which menstruation ceases, begins as early as 40 years of age. Women will typically complete the transition to menopause at an average age of 52 years (NAMS, 2012, 2014). The transition period to menopause is

often referred to as the *perimenopausal* state. This transition period varies greatly between women, but it may last up to 10 years and is associated with irregular menstrual cycles as well as significant fluctuations in circulating ovarian hormone levels and accelerated follicular depletion (Burger, Hale, Dennerstein, & Robertson, 2008; Harlow & Paramsothy, 2011; Richardson & Nelson, 1990). Menopause is primarily driven by follicular depletion, which can alter normal functioning of the HPG axis (NAMS, 2014). At menopause, the ovarian follicle reserve is depleted, resulting in cessation of the menstrual cycle; menopause is consequently characterized by a marked decrease in the production and release of circulating ovarian hormones, including estrogens and progesterone (NAMS, 2014). Menopause is confirmed retrospectively after one year without menses (NAMS, 2014). The predominant circulating ovarian-derived hormones after menopause are androgens, and the predominant circulating estrogen is estrone, much of which is released from the adrenal cortex (Lobo, 2007; Vermeulen, 1976). Circulating levels of LH and FSH increase in response to the disrupted feedback from the hypothalamus and the anterior pituitary following low levels of estrogens and progesterone.

Rodent: Estropause

Age-related reproductive senescence in the female rodent is termed *estropause*. Whereas the onset of menopause is mainly dictated by a decrease in follicle reserve, estropause is primarily governed by intrinsic alterations in the HPG axis (S. E. Mennenga & Bimonte-Nelson, 2015; Wise, 1982). An assessment of circulating hormone profiles of estradiol, progesterone, LH, and FSH, and of the median eminence profile of luteinizing

hormone releasing hormone (LHRH), in proestrus middle-aged rats compared to proestrus young rats, showed that before the pre-ovulatory LH surge, young and middle-aged rats had similar hormone profiles (Wise, 1982). However, middle-aged rats had lower LHRH, estradiol, and progesterone levels prior to ovulation relative to young rats, and the LH surge in middle-aged rats was temporally delayed compared to that of young rats (Wise, 1982). These data suggest that as aging ensues in the rat, the function and responsiveness of the HPG axis slows down or is otherwise disrupted, altering communication with the ovary.

A female rodent in estropause is chronically anovulatory and exhibits either moderate circulating estrogen levels and low progesterone levels reflective of a *persistent estrus* phase, or low estrogen and high progesterone circulating levels reflective of a *repetitive pseudopregnancy* state (S. E. Mennenga & Bimonte-Nelson, 2015; Westwood, 2008). Eventually, many female rodents enter a state of *anestrus* which is marked by low circulating levels of estrogens and progesterone (S. E. Mennenga & Bimonte-Nelson, 2015; Westwood, 2008). Of note, the terms anestrus and persistent diestrus are often used interchangeably in the literature; in this chapter, this phase will be referred to as anestrus. A longitudinal study evaluating reproductive senescence in female rats as they progressed from young adulthood (4 months old) to aged (18-22 months old) revealed a trend of irregular cycles during the transition between normal to senescent reproductive states, with rats entering either persistent estrus followed by irregular cycles and sometimes anestrus, or entering immediately into anestrus (LeFevre & McClintock, 1988). LH and FSH levels during estropause can differ depending on whether an animal is in persistent estrus, repetitive pseudopregnancy, or an anestrus state (Huang, Steger, Bruni, & Meites,

1978). For example, aged rats in the persistent estrus state have higher FSH levels than aged rats in either anestrus or repetitive pseudopregnancy states, and higher FSH levels than young proestrus and estrus rats (Huang et al., 1978). FSH levels in anestrus and repetitive pseudopregnant aged rats are lower than those of young rats in proestrus or estrus cycle phases (Huang et al., 1978). LH levels are similar between aged rats in persistent estrus and young rats in proestrus or estrus (Huang et al., 1978). LH levels are also similar between aged rats in repetitive pseudopregnancy and young rats in diestrus (Huang et al., 1978). Interestingly, in mice, both LH and FSH levels are reportedly higher in aged mice compared to young normally cycling C57BL/6 mice (Parkening, Collins, & Smith, 1980).

Animal models of menopause

Animal models of menopause have been used for decades in preclinical experiments to study the endocrine system and changes thereof. Despite the reproductive senescence differences mentioned above between rodents and humans, animal models have provided tremendous insight into hormone-brain-behavior interactions. In the rodent, much like in the human, the endocrine system contains a vast and complex milieu of hormone interactions whose tight regulation provides a homeostatic environment. As discussed above, the circulating ovarian hormone profile differs between reproductively senescent women and female rodents. In a menopausal woman, there is a marked decrease in circulating ovarian hormone levels of estrogens and progesterone, and androgens become the predominant circulating ovarian hormones; in an estropausal rodent, most animals exhibit either consistently moderate levels of estrogens with low

levels of progesterone, or low levels of estrogens with high levels of progesterone. Due to these distinct differences, several types of rodent menopause models have been developed to mimic the trajectory and hormone profile of menopause as closely as possible (Figure 2). These models allow for systematic preclinical evaluations of menopause and menopausal hormone therapies on normal system functions, including, but not limited to, brain functions such as cognition. It should be noted, however, that no model of menopause in rodents is flawless, and indeed no animal model can be truly perfect. Each model discussed herein provides certain advantages, alongside some drawbacks, for experimental designs aimed at understanding how the ever-changing ovarian hormone profile across a female's reproductive lifespan impacts cognitive function.

Ovary intact

The *ovary intact* rodent model is useful in studying and understanding the impact of hormone fluctuations across the normal reproductive cycle on the brain and its functions (Stackman et al., 1997; Quinlan et al., 2010; Woolley and McEwen, 1992; Spencer et al., 2010; Warren et al., 1995). For instance, the classic study by Woolley and McEwen (1992) showed that rats in proestrus, the estrous phase with the highest E2 levels, exhibited an increased density of synapses on dendritic spines in the hippocampal CA1 region compared to rats in estrus, the estrous phase in which E2 levels are lower. Long-term potentiation, the leading molecular model of memory formation, is enhanced during proestrus relative to estrus in rats (Warren, Humphreys, Juraska, & Greenough, 1995). Of note, however, there are important caveats to investigating how circulating

ovarian hormones alter the brain and its functions in ovary intact rodents. Although this model allows changes across the estrous cycle to be observed, such as alterations in hippocampal microstructure and function, the fluctuations in numerous ovarian hormones across the cycle make it difficult to decipher the specific hormone responsible for a given effect. Moreover, exogenously administering hormones to a gonadally-intact animal in which hormones are actively cycling can dysregulate the naturally functioning endocrine system, further complicating the interpretation of which particular hormone/s modulate or drive an observed effect. However, there are creative ways to get around these limitations. For example, in a series of studies, Woolley and McEwen combined estrous cycle data with add-back exogenous administration in the *ovariectomy (Ovx)* rodent model to parse the contributions of E2 and progesterone. In the Ovx model, discussed in detail below, the main source of circulating ovarian hormones – the ovaries – are surgically removed. Specific hormone/s of interest can be added back to this 'blank' circulating gonadal hormone milieu to examine their direct effects. It is important to keep in mind that even with Ovx, ovarian hormones (e.g., progesterone and E2) can still be produced in other tissues such as the brain (Micevych and Sinchak, 2008; Kretz et al., 2004, Tuscher et al., 2016). Nevertheless, the Ovx model is an effective method to investigate hormone effects on learning and memory specific to a circulating ovarian hormone milieu of interest. In this manner, Woolley and McEwen were able to show that following the loss of circulating ovarian hormones, spine density in the hippocampal CA1 region gradually decreased after Ovx (C S Woolley & McEwen, 1993). Acute exogenous administration of an estrogen, estradiol benzoate, 3 days after Ovx temporarily increased spine density in the hippocampal CA1 region compared to Ovx

controls, peaking at 2-3 days following injection (C S Woolley & McEwen, 1993). However, acute exogenous administration of progesterone following estradiol administration produced a transient increase in spine density in the hippocampal CA1 region during the first 6 hours, followed by a sharp decline, as compared to estradiol alone (C S Woolley & McEwen, 1993). Further, blocking progesterone receptors during proestrus in ovary-intact rats attenuated the spine density loss seen from proestrus to estrus (Woolley and McEwen, 1993). These results indicate that progesterone is critical for the natural recession of E2-induced spine growth. Thus, these scientists more specifically isolated the contributions of estradiol and progesterone by using the ovary intact rodent model to uncover structural changes in the hippocampus across the estrous cycle, and cleverly assimilated this novel information with the Ovx model to determine how each hormone drives these effects.

Ovary intact middle-aged or aged rodents are not often used to model the neurobehavioral effects of estrogen-based hormone therapy in menopause because estropause is driven by HPG axis dysregulation, whereas menopause is driven by the depletion of ovarian follicle supplies. The dissimilar circulating ovarian hormone profiles in estropause and menopause must be acknowledged and accounted for when using an ovary intact model to address questions regarding the effects of hormone therapies at reproductive senescence. However, of note, ovary intact middle-aged and aged rodents do provide an important model of HPG axis dysregulation, which can be used to gain insights into how changes in HPG axis function impact systemic functions, including cognition. Indeed, numerous scientists have effectively used ovary-intact aging rodents as a model, and have dedicated their life's work to understanding the mechanisms driving

menopause, such as the role of HPG axis function (Downs & Wise, 2009; Kermath & Gore, 2012; Morrison, Brinton, Schmidt, & Gore, 2006; Wise, 1982).

Bilateral ovariectomy (Ovx)

Ovx is the classic technique in rodents used to observe effects of ovarian hormone loss. Ovaries in rodents, much like in humans, are bilaterally located in the peritoneal cavity. In rodents, one ovary is connected to each end of a bifurcated uterine horn, which acts as the uterus (Figure 2). During bilateral Ovx, the ovaries, oviducts (which are akin to the fallopian tubes in women), and uppermost tips of the uterine horns are ligated and excised (Hiroi et al., 2016; Koebele & Bimonte-Nelson, 2016; M. E. Olson & Bruce, 1986). With the removal of the ovaries, there is an abrupt cessation of the estrous cycle and a drop in endogenous circulating ovarian hormones (e.g. estrogens, progesterone, and androgens), creating a 'blank' circulating hormonal slate, although some ovarian hormones can still be produced in other tissues (e.g., the brain), to which hormones of interest can exogenously be added back to systematically assess the effects of factors such as dose, route, and/or duration of treatment administration on various system functions. This model has allowed for tremendous discovery in the field of behavioral neuroendocrinology because bilateral Ovx not only produces a drastic decrease in circulating ovarian hormone levels, but also an increase in FSH and LH levels similar to those seen in menopausal women after surgical removal of ovaries (oophorectomy). By the age of 60, more than one third of women in the U.S. undergo hysterectomy surgery, which is the removal of the uterus; an estimated 600,000 women in the U.S. undergo hysterectomy surgeries each year, half of which are accompanied by oophorectomy

(Whiteman et al., 2008). Oophorectomy and hysterectomy often occur together, a trend that has increased over time (Lowder et al., 2010). Typically, women undergo oophorectomy to combat ovary-related abnormalities such as onset of ovarian cancer (Asante et al., 2010; Erekson, Martin, & Ratner, 2013), reduction of the risk for breast cancer development (J. E. Olson, Sellers, Iturria, & Hartmann, 2004), and treatment for symptoms occurring with severe premenstrual syndrome (Cronje, Vashisht, & Studd, 2004). As such, bilateral Ovx is considered a fitting technique for modeling surgical menopause, as it removes the main source of endogenous ovarian hormones in a manner similar to oophorectomy. Thus, Ovx provides an excellent model for methodically examining the effects of both surgical menopause and effects of specific hormone therapy regimens on cognition.

A major benefit of the Ovx model is that it allows researchers to delve into questions regarding the *window of opportunity theory* for efficacy of menopausal hormone therapies. The window of opportunity theory posits that there is a critical period after ovarian hormone loss in which hormone therapy can have the most beneficial therapeutic effect on the brain and cognition (Linda A Bean et al., 2015; J. M. Daniel, Witty, & Rodgers, 2015; Maki, 2013; W. A. Rocca, Grossardt, & Shuster, 2011; M. Singh, Simpkins, Bimonte-Nelson, & Brinton, 2013). Evidence in support of this theory comes from studies showing that if initiation of hormone therapy is delayed well after the onset of menopause in women or Ovx in rats, then the beneficial effects of estrogen treatments on cognition in both humans (Rocca et al., 2010, 2011) and rats (Jill M. Daniel, Hulst, & Berbling, 2006; R. B. Gibbs, 2000) are attenuated. In one such study, middle-aged Ovx rats received tonic subcutaneous E2 treatment immediately following

Ovx surgery, or 5 months after Ovx surgery (Daniel et al., 2006). Findings from this study revealed that E2 treatment immediately following Ovx resulted in better spatial working memory compared to vehicle controls, whereas E2 treatment initiated 5 months following Ovx yielded no difference in spatial working memory compared to vehicle controls (Jill M. Daniel et al., 2006). Another study showed that rats receiving acute E2 treatment after prolonged hormone deprivation following Ovx (~10 weeks) exhibited decreased apical spine density in the CA1 region of the hippocampus compared to rats receiving exogenous intermittent E2 treatment beginning about a week after Ovx (McLaughlin, Bimonte-Nelson, Neisewander, & Conrad, 2008). Of note, both of these E2 treatment regimens increased apical spine density in the CA1 region of the hippocampus relative to vehicle control (McLaughlin et al., 2008). Collectively, these findings suggest that there is diminished sensitivity of hippocampal morphology and mnemonic efficacy to E2 treatment following delayed onset of estrogen treatment. The Gibbs laboratory further elaborated on the critical window of opportunity theory by proposing a cholinergic basis for the theory. Their behavior findings were consistent, demonstrating that while E2 replacement directly after, or 3 months after, Ovx improved spatial working memory in middle-aged rats, E2 replacement 10 months after Ovx did not show improvements in spatial working memory (R. B. Gibbs, 2000). Moreover, findings from several studies showed a detrimental impact of Ovx on cholinergic neurons of the basal forebrain in rats, and a decrease in E2's ability to oppose this impact as a function of both age and time since the loss of ovarian hormones (R.B. Gibbs, 2010; R B Gibbs, 1998). Taken together, research using the Ovx model in rodents has provided an important method of testing the validity of the window of opportunity theory and the putative

neurobiological mechanisms underlying this window. Importantly, this work has illustrated that the timing of exogenous hormone treatment initiation following loss of circulating ovarian hormones greatly impacts its efficacy in tests of spatial learning and memory.

The Ovx rodent model also allows researchers to systematically address the question of whether age of menopause onset affects cognitive function. Although the average age of menopause is 52 years, the age of menopause onset varies greatly (typically from 40 to 58 years of age) (NAMS, 2014), yet little is known about whether age of onset impacts cognitive function. This issue is just beginning to be investigated. One study showed that there are varying cognitive effects of early versus late menopause in women, independent of hormone therapy; women who underwent menopause later in life (between 51-63 years of age) performed better on the Mini-Mental State Exam (MMSE), a common test used to assess global cognitive function, than women who underwent menopause earlier in life (between 16-40 years of age) (McLay, Maki, & Lyketsos, 2003). Another study demonstrated that women who underwent menopause at or before 40 years of age were impaired in verbal fluency and visual memory tasks compared to women who underwent menopause after 50 years of age (Ryan et al., 2014). The Ovx rodent model has been effectively used to systematically determine the impact of age of ovarian hormone loss on cognition, as well as the cognitive response to exogenous hormone treatment, discussed in detail later in this chapter.

Regarding limitations, it is important to note that the Ovx rodent model is a binary all-or-nothing model. That is, the cessation of circulating ovarian hormones is abrupt after surgical removal of the ovaries, much like turning off an electrical switch. In

contrast, ovarian hormone loss with menopause in women is a gradual process that occurs against a backdrop of natural aging. Thus, the Ovx model alone does not provide an opportunity to investigate the effects on system functions with the natural transition to menopause and despite closely modeling surgical menopause, Ovx is not translationally representative of the transitional natural menopause that most women will experience (Whiteman et al., 2008). In general, most women will experience transitional menopause, with the transition phase lasting up to 10 years prior to the onset of menopause with associated irregular fluctuations of circulating ovarian hormone levels as well as several undesired symptoms (Burger et al., 2008; Harlow & Paramsothy, 2011). Thus, an informative additional model of menopause would allow for investigation of changes across the transitional period of natural menopause and subsequent effects of menopausal hormone therapy.

Unilateral ovariectomy (ULO)

To circumvent the disadvantage of the abrupt loss of circulating ovarian hormones seen with the Ovx rodent model, researchers have developed clever alternatives to design models of menopause that allow the endocrine system to function at partial capacity. Instead of bilaterally removing both ovaries, one ovary alone can instead be removed for *unilateral ovariectomy (ULO).* The ULO model presents an interesting variant of the Ovx model (Figure 2). Six hours after ULO was performed in 2-3 month old rats experiencing metestrus at the time of surgery, there was a surge in FSH levels in plasma, a characteristic of menopausal status in women, as well as in the pituitary (Otani & Sasamoto, 1982). Plasma FSH markedly increased within 6 hours, plateaued at 12

hours, and dropped to normal levels by 24 hours following surgery, compared to bilaterally ovary intact rats, whereas plasma LH levels did not differ from bilaterally ovary intact rats (Otani & Sasamoto, 1982). FSH in the pituitary also showed a significant increase after 6 hours that persisted until the animals were in proestrus when compared to bilaterally ovary intact rats. This surge in both plasma and pituitary FSH was also seen when ULO surgery was performed during the diestrus phase (Otani & Sasamoto, 1982). The ULO animals exhibited approximately double the largest measured follicles compared to those of bilaterally ovary intact rats, likely due to the FSH rise (Otani & Sasamoto, 1982). The FSH increase is believed to occur as a compensatory mechanism for the loss of one ovary, which the authors posited was due to a lack of inhibin-like substances (Otani & Sasamoto, 1982). In women, unilateral oophorectomy has been shown to impact relative risk of dementia. Hysterectomy alone increased the risk of dementia or cognitive impairment, an effect that was further increased with the addition of unilateral oophorectomy, with an even greater elevation in risk when hysterectomy was combined with bilateral oophorectomy (Walter A. Rocca, Grossardt, Shuster, & Stewart, 2012). Collectively, these findings indicate that unilateral removal of an ovary provides a translationally-relevant model of hormonal changes during menopause, and could provide meaningful insights into the putative altered risk of cognitive change with natural and/or surgical menopause. However, this model has been very infrequently used in behavioral neuroendocrinology, so little is known about its effects on cognition and brain function in rodents.

4-vinylcyclohexene diepoxide (VCD)

Given that the majority of women will undergo transitional menopause, it is noteworthy that an animal model has been developed that recapitulates the natural, transitional menopause that women typically experience. In women, menopause is induced by follicular depletion, which accelerates in the decade preceding menopause (Richardson & Nelson, 1990). Humans and rodents have four different types of ovarian follicles: primordial, primary, secondary, and antral follicles, which mature during the follicular phase of the female cycle (Koebele & Bimonte-Nelson, 2016; Kronenberg et al., 2008). Within each cycle, a follicle progresses from primordial to primary, to secondary, and to antral, after which it becomes preovulatory, and a mature egg is released from the ovary (Koebele & Bimonte-Nelson, 2016; Kronenberg et al., 2008). In rodents, *4-vinylcyclohexene diepoxide (VCD),* a metabolite of vinylcyclohexene (VCH), induces ovarian follicular depletion via accelerated atresia of primordial and primary ovarian follicles, while leaving the ovaries intact (Flaws et al., 1994; Springer et al., 1996; Kao et al., 1999; Hoyer et al., 2001). If VCD treatment is initiated before the rodents undergo estropause, a form of reproductive senescence ensues that is remarkably similar to that of women who undergo transitional menopause. Follicular depletion can be induced in mice (Kao et al., 1999; L. P. Mayer, Devine, Dyer, & Hoyer, 2004) and in rats (Kao et al., 1999; Loretta P. Mayer et al., 2002) via a series of intraperitoneal injections of VCD. More recently, protocols have included oral consumption of food bait containing a combination of VCD and triptolide (Dyer et al., 2013). Triptolide is a type of herbal Chinese medicine synthesized from *Trypterygium wilfordii,* also known as the thunder god vine, which is known to accelerate follicular depletion (Dyer et al., 2013).

The most commonly utilized doses of VCD to date are 80 mg/kg/day or 160 mg/kg/day via intraperitoneal injection for a total of 15 days. These two doses and regimens are effective at depleting ovarian follicles with reduced incidence of health concerns in rats and mice compared to other tested doses and regimens of VCD injections (Acosta et al., 2010; Acosta, Mayer, Talboom, Tsang, et al., 2009; Koebele et al., 2017; Thompson et al., 2005). Compared to vehicle controls, rats receiving daily intraperitoneal injection of 80 mg/kg VCD for 30 days showed evidence of initial primordial and primary follicle depletion (Loretta P. Mayer et al., 2002). By 60 days after the first VCD injection, growing follicles started to deplete, and by 120 days after the first VCD injection, antral follicles started to deplete (Loretta P. Mayer et al., 2002). In a study evaluating 30, 60, 120, 240, and 360 post-initial VCD injection day timepoints, rats showed heightened levels of FSH relative to controls by 120 days after the first VCD injection, and levels remained elevated until 360 days after VCD initiation (Loretta P. Mayer et al., 2002). Of note, the VCD model achieves follicular depletion and elevated FSH levels within 4 months of treatment initiation, and the transition to menopause in women can take up to a decade which is akin to about 4.6 months in a rat (Richardson and Nelson, 1990; Sengupta, 2013). On the other hand, if rats were given a single bolus of VCD, there was growth, rather than depletion, of primary follicles compared to controls (Borman et al., 1999). One interpretation of these findings is that the first response to VCD is protective in nature; however, this effect is not yet completely understood.

It is critical to note that there are differences in the effects of VCD/VCH treatment between mice and rats. Mice are far more sensitive to VCH-induced follicular depletion, which researchers believe is because mice have a greater capability to metabolize VCH to

VCD (Hoyer et al., 2001; Hoyer & Sipes, 2007; Kappeler & Hoyer, 2012). In rats, VCH results in little-to-no follicular depletion following treatment at the doses tested thus far, and therefore, VCD is considered the bioactive form of the compound in both rats and mice (Hoyer et al., 2001; Hoyer & Sipes, 2007). Mice also seem to be more responsive to VCD than rats. In one of the first studies comparing VCD treatment in mice versus rats, an 80 mg/kg intraperitoneal injection of VCD for 12 days resulted in greater overall primordial and primary follicular loss in mice than in rats compared to age-matched species-specific controls (Kao et al., 1999). In the same study, VCD-treated mice also showed earlier primordial follicular loss, beginning on day 8 of injections, whereas VCD-treated rats did not show significant primordial follicular loss until day 10 (Kao et al., 1999). However, once primordial follicular loss was detected, the rate of primordial follicular loss remained similar across both species, indicating that both species lost follicles at a comparable rate (Kao et al., 1999).

In mice that have received VCD, as with women undergoing transitional menopause, plasma gonadotropin (LH and FSH) levels increased, and plasma progesterone levels decreased, relative to mice that received vehicle; E2 levels became undetectable via radioimmunoassay (L. P. Mayer et al., 2004). Thus, since androstenedione levels only decreased by about one-third, the hormone profile of VCD-treated mice became androstenedione-rich, a characteristic which is similar in postmenopausal women (L. P. Mayer et al., 2004). Underscoring that VCD-treated ovaries can yield androgens, cells taken from the ovaries of VCD-treated mice could be induced in culture to produce androstenedione (L. P. Mayer et al., 2004). Research utilizing the VCD rat model showed that when compared to ovary-intact vehicle-treated

sham rats, VCD-treated rats showed increased levels of circulating FSH, and no differences in circulating LH and androstenedione levels, at subject sacrifice which occurred 141 days after the first VCD injection (Acosta et al., 2009a). Taken together, the VCD-induced rise in gonadotropins, coupled with an androstenodione-rich milieu, supports the tenet that the VCD rodent model is a translationally viable model of transitional menopause with the noted caveat that all models have their strengths and weaknesses. Indeed, although the VCD rodent model represents many aspects of the alterations in ovarian follicles and circulating hormone profiles occurring with transitional menopause, there are precautions and complexities of this model that must be considered. For instance, VCD is a chemical utilized in the creation of epoxy and other industrial byproducts (Chhabra, 1989). As such, its exogenous administration to rodents may lead to undesired effects, which, in turn, may inadvertently impact behavioral outcomes (Chhabra, 1989). Recent research has revealed that oral VCD given daily for 28 days can increase oxidative stress in the liver and kidneys, as well as elevate circulating and liver inflammatory factors, in male and female rats (Amos Olalekan Abolaji et al., 2016). Further investigation showed that oral VCD given daily for 28 days can also increase oxidative stress in the ovaries and uterine horns, raise apoptosis and inflammatory factors in the ovaries, as well as result in hormone dysregulation, in young-adult female rats (Amos O. Abolaji et al., 2016). The authors propose that these effects may be mechanisms through which VCD induces ovarian follicular depletion (Amos O. Abolaji et al., 2016). Nonetheless, these unintentional effects may confound the interpretation of the impact of ovarian hormone loss and hormone therapy on various brain and behavioral outcomes in VCD-treated rodents.

In conclusion, this section has discussed the strengths and weaknesses of several menopause models. One must choose carefully when selecting a menopausal model. Although all currently available rodent models of reproductive senescence offer opportunities for precise hormone manipulation, especially when combined with pharmacological manipulations or hormone-variant knock outs, for example, each has caveats that must be taken into account when interpreting results regarding the impact of menopause and hormone therapy on system functions, including cognition. Of note, all recapitulate enough key aspects of menopause to allow researchers to directly study the complexities of hormone interactions across reproductive aging. In preclinical research, strictly regimented experimental study designs can be conducted with proper control and randomization. Indeed, animal models are an important step in gaining a better understanding of the intricacies and implications of hormonal changes across the lifespan, and can provide the vital foundational knowledge necessary to stimulate innovative research which translates to the clinic.

Spatial memory

The hippocampus is a brain region involved in the formation of *spatial memories,* which are memories regarding one's environment and orientation in space (Leutgeb et al., 2005; O'Keefe & Dostrovsky, 1971; Rosenzweig, Redish, Mcnaughton, & Barnes, 2003). Numerous behavioral maze tasks have been developed to assess hippocampal-dependent spatial memory processing in rodents. These testing paradigms evaluate a rodent's ability to learn to navigate in the maze and remember one or multiple locations of reward in an environment that provides abundant cues located outside of the maze (extra-maze).

Spatial memory tasks can be designed to test either short-term or long-term memory. Short-term memory, whereby information is either updated and/or manipulated, is termed *working memory* (Baddeley, 2003; H. A. Bimonte-Nelson, Daniel, & Koebele, 2015; Olton & Papas, 1979). Long-term memory, whereby information remains constant over a long period of time, is termed *reference memory* (H. A. Bimonte-Nelson et al., 2015; Olton & Papas, 1979). Here, several of the commonly used tasks to assess these memory types are defined. For instance, in the *Morris water maze (MWM)* task, an animal must learn to find a submerged escape platform in a round tub filled with opaque water using spatial extra-maze cues that are placed throughout the testing room. Although the start location varies across trials and days, the platform location remains constant throughout testing, thus, this task tests spatial reference memory. Another task, the radial arm maze (RAM) was originally developed to test spatial working memory (Olton & Samuelson, 1976). The wheel-shaped apparatus consists of a circular central platform from which 8, 12, or 17 arms radiate. Each arm is baited at the end with a hidden cup in which food or water is placed for food- or water-restricted rodents to find. The maze is surrounded by extra-maze cues that animals can use to navigate. Once located, food and water rewards are not replaced, so the optimal strategy is to visit each arm only once. Because rodents do not enter the arms in the same sequence each day, this task tests spatial working memory. The RAM has been modified over the years to test both spatial reference and working memory simultaneously by baiting only half the arms with food (Olton & Papas, 1979). Because the same arms remain unbaited throughout trials and days, animals should never enter them; this information remains constant – these arms never have food, and thus, these arms assess spatial reference memory. In contrast, re-entries into

previously entered arms within a day are considered tests of spatial working memory because information must be updated – the previously entered arms no longer contain bait. More recently, a water-escape version of the RAM has been developed that merges the response-reinforcement contingencies of the MWM with the ability to measure spatial working and reference memory of the RAM (Bimonte & Denenberg, 1999; Hyde, Hoplight, & Denenberg, 1998). The water version of the RAM task is utilized in a similar manner; however, it is not reliant on appetitive reinforcement for the completion of the maze. For this task, typically an 8-arm tub is filled with opaque water and the ends of some arms contain an escape platform hidden underneath the surface of the water. The animal is trained to find the location of the hidden platform(s); once a platform is located, it is removed for the rest of that testing day. Thus, animals must update and remember the location/s from which they have already escaped. As in the dry-land RAM, optimal performance is obtained when animals use extra-maze cues for spatial navigation. Spatial reference and working memory are tested in the same way as the land RAM, with the platforms replacing baited food or water as the navigational target. The *delayed-match-to-position (DMP)* maze is used to test spatial working memory and *recent memory*, which is the short term retention of information, but does not involve the manipulation of information as does working memory. In this water-escape task, one out of many arms (this can vary in number, depending on the maze) contains a hidden platform, the location of which remains the same within a day, but varies across days. Similar to other water-escape maze tasks, the maze is filled with opaque water and is located in a room with numerous spatial cues that can be utilized for spatial navigation. The first trial is considered the information trial, in which the animal learns where the platform location is

for that day. The animal must return to the correctly platformed spatial location for the remainder of that day. This requires an animal to update the information regarding the platform location across days. Trial 2 is considered the working memory trial since information must be updated from the prior day. The remainder of the trials within that same day are not considered working memory, but are considered recent memory, since nothing is being updated but short term retention must occur to solve the task successfully. This task can also utilize food reward in a dry walk-through maze, such as the classic T-maze (three perpendicular arms in the shape of a T) and Y-maze (three non-perpendicular arms in the shape of a Y). Here, as in the water-escape DMP version, the reinforcer is alternated (e.g., the platform arm is alternated in the water-escape version and the baited arm is alternated in the dry-land version). A delay period between trials for any of these tasks can be implemented to test for spatial memory retention. Additional details on the mazes and the maze protocols can be found in The Maze Book: Theories, Practice, and Protocols for Testing Rodent Cognition (H. A. Bimonte-Nelson, 2015a).

Chronic estrogen therapy and spatial memory

The abrupt decrease in circulating ovarian hormone levels after Ovx in young adult or middle-aged animals is often associated with compromised spatial learning and memory (Bimonte & Denenberg, 1999; Feng, Cheng, & Zhang, 2004; R. B. Gibbs & Johnson, 2008; Alicja L Markowska & Savonenko, 2002; Talboom, Williams, Baxley, West, & Bimonte-Nelson, 2008). Although estrogen supplementation can benefit cognition after Ovx, the learning and memory effects of estrogen-based hormone therapy are largely dependent on type of estrogen used. In addition, preclinical studies examining

the role of estrogens in spatial memory reveal multiple other factors that profoundly impact the efficacy of estrogen treatment. These include type of menopause model used, cyclic versus tonic administration, age at treatment initiation (young adult: 4-8 months, middle-aged: 11-14 months, and aged: >17 months), and timing of supplementation relative to length of prior hormone loss.

CEE and its equine-specific components

As previously mentioned, CEE are the hormones used in the name brand Premarin, a hormone therapy formulation prescribed to menopausal women to manage symptoms associated with menopause. A handful of preclinical studies have addressed the effects of CEE on cognitive function using rodent models, with a focus on how CEE dose, administration regimen, and type of menopause influences CEE's cognitive effects. Overall, studies suggest that CEE treatment in Ovx middle-aged rats can have a beneficial effect on spatial working memory, spatial reference memory, and delayed retention of spatial memory (Acosta, Mayer, Talboom, Zay, et al., 2009; E. Engler-Chiurazzi et al., 2011). Moreover, following behavioral testing, CEE-induced changes in the transcription of genes in the hippocampus that are involved in cognitive function and plasticity were found, providing biological plausibility for the impact of CEE on spatial memory (E. Engler-Chiurazzi et al., 2011). The Bimonte-Nelson laboratory has administered cyclic subcutaneous injections (2 days on and 2 days off) of 10 μg CEE/day, 20 μg CEE/day, or 30 μg CEE/day to middle-aged Ovx rats for 18 days prior to behavior testing; the treatment regimen was continued until behavior testing was completed (Acosta, Mayer, Talboom, Zay, et al., 2009). The 20 μg CEE/day dose was chosen based

on the clinically used daily 0.625 mg CEE dose in Premarin, adjusting for differences in average body weight of a middle-aged woman and rat; of note, this dose was used in the Women's Health Initiative Memory Study (WHIMS) (Shumaker et al., 2003). Compared to the vehicle-treated control group, all CEE-treated groups exhibited enhanced spatial reference memory on the MWM task, and improved spatial working memory during the acquisition phase of the DMP plus maze task (Acosta, Mayer, Talboom, Zay, et al., 2009). In addition, the 30 μg CEE/day group also exhibited enhanced spatial localization of the platform location on the MWM relative to the vehicle control group (Acosta, Mayer, Talboom, Zay, et al., 2009). The beneficial cognitive effects of the 30 μg CEE/day (2 days on and 2 days off) treatment regimen were recently replicated in a study assessing young adult Ovx rats of a different strain (Sprague-Dawley, versus the previously used Fischer-344) on the water RAM and DMP plus maze tasks (Hiroi et al., 2016). Evaluating effects in a different strain is important, as the two strains inherently have different genetic backgrounds as do people, thus suggesting that these findings on estrogenic cognitive effects may be generalized to a genetically diverse population within a species. Additional evidence suggests that CEE-mediated memory performance is dependent on CEE dose. In one experiment, tonic CEE was administered to middle-aged Ovx rats via a subcutaneous osmotic pump which released CEE at doses based on the same calculations as Acosta et al (2009b), adjusting for body weight for the daily 0.625 mg CEE dose in Premarin. This design resulted in low, medium, and high CEE groups of 12 μg CEE/day, 24 μg CEE/day, and 36 μg CEE/day, respectively (E. Engler-Chiurazzi et al., 2011). The low CEE dose impaired spatial reference memory performance in the MWM task, as well as impaired spatial working memory performance in the DMP plus

maze task, when compared to the vehicle control group (E. Engler-Chiurazzi et al., 2011). The high CEE dose enhanced spatial memory retention in the water RAM following a 4 hour delay, and the two higher CEE doses (statistically combined) improved spatial memory retention in the DMP plus maze following a 6 hour delay, relative to the vehicle control group (E. Engler-Chiurazzi et al., 2011). Interestingly, an assessment of the circulating E1:E2 ratio for all CEE treated rats revealed a negative correlation between the E1:E2 ratio and working memory errors; indeed, as the ratio of E1:E2 increased, animals in this study tended to exhibit better working memory performance (E. Engler-Chiurazzi et al., 2011). The results indicated that when circulating E2 was present, high circulating E1 could be cognitively beneficial. Important findings from the Galea laboratory, wherein adult Ovx rats were administered daily subcutaneous injections of 10 μg CEE/day or 20 μg CEE/day, demonstrated that both the 10 μg and the 20 μg CEE/day doses impaired spatial working and reference memory on the land RAM (Barha & Galea, 2013). Spatial working and reference memory impairment due to the cyclic (daily) low CEE dose used here is consistent with the spatial working and reference memory impairment of the tonic low CEE dose evidenced in Engler-Chiurazzi et al (2011). The collected findings indicate that the age of the rats at CEE treatment, and whether CEE administration is cyclic or tonic, impacts efficacy on cognition. Cyclic CEE injections (2 days on/2 days off) with a break in between administration tended to enhance spatial memory on the MWM, water RAM, and DMP plus maze tasks in young adult and middle-aged Ovx rats; whereas, tonic CEE released through osmotic pumps at similar doses in middle-aged Ovx rats resulted in more variable efficacy, yielding impaired, null, or enhanced effects on spatial memory that had greater dependence on CEE dose. More

frequent injections (e.g., daily) of CEE in adult Ovx rats impaired spatial memory on the land RAM; of note, this daily injection regimen could yield tonic-like effects due to accumulation of the sesame oil/hormone substrate at the injection tissue sites.

Background hormone milieu also impacts cognitive efficacy of CEE treatment, as the type of menopause model used to evaluate the cognitive impact of CEE can markedly affect the direction of the outcome. In a study comparing Ovx and VCD models of menopause, menopause type before giving CEE hormone therapy was methodically manipulated. Female rats underwent either Ovx to model surgical menopause, or VCD to model transitional menopause incorporating gradual follicular depletion and retention of follicle-deplete ovaries, followed by 2 days on/2 days off 30 μg CEE/day subcutaneous injections at middle-age (Acosta et al., 2010). In comparison to respective vehicle controls, CEE treatment in Ovx rats enhanced, whereas CEE treatment in VCD rats impaired, spatial working and reference memory on the water RAM (Acosta et al., 2010). Additionally, following implementation of an 8-hour delay on the DMP plus maze task, CEE treatment did not impact spatial memory retention in VCD rats, but CEE treatment improved spatial memory retention in Ovx rats (Acosta et al., 2010). These data indicate that CEE has contrasting effects on spatial learning and memory that are dependent on whether the animal underwent surgical versus transitional menopause. With Ovx, there is an abrupt and complete loss of ovarian follicles and circulating ovarian hormone levels, whereas with VCD, there is a transitional decrease in ovarian follicles and in some circulating ovarian hormone levels over time. Thus, the effects on the brain and cognitive function may be different as a function of abrupt versus gradual change in ovarian hormone profile. Moreover, androstenedione is the predominant circulating ovarian

hormone in the VCD, but not the Ovx, model of menopause. The presence of androstenedione can also play a role in the cognitive effects of estrogen-based hormone therapy. Indeed, research has shown in multiple replicate studies that increasing circulating levels of androstenedione in VCD rodents are correlated with worse cognitive performance (Acosta et al., 2010; Acosta, Mayer, Talboom, Tsang, et al., 2009; B. W. Camp et al., 2012). Interestingly, blocking the conversion of androstenedione to E1 rescued the cognitive impairment seen with androstenedione treatment (Sarah E. Mennenga, Koebele, et al., 2015), indicating that E1 may be a contributing factor in the impairment observed when levels of circulating androstenedione are high.

The known estrogens in CEE, including sulfate ester forms of E1, E2, 17α-estradiol, equilin, 17β-dihydroequilin, 17α-dihydroequilin, equilenin, 17β-dihydroequilenin, 17α-dihydroequilenin, and Δ8,9-dehydroestrone, have varied functional and neuroprotective effects (Bhavnani, 2003; Bhavnani & Stanczyk, 2014; Brinton, Proffitt, Tran, & Luu, 1997; Talboom et al., 2010; Zhao & Brinton, 2006). These equine estrogens differ in binding affinity to estrogen receptors, which may contribute to the variation in their functional effects (Bhavnani, 2003; Lobo, 2007). For instance, *in vitro* analysis of these 10 estrogens in cultured basal forebrain neurons showed that eight of the estrogens, 17α-estradiol, E2, equilin, 17α-dihydroequilin, equilinen, 17α-dihydroequilenin, 17β-dihydroequilenin, and Δ8,9-dehydroestrone, had neuroprotective effects as measured by the ability to decrease glutamate excitotoxicity-induced damage to the neuronal plasma membrane (Zhao & Brinton, 2006). However, when examined for neuroprotective effects against damage induced by β-amyloid$_{25\text{-}35}$, only E2, E1, and Δ8,9-dehydroestrone were protective against the associated decrease in intracellular ATP

(Zhao & Brinton, 2006). Additionally, greater neuroprotective effects were observed following co-administration of E2 plus equilin, E2 plus Δ8,9-dehydroestrone, and equilin plus Δ8,9-dehydroestrone against decline in intracellular ATP compared to glutamate treatment alone, and in relation to individual administration of E2, equilin, or Δ8,9-dehydroestrone (Zhao & Brinton, 2006). Several preclinical studies have aimed to decipher the effects of various equine estrogens in CEE on spatial memory using animal models. The effects of tonic subcutaneous administration of Δ8,9-dehydroestrone or equilin, two CEE components not naturally found in women, in middle-aged Ovx rats were compared (Talboom et al., 2010). These two components were selected because they had been previously shown by the Brinton laboratory to have consistent and potent neuroprotective effects *in vitro* relative to the other equine estrogens in CEE (Zhao & Brinton, 2006). It was found that Δ8,9-dehydroestrone treatment enhanced spatial reference, working, and recent memory compared to vehicle control treatment, and that equilin treatment had no effect on spatial memory (Talboom et al., 2010). The lack of beneficial cognitive effects of equilin treatment is not consistent with its neuroprotective effects in culture. Together, these findings suggest that although *in vitro* studies can yield insight into the neuroprotective effects of estrogens, efficacy might not necessarily translate to behavioral outcomes *in vivo*. However, it is important to keep in mind that the cell culture work directly induced cell damage via glutamate or β-amyloid$_{25\text{-}35}$, whereas the animal work did not model cytotoxicity or a disease state as the pre-treatment model; rather, the Ovx rat model was utilized. This variation in study design could explain, in part, the divergent outcomes of the same estrogens.

Estrone (E1)

Evidence suggests that E1 may also impact cognitive function and brain health. In postmenopausal women, the primary circulating estrogen is E1 (Lobo, 2007). Circulating E2 can be metabolized into E1 and vice versa, and the affinity of E1 for estrogen receptors is lower than E2 (Kuhl, 2005). Interestingly, as discussed in greater detail above, there is a relationship between the ratio of circulating E1 to E2 and cognition, whereby higher E1:E2 is associated with enhanced maze scores (E. Engler-Chiurazzi et al., 2011). Because the sulfate ester of E1 comprises roughly 50% of the CEE that is used in menopausal hormone therapy (Bhavnani, 2003; Bhavnani & Stanczyk, 2014; Lobo, 2007), it is important to understand the effects of E1 on cognition.

Several studies have examined the cognitive effects of E1 alone. The Galea laboratory first examined the effects of one single subcutaneous injection of 0.3 μg, 1 μg, or 10 μg of E1 in adult Ovx rats 30 minutes prior to contextual fear conditioning (Barha, Dalton, & Galea, 2010). Although no dose of E1 enhanced contextual fear conditioning, 1 μg E1 impaired memory relative to vehicle controls (Barha et al., 2010), suggesting a detrimental effect of this dose. Similarly, a tonic subcutaneous dose of 2.6 μg/day, 4 μg/day, or 8 μg/day of E1 in Ovx middle-aged rats was subsequently compared, and it was found that the 8 μg E1/day dose impaired spatial working memory and retention on the DMP plus maze task compared to vehicle controls, whereas the lower doses had no effects on memory (E. B. Engler-Chiurazzi et al., 2012). The Galea laboratory also investigated the effects of daily E2 (10 μg) or E1 (10 μg) treatment for 20 days on spatial reference memory (as tested on the MWM) as well as hippocampal neurogenesis and activation in adult Ovx rats (McClure, Barha, & Galea, 2013). Although they found no

treatment differences in spatial reference memory, E2 treatment yielded higher, and E1 treatment yielded lower, cell survival levels in the dentate gyrus of the hippocampus relative to controls (McClure et al., 2013). Taken together, these studies indicate that both chronic and isolated-acute E1 treatment regimens can impair spatial or hippocampal-mediated cognitive performance on a dose-dependent basis in a surgical menopause background as modeled in Ovx rats, with differential effects on the hippocampus with E1 versus E2 treatments. Additional studies are needed to fully understand the effects of exogenously administered E1, as well as circulating E1 levels, on memory, including how varied E1:E2 ratios impact the brain and its functioning.

17β-estradiol (E2)

E2 is the most potent naturally circulating estrogen in mammals, and it is also a component in several hormone therapy formulations. E2 is the most widely studied estrogen in preclinical studies; thus, there are numerous studies addressing the role of E2 in spatial learning and memory. Many of these studies indicate that E2 treatment enhances spatial memory in Ovx young and middle-aged rats when tested on the MWM (Heather A. Bimonte-Nelson, Francis, Umphlet, & Granholm, 2006; Lowry, Pardon, Yates, & Juraska, 2010; Talboom et al., 2008) and on the water and land versions of the RAM (Bimonte & Denenberg, 1999; Jill M Daniel, Fader, Spencer, & Dohanich, 1997; V N Luine, Richards, Wu, & Beck, 1998; Rodgers, Bohacek, & Daniel, 2010). However, although the overall consensus in the field seems to be that E2 is beneficial to memory, the story does not appear to be so straightforward. As with CEE, multiple factors impact whether exogenous E2 treatment will yield beneficial, null, or detrimental effects on

hippocampal memory. These factors include, but are not limited to, the dose, regimen, and age of E2 administration, as well as the time between Ovx and E2 administration (Barha & Galea, 2010; Heather A. Bimonte-Nelson et al., 2006; Jill M. Daniel et al., 2006; Frick, 2009; R. B. Gibbs, 2000; Alicja L Markowska & Savonenko, 2002; Talboom et al., 2008).

Rodent studies evaluating the effects of E2 on cognitive and brain function indicate that these effects are dose-dependent (for review: Barha and Galea, 2010; Frick, 2010). One study found, for example, that tonic subcutaneous administration of 0.25 mg E2, but not 0.50 mg E2, via 60-day release pellets in middle-aged Ovx rats enhanced spatial reference memory on the MWM (Heather A. Bimonte-Nelson et al., 2006). Additionally, in adult Ovx rats, a single subcutaneous injection of 0.3 μg E2 enhanced contextual fear conditioning, whereas 1.0 μg or 10.0 μg E2 impaired contextual fear conditioning, relative to controls (Barha et al., 2010). The age at E2 administration can also play a role in the effect of E2 on spatial memory. Indeed, in young and middle-aged Ovx rats, tonic subcutaneous E2 treatment enhanced spatial reference memory on the MWM, but had no effect in aged Ovx rats (Talboom et al., 2008).

Another important factor to consider is that E2 administration regimen can influence the direction of E2's impact on cognitive function. For example, relative to vehicle controls, tonic E2 treatment enhanced spatial working memory in aged Ovx rats only when the animals were primed with cyclic E2 subcutaneous injections (Alicja L Markowska & Savonenko, 2002). In another study evaluating middle-aged Ovx rats, cyclic E2 injections (bi-weekly), and low dose tonic E2 implants, but not high dose tonic E2 implants, enhanced spatial reference memory on the MWM compared to vehicle

controls (Heather A. Bimonte-Nelson et al., 2006). A study evaluating aged Ovx mice on the water RAM found that three months of continuous E2 (injections every day) did not impact spatial reference and working memory, and intermittent E2 (injections every 4 days) impaired spatial reference memory and tended to impair spatial working memory, as compared to vehicle controls (Gresack & Frick, 2006). In addition, accumulating research suggests that there are significant effects of the critical window of opportunity, the period of time between ovarian hormone loss and initiation of hormone treatment administration, which can influence E2-induced cognitive efficacy. For example, a study of middle-aged rats from the Gibbs laboratory provides a clear illustration of this point. Specifically, E2 treatment initiated immediately or 3 months following Ovx enhanced spatial working memory on a DMP task, whereas E2 treatment initiated 10 months following Ovx had no effect on memory (R. B. Gibbs, 2000). In another study, middle-aged Ovx rats that received E2 treatment immediately following Ovx surgery exhibited better spatial working memory in the land RAM, whereas those that received E2 treatment 5 months following Ovx surgery did not show a benefit, compared to controls (Jill M. Daniel et al., 2006). Collectively, the abundant preclinical work systematically evaluating E2 treatment effects on spatial cognition suggests that E2 can play a beneficial role in cognition. However, as illustrated above, multiple factors may render the efficacy of E2 null or detrimental.

Synthetic estrogens

Synthetic estrogens are commonly used in both contraceptive and menopausal hormone therapy formulations. Preclinical studies of these estrogens provide additional

support that multiple factors surrounding estrogen treatment administration can influence estrogens' effects on spatial learning and memory. Ethinyl estradiol has been evaluated for both dose and administration regimen effects in the rodent Ovx model. For example, the Bimonte-Nelson laboratory recently assessed two doses of tonic, subcutaneous ethinyl estradiol administration in young adult Ovx rats on the spatial water RAM and MWM; the high ethinyl estradiol dose (0.3 μg/day) impaired spatial working memory on the water RAM relative to vehicle controls and a low ethinyl estradiol dose (0.125 μg/day), but did not affect spatial reference memory on the MWM (Sarah E. Mennenga, Gerson, Koebele, et al., 2015). Moreover, in another study, the effects on spatial memory of cyclic subcutaneous ethinyl estradiol in doses of 0.125 μg/day, 0.18 μg/day, or 0.3 μg/day in young adult Ovx rats were tested (Sarah E. Mennenga, Gerson, Koebele, et al., 2015). Consistent with previous studies, the 0.3 μg/day dose impaired spatial working memory in the water RAM relative to vehicle, 0.125 μg/day, and 0.18 μg/day (Sarah E. Mennenga, Gerson, Koebele, et al., 2015). All three cyclic ethinyl estradiol treatments impaired spatial reference memory on the MWM task (Sarah E. Mennenga, Gerson, Koebele, et al., 2015). Others have also tested the effects on spatial memory of another E2 synthetic analog, estradiol benzoate, using the rodent Ovx model. The lowest subcutaneous injection dose of 0.32 μg estradiol benzoate/day enhanced spatial working memory in the land RAM compared to vehicle controls, whereas the higher doses of 1 μg/day and 5 μg/day impaired spatial working memory compared to vehicle controls (Holmes, Wide, & Galea, 2002). Another study administered tonic low or high doses of estradiol benzoate via subcutaneous silastic capsules, resulting in estradiol plasma levels of ~40 pg/ml or >200 pg/ml, respectively, to young, middle-aged, and aged Ovx rats

(Foster, Sharrow, Kumar, & Masse, 2003). Results showed an age by dose interaction for retention on a spatial discrimination task (Foster et al., 2003), such that enhanced retention scores were exhibited for the vehicle-treated young rats, low estradiol benzoate-treated middle-aged rats, and high estradiol benzoate-treated aged rats; retention scores were not impacted for low or high estradiol benzoate-treated young rats, vehicle or high estradiol benzoate-treated middle-aged rats, and vehicle or low estradiol benzoate-treated aged rats (Foster et al., 2003). Thus, there is evidence indicating that, similar to natural estrogens, synthetic estrogens can also impact learning and memory. Although there is limited work done in this research domain, results thus far suggest that the directionality of the cognitive impact of synthetic estrogens is governed not only by the type of synthetic estrogen, but also by experimental factors such as age, treatment dose, and treatment regimen.

In summary, preclinical research spanning the last three decades has revealed a great deal regarding exogenous treatment with estrogens and subsequent effects on spatial memory. It is now understood that estrogens do modulate spatial memory, although several factors impact the directionality of these estrogenic effects, including the type, dose, duration, and frequency of estrogen treatment, as well as the age and memory type evaluated. However, the picture is not yet complete, and replication and extension of many of the existing studies are needed. Additional research is required to determine the critical factor variants driving the decision points to dictate whether a woman should start estrogen therapy, and if so, when – for example, during the transition to menopause or postmenopause, immediately following or after a delayed period of surgical ovarian loss, or never unless symptoms occur – as well as what type of estrogen would be best for a

woman with a particular circulating hormone background milieu. It is possible that there is an estrogen formulation that is yet to be created, discovered, or evaluated that would be beneficial in a broad range of female phenotypes, thereby yielding an optimal hormone therapy profile for overall women's health during aging. Furthermore, when considering estrogens as hormone therapy, interactions with progestogens should be carefully considered. Indeed, hormone therapy is often provided as an estrogen plus progestogen combination formulation. The following section discusses what is currently known regarding progestogen, as well as estrogen plus progestogen, treatments and their effects on spatial learning and memory.

Progestogens, and estrogen plus progestogen hormone combinations

The term *progestogens* is used to describe both endogenous progesterone and synthetic variations of progesterone, known as *progestins*. Some examples of progestins are medroxyprogesterone acetate (MPA) and levonorgestrel (Levo), two compounds found in both birth control formulations and hormone therapy. When estrogens are given exogenously to a woman with an intact uterus, a progestogen component is necessary to oppose the heightened risk for developing endometrial hyperplasia and cancer with unopposed estrogen exposure (NAMS, 2012). The WHIMS indicated that CEE plus MPA combination hormone therapy did not have beneficial effects on cognition, especially if deficiencies in cognitive abilities were present prior to initiation of treatment (Espeland et al., 2004; Rapp et al., 2003; Resnick et al., 2006; Shumaker et al., 2003). Specifically, exogenous administration of the CEE plus MPA hormone combination in postmenopausal women 65 years or older did not enhance cognition compared to

exogenous administration of a placebo (Rapp et al., 2003). Furthermore, a clinically meaningful increase in risk for cognitive decline was observed for postmenopausal women taking this combination hormone therapy (Rapp et al., 2003). Postmenopausal women taking CEE plus MPA had twice the risk for developing probable dementia, and were not protected against mild cognitive impairment, compared to those taking a placebo (Shumaker et al., 2003). There is evidence that the MPA component of hormone therapy is detrimental to the brain and its functioning. For example, *in vitro* MPA treatment was not neuroprotective, and when combined with E2, MPA attenuated the neuroprotective effects of E2 against glutamate toxicity (Nilsen & Brinton, 2002). The Bimonte-Nelson laboratory has demonstrated that MPA can impair spatial working memory in both middle-aged and aged Ovx rats, and that this effect may be non-reversible, as cognitive impairments continued even when MPA treatment was stopped for 4 months prior to behavior testing and circulating MPA levels were not detectable (Braden et al., 2010, 2011). The Kronos Early Estrogen Prevention Study (KEEPS) included an ancillary study, the Cognitive and Affective Study (KEEPS-Cog), to address global cognitive function in women within 6-36 months of their last menstrual period taking CEE or E2 hormone therapy in combination with micronized progesterone. Given the timing of treatment to menopause, this study evaluated women ranging from the late menopausal transition to early postmenopause (Gleason et al., 2015). Neither combination hormone therapy regimens benefitted or impaired cognition over a four-year period compared to placebo treatment, although the CEE/micronized progesterone combination improved some depression and anxiety measures (Gleason et al., 2015). Although this study would seem to suggest little cognitive benefit of CEE/micronized

progesterone and E2/micronized progesterone, preclinical studies examining clinically-relevant hormone combination treatments, although largely focusing on E2 rather than CEE, suggest that the addition of a progestogen component to an estrogen treatment can impact E2-induced beneficial cognitive effects. Outcomes seem to be heavily influenced by the type of estrogen and type of progestogen utilized, as well as the behavioral or neurobiological outcome examined. These findings are further elaborated upon below.

Progesterone

Accumulating evidence suggests that the addition of progesterone to a cognitively efficacious E2 treatment regimen can attenuate or obviate E2-induced benefits, although these effects depend on numerous variables, including, but not limited to, mode of E2 and progesterone administration, as well as progesterone dose. The Bimonte-Nelson laboratory found that in middle-aged Ovx rats, the addition of tonic progesterone (2 subcutaneous pellets with 200 mg released over 60 days) to cyclic E2 (10 µg subcutaneous injection every other week) or to tonic low dose E2 (subcutaneous pellet with 0.25 mg released over 60 days) attenuated E2-induced beneficial effects on spatial reference memory on the MWM (Heather A. Bimonte-Nelson et al., 2006). Indeed, the cyclic E2 plus tonic progesterone, and the tonic low dose E2 plus tonic progesterone, combination treatments resulted in spatial reference memory that was comparable to age-matched vehicle controls; in contrast, cyclic E2 alone and low dose tonic E2 alone treatments enhanced spatial reference memory (Heather A. Bimonte-Nelson et al., 2006). Not all studies show that progesterone attenuates the benefits of estrogens, however. For example, in middle-aged Ovx rats, tonic progesterone and E2 combined (both via

subcutaneous implant for 28 days) enhanced spatial reference memory on the MWM in a manner similar to that of acute E2 alone (16.67 μg E2/kg subcutaneous injections for two days) and tonic E2 alone (subcutaneous E2 implant for 28 days) relative to controls (Markham, Pych, & Juraska, 2002). Moreover, Gibbs showed that when Ovx was performed at middle-age, rats receiving tonic E2 treatment immediately after Ovx, tonic E2 treatment 3 months after Ovx, or cyclic E2 plus progesterone treatment 3 months after Ovx, exhibited enhanced spatial working memory in the DMP task compared to Ovx rats receiving control treatment (R. B. Gibbs, 2000). Interestingly, spatial working memory in middle-aged rats receiving cyclic E2 plus progesterone treatment starting 10 months after Ovx was not different from that of non-hormone treated controls (R. B. Gibbs, 2000). These findings show that cyclic E2 plus progesterone combination treatment *can be* cognitively beneficial with the appropriate parameters. They support the existence of a critical window of opportunity following the loss of circulating ovarian hormones during which exogenously administered ovarian hormones can have potentially beneficial cognitive effects.

Additional studies utilizing acute E2 and progesterone combination hormone treatment further highlight the complexities of the cognitive effects of these two hormones. The Frick laboratory has shown that, in young Ovx mice, a single bolus intraperitoneal injection of progesterone (10 mg/kg or 20 mg/kg) given immediately after training improved non-spatial object recognition memory consolidation tested 48 hours later compared to vehicle-treated controls (L. L. Harburger, Pechenino, Saadi, & Frick, 2008). Similarly, effects were observed in young Ovx mice after dorsal hippocampal infusion of 0.01, 0.1, or 1 μg/μl progesterone (Fortress, Heisler, & Frick, 2015; P.T. Orr,

Rubin, Fan, Kent, & Frick, 2012; Patrick T Orr, Lewis, & Frick, 2009). Intraperitoneal injections of progesterone also enhanced object recognition memory consolidation in middle-aged and aged Ovx mice in a dose-dependent manner (Lewis, Orr, & Frick, 2008). A 20 mg/kg dose of progesterone also enhanced spatial reference memory consolidation in the MWM among aged Ovx mice, but no dose of progesterone affected memory in this task among middle-aged or young Ovx mice (L. L. Harburger et al., 2008; Lewis et al., 2008), suggesting a potentially important influence of aging on the ability of progesterone to regulate spatial reference memory. On the other hand, the Bimonte-Nelson laboratory has shown that progesterone (0.7 mg/day via daily subcutaneous injection) administered for 13 days before testing impaired spatial working memory on the water RAM in middle-aged Ovx rats when compared to vehicle-treated controls (B. Blair Braden et al., 2015), indicating that spatial working memory is sensitive to chronic progesterone treatment. In aged Ovx mice, when paired with acute E2 (0.2 mg/kg via intraperitoneal injection) treatment, the high dose progesterone (20 mg/kg via acute intraperitoneal injection) eliminated the beneficial effects of E2 on spatial reference memory in the MWM (Lauren L. Harburger, Bennett, & Frick, 2007). The low dose of progesterone (10 mg/kg via acute intraperitoneal injection) did not significantly attenuate the beneficial effects of E2 (Lauren L. Harburger et al., 2007). This research demonstrates that the progesterone dose can influence progesterone and estrogen/progesterone combination treatment outcomes for cognition.

Studies on E2 plus progesterone hormone treatments have also examined impacts on neurobiological mechanisms thought to relate to cognitive function. The Bimonte-Nelson laboratory has demonstrated that the addition of progesterone attenuated the E2-

induced increase in neurotrophin protein levels. Specifically, tonic E2 (subcutaneous pellet with 1.5 mg/60 days) treatment in aged Ovx rats increased brain derived neurotrophic factor, nerve growth factor, and neurotrophin-3 levels in the entorhinal cortex, a brain region involved in spatial memory, compared to age-matched Ovx controls (H. A. Bimonte-Nelson, Nelson, & Granholm, 2004; Steffenach, Witter, Moser, & Moser, 2005). The addition of tonic progesterone (2 subcutaneous pellets with 200 mg/60 days) obviated these E2-induced increases, whereby neurotrophin levels in the entorhinal cortex following E2 plus progesterone treatment did not differ from those observed in age-matched Ovx controls (H. A. Bimonte-Nelson et al., 2004). Progesterone may reduce the beneficial effects of E2 on memory by interfering with the biochemical effects of E2. For example, dorsal hippocampal activation of the extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK) signaling cascade, particularly the ERK2 isoform of ERK, is necessary for E2 (administered via acute intraperitoneal injection or acute intracerebroventricular infusion in the dorsal third ventricle) to enhance object recognition memory consolidation in young Ovx mice (Fernandez et al., 2008; L. L. Harburger, Saadi, & Frick, 2009). Interestingly, dorsal hippocampal infusion of progesterone has a biphasic effect on ERK2 activation whereby ERK2 activation increased 5 minutes, but decreased 15 minutes, after infusion; blocking activation of ERK2 also attenuated the beneficial effects of progesterone alone on objection recognition consolidation memory (Orr et al., 2012). When acute intraperitoneal progesterone injections of 5, 10, or 20 mg/kg were given in addition to 0.2 mg/kg E2 treatment, the E2-induced increase in dorsal hippocampal ERK2 activation was no longer present; this was true for all progesterone doses co-administered with E2 (L. L.

Harburger et al., 2009). However, only the 5 mg/kg dose of progesterone attenuated E2's effects on object recognition; E2 could still enhance object recognition when co-administered with 10 or 20 mg/kg progesterone (L. L. Harburger et al., 2009). These findings suggest that the memory-enhancing effects of E2 combined with the higher progesterone doses were not associated with ERK2 activation, at least at the same time point observed after E2 alone, indicating that while progesterone alters the biochemical effects of E2 this alteration does not necessarily mediate the memory enhancing effects of E2. The findings also suggest that the ratio of E2 to progesterone is important for behavioral outcomes such as those tested here for non-spatial memory.

E2 plus progesterone combination hormone treatment effects have also been studied in the context of diet and exposure to drugs of abuse. For instance, a study testing the cognitive effects of methamphetamine found that Ovx rats administered chronic E2 (1 mg/kg via daily intraperitoneal injection) or progesterone (8 mg/kg via daily intraperitoneal injections) showed attenuated methamphetamine-induced spatial reference memory deficits on the MWM (Ghazvini et al., 2016). The combination of E2 and progesterone, however, fully opposed the benefits of either hormone alone for these methamphetamine-induced impairments (Ghazvini et al., 2016). Other factors, such as diet and general health, can impact the manner in which E2 and progesterone interact. In a study in middle-aged Ovx rats investigating the interactions between dietary calcium intake and hormone therapy, chronic E2 treatment (10 µg every 48 hours via subcutaneous injections for 128 days) with or without progesterone (500 µg four hours before testing) enhanced learning on the land RAM only when dietary calcium was standard as in regular rat chow; when dietary calcium was decreased, E2 alone still

facilitated learning, but the combination E2 plus progesterone therapy did not (Sato et al., 2004). These examples speak to the breadth of factors that researchers should consider during the scientific study of hormone therapy; with more research, it could be revealed that numerous factors such as diet and drug exposures are critical contributors to cognitive efficacy of hormone therapy.

Another important area of research regarding the study of hormone therapy is in relation to neurodegenerative disease, specifically Alzheimer's disease (AD). Some studies suggest that estrogens may help to improve cognition in patients with AD (Asthana et al., 1999, 2001; Wharton et al., 2011; Wroolie et al., 2015; for review see: Henderson, 2006), and to both improve cognition and reduce neuropathology in rodent models of AD (Amtul, Wang, Westaway, & Rozmahel, 2010; Carroll et al., 2007; Carroll, Rosario, Villamagna, & Pike, 2010). Using the triple transgenic mouse AD model (3xTg-AD), the Pike laboratory showed that tonic E2 alone treatments immediately after Ovx improved recent memory as tested on the Y-maze; neither tonic nor cyclic progesterone treatment impacted performance relative to vehicle treatment (Carroll et al., 2007; Carroll et al., 2010). It is unclear what impact combination E2/progesterone treatment has on this novelty memory task in the triple transgenic AD mouse model, as tonic progesterone did not reverse E2's beneficial effects in the first study (Carroll et al., 2007), but did reverse E2's effects in the second study (Carroll et al., 2010). Of note, when progesterone was administered cyclically instead of tonically, it did not reverse E2's cognitive enhancements (Carroll et al., 2010), indicating that tonic versus cyclic mode of administration might impact outcome of combination hormone therapy, at least in this AD mouse model. Two key markers for AD-like neuropathology

in the AD mouse model are increased levels of ß-amyloid accumulation and increased hyperphosphorylation of the tau protein. In both studies, E2 treatment decreased ß-amyloid accumulation compared to controls, whereas the combination of E2 plus tonic progesterone treatment was ineffective (Carroll et al., 2007; Carroll et al., 2010), and the combination of E2 plus cyclic progesterone treatment maintained the E2-induced decrease in ß-amyloid accumulation (Carroll et al., 2010). Further, E2 plus tonic progesterone (Carroll et al., 2007; Carroll et al., 2010), as well as E2 plus cyclic progesterone (Carroll et al., 2010), decreased hyperphosphorylated tau, even though E2 alone had either a null effect (Carroll et al., 2007) or beneficial effect (Carroll et al., 2010) on hyperphosphorylated tau. Collectively, findings from these studies indicate that the regimen of progesterone treatment, either cyclic or tonic, may influence cognitive function and expression of neuropathological hallmarks in rodent models of AD, although relationships between these neuropathological markers and cognition remain unclear.

Synthetic progestins

The implications of the findings from the WHIMS brought new light to the investigation of the complexities in combination hormone therapy, including, but not limited to, its effects on cognitive function. MPA alone treatment has been shown by the Bimonte-Nelson and other laboratories to impair spatial and novelty memory in ages ranging from young adulthood to late middle age in the rat (B. B. Braden et al., 2011; B. Blair Braden et al., 2017, 2010; Okojie & Oyekunle, 2014). Although the effect of MPA alone has proven to be robust, cognitive effects from combination E2/MPA studies are

still not fully understood. Some research suggests that the combination of E2 and MPA can be beneficial for cognitive performance. One study investigated the effects of chronic oral E2 (47 µg/kg/day via drinking water), cyclic oral E2 (47 µg/kg/day via drinking water) given three out of every four days, and chronic oral E2 plus tonic MPA (1.5 mg via a 90 day subcutaneous pellet) in rats that received Ovx (Chisholm & Juraska, 2012). Ovx occurred at 12-13 months old, hormone treatment began immediately after, and behavior testing was initiated at 17-18 months old (Chisholm & Juraska, 2012). The chronic oral E2 plus MPA combination enhanced learning of a T-maze alternation task compared to Ovx rats receiving vehicle only, chronic oral E2 only, or cyclic oral E2 only (Chisholm & Juraska, 2012). This beneficial effect of combined E2/MPA hormone treatment may be specific to a task such as the alternation test used in the study, as animals can use a variety of navigational strategies to solve the task. Remarkably, research has demonstrated that the proestrus phase of the estrous cycle (high circulating estrogens) biases female rats towards hippocampal-based navigation strategies, while the estrus phase (low circulating estrogens) biases female rats towards non-hippocampal-based navigation strategies (Korol et al., 2004). Further research has demonstrated that progesterone in combination with estradiol benzoate can shift rats towards non-hippocampal navigation strategies (Korol and Pisani, 2015). Indeed, when a spatial task was utilized, the same chronic oral E2 plus tonic MPA combination treatment impaired performance, at similar experimental timeframes. Specifically, Ovx rats given the E2/MPA combination exhibited a greater swim distance on the MWM compared to those given chronic oral E2 only or cyclic oral E2 only (Lowry et al., 2010). Together, experiments evaluating MPA alone are uniform in showing consistent cognitive

impairments at a broad range of ages. The cognitive outcome of adding MPA to E2 treatment seems to be mediated, in part, by type of memory examined; for a spatial memory task, the combination E2/MPA treatment impaired cognitive function compared to E2 alone, whereas for a task that is ambiguous in its spatiality, the combination E2/MPA treatment enhanced cognitive function relative to E2 alone.

Ethinyl estradiol and Levo are clinically available in combination for oral contraceptive use; however, they are discussed here because they could potentially be used for menopausal hormone therapies (individually or together), and there is a population of women who utilize oral and other hormonal contraceptives during the transition to menopause. To my knowledge, there has been only one preclinical study investigating the impact of chronic ethinyl estradiol and Levo in combination on cognition. This study demonstrated that three weeks of daily subcutaneous treatment with low doses of either ethinyl estradiol (10 µg subcutaneous injection) or Levo (20 µg subcutaneous injection) alone, or a low dose ethinyl estradiol and Levo combination (10:20 µg), impaired non-spatial novel object recognition memory in young ovary intact rats compared to vehicle treatment (Simone et al., 2015). High dose treatment of ethinyl estradiol (30 µg) alone improved memory compared to controls on the same task, whereas high dose Levo (60 µg) and high dose ethinyl estradiol plus Levo combination (30:60 µg) treatments had no effect compared to control treatment (Simone et al., 2015). Thus, when the treatment doses were high, the addition of Levo to the cognitively beneficial ethinyl estradiol attenuated the effects of ethinyl estradiol on non-spatial memory. When tested on a novel context recognition task, assessing spatial memory, only the low dose Levo (20 µg) alone treatment resulted in improved memory compared

to vehicle controls (Simone et al., 2015). This beneficial cognitive effect of Levo alone treatment was replicated in two separate studies from the Bimonte-Nelson laboratory, where tonic treatment and cyclic treatment with Levo in middle-aged Ovx rats enhanced spatial working memory on the water RAM (B. Blair Braden et al., 2017; Prakapenka et al., 2018). Interestingly, the beneficial effects of Levo were not maintained when combined with E2. Indeed, both E2 and Levo enhanced spatial working memory on the water RAM when they were each given alone, but did not yield benefits on spatial working memory when given together (Prakapenka et al., 2018). Levo is one of the only synthetic progestins tested to date in preclinical models that exhibits some beneficial cognitive effects. These beneficial effects occurred in both young ovary-intact and middle-aged Ovx rats. Further systematic experimental evaluations identifying potential impacts of Levo in combination with clinically-utilized estrogens, including naturally occurring and synthetic versions, will help elucidate whether there is a broader efficacy for this clinically relevant hormone combination, potentially providing a wider range of hormone therapy combination options for menopausal women.

Conclusions

Preclinical investigations are vital to understanding the role of estrogen-containing hormone therapy, including as a sole treatment and as estrogenic plus progestogenic hormone therapy combinations. Using representative rodent models of menopause, such basic science research can yield great insight into how these treatments impact the brain and its functions, including cognition. These models provide unique opportunities to directly manipulate multiple factors implicated in clinically-relevant

exogenous hormone treatments to achieve the goal of understanding how hormones impact system functions, and to optimize regimens. Indeed, rodent studies of the behavioral and neurobiological effects of hormones highlight the intricate complexities governing the roles of ovarian hormones, and their combinations, in cognitive function. Although preclinical research suggests that estrogen-based hormone therapies can benefit cognition following the loss of circulating ovarian hormones, these findings are heavily dependent on study design and the specific factors addressed within a study. That one can rigorously control experimental designs with animal models has allowed researchers to methodically and systematically test effects of varied hormone exposures after different types of ovarian hormone loss. Results have indicated that the effects of estrogens on cognitive and brain function are reliant on a myriad of factors, including type of estrogen used, estrogen dose, route of administration, age at treatment, type of menopause model, and time of treatment initiation in relation to hormone deprivation history. Interactions between ovarian hormones, specifically those between estrogens and progestogens, and between different types of estrogens, differentially affect cognitive function. Again, several factors govern these interactions, such as the ratio between estrogen and progestogen treatment, the specific type of estrogen or progestogen utilized, as well as other treatment regimen variables. Furthermore, rodent studies reveal additional complexities when interpreting specific effects of estrogen-based hormone therapies on cognition, such as diet or history of drug abuse.

Bridging preclinical and clinical research is critical to translating findings from bench to bedside to result in discoveries that will truly impact women's health. One of the most critical ways to facilitate translation is to ensure that preclinical findings are noted

and utilized as being a liaison to bridge and drive clinical study design, and also that purposeful efforts are made to methodically evaluate potentially important clinical therapies and factors as directly as possible in preclinical studies. This includes using animal models to test hormone therapy individual components and combinations as used in women, encompassing effects mapped on to the timepoints relative to age and menopause status as closely as possible. While there are strengths and weaknesses to every preclinical model, acknowledging these caveats while still aiming to optimize translation is key to successful scientific discovery and translation across species. Overall, using these strategies can aid in clarifying the effects of menopausal hormone therapy on the many systems and functions important for women's overall health, and can potentially lead to the development of targeted hormone therapies aimed at optimizing the quality of life for women experiencing symptoms associated with menopause. Of further note, collaborative efforts will be critical to broadening and extending understanding of the multi-dimensional effects of ovarian hormone loss and replacement, including work spanning natural and synthetic therapeutic options. Engaging a consortium of experts extending outside of the behavioral neuroendocrinology field is the optimal strategy to facilitate the mapping of hormone neuromechanism pathways that might impact brain functioning, including cognition. Subsequently, once potential pathways and associated targets are identified, scientists can engineer with the help of novel tools such as brain- or receptor- targeted delivery platforms to administer hormones to desired foci of interest in order to achieve optimal therapeutic efficacy. As the decades have passed and research has shed light on the many systems impacted by hormones, it has been learned that factors that were once considered

nuances of a hormone effect are, in fact, clear drivers of these effects. For continued progress in the field of translational behavioral neuroendocrinology, research must continue to discover, acknowledge, and respect these complexities, and push forward with a multi-pronged approach using classic techniques complimented by innovative and novel methods. Purposeful collaborative efforts between clinicians and basic scientists will break barriers and ultimately translate findings to yield optimal hormone therapy efficacy and outcomes for successful cognitive and brain aging in women.

Goals of this dissertation

The overarching goal of this dissertation work was to approach optimization of E2 treatment, whereby maximal beneficial cognitive effects with minimal peripheral stimulation, particularly at the uterine horns, would be achieved, utilizing several distinct drug delivery strategies. The rat model of surgical menopause, Ovx, was implemented across all studies, and the same timeline between Ovx, treatment, and behavioral battery initiation was utilized. The first strategy was to evaluate an E2 plus progestogen hormone combination treatment that contained the cognitively-beneficial E2 plus a cognitively-beneficial progestogen. Chapter 2 examined the impact of subcutaneously administered E2 plus Levo hormone combination treatment on spatial learning and memory, as well as on uterine stimulation. Then, Chapter 3 further evaluated the effect of subcutaneously administered E2 plus Levo hormone combination treatment on spatial learning and memory, as well as on uterine stimulation, as a function of clinically relevant E2 to Levo dose ratios. The second strategy was to encapsulate E2 in polymeric nanoparticles to harness the sustained release, decreased metabolism, and potential for tissue-specific

targeting delivery advantages following agent encapsulation in nanoparticles. Chapter 4 assessed the cognitive effects of E2 encapsulated poly (lactic-co-glycolic) acid (PLGA) nanoparticles relative to blank PLGA nanoparticles and relative to free E2, provided that blank PLGA and the sesame oil vehicle for free E2 did not differ in cognitive outcomes. Additionally, impact on uterine horn stimulation was evaluated. The third approach combined both route of administration as well as addition of an agent carrier delivery strategies to target the delivery of E2 to the brain, but minimize its delivery to the uterine horns. Specifically, intranasal administration of E2 was implemented to achieve greater brain-to-uterine horn delivery relative to the subcutaneous route of administration, and cyclodextrins were used to solubilize E2 as well as to increase the delivery of E2 to brain regions involved in cognition relative to free E2. Thus, Chapter 5 addressed the impact of intranasal administration of E2 on spatial learning and memory as well as uterine horn stimulation following either free E2 intranasal treatment or an E2-cyclodextrin intranasal treatment that yielded greatest uptake in the dorsal hippocampus. Chapter 6 summarized the findings from the studies evaluating the three delivery approaches outlined above, provides limitations of these studies, as well as takeaways and future directions.

CHAPTER 2

17β-ESTRADIOL PLUS A COGNITIVELY-BENEFICIAL PROGESTOGEN, LEVONORGESTREL

This chapter was published in Neurobiology of Aging in January, 2018, and was titled:

Contrasting effects of individual versus combined estrogen and progestogen regimens as working memory load increases in middle-aged ovariectomized rats: one plus one does not equal two

A.V. Prakapenka, R. Hiroi, A.M. Quihuis, C. Carson, S. Patel, C. Berns-Leone, C. Fox, R.W. Sirianni, H.A. Bimonte-Nelson

Contribution: I am the first author of this document and was the primary graduate student principal investigator on this project. Under the mentorship of Heather A. Bimonte-Nelson and Rachael W. Sirianni, I designed and carried out the study with the assistance of our laboratory teams. I am the first-author for the manuscript, and I received great help from my co-authors in carrying out the study as well as editing the manuscript.

Introduction

Menopause, defined as the cessation of menses for at least one year, is marked by a reduction in levels of ovarian hormones, including estrogens and progesterone. This decrease in circulating levels of ovarian hormones can lead to the onset of several undesired physiological symptoms, including hot flashes, vaginal atrophy, and osteoporosis (Al-Safi & Santoro, 2014; NAMS, 2014). In women, several domains of memory performance, as

well as focus and concentration, are also sensitive to changes in ovarian hormone levels, and have been associated with menopausal status (Maki, 2012). The presence and severity of these symptoms vary amongst women, and these symptoms can greatly impact a woman's quality of life; as a result, some women choose to take hormone therapy to ameliorate their symptoms. Thus, it is imperative to acquire a thorough understanding of how alterations in levels of ovarian hormones, and exogenously administered hormones, impact issues associated with menopause, such as changes in memory. Moreover, elucidating the roles of ovarian hormone loss and hormone therapies on the brain and its functions could lead to novel hormone therapy options that are tailored to alleviating specific symptoms associated with menopause.

17β-estradiol (E2) is the most potent, naturally circulating estrogen in mammals, and it is commonly used as the estrogenic component in hormone therapy for menopause. As early as the 1950s, studies have suggested a beneficial role of estrogens in cognitive and related molecular processes of the central nervous system (e.g., Bimonte and Denenberg, 1999; Caldwell and Watson, 1952; Komnenich et al., 2013; Matsumoto et al., 1985; Singh et al., 1995; Woolley and McEwen, 1993). Today, there are an extensive array of studies aimed at understanding the effects of E2 on learning and memory in humans as well as in animal models (Frick, 2015; Koebele & Bimonte-Nelson, 2015; Korol & Pisani, 2015; Maki, 2012; S. E. Mennenga & Bimonte-Nelson, 2013; Sherwin, 2006). The ovariectomy (Ovx) model in rodents, whereby the primary source for circulating ovarian hormones, the ovaries, are surgically removed, provides a low circulating ovarian hormone profile or a 'blank hormonal slate'. Although some ovarian hormones (e.g., E2 and progesterone) can also be synthesized in the brain (Kretz et al., 2004; Micevych & Sinchak,

2008; J. J. Tuscher et al., 2016), the Ovx rodent model can be employed to study the cognitive effects of exogenously administered hormone regimens that aim to achieve a specific circulating hormone profile. In Ovx rats, E2 treatment enhanced cognitive performance on a multitude of learning and memory behavioral paradigms, such as the radial-arm maze (Bimonte & Denenberg, 1999; Jill M. Daniel et al., 2006; Jill M Daniel et al., 1997; Fader, Johnson, & Dohanich, 1999; R. B. Gibbs & Johnson, 2008; V N Luine et al., 1998; Rodgers et al., 2010), Morris water maze (MWM; Heather A. Bimonte-Nelson et al., 2006; El-Bakri et al., 2004; Feng et al., 2004; Kiss et al., 2012; Lowry et al., 2010; McLaughlin et al., 2008; Talboom et al., 2008), delayed match-to-position T-maze (R. B. Gibbs, 2000; R B Gibbs, 1999; Robert B. Gibbs, 2007; Robert B. Gibbs, Gabor, Cox, & Johnson, 2004; Robert B Gibbs, 2002), and object placement (Conrad, McLaughlin, Huynh, El-Ashmawy, & Sparks, 2012; Frye, Duffy, & Walf, 2007; Victoria N. Luine, Jacome, & Maclusky, 2003; McLaughlin et al., 2008).

In the brain, E2 can modulate the MAPK/ERK pathway, which is involved in the formation of different memory types (Atkins, Selcher, Petraitis, Trzaskos, & Sweatt, 1998; Blum, Moore, Adams, & Dash, 1999; Schafe et al., 2000). When the MAPK/ERK signaling pathway is activated, the signal travels from a cell receptor (e.g., estrogen receptors) to the nucleus DNA via a sequence of proteins, including the activated extracellular signal-regulated kinase 1 and 2 (Erk1/2) (Ciccarelli and Giustetto, 2014; Koebele and Bimonte-Nelson, 2017; Witty et al., 2012). Research indicates that, in the dorsal hippocampus, MAPK/ERK activation is essential for long-term memory formation, as well as for E2-induced beneficial effects on memory consolidation (Blum et al., 1999; L. Fan et al., 2010; Fernandez et al., 2008; L. L. Harburger et al., 2009). Indeed, there is increased expression

of activated Erk2 in the dorsal hippocampus following E2 treatment (Fernandez et al., 2008; L. L. Harburger et al., 2009; Witty, Gardella, Perez, & Daniel, 2013). Blocking this E2-induced increase in Erk2 activation attenuated the beneficial cognitive effects of E2 on the object recognition task (L. Fan et al., 2010; Fernandez et al., 2008). E2 treatment can also increase the expression of neurotrophins that are associated with learning and memory, including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin 3 (NT-3), in regions of the brain that are involved in cognitive function (i.e. entorhinal cortex and hippocampus) (H. A. Bimonte-Nelson et al., 2004; Kiss et al., 2012; Zhou, Zhang, Cohen, & Pandey, 2005). Neurotrophins and the MAPK/ERK pathway have been implicated in learning and memory as well as neuroplasticity (Bechara, Lyne, & Kelly, 2014; Gooney, Shaw, Kelly, O'Mara, & Lynch, 2002; McGauran et al., 2008). Taken together, these studies highlight the significant role of E2 in cognitive function that is mediated through its role in several defined pathways associated with neuroplasticity and memory.

For women with an intact uterus, estrogen-based hormone therapies must also include a progestogen component to offset the increased risk for developing endometrial hyperplasia and cancer following exposure to unopposed estrogens (NAMS, 2012). Progestogens are a class of steroid hormones, and include natural progesterone and progestins (synthetic progestogens), which bind to the progesterone receptor. There is preclinical evidence that some progestogens can also offset the cognitive benefits of E2 (Heather A. Bimonte-Nelson et al., 2006; L. L. Harburger et al., 2009; Lauren L. Harburger et al., 2007; Lowry et al., 2010). For instance, studies testing combination hormone therapies have shown that the addition of progesterone to E2 treatment reversed the

enhancing cognitive effects of E2 on the spatial reference memory MWM task in Ovx rodents (Heather A. Bimonte-Nelson et al., 2006; Lauren L. Harburger et al., 2007; Lowry et al., 2010). There is also preclinical evidence that the addition of progesterone can attenuate E2-induced changes in several neuromolecular mechanisms in the brain that are essential for cognitive function. For example, in the entorhinal cortex, the E2-induced increases in BDNF, NGF, and NT-3 levels were obviated with the addition of progesterone (H. A. Bimonte-Nelson et al., 2004). In the dorsal hippocampus, progesterone in combination with E2 treatment attenuated the E2-induced increase in activated Erk2 expression (L. L. Harburger et al., 2009). These findings indicate that the addition of a progesterone component in hormone therapy to oppose undesired E2 stimulation in the periphery may not be cognitively beneficial, and that it can attenuate associated E2-induced benefits.

Since progesterone has low systemic bioavailability with oral and transdermal delivery, synthetic progestogens are often used for both contraceptive and hormone therapy purposes (Du, Sanchez, Kim, & Azen, 2013; Kuhl, 2005; Pickar, Bon, Amadio, Mirkin, & Bernick, 2015). Medroxyprogesterone acetate (MPA) is a synthetic progestogen that is commonly prescribed for birth control (Depo-Provera), as well as used in combination with an estrogen for menopausal hormone therapy. The Bimonte-Nelson laboratory found that exogenous treatment with MPA alone in female rats impaired cognitive function (B. B. Braden et al., 2011; B. Blair Braden et al., 2017, 2010). Furthermore, a study testing the effects of a tonic MPA and E2 hormone combination showed that subcutaneous MPA (via a pellet) plus oral E2 (via drinking water) treatment resulted in impaired learning on the MWM in middle-aged Ovx rats compared to chronic E2, chronic E2 plus progesterone, or

cyclic E2 (Lowry et al., 2010). Additionally, subcutaneous tonic administration of progesterone or of MPA in adult Ovx rats blocked the neuroprotective effects of E2 following excitotoxic lesion with kainate (Rosario, Ramsden, & Pike, 2006). These studies further indicate that the role of E2 on the brain and cognitive function can be altered by the addition of a progestogen, and the magnitude of this effect may be governed by the type of progestogen administered (e.g., progesterone versus MPA).

Research done thus far supports the hypothesis that MPA has detrimental effects on cognition, alone and in combination with estrogen; there are other FDA-approved progestogens that satisfy the uterus opposing effects that have not been cognitively profiled. An important goal to aid women's health is to find a progestogen that will accomplish uterine protection while not imposing negative cognitive effects. Levonorgestrel (Levo) is a synthetic progestogen utilized in multiple contraceptives, such as intrauterine devices (i.e. Mirena) and emergency contraception (i.e. Plan B), as well as in combination with estrogens in oral birth control pills such as Lutera, Aviane, Seasonique, and Seasonale. In menopausal hormone therapy, Levo is combined with E2 in the transdermal patch, Climara Pro. The contraceptive efficacy of Levo, and of combination estrogen plus Levo, hormone formulations are well established. However, research has only just begun to address the potential effects of Levo on cognitive performance, and only one preclinical study has evaluated such impact in the context of aging. The Bimonte-Nelson laboratory has demonstrated that daily subcutaneous administration of 0.6 μg Levo, a dose that is equivalent to the clinically available Climara Pro patch when accounting for body weight, enhanced working memory performance on the water radial-arm maze (WRAM) relative to administration of the vehicle control in

middle-aged, Ovx rats (B. Blair Braden et al., 2017). This is especially exciting, as this is the first progestogen given chronically shown to benefit memory in a preclinical model of menopause. However, whether these benefits will hold when given in combination with an estrogen is yet to be determined. This question is critically important given the strong clinical use of combined regimens for menopausal hormone therapy.

The current study examined the effect of E2 + Levo hormone combination treatment on cognitive function, with interpretations relative to vehicle control treatment, as well as relative to E2 alone and Levo alone treatments. The intent was to study these regimens in the context of older age and ovarian hormone loss. Thus, treatments were administered to middle-aged Ovx rats. The 0.6 μg Levo dose was carefully chosen based on prior studies that showed enhancing cognitive effects of Levo alone at this dose (B. Blair Braden et al., 2017); I was interested in examining whether a cognitively enhancing Levo dose when given alone would also enhance cognitive function when given in combination with E2. First, cognitive function was assessed using a battery of behavioral tasks to test spatial learning and memory using the WRAM (working and reference memory) and the MWM (reference memory), including a control behavioral task (visible platform). Second, following behavioral testing, activated Erk1 and Erk2 levels in brain regions that are involved in cognitive function were evaluated, including the frontal cortex, dorsal hippocampus, CA1/CA2 ventral hippocampus, entorhinal cortex, and perirhinal cortex. Given that the E2 and Levo regimens tested here were based on prior effects benefitting cognition in the Bimonte-Nelson laboratory, I hypothesized that E2 alone and Levo alone treatments would yield favorable cognitive effects. Because Levo is often used in combination with estrogens in clinical formulations, and because Levo is the first

progestogen shown to initiate beneficial cognitive effects when given alone in the Bimonte-Nelson laboratory, I sought to determine whether its beneficial effects would hold when given combined with estrogen. Thus, here, I ask: in a surgical menopause model, will the combination of two cognitively-enhancing hormones result in strengthened beneficial effects, or in null or attenuating effects, on learning and memory performance?

Methods

Animals

Forty middle-aged, 11-month old, Fischer-344 CDF virgin female rats from the National Institute on Aging, Harlan Laboratories (Indianapolis, IN) were used based on prior publications (B. Blair Braden et al., 2017; Chisholm & Juraska, 2012; Rodgers et al., 2010). Rats were pair housed on a 12-hour light/dark cycle, and food (Teklad global 18% protein rodent diet, Envigo) and water were *ad libitum*. All procedures were done with approval from the Arizona State University IACUC and followed the standards set by the National Institutes of Health.

Ovariectomy (Ovx)

All rats underwent Ovx surgery from the dorsolateral aspect under acute isoflurane inhalation anesthesia. Each rat was administered a single subcutaneous injection of Rimadyl (5 mg/kg) for pain. After dorsolateral incisions were made in the skin and peritoneum, a ligature was applied to the tip of each uterine horn and each ovary was removed. The muscle and skin were sutured (Coated VICRYL Suture, Ethicon) and rats were subcutaneously administered saline (2 ml) to prevent dehydration.

Treatment administration

Treatment administration started 21 days after Ovx surgery, and continued until the end of the study (see Figure 3A for the study timeline). Rats were randomly assigned to receive either a daily subcutaneous injection of sesame oil as control (Vehicle, n = 10) or a hormone injection of 3 μg E2 (E2-Only, n = 10), 0.6 μg Levo (Levo-Only, n = 9), or a combination of 3 μg E2 and 0.6 μg Levo (E2 + Levo, n = 10), in 0.1 ml of sesame oil. All treatment injections were done between 7:00-8:00 am; behavioral tasks were initiated half an hour after the last treatment injection, and treatment groups were counterbalanced throughout the day to account for timing differences between treatment injection and behavioral testing. One animal from the Levo-Only group was excluded from all analyses due to premature death, the cause of which was not related to experimental conditions. The dose for Levo-Only treatment was based on published work from the Bimonte-Nelson laboratory where a daily subcutaneous injection of 0.6 μg of Levo enhanced performance on the WRAM in middle-aged Ovx rats (B. Blair Braden et al., 2017). After 20 days of treatment, all animals started testing on a battery of behavioral tasks to assess cognitive performance. The timing of treatment initiation and duration prior to behavior testing was methodically decided based on prior studies from the Bimonte-Nelson laboratory that have shown effects of hormone treatment on cognitive performance (Heather A. Bimonte-Nelson et al., 2006; E. Engler-Chiurazzi et al., 2011; Sarah E. Mennenga, Gerson, Koebele, et al., 2015; Talboom et al., 2008). Throughout the span of the study, body weights were measured weekly to evaluate the expected increase in body weight as a result of Ovx, followed by the expected decrease in body weight for animals that received an estrogen-

containing treatment (Figure 3B; Geary et al., 1994; McLaughlin et al., 2008; Mennenga and Bimonte-Nelson, 2013).

Water radial-arm maze (WRAM)

The first behavioral task was the win-shift WRAM used to examine spatial working and reference memory (H. A. Bimonte-Nelson, 2015d; Bimonte & Denenberg, 1999; B. Blair Braden et al., 2017; S. E. Mennenga & Bimonte-Nelson, 2015; Sarah E. Mennenga, Koebele, et al., 2015). WRAM testing started on day 21 of treatment administration and lasted for 13 days. The maze was an 8 arm apparatus, and 4 out of the 8 arms contained hidden platforms. Each arm's dimensions were 38.1 cm x 12.7 cm, and platforms were 10 cm in diameter. The maze was filled with water made opaque with nontoxic black paint that was kept between 18-20^{o}C for the duration of testing, and spatial cues were set up around the room to aid in spatial navigation. The room was 248 cm x 243 cm in size.

Each subject was randomly assigned a set of platform locations, which were kept fixed for the duration of testing for a subject. For each trial, subjects were dropped off in the start arm and allowed a maximum of 3 min to find a platform; once on the platform, subjects remained there for 15s before being placed in a heated testing cage for a 30s inter-trial interval (ITI). A trial was completed when a platform was found. During the ITI, the just-located platform was removed from the maze, and the maze water was cleaned of debris with a fishnet. At the end of the 30s ITI, the next trial was started. There were a total of 4 trials per day (one trial per platform). There was an increase in working memory load across trials as the memory system was increasingly taxed with the removal of each

additional found platform. On the last day of testing, a 6-hour delay between trials 2 and 3 was implemented to examine delayed memory retention.

Performance on the WRAM for each subject was determined by scoring the orthogonal measures of working and reference memory. Briefly, the first entry into a non-platformed arm within a day was defined as a reference memory (RM) error. A re-entry into a RM (non-platformed) arm within the same day was defined as a working memory incorrect (WMI) error. An entry into a previously platformed arm within a day was defined as a working memory correct (WMC) error.

Morris water maze (MWM)

The day after WRAM testing was completed, all animals began testing on the MWM to evaluate spatial reference memory performance (H. A. Bimonte-Nelson, 2015b; Talboom et al., 2014, 2008). The MWM apparatus was a circular tub, 188 cm in diameter, filled with water (18-20^{o}C) that was made opaque with nontoxic paint. One platform, 10 cm in diameter, was submerged in the northeast (NE) quadrant of the tub. The location of the platform remained constant across all trials and days of testing. There were abundant spatial cues set up around the room to aid in spatial navigation. The room was 348 cm x 337 cm in size. Each animal received 4 trials per day for each of the 5 testing days. The subject was dropped off at one of four starting locations (north, south, east, or west) at the start of each trial and was allowed 60s to locate the platform. The order of the drop off starting locations was randomized across days, but kept constant for all animals across 4 trials within a day. Once the platform was found, the subject remained on the platform for 15s and was then placed back into a heated cage for a 5-8 min ITI. If the platform was not

found within the allotted 60s trial time, the subject was led to the platform and remained there for 15s before being placed back into a heated cage for a 5-8 min ITI. Each rat's swim path was recorded using the Ethovision tracking system (Noldus Instruments, Wageningen, The Netherlands), and total swim distance to the platform was analyzed. On the last day of testing, a probe trial was administered as an additional 5^{th} trial to test for spatial localization. For the probe trial, the platform was removed and the subject was allowed to swim for a total of 60s after being dropped off from the furthest drop off location (west) in relation to where the platform was located (NE quadrant).

Visible platform

To confirm their capability to perform the procedural components of a water-escape task, animals were tested on the visible platform task the day after completion of MWM testing (Bimonte-Nelson, 2015a; Mennenga et al., 2015a, 2015c). The visible platform task was composed of a rectangular tub, 100 cm x 60 cm, filled with clear water kept at 18-20^{o}C; a black platform, 10 cm in diameter, remained 4 cm above the water surface throughout testing. A curtain was used to block obvious spatial cues located in the area distal from the maze. The room was 168 cm x 155 cm in size. There were a total of 6 trials per animal for the one day of testing, with a 90s maximum trial time. Once the platform was found, subjects were given 15s on the platform and then placed back into a heated cage (ITI was 5-8 min). The drop off location remained constant throughout testing, and the platform location was varied semi-randomly between three distinct locations.

Blood serum analysis

The day after visible platform testing, animals were euthanized with isoflurane, starting at the regular testing time and in the same order in which they were tested. Blood was collected via cardiocentesis, allowed to clot at 4°C (Vacutainer 367986, Becton Dickinson and Company, Franklin Lakes, NJ, USA), and centrifuged at 3000 rpm for 20 min at 4°C to obtain blood serum. Serum was stored at -20°C until analysis. Circulating E2 and estrone levels were determined by radioimmunoassay at the Core Endocrinology Laboratory of the Pennsylvania State University, College of Medicine. Specifically, E2 and estrone levels were measured in duplicate using a double antibody liquid-phase radioimmunoassay (Beckman Coulter, Brea, CA) as previously reported (E. B. Engler-Chiurazzi et al., 2012; Koebele et al., 2017; Sarah E. Mennenga, Koebele, et al., 2015). For the E2 assay, E2-specific antibodies were used with ^{125}I-labeled E2 as the tracer. The E2 assay had a functional sensitivity of 4 pg/ml. Inter-assay coefficients of variation at a mean level of 6 pg/ml E2 averaged 8%, and intra-assay coefficients of variation averaged 6%. For the estrone assay, estrone-specific antibodies were used with ^{125}I-labeled estrone as the tracer. The estrone assay had a functional sensitivity of 16 pg/ml. Inter-assay coefficients of variation for estrone at a mean level of 90 pg/ml averaged 11%, and intra-assay coefficients of variation averaged 8%.

Uterine horn weights

It is known that uterine horn weight is impacted by the presence of ovarian hormones (e.g., Engler-Chiurazzi et al., 2012; Mennenga et al., 2015b; Westerlind et al., 1998). To confirm complete Ovx as well as E2 exposure, and to assess whether the addition

of Levo impacted the expected E2-induced increases, uterine horns were inspected and removed at sacrifice, trimmed of visible fat, and weighed (wet weight).

Brain dissection

Immediately following cardiocentesis, the brain was rapidly dissected. The Rat Brain Atlas (Paxinos & Watson, 1998) was used as a reference for plate designations. The frontal cortex was taken from the dorsal aspect of the brain (plates 5-14). The brain was then cut across the coronal plane to gain access to the dorsal hippocampus (plates 33-35), and the CA1/CA2 ventral hippocampus, entorhinal cortex, and perirhinal cortex (plates 39-42). Brain regions were frozen and stored at -70^{o}C until western blot analyses.

Western blots

Activated Erk1/2 expression levels in the frontal cortex, dorsal hippocampus, CA1/CA2 ventral hippocampus, entorhinal cortex, and perirhinal cortex (all left hemisphere) were analyzed using western blots (P.T. Orr et al., 2012). Samples were suspended in 1:50 w/v RIPA buffer (150mm NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Na deoxycholate, 50 mm Tris, protease inhibitor (cat# 5892791001, Millipore-Sigma), and phosphatase inhibitor (cat# 524625, Millipore-Sigma)), homogenized with a probe sonicator (Ultrasonic Processor, Cole Parmer, IL, USA), and centrifuged at 10,000 rpm for 10 min at 4^{o}C. The BCA protein assay (ThermoFisher Scientific, Pittsburgh, PA, USA) was used to determine protein concentration. Brain homogenates were run on 4-12% NuPAGE Bis-Tris gel using the SureLock mini-cell (Invitrogen, Carlsbad, CA, USA) and blotted to an Immobilon PVDF membrane. All samples were loaded at the same protein

concentration per brain region, and all gels were counterbalanced by treatment group, with a total of three gels run per brain region. The western blot was blocked in 10% non-fat milk for 1 hour, and incubated overnight in anti-phospho p44/p42 Erk1/2 primary antibody (1:2000, Cell Signaling) at 4^{o}C. The blot was then incubated with anti-rabbit HRP (1:2000, Cell Signaling) for 1 hour at room temperature, and visualized using chemiluminescence (LumiGlo and Peroxide, Cell Signaling) in a film developer (Konica SRX-101A Film Processor, Tokyo, Japan). After imaging, the blot was stripped in 0.2M NaOH and re-probed for anti-total p44/p42 Erk1/2 (1:1000, Cell Signaling). Densitometry was performed using ImageJ software. Activated Erk1/2 levels were expressed as phosphorylated Erk1/2 expression normalized to total Erk1/2 expression.

Statistical analyses

Behavioral measures obtained from each maze were analyzed separately, using two-tailed tests unless otherwise specified. Alpha was set at $p < 0.05$ for all statistical analyses, and Fisher's PLSD tests were used for post hoc analyses.

To evaluate overall WRAM learning across all days, an omnibus repeated measures ANOVA was run to examine the Day main effect (days 2-12) for each of the three error types (WMC, WMI, and RM errors). Next, based on prior publications (H. A. Bimonte-Nelson et al., 2015; B. Blair Braden et al., 2017), WRAM testing days were blocked into three blocks: Days 2-5 (Block 1), Days 6-9 (Block 2), and Days 10-12 (Block 3). Each block was analyzed separately using repeated measures ANOVA for each of the three error types, as done previously (H. A. Bimonte-Nelson et al., 2015; B. Blair Braden et al., 2017). To determine the effects of hormone treatment on WRAM performance, the independent

variable was Treatment, and the repeated measures were Trials nested within Days. In the case of a significant Trial x Treatment interaction, Trials 3 and 4 were analyzed separately to test the higher memory load trials, with Treatment as the independent variable and Days as the repeated measures. For the WRAM delay, each treatment group was evaluated separately for WMC, WMI, and RM errors using one-tailed repeated measures ANOVA since the delay period typically impairs performance on the WRAM, as shown in prior findings (B. Blair Braden et al., 2015; Hiroi et al., 2016). Specifically, to analyze performance following the 6-hour delay, the post-delay trials, Trials 3 and 4 on Day 13 (delay performance) were averaged and compared to the average of the baseline trials, Trials 3 and 4 on Day 12 (baseline performance), as done previously (Camp et al., 2012; Engler Chuirazzi et al., 2011; Mennenga et al., 2015; 2014).

MWM Total Swim Distance data were analyzed using repeated measures ANOVA. The independent variable was Treatment, and the repeated measures were Trials nested within Days. For the probe trial, repeated measures ANOVA was used to compare Percent Swim Distance in the NE quadrant (the previously platformed quadrant) to the quadrant that was located diagonally opposite to the NE quadrant (SW, southwest) to confirm spatial localization of the platform location (H. A. Bimonte-Nelson, 2015b; H. A. Bimonte-Nelson et al., 2015).

For the visible platform analysis, Time to Platform was analyzed using repeated measures ANOVA. The independent variable was Treatment and the repeated measures were Trials (6 trials).

For blood serum levels of E2 (pg/ml) and estrone (pg/ml), and uterine horn weights (g), a one-way ANOVA was used with Treatment as the independent variable. A one-way

ANOVA was also used to analyze activated Erk1 and Erk2 expression in each brain region, with Treatment as the independent variable and activated Erk expression (phosphorylated Erk normalized to total Erk) as the dependent variable. To examine relationships between activated Erk expression and cognitive performance, Pearson *r* correlations were run between activated Erk1 and activated Erk2 expression in each brain region and WMC, WMI, and RM error measures for Block 1 of WRAM, the block of testing where main behavioral effects were seen. To account for multiple correlations, a false discovery rate (FDR) threshold of 0.1 was used; both uncorrected (*P*) and FDR-corrected (*Q*) statistics are reported (Benjamini & Hochberg, 1995).

Results

Water radial-arm maze (WRAM)

Spatial working and reference memory performance were measured using the WRAM. To analyze overall learning across all days of testing, there was a main effect of Day for each memory measure, whereby errors decreased for WMC [$F_{(10,35)} = 7.042$, $p < 0.0001$], WMI [$F_{(10,35)} = 12.603$, $p < 0.0001$; Figure 4A], and RM [$F_{(10,35)} = 8.176$, $p < 0.0001$] across days 2-12 of testing, demonstrating learning of the WRAM task for all three measures of memory (data not shown). For each memory measure, there was no significant Treatment x Day interaction for Days 2-12, suggesting that groups did not differ in WRAM learning across the entire learning curve. In Block 1, there was a main effect of Treatment for WMI errors [$F_{(3,35)} = 3.597$, $p < 0.05$; Figure 4B]. Post hoc analysis of this measurement collapsed across all trials revealed fewer errors in the E2-Only [$p < 0.05$] and Levo-Only [$p < 0.01$] treatment groups relative to the Vehicle control group, while there were no

significant differences between the Vehicle and E2 + Levo groups. These results indicate that the individual hormone treatments, E2-Only and Levo-Only, enhanced WMI performance during task acquisition, while the combination treatment did not. Additionally, post hoc analyses for Block 1 for WMI revealed that the E2 + Levo group made more errors than the Levo-Only group [$p < 0.05$], demonstrating that the E2 + Levo hormone combination treatment impaired acquisition of the WMI measure relative to Levo-Only treatment; there was a marginal trend for the E2 + Levo group to make more errors than the E2-Only group [$p < 0.1$], suggesting that the E2 + Levo hormone combination treatment tended to impair acquisition of the WMI measure relative to E2-Only treatment. For Block 1, there was also a significant Treatment x Trial interaction [$F_{(9,105)} = 2.23$, $p < 0.05$; Figure 4C] for WMI errors. Post hoc analyses showed that E2-Only [$p < 0.01$; Figure 4D], Levo-Only [Figure 4D], and E2 + Levo [$p < 0.01$; Figure 4D] treatment groups made fewer WMI errors compared to the Vehicle control group on Trial 3, the moderate working memory load trial. On Trial 4, the highest working memory load trial, post hoc analyses revealed that the E2 + Levo group made more WMI errors than the E2-Only [$p < 0.05$; Figure 4E] and Levo-Only [$p < 0.05$; Figure 4E] groups, signifying that the combination hormone treatment impaired the ability to handle a high working memory demand relative to the groups treated with either hormone alone. Due to these divergent cognitive effects of the E2 + Levo treatment across trials, a post hoc decision was made to test the interaction between the high working memory load trials, Trial 3 and 4, and E2 + Levo treatment group WMI performance relative to Vehicle control. This interaction was indeed significant [$F_{(1,18)} = 6.67$, $p < 0.05$], indicating that the cognitive impact of the E2 + Levo hormone combination treatment is modulated by demand in working memory load

(Figure 4F). The Treatment main effects for WMC or RM for Blocks 1, 2 and 3, and for WMI for Blocks 2 and 3, were not significant (data not shown).

Following the 6-hour delay on Day 13, the Vehicle control group [$F_{(1,9)} = 4.509$, $p < 0.05$] and Levo-Only group [$F_{(1,8)} = 0.5099$, $p < 0.05$] made more WMC errors on the post delay trials relative to baseline trials, indicating forgetting across the delay period (Figure 5). E2-Only and E2 + Levo groups did not significantly differ in WMC errors for post delay trials compared to baseline trials (Figure 5), suggesting that these groups did not show significant forgetting across the delay period. No differences were seen for WMI and RM error measures for each treatment group on the post-delay trials relative to baseline trials (data not shown).

Morris water maze (MWM)

The MWM was used to evaluate spatial reference memory performance. There was a main effect of Day across all five days of testing, with Total Swim Distance scores decreasing across days [$F_{(4,35)} = 83.997$, $p < 0.0001$; Figure 6A]. The lack of a significant Treatment x Day interaction indicates that the groups did not differ in learning trajectory across days. The Treatment main effect for swim distance to the platform was not significant. For the probe trial, there was a main effect of Quadrant, with a greater percent swim distance spent in the NE target quadrant, the previously-platformed quadrant, relative to the opposite, SW quadrant [$F_{(1,35)} = 246.174$, $p < 0.0001$; Figure 6B], indicating spatial localization of the platform location. The lack of a significant Treatment x Quadrant interaction suggests that the groups did not differ in their pattern of spatial localization by the end of testing.

Visible platform

Motor and visual competence to solve a water-escape maze task was evaluated using the visible platform test. There was a main effect of Trial [$F_{(5,35)} = 5.609$, $p < 0.0001$], with an 8.6s average latency to the platform across trials (data not shown). During the last trial of testing, each animal reached the platform in under 18s, confirming the ability to complete a water-escape maze task. Neither the Treatment, nor the Treatment x Trial interaction, was significant, indicating that the groups did not differ in the ability to learn and perform the procedural components of a water-escape maze task.

Blood serum analysis

E2 and estrone levels in blood serum, collected at sacrifice, were analyzed. For E2 levels, there was a main effect of Treatment [$F_{(3,34)} = 10.691$, $p < 0.0001$], with post hoc analyses showing higher E2 levels for the E2-Only [$p < 0.0001$] and E2 + Levo [$p < 0.0001$] groups compared to the Vehicle group, as well as higher E2 levels for the E2-Only [$p < 0.0001$] and E2 + Levo [$p < 0.0001$] groups compared to the Levo-Only group (Figure 7A). For estrone levels, there was a main effect of Treatment [$F_{(3,35)} = 25.014$, $p < 0.0001$], with higher estrone levels for both E2-Only [$p < 0.0001$] and E2 + Levo [$p < 0.0001$] groups relative to the Vehicle group, as well as higher estrone levels for both E2-Only [$p < 0.0001$] and E2 + Levo [$p < 0.0001$] groups relative to the Levo-Only group (Figure 7B). These results verify systemic presence of E2 and estrone in the groups receiving exogenous E2 treatment.

Uterine horn weight

There was a main effect of Treatment [$F_{(3,35)} = 58.915$, $p < 0.0001$] for uterine horn weights; higher weights were seen in the E2-Only [$p < 0.0001$] and E2 + Levo [$p < 0.0001$] groups compared to the Vehicle group, and higher weights were seen in the E2-Only [$p < 0.0001$] and E2 + Levo [$p < 0.0001$] groups compared to the Levo-only group, as expected with estrogen exposure (Figure 7C; Engler-Chiurazzi et al., 2012; Mennenga et al., 2015b; Westerlind et al., 1998). There was no significant difference in uterine horn weights between the E2-Only group and the E2 + Levo group, suggesting that the addition of the currently-utilized Levo regimen to the E2 treatment did not impact the E2-induced increase in uterine horn weight at the time of uterine data collection.

Brain analysis

Western blots were performed to examine activated Erk1 and Erk2 expression in several regions of the brain that are indicated in learning and memory. There were no significant main effects of Treatment for activated Erk1 and Erk2 expression in the frontal cortex, dorsal hippocampus, CA1/CA2 ventral hippocampus, entorhinal cortex, or perirhinal cortex (data not shown). A correlation table summarizing the relationship between activated Erk1 and activated Erk2 expression in the frontal cortex and error measures for Block 1 of WRAM for each treatment group is presented in Table 1. After adjusting for multiple correlations using a set FDR threshold of 0.1, a relationship between cognitive performance and activated Erk2 expression was seen that was specific to animals treated with E2-Only, with higher levels of activated Erk2 associated with better performance. In particular, in the frontal cortex, there was a significant negative correlation

between activated Erk2 expression and Block 1 WMC errors within the E2-Only group [r(19) = -0.88, $P < 0.001$, $Q < 0.1$; Figure 8B], suggesting that the E2-treated animals that tended to have higher levels of activated Erk2 in the frontal cortex tended to make fewer WMC errors. In contrast to the relationship seen with E2-Only treatment, there was no significant correlation between activated Erk2 expression in the frontal cortex and Block 1 WMC errors within the E2 + Levo treatment group [r(19) = 0.26, $P = 0.47$, $Q = 0.87$; Figure 8D], suggesting that the addition of Levo obviated the E2-induced association between working memory performance and activated Erk2 levels in the frontal cortex. There was also no significant correlation between activated Erk2 expression in the frontal cortex and Block 1 WMC errors within the Vehicle control group [r(19) = 0.72, $P = 0.02$, $Q = 0.28$; Figure 8A] and within the Levo-Only treatment group [r(18) = -0.43, $P = 0.25$, $Q = 0.87$; Figure 8C]. There was no significant relationship between cognitive performance and activated Erk1 expression, nor between cognitive performance and activated Erk2 expression, in the dorsal hippocampus, CA1/CA2 ventral hippocampus, entorhinal cortex, or perirhinal cortex (correlation tables not shown).

Discussion

The current study demonstrated that E2-Only and Levo-Only treatments enhanced working memory performance during acquisition of the WRAM, as measured by WMI errors collapsed across all trials, and that the combination of E2 + Levo attenuated these beneficial cognitive effects. In fact, at the highest demand working load (trial 4) during acquisition, the E2 + Levo combination impaired performance, as compared to the E2-Only and Levo-Only groups. Thus, even a progestin that was shown here and in prior work

(Braden et al., 2017) to enhance the ability to handle an increasing working memory load on the WRAM task when given alone, can reverse the beneficial effects of E2 at a high memory demand. Additionally, findings showed that there was a distinct relationship between activated Erk2 expression in the frontal cortex and working memory performance within the E2-Only treatment group, which was mitigated by the addition of Levo to the E2 treatment. However, Levo did not uniformly attenuate the benefits of E2. At a working memory load that was less demanding, the addition of Levo did not reverse the cognitive benefits of E2 relative to vehicle treatment, as determined by trial 3 for WMI errors on the WRAM. Indeed, similar to each hormone treatment given alone, the hormone combination treatment benefitted this moderate working memory load trial performance as compared to the vehicle treatment.

Overall, the present study found that all hormone treatment groups were able to learn spatial working and reference memory tasks, as shown by their performance across all days on the WRAM (Days 1-12, collapsed across the 4 trials) and the MWM (Days 1-5, collapsed across the 4 trials). During the acquisition phase of the WRAM (Block 1 of testing), when rats were initially learning the rules of the task, the E2-Only and Levo-Only treatment groups made fewer WMI errors across all four trials compared to the vehicle group, indicating that E2-Only and Levo-Only enhanced working memory performance during the learning phase of the task relative to control. With reference to prior publications testing E2 (using various regimens, doses, and rat ages), in general, results here are consistent with previously published findings suggesting enhanced cognitive performance with E2 only treatment (Bimonte & Denenberg, 1999; Jill M. Daniel et al., 2006; Jill M Daniel et al., 1997; Fader et al., 1999; R. B. Gibbs & Johnson, 2008; V N Luine et al.,

1998; Rodgers et al., 2010). The results are also consistent with previously published findings of enhanced cognitive performance with Levo only treatment (B. Blair Braden et al., 2017; Simone et al., 2015). When evaluating all four trials combined, the combination of E2 + Levo obviated the working memory benefits of E2-Only and Levo-Only. There has been only one other published preclinical study testing an estrogen/Levo combination, whereby the synthetic estrogen ethinyl estradiol (EE) plus Levo was tested in a different model and behavior paradigm: the young ovary-intact rat tested in object memory (Simone et al., 2015). However, even with the important differences between the studies, similar results were reported, with the EE plus Levo hormone combination treatment attenuating EE-induced improvements in novel object memory and Levo-induced enhancements in visuospatial memory in ovary-intact young rats (Simone et al., 2015). Preclinical studies testing other combination hormone therapies have also shown that, in Ovx rodents, the addition of natural progesterone to E2 treatment can reverse the enhancing cognitive effects of E2 (Heather A. Bimonte-Nelson et al., 2006; Lauren L. Harburger et al., 2007; Lowry et al., 2010). It has also been shown that the addition of MPA to an E2 treatment resulted in impaired learning on the MWM compared to E2 treatment alone (Lowry et al., 2010).

Interestingly, results from this study showed that working memory demand influenced the direction of hormone treatment effects on cognitive performance specifically during the acquisition phase of the WRAM. On Trial 3, when the working memory load was moderate, the E2-Only, Levo-Only, and E2 + Levo treatment groups made fewer WMI errors than the vehicle group, suggesting that all hormone treatments enhanced performance relative to control treatment during this moderate demand trial. However, on Trial 4, when the working memory load was highest, the E2 + Levo treatment

group made more WMI errors compared to E2-Only and Levo-Only treatment groups, revealing that this estrogen/progestin combination treatment impaired high demand working memory ability relative to each individual hormone treatment. This is additionally represented in Figure 4F, with effects clearly illustrated by the interaction between the moderate and the high working memory load trials (Trials 3 and 4) and Treatment (E2 + Levo group versus the vehicle group). Taken together, these results indicate that the hormones E2 and Levo impact cognitive function in a model of surgical menopause, with the direction of this mnemonic impact dependent on: 1) whether E2 and Levo are administered alone as an individual exogenous regimen, or together as a hormone combination exogenous regimen, and 2) cognitive demand. Each individual hormone regimen and the combined hormone regimen enhanced cognitive performance when the working memory demand was moderate; however, when the working memory demand was high, these two hormones in combination impaired performance compared to each hormone alone. These findings are in accordance with previous studies demonstrating distinct hormone effects across an increase in working memory demand, including with estrogens (Bimonte & Denenberg, 1999; Hiroi et al., 2016; Sarah E. Mennenga, Gerson, Koebele, et al., 2015), progestogens (B. B. Braden et al., 2011; B. Blair Braden et al., 2017, 2015), and androgens (Bryan W. Camp et al., 2012; Sarah E. Mennenga, Koebele, et al., 2015).

When a 6-hour delay period was implemented for the WRAM, neither of the E2-treated groups (E2-Only and E2 + Levo) differed in WMC errors between the post-delay trials and the baseline trials. This suggests that exogenous E2 treatment protected from delay-induced impairment in performance on working memory. Of note, the addition of

Levo to E2 did not reverse the protective effects of E2-Only against delay-induced impairment in working memory performance. However, the vehicle and Levo-Only treatment groups exhibited impaired performance, where they made more WMC errors on the post-delay trials relative to their baseline performance. Together, these findings demonstrate a potential protective effect of E2 on cognitive function across the delay period that was not seen with the vehicle and the Levo-Only treatment, regardless of whether Levo is on board with the E2 treatment or not. These results are consistent with previous studies where E2 enhanced cognitive performance following the implementation of a delay period (Lauren L. Harburger et al., 2007; Talboom et al., 2008). For instance, one study trained aged Ovx mice on the MWM and then immediately administered vehicle, E2, or E2 plus a low or high dose of progesterone treatment (Lauren L. Harburger et al., 2007). Following a 24 hour delay period, results showed that the E2 treatment enhanced performance (Lauren L. Harburger et al., 2007). The addition of a low dose of progesterone did not alter the beneficial cognitive effects of E2 on post-delay performance, but the addition of a high dose of progesterone attenuated the beneficial cognitive effects of E2 on post-delay performance (Lauren L. Harburger et al., 2007). Another study from the Bimonte-Nelson laboratory also showed that E2 treatment had beneficial effects on overnight forgetting on the MWM task compared to vehicle control (Talboom et al., 2008). Collectively, these results reveal a protective role of E2 in cognitive function following the implementation of a delay period.

The hormone impact was working memory-specific in the current study. For the spatial reference memory MWM, all treatment groups decreased in swim distance to the platform across all days of testing. However, there were no treatment differences in the

swim distance to the platform, suggesting similar spatial reference memory performance on this task. On the probe trial for MWM, all treatment groups swam a greater percent distance in the NE target quadrant, which previously contained the platform, compared to the opposing SW quadrant. Thus, results from the MWM task showed that all groups were able to effectively learn the spatial reference memory task and spatially localize to the platform location in a similar pattern. It is important to note that the MWM was administered following the WRAM. Some studies suggest that previous cognitive experience can impact learning and memory performance (A L Markowska, 2002; Talboom et al., 2014). Thus, prior learning experience may have affected learning and memory performance on the MWM in the present study, and may be in part contributing to the lack of significant treatment effects on the MWM. However, these data are consistent with the lack of a treatment effect for the spatial reference memory measure of the WRAM in the current study.

The visible platform task tests the ability of animals to effectively perform the procedural components of a water-escape maze task. There were no treatment differences and no treatment by trial interactions on the visible platform task, indicating that all groups had similar capabilities required to effectively complete a water-escape maze task. Therefore, the interpretations of treatment effects on cognitive performance in the present study are not impacted by the motor and visual capabilities of the animals.

In the current study, E2 exposure was confirmed by showing that E2 treatment elevated circulating levels of E2 and its metabolite, estrone, relative to vehicle treatment, and by demonstrating that uterine horn weight increased with E2 treatment as compared to vehicle treatment. In women, the addition of a progestogen is meant to offset the uterine

stimulation induced by estrogen, and this has been seen in rat models (Armstrong, 1968; Creasy, Kafrissen, & Upmalis, 1992; S. E. Mennenga & Bimonte-Nelson, 2015). However, in the current study, the addition of Levo to E2 treatment did not significantly reduce E2-induced increase in uterine horn weight. This may be explained in part by the 5:1 E2 to Levo ratio used in the present study, which was based on the dose of Levo that has previously been shown to enhance spatial learning and memory when administered alone. Additional investigation is warranted to address whether decreasing the E2 to Levo ratio, including to the 3:1 ratio used in Climara Pro, can significantly reduce uterine horn stimulation.

In the frontal cortex, the present study found a relationship between working memory performance and activated Erk2 expression for the E2-Only group, where animals that tended to perform better on the WMC measure tended to have higher activated Erk2 expression. This suggests that there is a unique relationship between working memory performance and activated Erk2 expression in the presence of E2 in the frontal cortex, a region that is heavily involved in normal working memory function (Funahashi & Kubota, 1994). It is noteworthy that the beneficial cognitive effects of E2 in this study were specific to working memory, and here the activation of a signaling pathway implicated in cognitive function was linked particularly to E2-Only treatment and its effects in a region of the brain that plays a significant role in processing working memory information. In contrast to the relationship seen with E2-Only treatment, there was no relationship between cognitive performance as measured by WMC errors made and activated Erk2 expression within the E2 + Levo hormone combination treatment group, indicating that the addition of Levo obviated the E2-induced association between cognitive performance and activated Erk2

levels in the brain. Additionally, there was a relationship trend within the Vehicle group between activated Erk2 expression in the frontal cortex and WMC errors, whereby higher activated Erk2 levels correlated with higher WMC errors, indicating that E2-Only treatment may have reversed the relationship between activated Erk2 expression and working memory performance in the control group; this correlation was statistically significant before the correction, but it was not statistically significant after correcting for multiple correlations. Although the relationship between cognitive performance and activated Erk2 levels in the frontal cortex was specific to the WMC measure and the beneficial cognitive effects of E2-Only treatment were specific to the WMI measure, it is important to note that these measures of working memory performance are orthogonal and may be governed by different neurological pathways and brain regions. For example, a hippocampal lesion study found that a complete hippocampal lesion in male rats resulted in increased WMC errors compared to WMI errors when tested on an 8-arm radial arm maze, but no differences were seen between WMC errors and WMI errors in control rats and in rats with partial hippocampal lesions (Jarrard, Luu, & Davidson, 2012). Thus, the type of memory affected, and the directionality of the cognitive effect following hormone treatment, may be governed by the brain regions and neural pathways that are specific to the hormones examined. There were no treatment-induced differences in activated Erk1 and Erk2 expression in the dorsal hippocampus, CA1/CA2 ventral hippocampus, frontal cortex, entorhinal cortex, or perirhinal cortex. It is important to highlight that the treatment regimen in this study was a chronic and cyclic low dose injection, whereas studies that have shown E2-induced increase of activated Erk2 expression in the dorsal hippocampus

are typically acute or injected at a higher or tonic dose (Fernandez et al., 2008; L. L. Harburger et al., 2009; Witty et al., 2013).

The growing preclinical and clinical evidence indicates great complexity in the cognitive effects of ovarian hormone loss and hormone therapy. This drives the field toward opening new avenues of study to help scientist understand which factors play into this complexity in order to truly understand hormone-related impacts. Many publications indicate that ovarian hormone loss in women, and in rodents, is associated with changes in memory performance across multiple domains of memory, that subsequent E2 treatment can result in beneficial effects on cognitive function, and that the addition of a progestogen can attenuate these E2-induced cognitive benefits (Frick, 2015; Koebele & Bimonte-Nelson, 2015; Korol & Pisani, 2015; Maki, 2012; S. E. Mennenga & Bimonte-Nelson, 2013; Sherwin, 2006). There are notable exceptions to these outcomes, as the extent and direction of hormone therapy effects are sensitive to a myriad of variables. The details and parameters of how such factors impact outcomes are just beginning to be understood (Koebele & Bimonte-Nelson, 2015, 2017; Korol & Pisani, 2015). For example, age is a critical factor affecting the efficacy of estrogens on cognitive and brain function, with diminished or lost efficacy as aging ensues (L.A. Bean, Ianov, & Foster, 2014; Foster et al., 2003; R.B. Gibbs, 2010; Koebele & Bimonte-Nelson, 2017; Maki, 2013; S. E. Mennenga & Bimonte-Nelson, 2013; Talboom et al., 2008). Thus, the hormone treatment effects observed in the present study in middle-aged rats may be more pronounced if tested in young rats, and may be attenuated in aged rats.

Several large-scale clinical studies have set out with the common goal to further understand the cognitive impact of menopause and hormone therapies taken by women

(Gleason et al., 2015; Greendale, Derby, & Maki, 2011; Karlamangla, Lachman, Han, Huang, & Greendale, 2017; Rapp et al., 2003; Shumaker et al., 2003). Only one human study thus far has specifically evaluated the impact estrogen and Levo have on cognitive function. In this study, the estradiol valerate plus Levo oral hormone therapy, Kilomonorm, was assessed; after two months, Kilomonorm improved concentration, speed of cognitive function, and short-term memory in peri- and post- menopausal women that either had received a hysterectomy (n = 6), ovariectomy (n = 14), hysterectomy plus ovariectomy (n = 6), or no surgical manipulations (n = 52) (Rudolph et al., 2000). In the current study, the cognitive effects of the estrogen plus Levo treatment in surgically menopausal rats were contingent on the level of cognitive demand and memory type evaluated. Thus, systematically designed studies of clinically-relevant hormone formulations and their effects in animal models of menopause are critical; information gained from such studies can inform future human study designs as steps are taken to further understand the complex interactions between hormones and various memory domains.

In conclusion, to my knowledge, this study is the first to examine the effect of an E2 + Levo hormone combination treatment, as well as E2-Only and Levo-Only treatments, on cognitive function in a preclinical model of surgical menopause. Hormone therapy, which can contain only estrogens, only progestogens, or an estrogen plus progestogen combination, is used to decrease the onset and severity of undesired changes associated with menopause. Thus, it is critical to not only examine the individual effects of hormones on symptoms associated with menopause, including the impact on learning and memory, but also how the combination of both an estrogen and a progestogen impacts these symptoms. Results showed that E2 and Levo each have beneficial effects on spatial

working memory when administered separately, replicating prior studies (Heather A. Bimonte-Nelson et al., 2006; Bimonte & Denenberg, 1999; B. Blair Braden et al., 2017; Talboom et al., 2008). However, the E2 + Levo hormone combination impaired spatial working memory relative to either of the hormones alone when the spatial working memory task was highly taxing. Furthermore, a relationship between activated Erk2 levels in the frontal cortex and spatial working memory following E2-Only treatment was observed. These findings are significant as they highlight opposing effects of an estrogen and progestogen hormone combination treatment, and raise further questions regarding which underlying neurobiological mechanisms are responsible for the individual cognitive enhancements of E2-Only and Levo-Only, and for the negating effects when the two hormones are administered together. This study illustrates that although an estrogen and a progestogen can be cognitively beneficial when administered alone, the clinically used combination of the same estrogen and progestogen does not necessarily result in added benefits, and can in fact yield impairments. Indeed, in the context of hormone therapy, one plus one does not always equal two.

CHAPTER 3

17β-ESTRADIOL PLUS LEVONORGESTREL HORMONE TREATMENT: MEMORY IMPAIRMENTS ARE DEPENDENT ON HORMONE RATIO

This chapter will be formatted and submitted for publication in Hormones and Behavior.

Contribution: I am the first author of this document and was the primary graduate student principal investigator on this project. This study was part of an undergraduate honors thesis, and I mentored the student through the design, procedures, data analyses, and writing aspects of the project. The primary mentor on this project was Heather A. Bimonte-Nelson, and we received assistance from our laboratory team in carrying out the study.

Introduction

The average age of the United States (U.S.) population is increasing. According to projections by the U.S. Census Bureau, in the next several decades there will be a higher proportion of older people, defined as 65 years old and over, comprising the overall population, with an estimated increase of 9% in the number of older people from years 2014 to 2060 (Colby & Ortman, 2015). Indeed, one in five people are predicted to be aged 65 and over by the year 2030 (Colby & Ortman, 2015). Of note, around the average age of 52 years, women undergo menopause and may experience associated undesired symptoms, including poor memory performance, poor concentration, vasomotor symptoms, osteoporosis, and vaginal dryness (Maki, 2012; NAMS, 2014). As the aging population

continues to grow, more women will be living in a menopausal state, and will do so for longer periods of time. Thus, in order to maintain a high quality of life for aging women, it is imperative to develop a complete understanding of the basic science behind the changes that can occur, such as with cognitive function, across the transition to menopause as well as post-menopause.

Menopause, confirmed after at least one year of a lack of menses, is associated with a marked reduction in the circulating levels of ovarian hormones, including estrogens and the natural progestogen, progesterone (NAMS, 2014). Rodent models of menopause have been extensively utilized over the last several decades to parse out the effects of decreased circulating ovarian hormone levels on systemic functions, such as cognitive function, as well as the effects of subsequent exogenous hormone administration (Prakapenka et al., *in review*). In the clinic, multiple hormone therapy options are available for women to help alleviate the presence and severity of menopause-associated symptoms. Hormone therapies vary in type of hormone (estrogens, progestogens, or estrogen plus progestogen combinations), dose of hormone, as well as route of hormone administration (Prakapenka et al., *in review*). One hormone widely studied regarding its pharmacokinetics and pharmacodynamics in relation to cognitive function is 17β-estradiol (E2), the most potent endogenous estrogen in women and rodents that is often used in hormone therapy formulations (Frick, 2015; Koebele & Bimonte-Nelson, 2015; Korol & Pisani, 2015; Maki, 2012; S. E. Mennenga & Bimonte-Nelson, 2013; Sherwin, 2006). In rodent models, E2 has been repeatedly shown to improve cognition following the loss of circulating ovarian hormones with ovariectomy (Ovx), the surgical removal of the ovaries, across several distinct behavioral paradigms, including the Morris water maze (MWM; Heather A.

Bimonte-Nelson et al., 2006; El-Bakri et al., 2004; Feng et al., 2004; Kiss et al., 2012; Lowry et al., 2010; McLaughlin et al., 2008; Talboom et al., 2008), radial-arm maze (Bimonte & Denenberg, 1999; Jill M. Daniel et al., 2006; Jill M Daniel et al., 1997; Fader et al., 1999; R. B. Gibbs & Johnson, 2008; V N Luine et al., 1998; Rodgers et al., 2010), object placement task (Conrad et al., 2012; Frye et al., 2007; Victoria N. Luine et al., 2003; McLaughlin et al., 2008), and delayed match-to-position T-maze (R. B. Gibbs, 2000; R B Gibbs, 1999; Robert B. Gibbs, 2007; Robert B. Gibbs et al., 2004; Robert B Gibbs, 2002). If a woman has her uterus, estrogen-containing hormone therapy must also contain an opposing progestogen to reduce the risk of endometrial hyperplasia and cancer associated with unopposed estrogen (NAMS, 2012). Rodent studies currently suggest that when combined with certain progestogens, the beneficial effects of E2 on cognition can be attenuated (Heather A. Bimonte-Nelson et al., 2006; Lauren L. Harburger et al., 2007; Lowry et al., 2010; Prakapenka et al., 2018). However, there are a multitude of different synthetic progestogens clinically-available that differ not only by type of activity (progestogenic, androgenic, estrogenic, etc.) but also by potency (i.e. affinity to receptors; Kuhl, 2005), and many of them have yet to be evaluated for cognitive effects when administered alone or in combination with an estrogen in middle-aged Ovx rodents.

We have recently shown that when administered alone, E2 and the synthetic progestogen, levonorgestrel (Levo) each improved spatial working memory on the water radial-arm maze (WRAM) relative to vehicle in middle-aged Ovx rats (Prakapenka et al., 2018). However, the combination hormone treatment E2 plus Levo attenuated the beneficial cognitive effects of E2 alone and of Levo alone, and did not reduce the E2-induced increase in uterine horn weight – a marker of E2 exposure and stimulation at the

uterine horns (Prakapenka et al., 2018). The E2 and Levo doses in that study were based on the doses from prior work in the Bimonte-Nelson laboratory that yielded enhancing effects on cognition by each individual hormone alone, resulting in a 5:1 E2 to Levo dose ratio. The clinically available Climara Pro transdermal patch that contains both E2 and Levo employs a 3:1 E2 to Levo dose ratio to treat symptoms associated with menopause. Of note, studies show that several factors can impact the direction of hormone effects on cognition, including the dose ratio of two hormones used in a treatment (L. L. Harburger et al., 2009; Koebele & Bimonte-Nelson, 2015; Jennifer J. Tuscher, Fortress, Kim, & Frick, 2015). For instance, in a study evaluating E2 plus progesterone combination treatment in Ovx mice, when the E2 dose was kept constant, only the addition of the lowest tested progesterone dose attenuated the beneficial effects of E2 on the novel object recognition task, an effect that did not occur with the addition of the two higher doses of progesterone (L. L. Harburger et al., 2009). In fact, even though Levo is not considered to have estrogenic effects because it does not bind to estrogen receptors, recent *in vitro* work has shown that Levo can increase the mRNA expression for estrogen receptors in neuron cultures (Jayaraman & Pike, 2014; Kuhl, 2005). Thus, the clinically-utilized E2 and Levo hormone combination treatment must be evaluated further to determine if altering the dose ratio of E2 to Levo will yield cognitively enhancing effects or if the previously observed cognitive effects persist across a clinically relevant ratio of E2 to Levo doses.

The aim of the present study was to examine the cognitive effects of three E2 plus Levo hormone combination treatments, including the combination dose ratio used in Climara Pro and the combination dose ratio from my prior study. I hypothesized that varying the dose ratios of E2 to Levo by increasing the Levo dose while keeping E2 dose

constant would have differential effects on cognition following a decrease in circulating ovarian hormone levels. To evaluate this, for the current study, middle-aged Ovx rats received daily subcutaneous injections of either a vehicle control, E2 alone control, or E2 plus Levo at three different dose ratio combinations, keeping the E2 dose constant across all E2-containing treatments. All rats then underwent testing on a behavioral battery to assess spatial learning and memory, and uterine horn weight was collected at the end of the study to evaluate opposing effects of Levo on E2-induced stimulation at the uterine horns.

Methods

Animals

50 11-month old Fischer-344-CDF virgin female rats were used (National Institute on Aging, Harlan Laboratories, Indianapolis, IN, USA). The rats were pair housed and received food and water ad libitum. The colony room was kept on a 12-hour light/dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards.

Ovariectomy (Ovx)

At 11 months old, all rats received Ovx surgery to remove their ovaries as previously described (B. Blair Braden et al., 2017; Prakapenka et al., 2018). Briefly, rats were anesthetized via isoflurane inhalation, received bilateral dorsolateral incisions in the skin and peritoneum, and the ovaries and tips of the uterine horns were ligatured and removed. Muscle and skin were then sutured closed. For pain, rats received a subcutaneous injection of Rimadyl (5 mg/ml/kg) and to prevent dehydration, rats received a subcutaneous

injection of sterile saline (2 ml).

Hormone treatments

Twenty-one days following Ovx surgery, rats received daily subcutaneous treatment for the rest of the study (see timeline in Figure 9). All rats were randomly assigned to either the Vehicle (0.1 ml sesame oil, n=10) control group, the E2 Only (0.3 μg E2, n=10) control group, or one of the hormone combinations groups: E2 + Low Levo (0.3 μg E2 plus 0.06 μg of Levo, n=10), E2 + Med Levo (0.3 μg E2 plus 0.1 μg of Levo, n=10), or E2 + High Levo (0.3 μg E2 plus 0.6 μg of Levo). The doses for low, medium, and high Levo were determined based on their ratio to the constant E2 dose and prior findings, as summarized in Table 3. Specifically, the E2 + Low Levo dose ratio (5:1 E2 to Levo) was the same dose ratio previously tested within the Bimonte-Nelson laboratory (Prakapenka et al., 2018), the E2 + Med Levo dose ratio (3:1 E2 to Levo) was the same dose ratio as in the clinically used Climara Pro transdermal patch, and the E2 + High Levo dose ratio (1:2 E2 to Levo) was obtained by using the dose of Levo treatment that has been repeatedly found to have beneficial cognitive effects when given alone in middle-aged Ovx rats (B. Blair Braden et al., 2017; Prakapenka et al., 2018).

Vaginal smears

Vaginal smears were done 16 days following treatment initiation for three consecutive days to confirm Ovx, as well as E2, exposure based on vaginal cytology as previously described (S. E. Mennenga & Bimonte-Nelson, 2015). Briefly, rats were held by the base of the tail and the rear end was gently lifted to expose the anogenital area. A

sterile cotton-tipped applicator, moistened with sterile saline, was inserted about 1 cm into the vaginal canal and the vaginal walls were swabbed for cell collection. The cotton swab was then rolled along a blank microscope slide to transfer the cells onto the slide, the cell types were examined under a light microscope (10x), and cell types and cycle phase were recorded.

Water radial-arm maze (WRAM)

Rats were tested on the water radial-arm maze (WRAM) after 21 days of treatment administration to assess spatial working and reference memory, as done previously (H. A. Bimonte-Nelson, 2015d; Bimonte & Denenberg, 1999; Prakapenka et al., 2018). The WRAM was an 8-armed apparatus consisting of evenly spaced arms (each arm 38.1 cm x 12.7 cm) extending outward from a central arena. Non-toxic black paint was used to create opaque water, kept at 18-20°C, that filled the maze. Four out of the 8 arms contained hidden platforms (10 cm in diameter) that were kept submerged under water. The platform locations for each rat remained constant across a total of 13 days of testing, but varied across rats. Abundant extra-maze cues were present in the room to aid in spatial navigation. There were a total of four trials per day, one trial per platform, with the same drop-off location for each trial. Each trial was considered over when a rat either found a platform or 3 minutes passed for the maximum allotted trial time, and the rat was then led to the nearest platform. When found, the rat was allowed 15 seconds on the platform and was then placed in a heated testing cage for an inter-trial interval (ITI) of 30 seconds. The ITI provided time to remove the just-found platform and clean the maze of any debris while re-distributing olfactory cues. The next trial began following the ITI, and the trial process was repeated

until all platforms had been found. In this manner, increase in working memory load within a day was evaluated as rats had to update the location of the platform just found, that it was no longer there, and remember which platform locations remained. On day 13 of WRAM testing, a 6-hour delay was implemented between the second and third trials for each rat to assess delayed memory retention. Performance on the WRAM was evaluated by scoring error arm entries. These error entries were further split into three orthogonal memory measures: reference memory (RM) error was committed when an arm that never contained a platform was entered, working memory incorrect (WMI) error was committed when an arm that never contained a platform was re-entered within a day, and a working memory correct (WMC) error was committed when an arm that previously contained a platform was entered within a day (H. A. Bimonte-Nelson, 2015d).

Morris water maze (MWM)

Spatial reference memory was evaluated on the MWM the day following WRAM delay, as was done previously (H. A. Bimonte-Nelson, 2015b; Prakapenka et al., 2018). The maze was a circular pool 188 cm in diameter in a room with abundant extra-maze cues to assist with spatial navigation, and the maze was filled with 18-20°C opaque water. A single platform (10 cm in diameter) was hidden beneath the surface of the water in the Northeast (NE) quadrant of the maze. The platform location remained the same for all rats for 4 trials per day, and 5 days of testing. The drop-off locations (North, South, West, East) varied across trials, and the order of the drop-off locations varied each day. Once the rat was released into the water, the trial started and a maximum of 60 seconds were allowed to find the platform. After the platform was located, or after the rat was led to it after 60

seconds, the rat remained on the platform for 15 seconds before being placed into a heated testing cage. The ITI was approximately 5-8 minutes, during which the water was cleaned of any debris and olfactory cues were re-distributed. MWM performance was recorded by a camera suspended above the maze, and EthoVision tracking system (Noldus Instruments, Wageningen, The Netherlands) was used to determine swim distance. On the 5th day of testing, a 5th trial was added as the probe trial during which the platform was removed and rats were dropped off from the West location, the furthest location from where the platform would have been located (Northeast quadrant). Each rat was allowed to swim for a full 60-second period, and the percentage of total distance that the rats swam in the Northeast quadrant, the target quadrant that previously contained the platform during all other trials, relative to the opposite Southwest quadrant was measured to evaluate spatial localization to the platform.

Visible platform

The non-spatial visible platform task was administered the day after MWM was completed to examine motor and visual competence (H. A. Bimonte-Nelson, 2015c). The visible platform was a rectangular tub (100 cm x 60 cm) filled with clear water that was kept at a temperature of 18-20°C. A visible black platform (10 cm in diameter) was placed in the tub and positioned 4 cm above the water. Opaque curtains surrounded the maze, blocking any potential extra-maze cues. Once released at the drop-off location, each rat had 90 seconds to find the platform for each trial; once found, the rat remained on the platform for 15 seconds and was then placed into a heated testing cage for an ITI of 5-8 minutes. Each rat was tested for 6 trials across one day. The drop-off location remained the

same across all trials, but the location of the platform varied semi-randomly across 3 different locations.

Uterine horn weights

The wet weights of uterine horns were collected to confirm Ovx, E2 exposure, as well as potential opposing effects of Levo on E2's uterine-stimulating effects (E. B. Engler-Chiurazzi et al., 2012; Sarah E. Mennenga, Gerson, Koebele, et al., 2015; Prakapenka et al., 2018; Westerlind et al., 1998). Specifically, one day after the completion of the visible platform task and following treatment administration, rats underwent euthanasia under isoflurane anesthesia, at which point uterine horns were removed, inspected, trimmed of visible fat, and weighed.

Statistical analyses

Repeated measures ANOVA was used to evaluate behavioral outcomes, and alpha level was set at 0.05. Two animals were excluded from all behavioral analyses (one from the Vehicle group and one from the E2 Only group) due to pathological findings at autopsy. Each maze was analyzed separately.

For the WRAM, errors made on each memory measure (WMC, WMI, and RM) were analyzed separately for Days 2-7, the acquisition phase, and Days 8-12, the asymptotic phase (H. A. Bimonte-Nelson et al., 2015; B. Blair Braden et al., 2017). For all analyses, planned comparisons were performed to compare Vehicle versus E2 Only, Vehicle versus E2 + Low Levo, Vehicle versus E2 + Med Levo, and Vehicle versus E2 + High Levo groups. The independent variable was Hormone and the repeated measures was

Trials nested within Days, and the dependent variable was Errors. If there was a significant Trial x Hormone interaction, the higher working memory load trials, trials 3 and 4, were analyzed separately using the same planned comparison groups. Additionally, linear regression analyses were done to assess the relationship between WMC, WMI, and RM errors and Levo dose. For WRAM delay, errors made on each memory measure (WMC, WMI, and RM) on the post-delay trial, Trial 3 on Day 13, were compared to the baseline trial, Trial 3 on Day 12, within each treatment group (B. Blair Braden et al., 2017).

For the MWM maze, total swim distance across 5 days was analyzed. As was done for WRAM, planned comparisons were performed to compare Vehicle versus E2 Only, Vehicle versus E2 + Low Levo, Vehicle versus E2 + Med Levo, and Vehicle versus E2 + High Levo groups. The independent variable was Hormone and the repeated measures were distance swam on Trials nested within Days. The probe trial was analyzed separately to assess spatial localization for each treatment group using a repeated measures ANOVA to evaluate percent swim distance in the previously platformed quadrant (Northeast; NE) compared to percent swim distance in the directly opposite quadrant (Southwest; SW).

For visible platform performance, the same planned comparisons were performed as for the WRAM and MWM to compare Vehicle versus E2 Only, Vehicle versus E2 + Low Levo, Vehicle versus E2 + Med Levo, and Vehicle versus E2 + High Levo groups using repeated measures ANOVA. The independent variable was Hormone and the repeated measure was time to platform on Trials.

Uterine horn weights were analyzed using one-way ANOVA, with Hormone as the independent variable and uterine horn weight as the dependent variable. Planned comparisons were performed to compare Vehicle versus E2 Only, Vehicle versus E2 +

Low Levo, Vehicle versus E2 + Med Levo, and Vehicle versus E2 + High Levo groups. Uterine horn weight was expected to increase with E2 treatment, and the addition of Levo was anticipated to decrease uterine horn weight compared to E2 alone; thus, additional planned comparisons were performed to examine E2 versus E2 + Low Levo, E2 versus E2 + Med Levo, and E2 versus E2 + High Levo groups. Two animals were excluded from all uterine horn weight analyses due to uterine horn pathology (e.g. cysts).

Results

Vaginal smears

All animals receiving Vehicle treatment exhibited mostly blank smears with few cells present across 3 consecutive days (Figure 10), indicating lack of ovarian hormone stimulation and cyclicity, and confirming complete Ovx (Goldman et al., 2007; S. E. Mennenga & Bimonte-Nelson, 2015). All animals receiving E2 Only, E2 + Low Levo, E2 + Med Levo, or E2 + High Levo treatments had smears nearly identical to each other, containing numerous cells overall that mostly consisted of cornified cells (Figure 10). This confirmed the presence of circulating E2 for all hormone treatment groups, and suggests that the presence of Levo did not alter this measurement of vaginal cytology (Goldman et al., 2007; S. E. Mennenga & Bimonte-Nelson, 2015).

Water radial-arm maze (WRAM)

The WRAM evaluated spatial working and reference memory. For all memory measures on the acquisition phase (Days 2-7), when the rats were learning the rules of the task, there were no Hormone effects or Trial x Hormone interactions for any comparisons

(Figure 11A). For WMC errors made on the asymptotic phase (Days 8-12), there was a main effect of Hormone for the Vehicle versus E2 + High Levo comparison [$F_{(1,17)} = 5.504$, $p < 0.05$; Figure 11B], with the E2 + High Levo group making more errors than the Vehicle group, suggesting that the E2 + High Levo treatment impaired working memory. There was a marginal effect of Hormone for the Vehicle versus E2 + Med Levo comparison [$F_{(1,17)} = 3.381$, $p < 0.1$; Figure 11B], with the E2 + Med Levo group tending to make more WMC errors than the Vehicle group. There were no Trial x Hormone interactions between the Vehicle versus E2 + High Levo and Vehicle versus E2 + Med Levo comparisons for WMC errors. For WMC errors on the asymptotic phase for the Vehicle versus E2 + Low Levo and the Vehicle versus E2 Only group comparisons there were no significant effects of Hormone or Trial x Hormone interactions. For WMI errors made on the asymptotic phase, there was a main effect of Hormone for the Vehicle versus E2 + High Levo comparison [$F_{(1,17)} = 5.241$, $p < 0.05$; Figure 11B], where the E2 + High Levo group made more errors than the Vehicle group. There was also a Trial x Hormone interaction for the Vehicle versus E2 + High Levo comparison [$F_{(3,51)} = 6.318$, $p < 0.01$; Figure 11C], where the E2 + High Levo group made more errors than the Vehicle group on Trial 4 [$t(17) = 2.96$, $p < 0.01$; Figure 11C], the highest working memory load trial. There was no main effect of Hormone for the Vehicle versus E2 + Med Levo comparison, but there was a Trial x Hormone interaction [$F_{(3,51)} = 3.162$, $p < 0.05$; Figure 11C], with the E2 + Med Levo group making somewhat more errors than the Vehicle group on Trial 4 [$t(17) = 1.834$, $p < 0.1$; Figure 11C], the highest working memory load trial. There were no significant main effects of Hormone or Trial x Hormone interactions for WMI errors made on the asymptotic phase for the Vehicle versus E2 + Low Levo, and the Vehicle versus E2 Only,

group comparisons. For RM errors, there was a marginal effect of Hormone, and no Trial x Hormone interaction, for the Vehicle versus E2 + High Levo comparison [$F_{(1,17)} = 3.636$, $p < 0.1$; Figure 11B], where the E2 + High Levo group tended to make more errors than the Vehicle group. There were no significant effects of Hormone or Trial x Hormone interactions for RM errors on the asymptotic phase for the Vehicle versus Med Levo, Vehicle versus E2 + Low Levo, and Vehicle versus E2 Only comparisons.

It was noted that as the Levo dose increased when combined with a constant E2 dose, performance on the WRAM became progressively impaired for all memory measures on the asymptotic phase. This relationship between treatment group and performance was supported by a linear regression analysis. The linear regression analysis showed a significant positive linear trend across Levo dose for WMI errors [$r = 0.9144$; $F_{(1,3)} = 15.31$, $p < 0.05$; Figure 12] and a significant positive linear trend across Levo dose for RM errors [$r = 0.9048$; $F_{(1,3)} = 13.54$, $p < 0.05$; Figure 12]. There was also a marginal positive linear trend across Levo dose for WMC errors [$r = 0.8755$; $F_{(1,3)} = 9.85$, $p = 0.05$; Figure 12]. These results indicate that, for all memory measures, the number of errors made increased as the dose of Levo added to a constant E2 dose increased, suggesting that higher Levo, in combination with E2 and with the parameters evaluated in this study, is associated with poorer spatial working and reference memory.

Analysis of the 6-hour delay revealed that more WMC errors were made on the post-delay trial, Trial 3 on Day 13, compared to the baseline trial, Trial 3 on Day 12, by the E2 + Med Levo [$F_{(1,9)} = 5.444$, $p < 0.05$; Figure 13] and by the E2 + High Levo [$F_{(1,9)} = 16.000$, $p < 0.01$; Figure 13] groups, suggesting delay-induced forgetting. The E2 Only group [$F_{(1,8)} = 4.500$, $p < 0.1$; Figure 13] and the E2 + Low Levo group [$F_{(1,9)} = 3.462$, $p <$

0.1; Figure 13] tended to make more WMC errors on the post-delay trial compared to the baseline trial. The Vehicle group did not reach statistical significance in WMC errors made on the post-delay trial compared to the baseline trial, indicating that they did not exhibit delay-induced forgetting (Figure 13). There were no differences for the WMI and RM errors made on the post-delay trial compared to the baseline trial within each treatment group.

Morris water maze (MWM)

Spatial reference memory was examined on the MWM. There were no significant effects of Hormone, and no Hormone x Day interactions, for swim distance to the platform across the 5 days of testing for the Vehicle versus E2 Only, Vehicle versus E2 + Low Levo, Vehicle versus Med Levo, and Vehicle versus High Levo group comparisons (Figure 14A), suggesting that there were no differences in learning between the groups. A main effect of Quadrant was seen for all group comparisons for the probe trial (Figure 14B), where percent swim distance was greater in the target NE quadrant compared to the opposite SW quadrant for Vehicle [$F_{(1,8)} = 46.365$, $p < 0.0001$], E2 Only [$F_{(1,8)} = 91.826$, $p < 0.0001$], E2 + Low Levo [$F_{(1,9)} = 41.596$, $p < 0.0001$], E2 + Med Levo [$F_{(1,9)} = 66.161$, $p < 0.0001$], and E2 + High Levo [$F_{(1,9)} = 62.705$, $p < 0.0001$] treatment groups. This indicates that all treatment groups were able to learn and spatially localize to the platform location by the end of testing.

Visible platform

For Trials 1-6 of the visible platform task, there was a main effect of Trial for each of the planned comparisons: Vehicle versus E2 Only [$F_{(5,80)} = 2.376$, $p < 0.05$; Figure 15], Vehicle versus E2 + Low Levo [$F_{(5,85)} = 3.341$, $p < 0.01$; Figure 15], Vehicle versus E2 + Med Levo [$F_{(5,85)} = 2.888$, $p < 0.05$; Figure 15], and Vehicle versus E2 + High Levo [$F_{(5,85)} = 2.953$, $p < 0.05$; Figure 15], indicating that there was a decrease in latency to find the platform across trials. There were no main effects of Hormone for the Vehicle versus E2 Only, Vehicle versus E2 + Low Levo, or Vehicle versus E2 + Med Levo comparisons, indicating that there were no treatment differences in visual and motor ability to solve a water escape task. There was a main effect of Hormone for the Vehicle versus E2 + High Levo comparison [$F_{(1,17)} = 4.785$, $p < 0.05$; Figure 15], whereby the Vehicle group exhibited a greater latency to platform than the E2 + High Levo group. However, when only Trials 4-6 were analyzed, there was no main effect of Hormone for this comparison, nor was there a main effect for any of the other planned comparisons during these latter trials. This indicated that in the latter portion of visible platform testing, when the rats were expected to have learned the components of the visible platform task, there were no treatment differences. In fact, by the last trial, Trial 6, the mean escape time of all rats combined was 5.79 seconds, demonstrating that they possessed the appropriate procedural components necessary to solve a water escape task.

Uterine horn weights

Uterine horn weights were expected to increase with E2 treatment and decrease with the addition of Levo, if Levo in fact attenuated E2-induced stimulation with the tested

regimen (E. B. Engler-Chiurazzi et al., 2012; Sarah E. Mennenga, Gerson, Koebele, et al., 2015; Prakapenka et al., 2018; Westerlind et al., 1998). There was a main effect of Hormone for the Vehicle versus E2 Only [$F_{(1,16)}$ = 210.607, p < 0.0001; Figure 16A], Vehicle versus E2 + Low Levo [$F_{(1,18)}$ = 145.429, p < 0.0001; Figure 16A], Vehicle versus E2 + Med Levo [$F_{(1,18)}$ = 194.697, p < 0.0001; Figure 16A], and Vehicle versus E2 + High Levo [$F_{(1,18)}$ = 420.794, p < 0.0001; Figure 16A] group comparisons, with the Vehicle group exhibiting lower uterine horn weights compared to each treatment group that contained an E2 component. These results suggest that all E2 treatments did stimulate the uterine horns compared to vehicle control, regardless of the presence of Levo. Interestingly, there was a main effect of Hormone for the E2 Only versus E2 + Med Levo comparison [$F_{(1,16)}$ = 6.305, p < 0.05; Figure 16B], and for the E2 Only versus E2 + High Levo comparison [$F_{(1,16)}$ = 5.313, p < 0.05; Figure 16B], with lower uterine horn weights seen with the E2 + Med Levo and E2 + High Levo treatments relative to E2 Only. This indicates that the medium and high doses of Levo were high enough to oppose E2's stimulating effects on the uterine horns. It must be noted, however, that while the E2 + Med Levo and the E2 + High Levo treatments reduced uterine horn weight relative to E2 Only treatment, both combinations still increased uterine horn weight relative to Vehicle suggesting that the addition of these doses of Levo did not result in complete protection from E2-induced uterine stimulation.

Discussion

The two hormones evaluated in the present study, E2 and Levo, can each enhance spatial learning and memory when given alone in middle-aged Ovx rats (B. Blair Braden

et al., 2017; Prakapenka et al., 2018). Here, dose ratio-dependent cognitive and uterine stimulating effects of three E2 plus Levo hormone combination treatments – 5:1 (E2 + Low Levo), 3:1 (E2 + Med Levo), and 1:2 (E2 + High Levo) E2 to Levo dose ratios – were evaluated in middle-aged Ovx rats by systematically increasing the dose of Levo while maintaining constant E2 dose across all treatments. The combination treatments included the dose ratio used in the clinically-available Climara Pro as well as the combination dose ratio from my prior work (Prakapenka et al., 2018). Overall, I discovered that although the addition of the two highest Levo doses that were tested herein (E2 + Med Levo and E2 + High Levo) decreased E2-induced stimulation at the uterine horns, these doses impaired spatial learning and memory. On the other hand, the addition of the low dose of Levo (E2 + Low Levo) had no impact on spatial learning and memory, and yielded no change in E2-induced stimulation at the uterine horns, replicating prior findings (Prakapenka et al., 2018).

Performance on the WRAM was divided into two phases, the acquisition phase and the asymptotic phase, to evaluate spatial working and reference learning and memory. All treatment groups performed similarly during the acquisition phase of the WRAM, when the rules of the task were being learned, across all three memory measures (WMC, WMI, RM). For the asymptotic phase, when the rules of the task should have been already learned, the E2 + High Levo treatment group, which contained a cognitively beneficial dose of Levo when given alone, made more WMC and WMI errors than the Vehicle, and tended to make more RM errors than the Vehicle. Of note, the E2 + High Levo group made more WMI errors than the Vehicle when working memory was most taxed, on the highest working memory load trial evaluated, for the asymptotic phase of the WRAM. These

findings suggest that the addition of a cognitively beneficial dose of Levo to E2 in a 1:2 E2 to Levo dose ratio impaired spatial working and reference memory. The E2 + Med Levo group, the dose ratio representative of the clinically available Climara Pro hormone therapy, tended to make more WMC errors than the Vehicle, as well as more WMI errors than the Vehicle when working memory was taxed, indicating a trend in impairment for this clinically-relevant hormone combination ratio. The E2 + Low Levo group that employed the same combination dose ratio as my prior study (Prakapenka et al., 2018), as well as the E2 Only group, did not differ in WMC, WMI, and RM errors made compared to the Vehicle during the asymptotic phase of the WRAM, indicating that these two treatment groups did not impact spatial working and reference memory. Collectively, results revealed that the incremental increase in Levo dose, when combined with the constant E2 dose, was associated with an increase in errors made for all three memory measures. Indeed, linear regression analyses confirmed that as Levo dose increased when combined with E2, animals tended to make more WMC, WMI, and RM errors on the asymptotic phase of the WRAM, resulting in progressively greater spatial working and reference memory impairment across increasing Levo dose. Furthermore, following a 6-hour delay period, the E2 + Med Levo and E2 + High Levo groups made more WMC errors on the post-delay trial relative to the baseline trial, suggesting delay-induced forgetting. These delay findings support the detrimental cognitive effects evidenced during the asymptotic phase of the WRAM, with treatments containing a constant E2 dose combined with a medium and a high Levo dose in a 3:1 and 1:2 E2 to Levo dose ratio, respectively. Taken together, the WRAM results indicate a dose ratio-dependent effect of the E2 plus Levo combination treatment on cognition where higher Levo, in combination with E2 and

with the parameters evaluated in this study, was associated with poorer spatial working and reference memory in middle-aged Ovx rats.

The molecular and signaling pathways in the brain are crucial players in successful learning and memory, as well as hormone-induced effects on learning and memory (Atkins et al., 1998; Frick, 2015; Koebele & Bimonte-Nelson, 2017). It is very likely that the cognitive effects evidenced on the WRAM in the present study are mediated by the effects that E2 and Levo can have on one or more of these pathways, and the dose-ratio dependent interaction between these effects. For instance, activated extracellular-signal regulated kinase 2 (Erk 2) is involved in, and required for, E2-induced beneficial memory effects (L. Fan et al., 2010; Fernandez et al., 2008). I have shown that activated Erk 2 expression in the frontal cortex of E2-treated animals positively correlated with spatial working memory, but that this relationship was attenuated by the addition of Levo to the E2 treatment (Prakapenka et al., 2018). Thus, E2 and Levo can interact to exhibit contrasting effects in the brain. Understanding how E2 and Levo interact in the brain, and the potential relationship between this interaction to cognitive outcomes, is complex. Studies have evaluated the role of Levo alone, as well as E2 plus Levo, on brain processes, but the findings from these studies are contradictory. On one hand, Levo is thought to have no estrogenic effects as Levo itself does not bind to the estrogen receptors (Kuhl, 2005). On the other hand, studies indicate that Levo may in fact yield estrogenic activity indirectly via the binding of its derivatives to estrogen receptors, or via its capability to increase estrogen receptors' mRNA expression (Jayaraman & Pike, 2014; Santillán et al., 2001). This Levo-mediated estrogenic activity may be working in concert with E2 to yield the learning and memory changes seen in this study, particularly since the cognitively

beneficial effects of estrogens are dependent on both estrogen dose (more estrogen is not always cognitively beneficial) as well as estrogen type, which vary in binding affinity to estrogen receptors (Engler-Chiurazzi et al., 2012; Koebele and Bimonte-Nelson, 2015; Prakapenka et al., 2018, *in review*). In neuron culture, the addition of Levo to E2 treatment did not attenuate the neuroprotective effects of E2 alone, and of Levo alone, as measured by neuron survival, which does not support the impairing cognitive effects found in the present study (Jayaraman & Pike, 2014). However, another study showed that in young adult Ovx rats, Levo treatment increased markers of cell proliferation in the hippocampus when combined with E2 treatment (1:1 E2 to Levo dose ratio), but, this same E2 plus Levo combination treatment also increased cell apoptosis (Liu et al., 2010). In this respect, the increasingly impairing spatial memory effects evidenced in the present study may, in part, be explained by the apoptotic effects of E2 plus Levo combination treatment. In sum, although much research has been done to gain insight into how Levo, and Levo together with E2, impact brain processes, the existing evidence to date is conflicting. Future studies are needed with the primary focus on systematically examining the putative drivers of the complex effects of Levo and E2 plus Levo treatments in the brain, how these effects map on to cognitive measures, and the role that the ratio of E2 and Levo doses plays in both brain and behavioral outcomes.

The classic water maze task to evaluate spatial reference memory, the MWM, did not reveal any differences between the treatment groups. For the probe trial, all treatment groups swam more distance in the target NE quadrant, the quadrant that previously contained a platform, compared to the opposite quadrant, a quadrant that never contained a platform, indicating that rats learned the task and spatially localized to the platform

location. The lack of differences between treatments on the MWM is consistent with my prior study evaluating E2 alone, Levo alone, and E2 plus Levo where no treatment effects were found on the MWM as well (Prakapenka et al., 2018). This may be in part due to the MWM being administered immediately after the WRAM, as the prior cognitive experience on the WRAM may have inadvertently impacted learning and memory effects on the MWM (A L Markowska, 2002; Talboom et al., 2014). There were also no treatment differences on the visible platform task, the control task intended to examine visual and motor capabilities of each treatment group to solve a water-escape task. Specifically, results revealed no difference in time to reach the visible platform between the vehicle and each treatment group during the latter three trials of testing. Indeed, all rats swam to the platform in under six seconds on the last trial of the task, demonstrating that the appropriate procedural components necessary to solve a water escape task were present across all treatment groups.

It should be addressed that the E2 treatment in the present study did not show cognitive benefit on the WRAM and on the MWM tasks, which does not support the expected beneficial role of E2 on cognitive function. This finding highlights the difficulty in identifying the parameters of exogenous E2 exposure that can consistently yield a cognitive benefit in a rodent model of menopause, a topic of much discussion and debate in the literature (Koebele and Bimonte-Nelson, 2015; Prakapenka et al., *in review*). In fact, whether exogenous E2 treatment will yield beneficial, null, or detrimental effects on hippocampal-based memory in rodent models involves multiple factors, which include the time between Ovx and E2 administration as well as the dose, regimen, and age of E2 administration (Barha & Galea, 2010; Heather A. Bimonte-Nelson et al., 2006; Jill M.

Daniel et al., 2006; Frick, 2009; R. B. Gibbs, 2000; Alicja L Markowska & Savonenko, 2002; Talboom et al., 2008). The lack of an E2 only treatment effect on spatial learning and memory evaluated within this study at the examined dose and regimen parameters confirm E2's complex effects on cognition.

Uterine horn weight can be used as a marker of hormone stimulation at the uterine tissue in rodents (E. B. Engler-Chiurazzi et al., 2012; Sarah E. Mennenga, Gerson, Koebele, et al., 2015; Prakapenka et al., 2018; Westerlind et al., 1998). As predicted and previously reported, uterine horn weight was greater in all E2-containing treatment groups compared to Vehicle (Prakapenka et al., 2018). In support, vaginal cytology evaluations confirmed the presence of E2 in all E2-containing treatment groups, and lack of hormone stimulation in the Vehicle group. The addition of the low dose of Levo did not counteract E2-induced uterine horn weight, replicating my prior findings with the same E2:Levo dose ratio (Prakapenka et al., 2018). Interestingly, the addition of the medium and of the high Levo doses reduced uterine horn weight, albeit not back to the weight of the Vehicle group which can in part be due to the possible indirect estrogenic activity of Levo, such as the observed increase in mRNA expression for estrogen receptors with Levo treatment or the ability of Levo derivatives to bind estrogen receptors (Jayaraman & Pike, 2014; Santillán et al., 2001). In the clinic, opposing uterine stimulation is the primary purpose for including the progestogen component in estrogen-containing hormone therapy for menopausal women. Here, I show that the addition of Levo doses that opposed the effects of a constant E2 dose on uterine horn weight also impaired spatial learning and memory. Taken together, findings from this study suggest that the progestogen component of hormone therapy is a critical

factor that must be considered for optimal results in terms of not only the progestogen's uterine effects, but also its effects on brain and cognitive outcomes.

In conclusion, the present findings provide clinically-relevant insights into the cognitive effects of E2 plus Levo hormone combination treatments using a rodent model of surgical menopause. At the evaluated doses of E2 and of Levo, long-term E2 plus Levo combination treatment did not provide a cognitive benefit. Furthermore, the E2 plus Levo hormone combination treatments with a 3:1 and 1:2 E2 to Levo dose ratio impaired spatial learning and memory compared to vehicle control treatment. In fact, there was a linear dose-response relationship, where an increasing Levo dose in combination with a constant E2 dose resulted in increasingly detrimental cognitive effects. Yet, the same E2 plus Levo hormone combination treatments of a 3:1 and 1:2 E2 to Levo dose ratio reduced E2-induced stimulating effects at the uterine horns. Provided that women do take estrogen plus progestogen hormone therapy when the uterus is present, and that the addition of Levo to E2, a clinically available hormone combination, reduced E2-induced uterine stimulation at the hormone combination dose ratios that also impaired cognition, the search must continue for estrogen plus progestogen parameters that would yield optimal cognitive effects alongside uterine effects. Indeed, the progestogen is a necessity in hormone therapy in terms of its protection to the uterus, and additional knowledge regarding individual hormone and combination hormone treatments and their specific cognitive, neural, and uterine effects is required to harness the full potential and beneficial effects of progestogens as a valuable component in hormone therapy for menopausal women.

CHAPTER 4

THE COGNITIVE EFFECT OF 17β-ESTRADIOL POLY (LACTIC-CO-GLYCOLIC ACID) NANOPARTICLE TREATMENT IN MIDDLE-AGED OVARIECTOMIZED RATS

This chapter will be formatted and submitted to Behavior Research Methods. Included in this chapter are citations of a review paper that I was invited to write on the formulation parameters for poly (lactic-co-glycolic acid) micro- and nano- carriers, such as composition, molecular weight, and type of solvent used, and how they can be altered to systematically manipulate the pharmacokinetic and pharmacodynamic profiles of 17β-estradiol. This review was published as part of a special edition on Reproductive tissue engineering in Annals of Biomedical Engineering (Prakapenka, Bimonte-Nelson, & Sirianni, 2017).

Contribution: I am the first author of this document and was the primary graduate student PI on this project. Under the mentorship of Heather A. Bimonte-Nelson and Rachael W. Sirianni, I designed and carried out the study with the assistance of our laboratory teams.

Introduction

The use of poly (lactic-co-glycolic) acid (PLGA) nanoparticles as drug carriers to improve a drug's therapeutic efficacy has been extensively investigated across a variety of drugs and applications over the last several decades (Chasin & Langer, 1990; Cook et al., 2015; Danhier et al., 2012; Fazil, Shadab, Baboota, Sahni, & Ali, 2012; Mir, Ahmed,

& Rehman, 2017; Prakapenka et al., 2017). The PLGA polymer is approved by the U.S. Food and Drug Administration (FDA) for use in the clinic and it is considered to be of generally low toxicity as well as biocompatible and biodegradable (J. M. Anderson & Shive, 1997; Chasin & Langer, 1990; Danhier et al., 2012). In fact, since as early as 1974, PLGA-based materials – such as surgical sutures – have been used in clinical settings (Conn, Oyasu, Welsh, & Beal, 1974; Ulery, Nair, & Laurencin, 2011). Once a drug is encapsulated in PLGA nanoparticles, its activity following systemic administration can be modulated by modifying the release of the drug from the nanoparticles (Chasin & Langer, 1990; Prakapenka et al., 2017). Furthermore, the size as well as the surface of PLGA nanoparticles can be altered to achieve desired interactions between PLGA nanoparticles and tissue compartments, targeting drug activity to those tissue compartments (Kreuter, 2014). Indeed, there are several methods for synthesizing PLGA nanoparticles, and by altering various formulation parameters of the synthesizing process (e.g. PLGA composition, molecular weight, type of solvents used), it is possible to achieve specific particle size, drug loading, rate of degradation, and drug release (Makadia & Siegel, 2011; Mir et al., 2017; Prakapenka et al., 2017). Although a plethora of studies exist that evaluate and characterize both the fabrication of drug-encapsulated PLGA nanoparticles and how specific parameters contribute to certain desired outcome measures, behavior outcome measures (e.g. learning and memory) of drug-encapsulated PLGA nanoparticles have not been as heavily investigated. Of note, a limited number of studies have encapsulated the ovarian-derived hormone 17β-estradiol (E2) in PLGA nanoparticles, and to the best of my knowledge, no studies so far have evaluated E2

PLGA nanoparticle treatment effects on spatial learning and memory outcomes (Prakapenka et al., 2017).

It is understood that ovarian-derived hormones, including E2, can impact learning and memory (Koebele & Bimonte-Nelson, 2017; Victoria N. Luine, 2014). When considering the estrogens found endogenously in women and in rats, E2 is the most potent (Kuhl, 2005). Interestingly, only roughly 1-2% of systemically circulating E2 is freely available to have activity, as it often appears bound to serum hormone binding globulin or albumin (D. C. Anderson, 1974; Kuhl, 2005). Additionally, when E2 is exogenously administered, it is metabolized and cleared rapidly from the system (Kuhl, 2005). Nevertheless, across a woman's lifespan, E2 is heavily involved in reproductive cycle regulation, as well as development and maintenance of the reproductive system. At menopause, a woman will experience a decrease in ovarian-derived hormone levels, including E2, and she may experience several undesired symptoms (e.g. vasomotor symptoms, cognitive changes; NAMS, 2014). Hormone therapy can be prescribed to combat menopause-associated symptoms, and it commonly contains E2 as the estrogen component. In women with a uterus present, all E2-based hormone treatments must be accompanied by an opposing progestogen to offset undesired E2 exposure in the uterine tissue (NAMS, 2017). Studies evaluating learning and memory have shown that, following ovarian hormone loss, E2 can have a cognitively beneficial role in humans, and when evaluating these effects in rodent models of menopause (Frick, 2015; Koebele & Bimonte-Nelson, 2015; Korol & Pisani, 2015; Maki, 2012; S. E. Mennenga & Bimonte-Nelson, 2013; Sherwin, 2006). The required addition of an opposing progestogen, however, can attenuate the cognitively beneficial role of E2, limiting the therapeutic potential of E2

(Heather A. Bimonte-Nelson et al., 2006; L. L. Harburger et al., 2009; Lauren L. Harburger et al., 2007; Lowry et al., 2010). Thus, although E2-containing hormone therapies are FDA-approved, safe, and efficacious, E2 delivery may be optimized to achieve greater therapeutic potential of the hormone, such as enhanced beneficial effects on cognition. Indeed, several studies have begun exploring strategies for E2 delivery optimization, and have characterized effective E2 encapsulation in PLGA nanoparticles, resulting in distinct pharmacokinetic and pharmacodynamic profiles that are different than that of free E2 (Prakapenka et al., 2017). E2 PLGA nanoparticles can be further modified to increase E2 delivery to the brain (Mittal, Carswell, Brett, Currie, & Kumar, 2011), and thus potentially maximize the beneficial cognitive effects while reducing undesired peripheral effects when compared to free E2. However, as the effects of E2 PLGA on spatial learning and memory are currently unknown, it is critical to first confirm that the beneficial cognitive effects of E2 when encapsulated in PLGA nanoparticles can be achieved following the loss of ovarian hormones, and that blank PLGA nanoparticles do not impact learning and memory, prior to pursuing further engineering strategies to achieve brain-targeted delivery of E2 for cognitive therapy.

In the present study, E2 was encapsulated in PLGA nanoparticles and assessed for effects on spatial learning and memory. Specifically, middle-aged rats underwent ovariectomy (Ovx), whereby the ovaries were surgically removed, to achieve a blank circulating ovarian-derived hormone profile. Then, rats received weekly subcutaneous oil-control, free E2, blank PLGA, or E2 PLGA treatment, and were tested on a behavioral battery evaluating spatial learning and memory. Uterine stimulation was determined at the end of the study by comparing uterine horn weights. I hypothesized that weekly delivery

of E2 from PLGA nanoparticles would enhance cognition in middle-aged, Ovx rats relative to free E2, and relative to blank PLGA nanoparticles. I did not anticipate vehicle differences between oil-control, the vehicle for free E2, and blank PLGA nanoparticles, the vehicle for E2 PLGA.

Methods

Materials

All reagents for nanoparticle preparation and characterization, including dichloromethane (DCM), poly(vinyl alcohol) (PVA), methanol, phosphate buffered saline (PBS), and dimethyl sulfoxide (DMSO), were obtained from Sigma-Aldrich. 50:50 poly(DL-lactide-co-glycolide) (PLGA, ester terminated) was purchased from Durect Corporation (Cupertino, CA, USA) and 17β-estradiol (E2) was purchased from Sigma-Aldrich.

PLGA nanoparticle preparation

PLGA nanoparticles were prepared under endotoxin free conditions using the single-emulsion technique that was previously outlined by McCall and Sirianni (2013). Briefly, to make blank PLGA nanoparticles (blank-PLGA), 200 mg of PLGA was dissolved in 2 mL of DCM:methanol (4:1) and added drop-wise to 5% PVA, the aqueous phase, on vortex to create an emulsion. The emulsion was then ultrasonicated on ice 3 times in 10 second intervals (40% amplitude, Fisher Scientific Model 705 Sonic Dismembrator), added to 84 mL of 0.3% PVA, and allowed to harden over 3 hours. Particles were then washed 3 times with endotoxin free water by centrifugation at 25,000

rcf for 20 minutes at 4^0C (Beckman L8-80M Ultracentrifuge, F0630 rotor). Prior to lyophilization and storage (at -80^0C), 75 mg of trehalose was added to the particles. To prepare E2-containing PLGA nanoparticles (E2-PLGA), 12 mg of E2 and 200 mg of PLGA were dissolved in 2 mL of DCM:methanol (4:1) and processed using the same technique as blank-PLGA.

Nanoparticle characterization

Size and morphology of blank-PLGA and E2-PLGA nanoparticles were determined using scanning electron microscopy (SEM, FEI XL30) following the protocol described by McCall & Sirianni, 2013. The average diameter of each nanoparticle batch was measured using ImageJ (National Institutes of Health). The hydrodynamic diameter and polydispersity for the nanoparticles was determined using dynamic light scatting (DLS, NanoBrook 90Plus Zeta particle analyzer, Brookhaven Instruments, Hotsville, NY).

To calculate E2 concentration within E2-PLGA, E2-PLGA nanoparticles were dissolved in DMSO at 1 mg/mL and the fluorescence of the sample was compared to a control curve constructed using blank-PLGA nanoparticles spiked with known E2 concentrations (280 nm/310 nm excitation/emission). E2 loading was then determined by dividing E2 concentration by PLGA concentration. A release profile for E2 was obtained by adding 10 mg/mL E2-PLGA in 1x PBS to Slide-A-Lyzer Dialysis Cassette (3,500 MWCO) and submerging the cassette in 4 L of 37^0C 1x PBS release medium. For each time point, 20 μL of E2-PLGA sample were added to 180 μL of DMSO to obtain a 1 mg/mL concentration, and the fluorescence of the samples was read in triplicate (280

nm/310 nm excitation/emission). The release medium was changed at 2.5, 7.5, and 21.5 hours to assure sink conditions.

Animals

Fischer-344 CDF 11-month old virgin female rats were ordered from the National Institute on Aging, Harlan Laboratories (Indianapolis, IN). Food and water were *ad libitum* and a 12-hour light/dark cycle was imposed. Rats were pair-housed throughout the duration of the study. All procedures were approved by the Arizona State University IACUC and adhered to the standards set by the National Institutes of Health. Figure 18 depicts the timeline of experimental manipulations throughout the duration of the behavior study.

Ovariectomy (Ovx)

Ovx surgery was performed on all rats under anesthesia using acute isoflurane inhalation as previously done (B. Blair Braden et al., 2017; S. E. Mennenga et al., 2015; Prakapenka et al., 2018). Briefly, rats received dorsolateral incisions to the skin and muscle, each ovary was removed following ligature to the tip of the uterine horn, and the skin and muscle were sutured closed. All rats received a subcutaneous injection of Rimadyl (5 mg/mL/kg) for pain prior to start of surgery, as well as a subcutaneous injection of saline (2 mL) to prevent dehydration.

Treatment administration

Twenty-one days after Ovx, weekly subcutaneous treatment administration was initiated. Twenty rats were randomly assigned to receive 0.1 mL of either sesame oil (oil-

control, n = 10) or 3 μg of free E2 (free-E2, n = 10) in sesame oil, and twenty rats were randomly assigned to receive 0.1 mL of blank-PLGA (polymer weight matched to E2-PLGA, n = 10) suspended in saline or 3 μg of E2 encapsulated in PLGA (E2-PLGA, n = 10) suspended in saline. The oil-control and blank-PLGA treatments served as the vehicle controls in this study.

Behavioral assessment of cognitive function

Water radial-arm maze (WRAM)

On the day of the fourth treatment administration, all animals were tested on the win-shift WRAM task for 13 days to evaluate spatial working and reference memory (H. A. Bimonte-Nelson, 2015d; Bimonte & Denenberg, 1999; B. Blair Braden et al., 2017; S. E. Mennenga & Bimonte-Nelson, 2015; Sarah E. Mennenga, Koebele, et al., 2015). The maze was located in a room that was set up with abundant spatial cues to aid in spatial navigation. This maze contained 8 arms, with 38.1 cm x 12.7 cm dimensions for each arm and was filled with opaque water kept at 18-20^{o}C. Hidden platforms (10 cm in diameter) were submerged in 4 out of the 8 arms. A pre-determined set of platform locations, which were kept fixed throughout all 13 days of testing, were randomly assigned to each animal. An animal was allowed 3 minutes per trial to find a platform. The trial started when the animal was dropped off at the start arm location and the trial ended when the animal either found or was led to the platform (after 3 minutes passed). Animals were kept on the platform for 15 seconds after which they were placed in a heated testing cage. During the 30 second inter-trial interval (ITI), the just-found platform was taken out of the maze and the water was cleaned with a fishnet to remove debris and redistribute any olfactory cues;

the next trial was started at the end of the ITI. In this manner, there is one trial per platform (4 trials per day), resulting in increased strain on the memory system with each additional platform that is found and removed, leading to an increase in working memory load as trials progress. A 6-hour delay was implemented between trials 2 and 3 on the last day of testing, day 13, to assess delayed memory retention. To examine performance on the WRAM, error arm entries were recorded and scored on orthogonal measures of reference and working memory. A reference memory (RM) error was defined as the first entry, within a day, into a non-platformed arm. A working memory incorrect (WMI) error was defined as re-entry, within a day, into a non-platformed arm. A working memory correct (WMC) error was defined as entry, within a day, into an arm that was previously platformed.

Morris water maze (MWM)

MWM testing started the day following completion of WRAM testing to examine spatial reference memory performance, and continued for 5 days (H. A. Bimonte-Nelson, 2015b; Talboom et al., 2014, 2008). The maze was a 188 cm in diameter circular tub located in a room filled with spatial cues to aid in spatial navigation. One platform (10 cm in diameter) was submerged in 18-20^{o}C opaque water in the northeast quadrant of the tub; the platform location was kept constant for all trials across each day of testing. There were four starting locations (north, south, east, or west), one per trial with a total of 4 trials per day. The trial started when an animal was dropped off at one of these locations and the trial was completed when the animal either found the platform or was led to the platform, after the maximum trial time of 60 seconds. Each animal was kept on the platform for 15 seconds

and then returned back to a heated cage. The ITI was 5-8 minutes. The Ethovision tracking system (Noldus Instruments, Wageningen, The Netherlands) was used to determine each animal's swim distance. A probe trial was added on the last day of testing as an additional 5^{th} trial to examine spatial localization, during which the platform was removed and each animal's swim distance in each quadrant during a total of 60 seconds was measured.

Visible platform

The one-day visible platform task, initiated the day after MWM testing, was used to evaluate the motor and visual ability of each animal to complete a water-escape maze task (Bimonte-Nelson, 2015a; Mennenga et al., 2015a, 2015c). The maze was a 100 cm x 60 cm rectangular tub that was filled with 18-20^{o}C clear water, and one black platform (10 cm in diameter) was placed in the tub so that it would be 4 cm above the surface of the water. All obvious spatial cues surrounding the maze were blocked using a curtain. Each animal was allowed 90 seconds per trial to find the platform, the location of which varied semi-randomly between trials, with a total of 6 trials and an ITI of 5-8 minutes. The trial started when an animal was dropped off from a set location and the trial ended when the animal either found the platform or was led to the platform after 90 seconds. Each animal was given 15 seconds on the platform before being placed into a heated testing cage.

Confirming systemic presence of E2

Blood serum analysis

Double antibody liquid-phase radioimmunoassay (Beckman Coulter, Brea, CA), performed by the Core Endocrinology Laboratory of the Pennsylvania State University,

College of Medicine, was used to determine circulating E2 and estrone, a metabolite of E2, levels in blood serum (Engler-Chiurazzi et al., 2012; Koebele et al., 2017; Sarah E. Mennenga et al., 2015c; Prakapenka et al., *in review*). At sacrifice, blood was collected via cardiocentesis following euthanasia with isoflurane on the day of the 7th treatment injection in the same order as behavior testing occurred. To obtain serum, blood was allowed to clot at 4°C (Vacutainer 367986, Becton Dickinson and Company, Franklin Lakes, NJ, USA) and then centrifuged for 20 minutes at 3000 rpm at 4°C. Serum was stored at -20°C until radioimmunoassay analysis. E2-specific antibodies were used with ^{125}I-labeled E2 as the tracer for the E2 assay with a functional sensitivity of 4 pg/ml. Inter-assay coefficients of variation at a mean level of 6 pg/ml E2 averaged 8%. Estrone-specific antibodies were used with ^{125}I-labeled estrone as the tracer for the estrone assay with a functional sensitivity of 16 pg/ml. Inter-assay coefficients of variation for estrone at a mean level of 90 pg/ml averaged 11%.

Uterine horn weights

Ovarian hormones impact uterine horn weight (E. B. Engler-Chiurazzi et al., 2012; Sarah E. Mennenga, Gerson, Koebele, et al., 2015; Prakapenka et al., 2018; Westerlind et al., 1998). To obtain uterine horn weight, uterine horns were removed and trimmed of visible fat at sacrifice; the wet weight of uterine horns was then used to confirm Ovx and to assess E2 exposure.

Statistical analyses

For all statistical analyses, alpha was set at $p < 0.05$. Repeated measures ANOVA

was run separately to test for a main effect of Day and Treatment x Day interaction on the WRAM and on the MWM to determine learning. For the WRAM, each memory measure (WMC, WMI, and RM errors) was evaluated across days 1-12 of testing. For the MWM, Total Swim Distance was evaluated across days 1-5 of testing. Additionally, for the probe trial, each treatment group was analyzed separately using a repeated measures ANOVA to evaluate percent swim distance in the previously platformed quadrant (Northeast; NE) compared to percent swim distance in the directly opposite quadrant (Southwest; SW). For the visible platform, a repeated measures ANOVA was run to test for a main effect of Trial, a main effect of Treatment, and a Trial x Treatment interaction to assess learning and the ability to perform a water-escape task for all treatment groups.

For WRAM treatment effects, WRAM testing was split into three blocks, Block 1 (days 2-5), Block 2 (days 6-9), and Block 3 (days 10-12). If the vehicle control groups (oil-control and blank-PLGA) did not differ, I expected to collapse the two groups into one vehicle group. Thus, a planned comparison repeated measures ANOVA was run for each Block to directly compare oil-control and blank-PLGA treatments to evaluate vehicle effects. Block 3 did in fact exhibit an effect of Vehicle, and Vehicle x Trial interaction, for WMC and WMI measures, discussed in detail in the results section. Due to the Vehicle affect, additional analyses included planned comparison to only compare free-E2 to its respective vehicle control (oil-control) and E2-PLGA to its respective vehicle control (blank-PLGA) for WMC, WMI, and RM errors made on each Block of testing. In the case of a significant Treatment x Trial interaction, Trial 3 and Trial 4 were analyzed separately to evaluate performance on the moderate and high working memory load trials, respectively. For all analyses, Vehicle or Treatment were the independent variables and

Trials nested within Days were the repeated measures. For WRAM delay, errors made on the WMC, WMI, and RM memory measures on the post-delay trial, Trial 3 on Day 13, were compared to the baseline trial, Trial 3 on Day 12, within each treatment group as was done previously (B. Blair Braden et al., 2017). For MWM, planned comparisons were done to analyze Total Swim Distance between free-E2 and oil-control as well as E2-PLGA and blank-PLGA across the 5 days of testing. Treatment was set as the independent variable and Trials nested within Days were set as the repeated measures.

Planned comparison one-way ANOVAs were used separately for E2 and estrone blood serum levels and uterine horn weights to directly compare blank-PLGA and oil-control (vehicle effect), free E2 and oil-control, E2-PLGA and blank-PLGA, as well as E2-PLGA and free E2. Treatment was set as the independent variable and E2 Level, Estrone Level, and Uterine Horn Weight were set as the dependent variables.

Results

Nanoparticle characterization

E2 loading in PLGA nanoparticles was 4.45%. SEM image analysis revealed that the blank-PLGA and E2-PLGA nanoparticles were spherical with an average diameter of 175 ± 34 nm and 158 ± 30 nm, respectively (Figure 17A). DLS analysis revealed that the average hydrodynamic diameter for blank-PLGA and E2-PLGA nanoparticles was 232 ± 2.1 nm and 256 ± 2.3 nm, respectively, and the polydispersity index, a measure of molecular mass distribution, for blank-PLGA and E2-PLGA nanoparticles was 0.105 ± 0.04 and 0.158 ± 0.02, respectively (Figure 17C). All measurements are expressed as mean ± standard deviation. The surface charge for both batches of nanoparticles was close to

neutral, -1.91 for blank-PLGA and -0.59 for E2-PLGA (Figure 17C). The release profile of E2 from PLGA nanoparticles exhibited a rapid release of E2 within the first 8 hours (~80% of E2 released), followed by a slow sustained release. Completed release of E2 was observed after about 48 hours of incubation (Figure 17B).

Behavioral assessment of cognitive function

Water radial-arm maze (WRAM)

The WRAM measures spatial working and spatial reference memory. For each memory measure, there was a main effect of Day, indicating learning of the WRAM as illustrated by a decrease in WMC [$F_{(11,36)} = 5.676$, $p < 0.0001$; Figure 19], WMI [$F_{(11,36)} = 9.840$, $p < 0.0001$; Figure 19], and RM [$F_{(11,36)} = 7.816$, $p < 0.0001$; Figure 19] errors across days 1-12 of testing. There were no significant Treatment x Day interactions for each memory measure, demonstrating that WRAM learning for all days of testing did not differ based on treatment groups.

First, performance on Blocks 1, 2, and 3 of the WRAM was evaluated between the two vehicle control groups, oil-control and blank-PLGA, across the three memory measures, WMC, WMI, and RM. No main effects of Vehicle, or Vehicle x Trial interactions, were seen for WMC, WMI, and RM errors made on Blocks 1 and 2. However, results for the WMC measure on Block 3, the asymptotic phase evaluating memory retention, revealed a significant main effect of Vehicle [$F_{(1,18)} = 8.170$, $p < 0.05$; Figure 20A] and a Vehicle x Trial interaction [$F_{(2,36)} = 7.803$, $p < 0.01$; Figure 20A], where the oil-control group made more errors than the blank-PLGA group. Further analyses indicated that this Vehicle effect was particularly pronounced on Trial 4 [$p < 0.01$], the highest

working memory load trial evaluated on this version of the WRAM. For the WMI measure on Block 3, there was a marginal effect of Vehicle [$F_{(1,18)} = 4.296$, $p = 0.05$; Figure 20B] and a significant Vehicle x Trial interaction [$F_{(3,54)} = 4.056$, $p < 0.05$; Figure 20B] where the oil-control group tended to make more errors than the blank-PLGA group, and this effect was significant on the highest working memory load trial, Trial 4 [$p < 0.05$]. There was no significant effect of Vehicle and no Vehicle x Trial interaction for the RM measure on Block 3 (Figure 20C). Taken together, the two control vehicles exhibited significantly different effects on spatial working memory on Block 3, the asymptotic phase of the WRAM when rules of the task should be learned.

Following analysis of vehicle effects, planned comparisons were performed only to evaluate E2-PLGA versus its vehicle (blank-PLGA) as well as free E2 versus its vehicle (oil-control). This analysis revealed no main effect of Treatment, but a significant Treatment x Trial interaction, for WMC errors on Block 1 of testing [$F_{(2,36)} = 4.486$, $p < 0.05$; Figure 21A], or the acquisition phase during which the rules of the task were being learned, between E2-PLGA and blank-PLGA groups where the E2-PLGA group made fewer WMC errors on Trial 3, the moderate working memory load trial, compared to the blank-PLGA group [$p < 0.05$; Figure 21B]. There were no Treatment differences and no Treatment x Trial interactions on Blocks 2 and 3 for WMC measures (data not shown), as well as Blocks 1, 2 and 3 for WMI and RM measures, between E2-PLGA and blank-PLGA groups (see Figure 21A for Block 1 Treatment x Trial interactions). There were no Treatment differences and no Treatment x Trial interactions on Blocks 1, 2, and 3 for WMC, WMI, RM measures between free E2 and oil-control groups (data not shown). Thus, a main effect of vehicle was observed where the blank PLGA nanoparticle group improved

spatial working memory compared to the sesame oil group. Additionally, data suggest that the E2-PLGA treatment did enhance spatial working memory compared to its blank-PLGA control during the acquisition phase (Block 1) of the WRAM, but only when working memory load was moderate.

On day 13 of WRAM, a 6-hour delay was implemented between Trials 2 and 3. Errors made on the baseline trial, Trial 3 on Day 12, were compared errors made on the post-delay trial, Trial 3 on Day 13, within each treatment group. For WMC errors, oil-control [$F_{(1,9)} = 6.612$, $p < 0.05$; Figure 22], free E2 [$F_{(1,9)} = 18.447$, $p < 0.01$; Figure 22], E2-PLGA [$F_{(1,9)} = 6.688$, $p < 0.05$; Figure 22] treatment groups made errors on the delay trial compared to the baseline trial, indicating delay-induced forgetting. The blank-PLGA group appeared to make more WMC errors on the delay trial compared to the baseline trial, although this did not reach statistical significance. For WMI and RM errors, there were no differences within each treatment group between errors made on the delay trial compared to the baseline trial (data not shown).

Morris water maze (MWM)

Analysis of spatial reference memory performance across the five days of testing on the MWM revealed a main effect of Day, with decreasing Total Swim Distance scores across days [$F_{(4,36)} = 78.929$, $p < 0.0001$; Figure 23A]. The groups had a similar learning profile across days as there was no Day x Treatment significant effect. Planned comparisons revealed a marginal effect of treatment, and no Treatment x Day interaction, across the 5 days of testing between the blank-PLGA group and the E2-PLGA group [$F_{(1,18)} = 3.181$, $p < 0.1$; Figure 23B], suggesting that there was a trend for the E2-PLGA group to

swim a shorter distance to the platform than the blank-PLGA group. There was no effect of Treatment and no Treatment x Day interaction across the 5 days of testing between the oil-control and free E2 groups. For the probe trial, oil-control [$F_{(1,9)} = 61.563$, $p < 0.0001$], free E2 [$F_{(1,9)} = 14.357$, $p < 0.01$], blank-PLGA [$F_{(1,9)} = 69.291$, $p < 0.0001$], and E2-PLGA [$F_{(1,9)} = 108.477$, $p < 0.0001$] groups swam a greater percent distance in the target northeast quadrant where the platform was previously located compared to the opposite southwest quadrant that never contained the platform, suggesting that each treatment group was able to spatially localize to the platform location (data not shown).

Visible platform

The visible platform task was used to confirm visual and motor capability to complete a water-escape maze task. Across all six trials, there was a main effect of Trial [$F_{(5,36)} = 3.225$, $p < 0.01$], and the average latency to platform for all subjects across the six trials was 8.9 seconds (data not shown). There was no significant Trial x Treatment interaction and a marginal Treatment effect for all six trials [$F_{(3,36)} = 2.658$, $p < 0.1$]. No Treatment effect was seen for the last three trials tested, suggesting that all groups exhibited similar ability to perform on a water-escape maze task, especially by the latter half testing.

Confirming systemic presence of E2

Blood serum analysis

Blood serum levels of E2 and estrone were obtained to determine circulating levels of free E2 and its ability to be metabolized following weekly subcutaneous administration. Specifically, blood serum was collected in order of behavior testing the day of the 7th

weekly treatment administration. Planned comparison analyses revealed a main effect of Treatment for E2 levels between blank-PLGA and E2-PLGA groups [$F_{(1,18)} = 6.475$, $p < 0.05$; Figure 24A] as well as a main effect of Treatment for E2 levels between oil-control and free E2 groups [$F_{(1,18)} = 6.316$, $p < 0.05$; Figure 24A], where the E2-PLGA and free E2 groups had higher E2 levels than their respective controls. There was a main effect of Treatment for estrone levels between blank-PLGA and E2-PLGA groups [$F_{(1,18)} = 20.451$, $p < 0.001$; Figure 24B] and a main effect of Treatment for estrone levels between oil-control and free E2 groups [$F_{(1,18)} = 11.153$, $p < 0.01$; Figure 24B], where the E2-PLGA and free E2 groups had higher estrone levels than their respective controls. There were no Treatment effects between the E2-PLGA and free E2 groups in E2 levels or in estrone levels, suggesting that there was no difference in circulating free E2, or in its metabolism to estrone, between free versus nanoparticle encapsulated weekly subcutaneous administration of E2.

Uterine horn weight

Uterine horn weights were obtained the same day as blood serum as a marker for uterine stimulation as a function of treatment. There was a main effect of Treatment between the blank-PLGA and E2-PLGA groups [$F_{(1,18)} = 9.52$, $p < 0.01$; Figure 25] as well as a main effect of Treatment between oil-control and free E2 groups [$F_{(1,18)} = 31.458$, $p < 0.0001$; Figure 25], where the E2-PLGA and free E2 groups had higher uterine horn weights than their respective controls. Interestingly, there was also a main effect of Treatment between the E2-PLGA and free E2 groups [$F_{(1,18)} = 6.230$, $p < 0.05$; Figure 25], where the E2-PLGA group had higher uterine horn weights than the free E2 group,

indicating that E2 encapsulated in nanoparticles resulted in greater uterine horn stimulation than free E2.

Discussion

The present study evaluated the cognitive and uterine effects of E2 PLGA nanoparticle treatment relative to free E2, as well as blank PLGA nanoparticle treatment in middle-aged, Ovx rats. E2 encapsulation in PLGA nanoparticles enhanced spatial working memory on the WRAM, and exhibited a trend for enhanced spatial reference memory on the MWM compared to blank PLGA nanoparticles. Interestingly, there was a vehicle effect, whereby the blank PLGA nanoparticle group made fewer errors than the oil-control group, suggesting that either the blank PLGA nanoparticle treatment improved memory or the oil-control group impaired memory on the WRAM. The experiment as designed cannot determine which interpretation of the vehicle effect is supported; however, future research could decipher which interpretation of the vehicle effect would be supported. Consequently, E2 PLGA nanoparticle versus free E2 treatment effects on learning and memory could not be directly evaluated in this study. It is important to note that all groups performed similarly on the control visible platform task, with an average time to platform across all six trials of 8.9 seconds. When peripheral exposure to E2 was assessed, both E2-treated groups exhibited increased uterine horn weight compared to their respective vehicle controls, and E2 PLGA nanoparticle treatment had greater uterine horn weight than free E2 treatment, indicating potentially increased or sustained exposure of E2 at the uterine horns with nanoparticle encapsulation.

The WRAM is often used within the Bimonte-Nelson laboratory's behavioral battery to evaluate hormone effects on spatial working and reference memory in middle-

aged, Ovx rats (Acosta et al., 2010; B. Blair Braden et al., 2017; Prakapenka et al., 2018). In the present study, all rats learned the task and their learning trajectories across the 12 days of testing did not differ by treatment. WRAM performance was further evaluated across Blocks 1 (days 2-5), 2 (days 6-9), and 3 (days 10-12) of testing. Intriguingly, a vehicle effect was observed on Block 3 of testing, the asymptotic phase evaluating spatial memory retention. This effect revealed that blank PLGA nanoparticle treatment, the vehicle for E2 PLGA nanoparticle treatment, resulted in fewer WMC and WMI errors made compared to the oil-control treatment, the vehicle for free E2 treatment; the vehicle effect was primarily driven by performance on Trial 4, when working memory demand was highest. Following a 6-hour delay, analyses revealed that all treatment groups exhibited delay-induced forgetting on the WMC measure, although the blank PLGA treatment group did not reach statistical significance on the delay trial compared to the baseline trial, further supporting vehicle-specific differences on spatial working memory. The vehicle effect was surprising, as I did not anticipate the blank PLGA nanoparticles, suspended in sterile saline, to impact spatial learning and memory differently from the sesame oil. For instance, cholesterol can be neuroprotective against corticosterone-induced reduction in apical dendritic branching in the CA3 region of the hippocampus in gonadectomized male and female rats and sesame oil can reduce cholesterol levels, suggesting potential impairing effects of sesame oil (Ortiz et al., 2013; Satchithanandam et al., 1996). However, a thorough literature search indicated that on spatial memory measures, saline and sesame oil did not differ in effects and were typically combined as one control group for treatment comparisons (Abdulla, Calaminici, Stephenson, & Sinden, 1993; Tarbali & Khezri, 2016). Assuming similar effects of saline and sesame oil on

learning and memory, the PLGA characteristics may be the ones at play in the distinct vehicle effects on memory evidenced in the present study. Indeed, following systemic administration, PLGA degrades into lactic acid and glycolic acid, and recent studies suggest that lactic acid plays a critical role in long-term memory formation, spatial working memory, as well as neuroplasticity (Newman, Korol, & Gold, 2011; S. Sun, Li, Chen, & Qian, 2017; Suzuki et al., 2011; Yang et al., 2014). Additionally, PLGA nanoparticles may elicit a foreign body immune response following administration (Fournier, Passirani, Montero-Menei, & Benoit, 2003; Semete et al., 2010), and the immune system is involved in maintaining spatial learning and memory (Yirmiya & Goshen, 2011; Ziv et al., 2006). However, future studies are needed to systematically address these hypotheses. Existing studies evaluating drug-encapsulated PLGA nano- and micro-particle effects on learning and memory that included both saline as well as blank nanoparticle control groups revealed no vehicle effects on cognitive performance on the passive-avoidance task (Khalin et al., 2016; Wang et al., 2007). A recent study that included both saline and blank PEGylated poly(anhydrite) nanoparticle control groups on the spatial MWM did not report statistical analyses for the vehicle comparison even though the learning curves between the saline and blank nanoparticle groups do appear to be different (Moreno et al., 2018). Importantly, many of the studies that evaluated drug-encapsulated nanoparticle effects on learning and memory did not include both a saline and a blank nanoparticle control group; thus, based on these studies it cannot be definitively concluded whether or not the addition of the blank nanoparticle impacts memory differently from the saline control (Chu, Tian, Liu, Li, & Li, 2007; S. Fan et al., 2018; Joshi, Chavhan, & Sawant, 2010; Sánchez-López et al., 2018; D. Sun et al., 2016; Tiwari et al., 2014). Taken together, findings from the present study

underscore the critical importance of including proper controls and statistical analyses when evaluating novel delivery systems as it is possible that the addition of a seemingly inert carrier may indeed influence learning and memory outcomes on its own.

Due to the exhibited vehicle effects, WRAM performance was further evaluated only to compare E2 PLGA treatment relative to its control, as well as free E2 treatment relative to its control. On Block 1 of testing – the acquisition block, when rats were learning the rules of the task – a trial by treatment interaction revealed that E2 PLGA nanoparticle treatment resulted in fewer WMC errors relative to blank PLGA nanoparticle treatment on Trial 3, when working memory load was moderate. These findings are consistent with literature, and support a cognitively beneficial role of E2 following a decrease in circulating levels of ovarian-derived hormones (Bimonte & Denenberg, 1999; Jill M. Daniel et al., 2006; Jill M Daniel et al., 1997; Fader et al., 1999; R. B. Gibbs & Johnson, 2008; V N Luine et al., 1998; Prakapenka et al., 2018; Rodgers et al., 2010). Interestingly, free E2 treatment did not differ from its control on any of the three memory measures evaluated across the three blocks of WRAM testing. Although due to the exhibited differences in vehicles, E2 PLGA and free E2 treatments could not be directly compared for cognitive outcomes on the WRAM, the findings overall indicate that E2 encapsulation in PLGA nanoparticles did enhance the hormone's beneficial effects on cognition when a weekly subcutaneous treatment regimen was implemented, as E2 PLGA improved spatial working memory compared to its control whereas free E2 did not differ from its control, supporting my hypothesis that weekly delivery of E2 from PLGA nanoparticles will enhance cognition in middle-aged, Ovx rats relative to blank PLGA nanoparticles, and relative to free E2.

The second learning and memory task implemented within the behavioral battery of the present study, the MWM, evaluated spatial reference memory. Once again, all rats learned the task and exhibited a similar learning trajectory across all days of testing. Additionally, probe trial evaluation indicated that all treatment groups spatially localized to the platform location. There was a trend for the E2 encapsulated PLGA treatment group to swim a shorter distance to the platform across the 5 days of testing relative to its control, although the effect did not reach statistical significance. The free E2 treatment did not differ in swim distance to platform relative to its control. Overall, MWM data suggest that weekly subcutaneous E2 PLGA treatment tended to improve spatial reference memory, whereas weekly subcutaneous free E2 treatment did not. In Ovx rats, higher levels of circulating E2 correlate with better performance on the MWM task (Talboom et al., 2008). Thus, the moderate beneficial effects of E2-PLGA treatment on the MWM in the present study may in part be due to a greater presence of the hormone in cognitively-involved brain regions than free E2, as circulating E2 levels did not differ (discussed below), and postulate that additional investigation of E2 PLGA treatment as a function of treatment regimen as well as E2 dose may result in strengthened cognitive effects of E2 PLGA treatment.

Our overarching goal of optimizing the delivery of E2 for cognitive therapy was to enhance E2-mediated memory effects while minimizing exposure of the hormone to peripheral tissue. Following E2 PLGA and free E2 treatments, circulating levels of E2 and of estrone, a metabolite of E2, were higher relative to each treatment's respective controls, confirming the presence of E2 and its metabolite in the periphery. E2 and estrone circulating levels did not differ between E2 PLGA and free E2 treatment groups, indicating that E2 encapsulation in PLGA nanoparticles did not impact circulating levels of E2 or of

the hormone's metabolism to estrone. In terms of uterine stimulation, both E2 PLGA and free E2 increased uterine horn weight compared to their respective controls, confirming E2 exposure at the uterine horns as estrogen exposure was expected to increase uterine horn weight in Ovx rats (Prakapenka et al., 2018; Westerlind et al., 1998). Further, E2 PLGA increased uterine horn weight compared to free E2, suggesting prolonged exposure of the uterine horns to E2 following its delivery when encapsulated in PLGA nanoparticles. Thus, uterine stimulation findings from the current study indicate that E2 encapsulated in PLGA nanoparticles increased the hormone's uterine effects compared to free E2.

In sum, despite an apparent effect of vehicle on spatial working memory, the implementation of PLGA nanoparticles for E2 delivery enhanced the cognitively-beneficial effects of the hormone. Indeed, weekly E2 PLGA treatment resulted in improved spatial working memory on the WRAM task, and showed a trend in improved spatial reference memory on the MWM task, relative to control whereas free E2 did not impact spatial learning and memory relative to control. However, E2 PLGA treatment also resulted in enhanced effects of the hormone on uterine stimulation relative to free E2, suggesting that the use of PLGA nanoparticles, at least in a non-modified formulation, is not ideal for use in cognitive therapy. Nevertheless, the established baseline of cognitive and uterine effects following E2 PLGA treatment versus free E2 treatment provided within this study sets the stage for future work focused on modifying E2 PLGA formulations to target E2 delivery to the brain, and to indirectly decrease circulating levels and uterine horn exposure to the hormone once a lower treatment dose yielding the same cognitive outcomes as free E2 is achieved. And, importantly, I have shown that the evaluation of vehicle controls when implementing novel drug delivery platforms or outcome measures is critical, and can

impact interpretation of results, as the carrier itself may exhibit effects on evaluated outcome measures. In consideration of improving E2 treatment for potential use as a cognitive therapy, alternate drug delivery strategies must also be evaluated, such as addition of a specific targeting moiety, route of administration, or use of other drug carrier types. Undeniably, the merging of behavioral neuroendocrinology with biomedical engineering is an exciting, and still novel, avenue that can yield hormone therapy options optimized for desired therapeutic efficacy, such as treatment of a specific symptom with minimized peripheral burden, and will ultimately lead to improved quality of life for women across their lifespans.

CHAPTER 5

COGNITIVE EFFECTS OF INTRANASAL 17β-ESTRADIOL-CYCLODEXTRIN TREATMENT IN MIDDLE-AGED OVARIECTOMIZED RATS

This chapter will be formatted and submitted as a manuscript for publication to a peer-reviewed journal.

Contribution: I am the first author of this document and was the primary graduate student PI on this project. Under the mentorship of Heather A. Bimonte-Nelson and Rachael W. Sirianni, I designed and carried out the study with the assistance of our laboratory teams.

Introduction

Transitional menopause is a natural aspect of aging in women and occurs at an average age of 52 years (NAMS, 2014). Surgical menopause occurs following the surgical removal of reproductive tissues (e.g., the ovaries). The onset of either transitional or surgical menopause is associated with an overall decrease in circulating ovarian hormone levels, including two of the endogenously present steroid hormone classes, estrogens and progestogens. With menopause and the associated changes in circulating hormone milieu, several undesired symptoms may be present, such as changes in cognitive functions as well as vaginal atrophy, hot flashes, and osteoporosis (Maki, 2012; NAMS, 2014). Multiple hormone therapy options are available for women to decrease the presence and severity of some of these symptoms, and these therapies are often administered by oral or transdermal routes (Kuhl, 2005; Prakapenka et al., *in review*).

The two main components in hormone therapy are estrogens and progestogens. The type of hormone, the dose ratios of hormone combinations, as well as type and length of hormone administration can all vary when prescribed for hormone therapy (Kuhl, 2005; Prakapenka et al., *in review*). Thus, it is imperative to understand how menopause-associated changes in ovarian hormone milieu, as well as subsequent exogenous hormone therapy treatments, impact women's health, including brain health, and to explore alternate delivery platforms that may ultimately capitalize and improve on the therapeutic efficacy of hormone therapies.

One extensively studied ovarian hormone within the field of behavioral neuroendocrinology is 17β-estradiol (E2). E2 is the most potent natural estrogen in women and in rodents, it is a common estrogenic component in hormone therapy, and it can exhibit beneficial cognitive effects following a decrease in endogenously circulating ovarian hormones (Frick, 2015; Koebele & Bimonte-Nelson, 2015; Kuhl, 2005; Maki, 2012; S. E. Mennenga & Bimonte-Nelson, 2013; NAMS, 2012; Prakapenka et al., 2018). Through extensive evaluations, it is now understood that a myriad of factors, such as route of administration, regimen, dose, and background circulating hormone profile, can influence whether E2 will have beneficial, neutral, or even detrimental cognitive effects (Koebele & Bimonte-Nelson, 2015; Prakapenka et al., *in review*). The ovariectomy (Ovx) rodent model, whereby ovaries are surgically removed, has been instrumental in systematically controlling for and examining these factors as well as the corresponding observed changes in cognitive function (S. E. Mennenga & Bimonte-Nelson, 2013; Prakapenka et al., *in review*). For instance, I have recently showed that a daily subcutaneous injection of E2 enhanced spatial working memory on the water radial arm

maze (WRAM) in middle-aged, Ovx rats, and increased uterine horn weight, a marker of uterine exposure to estrogens (Prakapenka et al., 2018). Of note, although this specific E2 treatment regimen was cognitively-beneficial, its administration yielded increased peripheral uterine stimulation – an undesired effect. In the clinic, estrogen-only hormone therapy is associated with an increase in the risk for developing endometrial hyperplasia and cancer; thus, minimal uterine exposure to estrogens when administered alone is desired (NAMS, 2014). Although progestogens can and are used in the clinic to oppose these estrogen-associated risks in women that have an intact uterus, Ovx rodent models indicate that the addition of a progestogen can also oppose the cognitively-beneficial effects of estrogens (Heather A. Bimonte-Nelson et al., 2006; Lauren L. Harburger et al., 2007; Lowry et al., 2010; Maki, 2012; NAMS, 2012). Indeed, when evaluating a clinically relevant estrogen plus progestogen hormone combination, I found that the addition of a cognitively-beneficial synthetic progestogen, levonorgestrel (Levo), to a cognitively-beneficial dose of E2 at a 5:1 E2 to Levo dose ratio attenuated the beneficial effects of E2 and of Levo when each were given alone (Prakapenka et al., 2018). This hormone combination treatment did not decrease uterine horn weight compared to E2 alone treatment (Prakapenka et al., 2018). Further analyses revealed that the addition of Levo at a 3:1 and a 1:2 E2 to Levo dose ratio did in fact decrease uterine horn weight compared to E2 alone treatment; yet, these two hormone combination treatments also impaired spatial working and reference memory (Chapter 3). Taken together, existing studies posit value in developing a targeted delivery platform that can achieve an increased uptake of estrogen in regions of the brain involved in cognitive function, while

minimizing estrogen exposure at the uterus, as it would maximize the beneficial role of estrogens, such as E2, in learning and memory.

The intranasal route of administration can achieve greater delivery to the brain versus peripheral tissue compared to several other routes of administration (Nonaka et al. 2008). In regard to E2 delivery, a clinically available intranasal spray of E2, Aerodiol, was designed for menopausal hormone therapy but was discontinued for industrial reasons, with no severe side effects reported (Pelissier et al., 2001; Studd et al., 1999). Indeed, clinical evaluations of Aerodiol concluded that a daily administration of Aerodiol was well tolerated and effective at reducing symptoms associated with menopause in women (Pelissier et al. 2001; Studd et al. 1999). Further, intranasal E2 can circumvent first-pass E2 metabolism as compared to oral administration, as well as can achieve cyclic E2 circulating levels, a hormone profile that more closely represents the natural fluctuations in circulating E2 levels in both women and female rodents (M. E. Davis & Brewster, 2004; Studd et al., 1999). To the best of my knowledge, the cognitive effects of E2 following intranasal administration have not yet been evaluated, and additional assessments are needed to better understand the peripheral burden of intranasally administered E2, particularly at the uterine tissue, in order to help determine whether it could have an overall efficacious profile as a hormone therapy.

A major limitation in administering E2 via the intranasal route is the hormone's low solubility. In Aerodiol, E2 was solubilized with the addition of randomly methylated β-cyclodextrin. Cyclodextrins (CDs) are a group of compounds composed of sucrose molecules bound together in a ring, forming a hydrophobic core and a hydrophilic surface. Due to this donut-shape structure, CDs are commonly used in drug delivery to

solubilize hydrophobic compounds, such as E2, by creating a drug-inside-CD complex. CDs differ based on the number of sucrose chains (6-, 7-, and 8- chains), and their properties can be altered to enhance desired CD characteristics, such as increased solubility. For instance, the 2-hydroxypropyl β-CD is used to produce a commercially available 'water-soluble E2' (Sigma-Aldrich). Of note, this E2-CD complex has been previously utilized in behavioral rodent paradigms to assess the role of E2 in cognitive function (Fernandez et al., 2008; L. L. Harburger et al., 2009; Sinopoli, Floresco, & Galea, 2006). Furthermore, intranasal rodent studies suggest that the addition of CDs can enhance uptake of hydrophobic agents in the brain (e.g. ~2-fold for I-GALP, a galanin-like peptide) as well as impact regional brain distribution as a function of CD type (N Nonaka et al., 2008; Naoko Nonaka et al., 2012). Thus, based on prior studies evaluating intranasal delivery as well as the role of CDs in intranasal delivery to the brain, the combination of intranasal administration plus the addition of a CD is a promising avenue that may be utilized to achieve increased E2 uptake in the brain, minimal peripheral E2 exposure, and even targeted delivery to specific regions in the brain. Particularly, the brain regions of interest are the frontal cortex and the dorsal hippocampus; these two regions are heavily involved in learning and memory and are necessary for E2-induced beneficial cognitive effects (L. Fan et al., 2010; Fortress, Fan, Orr, Zhao, & Frick, 2013; Prakapenka et al., 2018). Here, I propose to evaluate a potentially novel delivery platform – intranasal delivery of E2 as a function of CD type – for E2-based hormone therapy targeted for greater cognitive effects and lower uterine effects.

In the present work, I tested the overarching hypothesis that intranasal administration of an E2-CD complex targeting estrogen delivery to the frontal cortex and

to the dorsal hippocampus in middle-aged Ovx rats will result in enhanced cognitive function and minimal uterine horn stimulation compared to vehicle control and compared to free E2. In Study 1, the brain-distribution and biodistribution profiles of radiolabeled E2, alone or complexed with CDs, in middle-aged, Ovx rats following intranasal administration was determined. I predicted that varying the CD type in the E2-CD complex formulations would differentially impact E2 uptake in specific regions of the brain following intranasal administration. Next, in Study 2, daily intranasal administration of free E2 and of an E2-CD combination that yielded greatest E2 delivery to cognitively-involved brain regions were evaluated on a behavioral battery assessing spatial working and spatial reference memory in middle-aged, Ovx rats. At the completion of the study, uterine horn weights were collected to assess E2-induced effects on uterine stimulation based on treatment type. I predicted that the E2-CD complex would improve spatial learning and memory, and have minimal uterine stimulation, relative to vehicle, and relative to free E2.

Methods - Study 1: Brain-distribution and biodistribution of free E2 and of E2-CD complexes following intranasal administration

Animals

Sixty-two female Fischer-344 CDF virgin rats were ordered from the National Institute on Aging, Harlan Laboratories (Indianapolis, IN). The ages ranged from 9-11 months old due to limited availability of ages at the NIA colony. Once arrived, rats were pair-housed, kept on a 12-hour light/dark cycle, and provided food and water *ad libitum*.

All procedures were approved by the Arizona State University IACUC and adhered to the standards set by the National Institutes of Health. See Figure 26 for the study timeline.

Ovariectomy (Ovx)

All rats received Ovx surgery under acute isoflurane anesthesia, as previously described (B. Blair Braden et al., 2017; S. E. Mennenga et al., 2015; Prakapenka et al., 2018). Specifically, following dorsolateral incisions to the skin and muscle, the tip of each uterine horn was ligatured, and the ovaries plus the tip of the uterine horns were excised. Muscle and skin were then sutured and stapled closed, respectively. Subcutaneous Rimadyl (5 mg/mL/kg) was given for pain, and subcutaneous saline (2 mL) was given to prevent dehydration. One rat died due to surgical complications.

Treatments

Approximately three weeks following Ovx surgery (20 ± 2 days after Ovx), 59 rats were randomly assigned to receive a single intranasal administration of one of the five treatment formulations outlined in Table 4. Two rats did not receive treatment and served as background radioactivity controls for later tissue processing. The five treatments were administered to each rat at 0.04 mCi of tritiated E2 per kg body weight. The formulations included free E2 (Treatment A), E2-randomly methylated β-CD complex (Treatment B), E2-2-hydroxypropyl β-CD complex (Treatment C), E2-β-CD complex (Treatment D), and E2-γ-CD complex (Treatment E). All treatments were solubilized in 0.9% saline with the exception of Treatment A, which was solubilized using 20% PEG solution in 0.9% saline; the pH was kept at 5.5 for all treatments to

match the pH range of a rat's nasal cavity and to minimize irritation following intranasal administration in awake rats (A. Singh, Singh, & Madhav, 2012). Tritiated E2, dissolved in ethanol, was purchased from American Radiolabeled Chemicals (cat # ART 0820). Treatment A served as the free E2 comparison, without the addition of a CD carrier. Treatment B utilized the CD that was used in the clinically available Aerodiol intranasal spray (Pelissier et al., 2001; Studd et al., 1999); tritiated E2 was combined with the CD that was dissolved in 96% ethanol at a 1:2 E2 to CD molar ratio. Treatment C utilized the CD that has been evaluated in behavioral studies of E2's effects on learning and memory, as well as neuromolecular mechanisms (Fernandez et al., 2008; L. L. Harburger et al., 2009; Sinopoli et al., 2006); tritiated E2 was combined with the CD that was dissolved in 96% ethanol at a 1:2 E2 to CD molar ratio. Treatment D utilized the base β-CD of the two CD derivatives from Treatments B and C; tritiated E2 was combined with the CD that was dissolved in 50% ethanol at 50^0C at a 1:4 E2 to CD molar ratio and sonicated for 30 minutes. Treatment E utilized γ-CD, which has a greater internal cavity size than that of β-CD (M. E. Davis & Brewster, 2004); tritiated E2 was combined with the CD that was dissolved in 50% ethanol at 50^0C at a 1:5 E2 to CD molar ratio and sonicated for 30 minutes. After the five treatment formulations were generated, each formulation was aliquoted, ethanol was evaporated, and treatments were stored at 4^0C. On the day of treatment administration, each rat was weighed, gently wrapped in a towel while exposing the nostrils, and administered 5 μl of a treatment per nostril for a total of 10 μl at 0.04 mCi/kg tritiated E2, while gently holding onto the jaw area of the rat's head to immobilize it. For three days prior to treatment administration, all rats were habituated to being handled for intranasal administration (but not administered anything).

Tissue collection

At 0.5, 2, or 6 hours following intranasal administration (n = 3-4 rats per treatment per time point), isoflurane was used to anesthetize the rat, whole blood was collected from the right atrium of the heart and frozen (-20^{0}C), and the rat was perfused with heparinized 1x PBS (10 units of heparin per ml of 1x PBS) through the left ventricle of the heart. Following perfusion, the brain was rapidly excised and dissected to collect the olfactory bulbs, trigeminal nerves, frontal cortex, cingulate cortex, basal forebrain, striatum, dorsal hippocampus, hypothalamus, amygdala, entorhinal cortex, perirhinal cortex, and the CA1/CA2 region of the ventral hippocampus. The tissue was weighed and stored at -20^{0}C until later processing. Uterine horns were removed from the body cavity, trimmed of visible fat, weighed, and stored at -20^{0}C until later processing. All samples were collected as a whole area for each region and tissue of interest and then processed for radioactivity detection using the liquid scintillation technique to obtain the desired radioactivity levels per mg of sample. Of note, alternate methods of radioactivity detection are available, such as film radiography of a section or slice of a region or tissue of interest, which can allow for more accurate spatial representation of radioactivity levels within a sample (e.g. region or cell localization) rather than the overall radioactivity level as a function of sample weight. For the purpose of this study, the goal was to determine and compare E2 delivery in areas of interest which differed in size (e.g., dorsal hippocampus versus uterine horns), and the homogenization plus liquid scintillation technique allowed for such comparisons.

Tissue processing

Solvable Solution (PerkinElmer) was used to homogenize whole blood, brain tissue, and uterine horns. For brain tissue, 0.005 ml of Solvable per mg of tissue was added to each sample and then incubated overnight at 50^0C. The next day, 0.2 ml of 30% hydrogen peroxide was added to each sample and incubated for 30 min at 50^0C for de-colorization. Then, Hionic-Fluor scintillation cocktail (PerkinElmer) was added to each sample and allowed 1 hour for light and temperature to adjust prior to reading the radioactivity of each sample in triplicate on the LS 6500 Multi-Purpose Liquid Scintillation Counter. For whole blood, 0.005 ml of Solvable per mg of whole blood was added to each sample and then incubated overnight at 50^0C. The next day, 0.2 ml of 30% hydrogen peroxide was added in 50 µl increments to minimize foaming to 500 µl of each sample and incubated for 30 min at 50^0C for de-colorization. Next, Hionic-Fluor scintillation cocktail (PerkinElmer) was added to each sample and allowed 1 hour for light and temperature to adjust prior to reading the radioactivity of each sample in triplicate. For uterine horns, each uterine horn was finely minced, weighed, and 0.005 ml of Solvable per mg of tissue was added to each sample and then incubated overnight at 50^0C. The next day, 0.2 ml of 30% hydrogen peroxide was added in 100 µl increments to minimize foaming to 500 µl of each sample and incubated for 30 min at 50^0C for de-colorization. Hionic-Fluor scintillation cocktail (PerkinElmer) was then added to each sample and allowed 1 hour for light and temperature to adjust prior to reading the radioactivity of each sample in triplicate. The tissue and whole blood from the rats that did not receive any treatment was processed in the same exact way and radioactivity was read at the same time as the respective samples were processed. All data were expressed

as CPM/mg of tissue after subtracting the background CPM/mg obtained from the control rats (rats that received no treatment).

Methods - Study 2: Cognitive evaluation of daily intranasal E2-CD treatment relative to free E2 and vehicle

Animals

Forty female Fischer-344 CDF virgin rats were ordered from the National Institute on Aging, Harlan Laboratories (Indianapolis, IN). Ages ranged from 9-10 months old. All rats were pair-housed and kept on a 12-hour light/dark cycle for the duration of the study. Food and water were provided *ad libitum*. All procedures were approved by the Arizona State University IACUC and adhered to the standards set by the National Institutes of Health. See Figure 26 for the study timeline.

Ovariectomy (Ovx)

Ovx surgery was performed on all rats following the same procedure as outlined in Study 1 methods. The first body weight measurement was collected immediately prior to Ovx, and body weight was monitored on a weekly basis for the rest of the study to monitor animal health as well to confirm Ovx and E2 exposure; body weight was expected to increase following Ovx and to be greater with vehicle control treatments compared to E2-containing treatments (Diz-Chaves et al., 2012; Prakapenka et al., 2018; Rogers, Perfield, Strissel, Obin, & Greenberg, 2009).

Treatment administration

Three weeks following Ovx surgery, all rats were randomly assigned to receive daily intranasal administration of one of four treatments that continued for the duration of the study (Figure 26). Treatments were counter-balanced so that 20 rats received intranasal administration from 7:10 - 7:30 am every day and 20 rats received intranasal administration at 9:10 - 9:30 am every day to account for future behavioral battery assessments which were estimated to take about 1 hour for 20 rats to complete (using 2 behavioral rooms). In this manner, all rats were behaviorally tested 0.5 - 1.5 hours following treatment administration, capitalizing on the treatment brain-distribution effects observed in Study 1. The four treatments followed the same synthesis and administration procedures as outlined in Study 1, and utilized non-radioactive E2 purchased from Sigma-Aldrich. The treatments included saline-PEG (20% PEG solution in saline) as the vehicle control for free E2, 1 μg/rat free E2, saline-CD (saline with 2-hydroxypropyl β-CD) as the vehicle control for the E2-CD complex, and 1 μg/rat E2-CD (E2-2-hydroxypropyl β-CD complex). 2-hydroxypropyl β-CD was chosen as the CD for the E2-CD complex treatment due to its greatest delivery of E2 to the dorsal hippocampus relative to free E2, as observed in Study 1 and discussed in more detail in the Results section; the amount of CD in saline-CD and in E2-CD vehicle groups were matched. The dose of E2 was determined to be 1 μg/rat/day based on the lowest dose of E2 in Aerodiol that effectively alleviated symptoms of menopause in women, adjusted for difference in body weight between women and rats (Studd et al. 1999).

Water radial-arm maze (WRAM)

On the fourth week of treatment administration, all rats started behavioral testing. The first task, the win-shift WRAM, tested spatial working and reference memory (H. A. Bimonte-Nelson, 2015d; Bimonte & Denenberg, 1999; B. Blair Braden et al., 2017; S. E. Mennenga & Bimonte-Nelson, 2015; Sarah E. Mennenga, Koebele, et al., 2015). The maze had 8 arms, with 38.1 cm x 12.7 cm dimensions for each arm, and the room had abundant spatial cues set up to aid in spatial navigation. The maze was filled with opaque water, and the temperature of the water was 18-20ºC. Four out of the 8 arms contained hidden platforms that were 10 cm in diameter, and the location of which was fixed throughout all days of testing for a rat, but varied across rats and within treatment groups. For each trial, there was a maximum time of 3 minutes allotted to solve the task by locating one of the platforms. To start a trial, a rat was dropped off at the same start arm location each time and the trial continued until the rat either found or was led to the platform (after 3 minutes passed). After 15 seconds on the platform, the rat was placed back into a heated testing cage. The inter-trial interval (ITI) was 30 seconds, during which the just-located platform was removed and a fishnet was used to clean the maze and redistribute olfactory cues. After the ITI, the next trial was started and the task continued until there were no more platforms left to find, resulting in four trials total per day for each rat. With each trial, the task became increasingly harder to solve as working memory demand was increasingly taxed as trials progressed. After 12 days of testing, on day 13, a delay period of 6 hours was added between trials 2 and 3 to evaluate delayed memory retention.

Performance on the WRAM was scored by recording and scoring number of error arm entries. Error entries were further divided into 3 memory measures evaluating

reference and working memory. A reference memory (RM) error was made when a rat entered a non-platformed arm within a day. A working memory incorrect (WMI) error was made when a rat re-entered a non-platformed arm within a day. A working memory correct (WMC) error was made when a rat entered a previously platformed arm within a day.

Visible platform

To confirm procedural components (e.g. visual, motor) to solve a water-escape task are present, the visible platform task was implemented on the last day of behavioral testing (Bimonte-Nelson, 2015a; Mennenga et al., 2015a, 2015c). Specifically, a rectangular tub, 100 cm x 60 cm, was set up in a room with all spatial cues blocked using a curtain around the maze. The maze contained 18-20^{o}C clear water with one black platform (10 cm in diameter) that was kept 4 cm above the water surface. The maximum trial time to find the platform was 90 seconds, with 6 trials total. The platform location was semi-randomly varied between trials, and the ITI was 5-8 minutes. The trial started when a rat was dropped off from a set location and the trial ended when the rat either found the platform or was led to the platform after 90 seconds. Once found, each rat was given 15 seconds on the platform before being placed into a heated testing cage. Performance on the visible platform task was measured as time, in seconds, to platform.

Uterine horn weights

Uterine horn weight served as a marker of uterine stimulation as it is well known that ovarian hormones impact uterine horn weight and can be effectively used to confirm Ovx and to assess E2 exposure (E. B. Engler-Chiurazzi et al., 2012; Sarah E. Mennenga,

Gerson, Koebele, et al., 2015; Prakapenka et al., 2018; Westerlind et al., 1998). The wet weight of uterine horns was collected at sacrifice after the uterine horns were excised and all visible fat was removed.

Statistical analyses

For Study 1 and Study 2 statistical analyses, alpha was set at $p < 0.05$. Study 1 analyses included one-tailed Student's t-tests to evaluate the hypothesis that the addition of a CD would increase the delivery of E2 to the dorsal hippocampus and to the frontal cortex relative to free E2 within each time point that was collected. An outlier was excluded from all analyses and figure representations as it was 5-fold greater than the next highest value within that treatment group (0.5 hr timepoint for Treatment B), although the interpretation of statistical outputs did not depend on whether the outlier was included or not in the analyses.

To evaluate changes in body weight across the 10 weeks of Study 2, a repeated measures ANOVA was run for Weeks 1-4, the weeks prior to treatment initiation, and Weeks 5-10, the weeks during treatment administration. The independent variable was Treatment, and the repeated measures were Weeks. Fisher's post hoc analyses were run when there was a significant main effect of Treatment.

For the WRAM, each memory measure (WMC, WMI, and RM errors) was evaluated separately on days 2-7, the acquisition phase, and on days 8-12, the asymptotic phase, as done previously (Hiroi et al., 2016; Sarah E. Mennenga, Gerson, Koebele, et al., 2015). A 2 x 2 factorial repeated measures ANOVA was used to evaluate performance, with Estrogen Treatment (No E2 or E2) and Vehicle Type (saline-PEG or saline-CD) as

factors. In the case of a significant main effect of Estrogen, main effect of Vehicle, or Estrogen x Vehicle interaction, additional repeated measures ANOVAs were conducted to directly evaluate Treatment effects for the appropriate two group comparison. A significant Trial x Estrogen, Trial x Vehicle, or Trial x Estrogen x Vehicle interaction led to further analyses on the highest working memory load trial that was evaluated on this version of the WRAM, Trial 4. For all analyses, Estrogen, Vehicle, or Treatment were set as the independent variables and Trials nested within Days were the repeated measures. For the WRAM delay, total errors made (WMC, WMI, and RM memory measures combined) on the post-delay trials (Trials 3 and 4 on Day 13) were compared to the baseline trials (Trials 3 and 4 on Day 12) within each treatment group to evaluate delayed memory retention.

For the visible platform, a repeated measures ANOVA was run to test for a main effect of Trial, a main effect of Treatment, and a Trial x Treatment interaction to assess simple learning and the ability to perform the procedural components of a water-escape task across treatment groups. Treatment was the independent variable and the repeated measures were Trials. A one-way ANOVA was run for uterine horn weights, and Fisher's post hoc analyses were used in the case of a significant Treatment effect. Treatment was the independent variable and Uterine Horn Weight was set as the dependent variable.

Results – Study 1: Brain-distribution and biodistribution of free E2 and of E2-CD complexes following intranasal administration

Representative spatial heat maps were created to illustrate the distribution of E2, as a function of CD type, across the examined brain regions and time (Figures 27-30). The evaluated brain regions included olfactory bulbs, trigeminal nerves, frontal cortex,

cingulate cortex, basal forebrain, striatum, dorsal hippocampus, hypothalamus, amygdala, entorhinal cortex, perirhinal cortex, and the CA1/CA2 region of the ventral hippocampus. In general, the greatest E2 delivery to each brain area was observed at the earliest time point collected, 0.5 hr, followed by a rapid decrease for each treatment formulation. Of note, there was strong variability in the data within a treatment formulation across each time point, as can be visualized by the quantitative graphs for each region (Figure 31), and at times resulted in a single point driving the overall average concentration (e.g. peak average concentration at the 2 hr time point rather than the 0.5 hr time point in the trigeminal nerve following intranasal free E2 administration). E2 concentration in the trigeminal nerves, one of the potential pathways for agent uptake into the brain with intranasal delivery, tended to have slower clearance across the three evaluated time points as compared to the olfactory bulbs, another potential pathway for agent uptake into the brain with intranasal delivery. Focusing on the two regions of interest, E2 payload in the dorsal hippocampus 0.5 hr following Treatment C administration was significantly greater than that of Treatment A - free E2 ($p < 0.05$). Furthermore, when comparing dorsal hippocampus to uterine horn E2 delivery, Treatment A resulted in ~2-fold increased E2 levels in dorsal hippocampus relative to uterine horns whereas Treatment C resulted in ~4-fold increased E2 levels in dorsal hippocampus relative to uterine horns (Figure 33 B). Interestingly, E2 levels in the dorsal hippocampus following Treatment A (free E2) did not exhibit rapid clearance across the collected time points as seen with Treatment C. No statistically significant differences in E2 delivery to the dorsal hippocampus were observed with the other three Treatment formulations, and at the other collected time points for all Treatment formulations, relative to free E2. There were no

statistically significant differences in E2 delivery to the frontal cortex with any E2-CD Treatment formulations relative to free E2.

E2 levels in whole blood were greatest at the 0.5 hr time point following intranasal administration for each Treatment group and cleared rapidly across time (Figures 32 and 33A). Although uterine horn E2 levels also were greatest at the 0.5 hr time point following intranasal administration for each Treatment group, the E2 levels did not exhibit as rapid clearance of E2 and were retained across the collected time points (Figure 32 and 33A). These findings are consistent with prior biodistribution studies evaluating the fate of exogenously administered tritiated E2, where E2 appeared to be retained for longer periods of time in estrogen-responsive tissues (Green, Luttge, & Whalen, 1969; Jehan, Srivasta, Akhlaq, Ahmad, & Setty, 1982; Jensen, 1963).

Results – Study 2: Cognitive evaluation of daily intranasal E2-CD treatment relative to free E2 and vehicle

Body weight

Average body weights were monitored for the duration of the study across each treatment group as a marker of health, and as a general marker of E2 presence (Prakapenka et al., 2018; Figure 34A). Across the first 4 weeks of the study, prior to the initiation of daily intranasal treatment, there was no main effect of Treatment for average body weight (Figure 34B). For the latter 6 weeks of the study, when daily intranasal treatment was administered, there was a main effect of Treatment for average body weight [$F_{(3,36)} = 4.276$, $p < 0.05$; Figure 34B], with free E2 and E2-CD exhibiting significantly lower body weights relative to their respective vehicle controls ($p < 0.05$),

confirming the presence of E2. Body weight between free E2 and E2-CD treatment groups was not significantly different.

Water radial-arm maze (WRAM)

The WRAM evaluated spatial working and spatial reference memory. The 12 days of testing were divided into the acquisition phase, days 2-7, when the rats were learning the rules of the task, and the asymptotic phase, days 8-12, after the rats should have learned the rules of the task. On the acquisition phase, a main effect of Estrogen was seen [$F_{(1,36)}$ = 5.161, $p < 0.05$; Figure 35A], whereby the groups with E2 made fewer RM errors than the groups with no E2. Additionally, there was a marginal Estrogen x Vehicle interaction [$F_{(1,36)}$ = 3.303, $p < 0.1$] for RM errors made on the acquisition phase (data not shown). Further analyses revealed that these effects were driven by a main effect of Treatment between saline-PEG and free E2 [$F_{(1,18)}$ = 8.452, $p < 0.01$; Figure 35B], where the free E2 group made fewer RM errors than the saline-PEG group. Further, there was a marginal Treatment effect between free E2 and E2-CD [$F_{(1,18)}$ = 3.349, $p < 0.1$], where the free E2 group tended to make fewer RM errors than the E2-CD group. Thus, on the acquisition phase, the free E2 treatment improved RM relative to its respective control, and tended to improve RM relative to the E2-CD treatment. There was a significant Estrogen x Vehicle interaction for WMI errors made on the asymptotic phase [$F_{(1,36)}$ = 5.414, $p < 0.05$; Figure 36A], as well as a significant Trial x Estrogen x Vehicle interaction [$F_{(3,108)}$ = 3.522, $p < 0.05$; Figure 36C]. Further analyses for WMI errors collapsed across all four trials revealed a main effect of Treatment between E2-CD and saline-CD [$F_{(1,18)}$ = 9.885, $p < 0.01$; Figure 36B], as well as between E2-CD and free E2 [$F_{(1,18)}$ = 4.449, $p < 0.05$; Figure 36B], where

the E2-CD group made more WMI errors compared to the saline-CD group and compared to the free E2 group. Additionally, on Trial 4, the highest working memory load trial evaluated on the WRAM in the present study, there was a significant Estrogen x Vehicle interaction for WMI errors [$F_{(1,36)} = 4.766$, $p < 0.05$; Figure 36C]. Further analyses for WMI errors made on Trial 4 alone revealed a main effect of Treatment between E2-CD and saline-CD [$F_{(1,18)} = 6.047$, $p < 0.05$; Figure 36D] and a marginal effect of Treatment between E2-CD and free E2 [$F_{(1,18)} = 3.157$, $p < 0.1$; Figure 36D], where the E2-CD group made more WMI errors than the saline-CD group and tended to make more WMI errors than the free E2 group. Together, these data indicate that the E2-CD treatment impaired working memory on the asymptotic phase of the WRAM, particularly at the high working memory demand. For the WRAM delay, there was no difference for total errors made (WMC, WMI, and RM memory measures combined) on the post-delay trials, Trials 3 and 4 on Day 13, compared to the baseline trials, Trials 3 and 4 on Day 12, for each treatment group, suggesting that no treatment group exhibited delay-induced forgetting following a 6-hour delay period (data not shown).

Visible platform task

The visible platform task assessed whether motor and visual abilities to complete a water-escape task were present across treatment groups. There was no main effect of Treatment and no significant Trial x Treatment for Latency to Escape, but there was a main effect of Trial [$F_{(5, 180)} = 7.770$, $p < 0.0001$], suggesting that each treatment group had a similar procedural capability to learn and complete a water-escape task. In fact, each

treatment group's average latency to platform across all 6 trials was under 9 seconds indicating strong performance.

Uterine horn weight

To evaluate the uterine effects of the E2 regimens tested here, uterine horn weight was examined across treatments. A main effect of Treatment was seen [$F_{(3,36)} = 58.043$, $p < 0.0001$; Figure 37], where both of the E2-treated groups had significantly higher uterine horn weights than their respective vehicle control groups ($p < 0.0001$). Additionally, the E2-CD group had greater uterine horn weight relative to the free E2 group ($p < 0.01$), suggesting that E2 did have uterine stimulating effects with both E2 treatments, and that these effects were more pronounced with the addition of the CD.

Discussion

The present work evaluated cognitive and uterine effects of free E2 and an E2-CD complex utilizing an intranasal administration in middle-aged, Ovx rats. In Study 1, I evaluated the distribution of tritiated E2 as a function of CD type in comparison to free E2 in the brain as well as in the periphery. Next, in Study 2, daily intranasal treatments of free E2 and of the E2-CD complex that resulted in greatest E2 uptake in the dorsal hippocampus were evaluated for cognitive and uterine effects. I originally hypothesized that intranasal administration of an E2-CD complex targeting estrogen delivery to the frontal cortex and to the dorsal hippocampus in middle-aged Ovx rats would result in enhanced cognitive function and minimal uterine horn stimulation compared to vehicle control and compared to free E2. Findings from Study 1 did not indicate a significant difference in E2 delivery to

the frontal cortex between free E2 and E2-CD complex formulations, but there was an increase observed in E2 delivery to the dorsal hippocampus when E2 was complexed with 2-hydroxypropyl β-CD relative to free E2 half an hour following intranasal administration. Thus, in Study 2, I evaluated whether intranasal administration of the E2-CD complex targeting estrogen delivery to the dorsal hippocampus in middle-aged Ovx rats would result in enhanced cognitive function and minimal uterine horn stimulation compared to vehicle control and compared to free E2. Intriguingly, Study 2 findings revealed that intranasally administered free E2 enhanced learning and memory on the WRAM but that the E2-CD complex, which was shown to exhibit greater E2 delivery to the dorsal hippocampus than free E2, impaired learning and memory. Furthermore, although both E2-containing treatment groups, free E2 and E2-CD complex, increased uterine horn weight relative to the respective vehicle control treatment groups, the E2-CD complex resulted in greater uterine horn weight than free E2, suggesting increased uterine stimulation by E2 with the addition of CD.

Although Study 1 evaluated E2 delivery across multiple regions in the brain, the primary focus was on E2 delivery across time to the frontal cortex and to the dorsal hippocampus as a function of CD type, as these regions are impacted by E2 and are associated with the cognitively-beneficial E2 effects (L. Fan et al., 2010; Fortress et al., 2013; Prakapenka et al., 2018; Meharvan Singh, Meyer, Millard, & Simpkins, 1994). Results showed that the E2-2-hydroxypropyl β-CD complex formulation increased E2 delivery to the dorsal hippocampus relative to free E2 half an hour following intranasal administration. E2 was rapidly cleared when delivered via the E2-2-hydroxypropyl β-CD complex whereas free E2 resulted in a more sustained presence of E2 in the dorsal

hippocampus over time. In relation to E2 levels in the uterine horns, free E2 resulted in ~2-fold greater dorsal hippocampus E2 levels and E2-2-hydroxypropyl β-CD complex resulted in ~4-fold greater dorsal hippocampus E2 levels. Furthermore, although E2 levels in whole blood and in the uterine horns were greatest at the half an hour time point following intranasal administration with both free E2 and the E2-2-hydroxypropyl β-CD complex, E2 levels in whole blood appeared to be cleared rapidly across time whereas E2 levels in uterine horns appeared to be retained and not cleared as rapidly across the collected time points. These findings support prior studies that suggest that E2 is retained for longer periods of time in estrogen-responsive tissues such as the uterine horns compared to circulating levels (Green et al., 1969; Jehan et al., 1982; Jensen, 1963). No differences were observed in frontal cortex E2 levels between free E2 and E2-CD complex formulations across the three collected time points; thus, this portion of the overarching hypothesis was not tested for cognitive outcomes.

Behavioral outcomes from Study 2 evaluating spatial working and reference memory on the WRAM revealed that when the rats were learning the rules of the task, during the acquisition phase, daily free E2 treatment improved reference memory performance relative to vehicle. The daily free E2 treatment group also tended to make fewer RM errors than the E2-CD complex group during the acquisition phase. On the WRAM, the RM measure is always capped at a maximum of four possible errors that can be made within a day as four out of the eight available arms never contain a platform, and a RM error entry is defined as a first entry into a non-platformed arm. Therefore, even with the inherent ceiling effect, the impact of the free E2 daily intranasal treatment on the reference memory measure was robust enough to reveal a significant beneficial effect.

The cognitively-beneficial effect of the intranasal free E2 treatment is consistent with prior literature that evaluated the effects of E2 treatments on tasks evaluating spatial reference memory (Bimonte-Nelson et al., 2006; El-Bakri et al., 2004; Feng et al., 2004; Kiss et al., 2012; Lowry et al., 2010; McLaughlin et al., 2008; Talboom et al., 2008). On the asymptotic phase, when the rules of the WRAM should have been learned, an estrogen by vehicle interaction was observed. Specifically, the E2-CD complex treatment impaired spatial working memory whereby the E2-CD complex group made more WMI errors than the CD vehicle control as well as than free E2, and this effect was present when working memory load was taxed (on the highest working memory load trial of the task). This finding contradicts my hypothesis, as I predicted that greater E2 delivery to the dorsal hippocampus would result in cognitively-beneficial effects that would be stronger than those of the free E2 treatment. It is possible that the E2 concentrations achieved with daily E2-CD complex treatment in the dorsal hippocampus may be too high to achieve beneficial effects of E2 on spatial learning and memory. Indeed, a recent study in naturally cycling women found that when E2 levels were supraphysiological, the effects of E2 on hippocampal activity exhibited an inverted-U shape, suggesting that there is a sweet spot for E2 levels in the hippocampus that would yield a desired outcome, at least in terms of hippocampal activity, and E2 levels less or greater than that sweet spot may in fact yield the opposite outcome (Bayer, Gläscher, Finsterbusch, Schulte, & Sommer, 2018). In support of this notion, rodent studies evaluating different doses of E2 treatment have shown cognitive impairments with higher doses of E2, while lower doses of E2 exhibited cognitive improvements (Barha et al., 2010; Heather A. Bimonte-Nelson et al., 2006). Taken together, the cognitive outcomes of the present

study provide a baseline for the evaluation of intranasally administered E2 on spatial learning and memory and warrant additional investigation into the dose of free E2, as well as E2-CD complexes, that could yield optimal beneficial cognitive effects following intranasal administration. It is also important to note that the visible platform task revealed that all treatment groups were able, and exhibited similar abilities, to complete a water-escape task, indicating that the treatment paradigms within the study were not detrimental to performance on a water-escape task.

Complete Ovx procedure and the presence of E2 in Study 2 were confirmed via body weight, as well as uterine horn weight, measurements. Following Ovx, all treatment groups exhibited rapid increases in average body weights that did not differ from each other, confirming successful Ovx as rodents tend to gain weight rapidly following the removal of both ovaries (Prakapenka et al., 2018; Rogers et al., 2009). After daily intranasal treatment initiation, which continued for the rest of the study, both of the E2-containing treatments continued to exhibit weight gain but at a lower rate than the two vehicle control groups, resulting in significantly lower average body weight with E2-containing treatments compared to their respective vehicle controls, and no difference in body weight between free E2 and E2-CD complex treatments. This E2-induced separation in body weight between free E2 and its vehicle control and E2-CD complex and its vehicle control was expected based on prior work and confirmed the presence of E2 following intranasal administration (Prakapenka et al., 2018). However, change in body weight is not a direct marker of peripheral E2 exposure, as studies have shown that estrogen receptors in the hypothalamus can mediate the observed changes in body weight (Xu et al., 2011). A more direct measure of peripheral exposure to E2 are uterine horn

weights, which are expected to decrease with Ovx and increase with E2 exposure (E. B. Engler-Chiurazzi et al., 2012; Sarah E. Mennenga, Gerson, Koebele, et al., 2015; Prakapenka et al., 2018; Westerlind et al., 1998). Indeed, in the present study, uterine horn weight was greater with free E2 and E2-CD complex treatments relative to their respective vehicle controls, confirming Ovx as well as E2 presence at the uterine tissue. Interestingly, uterine horn weights were greater with E2-CD complex treatment relative to free E2 treatment, suggesting potentially greater or longer exposure of the uterine tissue to E2 when the estrogen was complexed with the CD compared to without. Together, the uterine horn weight results indicate that the cognitively-beneficial daily free E2 intranasal treatment had lower uterine stimulation relative to the cognitively-impairing daily E2-CD complex treatment.

In conclusion, daily intranasal administration of the E2-CD complex that yielded the greatest E2 uptake in the dorsal hippocampus impaired spatial working memory and had the greatest effect on uterine stimulation in middle-aged, Ovx rats. However, daily intranasal free E2 treatment improved spatial reference memory and yielded lower uterine stimulation than E2-CD complex treatment. Thus, here, I identified a novel approach to evaluate the cognitive effects of E2 by implementing the previously clinically available intranasal route of administration, wherein daily E2 intranasal treatment improved memory in a low circulating ovarian hormone background. This work sets up the cognitive baseline for further evaluation of intranasally administered E2 in a rodent model of surgical menopause, such as varying the dose of intranasal E2 treatments as well as evaluating intranasal versus subcutaneous E2 treatments on both cognitive and uterine measures. In the clinic, intranasal E2 was effective at reducing

common symptoms of menopause (e.g, hot flashes) prior to discontinuation, however, its effects on cognition have not been assessed at that time (Dooley, Spencer, & Ormrod, 2001; Studd et al., 1999). The data in the present study suggest that intranasal E2 therapy should be re-visited for clinical effects, particularly focusing on its potential therapeutic effects on cognition in concert with treatment of other common symptoms of menopause (e.g. hot flashes). Additionally, the cognitively-beneficial effects of intranasal E2 provide novel avenues for further assessment of clinically-available estrogen plus progestogen combinations for cognitive therapy; indeed, the cognitive and uterine effects of combination hormone treatments where the estrogen is administered intranasally whereas the progestogen is administered either subcutaneously or locally (i.e. intrauterine devices) are currently unexplored. Combining and taking advantage of the inherent pharmacokinetic properties of different routes of administration may yield the optimal delivery platform for menopausal hormone therapy, whereby the beneficial cognitive effects of estrogens are maximized and the undesired uterine stimulation effects are minimized. Continuing the evaluation of the cognitive effects of intranasal E2 treatment, and the varying factors surrounding it, is critical as the information gained can lead to developments in hormone therapy formulations that could target particular symptoms, such as altered cognitive function, in menopausal women.

CHAPTER 6

GENERAL SUMMARY AND DISCUSSION

The introduction and conclusion sections of this chapter were adapted from an editorial that was written, and accepted, for an invited submission to the journal Aging based on the published findings from Chapter 2. The editorial was titled:

Memory and menopause: an unsolved puzzle

A.V. Prakapenka, H.A. Bimonte-Nelson

Contribution: The invited editorial was co-authored by Heather Bimonte-Nelson and me. I am the primary author of this chapter, and I received feedback on it from both of my mentors, Rachael Sirianni and Heather Bimonte-Nelson.

Menopause is a natural part of life that impacts every woman as aging ensues. Clinically, menopause in women is defined by a halt in menses for at least one year, during which time ovarian function and ovulation are attenuated (NAMS, 2014). The road to menopause can be transitional and natural, or surgical, if a woman undergoes surgical removal of parts of her reproductive system (e.g., ovaries, uterus). In both transitional and surgical menopause, there is ultimately a marked decrease in ovarian-derived circulating hormone levels. These steroid hormones, including estrogens and the natural progestogen, progesterone, not only maintain and support reproductive function, but also play key roles in numerous other bodily functions and systems, including brain-mediated functions such as cognition (Frick, 2015; Koebele & Bimonte-Nelson, 2017; Prakapenka et al., *in review*). Indeed, these two classes of steroid hormones are present,

and can even be synthesized, in the brain, as are respective receptors (Foster, 2012; Kretz et al., 2004; Micevych & Sinchak, 2008; Mitra et al., 2003). With menopause, a woman can experience a variety of symptoms that impact quality of life. Exogenously administered hormone therapy can be clinically used to prevent many undesirable menopause symptoms (e.g., vasomotor and sleep disturbances, vaginal and vulvar atrophy, osteoporosis). However, hormone therapy is not currently approved or recommended for aiding cognitive symptoms associated with menopause. Clinical data available to date do not definitively support hormone therapy benefits on cognition or dementia outcomes (NAMS, 2017). Amid the collection of imperfectly-fitting puzzle pieces that represent the uncertainty regarding whether hormone therapy yields positive cognitive effects, there have been exciting and illuminating scientific discoveries that have guided the direction of the field, allowing sections of the completed puzzle picture to emerge. If scientists think across bodily systems in an interdisciplinary fashion, if scientists think creatively, and if basic scientists, engineers, and physicians work together, the drive forward will continue with an escalating momentum, the puzzle will yield a more complete picture, and marked enrichments in the standard of care for women on the path to menopause, in menopause, and in post-menopause will ensue.

Studies over the last several decades indicate that estrogens and progestogens can impact learning and memory and related brain substrates, although behavioral neuroendocrinologists are still left with missing puzzle pieces of information regarding the optimal hormone milieu parameters that yield consistent and predictable beneficial impacts on brain and cognitive health. On numerous occasions, the Bimonte-Nelson laboratory and others have shown that the potent E2 can, but does not always, enhance cognition, and that

the addition of a progestogen can reverse the cognitive benefits and brain changes induced by E2 treatment (Heather A. Bimonte-Nelson et al., 2006; Frick, 2015b; L. L. Harburger et al., 2009; Koebele & Bimonte-Nelson, 2015; Prakapenka et al., 2018). Currently, available estrogen-containing treatment in the clinic must include an opposing progestogen for a woman that has her uterus to offset the estrogen-associated risk for endometrial hyperplasia and cancer (NAMS, 2014). Thus, although both efficacious and safe, hormone therapy formulations remain to be optimized, particularly in the domain of cognitive therapeutic effects. The present dissertation work strived to combine tools from behavioral neuroendocrinology with tools from biomedical engineering in an interdisciplinary fashion to develop a 17β-estradiol (E2) delivery platform for improved cognitive effects of E2 treatment while minimizing peripheral E2 burden. Specifically, the overarching goal of this dissertation work was to approach optimization of E2 treatment utilizing three distinct hormone delivery strategies - 1) combining E2 with a cognitively-beneficial progestogen, 2) encapsulating E2 in polymeric nanoparticles for subcutaneous administration, and 3) solubilizing E2 in cyclodextrin for intranasal administration. The rat model of surgical menopause, the surgical removal of the ovaries, or ovariectomy (Ovx), was implemented across all studies, and the same timeline between Ovx, treatment, and behavioral battery initiation was utilized.

Approach 1: Evaluation of estrogen plus progestogen hormone combination treatment that contained the cognitively-beneficial E2 plus a cognitively-beneficial progestogen.

In Chapters 2 and 3, the impact of a daily subcutaneous administration of E2 plus a cognitively-beneficial progestogen on spatial learning and memory, as well as on uterine

stimulation, was evaluated. The cognitively-beneficial progestogen was levonorgestrel (Levo), a synthetic progestogen used in the clinic for both hormone therapy and contraceptive purposes. In the first study, I replicated the beneficial cognitive effects of E2 and of Levo when administered alone in middle-aged, Ovx rats (Heather A. Bimonte-Nelson et al., 2006; Bimonte & Denenberg, 1999; B. Blair Braden et al., 2017; Talboom et al., 2008). Interestingly, I found that the E2 plus Levo hormone combination, at a 5:1 E2 to Levo dose ratio, attenuated the beneficial effects of E2 alone and Levo alone on spatial learning and memory. Indeed, this particular E2 plus hormone combination impaired spatial working memory relative to the individual E2 and Levo treatments when the working memory load was high. Furthermore, I observed a distinct relationship within the E2 alone treatment group between working memory performance and activated Erk2 expression in the frontal cortex. This E2-specific relationship was mitigated by the addition of Levo to the E2 treatment. When evaluating peripheral burden, it was found that uterine horn weight, which was used as a marker of uterine stimulation, was increased following treatments with E2 - both with E2 alone and E2 plus Levo treatment groups. The addition of Levo did not oppose E2-specific effects on uterine stimulation. The clinically available hormone therapy patch that contains both E2 and Levo combines the two hormones in a 3:1 E2 to Levo dose ratio. Thus, in the second study, I addressed how altering E2 to Levo dose ratios, by increasing Levo dose and keeping E2 dose constant, would impact both cognition and uterine stimulation. Specifically, dose ratio-dependent cognitive and uterine effects were evaluated following daily subcutaneous treatment with either a 5:1, 3:1, or 1:2 E2 to Levo dose ratio combinations. Results replicated findings from the first study, where the 5:1 E2 to Levo dose ratio combination treatment had no cognitive effects relative to

vehicle and did not oppose E2-specific uterine stimulation. The 3:1 and 1:2 E2 to Levo dose ratio combination treatments did decrease E2-specific uterine stimulation, but these two treatments impaired spatial learning and memory. Indeed, a significant linear relationship between memory and Levo dose was observed, where the addition of increasing Levo dose to E2 treatment tended to increase memory impairments. Taken together, findings from this translational approach to E2 delivery for optimized cognitive therapeutic effects indicated that even with the selection of a cognitively-beneficial progestogen, the beneficial cognitive effects of E2 alone are reversed when progestogen is added and can even result in impairment in combination formulations that oppose E2's effects at the uterine tissue.

Approach 2: E2 encapsulation in polymeric nanoparticles in order to harness the delivery advantages of nanoparticles, including sustained release, decreased metabolism, and potential for tissue-specific targeting.

Polymeric nanoparticles comprised of poly (lactic-co-glycolic) acid (PLGA) can be engineered to alter the activity and delivery of encapsulated agents. Chapter 4 aimed to evaluate the cognitive effects of weekly subcutaneous E2 encapsulated PLGA nanoparticle treatment in comparison to the blank PLGA nanoparticle and free E2 treatment, as well as the effects of these treatment formulations on uterine exposure to E2. Due to an observed vehicle effect between blank PLGA and sesame oil, the vehicle for free E2, I was not able to directly compare the effects of E2 PLGA to free E2 on spatial learning and memory. Results did show that E2 PLGA treatment improved learning and memory relative to its vehicle control, and free E2 did not differ in learning and memory relative to its vehicle

control. Additionally, although both of the E2-containing treatments increased uterine horn weight in respect to their vehicle controls, the cognitively enhancing E2 PLGA treatment exhibited greater uterine horn weight compared to free E2. Thus, the second delivery approach of this dissertation work underscored the critical importance of including proper controls in behavioral study design and revealed that E2 encapsulation in PLGA nanoparticles can have beneficial learning and memory effects following a treatment regimen when free E2 did not. Yet, E2 encapsulation in PLGA did increase E2 exposure at the uterine horns compared to free E2, suggesting that additional modifications to PLGA nanoparticles or an implementation of alternate delivery routes would be beneficial to achieve more optimal E2 presence in the brain and uterine horns to achieve desired cognitive therapeutic effects.

Approach 3: Intranasal administration of E2, combined with an agent carrier to solubilize E2, to target the delivery of E2 to the brain but minimize its delivery to the uterine horns.

The intranasal route of administration is thought to exhibit direct nose-to-brain agent delivery and can achieve greater agent uptake in the brain relative to peripheral tissues as compared to alternate routes of administration (Dhuria, Hanson, & Frey, 2010; Lochhead & Thorne, 2012; N Nonaka et al., 2008). The aim of the third approach was to determine the cognitive and uterine effects of daily intranasal E2 treatment. To solubilize E2 and to achieve greater E2 uptake in the brain, particularly in cognitive brain regions, four different cyclodextrins (CD) were evaluated as carriers for intranasal E2 delivery and compared to delivery of free E2 (solubilized in a 20% PEG saline solution). Brain-distribution data showed that E2 complexation with 2-hydroxypropyl β-CD increased

uptake of E2 in the dorsal hippocampus, an area involved in spatial learning and memory, half an hour following intranasal administration when compared to free E2. Interestingly, when evaluated for cognitive outcomes, the E2-2-hydroxypropyl β-CD complex impaired spatial learning and memory, even though this formulation was shown to have greater uptake in the dorsal hippocampus as opposed to free E2 and was therefore hypothesized to improve cognitive performance. On the other hand, the free E2 intranasal treatment improved spatial learning and memory, suggesting that E2 concentration in the brain, specifically in regions of the brain that are heavily involved in learning and memory, is a critical factor for E2's cognitive outcomes. Uterine horn weight data indicated that both free E2 and the E2-2-hydroxypropyl β-CD complex increased uterine horn weight relative to their vehicle controls, and that the cognitively impairing E2-2-hydroxypropyl β-CD complex formulation resulted in greater uterine horn weight as compared to the cognitively enhancing free E2. Together, these findings suggest that the intranasal route of administration is a promising delivery strategy for obtaining optimal cognitive therapeutic effects of E2 while minimizing peripheral burden. However, there are still a myriad of factors, including E2 treatment dose, that need to be evaluated in order to develop a better understanding of how these factors can impact the directionality of E2's cognitive effects following intranasal administration.

Conclusions

Developing estrogen-containing hormone therapy formulations that can yield beneficial, or at least neutral, effects on the brain and its functioning, with minimal undesired effects at the uterine tissue, could immensely expand the breadth of clinically-

available hormone-based treatment options across a wide variety of needs. This potential significance spans not only hormone therapy but also contraceptive use and treatment for hormone-related health conditions. The present dissertation work combined biomedical engineering with behavioral neuroendocrinology scientific approaches to evaluate three separate delivery strategies aimed at capitalizing on the beneficial cognitive effects of E2 while minimizing peripheral burden in a rodent model of menopause. Findings from the studies detailed here are comprehensively visualized in Figure 38, where the graph depicts uterine horn weights, a marker of uterine stimulation used across the studies, as a function of the evaluated E2 formulations. For each uterine horn weight bar, the green check-mark indicates improved spatial learning and memory relative to vehicle control, the brown null sign indicates neutral effects on spatial learning and memory relative to vehicle control, and the red 'x' denotes impaired spatial learning and memory relative to vehicle control. For the vehicle controls for each E2 formulation, the average uterine horn weights ranged from 0.135 – 0.184 g (data not shown in the figure); based on prior studies in the Bimonte-Nelson laboratory, uterine horn weights for ovary-intact middle-aged rats, on average, can range from 0.6 g to 0.9 g. Overall, lower stimulating effects at the uterine horns while maintaining cognitively-beneficial effects of E2 treatment following a decrease in circulating ovarian hormone levels were achieved by incorporating the intranasal route of administration for daily E2 treatment and by encapsulating E2 in polymeric nanoparticles for weekly subcutaneous administration. Additionally, several E2 formulations were identified that yielded impairing cognitive effects alongside lower uterine horn stimulation as well as impairing cognitive effects alongside greater uterine horn stimulation. For example, the addition of Levo to E2 treatment resulted in neutral or impairing cognitive

effects. At the 3:1 and the 1:2 E2 to Levo dose ratio treatment formulations, the addition of Levo reduced uterine horn weight compared to free E2 treatment although the weights remained greater than those of the vehicle control group. The greater uterine horn stimulation with E2 plus Levo treatments as compared to vehicle control could in part be explained by the ability of Levo to yield estrogenic activity indirectly via its capability to increase estrogen receptors' mRNA expression or the binding of its derivatives to estrogen receptors (Jayaraman & Pike, 2014; Santillán et al., 2001). Taken together, data from the present dissertation work can serve as the backbone for research in targeted E2 delivery aimed at optimizing the cognitive therapeutic effects of E2 in the context of menopause while minimizing peripheral burden.

The spatial working memory findings across the studies evaluated within this dissertation differ based on the phase of testing, acquisition or asymptotic, and on the trials, moderate working memory load (Trial 3) or highest working memory load (Trial 4) trials, during which E2 treatment effects were observed, which may confound the interpretation as well as the translational impact of the beneficial, neutral, or impairing cognitive effects. When analyzing WRAM data, working memory performance was initially evaluated across all 4 trials within the 12 days of testing with the goal of assessing the learning curve as well as potential trends in performance. Then, performance was blocked into specific phases (e.g., acquisition, asymptotic) of the learning curve such that the reported findings would reflect consistent trends in performance rather than isolation of a single effect. Further, the distinction in treatment effects observed on the moderate working memory load trial versus the highest working memory load trial within this dissertation highlights the ability to evaluate treatment effects that are dependent on working memory complexity

as evaluated by increase in working memory demand on the WRAM. For instance, a switch in working memory performance of a particular treatment group from Trial 3 to Trial 4 suggests that the increase in 1 unit of information in working memory was sufficient to alter the animals' performance in relation to their performance on the previous trial (Trial 3) as well as in relation to the other treatment groups on Trials 3 and 4. Indeed, in an 8-arm WRAM with 7 platformed arms, with the maximum highest working memory load of 7 information units (in contrast, a 4 information unit WRAM design was utilized within this dissertation), ovary-intact rats, low E2 dose treated Ovx rats, and moderate E2 dose treated Ovx rats exhibited improved working memory when the load contained 1 – 4 units of information, and only the moderate E2 dose treatment exhibited improved working memory when the load contained 6 units of information, indicating a working memory load impact on treatment effects as trials progressed and as the units of information within the working memory domain increased (Bimonte & Denenberg, 1999). Thus, in the context of the studies conducted within this dissertation, the difference in spatial working memory performance on Trial 3, the moderate working memory load trial, compared to the difference in spatial working memory performance on Trial 4, the highest working memory load trial, as a function of treatment yield impactful translational interpretations. These subtle changes in treatment effects within a particular working memory load and across an increase in working memory load can represent the difference in effect of a hormone therapeutic on executive function at moderate cognitive demand versus high cognitive demand in menopausal women.

It is important to note some limitations to the interpretation and translational application of data from this dissertation, which warrant additional investigations. For one,

the cognitive and uterine effects in the present studies were all evaluated within a rat model of surgical menopause, with the ovaries being surgically removed. However, most women undergo transitional menopause where ovaries remain intact, resulting in a different circulating ovarian hormone background profile. Studies show that type of menopause model in rats can indeed impact the directionality of a hormone's effects on cognitive outcomes (Acosta, Mayer, Talboom, Tsang, et al., 2009). Additionally, although uterine horn weight was used as a marker of uterine stimulation across all studies, it must be noted that the desired weight of the uterine horn weights for exogenous estrogen administration is not known. In fact, uterine horn weight can naturally fluctuate across a typical reproductive cycle in response to changes in endogenous circulating hormone milieu (Nequin, Alvarez, & Schwartz, 1979). Thus, it would be beneficial to determine an additional marker of uterine stimulation that could aid in the translational interpretation of E2-induced uterine stimulation. Furthermore, this dissertation focused on the delivery of E2, the most potent endogenous estrogen in women and in rodents (Kuhl, 2005). However, there are a multitude of different estrogens that still remain to be evaluated for their potential as a cognitive therapeutic, as they can differ in both brain activity as well as cognitive outcomes (E. B. Engler-Chiurazzi et al., 2012; Hiroi et al., 2016; Kuhl, 2005; Sarah E. Mennenga, Gerson, Koebele, et al., 2015; Prakapenka et al., *in review*). Indeed, additional alternate delivery strategies focused on targeted estrogen delivery, to achieve greater appropriately-regimented estrogen activity in the brain than in the periphery, and greater appropriately-regimented progestogen activity in the uterus than in the brain, must be explored. To be most efficacious, scientific strategies should be armed with knowledge regarding where and when during a lifetime hormone exposure is optimal, as well as what

type and concentration of hormone is most beneficial given specific hormonal historical contexts. Identifying the particular impactful components of these complex interactions is necessary to fill in the missing pieces of the menopause and memory puzzle, and can help scientists and clinicians optimize women's health across a lifetime.

References

Abdulla, F. A., Calaminici, M. R., Stephenson, J. D., & Sinden, J. D. (1993). Chronic treatments with cholinoceptor drugs influence spatial learning in rats. *Psychopharmacology*, *111*(4), 508–511.

Abolaji, A. O., Adedara, I. A., Abajingin, A. O., Fatunmibi, O. J., Ladipo, E. O., & Farombi, E. O. (2016). Evidence of oxidative damage and reproductive dysfunction accompanying 4-vinylcyclohexene diepoxide exposure in female Wistar rats. *Reproductive Toxicology*, *66*, 10–19.

Abolaji, A. O., Toloyai, P.-E., Odeleye, T. D., Akinduro, S., Teixeira Rocha, J. B., & Farombi, E. O. (2016). Hepatic and renal toxicological evaluations of an industrial ovotoxic chemical, 4-vinylcyclohexene diepoxide, in both sexes of Wistar rats. *Environmental Toxicology and Pharmacology*, *45*, 28–40.

Acosta, J. I., Mayer, L. P., Braden, B. B., Nonnenmacher, S., Mennenga, S. E., & Bimonte-Nelson, H. A. (2010). The cognitive effects of conjugated equine estrogens depend on whether menopause etiology is transitional or surgical. *Endocrinology*, *151*(8), 3795–3804.

Acosta, J. I., Mayer, L., Talboom, J. S., Tsang, C. W. S., Smith, C. J., Enders, C. K., & Bimonte-Nelson, H. A. (2009). Transitional versus surgical menopause in a rodent model: etiology of ovarian hormone loss impacts memory and the acetylcholine system. *Endocrinology*, *150*(9), 4248–4259.

Acosta, J. I., Mayer, L., Talboom, J. S., Zay, C., Scheldrup, M., Castillo, J., … Bimonte-Nelson, H. A. (2009). Premarin improves memory, prevents scopolamine-induced amnesia and increases number of basal forebrain choline acetyltransferase positive cells in middle-aged surgically menopausal rats. *Hormones and Behavior*, *55*, 454–464.

Al-Safi, Z. A., & Santoro, N. (2014). Menopausal hormone therapy and menopausal symptoms. *Fertility and Sterility*, *101*(4), 905–915.

Amtul, Z., Wang, L., Westaway, D., & Rozmahel, R. F. (2010). Neuroprotective mechanism conferred by 17beta-estradiol on the biochemical basis of Alzheimer's disease. *Neuroscience*, *169*, 781–786.

Anderson, D. C. (1974). Sex-hormone-binding globulin. *Clinical Endocrinology*, *3*(1), 69–96.

Anderson, J. M., & Shive, M. S. (1997). Biodegradation and biocompatibility of PLA and PLGA microspheres. *Advanced Drug Delivery Reviews*, *28*, 5–24.

Armstrong, D. T. (1968). Hormonal control of uterine lumen fluid retension in the rat. *Am J Physiol*, *214*(4), 764–771.

Asante, A., Whiteman, M. K., Kulkarni, A., Cox, S., Marchbanks, P. A., & Jamieson, D. J. (2010). Elective oophorectomy in the United States: trends and in-hospital complications, 1998-2006. *Obstetrics and Gynecology*, *116*(5), 1088–1095.

Asthana, S., Baker, L. D., Craft, S., Stanczyk, F. Z., Veith, R. C., Raskind, M. A., & Plymate, S. R. (2001). High-dose estradiol improves cognition for women with AD: results of a randomized study. *Neurology*, *57*(4), 605–612.

Asthana, S., Craft, S., Baker, L. D., Raskind, M. A., Birnbaum, R. S., Lofgreen, C. P., … Plymate, S. R. (1999). Cognitive and neuroendocrine response to transdermal estrogen in postmenopausal women with Alzheimer's disease: results of a placebo-controlled, double-blind, pilot study. *Psychoneuroendocrinology*, *24*, 657–677.

Atkins, C. M., Selcher, J. C., Petraitis, J. J., Trzaskos, J. M., & Sweatt, J. D. (1998). The MAPK cascade is required for mammalian associative learning. *Nature Neuroscience*, *1*(7), 602–609.

Baddeley, A. (2003). Working memory and language: an overview. *Journal of Communication Disorders*, *36*, 189–208.

Barha, C. K., Dalton, G. L., & Galea, L. A. M. (2010). Low doses of 17α-estradiol and 17β-estradiol facilitate, whereas higher doses of estrone and 17α- and 17β-estradiol impair, contextual fear conditioning in adult female rats. *Neuropsychopharmacology*, *35*, 547–559.

Barha, C. K., & Galea, L. A. M. (2010). Influence of different estrogens on neuroplasticity and cognition in the hippocampus. *Biochimica et Biophysica Acta - General Subjects*, *1800*, 1056–1067.

Barha, C. K., & Galea, L. A. M. (2013). The hormone therapy, Premarin, impairs hippocampus-dependent spatial learning and memory and reduces activation of new granule neurons in response to memory in female rats. *Neurobiology of Aging*, *34*, 986–1004.

Bayer, J., Gläscher, J., Finsterbusch, J., Schulte, L. H., & Sommer, T. (2018). Linear and inverted U-shaped dose-response functions describe estrogen effects on hippocampal activity in young women. *Nature Communications*, *9*(1), 1–12.

Beach, F. A., & Holz-Tucker, A. M. (1948). Effects of different concentrations of androgen upon sexual behavior in castrated male rats. *Journal of Comparative and Physiological Psychology, 42*(6), 433–453.

Bean, L. A., Ianov, L., & Foster, T. C. (2014). Estrogen receptors, the hippocampus, and memory. *Neuroscientist, 20*(5), 534–545.

Bean, L. A., Kumar, A., Rani, A., Guidi, M., Rosario, A. M., Cruz, P. E., … Foster, T. C. (2015). Re-opening the critical window for estrogen therapy. *The Journal of Neuroscience, 35*(49), 16077–16093.

Bechara, R. G., Lyne, R., & Kelly, Á. M. (2014). BDNF-stimulated intracellular signalling mechanisms underlie exercise-induced improvement in spatial memory in the male Wistar rat. *Behavioral Neuroscience, 275*, 297–306.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society, 57*, 289–300.

Bhavnani, B. R. (2003). Estrogens and menopause: pharmacology of conjugated equine estrogens and their potential role in the prevention of neurodegenerative diseases such as Alzheimer's. *Journal of Steroid Biochemistry and Molecular Biology, 85*, 473–482.

Bhavnani, B. R., & Stanczyk, F. Z. (2014). Pharmacology of conjugated equine estrogens: efficacy, safety and mechanism of action. *Journal of Steroid Biochemistry and Molecular Biology, 142*, 16–29.

Bimonte-Nelson, H. A. (2015a). *The Maze Book: Theories, Practice, and Protocols for Testing Rodent Cognition*. (H. A. Bimonte-Nelson, Ed.) (1st ed.). Humana Press.

Bimonte-Nelson, H. A. (2015b). The Morris maze protocol for rodents. In H. A. Bimonte-Nelson (Ed.), *The Maze Book: Theories, Practice, and Protocols for Testing Rodent Cognition* (1st ed., pp. 441–449). Humana Press.

Bimonte-Nelson, H. A. (2015c). The visible platform task for rodents. In H. A. Bimonte-Nelson (Ed.), *The Maze Book: Theories, Practice, and Protocols for Testing Rodent Cognition* (1st ed., pp. 451–454). Humana Press.

Bimonte-Nelson, H. A. (2015d). The water radial-arm maze: Four out of eight arms platformed protocol for rodents. In H. A. Bimonte-Nelson (Ed.), *The Maze Book: Theories, Practice, and Protocols for Testing Rodent Cognition* (1st ed., pp. 411–419). Humana Press.

Bimonte-Nelson, H. A., Daniel, J. M., & Koebele, S. V. (2015). The mazes. In H. A. Bimonte-Nelson (Ed.), *The Maze Book: Theories, Practice, and Protocols for Testing Rodent Cognition* (1st ed., pp. 37–72). Humana Press.

Bimonte-Nelson, H. A., Francis, K. R., Umphlet, C. D., & Granholm, A. C. (2006). Progesterone reverses the spatial memory enhancements initiated by tonic and cyclic oestrogen therapy in middle-aged ovariectomized female rats. *European Journal of Neuroscience*, *24*(1), 229–242.

Bimonte-Nelson, H. A., Nelson, M. E., & Granholm, A. E. (2004). Progesterone counteracts estrogen-induced increases in neurotrophins in the aged female rat brain. *Neuroreport*, *15*(17), 2659–2663.

Bimonte, H. A., & Denenberg, V. H. (1999). Estradiol facilitates performance as working memory load increases. *Psychoneuroendocrinology*, *24*(2), 161–173.

Bimonte, H. A., Mack, C. M., Stavnezer, A. J., & Denenberg, V. H. (2000). Ovarian hormones can organize the rat corpus callosum in adulthood. *Developmental Brain Research*, *121*(2), 169–177.

Blair, R. M., Fang, H., Branham, W. S., Hass, B. S., Dial, S. L., Moland, C. L., … Sheehan, D. M. (2000). The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. *Toxicological Sciences*, *54*(1), 138–153.

Blum, S., Moore, A. N., Adams, F., & Dash, P. K. (1999). A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial memory. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *19*(9), 3535–3544.

Borman, S. M., VanDePol, B. J., Kao, S., Thompson, K. E., Sipes, I. G., & Hoyer, P. B. (1999). A single dose of the ovotoxicant 4-vinylcyclohexene diepoxide is protective in rat primary ovarian follicles. *Toxicology and Applied Pharmacology*, *158*, 244–252.

Braden, B. B., Andrews, M. G., Acosta, J. I., Mennenga, S. E., Lavery, C., & Bimonte-Nelson, H. A. (2017). A comparison of progestins within three classes: differential effects on learning and memory in the aging surgically menopausal rat. *Behavioural Brain Research*, *322*, 258–268.

Braden, B. B., Garcia, A. N., Mennenga, S. E., Prokai, L., Villa, S. R., Acosta, J. I., … Bimonte-Nelson, H. A. (2011). Cognitive-impairing effects of medroxyprogesterone acetate in the rat: independent and interactive effects across time. *Psychopharmacology*, *218*(2), 405–418.

Braden, B. B., Kingston, M. L., Whitton, E., Lavery, C., Tsang, C. W. S., & Bimonte-Nelson, H. A. (2015). The GABAA antagonist bicuculline attenuates progesterone-induced memory impairments in middle-aged ovariectomized rats. *Frontiers in Aging Neuroscience*, *7*, 1–8.

Braden, B. B., Talboom, J. S., Crain, I. D., Simard, A. R., Lukas, R. J., Prokai, L., … Bimonte-Nelson, H. A. (2010). Medroxyprogesterone acetate impairs memory and alters the GABAergic system in aged surgically menopausal rats. *Neurobiology of Learning and Memory*, *93*, 444–453.

Brinton, R. D., Proffitt, P., Tran, J., & Luu, R. (1997). Equilin, a principal component of the estrogen replacement therapy premarin, increases the growth of cortical neurons via an NMDA receptor-dependent mechanism. *Experimental Neurology*, *147*, 211–220.

Burger, H. G., Hale, G. E., Dennerstein, L., & Robertson, D. M. (2008). Cycle and hormone changes during perimenopause: the key role of ovarian function. *Menopause (New York, N.Y.)*, *15*, 603–612.

Caldwell, B. M., & Watson, R. I. (1952). An evaluation of psychologic effects of sex hormone administration in aged women. *Journal of Gerentology*, *7*, 228–244.

Camp, B. W., Gerson, J. E., Tsang, C. W. S., Villa, S. R., Acosta, J. I., Blair Braden, B., … Bimonte-Nelson, H. A. (2012). High serum androstenedione levels correlate with impaired memory in the surgically menopausal rat: A replication and new findings. *European Journal of Neuroscience*, *36*(8), 3086–3095.

Carroll, J. C., Rosario, E. R., Chang, L., Stanczyk, F. Z., Oddo, S., LaFerla, F. M., & Pike, C. J. (2007). Progesterone and estrogen regulate Alzheimer-like neuropathology in female 3xTg-AD mice. *The Journal of Neuroscience*, *27*(48), 13357–13365.

Carroll, J. C., Rosario, E. R., Villamagna, A., & Pike, C. J. (2010). Continuous and cyclic progesterone differentially interact with estradiol in the regulation of Alzheimer-like pathology in female 3xtransgenic-Alzheimer's disease mice. *Endocrinology*, *151*(6), 2713–2722.

Chasin, M., & Langer, R. (1990). *Biodegradable Polymers as Drug Delivery Systems*. *Biodegradable Polymers as Drug Delivery Systems*. Marcel Dekker, Inc.

Chhabra, R. (1989). Toxicology and carcinogenesis studies of 4-vinyl-1-cyclohexene diepoxide in F344/N rats and B6C3F1 mice. *National Toxicology Program Technical Report Series*, *362*, 1–249.

Chisholm, N. C., & Juraska, J. M. (2012). Long term replacement of estrogen in combination with medroxyprogesterone acetate improves acquisition of an alternation task in middle aged female rats. *Behavioral Neuroscience*, *126*(1), 128–136.

Chu, D., Tian, J., Liu, W., Li, Z., & Li, Y. (2007). Poly(lactic-co-glycolic acid) microspheres for the controlled release of huperzine A: in vitro and in vivo studies and the application in the treatment of the impaired memory of mice. *Chemical & Pharmaceutical Bulletin*, *55*(4), 625–628.

Ciccarelli, A., & Giustetto, M. (2014). Role of ERK signaling in activity-dependent modifications of histone proteins. *Neuropharmacology*, *80*, 34–44.

Colby, S. L., & Ortman, J. M. (2015). Projections of the size and composition of the US population: 2014 to 2060. *Current Population Reports*, P25-1143. Retrieved from http://www.census.gov/content/dam/Census/library/publications/2015/demo/p25-1143.pdf

Conn, J., Oyasu, R., Welsh, M., & Beal, J. (1974). Vicryl (polyglactin 910) synthetic absorbalbe sutures. *The American Journal of Surgery*, *128*, 19–23.

Conrad, C. D., McLaughlin, K. J., Huynh, T. N., El-Ashmawy, M., & Sparks, M. (2012). Chronic stress and a cyclic regimen of estradiol administration separately facilitate spatial memory: Relationship with CA1 spine density and dendritic complexity. *Behavioral Neuroscience*, *126*(1), 142–156.

Cook, R. L., Householder, K. T., Chung, E. P., Prakapenka, A. V., Diperna, D. M., & Sirianni, R. W. (2015). A critical evaluation of drug delivery from ligand modified nanoparticles: Confounding small molecule distribution and efficacy in the central nervous system. *Journal of Controlled Release*, *220*.

Creasy, G. W., Kafrissen, M. E., & Upmalis, D. (1992). Review of the endometrial effects of estrogens and progestins. *Obsterical and Gynecological Survey*, *47*(9), 654–678.

Cronje, W. H., Vashisht, A., & Studd, J. W. W. (2004). Hysterectomy and bilateral oophorectomy for severe premenstrual syndrome. *Human Reproduction*, *19*(9), 2152–2155.

Danhier, F., Ansorena, E., Silva, J. M., Coco, R., Le Breton, A., & Préat, V. (2012). PLGA-based nanoparticles: An overview of biomedical applications. *Journal of Controlled Release*, *161*(2), 505–522.

Daniel, J. M., Fader, A. J., Spencer, A. L., & Dohanich, G. P. (1997). Estrogen enhances performance of female rats during acquisition of a radial arm maze. *Hormones and Behavior*, *32*(3), 217–225.

Daniel, J. M., Hulst, J. L., & Berbling, J. L. (2006). Estradiol replacement enhances working memory in middle-aged rats when initiated immediately after ovariectomy but not after a long-term period of ovarian hormone deprivation. *Endocrinology*, *147*(1), 607–614.

Daniel, J. M., Witty, C. F., & Rodgers, S. P. (2015). Long-term consequences of estrogens administered in midlife on female cognitive aging. *Hormones and Behavior*, *74*, 77–85.

Davis, M. E., & Brewster, M. E. (2004). Cyclodextrin-based pharmaceutics: past, present and future. *Nature Reviews Drug Discovery*, *3*(12), 1023–1035.

Davis, P. G., Chaptal, C. V., & McEwen, B. S. (1979). Independence of the differentiation of masculine and feminine sexual behavior in rats. *Hormones and Behavior*, *12*, 12–19.

Dhuria, S. V., Hanson, L. R., & Frey, W. H. (2010). Intranasal Delivery to the Central Nervous System: Mechanisms and Experimental Considerations. *Journal of Pharmaceutical Sciences*, *99*(4).

Diz-Chaves, Y., Kwiatkowska-Naqvi, A., Von Hülst, H., Pernía, O., Carrero, P., & Garcia-Segura, L. M. (2012). Behavioral effects of estradiol therapy in ovariectomized rats depend on the age when the treatment is initiated. *Experimental Gerontology*, *47*, 93–99.

Dooley, M., Spencer, C. M., & Ormrod, D. (2001). Estradiol-intranasal: a review of its use in the management of menopause. *Drugs*, *61*(15), 2243–2262.

Downs, J., & Wise, P. M. (2009). The role of the brain in female reporoductive aging. *Journal of Endocrinology*, *299*, 32–38.

Du, J., Sanchez, P., Kim, L., & Azen, C. (2013). Percutaneous progesterone delivery via cream or gel application in postmenopausal women: A randomized cross-over study of progesterone levels in. *Menopause*, *20*(11), 1–7.

Dyer, C. A., Raymond-Whish, S., Schmuki, S., Fisher, T., Pyzyna, B., Bennett, A., & Mayer, L. P. (2013). Accelerated follicle depletion in vitro and in vivo in Sprague-Dawley rats using the combination of 4-vinylcyclohexene diepoxide and triptolide. *Journal of Zoo and Wildlife Medicine*, *44*(4S), S9-17.

El-Bakri, N. K., Islam, A., Zhu, S., Elhassan, A., Mohammed, A., Winblad, B., & Adem, A. (2004). Effects of estrogen and progesterone treatment on rat hippocampal NMDA receptors: Relationship to Morris water maze performance. *J. Cell. Mol. Med.*, *8*(4), 537–544.

Engler-Chiurazzi, E. B., Talboom, J. S., Braden, B. B., Tsang, C. W. S., Mennenga, S., Andrews, M., … Bimonte-Nelson, H. A. (2012). Continuous estrone treatment impairs spatial memory and does not impact number of basal forebrain cholinergic neurons in the surgically menopausal middle-aged rat. *Hormones and Behavior*, *62*, 1–9.

Engler-Chiurazzi, E., Tsang, C., Nonnenmacher, S., Liang, W. S., Corneveaux, J. J., Prokai, L., … Bimonte-Nelson, H. A. (2011). Tonic Premarin dose-dependently enhances memory, affects neurotrophin protein levels and alters gene expression in middle-aged rats. *Neurobiology of Aging*, *32*, 680–697.

Erekson, E. A., Martin, D. K., & Ratner, E. S. (2013). Oophorectomy: the debate between ovarian conservation and elective oophorectomy. *Menopause (New York, N.Y.)*, *20*(1), 110–114.

Espeland, M. A., Rapp, S. R., Shumaker, S. A., Brunner, R., Manson, J. E., Sherwin, B. B., … Hays, J. (2004). Conjugated equine estrogens and global cognitive function in postmenopausal women: Women's Health Initiative Memory Study. *The Journal of the American Medical Association*, *291*(24), 2959–2968.

Eyster, K. M. (2016). The estrogen receptors: an overview from different perspectives. *Methods in Molecular Biology*, *1366*, 1–10.

Fader, A. J., Johnson, P. E. M., & Dohanich, G. P. (1999). Estrogen improves working but not reference memory and prevents amnestic effects of scopolamine on a radial-arm maze. *Pharmacology Biochemistry and Behavior*, *62*(4), 711–717.

Fan, L., Zhao, Z., Orr, P. T., Chambers, C. H., Lewis, M. C., & Frick, K. M. (2010). Estradiol-induced object memory consolidation in middle-aged female mice requires dorsal hippocampal extracellular signal-regulated kinase and phosphatidylinositol 3-kinase activation. *Journal of Neuroscience*, *30*(12), 4390–4400.

Fan, S., Zheng, Y., Liu, X., Fang, W., Chen, X., & Liao, W. (2018). Curcumin-loaded PLGA-PEG nanoparticles conjugated with B6 peptide for potential use in Alzheimer's disease. *Drug Delivery*, *25*(1), 1044–1055.

Fazil, M., Shadab, Baboota, S., Sahni, J. K., & Ali, J. (2012). Nanotherapeutics for Alzheimer's disease (AD): Past, present and future. *Journal of Drug Targeting*, *20*(2), 97–113.

Feng, Z., Cheng, Y., & Zhang, J. (2004). Long-term effects of melatonin or 17β-estradiol on improving spatial memory performance in cognitively impaired, ovariectomized adult rats. *J. Pineal Res, 37*, 198–206.

Fernandez, S. M., Lewis, M. C., Pechenino, A. S., Lauren, L., Orr, P. T., Gresack, J. E., … Frick, K. M. (2008). Estradiol-induced enhancement of object memory consolidation involves hippocampal Erk activation and membrane-bound estrogen receptors. *Journal of Neuroscience, 28*(35), 8660–8667.

Fitch, R. H., Cowell, P. E., Schrott, L. M., & Denenberg, V. H. (1991). Corpus callosum: demasculization via perinatal anti-androgen. *International Journal of Developmental Neuroscience*, *9*(1), 35–38.

Flaws, J. A., Doerr, J. K., Sipes, I. G., & Hoyer, P. B. (1994). Destruction of preantral follicles in adult rats by 4-vinyl-1-cyclohexene diepoxide. *Reproductive Toxicology*, *8*(6), 509–514.

Fortress, A. M., Fan, L., Orr, P. T., Zhao, Z., & Frick, K. M. (2013). Estradiol-induced object recognition memory consolidation is dependent on activation of mTOR signaling in the dorsal hippocampus. *Learning & Memory*, *20*(3), 147–155.

Fortress, A. M., Heisler, J. D., & Frick, K. M. (2015). The mTOR and canonical Wnt signaling pathways mediate the mnemonic effects of progesterone in the dorsal hippocampus. *Hippocampus*, *25*(5), 616–629.

Foster, T. C. (2012). Role of estrogen receptor alpha and beta expression and signalling on cognitive function during aging. *Hippocampus*, *22*(4), 656–669.

Foster, T. C., Sharrow, K. M., Kumar, A., & Masse, J. (2003). Interaction of age and chronic estradiol replacement on memory and markers of brain aging. *Neurobiology of Aging*, *24*(6), 839–852.

Fournier, E., Passirani, C., Montero-Menei, C. N., & Benoit, J. P. (2003). Biocompatibility of implantable synthetic polymeric drug carriers: Focus on brain biocompatibility. *Biomaterials*, *24*(19), 3311–3331.

Freeman, M. E. (2006). Neuroendocrine control of the ovarian cycle of the rat. In J. D. Neill (Ed.), *Knobil and Neill's Physiology of Reproduction* (Third Edit, pp. 2327–2388). Elsevier Inc.

Frick, K. M. (2009). Estrogens and age-related memory decline in rodents: what have we learned and where do we go from here? *Hormones and Behavior*, *55*(1), 2–23.

Frick, K. M. (2015). Molecular mechanisms underlying the memory-enhancing effects of estradiol. *Hormones and Behavior*, *74*, 4–18.

Frye, C. A., Duffy, C. K., & Walf, A. A. (2007). Estrogens and progestins enhance spatial learning of intact and ovariectomized rats in the object placement task. *Neurobiology of Learning and Memory*, *88*(2), 208–216.

Funahashi, S., & Kubota, K. (1994). Working memory and prefrontal cortex. *Neuroscience Research*, *21*, 1–11.

Geary, N., Trace, D., McEwen, B., & Smith, G. P. (1994). Cyclic estradiol replacement increases the satiety effect of CCK-8 in ovariectomized rats. *Physiology and Behavior*, *56*(2), 281–289.

Ghazvini, H., Khaksari, M., Esmaeilpour, K., Shabani, M., Asadi-Shekaari, M., Khodamoradi, M., & Sheibani, V. (2016). Effects of treatment with estrogen and progesterone on the methamphetamine-induced cognitive impairment in ovariectomized rats. *Neuroscience Letters*, *619*, 60–67.

Gibbs, R. B. (1998). Impairment of basal forebrain cholinergic neurons associated with aging and long-term loss of ovarian function. *Experimental Neurology*, *151*, 289–302.

Gibbs, R. B. (1999). Estrogen replacement enhances acquisition of a spatial memory task and reduces deficits associated with hippocampal muscarinic receptor inhibition. *Hormones and Behavior*, *36*(3), 222–233.

Gibbs, R. B. (2000). Long-term treatment with estrogen and progesterone enhances acquisition of a spatial memory task by ovariectomized aged rats. *Neurobiol. Aging*, *21*, 107–116.

Gibbs, R. B. (2002). Basal forebrain cholinergic neurons are necessary for estrogen to enhance acquisition of a delayed matching-to-position T-maze task. *Hormones and Behavior*, *42*(3), 245–257.

Gibbs, R. B. (2007). Estradiol enhances DMP acquisition via a mechanism not mediated by turning strategy but which requires intact basal forebrain cholinergic projections. *Hormones and Behavior*, *52*(3), 352–359.

Gibbs, R. B. (2010). Estrogen therapy and cognition: a review of the cholinergic hypothesis. *Endocrine Reviews*, *31*(2), 224–253.

Gibbs, R. B., Gabor, R., Cox, T., & Johnson, D. A. (2004). Effects of raloxifene and estradiol on hippocampal acetylcholine release and spatial learning in the rat. *Psychoneuroendocrinology*, *29*(6), 741–748.

Gibbs, R. B., & Johnson, D. A. (2008). Sex-specific effects of gonadectomy and hormone treatment on acquisition of a 12-arm radial maze task by Sprague Dawley rats. *Endocrinology*, *149*(6), 3176–3183.

Gleason, C. E., Dowling, N. M., Wharton, W., Manson, J. A. E., Miller, V. M., Atwood, C. S., … Asthana, S. (2015). Effects of hormone therapy on cognition and mood in recently postmenopausal women: findings from the randomized, controlled KEEPS-Cognitive and Affective Study. *PLoS Medicine*, *12*(6), 1–25.

Goldman, J. M., Murr, A. S., & Cooper, R. L. (2007). The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Research. Part B, Developmental and Reproductive Toxicology*, *80*, 84–97.

Gooney, M., Shaw, K., Kelly, A., O'Mara, S. M., & Lynch, M. A. (2002). Long-term potentiation and spatial learning are associated with increased phosphorylation of TrkB and extracellular signal-regulated kinase (ERK) in the dentate gyrus: Evidence for a role for brain-derived neurotrophic factor. *Behavioral Neuroscience*, *116*(3), 455–463.

Green, R., Luttge, W. G., & Whalen, R. E. (1969). Uptake and retention of tritiated estradiol in brain and peripheral tissues of male, female and neonatally androgenized female rats. *Endocrinology*, *85*(2), 373–378.

Greendale, G. A., Derby, C. A., & Maki, P. M. (2011). Perimenopause and cognition. *Obstetrics and Gynecology Clinics of North America*, *38*(3), 519–535.

Gresack, J. E., & Frick, K. M. (2006). Effects of continuous and intermittent estrogen treatments on memory in aging female mice. *Brain Research*, *1115*, 135–147.

Haim, S., Shakhar, G., Rossene, E., Taylor, A. N., & Ben-Eliyahu, S. (2003). Serum levels of sex hormones and corticosterone throughout 4- and 5-day estrous cycles in Fischer 344 rats and their simulation in ovariectomized females. *Journal of Endocrinology*, *26*, 1013–1022.

Harburger, L. L., Bennett, J. C., & Frick, K. M. (2007). Effects of estrogen and progesterone on spatial memory consolidation in aged females. *Neurobiology of Aging*, *28*(4), 602–610.

Harburger, L. L., Pechenino, A. S., Saadi, A., & Frick, K. M. (2008). Post-training progesterone dose-dependently enhances object, but not spatial, memory consolidation. *Behavioural Brain Research, 194*(2), 174–180.

Harburger, L. L., Saadi, A., & Frick, K. M. (2009). Dose-dependent effects of post-training estradiol plus progesterone treatment on object memory consolidation and hippocampal extracellular signal-regulated kinase activation in young ovariectomized mice. *Neuroscience, 160*(1), 6–12.

Harlow, S. D., & Paramsothy, P. (2011). Menstruation and the menopause transition. *Obstetrics and Gynecology Clinics of North America*, *38*(3), 595–607.

Henderson, V. W. (2006). Estrogen-containing hormone therapy and Alzheimer's disease risk: understanding discrepant inferences from observational and experimental research. *Neuroscience*, *138*, 1031–1039.

Hiroi, R., Weyrich, G., Koebele, S. V., Mennenga, S. E., Talboom, J. S., Hewitt, L. T., … Bimonte-Nelson, H. A. (2016). Benefits of hormone therapy estrogens depend on estrogen type: 17β-estradiol and conjugated equine estrogens have differential effects on cognitive, anxiety-like, and depressive-like behaviors and increase tryptophan hydroxylase-2 mRNA levels in dorsal raphe nucleus subregions. *Frontiers in Neuroscience*, *10*, 517.

Holmes, M. M., Wide, J. K., & Galea, L. A. (2002). Low levels of estradiol facilitate, whereas high levels of estradiol impair, working memory performance on the radial arm maze. *Behav Neurosci*, *116*(5), 928–934.

Hoyer, P. B., Devine, P. J., Hu, X., Thompson, K. E., & Sipes, I. G. (2001). Ovarian toxicity of 4-vinylcyclohexene diepoxide: a mechanistic model. *Toxicologic Pathology, 29*(1), 91–99.

Hoyer, P. B., & Sipes, I. G. (2007). Development of an animal model for ovotoxicity using 4-vinylcyclohexene: a case study. *Birth Defects Research. Part B, Developmental and Reproductive Toxicology*, *80*, 113–125.

Huang, H. H., Steger, R. W., Bruni, J. F., & Meites, J. (1978). Patterns of sex steroid and gonadotropin secretion in aging female rats. *Endocrinology, 103*(5), 1855–1859.

Hyde, L., Hoplight, B., & Denenberg, V. (1998). Water version of the radial - arm maze: learning in three inbred strains of mice. *Brain Research*, *785*(2), 236–244.

Jarrard, L. E., Luu, L. P., & Davidson, T. L. (2012). A study of hippocampal structure-function relations along the septo-temporal axis. *Hippocampus*, *22*(4), 680–692.

Jayaraman, A., & Pike, C. J. (2014). Differential effects of synthetic progestagens on neuron survival and estrogen neuroprotection in cultured neurons. *Molecular and Cellular Endocrinology*, *384*(1–2), 52–60.

Jehan, Q., Srivasta, S., Akhlaq, M., Ahmad, A., & Setty, B. S. (1982). Kinetics of distribution and retention of 3H-oestradiol-17 beta in rat tissues: a comparative study with free oestradiol and after its incorporation into liposomes. *Endocrinology*, *80*(1), 8–12.

Jensen, E. V. (1963). Comments on Dr. Pearlman's paper; Comparison of androgens and estrogens as to their fate in target tissues. *National Cancer Institute Monograph*, *12*, 317–322.

Joshi, S. A., Chavhan, S. S., & Sawant, K. K. (2010). Rivastigmine-loaded PLGA and PBCA nanoparticles: Preparation, optimization, characterization, in vitro and pharmacodynamic studies. *European Journal of Pharmaceutics and Biopharmaceutics*, *76*(2), 189–199.

Kao, S.-W., Sipes, I. G., & Hoyer, P. B. (1999). Early effects of ovotoxicity induced by 4-vinylcyclohexene diepoxide in rats and mice. *Reproductive Toxicology*, *13*(1), 67–75.

Kappeler, C. J., & Hoyer, P. B. (2012). 4-vinylcyclohexene diepoxide: a model for chemical ovotoxicity. *Systems Biology in Reproductive Medicine*, *58*(1), 57–62.

Karlamangla, A. S., Lachman, M. E., Han, W., Huang, M., & Greendale, G. A. (2017). Evidence for cognitive aging in midlife women: Study of women's health across the nation. *Plos One*, *12*(1), 1–13.

Kermath, B. A., & Gore, A. C. (2012). Neuroendocrine control of the transition to reproductive senescence: lessons learned from the female rodent model. *Neuroendocrinology*, *96*(1), 1–12.

Khalin, I., Alyautdin, R., Wong, T. W., Gnanou, J., Kocherga, G., & Kreuter, J. (2016). Brain-derived neurotrophic factor delivered to the brain using poly (lactide-co-glycolide) nanoparticles improves neurological and cognitive outcome in mice with traumatic brain injury. *Drug Delivery*, *23*(9), 3520–3528.

Kiss, Á., Delattre, A. M., Pereira, S. I. R., Carolino, R. G., Szawka, R. E., Anselmo-Franci, J. A., … Ferraz, A. C. (2012). 17β-Estradiol replacement in young, adult and middle-aged female ovariectomized rats promotes improvement of spatial reference memory and an antidepressant effect and alters monoamines and BDNF levels in memory- and depression-related brain areas. *Behavioural Brain Research*, *227*(1), 100–108.

Koebele, S. V., & Bimonte-Nelson, H. A. (2015). Trajectories and phenotypes with estrogen exposures across the lifespan: What does Goldilocks have to do with it? *Hormones and Behavior*, *74*, 86–104.

Koebele, S. V., & Bimonte-Nelson, H. A. (2016). Modeling menopause: the utility of rodents in translational behavioral endocrinology research. *Maturitas*, *87*, 5–17.

Koebele, S. V., & Bimonte-Nelson, H. A. (2017). The endocrine-brain-aging triad where many paths meet: Female reproductive hormone changes at midlife and their influence on circuits important for learning and memory. *Experimental Gerontology*, *94*, 14–23.

Koebele, S. V., Mennenga, S. E., Hiroi, R., Quihuis, A. M., Hewitt, L. T., Poisson, M. L., … Bimonte-Nelson, H. A. (2017). Cognitive changes across the menopause transition: A longitudinal evaluation of the impact of age and ovarian status on spatial memory. *Hormones and Behavior*, *87*, 96–114.

Komnenich, P., Lane, D. M., Dickey, R. P., & Stone, S. C. (1978). Gonadal hormones and cognitive performance. *Physiological Psychology*, *6*(1), 115–120.

Korol, D. L., & Pisani, S. L. (2015). Estrogens and cognition: Friends or foes? An evaluation of the opposing effects of estrogens on learning and memory. *Hormones and Behavior*, *74*, 105–115.

Kretz, O., Fester, L., Wehrenberg, U., Zhou, L., Brauckmann, S., Zhao, S., … Rune, G. M. (2004). Hippocampal synapses depend on hippocampal estrogen synthesis. *The Journal of Neuroscience*, *24*(26), 5913–5921.

Kreuter, J. (2014). Drug delivery to the central nervous system by polymeric nanoparticles: What do we know? *Advanced Drug Delivery Reviews*, *71*, 2–14.

Kronenberg, H. M., Melmed, S., Polonsky, K. S., & Larsen, P. R. (2008). *Williams Textbook of Endocrinology* (11th ed.). Elsevier.

Kuhl, H. (2005). Pharmacology of estrogens and progestogens: influence of different routes of administration. *Climacteric*, *8*, 3–63.

Kuhnz, W., Gansau, C., & Mahler, M. (1993). Pharmacokinetics of estradiol, free and total estrone, in young women following single intravenous and oral administration of 17 beta-estradiol. *Arzneimittelforschung*, *43*(9), 966–973.

Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Häggblad, J., Nilsson, S., & Gustafsson, J. A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology*, *138*(3), 863–870.

Labrie, F., Cusan, L., Gomez, J.-L., Côté, I., Bérubé, R., Bélanger, P., … Labrie, C. (2009). Effect of one-week treatment with vaginal estrogen preparations on serum estrogen levels in postmenopausal women. *Menopause (New York, N.Y.)*, *16*(1), 30–36.

Lees, B., & Stevenson, J. C. (2001). The prevention of osteoporosis using sequential low-dose hormone replacement therapy with estradiol-17β and dydrogesterone. *Osteoporosis International*, *12*, 251–258.

LeFevre, J., & McClintock, M. K. (1988). Reproductive senescence in female rats: a longitudinal study of individual differences in estrous cycles and behavior. *Biology of Reproduction*, *38*(4), 780–789.

Leutgeb, S., Leutgeb, J. K., Barnes, C. A., Moser, E. I., McNaughton, B. L., & Moser, M. (2005). Independent codes for spatial and episodic memory in hippocampal neuronal ensembles. *Science*, *309*, 619–623.

Lewis, M. C., Orr, P. T., & Frick, K. M. (2008). Differential effects of acute progesterone administration on spatial and object memory in middle-aged and aged female C57BL/6 mice. *Hormones and Behavior*, *54*(3), 455–462.

Liu, L., Zhao, L., She, H., Chen, S., Wang, J. M., Wong, C., … Brinton, R. D. (2010). Clinically relevant progestins regulate neurogenic and neuroprotective responses in vitro and in vivo. *Endocrinology*, *151*(12), 5782–5794.

Lobo, R. A. (2007). *Treatment of the Postmenopausal Woman: Basic and Clinical Aspects* (3rd ed.). Academic Press.

Lochhead, J. J., & Thorne, R. G. (2012). Intranasal delivery of biologics to the central nervous system. *Advanced Drug Delivery Reviews*, *64*(7), 614–628.

Lowder, J. L., Oliphant, S. S., Ghetti, C., Burrows, L. J., Meyn, L. A., & Balk, J. (2010). Prophylactic bilateral oophorectomy or removal of remaining ovary at the time of hysterectomy in the United States, 1979-2004. *American Journal of Obstetrics and Gynecology*, *202*(6), 538.e1–538.e9.

Lowry, N. C., Pardon, L. P., Yates, M. A., & Juraska, J. M. (2010). Effects of long-term treatment with 17 β-estradiol and medroxyprogesterone acetate on water maze performance in middle aged female rats. *Hormones and Behavior*, *58*, 200–207.

Luine, V. N. (2014). Estradiol and cognitive function: Past , present and future. *Hormones and Behavior*, *66*(4), 602–618.

Luine, V. N., Jacome, L. F., & Maclusky, N. J. (2003). Rapid enhancement of visual and place memory by estrogens in rats. *Endocrinology*, *144*(7), 2836–2844.

Luine, V. N., Richards, S. T., Wu, V. Y., & Beck, K. D. (1998). Estradiol enhances learning and memory in a spatial memory task and effects levels of monoaminergic neurotransmitters. *Hormones and Behavior*, *34*, 149–162.

Magoffin, D. A. (2005). Ovarian theca cell. *International Journal of Biochemistry and Cell Biology*, *37*, 1344–1349.

Makadia, H. K., & Siegel, S. J. (2011). Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. *Polymers*, *3*(3), 1377–1397.

Maki, P. M. (2012). Minireview: Effects of different HT formulations on cognition. *Endocrinology*, *153*(8), 3564–3570.

Maki, P. M. (2013). The critical window hypothesis of hormone therapy and cognition: a scientific update on clinical studies. *Menopause (New York, N.Y.)*, *20*(6), 695–709.

Manson, J. E., Bassuk, S. S., Harman, S. M., Brinton, E. a, Cedars, M. I., Lobo, R., … Santoro, N. (2006). Postmenopausal hormone therapy: new questions and the case for new clinical trials. *Menopause (New York, N.Y.)*, *13*(1), 139–147.

Markham, J. A., Pych, J. C., & Juraska, J. M. (2002). Ovarian hormone replacement to aged ovariectomized female rats benefits acquisition of the Morris water maze. *Hormones and Behavior*, *42*, 284–293.

Markowska, A. L. (2002). Protective effect of practice on cognition during aging: Implications for predictive characteristics of performance and efficacy of practice. *Neurobiology of Learning and Memory*, *78*(2), 294–320.

Markowska, A. L., & Savonenko, A. V. (2002). Effectiveness of estrogen replacement in restoration of cognitive function after long-term estrogen withdrawal in aging rats. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *22*(24), 10985–10995.

Matsumoto, A., Arai, Y., & Osanai, M. (1985). Estrogen stimulates neuronal plasticity in the deafferented hypothalamic arcuate nucleus in aged female rats. *Neuroscience Research*, *2*, 412–418.

Mattison, D. R., Karyakina, N., Goodman, M., & LaKind, J. S. (2014). Pharmaco- and toxicokinetics of selected exogenous and endogenous estrogens: a review of the data and identification of knowledge gaps. *Critical Reviews in Toxicology*, *44*(8), 696–724.

Mayer, L. P., Devine, P. J., Dyer, C. A., & Hoyer, P. B. (2004). The follicle-deplete mouse ovary produces androgen. *Biology of Reproduction*, *71*, 130–138.

Mayer, L. P., Pearsall, N. A., Christian, P. J., Devine, P. J., Payne, C. M., McCuskey, M. K., … Hoyer, P. B. (2002). Long-term effects of ovarian follicular depletion in rats by 4-vinylcyclohexene diepoxide. *Reproductive Toxicology*, *16*, 775–781.

McClure, R. E. S., Barha, C. K., & Galea, L. A. M. (2013). 17β-Estradiol, but not estrone, increases the survival and activation of new neurons in the hippocampus in response to spatial memory in adult female rats. *Hormones and Behavior*, *63*(1), 144–157.

McGauran, A. T., Moore, J. B., Madsen, D., Barry, D., O'Dea, S., Mahon, B. P., & Commins, S. (2008). A possible role for protein synthesis, extracellular signal-regulated kinase, and brain-derived neurotrophic factor in long-term spatial memory retention in the water maze. *Behavioral Neuroscience*, *122*(4), 805–815.

McLaughlin, K. J., Bimonte-Nelson, H. A., Neisewander, J. L., & Conrad, C. D. (2008). Assessment of estradiol influence on spatial tasks and hippocampal CA1 spines: Evidence that the duration of hormone deprivation after ovariectomy compromises 17β-estradiol effectiveness in altering CA1 spines. *Hormones and Behavior*, *54*(3), 386–395.

McLay, R. N., Maki, P. M., & Lyketsos, C. G. (2003). Nulliparity and late menopause are associated with decreased cognitive decline. *Journal of Neuropsychiatry and Clinical Neurosciences*, *15*, 161–167.

Mennenga, S. E., & Bimonte-Nelson, H. A. (2013). Translational cognitive endocrinology: Designing rodent experiments with the goal to ultimately enhance cognitive health in women. *Brain Research*, *1514*, 50–62.

Mennenga, S. E., & Bimonte-Nelson, H. A. (2015). The importance of incorporating both sexes and embracing hormonal diversity when conducting rodent behavioral assays. In H. A. Bimonte-Nelson (Ed.), *The Maze Book: Theories, Practice, and Protocols for Testing Rodent Cognition* (1st ed., pp. 299–321). Humana Press.

Mennenga, S. E., Gerson, J. E., Dunckley, T., & Bimonte-Nelson, H. A. (2015). Harmine treatment enhances short-term memory in old rats: Dissociation of cognition and the ability to perform the procedural requirements of maze testing. *Physiology and Behavior*, *138*, 260–265.

Mennenga, S. E., Gerson, J. E., Koebele, S. V., Kingston, M. L., Tsang, C. W. S., Engler-Chiurazzi, E. B., … Bimonte-Nelson, H. A. (2015). 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. *Psychoneuroendocrinology*, *54*, 1–13.

Mennenga, S. E., Koebele, S. V., Mousa, A. A., Alderete, T. J., Tsang, C. W. S., Acosta, J. I., … Bimonte-Nelson, H. A. (2015). Pharmacological blockade of the aromatase enzyme, but not the androgen receptor, reverses androstenedione-induced cognitive impairments in young surgically menopausal rats. *Steroids*, *99*, 16–25.

Micevych, P., & Sinchak, K. (2008). Estradiol regulation of progesterone synthesis in the brain. *Molecular and Cellular Endocrinology*, *290*(1–2), 44–50.

Mir, M., Ahmed, N., & Rehman, A. ur. (2017). Recent applications of PLGA based nanostructures in drug delivery. *Colloids and Surfaces B: Biointerfaces*, *159*, 217–231.

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

Mittal, G., Carswell, H., Brett, R., Currie, S., & Kumar, M. N. V. R. (2011). Development and evaluation of polymer nanoparticles for oral delivery of estradiol to rat brain in a model of Alzheimer's pathology. *Journal of Controlled Release*, *150*, 220–228.

Moreno, L. C. G. e. A. I., Solas, M., Martínez-Ohárriz, M. C., Muñoz, E., Santos-Magalhães, N. S., Ramirez, M. J., & Irache, J. M. (2018). Pegylated nanoparticles for the oral delivery of nimodipine: Pharmacokinetics and effect on the anxiety and cognition in mice. *International Journal of Pharmaceutics*, *543*(1–2), 245–256.

Morrison, J. H., Brinton, R. D., Schmidt, P. J., & Gore, A. C. (2006). Estrogen, menopause, and the aging brain: how basic neuroscience can inform hormone therapy in women. *The Journal of Neuroscience*, *26*(41), 10332–10348.

NAMS. (2012). The 2012 hormone therapy position statement of The North American Menopause Society (NAMS). *Menopause: The Journal of The North American Menopause Society*, *19*(3), 257–271.

NAMS. (2014). *Menopause Practice: A Clinician's Guide* (5th ed.). Mayfield Heights: The North American Menopause Society (NAMS).

NAMS. (2017). The 2017 hormone therapy position statement of the North American Menopause Society. *Menopause: The Journal of The North American Menopause Society*, *24*(7), 1–26.

Nequin, L. G., Alvarez, J., & Schwartz, N. B. (1979). Measurement of serum steroid and gonadotropin levels and uterine and ovarian variables throughout 4 day and 5 day estrous cycles in the rat. *Biology of Reproduction*, *20*, 659–670.

Newman, L. A., Korol, D. L., & Gold, P. E. (2011). Lactate produced by glycogenolysis in astrocytes regulates memory processing. *PLoS ONE*, *6*(12), e28427.

Nilsen, J., & Brinton, R. D. (2002). Impact of progestins on estrogen-induced neuroprotection: Synergy by progesterone and 19-norprogesterone and antagonism by medroxyprogesterone acetate. *Endocrinology*, *143*(1), 205–212.

Nonaka, N., Farr, S. A., Kageyama, H., Shioda, S., & Banks, W. A. (2008). Delivery of galanin-like peptide to the brain: targeting with intranasal delivery and cyclodextrins. *J Pharmacol Exp Ther*, *325*(2), 513–519.

Nonaka, N., Farr, S. A., Nakamachi, T., Morley, J. E., Nakamura, M., Shioda, S., & Banks, W. A. (2012). Intranasal administration of PACAP: Uptake by brain and regional brain targeting with cyclodextrins. *Peptides*, *36*(2), 168–175.

O'Keefe, J., & Dostrovsky, J. (1971). The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Research*, *34*, 171–175.

Okojie, A. K., & Oyekunle, O. A. (2014). Depo-Provera effects on Wistar rat performance in the Y-maze. *Metabolic Brain Disease*, *29*, 529–531.

Olson, J. E., Sellers, T. A., Iturria, S. J., & Hartmann, L. C. (2004). Bilateral oophorectomy and breast cancer risk reduction among women with a family history. *Cancer Detection and Prevention*, *28*, 357–360.

Olson, M. E., & Bruce, J. (1986). Ovariectomy, ovariohysterectomy and orchidectomy in rodents and rabbits. *The Canadian Veterinary Journal*, *27*(12), 523–527.

Olton, D. S., & Papas, B. C. (1979). Spatial memory and hippocampal function. *Neuropsychologia*, *17*, 669–682.

Olton, D. S., & Samuelson, R. J. (1976). Remembrance of places passed: Spatial memory in rats. *Journal of Experimental Psychology: Animal Behavior Processes, 2*(2), 97–116.

Orr, P. T., Lewis, M. C., & Frick, K. M. (2009). Dorsal hippocampal progesterone infusions enhance object recognition in young female mice. *Pharmacology Biochemistry and Behavior, 93*(2), 177–182.

Orr, P. T., Rubin, A. J., Fan, L., Kent, B. A., & Frick, K. M. (2012). The progesterone-induced enhancement of object recognition memory consolidation involves activation of the extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin (mTOR) pathways in the dorsal hippocampus. *Hormones and Behavior, 61*(4), 487–495.

Ortiz, J. B., McLaughlin, K. J., Hamilton, G. F., Baran, S. E., Campbell, A. N., & Conrad, C. D. (2013). Cholesterol and perhaps estradiol protect against corticosterone-induced hippocampal CA3 dendritic retraction in gonadectomized female and male rats. *Neuroscience, 246*, 409–421.

Otani, T., & Sasamoto, S. (1982). Plasma and pituitary hormone changes and follicular development after unilateral ovariectomy in cyclic rats. *Journal of Reproduction and Fertility, 65*, 347–353.

Parkening, T. A., Collins, T. J., & Smith, E. R. (1980). Plasma and pituitary concentrations of LH, FSH and prolactin in aged female C57BL/6 mice. *Journal of Reproduction and Fertility, 58*, 377–386.

Paxinos, G., & Watson, C. (1998). *The Rat Brain in Stereotaxic Coordinates* (4th ed.). New York: Academic Press.

Pelissier, C., De Kervasdoue, A., Chuong, V. T., Maugis, E. L., De Mouillac, F., Breil, M. H., … Rime, B. (2001). Clinical evaluation, dose-finding and acceptability of AERODIOL, the pulsed estrogen therapy for treatment of climacteric symptoms. *Maturitas, 37*, 181–189.

Pérez, S. E., Chen, E.-Y., & Mufson, E. J. (2003). Distribution of estrogen receptor alpha and beta immunoreactive profiles in the postnatal rat brain. *Developmental Brain Research, 145*(1), 117–139.

Phoenix, C. H., Goy, R. W., Gerall, A. A., & Young, W. C. (1959). Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. *Endocrinology, 65*, 369–382.

Pickar, J. H., Bon, C., Amadio, J. M., Mirkin, S., & Bernick, B. (2015). Pharmacokinetics of the first combination 17β-estradiol/progesterone capsule in clinical development for menopausal hormone therapy. *Menopause, 22*(12), 1308–1316.

Prakapenka, A. V., Bimonte-Nelson, H. A., & Sirianni, R. W. (2017). Engineering poly(lactic-co-glycolic acid) (PLGA) micro- and nano-carriers for controlled delivery of 17β-estradiol. *Annals of Biomedical Engineering*, *45*(7), 1697–1709.

Prakapenka, A. V., Hiroi, R., Quihuis, A. M., Carson, C., Patel, S., Berns-Leone, C., … Bimonte-Nelson, H. A. (2018). Contrasting effects of individual versus combined estrogen and progestogen regimens as working memory load increases in middle-aged ovariectomized rats: one plus one does not equal two. *Neurobiology of Aging*, *64*, 1–14.

Prakapenka, A. V., Pena, V., & Bimonte-Nelson, H. A. *(under review)*. Ovarian hormones, cognition, and reproductive aging: applications and implications for translating preclinical endocrine brain research to the clinic. In *Estrogens and Memory: Basic Research and Clinical Implications*.

Pritchett-Corning, K. R., Chang, F. T., & Festing, M. F. W. (2009). Breeding and housing laboratory rats and mice in the same room does not affect the growth or reproduction of either species. *Journal of the American Association for Laboratory Animal Science*, *48*(5), 492–498.

Quinlan, M. G., Duncan, A., Loiselle, C., Graffe, N., & Brake, W. G. (2010). Latent inhibition is affected by phase of estrous cycle in female rats. *Brain and Cognition*, *74*, 244–248.

Rapp, S. R., Espeland, M. A., Shumaker, S. A., Henderson, V. W., Brunner, R. L., Manson, J. E., … Bowen, D. (2003). Effect of estrogen plus progestin on global cognitive function in postmenopausal women. *The Journal of the American Medical Association*, *289*(20), 2663–2672.

Reginster, J. Y., Albert, A., Deroisy, R., Colette, J., Vrijens, B., Blacker, C., … Franchimont, P. (1997). Plasma estradiol concentrations and pharmacokinetics following transdermal application of Menorest® 50 or Systen® (Evorel®) 50. *Maturitas*, *27*, 179–186.

Resnick, S. M., Maki, P. M., Rapp, S. R., Espeland, M. A., Brunner, R., Coker, L. H., … Shumaker, S. A. (2006). Effects of combination estrogen plus progestin hormone treatment on cognition and affect. *Journal of Clinical Endocrinology and Metabolism*, *91*(5), 1802–1810.

Richardson, S., & Nelson, J. (1990). Follicular depletion during the menopausal transition. *Annals of the New York Academy of Sciences*, *592*(1), 13–20.

Rocca, W. A., Grossardt, B. R., & Shuster, L. T. (2010). Oophorectomy, menopause, estrogen, and cognitive aging: the timing hypothesis. *Neurodegenerative Diseases*, *7*, 163–166.

Rocca, W. A., Grossardt, B. R., & Shuster, L. T. (2011). Oophorectomy, menopause, estrogen treatment, and cognitive aging: clinical evidence for a window of opportunity. *Brain Research*, *1379*, 188–198.

Rocca, W. A., Grossardt, B. R., Shuster, L. T., & Stewart, E. A. (2012). Hysterectomy, oophorectomy, estrogen, and the risk of dementia. *Neurodegenerative Diseases*, *10*, 175–178.

Rodgers, S. P., Bohacek, J., & Daniel, J. M. (2010). Transient estradiol exposure during middle age in ovariectomized rats exerts lasting effects on cognitive function and the hippocampus. *Endocrinology*, *151*(3), 1194–1203.

Rogers, N., Perfield, J., Strissel, K., Obin, M., & Greenberg, A. (2009). Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. *Endocrinology*, *150*(5), 2161–2168.

Rosario, E. R., Ramsden, M., & Pike, C. J. (2006). Progestins inhibit the neuroprotective effects of estrogen in rat hippocampus. *Brain Research*, *1099*(1), 206–210.

Rosenzweig, E. S., Redish, A. D., Mcnaughton, B. L., & Barnes, C. A. (2003). Hippocampal map realignment and spatial learning. *Nature Neuroscience*, *6*(6), 609–615.

Rudolph, I., Zimmermann, T., Kaminski, K., Jandova, K., Borovsky, B., Ahrendt, H.-J., & Golbs, S. (2000). Changes in psychic and somatic well-being and cognitive capabilities of peri- and postmenopausal women after the use of a hormone replacement drug containing estradiol valerate and levonorgestrel. *Methods and Findings in Experimental and Clinical Pharmacology*, *22*, 51–56.

Ruoff, W. L., & Dziuk, P. J. (1994). Absorption and metabolism of estrogens from the stomach and duodenum of pigs. *Domestic Animal Endocrinology*, *11*(2), 197–208.

Ryan, J., Scali, J., Carrière, I., Amieva, H., Rouaud, O., Berr, C., … Ancelin, M.-L. (2014). Impact of a premature menopause on cognitive function in later life. *BJOG: An International Journal of Obstetrics and Gynaecology*, *121*(13), 1729–1739.

Sánchez-López, E., Ettcheto, M., Egea, M. A., Espina, M., Cano, A., Calpena, A. C., … García, M. L. (2018). Memantine loaded PLGA PEGylated nanoparticles for Alzheimer's disease: In vitro and in vivo characterization. *Journal of Nanobiotechnology*, *16*(1), 1–16.

Santillán, R., Pérez-Palacios, G., Reyes, M., Damián-Matsumura, P., García, G. A., Grillasca, I., & Lemus, A. E. (2001). Assessment of the oestrogenic activity of the contraceptive progestin levonorgestrel and its non-phenolic metabolites. *European Journal of Pharmacology*, *427*(2), 167–174.

Satchithanandam, S., Chanderbhan, R., Kharroubi, A. T., Calvert, R. J., Klurfeld, D., Tepper, S. A., & Kritchevsky, D. (1996). Effect of sesame oil on serum and liver lipid profiles in the rat. *International Journal for Vitamin and Nutrition Research*, *66*(4), 386–392.

Sato, T., Tanaka, K.-I., Ohnishi, Y., Teramoto, T., Irifune, M., & Nishikawa, T. (2004). Effects of estradiol and progesterone on radial maze performance in middle-aged female rats fed a low-calcium diet. *Behavioural Brain Research*, *150*, 33–42.

Schafe, G. E., Atkins, C. M., Swank, M. W., Bauer, E. P., Sweatt, J. D., & LeDoux, J. E. (2000). Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of pavlovian fear conditioning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *20*(21), 8177–8187.

Semete, B., Booysen, L. I. J., Kalombo, L., Venter, J. D., Katata, L., Ramalapa, B., … Swai, H. (2010). In vivo uptake and acute immune response to orally administered chitosan and PEG coated PLGA nanoparticles. *Toxicology and Applied Pharmacology*, *249*(2), 158–165.

Sherwin, B. B. (2006). Estrogen and cognitive aging in women. *Neuroscience*, *138*(3), 1021–1026.

Shumaker, S. A., Legault, C., Rapp, S. R., Thal, L., Wallace, R. B., Ockene, J. K., … Wactawski-Wende, J. (2003). Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women. *The Journal of the American Medical Association*, *289*(20), 2651–2662.

Simone, J., Bogue, E. A., Bhatti, D. L., Day, L. E., Farr, N. A., Grossman, A. M., & Holmes, P. V. (2015). Ethinyl estradiol and levonorgestrel alter cognition and anxiety in rats concurrent with a decrease in tyrosine hydroxylase expression in the locus coeruleus and brain-derived neurotrophic factor expression in the hippocampus. *Psychoneuroendocrinology*, *62*, 265–278.

Singh, A., Singh, A., & Madhav, N. (2012). Nasal cavity: a promising transmucosal platform for drug delivery and research approaches from nasal to brain targeting. *Journal of Drug Delivery & Therapeutics*, *2*(3), 22–33.

Singh, M., Meyer, E. M., Millard, W. J., & Simpkins, J. W. (1994). Ovarian steroid deprivation results in a reversible learning impairment and compromised cholinergic function in female Sprague-Dawley rats. *Brain Research*, *644*(2), 305–312.

Singh, M., Meyer, E. M., & Simpkins, J. W. (1995). The effect of ovariectomy and estradiol replacement on brain-derived neurotrophic factor messenger ribonucleic acid expression in cortical and hippocampal brain regions of female Sprague-Dawley rats. *Endocrinology*, *136*(5), 2320–2324.

Singh, M., Simpkins, J. W., Bimonte-Nelson, H. A., & Brinton, R. D. (2013). Window of opportunity for estrogen and progestin intervention in brain aging and Alzheimer's disease. *Brain Research*, *1514*, 1–2.

Sinopoli, K. J., Floresco, S. B., & Galea, L. A. M. (2006). Systemic and local administration of estradiol into the prefrontal cortex or hippocampus differentially alters working memory. *Neurobiology of Learning and Memory*. https://doi.org/10.1016/j.nlm.2006.04.003

Spencer, J. L., Waters, E. M., Milner, T. A., Lee, F. S., & McEwen, B. S. (2010). BDNF variant Val66Met interacts with estrous cycle in the control of hippocampal function. *Proceedings of the National Academy of Sciences*, *107*(9), 4395–4400.

Springer, L. N., Flaws, J. A., Sipes, I. G., & Hoyer, P. B. (1996). Follicular mechanisms associated with 4-vinylcyclohexene diepoxide-induced ovotoxicity in rats. *Reproductive Toxicology (Elmsford, N.Y.)*, *10*(2), 137–143.

Stackman, R. W., Blasberg, M. E., Langan, C. J., & Clark, A. S. (1997). Stability of spatial working memory across the estrous cycle of Long-Evans rats. *Neurobiol Learn Mem*, *67*, 167–171.

Steffenach, H., Witter, M., Moser, M., & Moser, E. I. (2005). Spatial memory in the rat requires the dorsolateral band of the entorhinal cortex. *Neuron*, *45*, 301–313.

Stocco, C., Telleria, C., & Gibori, G. (2007). The molecular control of corpus luteum formation, function, and regression. *Endocrine Reviews*, *28*(1), 117–149.

Studd, J., Pornel, B., Marton, I., Bringer, J., Varin, C., Tsouderos, Y., & Christiansen, C. (1999). Efficacy and acceptability of intranasal 17 β-oestradiol for menopausal symptoms: Randomised dose-response study. *Lancet*, *353*(9164), 1574–1578.

Sun, D., Li, N., Zhang, W., Zhao, Z., Mou, Z., Huang, D., … Wang, W. (2016). Design of PLGA-functionalized quercetin nanoparticles for potential use in Alzheimer's disease. *Colloids and Surfaces B: Biointerfaces*, *148*, 116–129.

Sun, S., Li, H., Chen, J., & Qian, Q. (2017). Lactic acid: No longer an inert and end-product of glycolysis. *Physiology*, *32*(6), 453–463.

Suzuki, A., Stern, S. a, Bozdagi, O., Huntley, G. W., Ruth, H., Magistretti, P. J., & Alberini, C. M. (2011). Astrocyte-neuron lactate transport is required for long-term memory formation. *Cell*, *144*(5), 810–823.

Talboom, J. S., Engler-Chiurazzi, E. B., Whiteaker, P., Simard, A. R., Lukas, R., Acosta, J. I., … Bimonte-Nelson, H. A. (2010). A component of Premarin® enhances multiple cognitive functions and influences nicotinic receptor expression. *Hormones and Behavior*, *58*, 917–928.

Talboom, J. S., West, S. G., Engler-Chiurazzi, E. B., Enders, C. K., Crain, I., & Bimonte-Nelson, H. A. (2014). Learning to remember: Cognitive training-induced attenuation of age-related memory decline depends on sex and cognitive demand, and can transfer to untrained cognitive domains. *Neurobiology of Aging*, *35*(12), 2791–2802.

Talboom, J. S., Williams, B. J., Baxley, E. R., West, S. G., & Bimonte-Nelson, H. A. (2008). Higher levels of estradiol replacement correlate with better spatial memory in surgically menopausal young and middle-aged rats. *Neurobiology of Learning and Memory*, *90*(1), 155–163.

Tarbali, S., & Khezri, S. (2016). Vitamin D3 attenuates oxidative stress and cognitive deficits in a model of toxic demyelination. *Iranian Journal of Basic Medical Sciences*, *19*(1), 80–88.

Thompson, K. E., Bourguet, S. M., Christian, P. J., Benedict, J. C., Sipes, I. G., Flaws, J. A., & Hoyer, P. B. (2005). Differences between rats and mice in the involvement of the aryl hydrocarbon receptor in 4-vinylcyclohexene diepoxide-induced ovarian follicle loss. *Toxicology and Applied Pharmacology*, *203*, 114–123.

Tiwari, S. K., Agarwal, S., Seth, B., Yadav, A., Nair, S., Bhatnagar, P., … Gupta, K. C. (2014). Curcumin-loaded nanoparticles potently induce adult neurogenesis and reverse cognitive deficits in Alzheimer's disease model via canonical Wnt/β-catenin pathway. *ACS Nano*, *8*(1), 76–103.

Tuscher, J. J., Fortress, A. M., Kim, J., & Frick, K. M. (2015). Regulation of object recognition and object placement by ovarian sex steroid hormones. *Behavioural Brain Research*, *285*, 140–157.

Tuscher, J. J., Szinte, J. S., Starrett, J. R., Krentzel, A. A., Fortress, A. M., Remage-Healey, L., & Frick, K. M. (2016). Inhibition of local estrogen synthesis in the hippocampus impairs hippocampal memory consolidation in ovariectomized female mice. *Hormones and Behavior*, *83*, 60–67.

U.S. FDA's Office of Women's Health. (2015). Menopause: medicines to help you. Retrieved March 5, 2017, from http://www.fda.gov/ForConsumers/ByAudience/ForWomen/ucm118627.htm

Ulery, B. D., Nair, L. S., & Laurencin, C. T. (2011). Biomedical applications of biodegradable polymers. *The Journal of Polymer Science Part B: Polymer Physics*, *49*(12), 832–864.

Vermeulen, A. (1976). The hormonal activity of the postmenopausal ovary. *Journal of Clinical Endocrinology and Metabolism*, *42*(2), 247–253.

Wang, C., Zhang, T., Ma, H., Liu, J., Fu, F., & Liu, K. (2007). Prolonged effects of poly(lactic-co-glycolic acid) microsphere-containing huperzine A on mouse memory dysfunction induced by scopolamine. *Basic and Clinical Pharmacology and Toxicology*, *100*(3), 190–195.

Warren, S. G., Humphreys, A. G., Juraska, J. M., & Greenough, W. T. (1995). LTP varies across the estrous cycle: enhanced synaptic plasticity in proestrus rats. *Brain Research*, *703*(1–2), 26–30.

Westerlind, K. C., Gibson, K. J., Malone, P., Evans, G. L., & Turner, R. T. (1998). Differential effects of estrogen metabolites on bone and reproductive tissues of ovariectomized rats. *J Bone Miner Res*, *13*(884–431), 1023–1031.

Westwood, F. R. (2008). The female rat reproductive cycle: a practical histological guide to staging. *Toxicologic Pathology*, *36*, 375–384.

Wharton, W., Baker, L. D., Gleason, C. E., Dowling, M., Barnet, J. H., Johnson, S., … Asthana, S. (2011). Short-term hormone therapy with transdermal estradiol improves cognition for postmenopausal women with Alzheimer's disease: results of a randomized controlled trial. *Journal of Alzheimer's Disease*, *26*(3), 495–505.

Whiteman, M. K., Hillis, S. D., Jamieson, D. J., Morrow, B., Podgornik, M. N., Brett, K. M., & Marchbanks, P. A. (2008). Inpatient hysterectomy surveillance in the United States, 2000-2004. *American Journal of Obstetrics and Gynecology*, *198*(1), 34.e1-34.e7.

Williams, C. L., Barnett, A. M., & Meck, W. H. (1990). Organizational effects of early gonadal secretions on sexual differentiation in spatial memory. *Behavioral Neuroscience*, *104*(1), 84–97.

Wise, P. M. (1982). Alterations in the proestrus pattern of median eminence LHRH, serum LH, FSH, estradiol and progesterone concentrations in middle-aged rats. *Life Sciences*, *31*, 165–173.

Witty, C. F., Foster, T. C., Semple-Rowland, S. L., & Daniel, J. M. (2012). Increasing hippocampal estrogen receptor alpha levels via viral vectors increases MAP kinase activation and enhances memory in aging rats in the absence of ovarian estrogens. *PLoS ONE*, *7*(12), 1–10.

Witty, C. F., Gardella, L. P., Perez, M. C., & Daniel, J. M. (2013). Short-term estradiol administration in aging ovariectomized rats provides lasting benefits for memory and the hippocampus: A role for insulin-like growth factor-I. *Neuroendocrinology*, *154*(2), 842–852.

Woolley, C. S., & McEwen, B. S. (1992). Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. *The Journal of Neuroscience*, *12*(7), 2549–2554.

Woolley, C. S., & McEwen, B. S. (1993). Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. *The Journal of Comparative Neurology*, *336*(2), 293–306.

Wroolie, T. E., Kenna, H. A., Williams, K. E., & Rasgon, N. L. (2015). Cognitive effects of hormone therapy continuation or discontinuation in a sample of women at risk for Alzheimer's disease. *American Journal of Geriatric Psychiatry*, *23*(11), 1117–1126.

Xu, Y., Nedungadi, T. P., Zhu, L., Sobhani, N., Irani, B. G., Davis, K. E., … Clegg, D. J. (2011). Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction. *Cell Metabolism*, *14*(4), 453–465.

Yang, J., Ruchti, E., Petit, J.-M., Jourdain, P., Grenningloh, G., Allaman, I., & Magistretti, P. J. (2014). Lactate promotes plasticity gene expression by potentiating NMDA signaling in neurons. *Proceedings of the National Academy of Sciences*, *111*(33), 12228–12233.

Yirmiya, R., & Goshen, I. (2011). Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain, Behavior, and Immunity*, *25*(2), 181–213.

Zhao, L., & Brinton, R. D. (2006). Select estrogens within the complex formulation of conjugated equine estrogens (Premarin®) are protective against neurodegenerative insults: implications for a composition of estrogen therapy to promote neuronal function and prevent Alzheimer's disease. *BMC Neuroscience, 7*, 24.

Zhou, J., Zhang, H., Cohen, R. S., & Pandey, S. C. (2005). Effects of estrogen treatment on expression of BDNF and CREB expression and phosphorylation in rat amygdaloid and hippocampal structures. *Neuroendocrinology, 81*(5), 294–310.

Ziv, Y., Ron, N., Butovsky, O., Landa, G., Sudai, E., Greenberg, N., … Schwartz, M. (2006). Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nature Neuroscience, 9*(2), 268–275.

Table 1.
U.S. FDA approved hormone therapy for treatment of symptoms associated with menopause[a].

Estrogen-only hormone therapy, which needs an opposing progestogen if the uterus is present		
Brand Name	**Hormone type**	**Route of administration**
Alora	Estradiol	Patch
Cenestin	Synthetic Conjugated Estrogens	Pill
Climara	Estradiol	Patch
Delestrogen	Estradiol Valerate	Injection (Shot)
Divigel	Estradiol	Gel
Elestrin	Estradiol	Gel
Enjuvia	Synthetic Conjugated Estrogens	Pill
Esclim	Estradiol	Patch
Estrace	Estradiol	Pill Vaginal Cream
Estraderm	Estradiol	Patch
Estrasorb	Estradiol	Skin Cream (Emulsion)
Estring	Estradiol	Vaginal Insert
EstroGel	Estradiol	Gel
Evamist	Estradiol	Skin Spray (Transdermal)
Femring	Estradiol Acetate	Vaginal Ring
Femtrace	Estradiol Acetate	Pill
Menest	Esterified Estrogen	Pill
Menostar (only used to prevent osteoporosis)	Estradiol	Patch
Minivelle	Estradiol	Patch
Ogen	Estropipate	Pill Vaginal Cream
Ortho-Est	Estropipate	Pill
Premarin	Conjugated Estrogens	Pill Vaginal Cream Injection (Shot)
Vagifem	Estradiol	Vaginal Tablet
Vivelle	Estradiol	Patch
Vivelle-Dot	Estradiol	Patch

Progestogen-only hormone therapy, which can also be taken in combination with estrogen-only therapy		
Prometrium	Micronized Progesterone	Pill
Provera	Medroxyprogesterone Acetate	Pill
Estrogen-progestogen combination hormone therapy		
Activella	Estradiol / Norethindrone Acetate	Pill
Angeliq	Estradiol / Drospirenone	Pill
Climara Pro	Estradiol / Levonorgestrel	Patch
Combipatch	Estradiol / Norethindrone Acetate	Patch
Femhrt	Ethinyl Estradiol / Norethindrone Acetate	Pill
Prefest	Estradiol / Norgestimate	Pill
Prempro	Conjugated Estrogen / Medroxyprogesterone Acetate	Pill

[a]Adapted from U.S. FDA Menopause: Medicines to Help You (U.S. FDA's Office of Women's Health, 2015).

Table 2.
Correlation matrix showing Pearson *r* correlations, for each treatment group, between activated Erk1 and activated Erk2 expression in the frontal cortex and error measures for Block 1 of WRAM, the block of testing where main behavioral effects were seen. To account for multiple correlations, a false discovery rate (FDR) threshold of 0.1 was used; both uncorrected (*P*) and FDR-corrected (*Q*) statistics are reported (Benjamini & Hochberg, 1995). Significant correlations following FDR-correction are noted with a * and are in **bold**; significant correlations prior to FDR-correction are noted with a # and are *italicized*.

		Vehicle		**E2-Only**		**Levo-Only**		**E2 + Levo**	
		Erk 1	**Erk2**	**Erk 1**	**Erk2**	**Erk 1**	**Erk2**	**Erk 1**	**Erk2**
RM errors	***Q***	0.8747	0.8909	0.9099	0.2820	0.8190	0.8747	0.8747	0.9099
	P	*0.0418#*	0.0769	0.7736	*0.0047#*	*0.0273#*	0.3107	0.2756	0.6570
	r	-0.6502	-0.5830	-0.1046	-0.8079	-0.7244	-0.3817	0.3823	0.1609
WMI errors	***Q***	0.8909	0.8909	0.9224	0.8747	0.8747	0.8747	0.8747	0.8747
	P	0.7260	0.5249	0.8302	0.2725	0.0890	0.1652	0.3522	0.1694
	r	0.1273	0.2288	0.0781	-0.3846	-0.5979	-0.5054	0.3297	0.4711
WMC errors	***Q***	0.8747	0.7160	0.8747	**0.0960***	0.8747	0.8747	0.8747	0.8747
	P	0.0634	*0.0179#*	0.1531	*0.0008#*	0.0862	0.2538	0.3389	0.4670
	r	0.6059	0.7242	-0.4873	-0.8803	-0.6021	-0.4252	0.3383	0.2607

Table 3.
Summary of treatment groups.

Group	Dose (μg)	Dose Ratio (E2:Levo)	Rationale for treatment
Vehicle	E2: none Levo: none	___	Vehicle control for all hormone treatments
E2 only	E2: 0.3 μg Levo: none	___	E2 only control for combination hormone treatments
E2 + Low Levo	E2: 0.3 μg Levo: 0.06 μg	5:1	E2:Levo dose ratio used in my prior study (Chapter 2)
E2 + Med Levo	E2: 0.3 μg Levo: 0.1 μg	3:1	E2:Levo dose ratio used in the clinically-available hormone therapy transdermal patch, Climara Pro
E2 + High Levo	E2: 0.3 μg Levo: 0.6 μg	1:2	E2:Levo dose ratio with a Levo dose that has been repeatedly shown to improve cognition when given alone

Table 4.
Summary of treatment groups for Study 1.

Treatment	Formulation	E2:CD molar ratio	Rationale for CD
A	free E2 – *dissolved in 20% PEG saline solution*	___	___
B	E2-randomly methylated β-CD – *dissolved in saline*	1:2	CD derivative found in Aerodiol intranasal spray
C	E2-2-hydroxypropyl β-CD – *dissolved in saline*	1:2	CD derivative used in behavioral studies evaluating estrogen effects on the brain
D	E2-β-CD – *dissolved in saline*	1:4	base CD for the derivatives used in treatments B and C
E	E2-γ-CD – *dissolved in saline*	1:5	CD with a larger inner cavity than β-CD

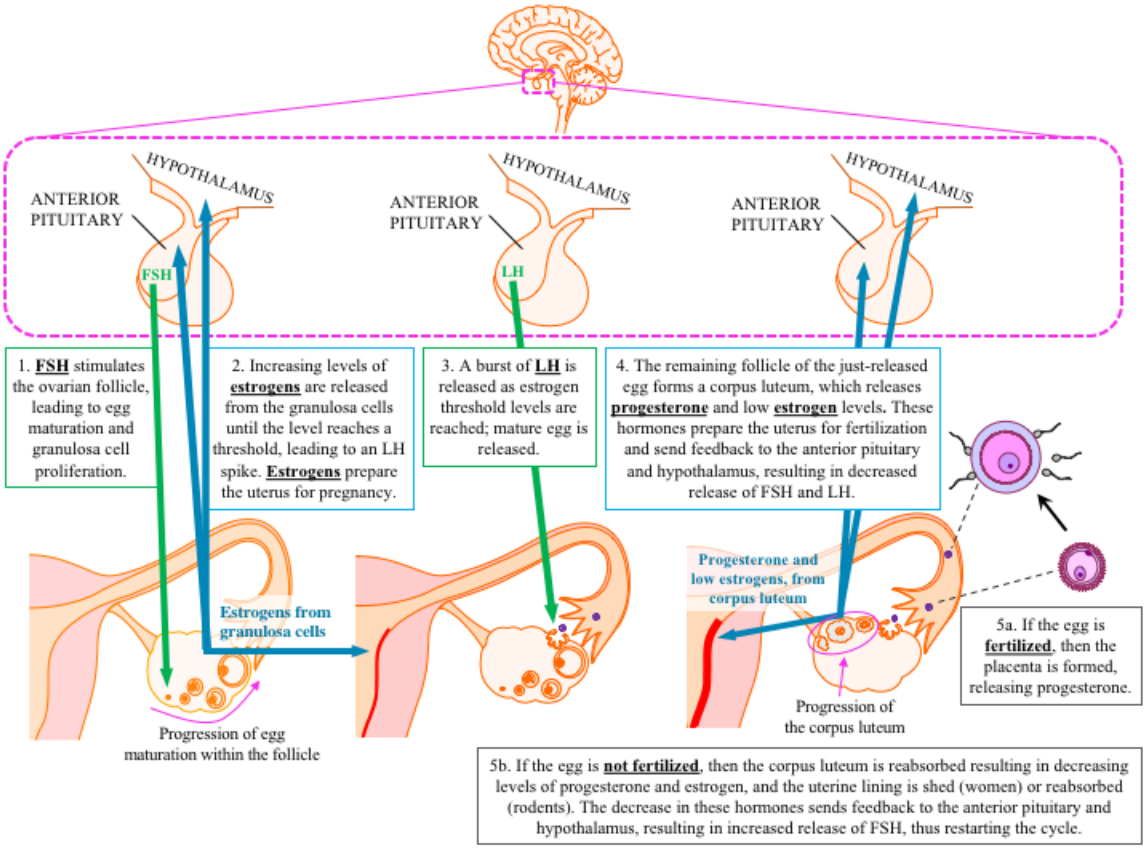


Figure 1. The reproductive cycle.

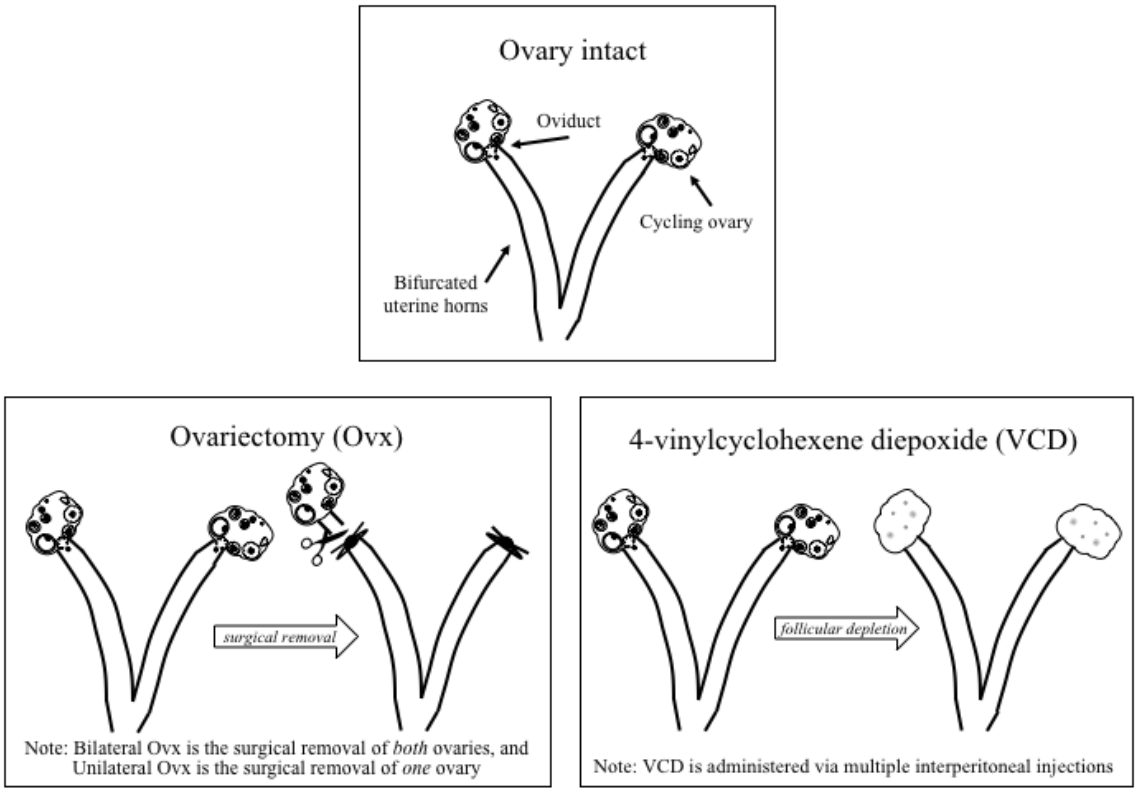


Figure 2. Rodent models of menopause.

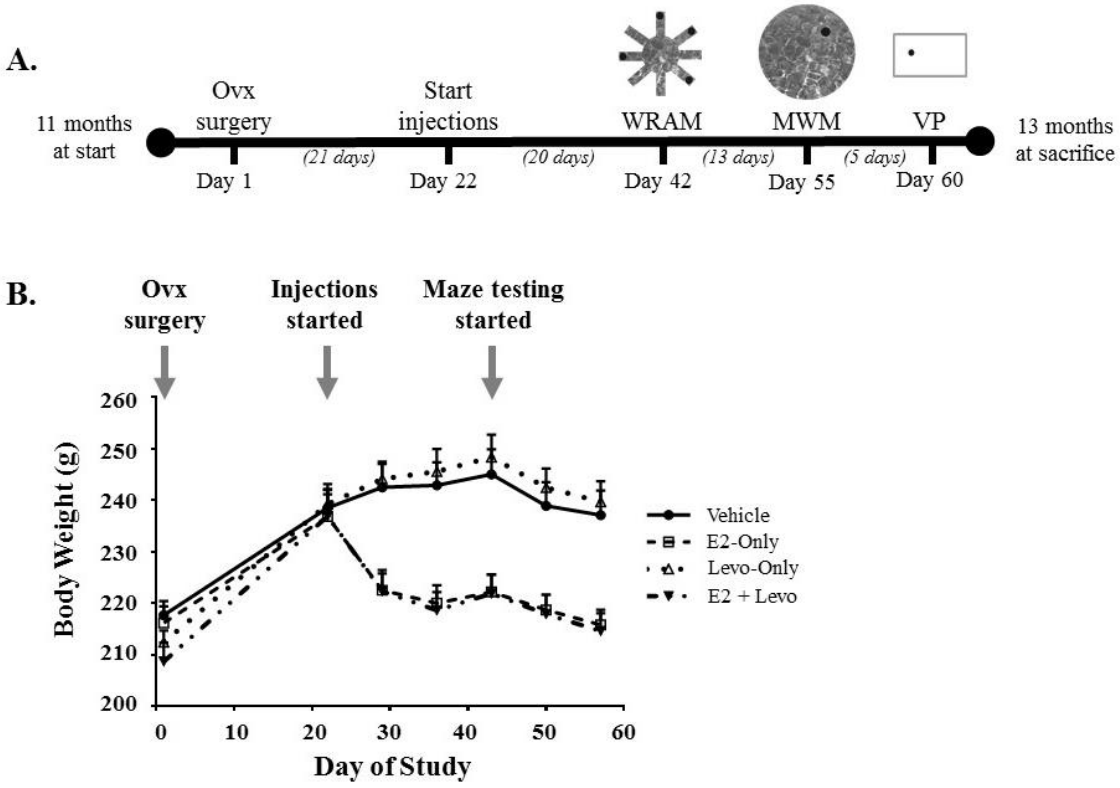


Figure 3. Study timeline and trajectory of rat body weight throughout the span of the study. (A) Study timeline depicts time periods between Ovx, start of treatment injections, and order of behavioral testing. (B) Changes in body weight accurately represent expected fluctuations as a result of Ovx, type of treatment, and maze testing.

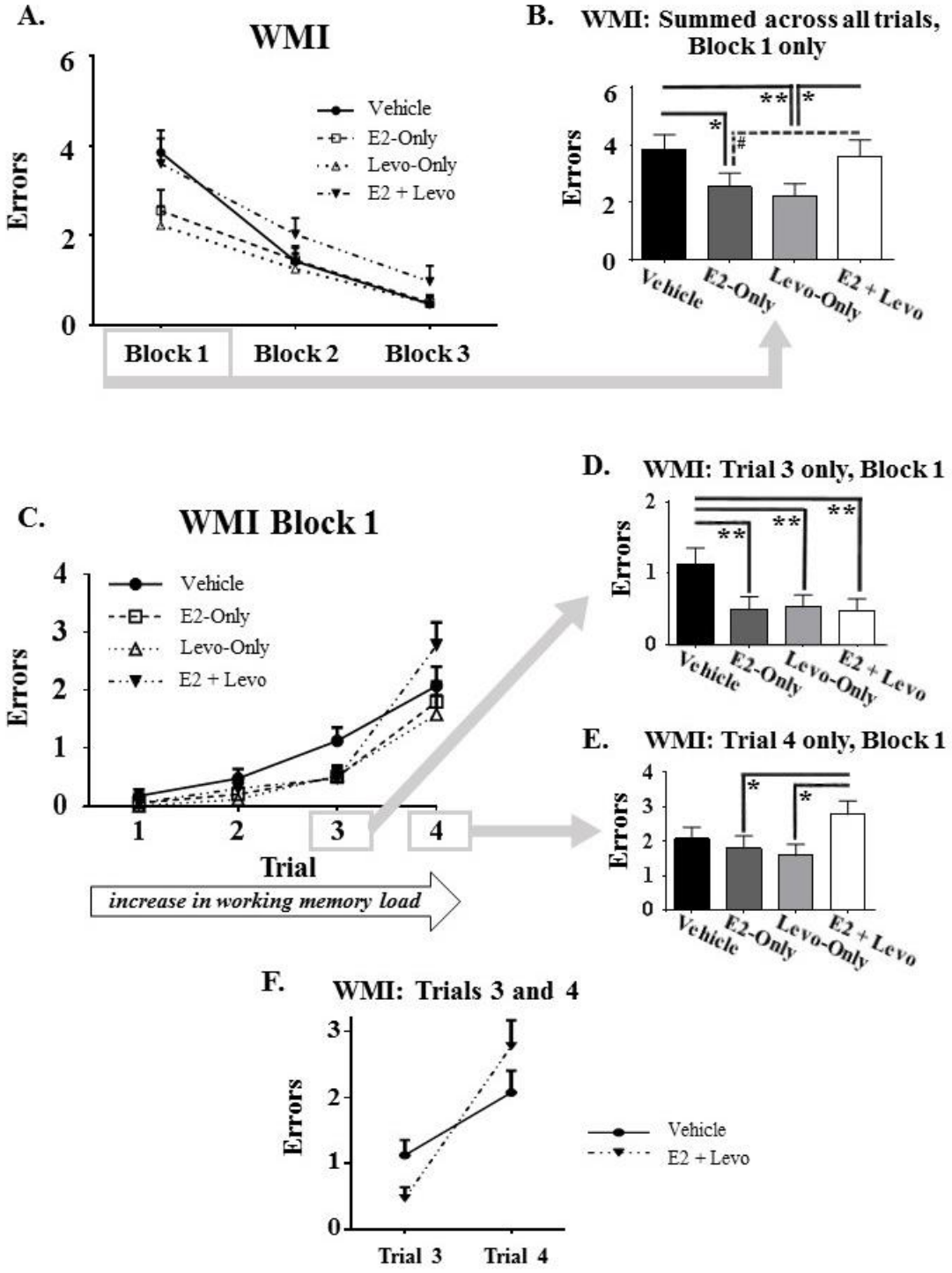


Figure 4. Water radial-arm maze (WRAM) performance. A) Learning curve for WMI errors, collapsed across trials for Blocks 1, 2, and 3. B) WMI Block 1 only, summed across trials, depicting a decrease in WMI errors for E2-Only and Levo-Only treatment groups relative to the Vehicle group. C) WMI errors across Trials 1-4 for Block 1 of testing. D) WMI errors on Trial 3 only, the moderate working memory load trial, for Block 1 of testing, illustrating that all hormone treatment groups made fewer WMI errors relative to the

Vehicle group. E) WMI errors on Trial 4 only, the high working memory load trial, for Block 1 of testing, depicting that the E2+Levo group made more WMI errors relative to E2-Only and Levo-Only groups. F) Treatment x Trial interaction, representing WMI errors made on the moderate and high working memory load trials (Trials 3 and 4, respectively) and Treatment (Vehicle and E2+Levo) for Block 1 of testing. The impact of E2 + Levo treatment on WMI performance was dependent on working memory demand. All errors are expressed as mean ± SEM. #$p < 0.1$, *$p < 0.05$, **$p < 0.01$.

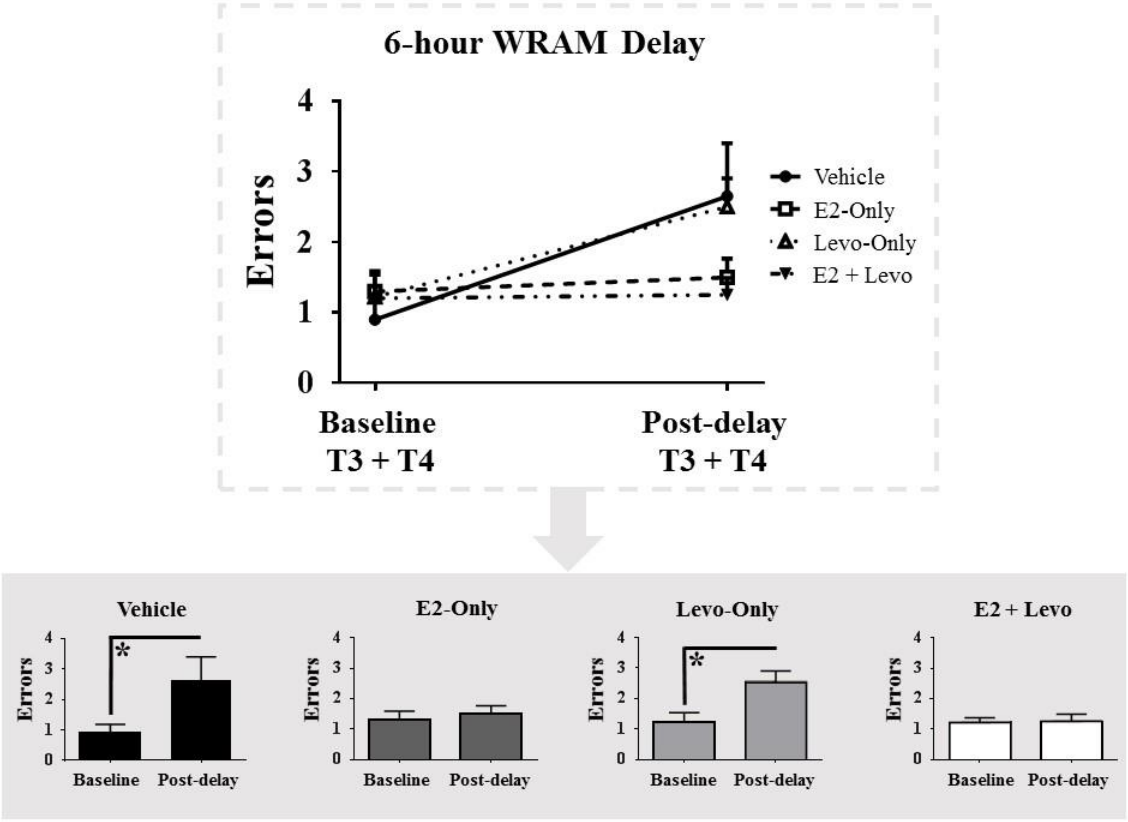


Figure 5. Water radial-arm maze (WRAM) performance following a 6-hour delay. Top graph shows WMC errors made on baseline trials (T3+4 on Day 12 of WRAM) and post-delay trials (T3+4 on Day 13 of WRAM) for all treatment groups. Bottom graphs illustrate WMC errors made on baseline trials and post-delay trials split by treatment group. All errors are expressed as mean ± SEM. *$p < 0.05$.

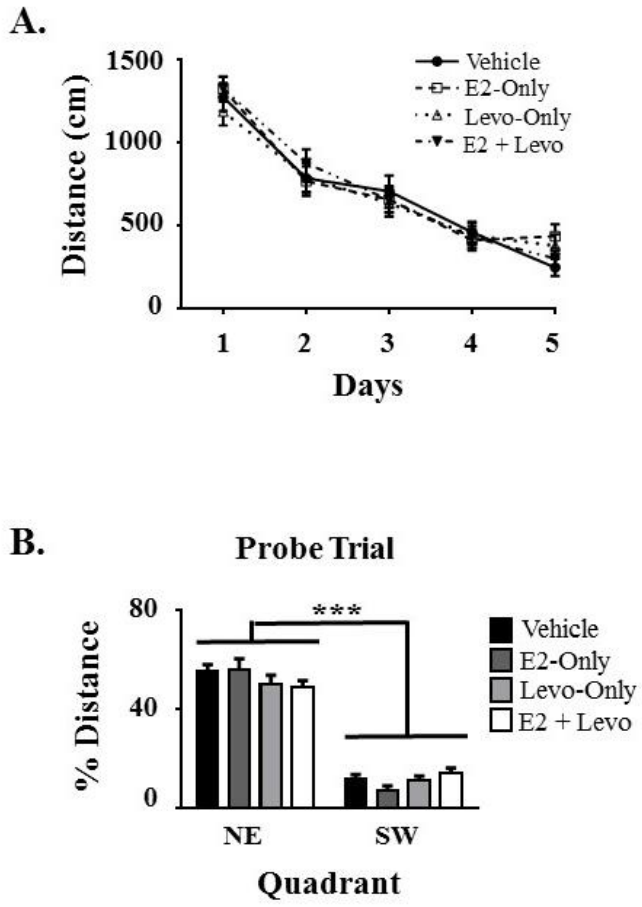


Figure 6. Morris water maze (MWM) performance. A) Swim distance to the platform for the 5 days of testing, illustrating that all treatment groups learned the task as depicted by the decrease in swim distance across days. B) Percent swim distance in the northeast (NE) quadrant that previously contained the platform compared to the opposing southwest (SW) quadrant that never contained a platform on the probe trial, confirming spatial localization of the platform by animals from all treatment groups. All measurements are expressed as mean ± SEM. ***$p < 0.0001$

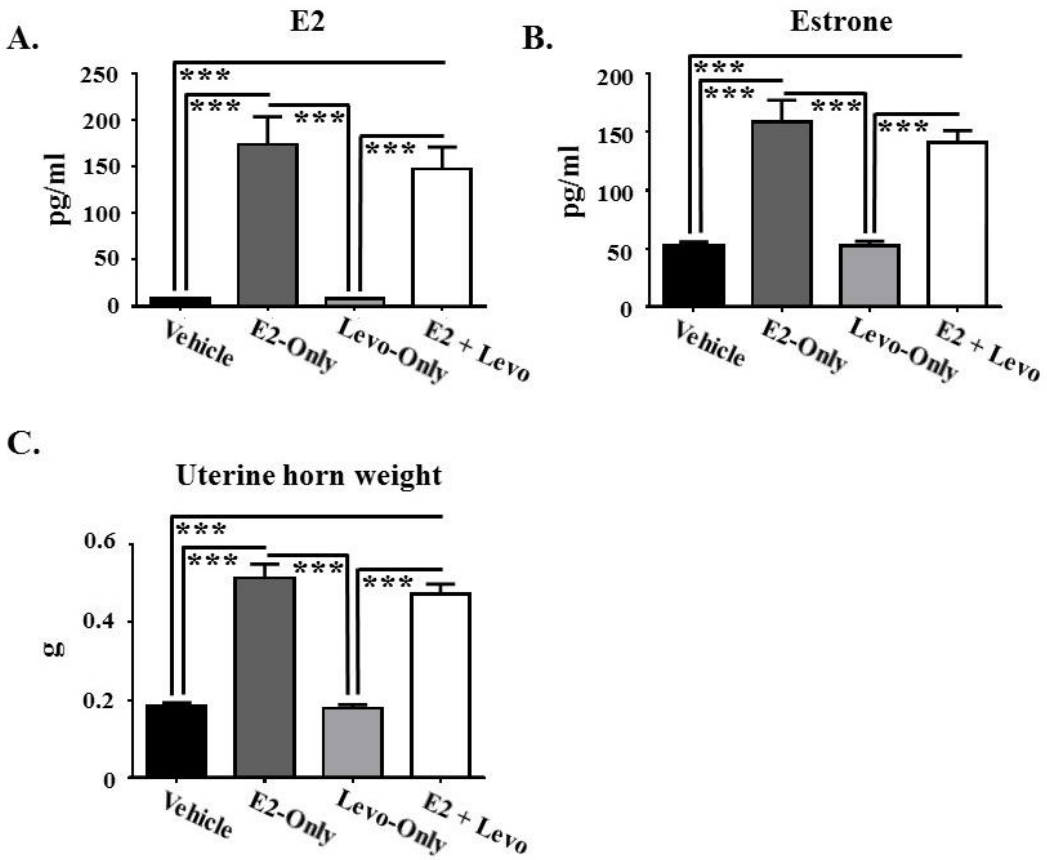


Figure 7. Circulating E2 and estrone levels and uterine horn weights. (A) E2 blood serum levels were increased in the E2-Only and E2+Levo treatment groups compared to the Vehicle group and compared to the Levo-Only group. (B) Estrone blood serum levels were elevated in the E2-Only and E2 + Levo treatment groups relative to the Vehicle group and relative to the Levo-Only group. (C) Uterine horn weight increased for the two E2 treated groups (E2-Only, and E2 + Levo) relative to the Vehicle group and relative to the Levo-Only group. All measurements are expressed as mean ± SEM. ***$p < 0.0001$

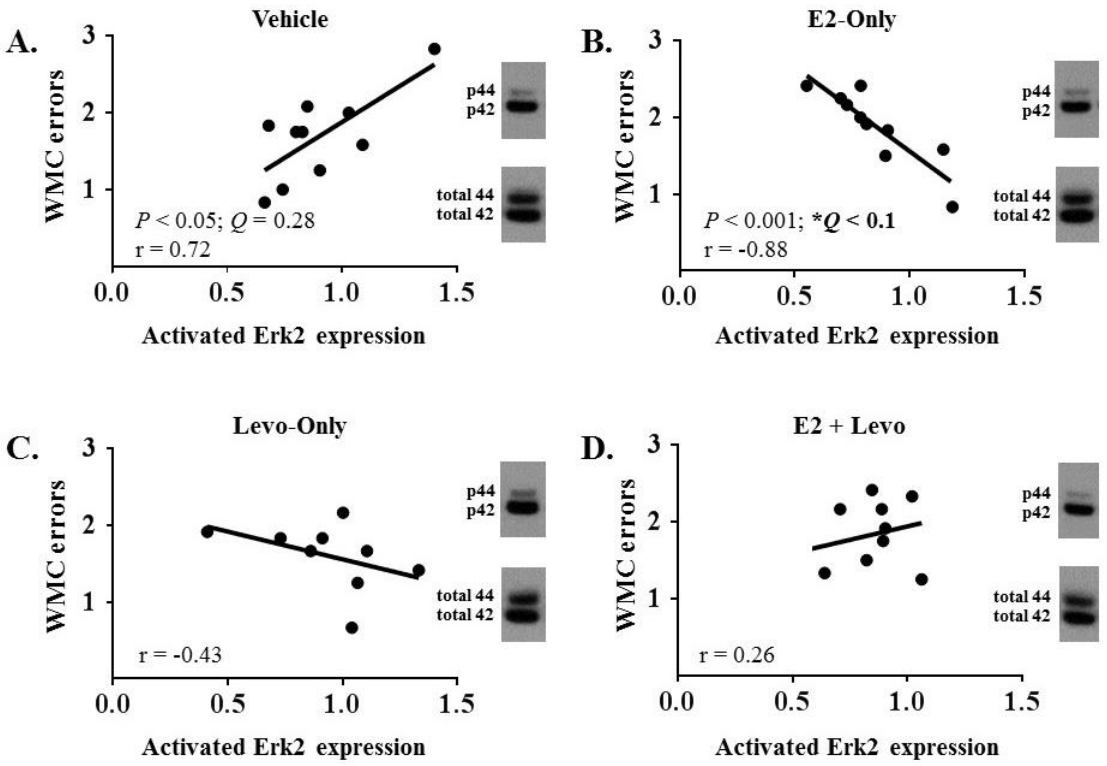


Figure 8. Pearson *r* correlations between Block 1 WMC errors and activated Erk2 expression in the frontal cortex. For each treatment group, a single sample was chosen to provide a representative image of the blots for phosphorylated Erk1 and Erk2 as well as total Erk1 and Erk2 in the frontal cortex. (A) Block 1 WMC errors did not correlate with activated Erk2 expression within the Vehicle group. (B) Block 1 WMC errors correlated with activated Erk2 expression within the E2-Only group. (C) Block 1 WMC errors did not correlate with activated Erk2 expression within the Levo-Only group. (D) Block 1 WMC errors did not correlate with activated Erk2 expression within the E2+Levo group. Of note, the false discovery rate-corrected statistics (*Q*) are reported here to account for multiple correlations. **Q* < 0.1. Activated Erk2 expression is expressed as phosphorylated Erk2/total Erk2.

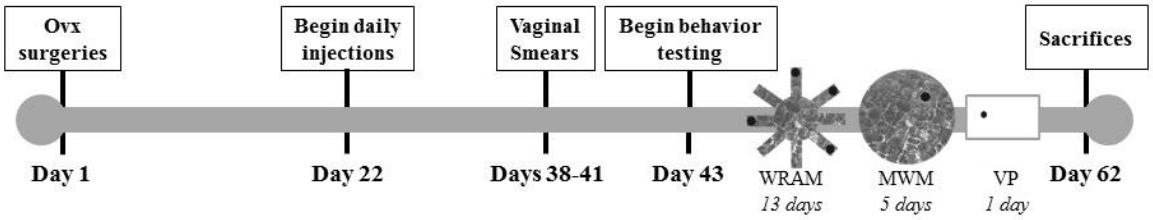


Figure 9. Study timeline. The study timeline illustrates the timing between Ovx, treatment initiation, vaginal smears, and the implemented behavioral battery.

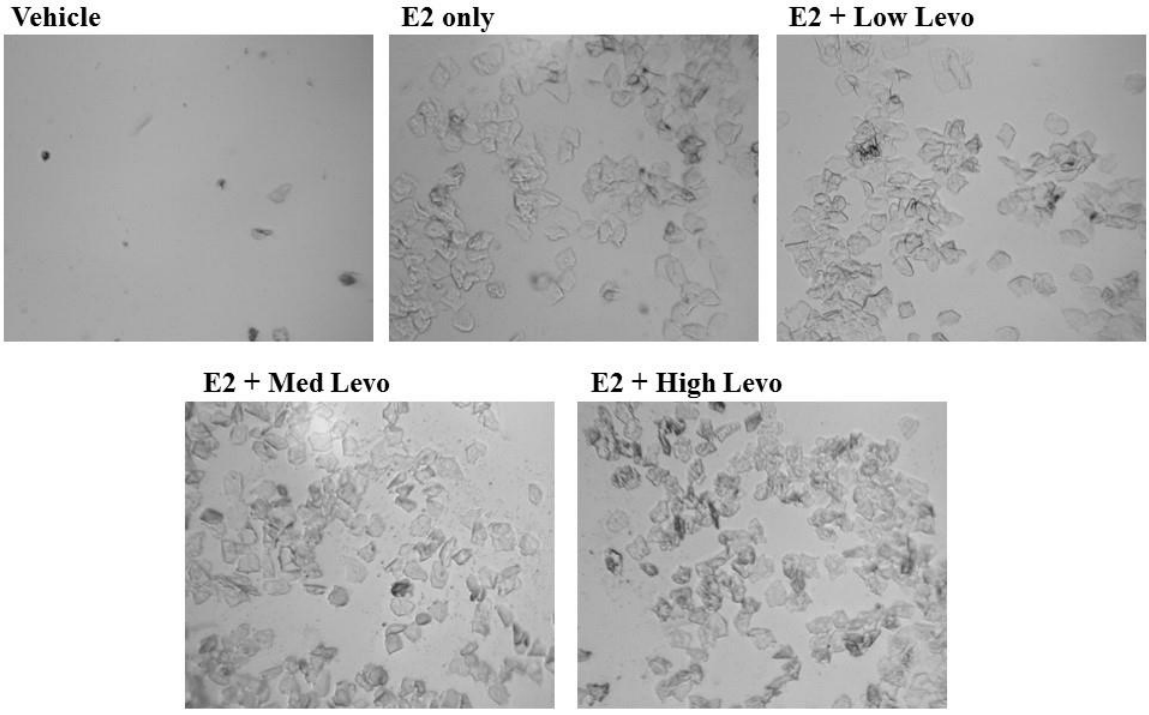


Figure 10. Representative images of vaginal cytology for each treatment group. The Vehicle group had mostly blank smears that contained few cells, confirming Ovx and lack of hormone stimulation. All E2-containing treatment groups had lots of cells in the smear that mostly consisted of cornified cells, a marker of E2 exposure. The addition of Levo to E2 did not appear to impact vaginal cytology.

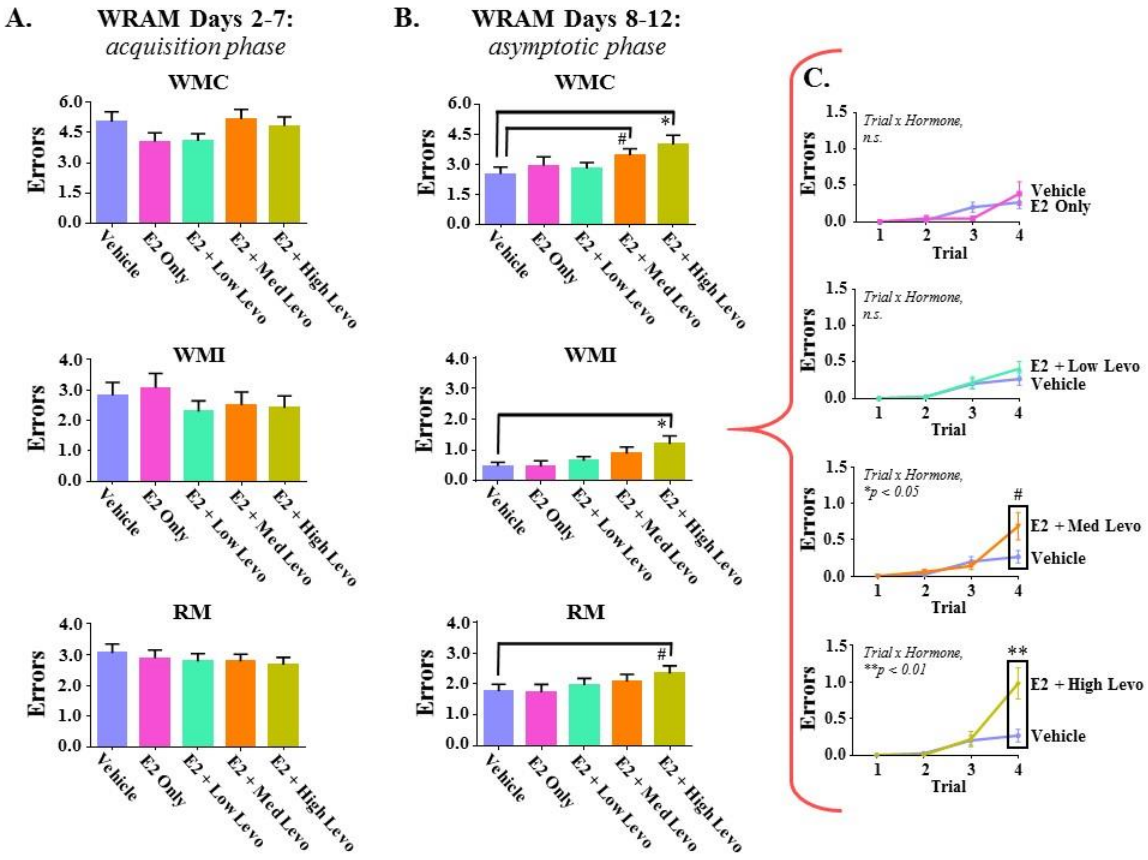


Figure 11. Errors made on the water radial-arm maze split by acquisition phase (days 2-7) and asymptotic phase (days 8-12). (A) There were no treatment differences for WMC, WMI, and RM errors made on the acquisition phase, when rules of the task were being learned. (B) On the asymptotic phase, when rules of the task should be learned, the E2 + High Levo group made more WMC and WMI errors than the Vehicle, and tended to make more RM errors than the Vehicle. The E2 + Med Levo group tended to make more WMC errors than the Vehicle. (C) A Trial x Hormone interaction for WMI errors revealed that the E2 + High Levo group made more WMI errors than the Vehicle group on Trial 4, the highest working memory load trial evaluated on the asymptotic phase of the WRAM. The E2 + Med Levo group tended to make more WMI errors than the Vehicle group on Trial 4 on the asymptotic phase of the WRAM. Data are presented as mean ± SEM. #$p < 0.1$, *$p < 0.05$, **$p < 0.01$.

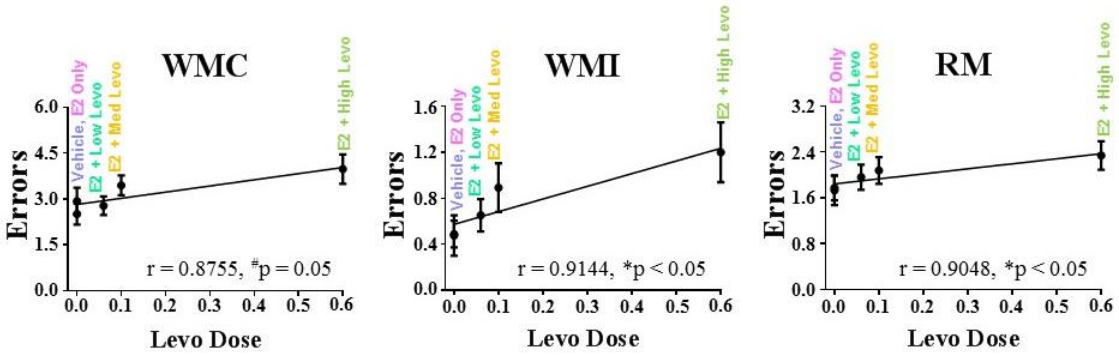


Figure 12. Linear regression analyses between Levo dose and errors made on the asymptotic phase of the water-radial arm maze. For WMC measure, errors tended to increase linearly as the Levo dose, in combination with the constant E2 dose, increased. For both WMI and RM measures, errors increased linearly as the Levo dose, in combination with the constant E2 dose, increased. Data are presented as mean ± SEM. #$p < 0.1$, *$p < 0.05$.

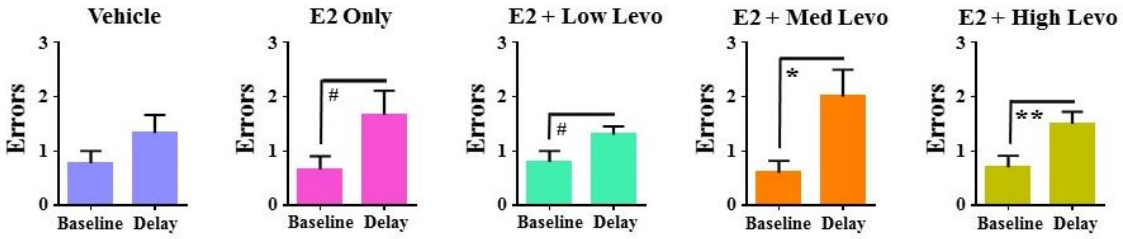


Figure 13. Effect of a 6-hour delay period on WMC errors made on the water radial-arm maze. The E2 + Med Levo and E2 + High Levo groups made more WMC errors on the post-delay (Delay) trial, Trial 3 on Day 13, compared to the baseline (Baseline) trial, T3 on Day 12, indicating delay-induced forgetting. The E2 Only and E2 + Low Levo groups tended to make more WMC errors on the Delay trial compared to the Baseline trial, and the Vehicle group did not statistically differ in WMC errors made on the Delay trial compared to the Baseline trial suggesting that these three treatment groups were not as impacted by the delay period. Data are presented as mean ± SEM. #$p < 0.1$, *$p < 0.05$, **$p < 0.01$.

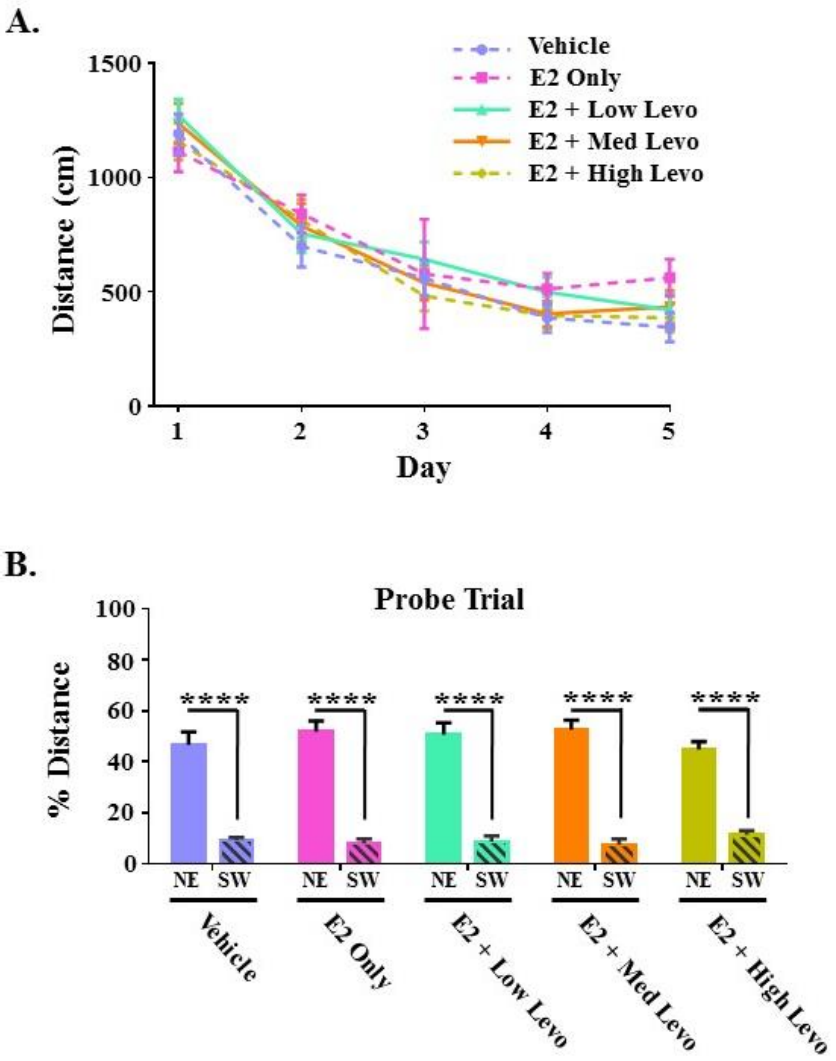


Figure 14. Morris water maze performance. (A) There were no treatment differences in distance to platform. (B) On the probe trial, all treatment groups swam a greater percent distance in the target NE quadrant, the quadrant that previously contained a platform, compared to the opposite SW quadrant, suggesting that all groups were able to spatially localize to the platform location. Data are presented as mean ± SEM. ****$p <$ 0.0001.

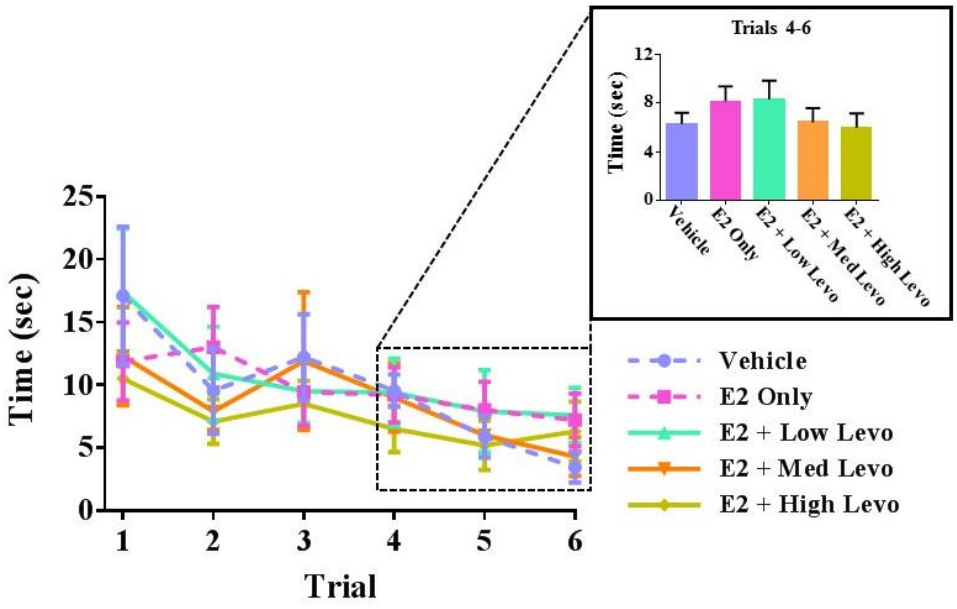


Figure 15. Visible platform performance. (A) All treatment groups decreased in time to platform across all 6 trials of testing. (B) No treatment differences were seen on the last 3 trials of testing, indicating that all treatment groups could perform and solve a water escape task in a similar manner. Data are presented as mean ± SEM.

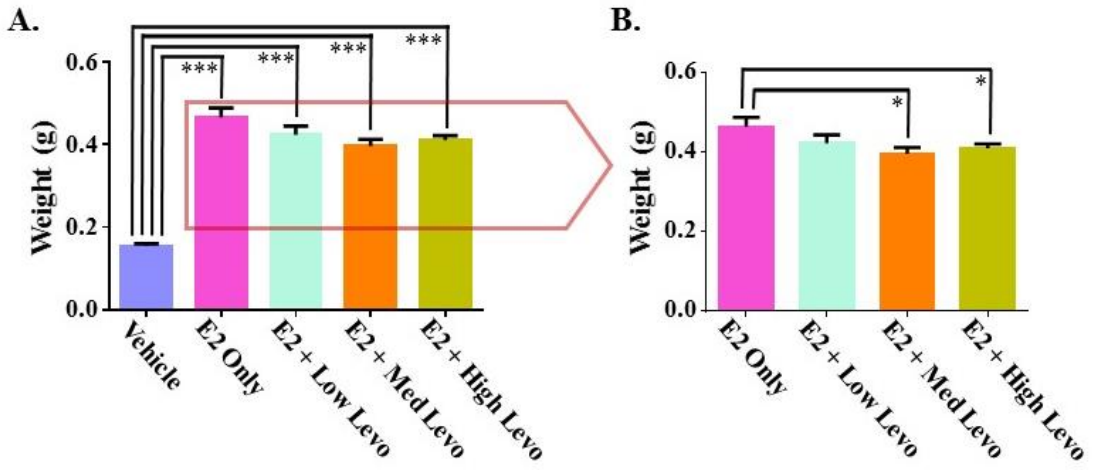
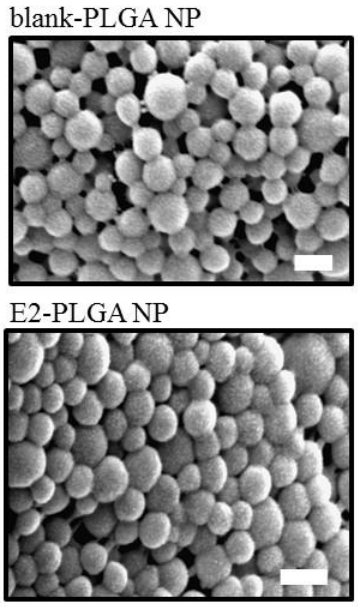
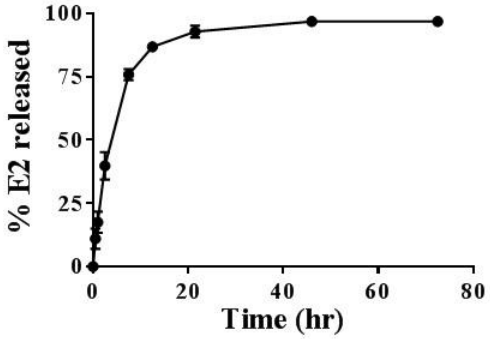


Figure 16. Uterine horn weights as a marker of uterine stimulation. (A) The E2 Only, E2 + Low Levo, E2 + Med Levo, and E2 + High Levo groups had greater uterine horn weight than the Vehicle group, suggesting E2-induced stimulation at the uterine horns (B) The addition of the medium and high Levo doses, the E2 + Med Levo and the E2 + High Levo groups, resulted in lower uterine horn weights than the E2 Only group, indicating opposing effects of Levo on E2-induced uterine stimulation at these E2:Levo dose ratio combinations. Data are presented as mean ± SEM. ***$p < 0.0001$, *$p < 0.05$.

A.



B.



C.

Particle Type	DLS Diameter (nm)	SEM Diameter (nm)	DLS Polydispersity	Zeta Potential (mV)
blank-PLGA	232 ± 2.1	175 ± 34	0.105 ± 0.04	-1.91
E2-PLGA	256 ± 2.3	158 ± 30	0.158 ± 0.02	-0.59

Figure 17. Blank-PLGA and E2-PLGA characterization. (A) Representative SEM images of blank-PLGA and E2-PLGA nanoparticles; scale bar is 200 nm. (B) E2 release profile from PLGA nanoparticles, n = 4 per time point. (C) Table summarizing the size, polydispersity, and zeta potential of each nanoparticle formulation. Data are presented as mean ± standard deviation.

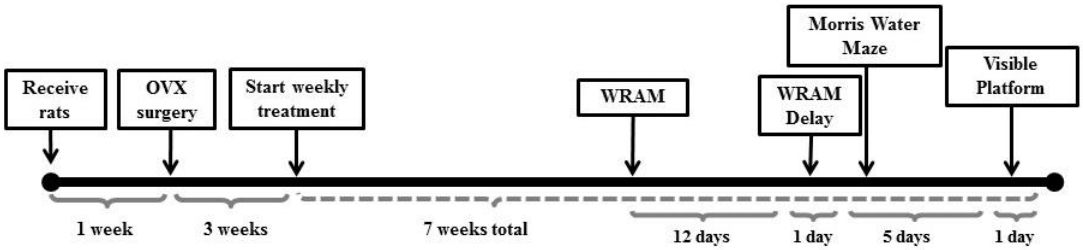


Figure 18. Study timeline. The study timeline illustrates the timing between Ovx surgery and initiation of weekly treatment injections, as well as between treatment initiation and behavior testing on the water radial-arm maze (WRAM), Morris water maze, and visible platform task.

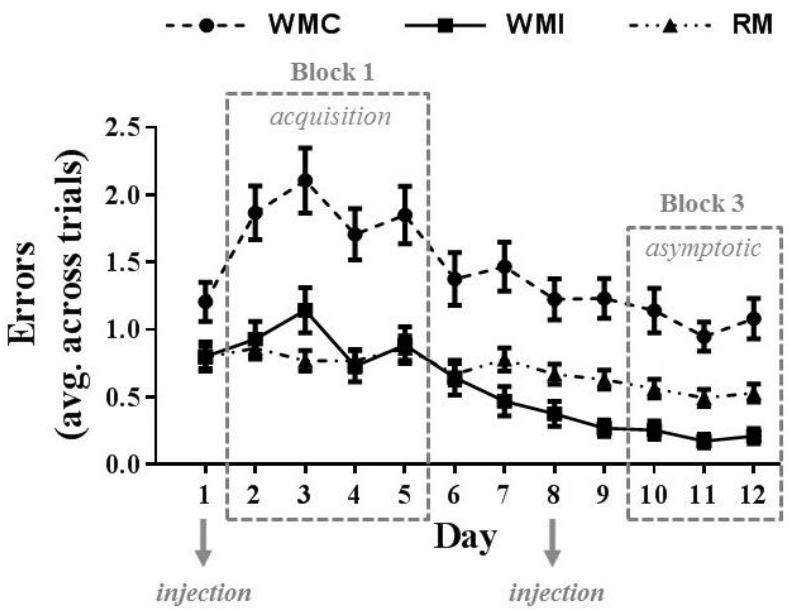


Figure 19. Water radial-arm maze learning curves for each memory measure. WMC, WMI, and RM errors made on each day of testing, collapsed across trials and treatment groups, are shown to visualize the learning curve for each memory measure. The days of weekly injection administration are noted. Data are presented as mean ± SEM.

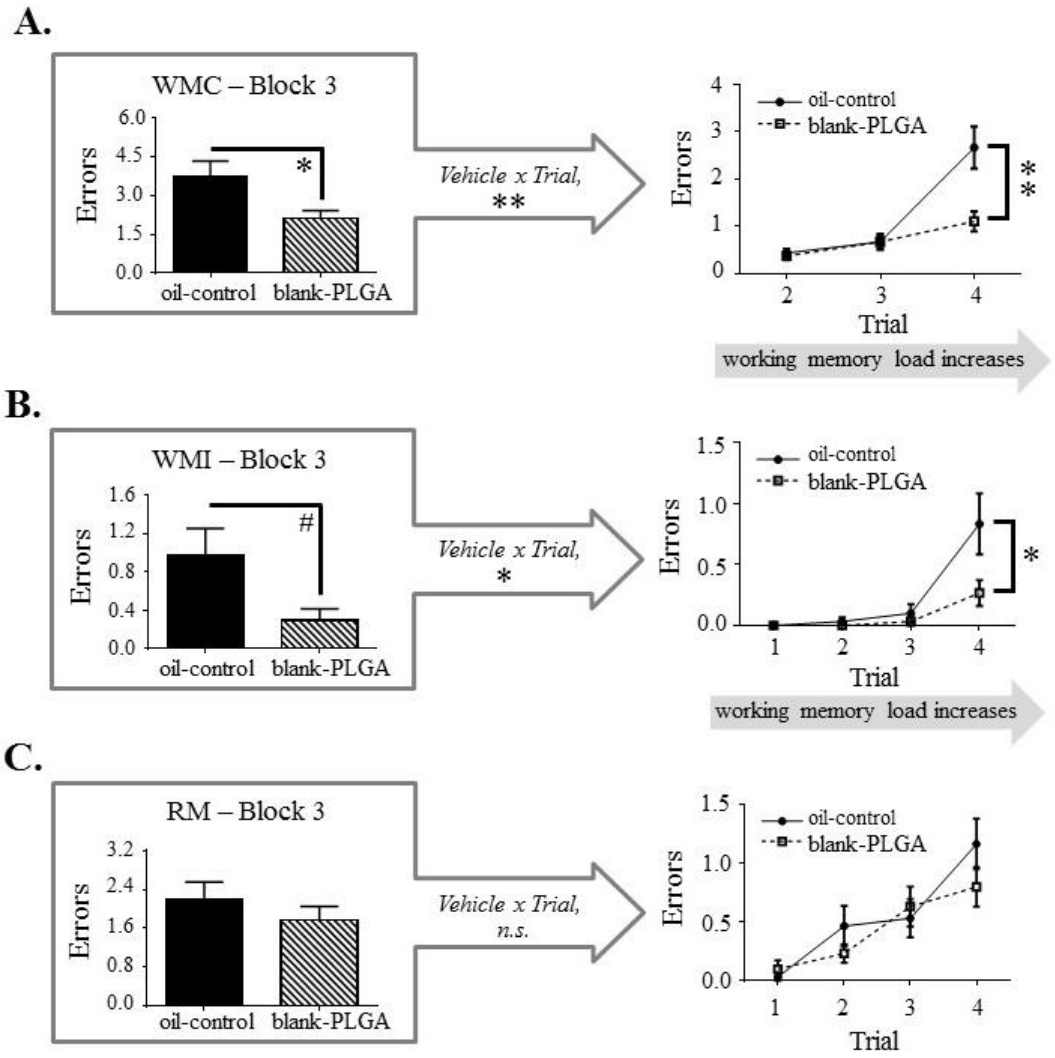


Figure 20. Water radial-arm maze: vehicle effect on the asymptotic phase. (A) WMC errors on Block 3 of testing, the asymptotic phase, where the blank-PLGA made fewer WMC errors than the oil-control group. The trial by vehicle graph illustrates that the blank-PLGA made fewer WMC errors than oil-control on Trial 4, the highest working memory load trial. (B) WMI errors on Block 3 of testing where the blank-PLGA tended to make fewer WMI errors than the oil-control group. The trial by vehicle graph illustrates that the blank-PLGA made fewer WMI errors than oil-control on Trial 4, the highest working memory load trial. (C) RM errors on Block 3 of testing, where no differences between the two vehicle control groups were observed. Data are presented as mean ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$, $\#p < 0.1$.

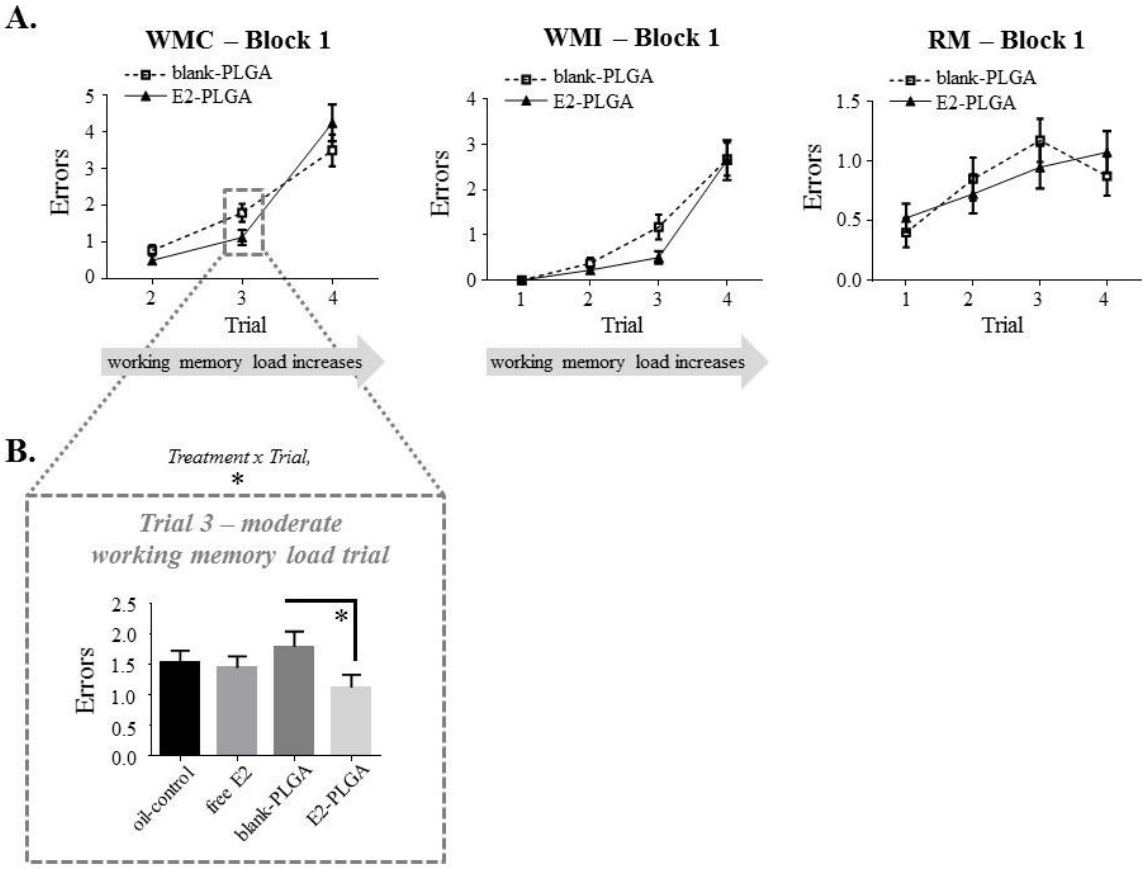


Figure 21. Water radial-arm maze: acquisition phase. (A) Trial by treatment graphs for WMC, WMI, and RM errors made on Block 1, the acquisition phase, for blank-PLGA and E2-PLGA. For WMC, there was a treatment x trial interaction, and further analyses revealed that on Trial 3, the moderate working memory load trial, E2-PLGA made fewer WMC errors than blank-PLGA, shown in (B). Data are presented as mean ± SEM. *$p <$ 0.05.

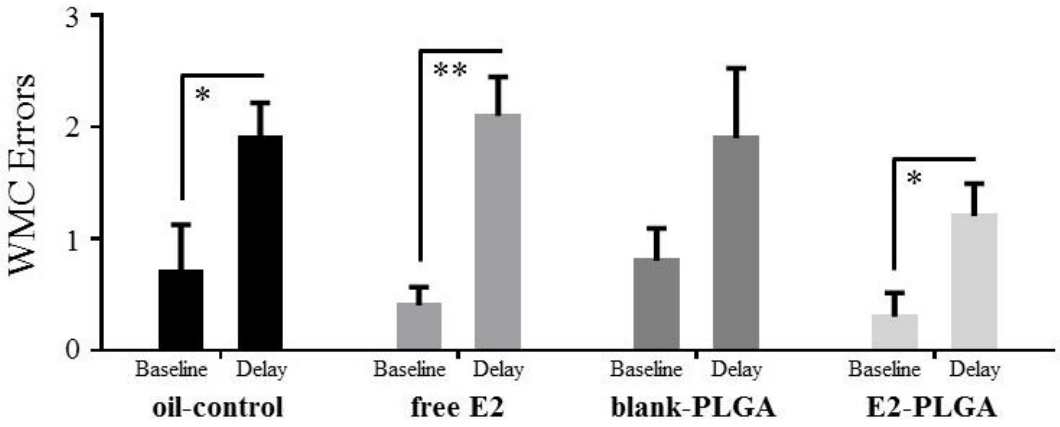


Figure 22. WMC errors made after a 6-hour delay on the water radial-arm maze. WMC errors made on the Delay trial, Trial 3 on Day 13, and WMC errors made on the Baseline trial, Trial 3 on Day 12, for each treatment group. Oil-control, free E2, and E2-PLGA groups made more WMC errors on the Delay trial relative to the Baseline trial, suggesting delay-induced forgetting. Differences in WMC errors made on the Delay trial relative to the Baseline trial by the blank-PLGA group did not reach statistical significance. Data are presented as mean ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$.

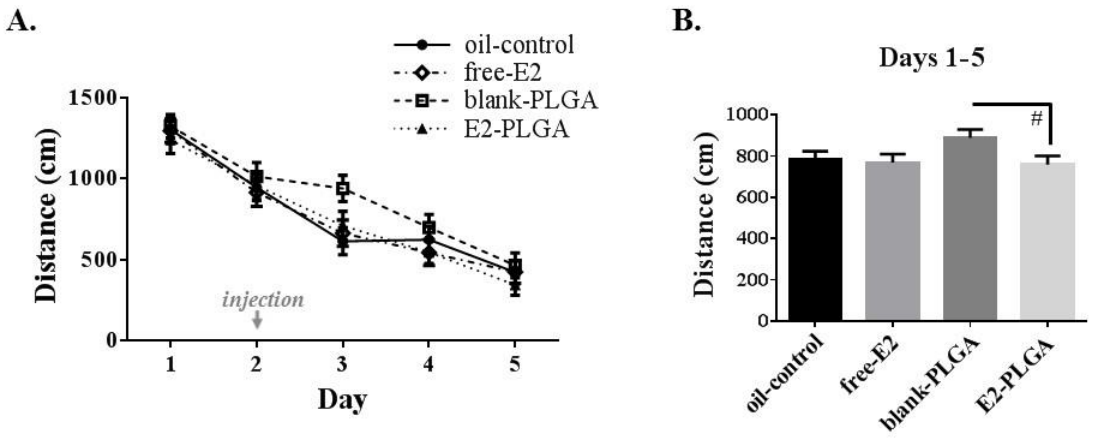


Figure 23. Morris water maze performance. (A) Distance to platform for each day of testing, collapsed across trials, depicting learning of the task by each treatment group. The day of treatment injection is noted on the x-axis. (B) Distance to platform collapsed across days and trials, noting that the E2-PLGA group tended to swim a shorter distance to platform than the blank-PLGA group. Data are presented as mean ± SEM. #$p < 0.1$.

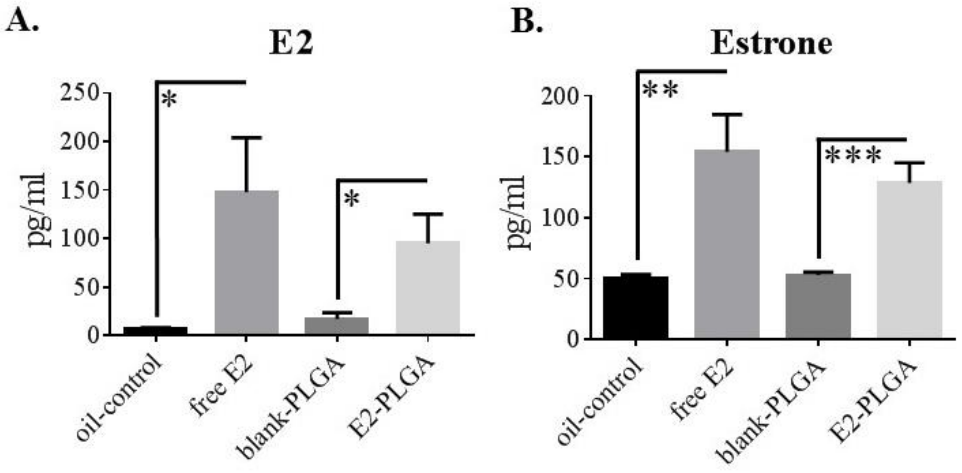


Figure 24. E2 and estrone blood serum levels. (A) E2 blood serum levels collected following the 7th treatment injection, with free E2 and E2-PLGA exhibiting greater E2 levels than oil-control and blank-PLGA, respectively. (B) Estrone blood serum levels collected following the 7th treatment injection, with free E2 and E2-PLGA exhibiting greater estrone levels than oil-control and blank-PLGA, respectively. Data are presented as mean ± SEM. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

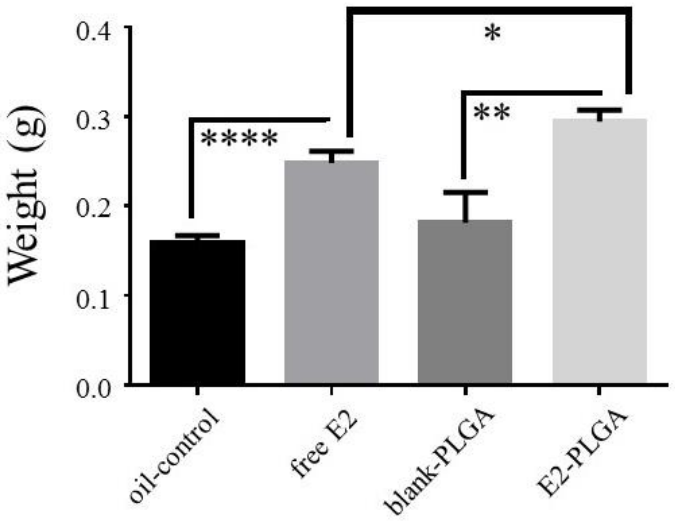


Figure 25. Uterine horn weights. Free E2 and E2-PLGA treatments increased uterine horn weight relative to oil-control and blank-PLGA, respectively, indicating E2-induced uterine stimulation. E2-PLGA had greater uterine horn weight compared to free E2, suggesting greater E2 exposure with the E2-PLGA treatment formulation. Data are presented as mean ± SEM. ****$p < 0.0001$, **$p < 0.01$, *$p < 0.05$.

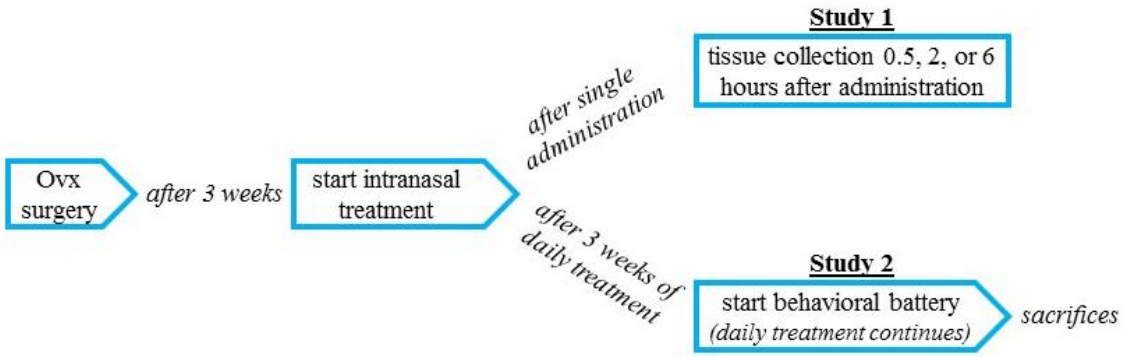


Figure 26. Study 1 and Study 2 timeline. The timeline highlights the similarities and distinctions between Study 1 and Study 2 in regards to Ovx, treatment, and tissue collection versus behavioral testing.

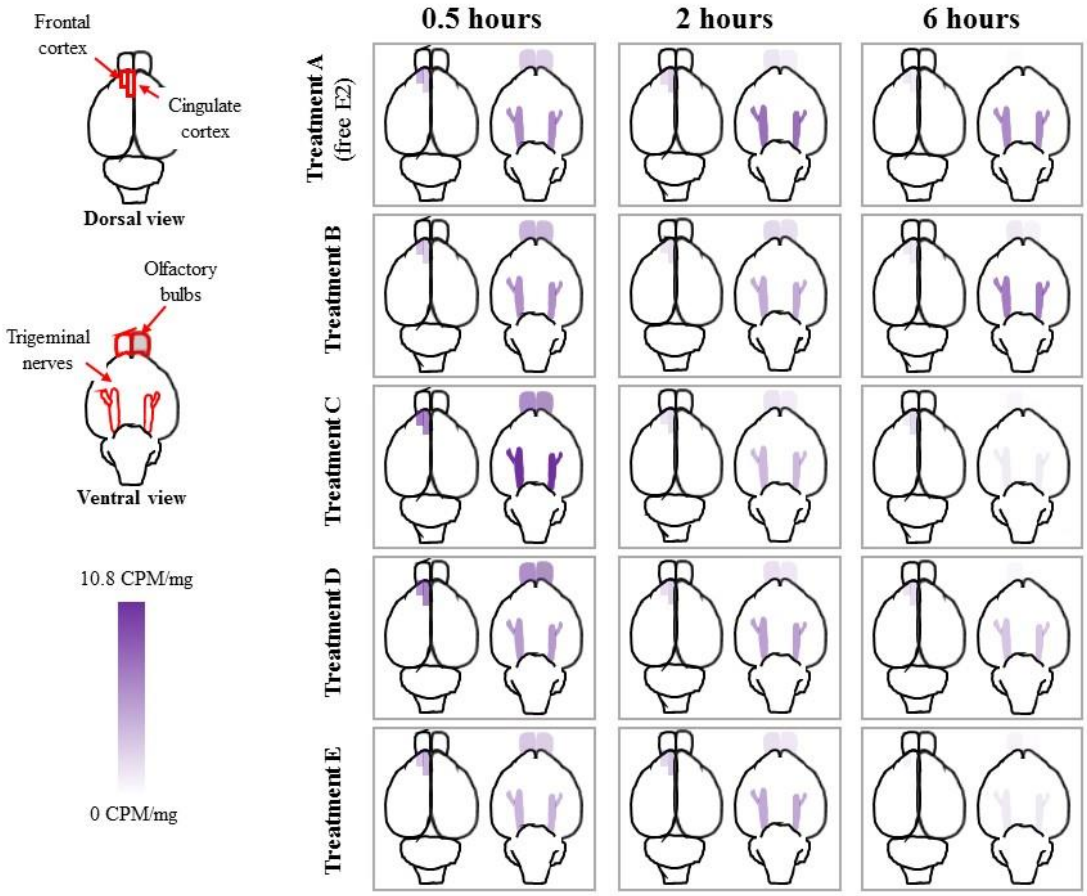


Figure 27. Spatial distribution of tritiated free E2 and E2-CD complex formulations in the brain across time – Part 1. Representative spatial heat maps illustrating the distribution of E2 across time and as a function of treatment formulation in the olfactory bulbs, trigeminal nerves, frontal cortex, and cingulate cortex.

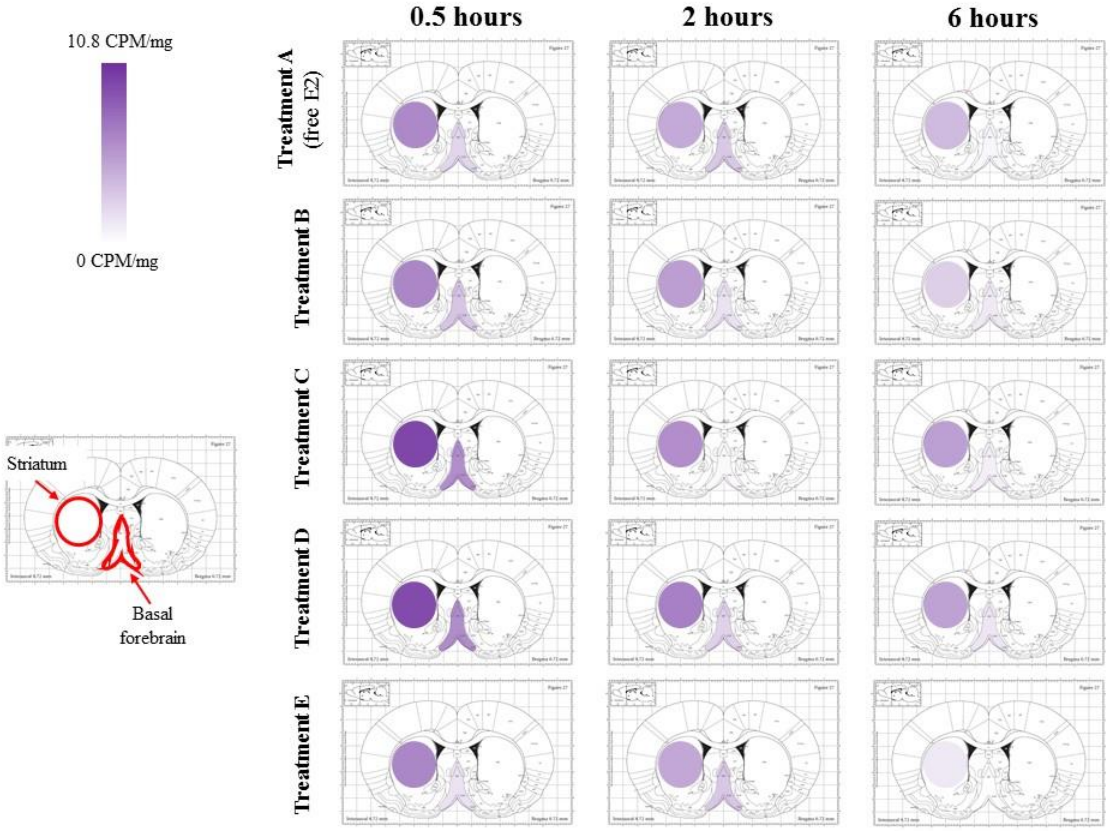


Figure 28. Spatial distribution of tritiated free E2 and E2-CD complex formulations in the brain across time – Part 2. Representative spatial heat maps illustrating the distribution of E2 across time and as a function of treatment formulation in the striatum and basal forebrain.

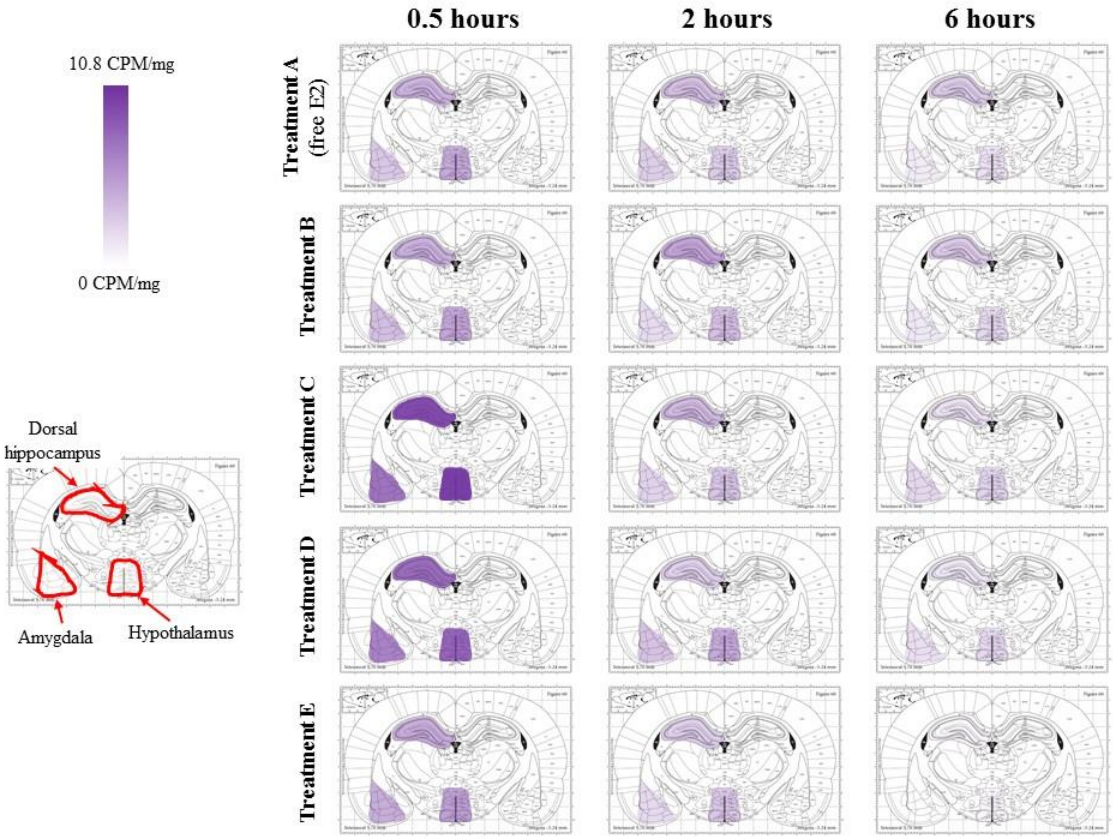


Figure 29. Spatial distribution of tritiated free E2 and E2-CD complex formulations in the brain across time – Part 3. Representative spatial heat maps illustrating the distribution of E2 across time and as a function of treatment formulation in the dorsal hippocampus, amygdala, and hypothalamus.

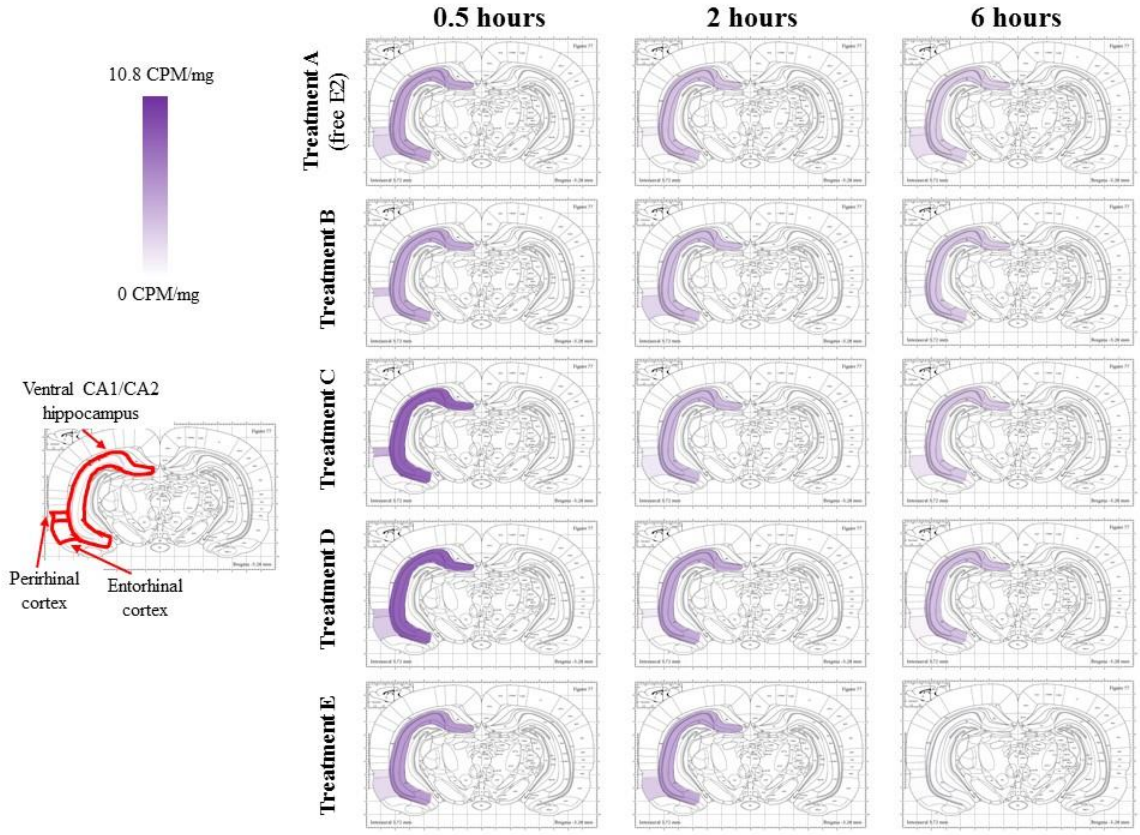
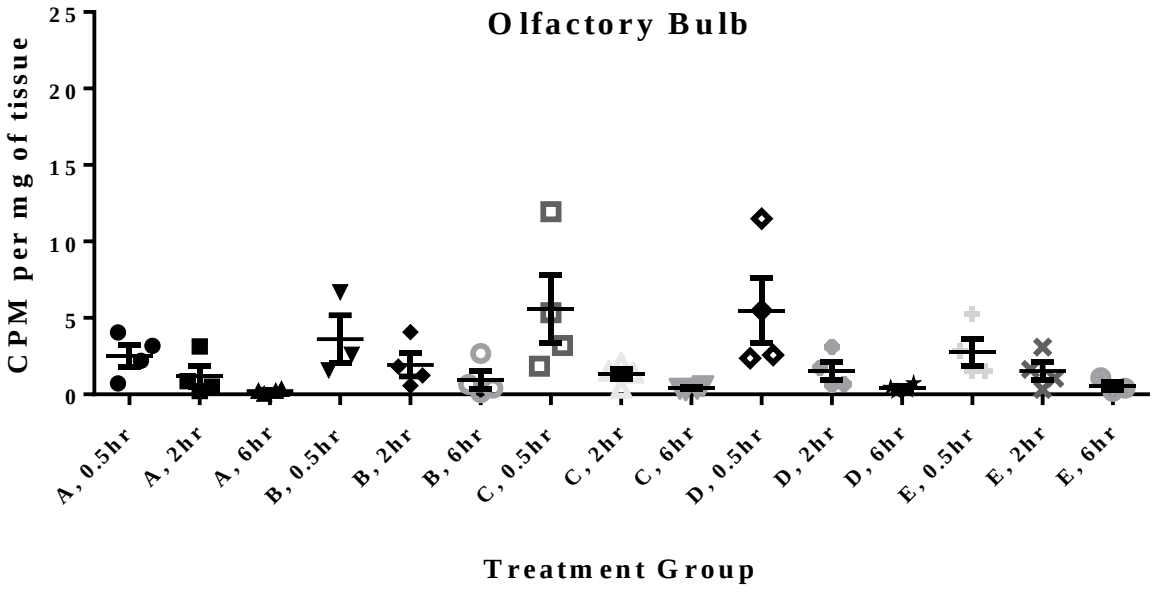
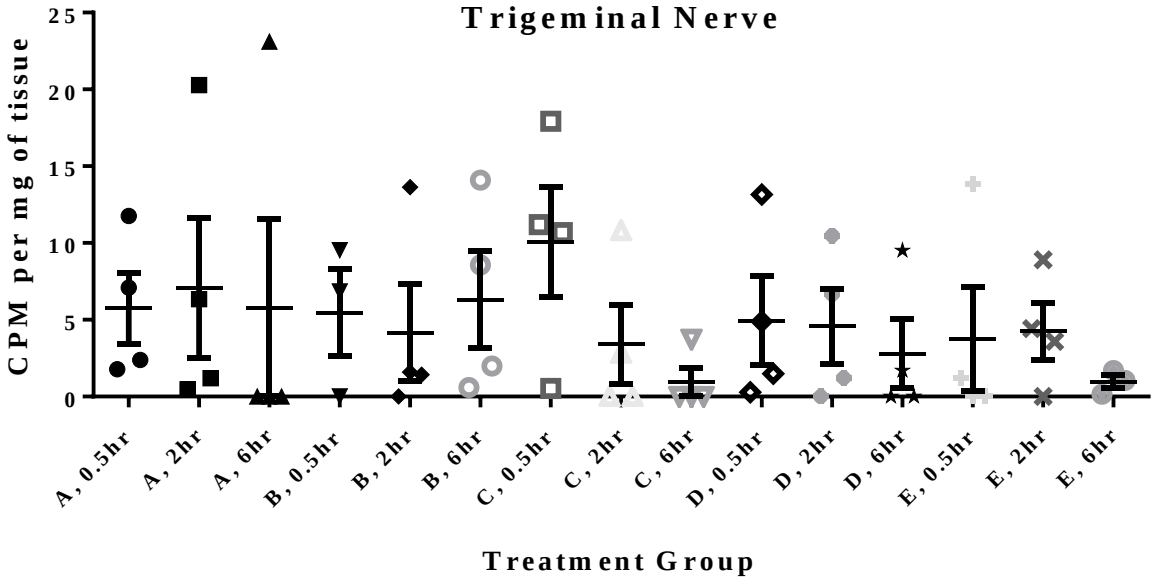
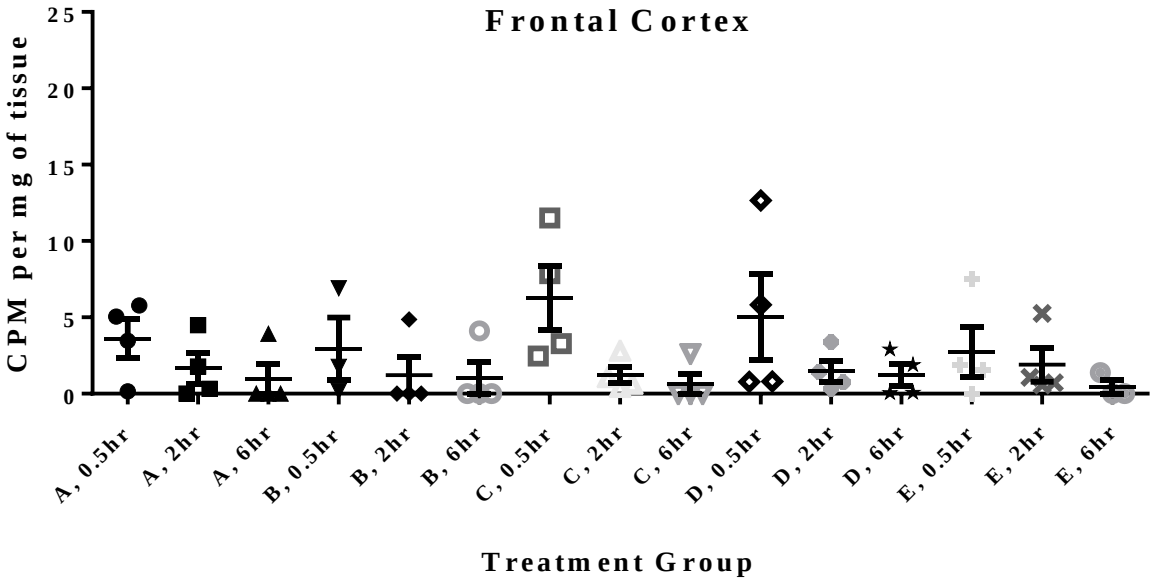
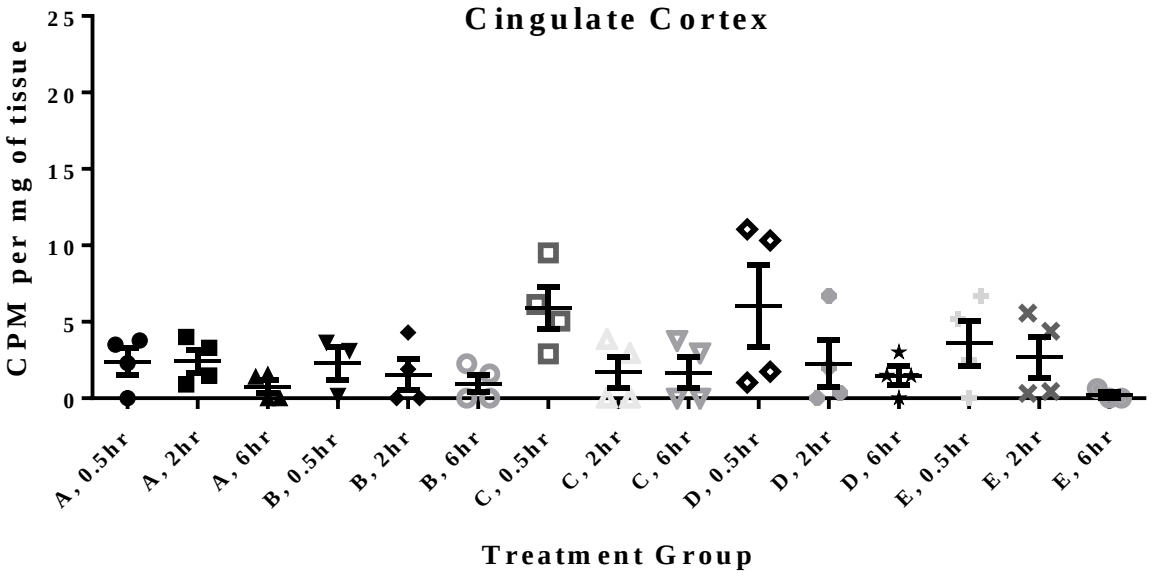


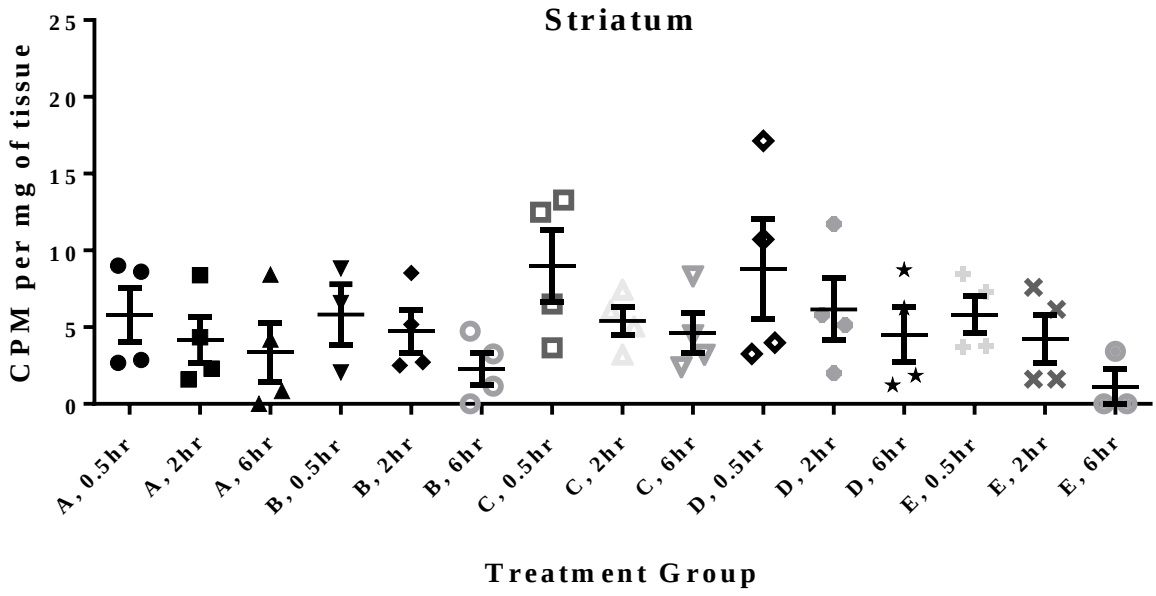
Figure 30. Spatial distribution of tritiated free E2 and E2-CD complex formulations in the brain across time – Part 4. Representative spatial heat maps illustrating the distribution of E2 across time and as a function of treatment formulation in the perirhinal cortex, entorhinal cortex, and ventral CA1/CA2 hippocampus.

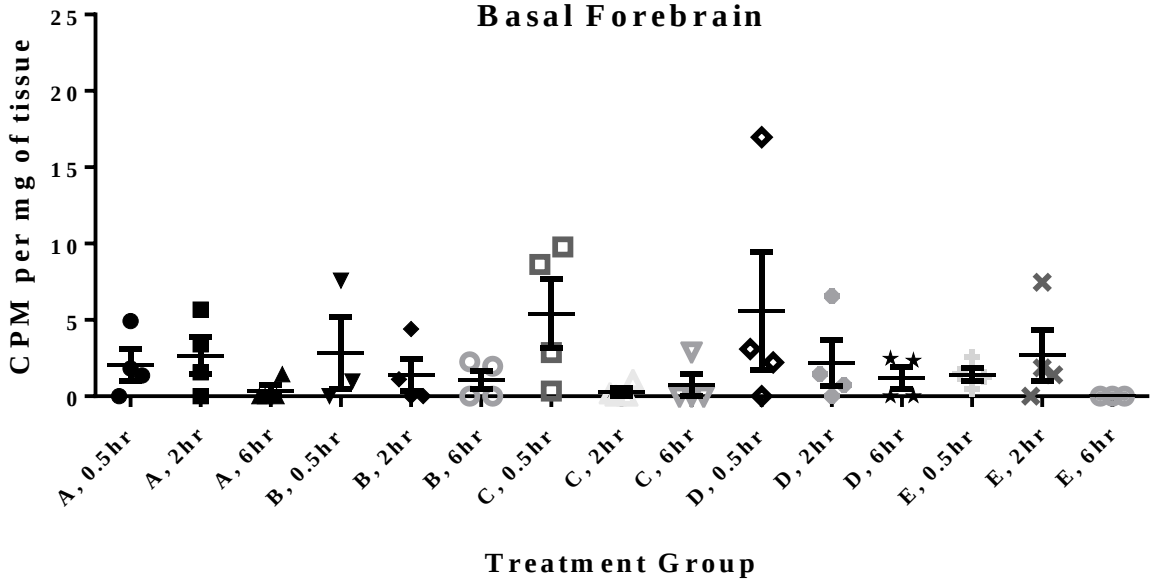

Olfactory Bulb
CPM per mg of tissue
25
20
15
10
5
0
A, 0.5hr
A, 2hr
A, 6hr
B, 0.5hr
B, 2hr
B, 6hr
C, 0.5hr
C, 2hr
C, 6hr
D, 0.5hr
D, 2hr
D, 6hr
E, 0.5hr
E, 2hr
E, 6hr
Treatment Group

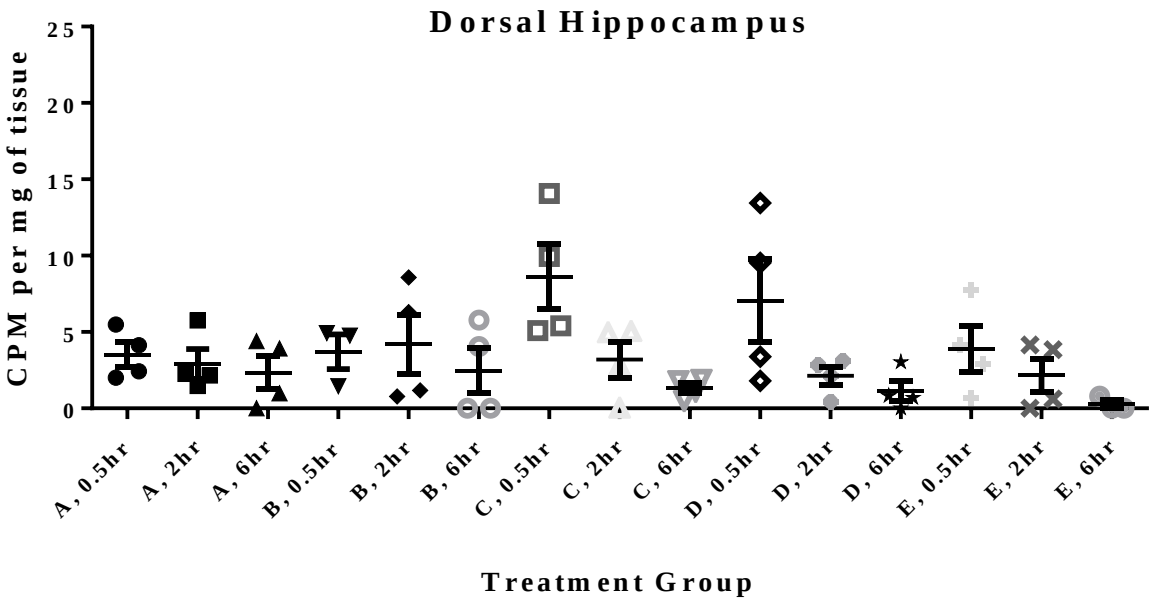

Trigeminal Nerve
CPM per mg of tissue
25
20
15
10
5
0
A, 0.5hr
A, 2hr
A, 6hr
B, 0.5hr
B, 2hr
B, 6hr
C, 0.5hr
C, 2hr
C, 6hr
D, 0.5hr
D, 2hr
D, 6hr
E, 0.5hr
E, 2hr
E, 6hr
Treatment Group

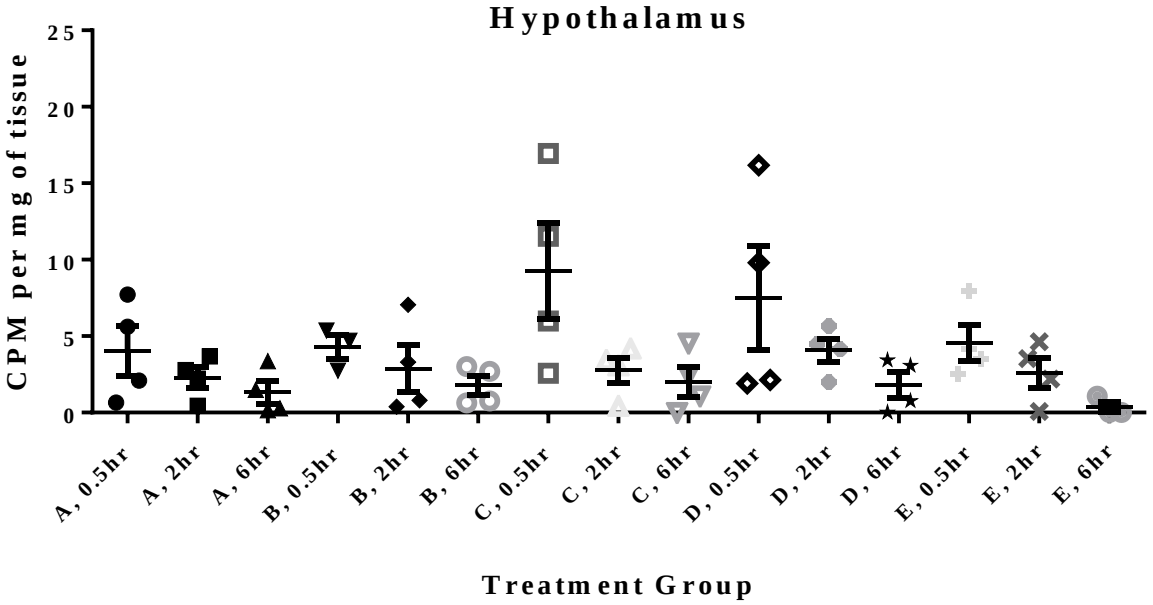

Frontal Cortex
CPM per mg of tissue
25
20
15
10
5
0
A, 0.5hr
A, 2hr
A, 6hr
B, 0.5hr
B, 2hr
B, 6hr
C, 0.5hr
C, 2hr
C, 6hr
D, 0.5hr
D, 2hr
D, 6hr
E, 0.5hr
E, 2hr
E, 6hr
Treatment Group

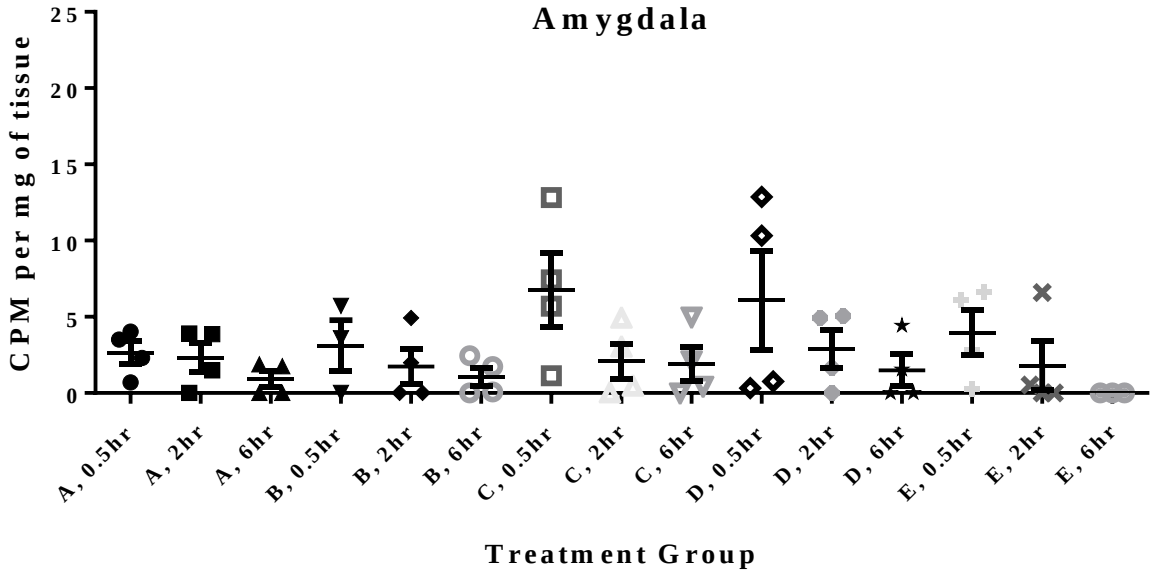

Cingulate Cortex
CPM per mg of tissue
25
20
15
10
5
0
A, 0.5hr
A, 2hr
A, 6hr
B, 0.5hr
B, 2hr
B, 6hr
C, 0.5hr
C, 2hr
C, 6hr
D, 0.5hr
D, 2hr
D, 6hr
E, 0.5hr
E, 2hr
E, 6hr
Treatment Group

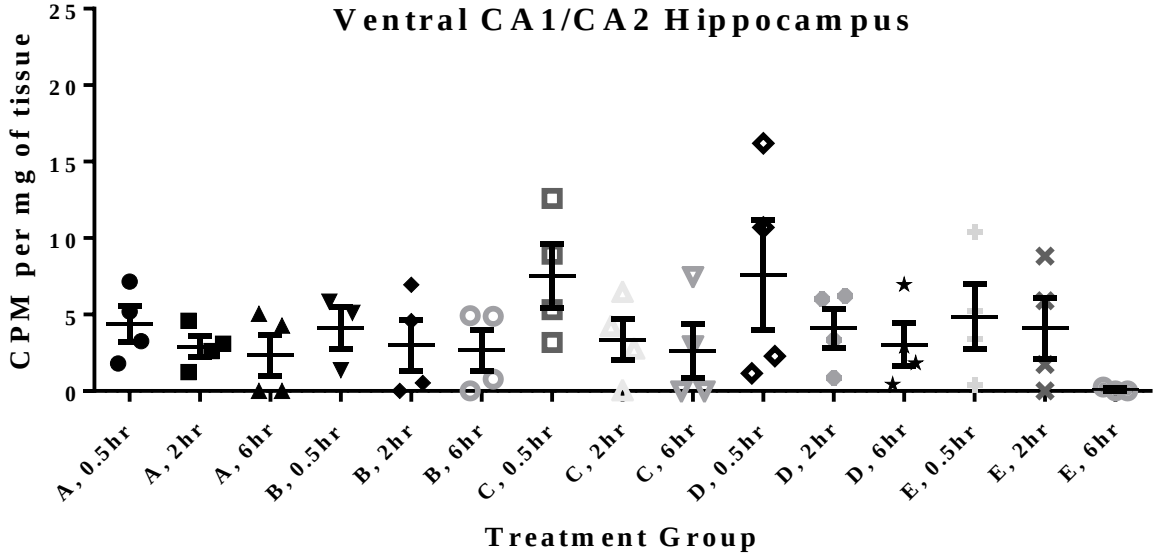

Striatum
CPM per mg of tissue
25
20
15
10
5
0
A, 0.5hr
A, 2hr
A, 6hr
B, 0.5hr
B, 2hr
B, 6hr
C, 0.5hr
C, 2hr
C, 6hr
D, 0.5hr
D, 2hr
D, 6hr
E, 0.5hr
E, 2hr
E, 6hr
Treatment Group


Basal Forebrain
CPM per mg of tissue
25
20
15
10
5
0
A, 0.5hr
A, 2hr
A, 6hr
B, 0.5hr
B, 2hr
B, 6hr
C, 0.5hr
C, 2hr
C, 6hr
D, 0.5hr
D, 2hr
D, 6hr
E, 0.5hr
E, 2hr
E, 6hr
Treatment Group


Dorsal Hippocampus
CPM per mg of tissue
25
20
15
10
5
0
A, 0.5hr
A, 2hr
A, 6hr
B, 0.5hr
B, 2hr
B, 6hr
C, 0.5hr
C, 2hr
C, 6hr
D, 0.5hr
D, 2hr
D, 6hr
E, 0.5hr
E, 2hr
E, 6hr
Treatment Group


Hypothalamus
CPM per mg of tissue
25
20
15
10
5
0
A, 0.5hr
A, 2hr
A, 6hr
B, 0.5hr
B, 2hr
B, 6hr
C, 0.5hr
C, 2hr
C, 6hr
D, 0.5hr
D, 2hr
D, 6hr
E, 0.5hr
E, 2hr
E, 6hr
Treatment Group


Amygdala
CPM per mg of tissue
25
20
15
10
5
0
A, 0.5hr
A, 2hr
A, 6hr
B, 0.5hr
B, 2hr
B, 6hr
C, 0.5hr
C, 2hr
C, 6hr
D, 0.5hr
D, 2hr
D, 6hr
E, 0.5hr
E, 2hr
E, 6hr
Treatment Group


Ventral CA1/CA2 Hippocampus
CPM per mg of tissue
25
20
15
10
5
0
A, 0.5hr
A, 2hr
A, 6hr
B, 0.5hr
B, 2hr
B, 6hr
C, 0.5hr
C, 2hr
C, 6hr
D, 0.5hr
D, 2hr
D, 6hr
E, 0.5hr
E, 2hr
E, 6hr
Treatment Group

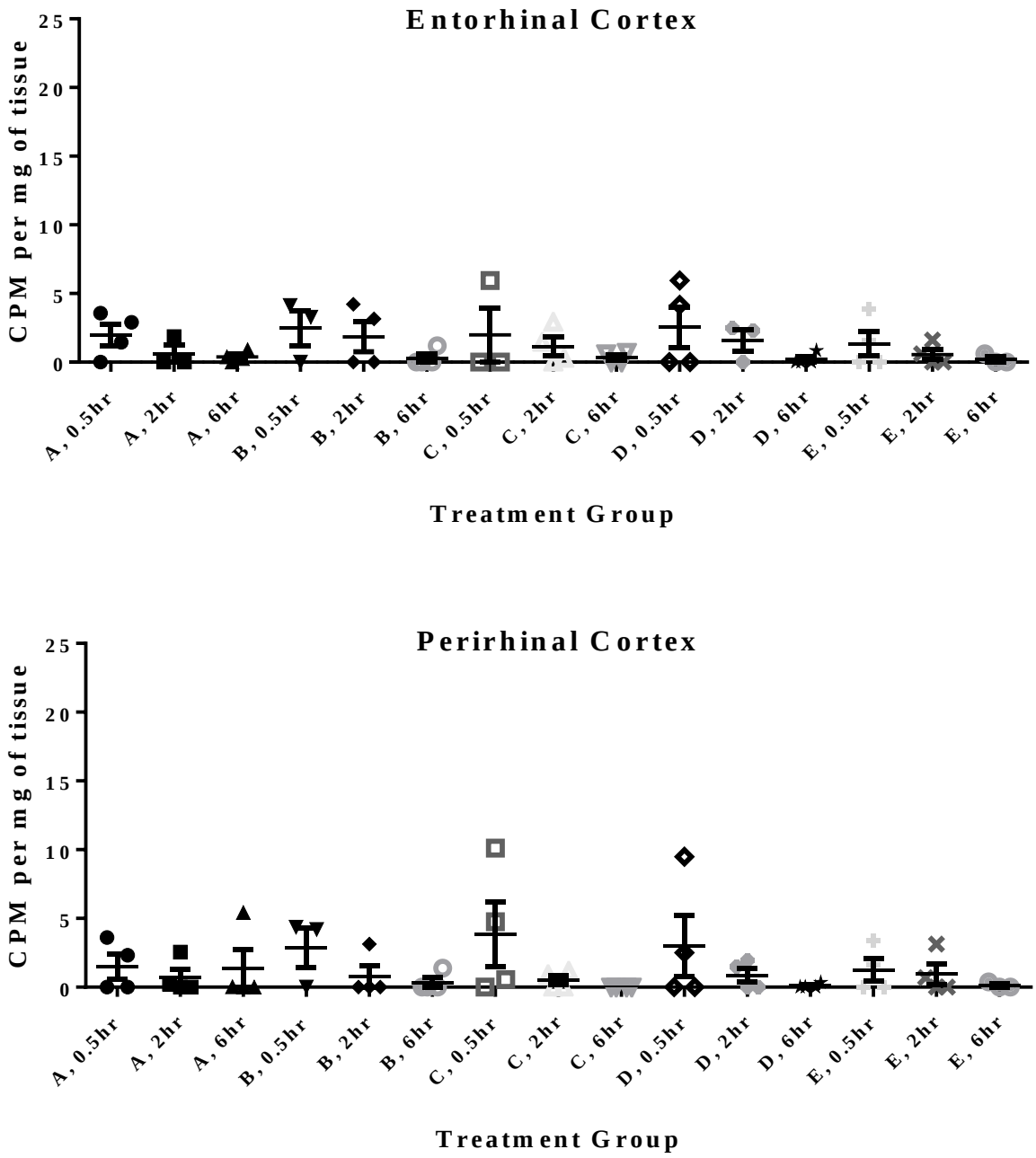


Figure 31. Brain distribution of tritiated free E2 and E2-CD complex formulations across time. Quantitative representation of E2 distribution across time and as a function of treatment formulation in the olfactory bulbs, trigeminal nerves, frontal cortex, cingulate cortex, basal forebrain, striatum, dorsal hippocampus, hypothalamus, amygdala, entorhinal cortex, perirhinal cortex, and the CA1/CA2 region of the ventral hippocampus.

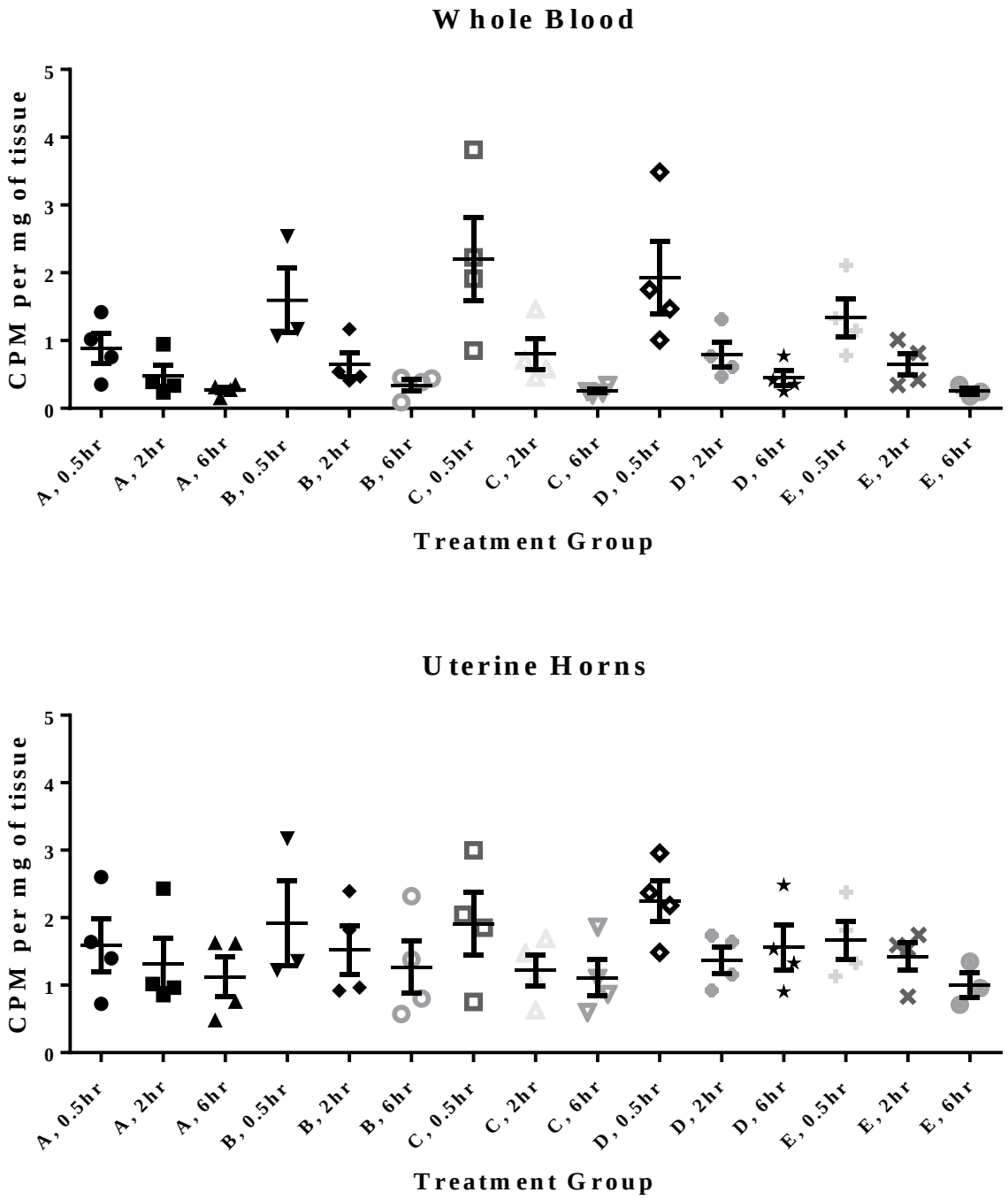


Figure 32. Biodistribution of tritiated free E2 and E2-CD complex formulations across time. Quantitative representation of E2 distribution across time and as a function of treatment formulation in whole blood and uterine horns.

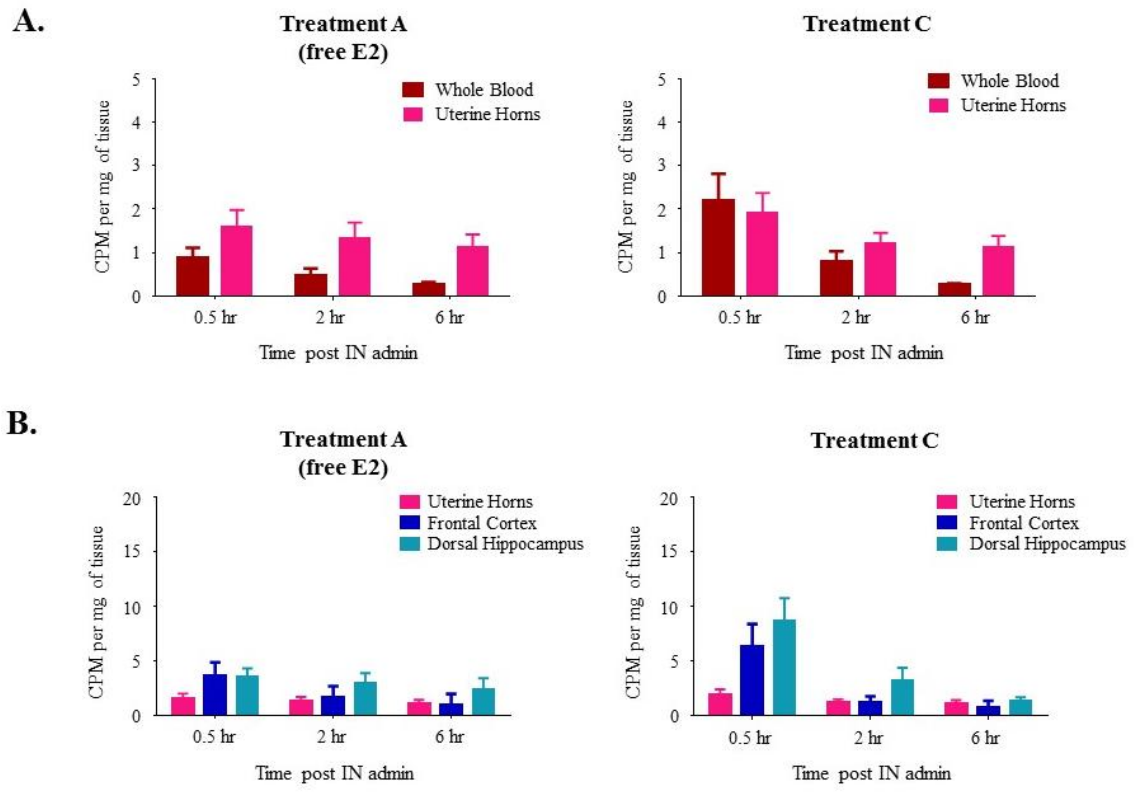


Figure 33. Tritiated E2 distribution in whole blood and uterine horns. (A) Tritiated E2 distribution across time in whole blood and uterine horns with Treatment A (free E2) and Treatment C formulations. E2 appears to be rapidly cleared from whole blood but sustained at the uterine horns with both treatments (B) Tritiated E2 distribution across time in frontal cortex, dorsal hippocampus, and uterine horns with Treatment A (free E2) and Treatment C formulations. E2 uptake in dorsal hippocampus is greater relative to uterine horns 0.5 hr following intranasal administration with Treatment C as compared to Treatment A. Data are presented as mean ± SEM.

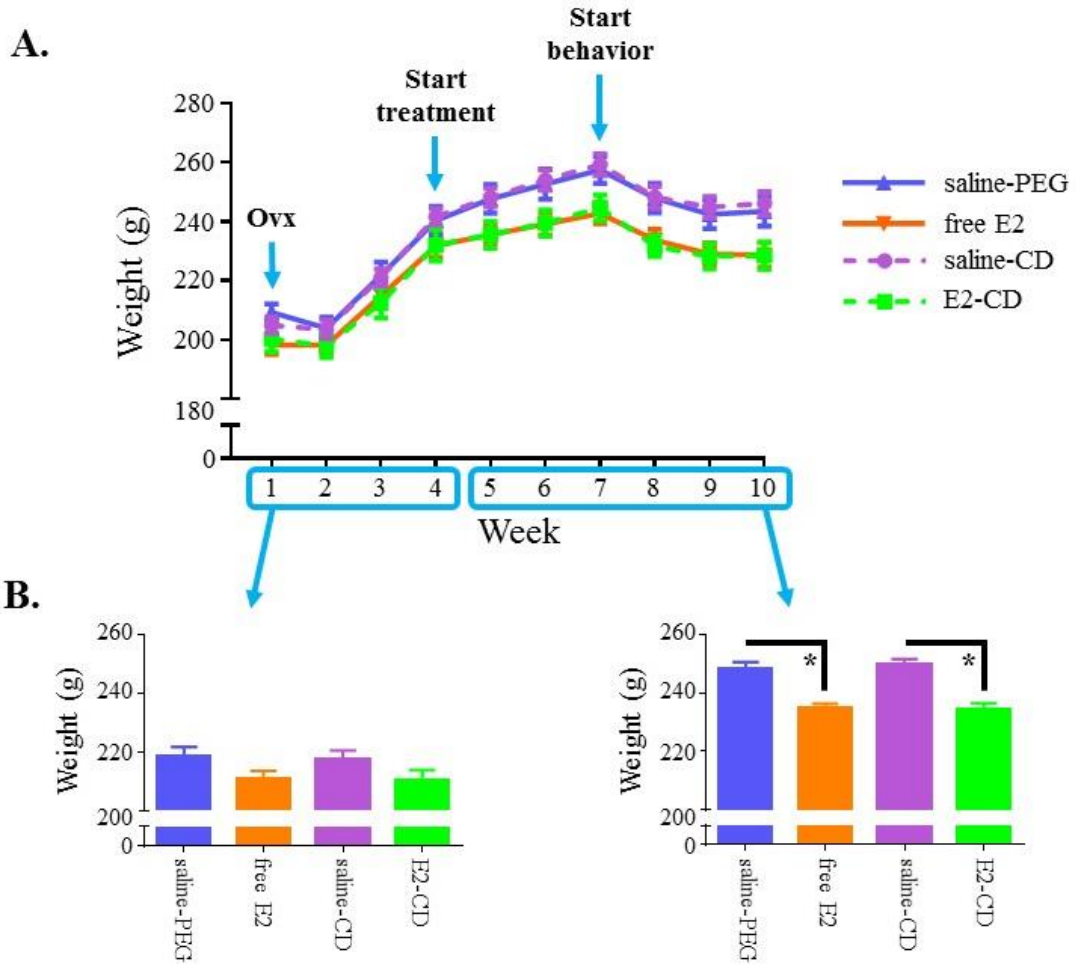


Figure 34. Weekly body weights. (A) Average weekly body weight for each treatment group throughout the duration of the study. Ovx, treatment initiation, and behavior initiation are noted on the graph. (B) Average body weight for weeks 1-4 of the study, prior to treatment initiation. (C) Average body weight for weeks 5-10 of the study, after treatment initiation, where E2-containing treatment groups exhibited lower average body weight than respective vehicle control groups. Data are presented as mean ± SEM. *$p <$ 0.05.

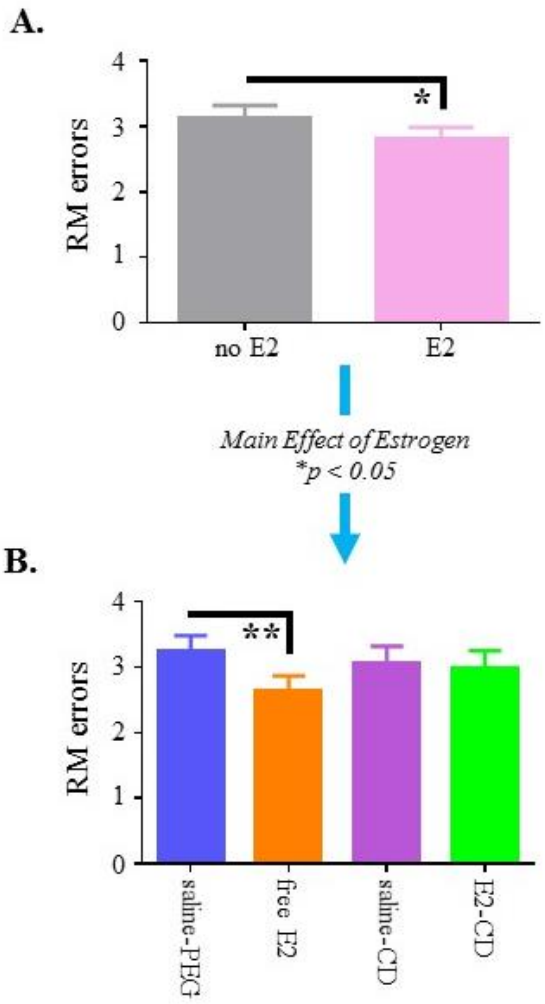


Figure 35. Water radial-arm maze performance on the acquisition phase. (A) RM errors made on the acquisition phase between E2-containing treatment groups and groups that did not contain E2, showing a main effect of E2. (B) RM errors made on the acquisition phase across the four treatment groups, where the free E2 group made less errors than its vehicle control, saline-PEG. Data are presented as mean ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$.

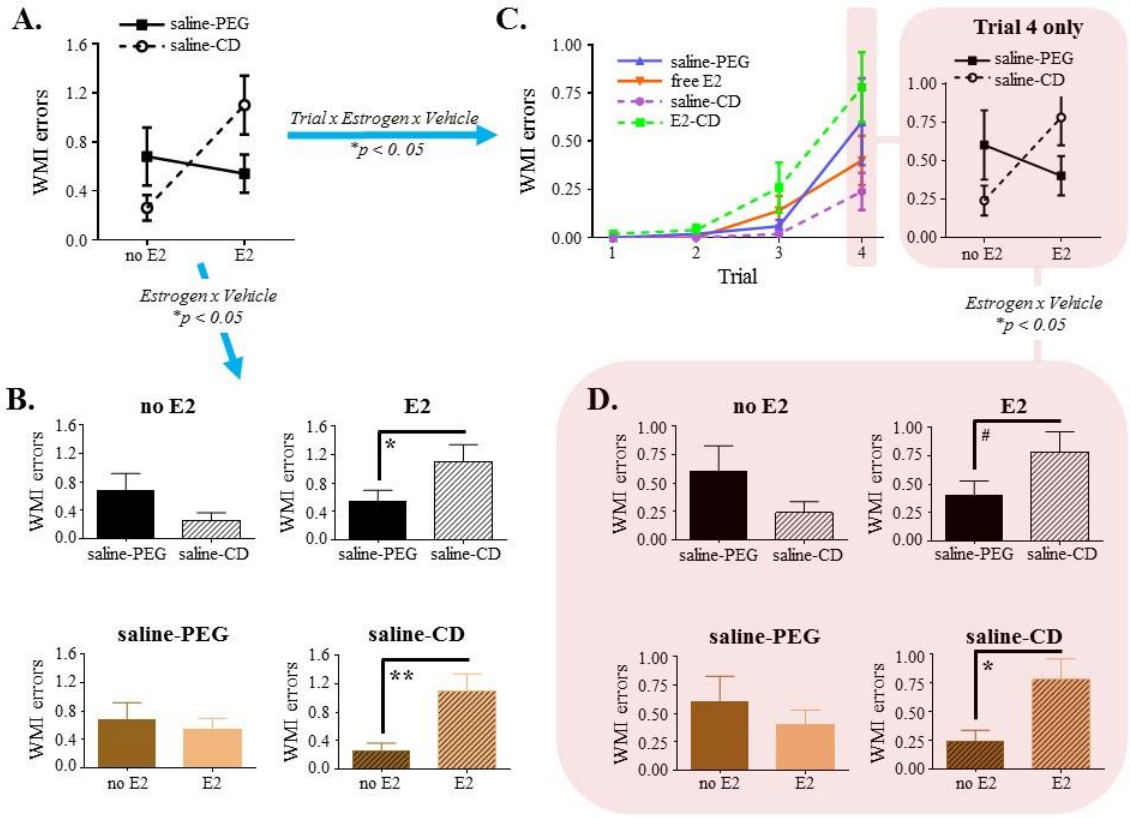


Figure 36. Water radial-arm maze performance on the asymptotic phase. (A) Estrogen by vehicle interaction for WMI errors made on the asymptotic phase. (B) Estrogen by vehicle interaction separated into four graphs, showing that the E2-CD group made more WMI errors than the free E2 group and than the saline-CD group. (C) Trial by estrogen by vehicle interaction for WMI errors made on the asymptotic phase, where an estrogen by vehicle interaction was observed on Trial 4, the highest working memory load trial evaluated on the task. (D) Trial 4 estrogen by vehicle interaction separated into four graphs, showing that the E2-CD group tended to make more WMI errors than free E2 group and made more WMI errors than saline-CD group. Data are presented as mean ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$, $\#p < 0.1$.

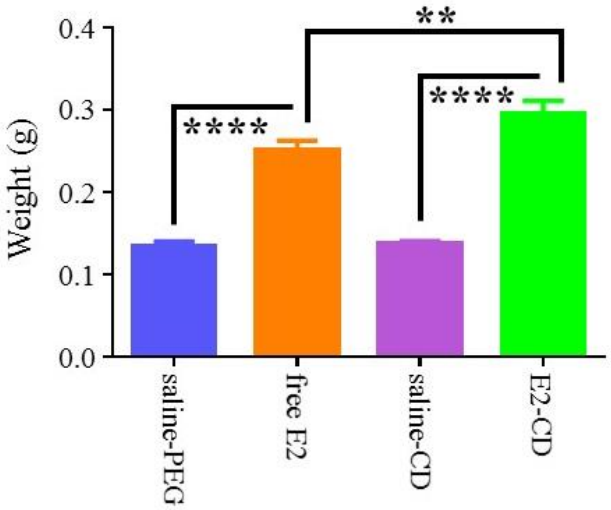


Figure 37. Uterine horn weights. Free E2 and E2-CD treatment had greater uterine horn weight than their respective vehicle controls, saline-PEG and saline-CD, confirming E2 presence at the uterine horns. E2-CD had increased uterine horn weight relative to free E2, suggesting greater uterine horn exposure to E2 with the addition of the CD carrier. Data are presented as mean ± SEM. ****$p < 0.0001$, **$p < 0.01$.

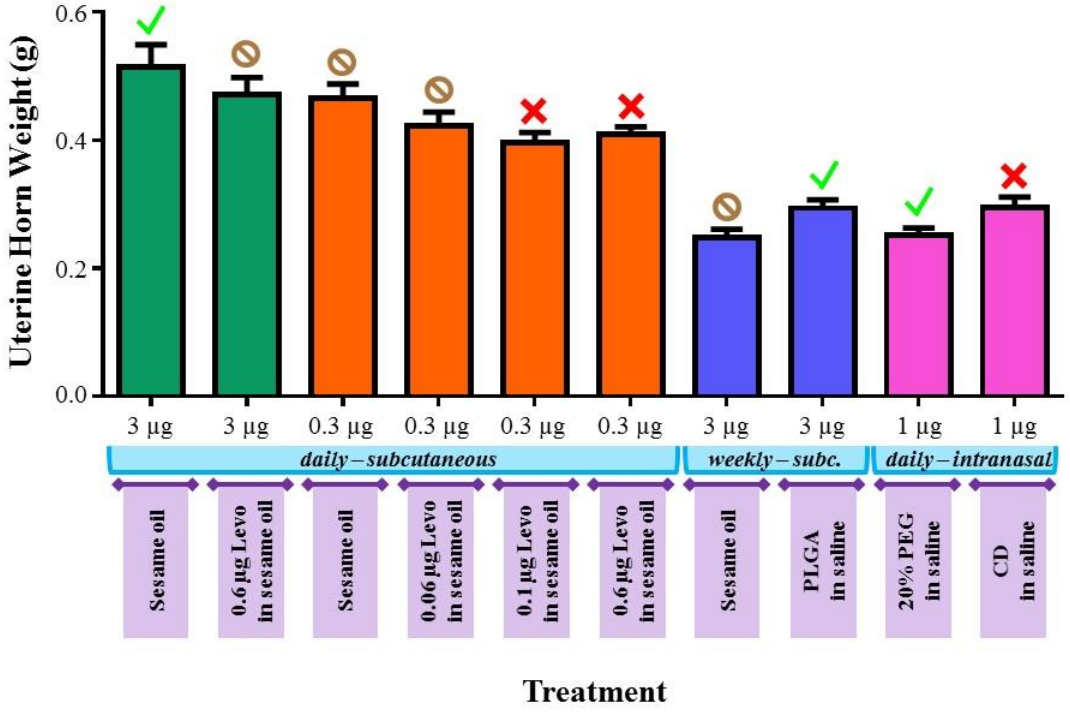


Figure 38. Summary of cognitive and uterine effects across the E2-containing formulations evaluated in the present dissertation. Uterine horn weight for each E2-containing formulation across all chapters – Chapter 2 (green bars), Chapter 3 (orange bars), Chapter 4 (blue bars), and Chapter 5 (pink bars). The dose of E2, regimen and type of treatment administration, as well as dose of Levo and type of vehicle are depicted on the x-axis. Above the bar for each E2-containing formulation, a green check-mark indicates improved spatial learning and memory relative to vehicle control, a brown null sign indicates neutral effects on spatial learning and memory relative to vehicle control, and a red 'x' denotes impaired spatial learning and memory relative to vehicle control. For reference, average uterine horn weights for all evaluated vehicle controls were between 0.135 – 0.184 g. Data are presented as mean ± SEM.

APPENDIX A

ESPRESSO MARTINI

ESPRESSO MARTINI

Ingredients

1 shot Vodka

¼ shot Simple syrup

¼ shot Kahlua

½ shot espresso

Garnish: coffee bean

Instructions

Combine the ingredients, shake with ice, and strain into a cocktail glass. Place a coffee bean on top as garnish.