Optimization of Menopausal Hormone Therapies for Cognitive and Brain Aging Using a Rat Model

by

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ABSTRACT

Each year, millions of aging women will experience menopause, a transition from reproductive capability to reproductive senescence. In women, this transition is characterized by depleted ovarian follicles, declines in levels of sex hormones, and a dysregulation of gonadotrophin feedback loops. Consequently, menopause is accompanied by hot flashes, urogenital atrophy, cognitive decline, and other symptoms that reduce quality of life. To ameliorate these negative consequences, estrogen-containing hormone therapy is prescribed. Findings from clinical and pre-clinical research studies suggest that menopausal hormone therapies can benefit memory and associated neural substrates. However, findings are variable, with some studies reporting null or even detrimental cognitive and neurobiological effects of these therapies. Thus, at present, treatment options for optimal cognitive and brain health outcomes in menopausal women are limited. As such, elucidating factors that influence the cognitive and neurobiological effects of menopausal hormone therapy represents an important need relevant to every aging woman. To this end, work in this dissertation has supported the hypothesis that multiple factors, including post-treatment circulating estrogen levels, experimental handling, type of estrogen treatment, and estrogen receptor activity, can impact the realization of cognitive benefits with Premarin hormone therapy. We found that the dose-dependent working memory benefits of subcutaneous Premarin administration were potentially regulated by the ratios of circulating estrogens present following treatment (Chapter 2). When we administered Premarin orally, it impaired memory (Chapter 3). Follow-up studies revealed that this impairment was likely due to the handling associated with treatment administration and the task difficulty of the

memory measurement used (Chapters 3 and 4). Further, we demonstrated that the unique cognitive impacts of estrogens that become increased in circulation following Premarin treatments, such as estrone (Chapter 5), and their interactions with the estrogen receptors (Chapter 6), may influence the realization of hormone therapy-induced cognitive benefits. Future directions include assessing the mnemonic effects of: 1) individual biologically relevant estrogens and 2) clinically-used bioidentical hormone therapy combinations of estrogens. Taken together, information gathered from these studies can inform the development of novel hormone therapies in which these parameters are optimized.

DEDICATION

Heather, thank you for being an amazing, wonderful mentor (aka: mom-ter). Thank you for teaching me. Thank you for challenging me. Thank you for being my advocate and my cheerleader. Thank you for supporting and encouraging me. Thank you for being an inspiration and a role model. Thank you for making me not just a better scientist; thank you for making me a better person. Thank you for everything.

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TABLE OF CONTENTS

CHAPTER Page

CHAPTER Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

CHAPTER 1

GENERAL INTRODUCTION AND BACKGROUND

Menopause and Models to Study Hormone Loss and Cognitive Aging

Typically occurring in the fifth decade of life, aging women will experience menopause, a transition from reproductive capability to reproductive senescence (Timiras et al., 1995). The menopausal transition is characterized by depleted ovarian follicles, declines in naturally circulating levels of sex hormones, such as estrogens and progesterone, and a dysregulation of gonadotrophin feedback loops marked by increasing levels of follicular stimulating hormone and lutenizing hormone (Rannevik et al., 1995). As a result of these changing hormone levels, menopause is accompanied by hot flashes, urogenital atrophy, cognitive decline (specifically learning and memory), and other symptoms that reduce quality of life (Freedman, 2002; Sherwin and Henry, 2008). These consequences of the menopausal transition become important when considering that life expectancy has increased over the past century, but the age of menopause has not changed (Hawkes, 2003). Thus, women now spend a larger proportion of life in this post-menopausal, hypoestrogenic state associated with memory deficits and other negative physiological consequences. As well, not only are women spending a longer portion of life post-menopausal, but also, the aging female population is growing. Indeed, by the year 2050, 90 million people are projected to be over 65 years of age; over half of this sizable population will be women (US Census, 2008). In both sexes, memory function declines during aging (Conrad and Bimonte-Nelson, 2010; Erickson and Barnes, 2003) and Alzheimer's Disease, more prevalent in women than men (Fratiglioni et al.,1997), is

characterized by memory deficits. Thus, there is an important medical need to develop optimal treatments for the cognitive and physiological changes of the menopausal transition and aging processes.

To address this important issue, a commonly used model to study age-related cognitive decline and memory changes following hormone loss and replacement is the middle-aged, ovariectomized (Ovx) rodent, made hormone deplete via the surgical removal of the ovaries. Many parallels exist between the aging menopausal woman and the aging Ovx rodent, making this an effective model for this field of study. In both women and female rodents, age-related deterioration of the hippocampus and declines in performance on hippocampal-dependent tasks has been well documented (Barnes et al., 1980; Burke and Barnes, 2006). Further, evidence suggests that ovarian hormones play a protective role in preventing the observed memory decline in both species. For instance, relative to women without a history of taking hormone treatments, post-menopausal women who took exogenous estrogen-containing hormone therapy (HT) show enhanced performance on spatial memory measures (Kimura, 1995; Smith et al., 2001). Similarly, aging Ovx rats given exogenous estrogens show enhancements on tests of spatial memory as compared to untreated control rats (Bimonte-Nelson et al., 2010; Frick, 2009). Given these similarities, the benefit of utilizing the aging rodent model is that rodents with a median lifespan of greater than 20 months are considered appropriate aging models (Nadon, 2004a), while aging humans take many decades to reach senescence. Thus, researchers studying aging in rodents can develop interventions to attenuate, halt, reverse, or even prevent age-related memory decline in a relatively short time span.

In addition to their rapid rate of aging, another benefit of the rodent model is that the physiological and cognitive consequences of surgically-induced menopause are similar among women and rats. For instance, following oophorectomy in women, circulating levels of ovarian hormones decline rapidly (Sherwin, 2007). Comparably, following surgical ovary-removal (Ovx) in rats, circulating estrogens and progesterone fall to low levels (Wise and Ratner, 1980). In both models, the sudden loss of ovarian hormones is associated with memory impairments (Acosta et al., 2013; Henderson and Sherwin, 2007; Rocca et al., 2010b). Despite the similarity in ovarian hormone profiles following surgical ovary removal in women and rats, an important limitation of this model is that a large majority of women undergo a transitional, rather than surgical, menopause. Indeed, the majority of women experience a transitional menopause, in which follicles deplete and hormone levels change over many years, while only portion of women experience oophorectomy, menopause via a surgical removal of the ovaries (Timiras et al., 1995). However, the 'blank' ovarian hormone profile induced by Ovx in the rat allows investigators to isolate and evaluate the potentially distinct cognitive and neurobiological impacts of unique ovarian hormones as well as hormone formulations. This methodological approach permits the optimization of current and future menopausal HTs for cognitive outcomes, a primary aim of this dissertation.

Menopausal Hormone Therapies, Estrogens, and Memory

Premarin

To alleviate menopausal symptoms, HT is given. Premarin (conjugated equine estrogens), a purified pregnant mare urine compound first developed by Wyeth, is the

most widely used estrogen-based menopausal HT in North America (Hersh et al., 2004). Although primarily composed of estrone (E1) sulfate, Premarin is a mixture of at least 10 estrogen sulfates, many of which are unique to horses and have yet to be evaluated for their cognitive impacts in a human or rodent model (Kuhl, 2005). After metabolism, the biologically active hormones in circulation are primarily E1 and the more potent 17β-estradiol (17β-E2), as well as equine-specific estrogens such as equilin and $\Delta^{8,9}$-dehydroestrone (Bhavnani, 2003; Kuhl, 2005). Despite Premarin being an effective treatment for relieving the negative vasomotor symptoms and vaginal atrophy of menopause (Freedman, 2002), clinical and preclinical findings of Premarin's cognitive effects are inconclusive. Cell culture models suggest that Premarin can be neuroprotective. Specifically, Premarin enhances neuronal growth and increases neuronal survival after experimentally-induced insult in vitro (Brinton et al., 2000a; Brinton et al., 2000b). Benefits have also been reported in the behaving, middle-aged Ovx rat. Premarin administered via an acute subcutaneous injection (Walf and Frye, 2008) or via cyclical subcutaneous injections (Acosta et al., 2009b) enhances object memory and spatial navigation memory. In middle-aged women, the findings regarding Premarin-containing HT are mixed, with some studies reporting cognitive benefits, and others not (Hogervorst et al., 2000; Sherwin and Henry, 2008). Among these negative reports is the large, double-blind, placebo-controlled Women's Health Initiative Memory Study, comprised of nearly 7,500 post-menopausal women (Shumaker et al., 2004). Findings showed a non-significant increased incidence of probable dementia and mild cognitive impairment in women 65+ given Premarin (Shumaker et al., 2004). In addition, Premarin+medroxyprogesterone acetate, given to women with an intact uterus to prevent

potentially cancer-causing uterine stimulation by estrogens, elevated probable dementia risk (Shumaker et al., 2003). Thus, loss of ovarian hormones is not optimal for cognition, but neither are the most commonly prescribed HTs. It is still unclear which parameters underlie the realization of cognitive and neurobiological benefits with Premarin.

Bioidentical Hormone Therapy

Following the surprising null and detrimental findings of the Women's Health Initiative and Women's Health Initiative Memory Study for cognitive as well as peripheral outcomes, there has been increased interest in "bioidentical" hormone therapy (BHT) options (Bhavnani and Stanczyk, 2012; Cirigliano, 2007). Bioidentical hormones are chemically identical to those hormones produced endogenously in women, but are derived from a variety of 'natural' or de novo synthetic sources (Cirigliano, 2007). To create BHTs, formulations are individually compounded by pharmacists to contain specific steroids in various dosages, including 17β-E2, E1, estriol (E3), progesterone, dehydroepiandrosterone, and testosterone (Cirigliano, 2007). Despite claims by some women's health clinicians and popular authors (Walker, 2001) and the common belief among aging women (Adams and Cannell, 2001) that BHT is a natural, safer and more efficacious alternative to Premarin-based conventional HTs, these bioidentical formulations are not routinely tested by the Food and Drug Administration or other regulatory agencies. Further, there is little research thus far on the safety and efficacy of BHTs and there is a paucity of objective evaluations of the long-term cognitive consequences of these formulations (Bhavnani and Stanczyk, 2012; Cirigliano, 2007). Thus, at present, conventional HTs, such as Premarin-based compounds, are recommended for the treatment of menopausal symptoms given that 'customized

compounded hormones have variable purity and potency…lack efficacy and safety data' and may even 'pose additional risks' (American College of Obstetricians and Gynecologists, 2012). As such, given that Premarin is not an optimal menopausal HT for cognitive and physiological outcomes and given the increased use of BHT options in the absence of methodical evaluations of their effectiveness, there is a pressing clinical need for evaluation of the components of BHTs. Characterizing the unique contributions of individual estrogens endogenous to women is an important first step towards optimizing menopausal HT options for women.

17β-estradiol

Since it was first shown to enhance cognition in elderly women (Caldwell and Watson, 1952), there has been much interest in the ability of 17β-E2, the most potent naturally circulating estrogen (Kuhl, 2005), to impact the brain and memory. Today, 17β-E2, a common component in conventional HTs and BHTs, is perhaps the most well-characterized estrogen for neurobiological and cognitive outcomes. We and others have shown that 17β-E2 enhances spatial working (Bimonte and Denenberg, 1999; Daniel et al., 1997; Gibbs, 1999; Hruska and Dohanich, 2007; Luine and Rodriguez, 1994), reference memory (Bimonte-Nelson et al., 2006; El-Bakri et al., 2004; Markham et al., 2002) and object memory (Luine et al., 2003) in young and middle-aged rodents. Yet, whether 17β-E2 will impart cognitive benefits seems to depend on a number of factors. One important factor may be the age at the time the treatment is initiated. Indeed, converging data suggest that cognitive responsiveness to estrogen stimulation seems to decline with age, especially on spatial reference memory tasks (Foster et al., 2003; Gresack et al., 2007; Talboom et al., 2008). For instance, the same dose of 17β-E2

treatment that effectively enhanced performance on the Morris water maze among 4 and 16 month old, Ovx rats was generally ineffective in 24 month olds (Talboom et al., 2008). However, some studies still report benefits of 17β-E2 administration in aged rodents, suggesting an interactive role for the route of administration and age in the realization of cognitive benefits with 17β-E2 (Frick et al., 2002; Markowska and Savonenko, 2002a). Optimization of the factors that influence whether 17β-E2 will impart memory benefits will improve current and future HT options.

Estrone

In addition to 17β-E2 and E3, E1 is an endogenous estrogen that naturally circulates in women (Kuhl, 2005). Prior to menopause, endogenous E1 circulates in approximately a 1:1 ratio with 17β-E2 (Rannevik et al., 1995). However, during the menopausal transition, levels of 17β-E2 decline to a greater extent than do levels of E1, changing the circulating E1 to 17β-E2 ratio to 2:1 (Rannevik et al., 1995). This shift to higher ratio of E1 to 17β-E2 may have a significant impact on cognitive ability. As well, E1 is a component of the tri-estrogen BHT, Triest (Cirigliano, 2007). Further, Premarin, the most commonly prescribed estrogen-based menopausal HT (Hersh et al., 2004), is over 50% E1-sulfate, (Kuhl, 2005). Following treatment with Premarin to peri- and post-menopausal women, and middle-aged, Ovx rats, circulating levels of E1 increase (Acosta et al., 2009b; Yasui, 1999). Yet, the cognitive and neurobiological impacts of E1 are unclear. One study in young adult, Ovx rats has shown that a single subcutaneous E1 injection impairs memory on the contextual fear conditioning task when given 30 minutes before training (Barha et al., 2009). Furthermore, although not all in vitro studies report

negative effects with E1 treatment (Zhao and Brinton, 2006), for most measures in which other estrogenic Premarin components (e.g., equilin and $\Delta^{8,9}$-dehydroestrone) were neuroprotective, E1 was ineffective. Thus, the lack of clarity regarding the cognitive and neurobiological impacts of this estrogen in middle-age rodents represents a gap in the literature.

Estrogens and Putative Mechanisms of Action

Interactions of Estrogen with its Receptors

Once thought to only mediate reproductive behavior, estrogens such as 17β-E2 and E1 are now understood to impact a variety of non-reproductive behaviors, including performance on learning and memory tasks (Bimonte-Nelson et al., 2010). At least some of estrogens' diverse cognitive effects are mediated by ligand interactions with the classical, nuclear estrogen receptor (ER). Discovered in uterine tissue (Toft and Gorski, 1966), ER-alpha (ERα) was the first nuclear ER that demonstrated binding specificity for 17β-E2 and for many decades, was thought to be the sole ER with which estrogens interacted. However, the discovery of a second nuclear ER in ovarian granulosa cells, ER-beta (ERβ), added clarity, and perhaps more complexity, to the understanding of estrogen interactions with its receptors and the impacts on cognition (Kuiper et al., 1996). Since the discovery of the second ER, converging data suggest distinct, and complex, biological roles for each receptor subtype. For instance, although both ERs are members of the nuclear receptor superfamily (Peterson, 2000), they differ in their chromosomal localizations and ligand-binding domains (Gustafsson, 1999). As well, the pattern of ER distribution in the body and brain is complex. Although uterine tissues primarily express

ERα (Kuiper et al., 1997), both ER subtypes are found in cognitive brain regions associated with learning and memory, such as the hippocampus and basal forebrain (Shughrue et al., 1997). Some data suggest a predominate role for ERβ in cognitive enhancement following 17β-E2 treatment. For instance, animals lacking in ERβ and given 17β-E2 treatment are impaired on the Morris water maze and Y-maze tasks (Liu et al., 2008; Rissman et al., 2002). Interestingly, other findings indicate the importance of ERα in memory function. Specifically, Foster and colleagues (2008), using a lentiviral vector, restored ERα expression in adult Ovx, ERα knockout mice, finding that these mice displayed enhanced spatial reference memory Morris water maze performance compared to ERα knockout controls.

Data from studies using selective estrogen receptor modulators (SERMs) add additional complexity to the understanding of the role of ER for learning and memory. For instance, in young adult, Ovx rats, some, but not all, studies report enhanced novel object memory with acute treatment of the ERα agonist, propylpyrazole triol (PPT) as well as with the ERβ agonist, diarylpropionitrile (DPN), suggesting that both ERs contribute to object recognition memory (Frye et al., 2007; Jacome et al., 2010; Walf et al., 2006). Additionally, SERMs have imparted mixed effects for spatial reference and working memory (Hammond et al., 2009; Rhodes and Frye, 2006). Thus, given the complexity of the cognitive impact of each ER, characterizing the role of ERs in memory is an important research direction that can lead to the development of estrogenic treatments that target ERs in ways that impart beneficial effects for memory.

In addition to the importance of characterizing the role of ERs in the memory performance of young adult animals, given that estrogen-containing menopausal treatments are commonly prescribed to middle-aged women, it is clinically relevant to evaluate the cognitive impact of ER stimulation in middle-age menopause models. Indeed, ER distribution changes with increasing age (Mehra et al., 2005; Yamaguchi-Shima and Yuri, 2007) and cognitive responsiveness to estrogen stimulation seems to decline, especially on spatial reference memory tasks among aging animals (Foster et al., 2003; Gresack et al., 2007; Talboom et al., 2008). Recently, it has been hypothesized that changes in ER ratios during aging may account for these observed findings, and predicted that increasing ERα expression may ameliorate age-related cognitive decline among Ovx rats (Foster, 2012). However, the only study to date to evaluate the cognitive impacts of systemic administration of SERMs in middle-aged rats reported subtle impairments of chronic administration of PPT on the spatial working memory delayed alternation (Neese et al., 2010). Thus, adding to this body of work characterizing the roles for cognitive outcomes following ER stimulation in middle-age represents an important area of study.

Estrogens, the Immune Response, and Cognition

The immune response is characterized by the organized actions of the innate and adaptive systems for the protection against invading foreign pathogens (Abbas and Lichtman, 2001; Bird et al., 2008; Sompayrac, 1999). When a physical barrier is penetrated by a pathogen, the innate immune system, characterized by phagocytes, Natural Killer cells, and complement proteins, is an initial, rapid, generalized immune response. In addition, the secondary, adaptive immune system, characterized by the humoral B cell response and the cell-mediated T cell response, is a delayed, specific

immune response that generates a memory for the invading pathogen. Using professional antigen presenting cells, these two systems coordinate to effectively neutralize and destroy anything not identified as 'the self'.

The immune response impacts learning and memory. For instance, the Shatz laboratory has identified the expression of MHC I in neurons of the hippocampus and other regions of the non-immunologically challenged brain (Corriveau et al., 1998; Huh et al., 2000). Further, NMDA-dependent long-term potentiation is enhanced, and long-term depression is abolished, in mice missing the cell surface MHC I or its CD3 receptor component, suggesting that MHC I plays an important role not only in the immune response but also in hippocampal plasticity (Huh et al., 2000). There is evidence to suggest that other immune system components, in addition to MHC I, are associated with behavioral alterations. For instance, chronic lipopolysaccharide-induced inflammation is associated with impairments on the spatial spontaneous alternation task, an effect thought to be mediated by an increase in activated microglia in cingulate cortex, entrohinal cortex, dentate gyrus, and hippocampus (Hauss-Wegrzynial et al., 2000). Cytokines also seem to impact behavioral output. Specifically, anxiety behaviors on the open field, elevated plus maze, and forced swim task are disrupted in tumor-necrosis factor-α knockout mice (Yamada et al., 2000). As well, the cytokine interleukin-1β suppresses hippocampal long-term potentiation, impairs contextual fear conditioning, and negatively impacts spatial reference memory performance on the Morris water maze (Pugh et al., 2001). In regard to autoimmune disorders, in mice that spontaneously develop lupus, behavioral changes, such as increased anxiety-like behaviors in the open field and cognitive inflexibility via increased perseverative behavior in the Morris water maze, are

observed (Sakic et al., 1992). Further, an induced chronic complex immune disease is associated with changes on the Lashley maze, suggesting that the immune system can specifically alter learning and memory (Hoffman et al., 1998). Together, these data suggest that the immune system can impact learning and memory and does so, in part, via interactions within the hippocampus.

Interestingly, estrogens affect both the immune response (Oertelt-Prigione, 2012; Zen et al., 2010) and learning and memory performance (Bimonte-Nelson et al., 2010). ERs are present on macrophages, dendritic phagocytes, blood mononuclear cells, B cells, and T cells (Gulshan et al., 1990; Hill et al., 2011; Mao et al., 2005; Samy et al., 2000; Weusten et al., 1986). As well, fluctuations in endogenous estrogen levels appear to regulate the immune response and immune disease states, although findings among studies are conflicting (Oertelt-Prigione, 2012). For example, while some studies report menstrual cycle stage-specific changes in $CD4^+$ T cells, regulatory T cells, Natural Killer cells, and cytokine secretion, others report alterations in the opposite direction, or no change at all. During pregnancy, when circulating levels of sex hormones increase, there is a shift in Th cytokine profiles such that Th1 cell-mediated cytokines are inhibited while Th2 humoral cytokines are enhanced (Zen et al., 2010). This shift provides a potential mechanism for the observed improvement in symptoms of Th1 cell-mediated autoimmune disorders such as rheumatoid arthritis and the development or worsening of Th2 humoral autoimmune disorders during pregnancy (Zen et al., 2010). Further, treatment with exogenous estrogens also impacts the immune system. For example, among post-menopausal women, use of Premarin-containing HTs was associated with numerous beneficial immunological effects, such as more circulating B-cells and

increased mitogen-induced T-cell proliferation (Porter et al., 2001). Similarly, in aged mice, pre- treatment with 17β-E2 prior to burn injury reduced circulating interleukin-6 levels and improved survival by 28% (Kovacs et al., 2004). That estrogens profoundly impact the immune system highlights a novel and potential therapeutic target with which learning and memory outcomes during female reproductive senescence and aging can be improved.

Estrogens and Markers of Brain Health and Function

Neurotrophins

Discerning the mechanism of the cognitive effects of Premarin and its estrogenic components could have important implications for future HTs. Neurotrophins may be one mechanism of estrogen-induced neuroprotection and/or mnemonic changes. Neurotrophins are important for the survival and maintenance of neurons (Davies, 1996; Granholm, 2000). Age-related changes in neurotrophin levels, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), have been reported in animal models, and both NGF and BDNF have been associated with cognitive function, including spatial memory (Bimonte, 2008b; Bimonte et al., 2003; Granholm, 2000). For example, Bimonte et al., (2003) reported in aged 21-23 month old female rats, higher levels of NGF and BDNF in the frontal cortex were correlated with more errors committed on the water radial arm maze. Treatment with 17β-E2, the most potent naturally circulating estrogen (Kuhl, 2005), significantly impacts neurotrophin systems in Ovx rats, increasing NGF and its receptor mRNA levels in basal forebrain and

hippocampus (McMillan et al., 1996; Pan et al., 1999) and elevating NGF and BDNF protein levels in cognitive brain regions such as entorhinal and frontal cortecies (Bimonte-Nelson et al., 2004).

Gene Expression

High throughput gene expression experiments can yield insight into mechanistic and molecular processes involved in how HTs impact learning and memory. A few evaluations have been performed to determine whether 17β-E2 influences hippocampal gene expression in both young and middle-aged rats, with effects most notably recognized within domains of synaptic plasticity and signaling, transcription, growth, and neuroprotection (Aenlle and Foster, 2010; Aenlle et al., 2009; Pechenino and Frick, 2009). For example, in both young and middle-aged Ovx rats, acute 17β-E2 resulted in increased expression of genes associated with synaptic structural components, and decreased expression of genes associated with oxidative phosphorylation and mitochondrial dysfunction (Aenlle and Foster, 2010). Given the absence of a mechanistic understanding of how Premarin acts in the brain to influence memory, gene expression represents a powerful tool to discern potential transcriptional mechanisms of action of Premarin.

The Cholinergic System

The basal forebrain cholinergic system is important for learning and memory, is susceptible to age-related changes, and is impacted by ovarian hormone removal and 17β-E2 replacement (for review, see Gibbs, 2010)**.** For example, in aged female rats with working memory impairments, less choline acetyltransferase (ChAT) protein activity was found in the vertical diagonal bands (vDB) of the basal forebrain, relative to younger

counterparts (Luine and Hearns, 1990), suggesting that lower levels of ChAT activity are associated with worse memory performance during aging. 17β-E2 seems to beneficially impact the basal forebrain cholinergic system, as well as cognitive performance. In adult Ovx rats, 17β-E2 treatment increased ChAT protein activity in the horizontal diagonal bands (hDB), as well as medial septum (MS) ChAT immunoreactive (ChAT-IR) neuron counts (Gibbs, 1997; Luine, 1985). Evidence from Gibbs' laboratory suggests that the beneficial effects of 17β-E2 treatment on cognition require a functioning basal forebrain cholinergic system; for example, 17β-E2 was ineffective at improving cognition in animals with basal forebrain lesions, and enhanced memory only in non-lesion controls (Gibbs, 2002, 2007). Similar to the effects of 17β-E2, Premarin treatment in middle-aged Ovx rats increased basal forebrain ChAT-IR neuron counts in the vDB, and concomitantly aided spatial working memory and Morris water maze overnight retention (Acosta et al., 2009b). Yet, although it has been established that 17β-E2 and Premarin impact the basal forebrain cholinergic system, an effect which is likely related to cognitive enhancements (for review, see Bimonte-Nelson et al., 2010; Gibbs, 2010), whether E1 also impacts basal forebrain cholinergic neurons, as do 17β-E2 and Premarin, is not known.

Goals of this Dissertation

The collective literature presented here have led us to propose that estrogens, or specific estrogenic compounds, can have profound impacts on memory and associated neural substrates, and that several factors likely influence the realization of beneficial mnemonic effects following HT. Thus, the body of work contained in this dissertation

aims to answer several important, translational questions regarding the impact of estrogens on the brain and cognition during aging. *First,* we wish to clarify the conflicting literature on the memory effects of the most commonly prescribed menopausal HT, Premarin, and in a series of experiments, we will evaluate the impact of route of Premarin administration on spatial memory and neurobiological outcomes. The first experiment will evaluate the impacts of continuous Premarin treatment on a battery of learning and memory tasks and compare these effects to those shown previously in our laboratory using cyclic subcutaneous injections. The second experiment will evaluate the mnemonic impacts of Premarin administered orally, the route in which women take this HT. Follow-up evaluations presented here will clarify the potentially interactive effects of experimental handling and estrogen-containing treatments. *Second,* we are interested in clarifying the role of ERα during middle-age and propose to use a selective ERα modulator as a tool to elucidate this role. *Third,* we aim to determine the mnemonic impacts of the unique estrogen, E1, the prominent endogenous estrogen following the menopausal transition, an estrogen released into circulation following Premarin administration and, an increasingly common component of BHT formulations.

CHAPTER 2

TONIC PREMARIN DOSE-DEPENDENTLY ENHANCES MEMORY, AFFECTS NEUROTROPHIN PROTEIN LEVELS AND ALTERS GENE EXPRESSION IN MIDDLE-AGED RATS

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Introduction

Conjugated equine estrogen, trade name Premarin (Wyeth Pharmaceuticals, Philadelphia, PA), has been administered since 1942 and is the most widely used estrogenic component of hormone therapy (HT) in North America (Segal, 1997; Sitruk-Ware, 2002). Premarin is given unopposed to women who have undergone surgical menopause including uterus removal (Segal, 1997; Sherwin, 1998). As well, Premarin is the estrogenic component of Prempro, the most prescribed combination HT for women with a uterus (Segal, 1997; Sherwin, 1998). Clinical findings assessing cognitive effects of Premarin-containing therapies have been inconclusive. Premarin-containing therapy has been reported to improve memory in case studies (Ohkura et al., 1995), non-randomized small quasi-experimental designs (Carlson and Sherwin, 1998) and small double-blind placebo-controlled studies (Campbell and Whitehead, 1977). Also, a randomized, double-blind placebo controlled crossover trial showed that Premarin treatment altered brain activation patterns in women during memory task performance (Shaywitz et al., 1999). Yet, findings from the large placebo-controlled Women's Health Initiative Memory Study, conducted by the National Institutes of Health, showed that Premarin treatment yielded a non-significant increased incidence of probable dementia

and mild cognitive impairment in women 65 and over (Espeland et al., 2004; Shumaker et al., 2004). Further, there was an elevated probable dementia risk, and no effect on mild cognitive impairment, in women taking Premarin+medroxyprogesterone (Shumaker et al., 2003). This combination therapy also had a negative effect on verbal memory, but a trend for positive effects on figural memory, in women 65 and over that were free of probable dementia (Resnick et al., 2006). Together, the clinical studies indicate that Premarin-containing therapy can result in both beneficial and detrimental actions on cognition in women.

Cognitive effects of estrogen replacement have been evaluated in animal models. In young and middle-aged ovariectomized (Ovx) rodents, 17β-estradiol (17β-E2) enhances spatial working memory (Bimonte and Denenberg, 1999; Daniel et al., 1997; Daniel et al., 2005; Fader et al., 1999; Gibbs, 1999; Hruska and Dohanich, 2007; Luine and Rodriguez, 1994) and spatial reference memory (Bimonte-Nelson et al., 2006; El-Bakri et al., 2004; Feng et al., 2004; Frick et al., 2002; Markham et al., 2002). Like the clinical findings testing Premarin, not all animal studies testing 17β-E2 have shown positive effects (Chesler and Juraska, 2000; Fernandez and Frick, 2004; Galea et al., 2002; Galea et al., 2001; Holmes et al., 2002; Singh et al., 1994). To date, 17β-E2 has been the primary type of estrogen used to test cognitive effects of HT in the animal model. 17β-E2 is the most potent naturally-circulating estrogen, followed by estrone (E1) and estriol (E3), in order of receptor affinity (Kuhl, 2005; Sitruk-Ware, 2002). Premarin is derived from the urine of pregnant mares, and is comprised of a complex mixture of estrogen sulfates that have been conjugated by the horse's liver before excretion in urine; many of the estrogens present in Premarin are unique to horses (Bhavnani, 1998).

Premarin contains the sulfates of at least ten estrogens, is over 50% E1 sulfate, 20-25% equilin sulfate, and has only trace amounts of 17β-E2; after metabolism, the resulting biologically active circulating hormones are primarily E1 and, after E1's conversion, 17β-E2, as well as equilin (Bhavnani, 2003; Sitruk-Ware, 2002). It is hypothesized that these three metabolites are primarily responsible for the estrogenic effects of Premarin (Sitruk-Ware, 2002). It is noted that there are other estrogens and related metabolites present in Premarin that could alter efficacy of 17β-E2 effects, and may initiate effects on their own; these hormones include, but are not limited to, $\Delta^{8,9}$-dehydroestrone, dihydroequilin-17β and equilenin (Kuhl, 2005). Therefore, the animal studies done thus far testing the cognitive effects of 17β-E2 cannot be directly compared to potential effects of Premarin.

Like the cognitive enhancements seen after 17β-E2 treatment given via subcutaneous injection (Bimonte-Nelson et al., 2006; Chesler and Juraska, 2000; Luine et al., 2003), we recently showed cognitive enhancements after Premarin treatment given via cyclic, intermittent subcutaneous injections in middle-aged Ovx rats (Acosta et al., 2009b). Specifically, with this regimen Premarin improved spatial working memory delayed match to sample plus-maze performance and attenuated overnight forgetting on the spatial reference memory Morris water maze. However, cyclic intermittent versus continuous (tonic) estrogen administration may influence realization of memory benefits. With continuous estrogen treatment, ERs become downregulated, while with cyclic intermittent estrogen treatment, ER recycling and other physiological changes occur that may enhance ultimate responsiveness for many parameters, including learning and memory (Blaustein, 1993; Brown et al., 1996; Kassis and Gorski, 1981; Rosser et al.,

1993). Women, including those enrolled in the Women's Health Initiative study, typically take HT as a daily oral continuous regimen, not intermittent in nature. The cognitive effects of continuous Premarin treatment have not been evaluated in an animal model.

In vitro studies provide evidence that Premarin, or components thereof, has positive effects on the brain. Premarin enhances neuronal growth and increases neuronal survival after experimentally-induced insult in in vitro preparations, including in cognitive brain regions (Brinton et al., 2000a). While these in vitro experiments provide compelling evidence that Premarin could result in brain changes ultimately leading to enhancement in brain functions such as learning and memory, direct evaluations testing continuous Premarin's effects on cognition have not been done in an animal model.

Discerning the mechanism of the potentially cognitive enhancing effects of Premarin could have wide implications for future research and treatments for optimizing HT. Neurotrophins may be one mechanism of estrogen-induced neuroprotection or mnemonic changes. Survival and maintenance of cholinergic neurons are dependent upon neurotrophins, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF); age-related neurotrophin changes have been reported in animal models, and NGF and BDNF have been associated with cognitive function (Bimonte et al., 2003, Granholm, 2000; Hall et al., 2000; Kesslak et al., 1998). 17β-E2 treatment significantly impacts neurotrophin systems in young and aged Ovx rats, increasing neurotrophin and its receptor mRNA levels in basal forebrain, frontal cortex and hippocampus (McMillan et al., 1996; Pan et al., 1999) and elevating NGF and BDNF protein levels in cognitive brain regions (Bimonte-Nelson et al., 2004). Whether Premarin induces cognitive change,

and whether such changes are related to neurotrophin alterations, has not yet been evaluated.

From a broader perspective, gene expression experiments can yield insight into mechanistic and molecular processes involved in nootropic HTs. Evaluations have been performed to determine whether 17β-E2 influences hippocampal gene expression. While these constitute a small handful of studies, and work has only been done in young mice, this nonetheless provides evidence that estrogens can impact gene expression (Malyala et al., 2004), with effects most notably recognized within domains of synaptic plasticity in the hippocampus (Aenlle et al., 2007; Pechenino and Frick, 2009). The present experiment capitalized on similar gene expression array procedures in the hippocampus to yield insight into potential mechanisms of action of Premarin in cognitively-characterized middle-aged Ovx rats.

The present study examined three Premarin doses in middle-aged rats after surgical menopause. The doses were based on the 0.625 mg/day dose commonly prescribed to women, and used in the Women's Health Initiative studies, altered only for body weight of the rat. We tested whether Premarin altered learning and memory using a battery of tasks designed to tap several memory domains, followed by BDNF, NGF and gene expression assays. Since this is the first cognitive study to continuously administer Premarin to the rodent, we also obtained vaginal smears, uterine weights and pituitary weights to confirm endocrine responsiveness. Further, since 17β-E2 and E1 levels are increased in menopausal women after Premarin treatment (Sitruk-Ware, 2002), we measured 17β-E2 and E1 blood levels in our surgically menopausal rats. This allowed for treatment verification and determination of circulating levels that could be correlated

with maze scores to aid in interpretation of potentially effective treatment regimens. Indeed, while higher circulating 17β-E2 replacement levels have been correlated with better spatial reference memory Morris water maze performance (Talboom et al., 2008), correlations have not been evaluated for working memory. Furthermore, relationships between maze performance and circulating E1, or the ratio of E1:17β-E2, have not been assessed in the rodent, but have correlated with neuropsychological test scores in menopausal women (Lebrun et al., 2005; Phillips and Sherwin, 1992; Sherwin, 1988; Wolf and Kirschbaum, 2002).

Materials and Methods

Subjects

Subjects were 37 middle-aged, 13 month old, inbred Fischer-344 female rats born and raised at the aging colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN). Inbred rats were selected given that their low genetic and physiological variability allowed us to utilize a relatively small sample size to generalize observed cognitive impacts of our treatments to the general population (Nadon, 2004b). Rats were acclimated for several days, and were pair housed with an identical treatment assigned cage-mate. Animals had access to food and water ad libitum, and were maintained on a 12-h light/dark cycle. All procedures were approved by the local Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards.

Ovariectomy and Treatment

Fourteen days before behavioral testing, all rats were anesthetized with acute isoflurane inhalation and received Ovx. Bilateral dorsolateral incisions were made in the skin and peritoneum, and the ovaries and tips of uterine horns were ligatured and removed. The muscle was then sutured and the skin stapled. At the time of Ovx, Alzet osmotic pumps (model 2ML4, Durect Corporation, Cupertino, CA) containing either proplyene glycol (Ovx-Vehicle), or proplyene glycol plus one of three doses of Premarin (Ovx-Premarin-Low, Ovx-Premarin-Medium, Ovx-Premarin-High), were implanted dorsally into the scruff of the neck. Premarin was manufactured by Wyeth Pharmaceuticals (Philadelphia, PA) but obtained from a commercial pharmacy via veterinary prescription. The doses used in the current study were based on the daily 0.625 mg Premarin dose commonly taken by women, and used in the Women's Health Initiative Memory Study. Using the average female weight of 70 kg (www.halls.md) for calculations, resulting in 0.00893 mg drug/kg body weight woman, we determined the Premarin-Medium dose (24 μg Premarin powder, which is 10.53% Premarin) to be the rat body weight equivalent of what is clinically prescribed (Espeland et al., 2004; Shumaker et al., 2003; Shumaker et al., 2004). To approximate the injected doses we previously found to influence memory (Acosta et al., 2009b), we included 12μg (Ovx-Premarin-Low) and 36μg (Ovx-Premarin-High) doses as well. Upon completion of both surgical procedures, rats were given rimadyl for pain and 2ml of saline. Two weeks following initial pump insertion and two days before behavioral testing began, the first pump was removed and a second pump, filled with the identical substrate to that given previously, was inserted in each rat in the same manner as the first pump insertion. Behavioral testing

began 16 days after hormone administration was first initiated via the first pump insertion.

Verification of Peripheral Estrogenic Stimulation

To confirm Ovx and Premarin treatment, vaginal smears were performed at various time intervals throughout the study. Smears were classified as either proestrus, estrus, metestrus or diestrus (Goldman et al., 2007).

Water Radial Arm Maze

Subjects were tested on the water radial arm maze. This is a complex win-shift task that requires spatial working and reference memory and utilizes water-escape onto hidden platforms as the reinforcer (Bimonte and Denenberg, 1999; Bimonte et al., 2002; Hyde et al., 1998). The 8-arm maze was filled with room temperature water tinted black with nontoxic paint. Four arms had hidden platforms at their ends. Each subject had different platform locations that remained fixed throughout the experiment. A subject was released from the start arm and had 3 min to locate a platform. If an animal did not locate the platform within the allotted time limit, it was gently guided to the platform using a black plastic rod. Once a platform was found, the animal remained on it for 15 sec, and was returned to its heated cage for 30 sec until its next trial. During the inter-trial interval, the just-chosen platform was removed from the maze. The animal was placed again into the start alley and allowed to locate another platform. A daily session consisted of these events repeated until all four platforms were located. Consequently, for each animal a daily session consisted of four trials per session, with the number of platformed arms

reduced by one on each subsequent trial, and one more item of information to be remembered after every trial. Hence, the working memory system was increasingly taxed as trials progressed within a testing day.

Error quantification and blocking procedures are based upon previous studies using the water radial arm maze (Bimonte and Denenberg, 1999; Bimonte et al., 2002; Hyde et al., 1998). Each subject was given one session a day for 11 consecutive days. The first day was considered training because the animal had no previous experience in the maze. Days 2-11 were testing sessions, blocked into two phases: the **initial phase** (days 2-6), and the **latter phase** (days 7-11). Behavioral testing took place between 0800 and 1400 hour. An arm entry was counted when the tip of a rat's snout reached a mark delineated on the outside of the arm (11 cm into the arm). Errors were quantified for each daily session using the orthogonal measures of working and reference memory errors (Jarrard, 1993), as done previously in water radial arm maze studies (Bimonte et al., 2002). Working memory correct errors were first and repeat entries into any arm from which a platform had been removed during that session. Reference memory errors were first entries into any arm that never contained a platform. Working memory ncorrect errors were repeat entries into reference memory arms. During initial and latter phases, performance was measured for each type of error separately, as well as the three error subtypes combined (total errors). On day 12, a 4-hour delay was imposed between trials two and three to assess retention of multiple items of spatial information (Luine and Rodriguez, 1994). The dependent measure for performance on the delay day was total errors on trials three and four, the trials after the 4-hour delay.

Morris Water Maze

The Morris water maze evaluates spatial reference memory. This win-stay task consisted of a round tub (188 cm in diameter) filled with room temperature water made opaque with black non-toxic paint, with a hidden platform (10 cm wide) (Bimonte-Nelson et al., 2006; Morris et al., 1982). A video camera above the maze tracked the rat's path during each trial and a tracking system (Ethovision, Noldus Instruments, Leesburg, VA) analyzed each path. The rat was placed in the maze from any of four locations (North, South, East, or West) and had 60 sec to locate the platform, which remained in a fixed location (Northeast quadrant). Once the rat found the platform, the trial was terminated. If an animal did not locate the platform within the allotted time limit, it was gently guided to the platform using a black plastic rod. After 15 sec on the platform, the rat was removed from the maze and placed into its heated cage until the next trial. The rats were given five trials a day for three days. The approximate inter-trial interval was five-eight min. Swim distance (cm) was the dependent variable for the testing, non-probe trial portion of this task. To evaluate whether rats localized the platform to the spatial location, after all test trials were completed on day three, a sixth trial was given. This sixth trial was a 60 sec probe trial whereby the platform was removed. Since rats that learned the platform location were expected to spend the greatest percent distance in the target quadrant (Bimonte-Nelson et al., 2006), we analyzed probe trial data by assessing group differences in percent distance (cm) in the target (where the platform was previously located; Northeast) and opposite (quadrant diagonally opposite to where the platform was previously located; Southwest) quadrants. To further evaluate search strategy, the probe trial was divided into two 30 sec epochs. The frequency of platform

crossings, e.g. the number of times an animal swam over the previously platformed location, was quantified for each epoch. This analysis strategy was chosen to yield insight into whether animals knew the general vicinity of the platform location (via targeting the platform quadrant) and/or the exact platform location (via crossing over the platform location, quantified as platform crossings). Additionally, we assessed swim speed (distance swum/trial time) during both 30 sec epochs during the probe trial, as it could be a measure of motor ability and partially account for group differences in performance.

Delayed Match to Sample Water Maze

The water-escape delayed match to sample plus maze is a task that assesses spatial working and recent memory (Frick et al., 1995; Markowska and Savonenko, 2002b). The apparatus had four arms (each 38.1 cm long and 12.7 cm wide) and was filled with room temperature water made opaque with black non-toxic paint**.** The maze had a hidden escape platform at the end of one of the four arms. The platform location changed every day, but was fixed within a day. Rats received six consecutive trials within a daily session. The first trial was the information trial where the rat was exposed to that day's platform location, the second trial was the working memory trial (Frick et al., 1995), and trials three through six were memory test trials. Rats were dropped off in a semi-randomly chosen start arm location, and were given a maximum of 90 sec to swim to the platform. If an animal did not locate the platform within the allotted time limit, it was gently guided to the platform using a black plastic rod. Once on the platform, the rat remained on it for 15 sec, followed by placement into a heated cage for a 30 sec inter-trial interval. An arm entry was counted when the tip of a rat's snout reached a mark

delineated on the outside of the arm (11 cm into the arm). Entry into an arm with no platform counted as an error, the dependent variable. After four days of testing with a 30 sec inter-trial interval between all trials, rats were tested with a 6-hour delay (day five) between the information trial (trial one) and the working memory trial (trial two) to assess delayed memory retention. There were no additional trials after the post-delay trial (e.g. trial two). Errors were defined as entry into an arm with no platform, and were quantified for trial two. In addition, to determine whether each group was affected by this delay test, performance of each group on trial two was compared to trial two at baseline (day four was used as baseline).

Brain Tissue Collection

All rats were sacrificed the day after testing ended. Animals were anesthetized with isoflurane (Vetone, Meridian, Indiana) and decapitated according to National Institutes of Health euthanasia guidelines. Brains were rapidly dissected by the same experimenter who was blind to treatment group status. Referring to Paxinos and Watson (1998), from the left hemisphere, frontal cortex, cingulate cortex, entorhinal cortex, dorsal and ventral hippocampus (CA1/2), perirhinal cortex, and temporal cortex were dissected for neurotrophin quantification; from the right hemisphere the dorsal hippocampus (CA1/2) was dissected for gene expression profiling. Enzyme-linked immunosorbent assay procedures were carried out on the left hemisphere, and gene expression profiling was carried out on the right hemisphere, to reduce potential inter-hemispheric variability, a procedure used by our laboratory and others (Bimonte, 2008a; French et al., 2006; Gobbo and O'Mara, 2004). Brain tissues were placed in pre-weighed

microcentrifuge tubes, quickly weighed, frozen on dry ice and stored in at -70 °C until analysis.

Peripheral Tissue Collection, Uterine Weights, and Pituitary Weights

To examine the effects of Premarin on uterine and pituitary tissues, at sacrifice the uteri of subjects were removed, trimmed of visible fat and immediately weighed (wet weight). Pituitaries were collected from the base of the skull following brain removal and weighed (wet weight).

Hormone Assays

After decapitation, serum was collected and stored at -4 °C until analysis. E1 and 17β-E2 levels were determined by liquid chromatography-tandem mass spectrometry according to previously published methods (Nelson et al., 2004). After dansyl chloride derivatization, samples were separated by fast gradient chromatography and then were injected in a tandem mass spectrometer after formation of positive ions with atmospheric pressure chemical ionization. Limits of quantification for E1 and 17β-E2 were 0.2 and 0.5pg/ml, respectively, with interassay CV's of 15% or less at the concentrations obtained for these steroids.

Neurotrophin Level Quantification

BDNF and NGF levels were assessed using commercially available assay kits from Promega (Madison, WI). Neurotrophin assay procedures were done as previously described (Bimonte, 2008a; Bimonte et al., 2003; Bimonte-Nelson et al., 2004; French et

al., 2006). In brief, flat-bottom plates were coated with the corresponding capture antibody, which binds the neurotrophin of interest. The captured neurotrophin was bound by a second specific antibody, which was detected using a species-specific antibody conjugated to horseradish peroxidase as a tertiary reactant. All unbound conjugates were removed by subsequent wash steps according to the Promega protocol. After incubation with chromagenic substrate, color change was measured in an enzyme-linked immunosorbent assay plate reader at 450 nm. Using these kits, growth factors can be quantified in the range of 4.7-300pg/ml and 7.8-500pg/ml, respectively. For each assay kit, cross-reactivity with other trophic proteins is < 2-3%.

Hippocampal Gene Expression

These gene expression procedures have been used in previous studies (Basu, 2006; Long et al., 2009). For gene expression, dorsal hippocampal tissues from the Ovx-Vehicle, Ovx-Premarin-Low and Ovx-Premarin-High rats that were used for maze testing were taken. However, for the final data analysis, three subjects from the Ovx-Vehicle group, three from the Ovx-Premarin-Low group and seven from the Ovx-Premarin-High group were used. This was due to a number of samples not progressing forward to data analysis because of stringent quality control measures. These samples did not meet the minimum cRNA amplification requirements for expression profiling, or they were dropped after evaluating quality control reports following array scanning. These samples did not pass at least one of the following criteria: a maximum 3'/5' GAPDH ratio of 3.0, at least 30% present calls, and/or a maximum scaling factor of 5.0. Values falling outside of these thresholds are indicative of sample degradation and thus a lower level of sample

quality. Thus some groups suffered more attrition than others and individuals represented in the groups compared varied. Total RNA was isolated from dorsal hippocampal samples using the Qiagen (Germantown, MD) RNeasy Mini Kit. Total RNA was eluted with 20μL of RNase-free water. RNA was precipitated by the addition of 1/10 volume 3M NaOAc (pH 5.2) and 2.5 volumes absolute ethanol. After mixing and incubation at -20 °C for 1 hour, the samples were spun at ≥12,000 g for 20 min at 4 °C. The resulting pellet was washed twice with 80% ethanol and air dried. Pellets were resuspended in 9.1μL of DEPC-treated water. Isolated total RNA from each sample was amplified, cleaned, and biotin-labeled using Affymetrix's (Santa Clara, CA) GeneChip Once-Cycle Target Labeling kit with a T7 promoter as per manufacturer's protocol. Amplified and labeled cRNA was quantitated on a spectrophotometer and run on a 1% TAE gel to check for an evenly distributed range of transcript sizes. 20μg of cRNA was fragmented to approximately 35 to 200 bp by alkaline treatment (200mM Tris-acetate, pH 8.2, 500 mM KOAc, 150 mM MgOAc) and run on a 1% TAE gel to verify fragmentation. Separate hybridization cocktails are made using 15μg of fragmented cRNA from each sample as per Affymetrix's protocol. Two hundred μL of each cocktail was separately hybridized to an Affymetrix Rat Genome 230 2.0 Array for 16 hours at 45 °C in the Hybridization Oven 640. Arrays were washed on Affymetrix's upgraded GeneChip Fluidics Station 450 using a primary streptavidin phycoerythrin stain, subsequent biotinylated antibody stain, and secondary streptavidin phycoerythrin stain. Arrays were scanned on Affymetrix's GeneChip Scanner 3000 7G with AutoLoader. Scanned images obtained by the Affymetrix GeneChip Operating Software v1.2 were used to extract raw signal intensity values per probe set on the array and calculate detection calls as absent, marginal, or

present. Assignment of detection calls was based on probe-pair intensities for which one probe was a perfect match of the reference sequence and the other was a mismatch probe for which the thirteenth base (of the 25 oligonucleotide reference sequence) was changed. Signals are calculated using the One-Step Tukey's Biweight Estimate and all raw chip data was scaled in Affymetrix GeneChip Operating Software to 150 using MAS5.0 to normalize signal intensities for inter-array comparisons.

Statistical Analyses

Since our interest was to determine whether each dose enhanced performance relative to the Vehicle group, all of our two-group comparisons were planned. Uterine, serum, and brain analyses were run via independent samples t-tests set *a priori*. Because the group comparisons represented *a priori* planned contrasts, each comparison was evaluated using an alpha level of 0.05 except when noted otherwise (Keppel and Wickens, 2004 page 115). For behavior assessments, for each dependent variable described above, data were analyzed separately for each maze with a repeated measures ANOVA with Treatment as the between variable, and Days, Blocks of Days, Trials, Epoch, and/or Quadrant as the within variable, as appropriate for the specific maze test.

Using Pearson r correlations, we correlated serum E1 and 17β-E2 levels (pg/ml), and the ratio of E1:17β-E2 (E1 divided by 17β-E2), with the following measures: water radial arm maze total errors for initial and latter phases, water radial arm maze total errors on post-delay trials, Morris water maze swim distance (cm), target quadrant percent distance (cm) on the Morris water maze probe trial, delayed match to sample maze working memory trial total errors on day 1-4, and delayed match to sample maze errors

after the delay. We also correlated these serum hormone levels to each other to aid interpretation of hormone profiles.

Gene expression analysis consisted of two comparisons: (1) Ovx-Vehicle versus Ovx-Premarin-Low animals, and (2) Ovx-Vehicle versus Ovx-Premarin-High animals. Within each comparison, all samples in the comparison were first evaluated based on Affymetrix detection calls. For the first comparison, those genes demonstrating at least one present call out of a total of six (three Ovx-Vehicle animals and three Ovx-Premarin-Low animals) calls were extracted in order to remove genes that did not show measurable levels of expression across both sample groups. Similarly, for the second comparison, those genes demonstrating at least one present call out of a total of ten calls (three Ovx-Vehicle animals and seven Ovx-Premarin-High) were identified. Following this detection call filter, the average expression signals were calculated for each group in both comparisons; those genes that have both average expression signals less than 100 in a comparison are removed (e.g. if the Ovx-Vehicle average signal and the Ovx-Premarin-Low average signal for a gene are both less than 100 in the first comparison, the gene is removed) because changes at consistently low levels overlap with background. The ratio of average expression signals for each probe represents the fold change. The Student's t-test (heteroscedastic, two-tailed) was used to calculate p-values for transcriptomic changes for each gene. MetaCore GeneGo software was used for pathway analysis of significant genes. This software takes an input list of genes and evaluates the genes against an annotated database of genes/proteins and maps, which represent known relationships between genes/proteins. The software database also incorporates Gene Ontology (GO) processes under which the input list of genes can also be organized. This

tool supports the identification of processes and systems that are likely to be affected by changes in expression of specific genes. In this study, genes that demonstrate the most significant changes ($p < 0.01$) are input into the pathway analysis software to determine what mechanisms are likely affected by respective transcriptomic changes. For comparison 1, 962 genes were input into GeneGo, whereas for comparison 2, 120 genes were evaluated. The top ten processes for each comparison are listed in Chapter 3-Table II and III and represent the processes most likely affected by changes in gene expression. The numerator of the ratio represents the number of genes from the input list that are contained in the process, while the denominator represents the total number of genes in the process.

Results

Vaginal Smears, Uterine Weights, Pituitary Weights, and Serum Estrogen Levels

With the exception of one Ovx-Premarin treated rat, all Ovx-Premarin treated rats exhibited estrus-like vaginal smears that had many cornified cells. The animal was excluded from the study. All Ovx-Vehicle rats showed continuous diestrus-like smears. Ovx-Premarin-Low, Ovx-Premarin-Medium and Ovx-Premarin-High treatments increased uterine weights relative to Ovx-Vehicle animals [Ovx-Vehicle vs. Ovx-Premarin-Low: $t(16)=8.52$; $p < 0.0001$; Ovx-Vehicle vs. Ovx-Premarin-Medium: $t(15)=7.29$; $p < 0.0001$; Ovx-Vehicle vs. Ovx-Premarin-High: $t(14)=8.77$; $p < 0.0001$] (Chapter 2-Figure 1a). Premarin also dose dependently influenced pituitary weights (Chapter 2-Figure 1b). Pituitary weights in the Ovx-Premarin-High group were significantly elevated compared to the Ovx-Vehicle treated group [$t(13)=3.05$; $p < 0.01$].

Premarin treatment dose-dependently increased circulating hormone levels. All doses of Premarin increased E1 levels as compared to Vehicle [Ovx-Vehicle vs. Ovx-Premarin-Low: $t(7)=3.23$; $p < 0.05$; Ovx-Vehicle vs. Ovx-Premarin-Medium: $t(8)=6.92$; $p < 0.0001$; Ovx-Vehicle vs. Ovx-Premarin-High: $t(8)=4.62$; $p < 0.005$] (Chapter 2-Figure 1c). Since there appeared to be differences between the Premarin-dosed groups, and knowing whether these groups differed would aid interpretation of the behavior findings, we performed post-hoc comparisons comparing the three Premarin doses to each other for E1 and 17β-E2. Both medium [Ovx-Premarin-Low vs. Ovx-Premarin-Medium: $t(11)=5.66$; $p < 0.0001$] and high [Ovx-Premarin-Low vs. Ovx-Premarin-High: $t(11)=3.69$; $p < 0.0036$] Premarin doses had higher E1 levels than low dose Premarin. Only the two higher doses significantly increased circulating 17β-E2 as compared to Ovx animals [Ovx-Vehicle vs. Ovx-Premarin-Medium: $t(10)=5.54$; $p < 0.0005$; Ovx-Vehicle vs. Ovx-Premarin-High: $t(10)=3.41$; $p < 0.0001$] (Chapter 2-Figure 1d). For E1, both medium (Ovx-Premarin-Low vs. Ovx-Premarin-Medium: $t(11)=4.11$; $p < 0.0017$) and high (Ovx-Premarin-Low vs. Ovx-Premarin-High: $t(11)=2.37$; $p < 0.037$) Premarin doses elevated 17β-E2 levels significantly more than low dose Premarin. Post-hoc comparisons indicated that Ovx-Premarin-Medium and Ovx-Premarin-High groups did not differ from each other for E1 or 17β-E2 levels.

E1 and 17β-E2 levels were positively correlated when all animals were included [$r(24)=0.885$, $p < 0.0001$] (Chapter 2-Figure 1e), as well as when the correlation was run with only the three Premarin treated groups [$r(19)=0.894$, < 0.0001]. To ensure that this significant correlation was not attributable to group differences in E1 and 17β-E2 levels due to the experimental manipulations, we centered the data by subtracting each animal's

score from the mean of the treatment group to which they belonged (Enders and Tofighi, 2007). We then replaced the original serum hormone measures with the centered values in the correlation analyses. The correlation remained significant [$r(24)=0.710$, $p < 0.0001$], suggesting that the relationship between E1 and 17β-E2 was not being carried by group membership (Chapter 2-Figure 1f).

Water Radial Arm Maze

For the Initial (Chapter 2-Figure 2a) and Latter (Chapter 2-Figure 2b) phases, there were no Treatment main effects, nor were there interactions with Treatment for any dependent variable (working memory Correct, reference memory, working memory incorrect, total errors). On day 12, after all animals had been trained on the task, a four hour delay was instilled between trials two and three, placing a higher memory demand for trials three and four. The Ovx-Premarin-High group exhibited better performance than the Ovx-Vehicle group for total errors committed across the post-delay trials three and four [$t(16)=2.86$; $p < 0.05$] (Chapter 2-Figure 2c). No other Premarin treated group differed in post-delay performance compared to animals receiving Vehicle.

Morris Water Maze

Chapter 2-Figure 3a shows the mean distance scores±SEM for each treatment group across the three days of Morris water maze testing. Premarin treatment did not alter overall spatial reference memory performance; there was no main effect of Treatment, nor did Treatment interact with Days or Trials for any comparison. For the probe trial, there was a Quadrant main effect for each Ovx-Premarin-treated vs. Ovx-Vehicle

comparison, with a higher percent distance spent in the target quadrant versus the opposite quadrant [Ovx-Vehicle vs Ovx-Premarin-Low: $F(1,15)=81.79$; $p < 0.0001$; Ovx-Vehicle vs. Ovx-Premarin-Medium: $F(1,14)=180.42$; $p < 0.0001$; Ovx-Vehicle vs Ovx-Premarin-High: $F(1,16)=109.87$; $p < 0.0001$] (Chapter 2-Figure 3b), thereby indicating that all groups, regardless of hormone status, localized the platform location by the end of testing.

The frequency of platform crossings during the probe trial was dose dependently influenced by Premarin treatment. Ovx-Premarin-Low treated rats made fewer platform crossings than the Ovx-Vehicle controls [Treatment main effect: $t(15)=2.36$; $p < 0.05$], suggesting that Ovx-Premarin-Low animals were less able to localize the platform location on the probe trial. However, neither the Ovx-Premarin-Medium nor High groups differed from Ovx-Vehicle controls in number of platform crossings. To assess potential changes in platform search strategy across the 60 sec probe trial, we divided the probe trial into two 30 sec epochs and assessed the frequency of platform crossings across these epochs (Chapter 2-Figure 3c). A significant Treatment x Epoch interaction was found between the Ovx-Vehicle and Ovx-Premarin-Low groups [$F(1,15)=6.54$; $p <0 .05$]. For the first 30 sec epoch, Ovx-Premarin-Low rats made fewer platform crossings than Ovx-Vehicle rats [$t(15)=3.42$; $p < 0.01$]; there were no group differences on the second 30 sec epoch. Neither the Ovx-Premarin-Medium nor the Ovx-Premarin-High animals differed from the Ovx-Vehicle animals during the first or second 30 sec epochs. There were no significant comparisons between any Premarin-treated group and the Vehicle group for swim speed during the total 60 second probe trial, nor for the first or second 30 sec epoch analyzed separately.

Delayed Match to Sample Water Maze

Testing with a 30-second inter-trial interval: For the working memory test trial (trial two) collapsed across acquisition days, the Ovx-Premarin-Low group made more errors than the Ovx-Vehicle group [Treatment main effect: $F(1,16)=6.24$; $p < 0.05$] (Chapter 2-Figure 4a). There were no group differences between the Ovx-Vehicle group and either of the two higher Premarin dose groups for the working memory trial. For memory test trials (trials 3-6), there was a Treatment x Day interaction for the Ovx-Vehicle and Ovx-Premarin-Low comparison [$F(3,48)=4.67$, $p < 0.01$] (Chapter 2-Figure 4a), an effect due to Ovx-Premarin-Low animals committing more errors than Ovx-Vehicle animals on day three [Treatment effect for Day 3: $F(1,16)= 5.23$; $p < 0.05$], but not on days 1, 2, or 4.

Testing with a 6-hour inter-trial interval: For the 6-hour delay, Ovx-Premarin-Medium rats committed fewer errors after the delay than Ovx-Vehicle rats [$t(15)=2.41$; $p < 0.05$], and Ovx-Premarin-High rats made somewhat fewer errors than Ovx-Vehicle rats, with a marginal effect [$t(14)=1.94$; $p = 0.07$; Chapter 2-Figure 4b]. When animals receiving the two highest Premarin doses were combined, this Ovx-Premarin-Medium+High group committed significantly fewer errors on the test trial after the 6-hour delay compared to the Ovx-Vehicle group [$t(22)=3.05$; $p < 0.01$] (Chapter 2-Figure 4b). When we evaluated baseline performance (trial two of day four, the day before the delay) relative to post-delay performance (trial two of day five) to determine which groups were affected by the delay, it became clear that low-dose Premarin treatment impaired performance independent of the delay. Indeed, on this baseline day Ovx-Premarin-Low rats made more total errors than Ovx-Vehicle rats [$t(16)=2.94$; $p < 0.01$]

and performance of the Ovx-Premarin-Low group did not change with the delay (pre-delay total errors on trial 2 vs. post-delay total errors on trial 2: $p > 0.36$). In contrast, Ovx-Vehicle rats were impaired by the delay [$F(1,8)=6.00$; $p < 0.05$], while the delay did not impair performance in Ovx-Premarin-Medium and -High rats (ps > 0.59).

Correlations of Circulating Estrogens with Maze Performance

Premarin-treated animals that had higher levels of E1 [$r = -0.672$, $p < 0.001$](Chapter 2-Figure 5a), or a higher E1:17β-E2 ratio [$r = -0.483$, $p < 0.05$] (Chapter 2-Figure 5b), tended to make fewer errors on the working memory trial of delayed match to sample testing. These significant correlations were not due to mean differences between treatment groups, as correlations each held up after data were centered to obviate effects of group membership [$r = -0.470$, $p < 0.05$; $r = -0.499$, $p < 0.05$, respectively](Chapter 2-Figure 5c and 5d). There were no other significant relationships between E1, 17β-E2, and maze performance.

Neurotrophin Levels

The effect of Premarin treatment on neurotrophins was assessed in cingulate cortex, frontal cortex, entorhinal cortex, dorsal and ventral hippocampus, perirhinal cortex and temporal cortex (Chapter 2-Table I). All doses of Premarin significantly increased BDNF in the cingulate cortex compared to Vehicle [Ovx-Vehicle vs. Ovx-Premarin-Low: $t(16)=2.32$; $p < 0.05$; Ovx-Vehicle vs. Ovx-Premarin-Medium: $t(15)=2.39$; $p < 0.05$; Ovx-Vehicle vs. Ovx-Premarin-High: $t(14)=2.36$; $p < 0.05$]. Similarly, the two highest Premarin doses increased NGF in the cingulate cortex; Ovx-

Premarin-Medium and Ovx-Premarin-High groups had higher NGF protein levels in cingulate cortex as compared to the Ovx-Vehicle group [Ovx-Vehicle vs. Ovx-Premarin-Medium: $t(15)=3.80$; $p < 0.01$; Ovx-Vehicle vs Ovx-Premarin-High: $t(14)=4.00$; $p < 0.01$]. Premarin-High treatment, but no other dose, significantly decreased BDNF levels in the perirhinal cortex compared to Vehicle [$t(14)= 2.20$; $p < 0.05$]. No dose of Premarin altered perirhinal NGF levels, or BDNF or NGF levels in frontal cortex, temporal cortex, entorhinal cortex, dorsal hippocampus or ventral hippocampus.

Hippocampal Gene Expression

Expression profiling analysis of dorsal hippocampi led to identification of genes that were differentially expressed across treatment groups. In a comparison of the Ovx-Vehicle and the Ovx-Premarin-Low groups, 962 genes demonstrated statistically significant ($p < 0.01$) changes, whereas in a comparison of the Ovx-Vehicle and the Ovx-Premarin-High groups, only 120 genes showed significant ($p < 0.01$) changes. Top genes are shown in Chapter 2-Table II. For the Ovx-Premarin-Low group, primarily uncharacterized or predicted genes demonstrated the greatest changes (Chapter 2-Table II: List 1). For the Ovx-Premarin-High group, top genes ($p < 0.01$, greatest folds) include Homer1 (homer homolog 1), Pdk2 (pyruvate dehydrogenase kinase, isoenzyme 2), and Prkd2 (protein kinase D2) (Chapter 2-Table II: List 2). MetaCore GeneGo pathway analysis of significant genes also pinpointed cellular processes that may be affected by transcriptomic changes. The top 10 processes for each comparative analysis are listed in Chapter 2-Table III.

Chapter Summary and Discussion

The current study is the first to evaluate continuous Premarin treatment for memory and associated brain variables using the rodent model. Here, we demonstrate that continuous Premarin treatment affects memory, neurotrophin protein levels and gene expression in the middle-aged Ovx rat. Confirming peripheral endocrine responsiveness, all three Premarin doses resulted in positive estrus-like vaginal smears and increased uterine weights, and the highest Premarin dose increased pituitary weights. There were also dose-related increases in serum E1 and 17β-E2 levels. These levels were within low physiological range for ovary-intact young and middle-aged rodents (Lerner et al., 1990; Page and Butcher, 1982). Accordingly, E1 and 17β-E2 levels increased to the low physiological range in women after 0.625 and 1.25 mg/tablet daily oral Premarin treatment (Gruber et al., 2002; O'Connell, 1995). Premarin is largely E1 sulfate, which gets converted to E1, and then to 17β-E2. Therefore, the Premarin-induced elevations in 17β-E2 correspond with the expected sequence of steroid conversion, even though Premarin itself contains only trace amounts of 17β-E2 (Kronenberg et al., 2008). In the current study, circulating E1 levels significantly increased with low-, medium- or high-dose Premarin treatment, while 17β-E2 levels significantly increased only after the medium- and high- dose treatments. Further, the medium- and high- dose regimens resulted in E1 and 17β-E2 levels that were significantly higher than the low-dose regimen. This suggests that the ratio of these two estrogens varies with Premarin dose, and provides a dissociation of hormone profiles of the subjects in this study. This affords us the opportunity to evaluate whether the ratio of E1:17β-E2 correlates with the assessed cognitive and brain variables.

The dose-dependent cognitive effects of Premarin in this study are likely related to the resulting circulating hormone levels. Relative to Vehicle, the lowest dose tested, 12μg daily, impaired spatial learning on two maze tasks, even though the tasks evaluated different types of spatial memory. Specifically, low-dose Premarin treatment impaired learning the Morris water maze (spatial reference memory) and the delayed match to sample plus maze (spatial working memory), but had no effect on the water radial arm maze (spatial working and reference memory). These effects are especially noteworthy given that the low-dose Premarin regimen was the only treatment that did not elevate circulating 17β-E2 levels relative to Ovx-Vehicle animals, while it did increase E1 (Figure 1). These E1 levels were very low physiological (Lerner et al., 1990; Page and Butcher, 1982); significantly lower than those resulting from the medium- or high- doses of Premarin given in the current study. It is therefore plausible that these low circulating E1 levels, in the presence of very low 17β-E2 levels (comparable to that of Ovx animals), impairs performance on tasks that assess only reference memory place learning, or only working memory place learning, but not a task that is more complex such as the water radial arm maze. This more difficult water radial arm maze task likely challenged the Ovx group more so than the tasks solely testing reference or working memory, resulting in less discrimination compared to the low-dose Premarin effects. The correlation we found between performance on the delayed match to sample working memory trial and E1, and with the E1:17β-E2 ratio, further supports this tenet. Indeed, as levels of E1 increased, and the ratio of E1:17β-E2 increased, animals tended to show better working memory performance on this measure. Taken together with the dissociation of dose-specific estrogenic profiles, results suggest that higher levels of E1, in the presence of

17β-E2 concentrations higher than that of Ovx levels, are beneficial for memory. To our knowledge there is no animal study evaluating the cognitive effects of circulating E1 levels, neither endogenous nor exogenous after hormone treatment, although some work has been done in humans in this regard. Higher circulating E1 and 17β-E2 levels both correlated with better verbal recall scores in oopherectomized women given estrogen-containing HT (Phillips and Sherwin, 1992), and several cognitive measures improved after estrogen or estrogen-androgen therapy in oopherectomized women, concordant with increases in circulating E1 and 17β-E2 levels (Sherwin, 1988). Other studies have correlated E1 and 17β-E2 with cognitive measures in menopausal women that have not been given estrogen therapy. Findings range from higher E1 or 17β-E2 levels corresponding to better cognitive scores or a lower frequency of mild cognitive impairment (Lebrun et al., 2005; Wolf and Kirschbaum, 2002) to no correlation (Almeida et al., 2005) to higher 17β-E2 levels corresponding to worse cognitive scores (Barrett-Connor and Goodman-Gruen, 1999). Interestingly, in the latter study, higher endogenous E1 levels were marginally related to better performance on a verbal memory test in menopausal women not on HT ($p=.07$, Barrett-Connor and Goodman-Gruen, 1999, p. 1291). While these studies provide support that circulating E1 and 17β-E2 levels relate to cognition, there has been no methodical assessment of whether the balance *between* E1 and 17β-E2 impacts the direction or efficacy of E1's cognitive effects. 17β-E2's presence should be presumed when referring to E1, and vice versa, due to interconversion of these two estrogens by oxidoreductase 17β-hydroxysteroid dehydrogenase (Khan et al., 2004). Thus, it could be argued one can truly not dissociate them. However, this does not

preclude estrogenic effects on brain functions due to: (1) total steroid level and/or (2) the balance of E1 and 17β-E2.

In vitro, Premarin induced neuroprotection against β-amyloid, hydrogen peroxide and glutamate-induced toxicity in neurons derived from cognitive brain regions including the hippocampus, basal forebrain and cortex; in several cases Premarin was effective at multiple doses (Brinton et al., 2000a; Brinton et al., 2000b). Other in vitro work showed that high nanomolar to micromolar E1 concentrations exerted dose-dependent neuroprotective effects on cultured neurons (Bae et al., 2000; Brinton et al., 1997; Regan and Guo, 1997; Zhao and Brinton, 2006), although other components of Premarin were more effective than E1 (Brinton et al., 1997). We do not know the physiological concentrations of E1 or the E1:17β-E2 ratio in the brains of our animals, or how the effects may be distributed across various brain networks mediating our effects. However, collectively, the findings suggest that E1 can exert neurotrophic properties and neuroprotection (Prokai and Simpkins, 2007), which could translate to enhanced brain function, at least in the presence of 17β-E2 concentrations that are higher than Ovx levels.

Using 17β-E2 treatment, dose dependent mnemonic effects are shown in rodent studies. High physiological 17β-E2 levels enhanced learning a place strategy on a plus maze in young rats (Korol and Kolo, 2002) and on the Morris water maze in young and middle-aged rats (Talboom et al., 2008). The water radial arm maze, Morris water maze, and delayed match to sample tests used in the current study were spatial tasks. While no Premarin dose used in this study enhanced learning of these tasks, it is possible that a higher Premarin dose would have resulted in higher 17β-E2 levels that could have

improved spatial task acquisition. Indeed, the 17β-E2 levels resulting from even our highest Premarin dose treatment were low physiological. The spatial learning enhancements noted previously were seen with 17β-E2 levels in the higher physiological range (Korol and Kolo, 2002; Talboom et al., 2008). In the current study, the two highest Premarin doses tested, 24- and 36μg daily, enhanced memory retention when subjected to an extended temporal challenge. Specifically, high-dose Premarin treatment improved retention of numerous items of information across a 4-hour delay on the water radial arm maze, and the two highest Premarin doses enhanced 6-hour retention of one item of information on the delayed match to sample task. Both of these tasks require working- or short-term memory. There was no effect of an overnight delay on the spatial reference memory Morris water maze, suggesting that continuous Premarin does not influence overnight forgetting. These findings suggest that the memory enhancing effects of Premarin are task specific and moreover, require a mnemonic challenge across hours to be manifested. Our findings that the two highest doses of Premarin improved memory retention correspond with other studies showing that a higher 17β-E2 dose may be necessary to enhance memory, especially in rats approaching old age. Specifically, we have shown that higher circulating 17β-E2 replacement levels correlate with better spatial reference memory in young and middle-aged Ovx rodents (Talboom et al., 2008). The necessity of a higher dose during aging may be especially poignant for memory retention (Foster et al., 2003). While findings are not yet reconciled, studies report that higher levels of 17β-E2 given via daily injection (Holmes et al., 2002), or an intermediate, but not high, 17β-E2 dose given via drinking water (Fernandez and Frick, 2004), impairs

spatial maze performance. Also of note, while it is hypothesized that 17β-E2 and E1 are two Premarin components largely responsible for the estrogenic effects of Premarin (Sitruk-Ware, 2002), there are other estrogens and metabolites present in Premarin that could alter efficacy of E1 effects and/or initiate effects on their own. Thus, although we found correlations between memory performance and E1, and the E1:17β-E2 ratio, cognitive effects due to Premarin treatment could also be related to metabolites of Premarin such as $\Delta^{8,9}$-dehydroestrone, dihydroequilin-17β or equilin (Kuhl, 2005).

Many factors other than dose and the resulting ratios of estrogens in circulation likely also play a role in estrogenic effectiveness on memory and the brain. The amount of time between hormone loss and subsequent treatment likely impacts efficacy of estrogenic therapy. Women who participated in the Women's Health Initiative Memory Study were between 65-79 years old, and many had experienced ovarian hormone deprivation for a substantial amount of time before receiving Premarin-containing treatment (Shumaker et al., 1998). In the rodent, 17β-E2 replacement initiated immediately after Ovx enhanced spatial memory performance in middle-aged rats, but imparted no benefit when given 5 months after Ovx (Daniel et al., 2006). Age-related changes in responsiveness may also influence the effectiveness of estrogen treatment. Aged Ovx rats were not responsive to the 17β-E2 replacement regimen that was effective in young and middle-aged Ovx rats (Talboom et al., 2008), concurring with Age x 17β-E2 replacement interactions for spatial memory shown by Foster and colleagues (2003). The current study controlled for these factors since time after Ovx and age were constant for all groups. However, whether Premarin-induced memory enhancements would have completely reversed any observed age-related memory retention decrements cannot be

determined from the current experiment, as young animals were not assessed for comparison. Future studies incorporating this comparison group would be helpful in determining extent of Premarin-induced improvements during aging.

Interestingly, we have previously shown in middle-aged Ovx rats that 10μg of Premarin, given via two injections 24 hours apart, followed by 48 hours without injection, enhanced learning of the delayed match to sample task used in the current study (Acosta et al., 2009b). In contrast, the 12μg continuous Premarin dose used herein *impaired* performance on this same measure. Intermittent cyclic vs. continuous regimens are a plausible explanation for the difference in findings. Differences in ER expression, with cyclic estrogen treatment facilitating ER recycling, and continuous estrogen treatment down-regulating ERs, indicate divergent neural mechanisms of action for cyclic and continuous administration that likely impact learning and memory changes (Blaustein, 1993; Brown et al., 1996; Kassis and Gorski, 1981; Rosser et al., 1993). There are age-related alterations in the number and activity of ERs, which could influence responsivity as aging ensues (Chakraborty and Gore, 2004). The animals in our current and prior (Acosta et al., in 2009b) Premarin studies were middle-aged. Data suggest an ER-dependent mechanism of 17β-E2-induced benefits on spatial memory (Zurkovsky et al., 2006). Thus, changes in ERs with age and with type of estrogen regimen could influence responsiveness to estrogen for spatial memory. Further, for 17β-E2, continuous treatment only enhanced memory when cyclic treatment was initiated first in older Ovx rats (Markowska and Savonenko, 2002).

In addition to Premarin-induced dose-dependent effects on cognition, we found dose-dependent effects on neurotrophin protein levels in the cingulate and perirhinal

cortices. In the cingulate cortex, all Premarin doses increased BDNF, while only the two highest doses increased NGF. In the perirhinal cortex, only the highest Premarin dose affected neurotrophin levels, decreasing BDNF. BDNF and NGF proteins are implicated in learning and memory (Backman et al., 1996; Fischer, 1987; Frick, 1997; Mizuno, 2000; Scali, 1994). Neurotrophins may play a role in estrogenic-induced memory changes, as indicated by the current study using Premarin and prior studies using 17β-E2. 17β-E2 replacement increased BDNF and NGF proteins in the entorhinal cortex in aged Ovx rats (Bimonte-Nelson et al., 2004) and increased levels of TrkA, the high-affinity neurotrophin receptor, in the basal forebrain (McMillan et al., 1996; Singer et al., 1998). The neurotrophin findings in the current study also implicate the cingulate gyrus and perirhinal cortex as potential sites of action for Premarin treatment. These brain regions play critical roles for cognition in rodents, including for spatial and object memory (Bachevalier and Nemanic, 2008; Cain, 2006; Lee et al., 2006; Lukoyanov, 2005; Ramos, 2008)**,** and in humans as shown for spatial tasks (Kinderman, 2004; Moffat, 2006) and for degenerative changes with Alzheimer's disease (Hirono, 1998; Liang, 2008; Reiman, 2004). It is currently unknown how or whether Premarin-induced growth factor changes are related to the altered memory functions seen after treatment. Hypotheses set forth include compensatory relational changes in the hippocampal/basal forebrain retrograde transport system, which could account for upregulation in some brain regions, but downregulation in others (Granholm, 2000).

In the current report, gene expression profiling of dorsal hippocampus identified mechanisms possibly involved with Premarin-induced memory changes. The dorsal hippocampus was chosen as the region of analysis since it has well-known links with

learning and memory, especially regarding spatial navigation (Jarrard, 1993; Morris et al., 1982). While implications of gene expression changes identified in the current study have yet to be determined, the genes listed in Table II represent molecular clues about the processes relating to Premarin-effects on the rat hippocampus. Of these, it is noted that high-dose Premarin treatment increased Homer1 expression. Homer1, which binds metabotropic glutamate receptors (Brakeman et al., 1997), is particularly interesting due to its previously implicated role in memory functions (Jaubert et al., 2007; Lominac, 2005; Szumlinski et al., 2005). Since this study is the first to assess Premarin effects on gene expression in the brain after cognitive testing, it is recognized that further studies are necessary to distinguish those transcripts that may be altered by Premarin treatment alone, versus those transcripts that are regulated by a physiological cascade of the improved memory due to Premarin treatment.

In conclusion, this is the first study testing continuous Premarin, the estrogen component of the most commonly utilized HT given to women since 1942, on a mempry test battery in an animal model. We found that Premarin can affect memory, with divergent effects depending on dose. In middle-aged Ovx rats, Premarin enhanced memory retention on two tasks at higher doses. Low-dose Premarin impaired some aspects of performance, specific to spatial platform localization and learning a working memory task, but had no effect on memory retention. Premarin-induced cognitive changes may relate to the ratio of E1 to 17β-E2, with higher levels associated with better performance, although it is recognized that other components of Premarin could account for Premarin-induced memory changes. Gene expression profiling identified Premarin-associated transcriptomic changes, which likely includes Homer1, and provides a

foundation for delineating the molecular processes affected by Premarin. These findings suggest that Premarin can impact memory and the brain, and that dosing should be recognized as a clinically relevant factor possibly affecting the direction and efficacy of cognitive outcome.

CHAPTER 3

ORALLY ADMINISTERED PREMARIN DOSE-DEPENDENTLY IMPAIRS PERFORMANCE ON AN 8-ARM, SPATIAL WORKING MEMORY TASK IN MIDDLE-AGED FEMALE RATS: RELATIONS WITH EXPERIMENTAL HANDLING ASSOCIATED WITH TREATMENT ADMINISTRATION

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Introduction

Premarin, administered in the form of a daily oral tablet, is the most commonly prescribed estrogen-based form of menopausal hormone therapy (HT; Hersh et al., 2005). Yet, among peri- and post-menopausal women, Premarin's mnemonic impact is still unclear, with some studies reporting benefits and others reporting null or even detrimental impacts on memory performance (Bimonte-Nelson et al., 2010; Hogervorst et al., 2000; Sherwin and Henry, 2008). Interestingly, studies thus far testing the cognitive effects of Premarin in rodents, in which treatments were administered via a subcutaneous route, have generally revealed favorable results (Acosta et al., 2009b, Engler-Chiurazzi et al., 2011; Walf and Frye, 2008). Together, these findings suggest that cognitive benefits can be realized with Premarin treatment and that isolating the factors that facilitate its beneficial effects on memory will be crucial in optimizing this HT for cognitive outcomes.

Route of treatment administration could be one factor that impacts the realization of cognitive benefits with Premarin treatment. Indeed, important metabolic differences exist between the various options for administering menopausal HTs (Sherwin and

Henry, 2008). For instance, estrogens administered orally are first metabolized in the gut and liver before being released into circulation (Kuhl, 2005). This results in a rapid rise in circulating estrogens and a high ratio of blood estrone (E1) compared to 17β-estradiol (17β-E2; Kuhl, 2005). As such, compared to non-oral routes, higher doses of orally administered estrogen-containing treatments are needed to impart equivalent actions in the body. Conversely, estrogen treatments administered via the transdermal or subcutaneous route are released at a slow rate in which the ratio of circulating estrogens is closer to 1:1 and does not fluctuate greatly across time (Gleason et al., 2005; Kuhl, 2005). Thus, characterizing the effects of orally administered represents an important area of research yet, while the impact of subcutaneous Premarin has been studied by our lab (Acosta et al., 2009b, Engler-Chiurazzi et al., 2011) and others (Walf and Frye, 2008), there has been no study testing the effects of orally administered Premarin on cognitive outcomes.

Here, we conducted a broad dose-response study to evaluate the mnemonic impact of orally administered Premarin. As subcutaneously administered Premarin (Acosta et al., 2009b, Engler-Chiurazzi et al., 2011) and orally administered estrogens (Fernandez and Frick, 2004) have been shown to impact cognition and the brain in middle-aged, ovariectomized (Ovx) rodents, we hypothesized that orally administered Premarin would also impact cognition and neurotrophins in this population. Given that Premarin, prescribed in form of an oral tablet, impairs cognition in peri- and post-menopausal women, we predicted that the oral route of Premarin administration to middle-aged rats would impair performance on our battery of memory tasks.

Study 1

Materials and Methods

Subjects

Subjects were 40 inbred Fischer-344 female rats born (14 month old) and raised at the aging colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN). Inbred rats were selected given that their low genetic and physiological variability allowed us to utilize a relatively small sample size to generalize observed cognitive impacts of our treatments to the general population (Nadon, 2004b). Animals were acclimated for several weeks at the Arizona State University animal facility, were pair housed with an identically treated cage-mate, had exposure to food and water ad-libitum, and were maintained on a 12-h light/dark cycle. All procedures were approved by the local Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards.

Ovariectomy and Treatment

The experimental timeline is presented in Chapter 3-Figure 1a. To remove ovarian-secreted hormones, all rats were anesthetized with acute isoflurane (Vetone, Meridian, Indiana) inhalation and rats received Ovx. Bilateral dorsolateral incisions were made in the skin and peritoneum, and the ovaries and tips of uterine horns were ligatured and removed. The muscle was then sutured and the skin stapled. Seventeen±1 days after surgery, rats began treatment administration. Based on a pilot study to determine the optimal method to give Premarin orally with limited handling, we determined that hand feeding via a needless syringe was most effective to ensure the entire product was ingested. Rats received either distilled water (Ovx-Vehicle) or one of the following doses

of Premarin dissolved in distilled water: 30μg (Ovx-Oral-30), 90μg (Ovx-Oral-90) or 180μg (Ovx-Oral-180). The results of our prior findings showing memory enhancements after 30μg/day subcutaneous injections (Acosta et al., 2009b), and after 36μg/day via subcutaneous Alzet osmotic pumps (Engler-Chiurazzi et al., 2011), led us to use the 90μg dose as the middle oral dose. The oral Premarin doses were estimated based on the reduced bioavailability of estrogens when given orally (Kuhl, 2005). The 30μg/day oral dose, approximating a subcutaneous dose of 10μg/day, is one third of the medium dose, and the 180μg/day dose, approximating a subcutaneous dose of 60μg/day, is two times the middle dose. Fourteen±1 days after Ovx (three days before oral drug administration began), animals were introduced to the oral handling procedure daily for three days. Handling consisted of enwrapping each rat up to the shoulders in a clean rag, lifting the rat in the air, and inserting a syringe (without needle attached) into the mouth. Each syringe contained 0.1 ml of the appropriate substrate followed by a 0.1 ml distilled water chaser, and the entire syringe tip was dipped in sweetened condensed milk before placement in the mouth. Rats were given daily oral administration of their assigned substrate until the day of sacrifice. Behavioral testing began 40±1 days following Ovx, and 24±1 days after hormone administration was initiated.

Verification of Peripheral Estrogenic Stimulation

Since few studies have assessed orally administered Premarin in the rat, we verified peripheral estrogenic stimulation via evaluation of traditional markers including vaginal smears (Goldman et al., 2007), uterine weights (Westerlind, 1998), and pituitary weights (Spady, 1999). Beginning approximately five days after Ovx surgeries, smears

were taken daily for five days to confirm lack of uterine stimulation. Next, to establish a temporal profile of estrogenic action for orally administered Premarin in the middle-aged rat, vaginal smears were taken daily for 13 days, beginning three days following the first oral Premarin treatment. Smears were classified as either proestrus, estrus, metestrus or diestrus (Goldman et al., 2007). Body weights were collected once per week and continued for the duration of the experiment, beginning 21 days after Ovx.

Delayed Match to Sample Water Maze

Forty±1 days after Ovx, rats were trained on the water-escape delayed match to sample maze, a task that assesses spatial working and recent memory (Frick et al., 1995; Markowska and Savonenko, 2002). The apparatus was a radial arm water maze with eight arms (each 38.1 cm long and 12.7 cm wide), filled with room temperature water made opaque with black non-toxic paint**.** The maze had a hidden escape platform at the end of one of the eight arms. The platform location changed every day, but was fixed within a day. Rats received six consecutive trials within a daily session. The first trial was the information trial where the rat was exposed to that day's platform location, the second trial was the working memory trial, and trials three through six were recent memory trials (Acosta 2009b; Engler-Chiurazzi et al 2011)**.** Each rat was dropped off in a semi-randomly chosen start arm location, and was given a maximum of 90 sec to swim to the platform. If an animal did not locate the platform within the allotted time limit, it was gently guided to the platform using a black plastic rod. Once on the platform, the rat remained on it for 15 sec, followed by placement into a heated cage for a 30 sec inter-trial interval. An arm entry was counted when the tip of a rat's snout reached a mark

delineated on the outside of the arm (11 cm into the arm). Entry into an arm with no platform counted as an error, the dependent variable. After eight days of baseline testing with a 30 sec inter-trial interval between all trials, rats were tested using a 6-hour delay (day 9) and 8-hour delay (day 10) to assess delayed memory retention. Since the second trial is the initial trial to test recall of the updated information, the delays were given between trial one and two to determine whether the increased inter-trial interval impacted, specifically, memory retention of one item of information. For the delayed retention trials, there were no additional trials after the test trial (e.g. post-delay trial two).

Morris Water Maze

Next, rats were trained on the Morris water maze, a task that evaluates spatial reference memory. This win-stay task consisted of a round tub (188 cm in diameter) filled with room temperature water, made opaque with black non-toxic paint, and with a hidden platform (10 cm wide) submerged just below the surface. The rat was placed in the maze from any of four locations (North, South, East, or West) and had 60 sec to locate the platform, which remained in a fixed location (Northeast quadrant). If an animal did not locate the platform within the allotted time limit, it was gently guided to the platform using a black plastic rod. After 15 sec on the platform, the rat was placed into its heated cage until the next trial. Rats were given five trials a day for three days. Animals were tested in squads (eight-nine rats in each squad) so that trial one was completed for each rat in the group, then trial two, etc., as done previously (Hyde et al., 2002; Schrott et al., 1992; Stavnezer et al., 2002). A video camera and tracking system (Ethovision, Noldus Instruments) tracked and analyzed each rat's path, with swim distance (cm) as the

dependent variable. To evaluate whether rats localized the platform to the spatial location, after the test trials, a 60 sec probe trial was given whereby the platform was removed. Since rats that learned the platform location were expected to spend the greatest percent distance in the target quadrant (Bimonte-Nelson et al., 2006), the dependent variable for the probe trial data was percent distance moved in the previously platformed quadrant.

Black/White Discrimination

Because many clinical studies have shown benefits of Premarin on measures of non-spatial, verbal memory such as the California Verbal Learning Test (Resnick et al., 1998), the memory phase from the Blessed Information Memory Concentration test and the Buschke's free and cued selective reminding test (Verghese, 2000), and word list recall (Kimura, 1995), rats were tested on a non-spatial black/white discrimination task to assess a non-spatial dimension of memory function. This win-stay, non-spatial, reference memory task is based on previously published protocols (Denenberg et al., 1992). The black Plexiglas maze (each arm was 38.1cm x 12.7cm) was filled with water made opaque with black non-toxic paint, and had a hidden platform at the end of one of the three arms. All spatial (extramaze) cues were blocked by curtains. For each rat, the hidden platform was paired with a black or white colored insert (counterbalanced across animals) in the arm of the maze. For any given rat, the platform and color stimulus remained paired throughout testing. The spatial location of the color stimulus was alternated across trials so that attention to any unblocked spatial cues would not support an effective maze solving strategy. Thus, the location of both the platform and paired

colored insert within the maze varied semi-randomly across trials. The start location was always in the arm with a gray colored insert, the spatial location of which was fixed across all days and trials. For a given trial, the rat was placed in the start arm and had 90 sec to locate the platform. If an animal did not locate the platform within the allotted time limit, it was gently guided to the platform using a black plastic rod. After 15 sec on the platform, the rat was placed into its heated cage until the next trial. Rats were given eight trials a day for 12 days. Animals were tested in squads (nine-ten rats in each squad) so that trial one was completed for each rat in the group, then trial two, etc., resulting in an approximate inter-trial interval of eight min, as done on other mazes (Schrott et al., 1992; Hyde et al., 2002; Stavnezer et al., 2002). An arm entry was counted when the tip of a rat's snout reached a mark delineated on the outside of the arm (11 cm into the arm). Entry into an arm with no platform counted as an error, which was the dependent measure. Errors were classified as first (initial entry into the unplatformed arm), repeat (repeated entries into the unplatformed arm), total (first and repeat errors combined) and start (first and repeat entries into the start arm) errors. In addition, each day, the number of correct first choices (where the rat entered the platformed arm) was quantified. After the final test trial, a 90 sec probe trial was given whereby the platform was removed from the maze. Arm entries, either into the previously platformed arm or into the previously unplatformed arm, was the dependent measure.

Brain Tissue Collection

All rats were sacrificed the day after maze testing ended. Animals were anesthetized with isoflurane and decapitated according to National Institutes of Health

euthanasia guidelines. Brains were rapidly dissected by an experimenter who was blind to treatment group status. Referring to Paxinos and Watson (1998), from the left hemisphere, anterior and posterior cingulate cortex, frontal cortex, dorsal hippocampus, entorhinal cortex, perirhinal cortex, and pituitary were dissected. For the anterior and posterior cingulate cortex, a strip of the dorsal cortex along the medial longitudinal fissure was removed and divided into two halves, with the anterior half comprising the anterior cingulate and the posterior half comprising the posterior cingulate; for the frontal cortex, the most medial 1.5 to 2 mm portion of the frontal cortex was dissected; for the dorsal hippocampus, the dentate gyrus and the alveus were excluded; for the entorhinal cortex, a 2-3 mm sample ventral to the hippocampus was taken; for the perirhinal cortex, a 1-1.5 mm sample surrounding the rhinal fissure was collected. Tissues were placed in pre-weighed microcentrifuge tubes, quickly weighed, frozen on dry ice, and stored in at -70 °C until analysis.

Peripheral Tissue Collection, Uterine Weights, and Pituitary Weights

At sacrifice, blood was taken via cardiocentesis. Uterine tissues were collected, trimmed of fat and connective tissue, and weighed as per previous methods (Acosta et al., 2009b). Following the removal of the brain from the skull cavity, pituitary was extracted from the base of the skull and placed into a pre-weighed microcentrifuge tube.

Hormone Assays

Serum levels of E1 and 17β-E2 were determined by liquid chromatography-Tandem Mass Spectrometry according to previously published methods (Nelson et al.,

2004). After dansyl chloride derivatization, samples were separated by fast gradient chromatography and then were injected in a tandem mass spectrometer after formation of positive ions with atmospheric pressure chemical ionization. Limits of quantification for E1 and 17β-E2 were 0.2 and 0.5 pg/ml, respectively, with interassay CV's of 15% or less at the concentrations obtained for these steroids.

Neurotrophin Level Quantification

Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) levels were assessed using commercially available assay kits from Promega (Madison, WI). Neurotrophin assay procedures were done as previously described (Bimonte et al., 2003; Bimonte-Nelson et al., 2004; Bimonte-Nelson et al., 2008; French, et al., 2006). In brief, flat-bottom plates were coated with the corresponding capture antibody, which binds the neurotrophin of interest. The captured neurotrophin was bound by a second specific antibody, which was detected using a species-specific antibody conjugated to horseradish peroxidase as a tertiary reactant. All unbound conjugates were removed by subsequent wash steps according to the Promega protocol. After incubation with a chromagenic substrate, color change was measured in an enzyme-linked immunosorbent assay plate reader at 450nm. Using these kits, BDNF and NGF can be quantified in the range of 4.7-300 pg/ml and 7.8-500 pg/ml, respectively. For each assay kit, cross-reactivity with other trophic proteins is < 2-3%.

Statistical Analyses

Pituitary weight, uterine weight, serum estrogen, and growth factor analyses were run via planned independent samples t-tests set a priori, comparing Vehicle to each Premarin treatment group. Since our interest was to determine whether each oral dose impacted these measures relative to the Vehicle group, all of our two-group comparisons were planned. Because the group comparisons represented a priori planned contrasts, each comparison was evaluated using an alpha level of .05, unless otherwise noted (Keppel and Wickens, 2004). For behavior assessments, data were analyzed separately for each maze with a one-way, repeated measures ANOVA with Treatment as the between variable and Blocks of Days, Days, Trials and/or Quadrant as the within variable, as appropriate for the specific maze test. This was done to allow interpretation of repeated measures effects in the context of potentially complex Treatment interactions.

Results

Vaginal Smears, Uterine Weights, Pituitary Weights, and Serum Estrogen Levels

For vaginal smears following Ovx but before treatment administration, all animals consistently exhibited diestrus-like vaginal smears composed of very few leukocytes and scattered cornified cells. After treatment administration began and just prior to behavioral testing initiation, all but one rat given Vehicle treatment exhibited continuous diestrus-like smears. This anomalous animal was excluded from the study. For Ovx-Oral-30 rats, approximately one third of rats within this group exhibited consistent smears that indicated positive vaginal stimulation. Among these rats, these estrus/metestrus-like smears, composed of numerous cornified and leukocytic cells with occasional epithelia

cells, were of stable profile (not cycling in cell type) across days. Approximately half of the Ovx-Oral-90 rats exhibited smears that indicated positive vaginal stimulation. Smears among these rats were cyclical, alternating between estrus/metestrus-like, and diestrus-like, smears. Ovx-Oral-180 rats exhibited primarily estrus/metestrus-like smears, composed of many cornified and leukocytic cells, again of stable profile.

Ovx-Oral-90 and Ovx-Oral-180 treatments increased uterine wet weights relative to Vehicle treatment [Vehicle vs. Ovx-Oral-90: $t(14)=3.22$; $p < 0.01$; Vehicle vs. Ovx-Oral-180: $t(13)=3.94$; $p < 0.005$] (Table 1). Premarin also dose dependently influenced pituitary weights (Table 1). Ovx-Oral-90 and Ovx-Oral-180 treatments increased pituitary weights relative to Vehicle treatment [Vehicle vs. Ovx-Oral-90: $t(15)=3.04$; $p < 0.01$; Vehicle vs. Ovx-Oral-180: $t(14)=2.28$; $p < 0.05$].

Premarin treatment dose-dependently increased circulating hormone levels. All oral Premarin doses increased E1 levels as compared to the Vehicle treatment [Vehicle vs. Ovx-Oral-30: $t(14)=3.93$; $p < 0.005$; Vehicle vs. Ovx-Oral-90: $t(15)=4.54$; $p < 0.0005$; Vehicle vs. Ovx-Oral-180: $t(14)=6.86$; $p < 0.0001$]. Chapter 3-Figure 2a shows mean±SEM serum levels of E1 for each treatment group. It is noteworthy that circulating E1 levels following 90μg oral Premarin administration were similar to those following 36μg of continuous subcutaneous Premarin administration (Engler-Chiurazzi et al., 2011).

All oral Premarin doses also increased circulating 17β-E2 levels as compared to the Vehicle treatment [Vehicle vs. Ovx-Oral-30: $t(14)=2.53$; $p < 0.05$; Vehicle vs. Ovx-Oral-90: $t(15)=3.52$; $p < 0.005$; Vehicle vs. Ovx-Oral-180: $t(14)=7.68$; $p < 0.0001$] (Chapter 3-Figure 2b). Again, we found similarities with our prior work; circulating 17β-

E2 levels following 90μg oral Premarin administration were similar to those following 36μg of continuous subcutaneous Premarin administration (Engler-Chiurazzi et al., 2011).

Delayed Match to Sample Water Maze

Testing with a 30-second inter-trial interval: Orally-administered Premarin dose-dependently impaired spatial working memory. We blocked testing days into two four-day blocks, and assessed Treatment x Block x Trial interactions for trials two through six. Any interactions with Days are not meaningful for this measure because an effect of Days in the context of multiple blocks of testing days only corresponds to unique days within a testing block; therefore, these were not reported.

There were no significant Treatment x Block interactions for trials two through six. Because the working memory trial (trial two) assesses a unique memory domain (Frick et al.,1995; Markowska and Savonenko, 2002) and because we have shown impacts of Premarin on the working memory trial in past studies (Acosta et al., 2009b; Engler-Chiurazzi et al., 2011), we assessed performance on the working memory trial and recent memory trials (trials three-six) separately. For the working memory trial, there was a Treatment x Block interaction [$F(3,31)=3.31$; $p < 0.05$]. To further assess this interaction, we analyzed performance during each testing block separately. There was a Treatment main effect for the first block [$F(3,31)=4.83$; $p < 0.01$]. To determine which Premarin dose impacted performance during the first testing block, we compared each dose to the Vehicle group. There was a Treatment main effect for the Vehicle vs. Ovx-

Oral-180 comparison [$F(1,14)=5.84$; $p < 0.05$], such that the Ovx-Oral-180 treatment increased total errors (Chapter 3-Figure 3a).

Testing with a 6-hour inter-trial interval: Orally administered Premarin did not impact delayed memory retention. To determine whether each group was affected by each delay, performance within each group on the working memory trial of each delay day was compared to the working memory trial on the final baseline day (day eight) via a repeated measures ANOVA with Day (test trial for delay day vs. test trial for baseline day) as the repeated measure. There were no significant Day main effects, indicating that no group was impaired by the initiation of a six- or eight-hour delay between the information and working memory trials. We also assessed whether Treatment interacted with working memory trial performance before and after the initiation of the delay challenge; there were no Treatment x Day interactions for either the six- or eight-hour delay comparisons with the final day of baseline.

Morris Water Maze

Orally-administered Premarin did not impact Morris water maze performance. There was a main effect of Days [$F(2,62)=97.21$; $p < 0.0001$] with decreasing distance scores across days. There was also a main effect of Trials [$F(4,124)=11.29$; $p < 0.0001$], with decreasing distance scores across trials (data not shown). There were no interactions between Treatment and Days and/or Trials. Because we have shown a benefit of overnight memory retention with subcutaneous 17β-E2 treatment (Talboom et al., 2008) and with subcutaneous Premarin treatment (Acosta et al., 2009b), we probed parameters that might yield insight into overnight memory retention. To do this, we assessed

performance on the final test trial of each day (trial five) versus the first trial of the following test day (trial one). For overnight forgetting, there were no Treatment x Trial interactions for the first (trial five on day one to trial one on day two) or second (trial five on day two to trial one on day three) overnight intervals, nor on both overnight intervals combined.

For the probe trial, we assessed group differences in percent swim distance (cm) in the target (where the platform was previously located) and opposite (quadrant diagonally opposite to where the platform was previously located) quadrants. There was a Quadrant main effect, with a greater percent distance in the target quadrant (Northeast) than the opposite quadrant (Southwest) [$F(1, 31)=215.23$; $p < 0.0001$], but no interaction between Treatment and Quadrant, indicating that Premarin exposure did not impact the ability to localize the platform beyond that of the Vehicle treatment (data not shown).

Black/White Discrimination

Orally-administered Premarin did not impact non-spatial reference memory. We blocked data into four three-day blocks. As with other behavioral tasks, any interactions with Days are not meaningful for this measure because an effect of Days in the context of multiple blocks of testing days only corresponds to unique days within a testing block; therefore, these were not reported. There were no interactions with Treatment and Blocks, and/or Trials. We have previously shown that Premarin can attenuate overnight forgetting on the reference memory Morris water maze (Acosta et al., 2009b). Because this black/white discrimination task is also a reference memory task, we evaluated

overnight forgetting for this task as well. There were no Treatment x Trial interactions for the overnight forgetting measures.

For performance on the final testing day, there were no group differences in the number of correct first choices including the probe trial. Similarly, there were no group differences for the percent of arm entries into the previously rewarded arm during the final probe trial.

Neurotrophin Levels

The effect of Premarin treatment on neurotrophins was assessed in anterior and posterior cingulate cortex, frontal cortex, hippocampus, entorhinal cortex, perirhinal cortex and pituitary (Chapter 3-Table 2). Ovx-Oral-180 increased NGF protein levels in the posterior cingulate cortex as compared to Vehicle treatment [$t(14)=2.36$; $p < 0.05$]. Ovx-Oral-180 treatment decreased NGF protein levels in the frontal cortex compared to Vehicle treatment [$t(14)=2.50$; $p < 0.05$], with no effect for any other dose for frontal cortex NGF. No dose of Premarin altered posterior cingulate or frontal cortex BDNF levels, or BDNF or NGF levels in anterior cingulate cortex, hippocampus, entorhinal cortex, perirhinal cortex or pituitary.

Study 2

In Study 1, orally-administered Premarin impaired spatial working memory. However, rodent handling associated with treatment administration can impact maze performance and obviate the spatial working memory benefits of 17β-E2 (Bohacek and Daniel, 2007). Thus, it was possible that the handling procedures we utilized to

administer the oral estrogen treatment were responsible for the cognitive effects we noted in Study 1. To systematically evaluate this, in Study 2, we probed the mnemonic influence of oral handling procedures on middle-aged, Ovx rats administered either subcutaneous Premarin or Vehicle. We predicted that Premarin treatment, given at a dose we have previously found to benefit memory (Engler-Chiurazzi et al., 2011), would only enhance performance in unhandled animals and that these treatment differences would be attenuated among the orally handled animals.

Materials and Methods

Subjects

Subjects were 40 inbred Fischer-344 female rats (14 month old) born and raised at the aging colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN). Acclimation and housing procedures were identical to those in Study 1.

Ovariectomy and Treatment

The experimental timeline is presented in Chapter 3-Figure 1b.To remove ovarian-secreted hormones, all rats were anesthetized with acute isoflurane inhalation and rats received Ovx as done in Study 1. Fourteen±1 days after Ovx surgery rats began treatment administration. Rats received either propylene glycol (Vehicle) or 36μg of Premarin dissolved in propylene glycol. The 36μg dose of Premarin was based on our prior findings showing memory enhancements after subcutaneous 30μg/day injections (Acosta et al., 2009) and 36μg/day via Alzet pumps (Engler-Chiurazzi et al., 2011). In parallel with other studies (Talboom 2011; Engler-Chiurazzi et al., 2011), Vehicle and

Premarin treatments were administered continuously using Alzet osmotic pumps (Model 2004; Durect Corporation, Cupertino, CA). Briefly, Premarin was dissolved in propylene glycol (Sigma, St. Louis, MO) and inserted into the pumps as per manufacturer's instructions. For the Vehicle group, pumps were filled with propylene glycol only. For pump insertion, under isoflurane anesthesia, a small incision was made in the dorsal scruff of the neck, and a subcutaneous pocket was created. One pump filled with Vehicle or Premarin was inserted into the pocket and the skin was stapled. We allowed four days for rats to recovery from pump insertion surgery and for dorsal neck incisions to heal. Handling procedures began 18±1 days after Ovx. Rats of both subcutaneous treatment groups were randomly assigned to receive either daily oral handling procedures or to remain in their home cages unhandled until sacrifice. Thus, the groups were: 1) unhandled, Vehicle-treated rats (Vehicle-Unhandled), 2) unhandled, Premarin-treated rats (Premarin-Unhandled), 3) oral handled, Vehicle-treated rats (Vehicle-Handled), and 4) oral handled, Premarin-treated rats (Premarin-Handled). There were 10 rats for each treatment group. Oral handling procedures were identical to those used in Study 1, except that each syringe contained only 0.2ml distilled water (no hormone treatments), and the entire syringe tip was dipped in sweetened condensed milk before placement in the rat's mouth. Given that the 2004 Alzet model pump secretes 0.25µl/hour of hormone for four weeks, in order to complete the battery of mazes used in Study 2, behavioral testing began 18 treatment administration and 14 days after handling, respectively, were initiated.

Verification of Peripheral Estrogenic Stimulation

We verified peripheral Premarin stimulation via evaluation of vaginal smears (Goldman et al., 2007), pituitary weights (Spady et al., 1999), and uterine weights (Westerlind et al., 1998), as done in Study 1. Fourteen±1 days after Ovx surgery, rats were vaginally smeared to confirm complete Ovx. Beginning 21 days following Ovx and eight days following treatment administration, vaginal smears were taken daily for two days to confirm treatment. Body weights were collected 14 and 21 days following Ovx.

Delayed Match to Sample Water Maze

Thirty-one days following Ovx, rats were trained on the 8-arm, spatial working memory, water-escape delayed match to sample maze, as done in Study 1. Rats were given six trials/day for eight days.

Morris Water Maze

Forty-three days following Ovx, rats were trained on the spatial reference memory, Morris water maze, as done in Study 1. Rats were given five trials/day for three days.

Black/White Discrimination

Forty-six days following Ovx, rats were trained on the three-arm, non-spatial reference memory, water-escape black/white discrimination task, as done in Study 1. In a pilot study, we noted that regardless of treatment status, rats for which the hidden

platform was paired with the black insert outperformed those paired with a white insert. Thus, for each rat in Study 2, the hidden platform was paired with a black arm insert, which remained constant across all nine days of testing. Rats were given eight trials/day for nine days.

Visible Platform

Fifty-five days following Ovx, rats were trained on the visible platform task. The visible platform task is used to confirm that our aging animals have maintained visual and motor competence and can perform the procedural components of a water escape task. A rectangular tub (99 cm x 58.5 cm) was filled with clear water. A black platform (10 cm wide) was positioned approximately 3.75 cm above the water surface (Hunter et al., 2003). Opaque curtains covered obvious extramaze cues. Animals were given six trials in one day. The drop off location remained the same across trials, and the platform location for each trial varied semi-randomly. Each rat had 90 sec to locate the platform, where the rat then remained for 15 sec before being placed back into its heated cage. If an animal did not locate the platform within the allotted time limit, it was gently guided to the platform. The inter-trial interval was approximately eight min. Latency (sec) to reach the platform was the dependent measure.

Peripheral Tissue Collection, Uterine Weights, and Pituitary Weights

Upon completion of behavioral testing, animals were sacrificed and blood, pituitary, and uterine tissues were collected, as done in Study 1.

Hormone Assays

Serum levels of E1 and 17β-E2 were conducted similarly to Study 1, according to previously published methods (Nelson et al., 2004).

Statistical Analyses

Pituitary, uterine, and serum analyses were run via planned independent samples t-tests set a priori with Treatment (Vehicle versus Premarin) as the between factor. For behavior assessments, data were analyzed separately for each maze with a two factor, repeated measures ANOVA with Treatment and Handling as the between variables and Blocks of Days, Days, Trials, and/or Quadrants as the within variable, as appropriate for the specific maze test. This was done to allow interpretation of Days and/or Trials repeated measures effects in the context of potentially complex Treatment and/or Handling interactions. Each comparison was evaluated using an alpha level of 0.05, unless otherwise noted.

Results

Vaginal Smears, Uterine Weights, Pituitary Weights, and Serum Estrogen Levels

Premarin administration induced peripheral estrogenic stimulation. Before treatment administration, all animals exhibited diestrus-like smears composed of few, primarily leukocytic cells. Following pump implantation, all Vehicle-treated rats exhibited diestrus-like smears, and, as we have found previously (Engler-Chiurazzi et al.,

2011), all Premarin-treated animals exhibited estrus/metestrus-like smears with many cornified cells.

Premarin increased uterine weights relative to Vehicle treatment [Treatment main effect: $F(1,37)=154.25$; $p < 0.0001$](Chapter 3-Table 1). In addition, Premarin increased pituitary weights relative to Vehicle treatment [Treatment main effect: $F(1,38)=42.27$; $p < 0.0001$] (Chapter 3-Table 1).

Premarin treatment increased circulating hormone levels. Premarin treatment increased E1 levels [$t(32)=62.20$; $p < 0.0001$] (Chapter 3-Figure 2c), and 17β-E2 levels [$t(35)=73.33$; $p < 0.0001$] (Chapter 3-Figure 2d) as compared to Vehicle treatment.

Delayed Match to Sample Water Maze

As we did in Study 1, we blocked testing days into two four-day blocks and assessed Treatment x Handling x Block x Trial interactions for trials two through six. However, there were no significant main effects nor interactions of Treatment and/or Handling with Block for trials two through six. Because we have shown that Premarin impacts the working memory trial on this task (Study 1; Engler-Chiurazzi et al., 2011), we assessed performance on the working memory trial (trial two), and recent memory trials (trials three through six), separately. For the working memory trial, there was a main effect of Handling for total errors [$F(1,36)=4.10$; $p = 0.05$] (Chapter 3-Figure 3b), such that handled animals made more errors than unhandled animals. Thus, handling procedures associated with oral treatment administration detrimentally impact spatial working memory performance.

Morris Water Maze

Neither Treatment nor Handling manipulations significantly impacted spatial reference memory performance or retention. For the analysis including all days and trials of testing, there was a Treatment x Handling x Trial interaction [$F(1,144)=2.49$; $p < 0.05$] (data not shown). To further clarify this interaction, as we have shown a benefit of overnight memory retention with subcutaneous 17β-E2 (Talboom et al., 2008) and with subcutaneous Premarin injections (Acosta et al., 2009b), as we did in Study 1, we assessed overnight forgetting. There were no Treatment and/or Handling x Trial interactions for the first (trial five on day one to trial one on day two) or second (trial five on day two to trial one on day three) overnight intervals, nor on both overnight intervals combined.

For the probe trial, all animals localized to the previously platformed quadrant. For percent distance moved in the target (Northeast) versus opposite (Southwest) quadrants, there was a Quadrant main effect, with a greater percent distance swum in the target (Northeast) than the opposite (Southwest) quadrant by all groups [$F(1,36)=449.51$; $p < 0.0001$] but no interaction between Quadrant and Treatment and/or Handling (data not shown).

Black/White Discrimination

Performance was analyzed during three-day blocks with all eight trials included. There was a Treatment x Block interaction [$F(2,72)=5.04$; $p < 0.001$]. For the third block, there was a Treatment x Handling x Trials interaction [$F(7,252)=2.84$; $p < 0.01$]. Higher order interactions with Trials are not meaningful for this task unless they are driven by

differences in overnight reference memory retention. Thus, as we did for Morris water maze performance, we assessed overnight retention during the third testing block. For the overnight intervals during the third testing block, there was a Treatment x Handling x Trials interaction [$F(1,36)=6.13$; $p < 0.05$] (Chapter 3-Figure 4). To further probe this interaction, we assessed the effect of Handling within each Treatment group separately. For the Premarin-treated animals, there was a Handling x Trials interaction [$F(1,18)=5.19$; $p < 0.05$], such that handling enhanced overnight retention of the color of the platformed arm (Chapter 3-Figure 4 insert).

For performance on the final testing day (including the probe trial), there were no group differences in the number of correct first choices. Similarly there were no group differences for the percent of arm entries into the previously rewarded arm during the probe trial.

Visible Platform

All animals, regardless of group, demonstrated visual and motor competence. The ANOVA revealed a Trials main effect [$F(5, 36)=11.03$; $p < 0.0001$], indicating that for all animals, latency to reach the visible platform decreased across trials (data not shown). There were no interactions between Treatment and/or Handling and Trials.

Chapter Summary and Discussion

Here, we evaluated the cognitive effects of orally administered Premarin, the most commonly prescribed menopausal HT (Hersh et al., 2005). In both Study 1 and 2, Premarin impacted markers of peripheral estrogenic stimulation. In Study 1, orally

administered Premarin increased uterine and pituitary weights, and the medium and high oral Premarin doses increased serum E1 and 17β-E2 levels in middle-aged Ovx rats. As well, 36μg subcutaneous Premarin similarly impacted these measures in Study 2. This indicates that our medium and high oral Premarin treatments and our 36μg subcutaneous Premarin treatment induced peripheral estrogenic stimulation, allowing a clear interpretation of Premarin-induced impacts on spatial cognition. The Ovx-Oral-180 dose impaired spatial working memory on the delayed match to sample task. That we found oral Premarin-induced impairments during the first testing block, but not when memory was challenged during the delay testing, suggests that these impairments were specific to initial task acquisition. Thus, the findings of Study 1 suggest that the realization of memory benefits with Premarin treatment may depend on the route of administration. Other studies evaluating orally administered estrogens suggest mixed nmemonic effects depending on task and memory type. For instance, oral-gavage administered estradiol valerate failed to enhance spatial reference memory Morris water maze performance in five month old Ovx rats (Aguiar et al., 2006). Similarly, while benefits were found with 17β-E2 administered via subcutaneous silastic implants, oral-gavage administered 17β-E2 failed to enhance water radial arm maze performance in this same age group (Garza-Meilandt et al., 2006). As well, 17β-E2 dissolved in drinking water imparted dose dependent effects in middle-aged, Ovx mice, such that 1,500nM dose (approximating 110μg/kg/day) impaired working memory performance on the water radial arm maze (Fernandez and Frick, 2004). Interestingly, the 1,000, 1,500, and the 2,500nM doses (corresponding to 70, 110, and 180μg/kg/day, respectively) enhanced object memory. Thus, converging evidence suggests that oral administration of estrogens imparts task-

specific memory effects and thus is not optimal for achieving consistent beneficial memory outcomes.

Neurobiological assessments of orally-administered Premarin in Study 1 revealed that the highest oral Premarin dose increased NGF levels in the posterior cingulate cortex and decreased NGF levels in the frontal cortex. That this dose increased NGF in the posterior, but not anterior, cingulate cortex clarifies and extends findings from Engler-Chiurazzi et al., (2011). In that study, increased NGF levels in the cingulate cortex (anterior and posterior combined) were found with 24- and 36-μg/day subcutaneously administered Premarin, doses that enhanced delayed match to sample working memory. These collective findings suggest that the mechanism for oral Premarin-induced working memory impairments may be related to the decrease in NGF in the frontal cortex, while Premarin-related memory enhancements may be related to NGF increases in whole cingulate. Orally administered 17β-E2 has also been shown to impact growth factors levels in brain. Specifically, the oral 1500nM 17β-E2 dose that impaired water radial arm maze performance also decreased frontoparietal NGF levels (Fernandez and Frick, 2004). Taken together, these findings identify a potential neurobiological mechanism for the detrimental impact of orally administered estrogen treatments.

In the context of findings from previous rodent studies reporting beneficial cognitive effects of subcutaneous Premarin (Acosta et al., 2009b; Engler-Chiurazzi et al., 2011; Walf and Frye, 2008), a limitation to the interpretation of Study 1 is the methodological differences in experimental handling associated with subcutaneous versus oral treatment administration. Indeed, rodent handling associated with treatment administration has been shown to interact with exogenous estrogen therapies such that

water radial arm maze benefits of 17β-E2 are masked by the enriching effects of two minutes/day of experimental handling (Bohacek and Daniel, 2007). Findings from Study 2 help to clarify the distinct cognitive impact of oral Premarin from that of the experimental handling associated with oral Premarin administration. For behavioral testing, oral-associated handling, rather than Premarin treatment, impaired spatial working memory on the delayed match to sample task. This suggests that the spatial working memory impairments following orally-administered Premarin observed in the current study were likely due to the detrimental cognitive impact of handling rather than Premarin treatment. As well, we found that among Premarin-treated animals, oral handling enhanced overnight retention on black/white discrimination, further supporting the hypothesis that handling influences memory independently of Premarin treatment.

Interestingly, in Study 2, Premarin did not impart working memory benefits on the delayed match to sample task, as we have previously observed (Acosta et al., 2009b, Engler-Chiurazzi et al., 2011). This lack of replication may be due to differences in the apparatus utilized across studies. Indeed, in Engler-Chiurazzi et al., (2011), the reported enhancements of subcutaneous Premarin were found using a 4-arm version of the delayed match to sample task. Yet, in the current studies, the impairments of Premarin were found using an 8-arm version of this same task. The addition of the arms likely made the task more complex. Of note, in women, estrogen-containing treatments have been shown to enhance cognition on some tasks, such as those requiring verbal and working memory, but not others (Sherwin and Henry, 2008), suggesting a role for task complexity in the realization of cognitive benefits with menopausal HTs. Exploring the potential interaction between task complexity and estrogen treatment will aid in the optimization of HT

options for women. To evaluate this interaction, in the next chapter, we methodically manipulate number of maze arms and assess cognitive efficacy of subcutaneous Premarin treatment in the rat model used in Engler-Chiurazzi and colleagues (2011) and Study 2.

Together, the findings from Studies 1 and 2 suggest that oral-associated handling exerts unique nmemonic effects that are distinct from those of Premarin, and that these effects impair spatial, but enhance non-spatial, memory. These findings suggest that the detriments associated with Premarin-treatment observed in the human population are likely not due to the oral route of Premarin administration. However, adding to the intricacy of Premarin's impact on the brain and cognition, these collective findings suggest that task complexity may be another important factor in the realization of memory benefits with Premarin-containing HT.

CHAPTER 4

SUBCUTANEOUS PREMARIN TREATMENT ENHANCES 4-ARM, BUT IMPAIRS 8-ARM, WORKING MEMORY PERFORMANCE: INTERACTIONS WITH ORAL AND ACCLIMATION HANDLING

Manuscript Status: In preparation

Introduction

The complex formulation, Premarin, is prescribed to middle-aged women for the treatment of negative menopausal symptoms, including hot flashes and vaginal atrophy (Timiras et al., 1995). Given, the well-established protective effect of estrogens on learning and memory (Bimonte-Nelson et al., 2010), interest in the ability of Premarin to attenuate, and even prevent age-related cognitive decline has increased. Although some clinical and preclinical findings regarding Premarin suggest that this hormone therapy (HT) can impart benefits for cognitive outcomes, evidence suggests that these beneficial outcomes depend on many factors including route of administration, dose, or experimental handling (Acosta et al., 2013). For instance, subcutaneous Premarin enhanced (Acosta et al., 2009b; Engler-Chiurazzi et al., 2011), whereas oral Premarin impaired, spatial working memory (Chapter 3-Study 1). In a follow-up study designed to isolate the mnemonic effects of handling from those of Premarin, rats that were administered either Vehicle, or the subcutaneous dose of Premarin (36μg/day) we had previously found to enhance memory (Engler-Chiurazzi et al., 2011), were randomly assigned to receive handling associated with oral treatment administration. We found that, regardless of Vehicle or Premarin treatment, handling associated with oral

administration of Premarin impaired spatial working memory (Chapter 3-Study 2). Thus, independent of Premarin treatment, it is likely that handling associated with oral treatment administration contributed to the working memory impairments observed in Chapter 3-Study 1. However, methodological differences in the difficulty of the tasks used could also partly account for the conflicting outcomes of these studies. Indeed, the spatial working memory enhancements of subcutaneous Premarin were found using a 4-arm version of the delayed match to sample task (Acosta et al., 2009b; Engler-Chiurazzi et al., 2011). Yet, the impairments of oral Premarin were found on an 8-arm version of this same task (Chapter 3). Determining whether task complexity underlies the realization of cognitive benefits with Premarin treatment will help to inform the optimal conditions in which to administer this HT.

Here, we aimed to clarify the potential interaction between task complexity and Premarin treatment by evaluating the impact of subcutaneous Premarin treatment on spatial working memory in middle-aged, surgically menopausal rats; we directly compared performance on a 4-arm version of the delayed match to sample task to an 8-arm version. As well, although experimental handling in rodents can impact memory performance, the direction of the effect varies across studies. For instance, handling associated with HT administration and acclimation enhanced memory performance among untreated rats, obviating the beneficial memory effects of 17β-estradiol (17β-E2; Bohacek and Daniel, 2007). Yet, handling associated with oral treatment of estrogen-containing HT can also impair memory (Chapter 3-Study 2). As such, we aimed to determine if the mnemonic effects of Premarin treatment differ depending on the type of handling experience. To do this, in addition to comparing Vehicle- and Premarin-treated

rats that experienced no handling or oral handling, we added a comparison group that experienced handling procedures designed to acclimate rodents, based on Bohacek and Daniel (2007). Among unhandled rats, we predicted that, compared to Vehicle, subcutaneous continuous Premarin would enhance performance on the 4-arm version, as we have shown previously (Engler-Chiurazzi et al., 2011), but impair performance on the more complex, 8-arm version. Further, we predicted that acclimation-like handling procedures would mask Premarin-induced benefits, similar to findings with 17β-E2 (Bohecek and Daniel, 2007), and that oral handling procedures would impair performance in both treatment groups, as reported in Chapter 3.

Materials and Methods

Subjects

Subjects were 53 inbred Fischer-344 female rats (14 month old) born and raised at the aging colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN). Inbred rats were selected given that their low genetic and physiological variability allowed us to utilize a relatively small sample size to generalize observed cognitive impacts of our treatments to the general population (Nadon, 2004). Animals were acclimated for several weeks at the Arizona State University animal facility, were pair housed with an identically treated cage-mate, had exposure to food and water ad-libitum, and were maintained on a 12-h light/dark cycle. All procedures were approved by the local Institutional Animal Care and Use Committee and adhered to National Institutes of Health standards.

Ovariectomy and Treatment

Thirty-two±1 days before behavioral testing ensued, all rats were anesthetized with acute isoflurane inhalation and received ovariectomy (Ovx). Bilateral dorsolateral incisions were made in the skin and peritoneum, and the ovaries and tips of uterine horns were ligatured and removed. The muscle was then sutured and the skin stapled. Fifteen±1 days after Ovx surgery, rats began treatment administration. Based on our prior findings showing memory enhancements after subcutaneous 30μg/day injections and 36μg/day via Alzet pumps (Durect Corporation, Cupertino, CA), we choose selected the 36μg/day dose. Thus, rats received either propylene glycol (Vehicle) or 36μg of Premarin dissolved in propylene glycol continuously via an Alzet osmotic pump. Handling procedures began 19±1 days after Ovx. Rats of both subcutaneous treatment groups were randomly assigned to remain in their home cages unhandled or to receive either 1) daily handling procedures typically associated with rat-experimenter acclimation or 2) daily handling procedures associated with oral treatment administration. Acclimation handling consisted of picking up the rat, allowing a rat to explore the top of a testing cart for 7.5 sec, followed by 7.5 sec of being nuzzled in the experimenter's arms and gently stroked similar to Bohacek and Daniel (2007). To make this handling equitable with the duration of oral handling used in previous studies (Chapter 3), the total duration of acclimation handling was 15 sec per day. As we have done previously (Chapter 3), orally handled rats were hand fed via a needleless syringe. Each syringe contained 0.2ml distilled water, and the entire syringe tip was dipped in sweetened condensed milk before placement in the mouth. The rat was then wrapped up in a hand towel such that only the head protruded, the syringe was inserted into the rat's mouth and the liquid was dispensed. The oral

handling process lasted approximately 15 sec for each rat. Rats were given daily oral handling or acclimation handling until the day of sacrifice. Behavioral testing began 17 and 13 days after treatment administration and handling were initiated, respectively. For behavioral testing, to control for the order in which rats were tested on the behavioral tasks, rats within each treatment and handling condition were further subdivided into groups based on test order. One set was tested first on the 4-arm maze followed the 8-arm maze, and the other set was tested on the 8-arm maze followed by the 4-arm maze. Thus, for each maze version, rats were either behavioral testing naive or behavioral testing experienced. In summary, the experimental groups were as follows: 1) Vehicle-treated, unhandled rats (Vehicle, N=9), 2) Premarin-treated, unhandled rats (Premarin, N=9), 3) Vehicle-treated, acclimation handled rats (Vehicle-Acclimation, N=9), 4) Premarin-treated, acclimation handled rats (Premarin-Acclimation, N=9), 5) Vehicle-treated, oral-administration handled rats (Vehicle-Oral, N=8), and 6) Premarin-treated, oral-administration handled rats (Premarin-Oral, N=8).

Verification of Peripheral Estrogenic Stimulation

We verified peripheral stimulation via evaluation of vaginal smears (Goldman et al., 2007), pituitary weights (Spady, 1999), and uterine weights (Westerlind, 1998). Fifteen±1 days after Ovx surgery, rats were smeared to confirm Ovx status. Beginning seven days following treatment administration, vaginal smears were taken daily for two days. Smears were classified as either proestrus, estrus, or metestrus, all of which are indicative of estrogenic stimulation, or diestrus, which is indicative of the absence of estrogenic stimulation (Goldman et al., 2007).

Delayed Match to Sample Water Maze

The water-escape delayed match to sample plus maze is a task that assesses spatial working and recent memory (Engler-Chiurazzi et al., 2011; Frick et al., 1995; Markowska and Savonenko, 2002b). Rats were trained on two versions of the task, a 4-arm and 8-arm version. The apparatus was a water radial arm maze with eight arms (each 38.1 cm long and 12.7 cm wide). For the 4-arm version, the apparatus had four arms available for entry (the other four arms were blocked)**.** For the 8-arm version, the apparatus had eight arms available for entry**.** In the 8-arm spatial task, the increased complexity included the potential interference from more arms, and their associated spatial locations, in which to enter. There were no other procedural differences between the two task versions. Each animal was tested on both task versions, in a counterbalanced order.

In each version of the task, the apparatus was filled with room temperature water made opaque with black non-toxic paint. For both the 4-arm and 8-arm versions, animals were required to locate one platform, the location of which was fixed within a day and changed across days, hence requiring working memory. Rats received six consecutive trials within a daily session. The first trial was the information trial where the rat was exposed to that day's platform location, the second trial was the working memory test trial, and trials three through six were memory test trials (Acosta et al., 2009b; Engler-Chiurazzi et al., 2011). Rats were dropped off in a semi-randomly chosen start arm location, and were given a maximum of 90 sec to swim to the platform. If an animal did not locate the platform within the allotted time limit, it was gently guided to the platform

using a black plastic rod. Once on the platform, the rat remained on it for 15 sec, followed by placement into a heated cage for a 30 sec inter-trial interval. An arm entry was counted when the tip of a rat's snout reached a mark delineated on the outside of the arm (11 cm into the arm). Entry into an arm with no platform counted as an error, the dependent variable. After eight days of testing with a 30 sec inter-trial interval between all trials, rats were tested with a six-hour delay (day nine) given between trial one and trial two. Since the second trial is the first trial to test recall of the updated information (working memory), the delays were given between trial one and trial two to determine whether the increased inter-trial interval impacted, specifically, working memory. There were no additional trials after the post-delay trial (e.g. trial two).

Visible Platform

The visible platform task is used to confirm that animals have maintained visual and motor competence and can perform the procedural components of a water escape task. A rectangular tub (99 cm x 58.5 cm) was filled with clear water. A black platform (10 cm wide) was positioned approximately 3.75 cm above the water surface (Hunter et al., 2003). Opaque curtains covered obvious extramaze cues. Animals were given six trials in one day. The drop off location remained the same across trials, and the platform location for each trial varied semi-randomly. Each rat had 90 sec to locate the platform, where the rat then remained for 15 sec before being placed back into its heated cage. If an animal did not locate the platform within the allotted time limit, it was gently guided to the platform. The inter-trial interval was approximately eight min. Latency (sec) to reach the platform was the dependent measure.

Peripheral Tissue Collection, Uterine Weights, and Pituitary Weights

The day following the completion of behavioral testing, rats were anesthetized with isoflurane and immediately decapitated. Blood was taken via cardiocentesis. Uterine tissues were collected, trimmed of fat and connective tissue, and weighed as per previous methods (Acosta et al., 2009b). Pituitary was extracted from the base of the skull following the removal of the brain from the skull cavity, and placed into pre-weighed microcentrifuge tubes.

Statistical Analyses

Pituitary and uterine weight analyses were run via planned one-way ANOVAs set a priori, with Treatment as the between factor. For delayed match to sample behavior assessments, to test replication of our prior findings regarding the working memory impact of subcutaneous Premarin treatment (Engler-Chiurazzi et al., 2011), to evaluate the potentially unique mnemonic impacts of Premarin within each handling condition, and to assess the potential interactive impact of prior testing experience, we conducted hypothesis-driven one way ANOVAs with Treatment as the between variable for each handled group. We collapsed across Days and evaluated Blocks of Days and Trials as the repeated variables for each handling group (unhandled, acclimation-typical, and oral) on each maze version (4-arm and 8-arm) separately. Data were collapsed across Days because any interactions with Days are not meaningful; an effect of Days in the context of multiple blocks of testing days only corresponds to unique days within a testing block. In the presence of significant Treatment interactions with one or more of the dependent

variables, follow-up analyses were conducted to further clarify the effects. For the visible platform task, we assessed performance on the final trial via one way ANOVAs with Treatment as the between variable for each handling group (unhandled, acclimation-typical, and oral). Each comparison was evaluated using an alpha level of .05, unless otherwise noted (Keppel and Wickens, 2004, p. 115).

Results

Vaginal Smears, Uterine Weights, and Pituitary Weights

Premarin treatment induced peripheral stimulation. At the time of pump insertion to initiate Premarin treatment, all but one of the animals showed diestrus-like smears, characterized by the presence of leukocytes and relatively low numbers of total cells. This one anomalous animal exhibited estrus-like smears, characterized by numerous cornified cells. This animal was excluded from all uterine weight, pituitary weight and behavioral data analyses. Following pump implantation, all Vehicle-treated animals showed diestrus-like smears. As expected Engler-Chiurazzi et al., 2011), all Premarin-treated animals showed estrus- or metestrus-like smears, with many cornified cells.

For uterine weights, one Vehicle-treated animal had a fluid filled cyst on the uterus and was excluded from the uterine weight data analysis. Because we have previously shown 36ug subcutaneous, continuous Premarin treatment to increase uterine and pituitary weights (Engler-Chiurazzi et al., 2011), we evaluated these effects using a one-tailed t-test. There was a Treatment main effect [$t(48)=11.09$; $p < 0.0001$], such that Premarin treatment increased uterine weights (Chapter 4-Table 1). For pituitary weights,

there was a Treatment main effect [$t(49)=1.70$; $p < 0.05$], such that Premarin treatment increased pituitary weights (Chapter 4-Table 1).

Delayed Match to Sample Water Maze

Evaluation of Difficulty of the 4-arm versus 8-arm Mazes

To verify that the 8-arm version of the delayed match to sample task was more complex and challenging than the 4-arm version, we compared the sum of total errors for all memory test trials (days one-eight, trials two-six) via an ANOVA with Maze (4-arm vs 8-arm) as the repeated measure. There was a Maze main effect [$F(1,51)=169.51$; $p < 0.0001$], such that more errors were committed on the 8-arm maze than on the 4-arm maze (data not shown). This finding confirms that the 8-arm version (Mean±SEM =62.60±3.23) was more challenging than the 4-arm version (Mean±SEM =18.31±1.25).

Treatment Memory Effects in Unhandled Rats

Testing with a 30-second inter-trial interval: For the 4-arm maze, there was a Treatment x Block x Trial interaction [$F(4,64)=4.37$; $p < 0.005$]. To further probe this finding, we assessed performance on each testing block separately. For the first testing block (Days 1-4), there was a Treatment x Trial interaction [$F(4,64)=3.92$; $p < 0.01$] (Chapter 4-Figure 1a). Because the working memory trial (trial two) assesses a unique memory domain and because we have shown impacts of Premarin on this trial in past studies (Acosta et al., 2009b; Engler-Chiurazzi et al., 2011), to follow up on this interaction, we assessed performance on the working memory trial (trial two) and recent memory trials (trials three-six) separately. There was a Treatment main effect for the working memory trial [$F(1,16)=5.44$; $p < 0.05$], such that Premarin enhanced

performance, with the Premarin group making fewer total errors than the Vehicle group (Chapter 4-Figure 1a). For the second testing block (Days 5-8), there were neither Treatment main effects nor a Treatment x Trial interaction.

For the 8-arm maze, there was a Treatment x Block interaction [$F(1,16)=6.28$; $p < 0.05$]. When we assessed this interaction on each testing block separately, during the first testing block (Days 1-4), there was a Treatment main effect [$F(1,16)=4.61$; $p < 0.05$], such that Premarin impaired performance with the Premarin group making more total errors than the Vehicle group (Chapter 4-Figure 2a). For the second testing block (Days 5-8), there were neither Treatment main effects nor a Treatment x Trial interaction (Chapter 4-Figure 2a).

Testing with a 6-hour inter-trial interval: We assessed treatment group differences in performance on the working memory trial on the final baseline day (30 sec inter-trial interval) compared to the post-delay trial (six-hour inter-trial interval), for both 4-arm and 8-arm task versions. When we assessed performance on the 4-arm maze, there were no Treatment x Day interactions (Figure 3a). For the 8-arm maze, there was a Treatment x Day interaction [$F(1,16)=15.89$; $p < 0.005$], such that Premarin-treated rats outperformed Vehicle-treated rats on baseline day eight but were impaired when memory was challenged by the delay on day nine (Chapter 4-Figure 3a).

Treatment Memory Effects in Acclimation Handled Rats

Testing with a 30-second inter-trial interval: When we assessed performance on the 4-arm maze, there were no Treatment main effects, nor were there interactions between Treatment and any repeated measures (Chapter 4-Figure 1b). As well, when we

assessed performance on the 8-arm maze, there were no Treatment main effects, nor were there interactions between Treatment and any repeated measures (Chapter 4-Figure 2b).

Testing with a 6-hour inter-trial interval: We assessed treatment group differences in performance on the working memory trial on the final baseline day (30 sec inter-trial interval) compared to the post-delay trial (six-hour inter-trial interval) for both 4-arm and 8-arm mazes. However, there were no Treatment x Day interactions for either the 4-arm and 8-arm maze (Chapter 4-Figure 3b). When we assessed performance on the working memory trial for the second four-day block of baseline (collapsed across days 5-8) compared to the post six-hour delay trial, there were no Treatment x Day interactions for either maze. When we assessed performance on the post-delay working memory trial, there were no main effects of Treatment for either maze.

Treatment Memory Effects in Oral Handled Rats

Testing with a 30-second inter-trial interval: When we assessed performance on the 4-arm maze, there were no Treatment main effects, nor were there interactions between Treatment and any repeated measures (Chapter 4-Figure 1c). As well, when we assessed performance on the 8-arm maze, there were no Treatment main effects, nor were there interactions between Treatment and any repeated measures (Chapter 4-Figure 2c).

Testing with a 6-hour inter-trial interval: We assessed treatment group differences in performance on the working memory trial on the final baseline day (30 sec inter-trial interval) compared to the post -delay trial (six-hour inter-trial interval) for both 4-arm and 8-arm mazes. However, there were no Treatment x Day interactions for either the 4-arm and 8-arm maze (Chapter 4-Figure 3c). We also assessed performance on the working memory trial for the second four-day block of baseline (collapsed across days 5-8)

compared to the post six-hour delay trial for both 4-arm and 8-arm mazes. Again, there were no Treatment x Day interactions for either maze. Finally, we assessed performance on the post-delay working memory trial. However, there were no main effects of Treatment for either maze.

Visible Platform

All animals, regardless of group, demonstrated visual and motor competence. On the final trial, there were no main effects of Treatment, indicating that for all animals, latency to reach the visible platform decreased across trials (data not shown).

Chapter Summary and Discussion

Here, we evaluated: 1) whether task complexity is a factor underlying the realization of memory benefits with exogenous Premarin, and 2) whether these effects of Premarin differ depending on handling experience. Subcutaneous Premarin treatment resulted in positive vaginal smears and increased uterine and pituitary weights. Among unhandled rats, 36μg subcutaneous Premarin enhanced working memory trial performance on the 4-arm, but impaired performance on the 8-arm, maze. Thus, our hypothesis that the impact of Premarin on memory would be modulated by task difficulty was supported. Providing further support for this hypothesis, in aging women, estrogen-containing menopausal therapies enhance cognition on some tasks, such as those requiring verbal and working memory, but not others (Sherwin and Henry, 2008).

Interestingly, the task-dependent effects of Premarin treatment were not observed among the acclimation and oral handled groups. That handling reversed the beneficial

effects of Premarin on memory extends work by Bohacek and Daniel (2007), in which radial arm maze benefits of 17β-E2 treatment were obviated by the enriching effects of two minutes/day of experimental handling. Here, approximately 15 sec of daily handling resulted in a similar attenuation of Premarin enhancements. Thus, the current findings, that handling masked Premarin-induced benefits, support our prediction. However, that handling also reversed the detrimental memory impacts of Premarin on the 8-arm delayed match to sample task was somewhat surprising given our previously reported impairments of oral handling on this same maze (Chapter 3-Study 2). In the context of a difficult task with a high memory demand, it is possible that both Premarin treatment and handling experience can each exert detrimental effects for memory performance, culminating in a significant impairment relative to unhandled, untreated controls. Together, these findings suggest that specific, optimized parameters regarding the handling experience and the difficulty of the cognitive task are necessary for the realization of Premarin-induced learning and memory benefits.

It is possible that the impairing effects of Premarin on the 8-arm maze were due to stress brought on by the difficulty of the task. Indeed, in the current study, the 8-arm task was associated with more total errors than the 4-arm maze, suggesting that the 8-arm version was more difficult and potentially more stressful than the 4-arm version. In middle-aged women, stress can obviate the beneficial effects of exogenous estrogen (Baker et al., 2012; Newhouse et al., 2010). For instance, oral hydrocortisone reversed benefits of chronic transdermal 17β-E2 on measures of verbal memory, working memory, and selective attention (Baker et al., 2012). In animals, although the interaction between sex and stress on cognitive outcomes is complex and likely depends on a number

of factors, findings from several studies suggest sex differences in cognitive outcomes following exposure to chronic stress such that males, but not females, are impaired on hippocampal-dependent memory tasks (Gillies and McArthur, 2010; Luine et al., 2007). For instance, a chronic restraint stress paradigm of 6 hour/day for 21 days impaired spatial Y-maze performance in adult male rats, but enhanced performance in female rats (Conrad et al., 2003). As well, exposure to chronic stress appears to enhance maze performance among estrogen-treated, Ovx rats (Luine, 2007). For instance, chronically stressed, 17β-E2-treated Ovx rats required fewer arm visits to complete the radial arm maze than non-stressed, 17β-E2-treated rats as well as stressed and non-stressed, cholesterol-treated rats (Bowman et al., 2002). The effect of Premarin treatment in chronically stressed, Ovx rats has not yet been evaluated. However, assuming that chronic stress and Premarin interact to influence memory among Ovx, female rats in a similar manner to that of the 17β-E2/stress interaction, if the added difficulty of the 8-arm task used here induced a stress response, we would expect that Premarin-treated rats show enhanced, rather than impaired, working memory. Thus, it is unlikely that differences in the stress associated with each task contributed to the current findings.

In conclusion, we found that among unhandled rats, Premarin enhanced working memory on the 4-arm delayed match to sample task, but impaired working memory on the 8-arm version. As well, acclimation and oral handling obviated the mnemonic effects of Premarin treatment in both maze versions. Together, these findings that the impacts of Premarin vary depending on handling experience and task difficulty, suggest that there are limited and very specific conditions in which Premarin will impart learning and memory benefits. Furthermore, that, within the same animal, the beneficial working

memory effects of Premarin are reversed when memory is challenged by a more difficult task suggest that this compound is not an ideal menopausal HT, and highlights the need to develop novel treatment options that are more optimal for cognitive outcomes. For example, a more beneficial HT option would enhance performance across a wide range of cognitive tasks, would be less sensitive to parameter alterations, and would impart cognitive benefits across a greater variety of conditions, such as task difficulty. Subsequent work in this dissertation will investigate some chemical and neurobiological mechanisms that could result in cognitive benefits versus impairments.

CHAPTER 5

CONTINUOUS ESTRONE TREATMENT IMPAIRS SPATIAL MEMORY AND DOES NOT IMPACT NUMBER OF BASAL FOREBRAIN CHOLINERGIC NEURONS IN THE SURGICALLY MENOPAUSAL MIDDLE-AGED RAT

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Introduction

Premarin (conjugated equine estrogens) has been given to menopausal women since 1942 (Stefanick, 2005), was the estrogenic component tested in the Women's Health Initiative Memory Study (Shumaker et al., 2004; Shumaker et al., 1998), and is the most widely prescribed estrogenic component of menopausal hormone therapy (HT) in the United States, even despite a decrease in use after the 2002 publication of clinical trial results (Hersh et al., 2004). Premarin has been shown to have both positive and negative effects on cognition in menopausal women (for review see Hogervorst et al., 2000; Sherwin and Henry, 2008), and can dose-dependently enhance memory in the middle-aged ovariectomized (Ovx) rat (Acosta et al., 2009b; Engler-Chiurazzi et al., 2011). Premarin is a complex estrogen formulation comprised of 50% estrone (E1) sulfate, and it contains the sulfates of at least ten other estrogens (Kuhl, 2005), many of which have yet to be individually evaluated for cognition in women or rodent models. Determining effects of the specific estrogen components of this complex formulation could help determine why it sometimes enhances, and why it sometimes impairs, cognition. Furthermore, it may identify a group of cognitively enhancing estrogens to be combined into optimal HT formulations for specific populations of women, as well as

identify estrogens detrimental to the brain and cognition to be excluded from future formulations. In women, 17β-estradiol (17β-E2), present only in trace amounts in Premarin, is the most potent naturally-circulating estrogen, followed by E1 and estriol (E3), in order of receptor affinity (Kuhl, 2005). 17β-E2 and E1 are biologically interconvertible; in vivo, they readily get converted into one another (Kuhl, 2005; Prokai-Tatrai and Prokai, 2005). Circulating levels of E1 increase following treatment with Premarin to menopausal and post-menopausal women (Yasui et al., 1999), and following administration of Premarin to middle-aged Ovx rats (Acosta et al., 2009b; Engler-Chiurazzi et al., 2011). Although we have shown that the Premarin components $\Delta^{8,9}$-dehydroestrone, and to a lesser extent, equilin, exert benefical cognitive effects in middle-aged, Ovx rats (Talboom et al., 2010), the cognitive impact of the principle circulating estogen following Premarin administration, E1, is unclear. We hypothesize that E1 will impair cognition in middle-aged Ovx rats. Indeed, one paper in young rats has shown a single subcutaneous E1 injection impairs contextual fear conditioning memory when given 30 minutes before training (Barha et al., 2009). Furthermore, although not all in vitro studies report negative effects with E1 treatment (Zhao and Brinton, 2006), for most measures in which other estrogenic Premarin components (e.g., equilin and $\Delta^{8,9}$-dehydroestrone) were neuroprotective in vitro, E1 was ineffective (Zhao and Brinton, 2006).

The basal forebrain cholinergic system is important for learning and memory, is susceptible to age-related changes, and is impacted by ovarian hormone removal and 17β-E2 replacement (for review see Gibbs, 2010). For example, in aged female rats, less choline acetyltransferase (ChAT) protein activity was found in the vertical diagonal

bands (vDB), relative to younger counterparts (Luine and Hearns, 1990). Also, in adult Ovx rats, 17β-E2 treatment increased ChAT protein activity in the hDB (horizontal diagonal bands; Luine, 1985), as well as ChAT-immunoreactive (ChAT-IR) neuron counts in the MS (Gibbs, 1997). Importantly, evidence from Gibbs' laboratory suggests that the effects of 17β-E2 on cognition require a functioning basal forebrain cholinergic system; for example, 17β-E2 was ineffective in animals with basal forebrain lesions, and enhanced memory only in non-lesion controls (2002, 2007). Although it has been established that 17β-E2 impacts the basal forebrain cholinergic system, an effect which is likely related to cognitive enhancements (for review see Bimonte-Nelson et al., 2010; Gibbs, 2010), there has been no study evaluating whether E1 impacts basal forebrain cholinergic neurons.

In the present study, we evaluated the cognitive impact of subcutaneously administered continuous E1 treatment in middle-aged Ovx rats, utilizing several spatial memory mazes previously shown to be sensitive to the effects of aging (Frick et al., 1995; Talboom et al., 2008), and hormone administration (Acosta et al., 2009b; Bimonte-Nelson et al., 2006; Engler-Chiurazzi et al., 2011; Walf et al., 2009), such that a potential pattern of E1's effects on specific memory types could be revealed. Several classic peripheral markers of estrogenic action, including vaginal smears and uterine weights, were measured to confirm effects of Ovx and E1 treatment. Lastly, we evaluated the impact of E1 on the basal forebrain cholinergic system by quantifying the number of ChAT-IR neurons in the medial septum (MS) and the hDB/vDB of the basal forebrain in the cognitively tested animals. Because 17β-E2 has been shown to impact ChAT protein activity (Luine, 1985) and ChAT-IR neuron counts (Gibbs, 1997) in the basal forebrain,

to aid in interpretation of potential E1 ChAT-IR effects, we evaluated ChAT-IR neuron numbers after treatment with 17β-E2 using the same quantification procedures as those used in the current study. Determining the impact of E1 on spatial memory and the cholinergic system will help to characterize the unique cognitive and neurobiological impacts of this estrogen, which is a primary circulating estrogen after administration of the commonly used HT, Premarin.

Materials and Methods

Subjects

We used 32 middle-aged (13 months old at the beginning of the study) Fischer-344 female rats born and raised at the National Institute on Aging colony at Harlan Laboratories (Indianapolis, IN). Inbred rats were selected given that their low genetic and physiological variability allowed us to utilize a relatively small sample size to generalize observed cognitive impacts of our treatments to the general population (Nadon, 2004b). Animals were pair-housed, acclimated for several weeks at Arizona State University, had exposure to food and water ad libitum, and were maintained on a 12-h light/dark cycle at 23°C. Experimental procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and adhered to Guidelines for the Care and Use of Laboratory Animals and NIH standards.

Ovariectomy and Treatment

Approximately 28 days before behavioral testing ensued, under isoflurane inhalant anesthesia, all rats underwent Ovx surgery to remove endogenous ovarian hormones. Dorsolateral incisions were made in the skin and peritoneum, and ovaries and

tips of uterine horns were ligated and removed. Rats were then separated into the following groups: Ovx with Vehicle only (polyethylene glycol)(Vehicle, n=9), Ovx plus 2.6μg/day of E1 (E1-Low, n=7), Ovx plus 4.0μg/day of E1 (E1-Med, n=8), and Ovx plus 8.0μg/day of E1 (E1-High, n=8). All hormones were purchased from Sigma (St. Louis, MO). The E1-Low dose was based on the most efficacious dose of 17β-E2 found in a dose response study conducted in our laboratory evaluating spatial working memory (unpublished observations). The E1-Med dose was based on findings from Beyer and colleagues (1976) in which 4.0μg/day of E1 induced lordosis behavior and increased uterine weights. To assess the cognitive and physiological impact of a broad range of E1 doses, the E1-High dose was double the E1-Med dose. Corresponding to published studies evaluating E1 and other estrogens (Barha et al., 2009; Talboom et al., 2010) in which subjects were given approximately one to two weeks between Ovx and hormone treatment, in the current study, hormone treatment began 19±1 days after Ovx. In parallel with other studies (Engler-Chiurazzi et al., 2011; Talboom et al., 2010), Vehicle and E1 treatments were administered continuously using Alzet osmotic pumps (Model 2004; Durect Corporation, Cupertino, CA). Briefly, E1 was dissolved in polyethylene glycol (Sigma, St. Louis, MO) and inserted into the pumps as per manufacturer's instructions. For the Vehicle group, pumps were filled with polyethylene glycol only. For pump insertion, under isoflurane anesthesia, a small incision was made in the dorsal scruff of the neck, and a subcutaneous pocket was created. One pump filled with Vehicle or the appropriate E1 dose was inserted into the pocket and the skin was stapled. Nine days after pump insertion surgery, cognitive testing began. All animals had Vehicle or E1 exposure for until sacrifice.

Verification of Peripheral Estrogenic Stimulation

To confirm the effects of Ovx as well as E1 treatments, we assessed several peripheral physiologic markers that routinely change with estrogen treatment. Notably, E1 has been found to impact peripheral tissues, including the uterus (Beyer et al., 1976). We therefore performed vaginal smears (Goldman et al., 2007) and measured uterine weights (Westerlind, 1998), the latter of which was done upon animal sacrifice. Smears were classified as proestrus, estrus, metestrus or diestrus (Acosta et al., 2009b; Engler-Chiurazzi et al., 2011; Goldman et al., 2007). Vaginal smears were conducted to confirm the lack of uterine stimulation and complete Ovx 18 days after Ovx, which was one day before E1 administration via pump implantation. Vaginal smears were also conducted daily for four days beginning five days after pump implantation.

Delayed Match to Sample Water Maze

The delayed match to sample water maze assessed spatial working memory. The maze had four arms (each 38.1 cm long and 12.7 cm wide) in a plus configuration, and was filled with room temperature water made opaque with black non-toxic paint. The maze had a hidden escape platform at the end of one arm. The platform location changed every day, but was fixed within a day. Rats received six consecutive trials within a daily session, for seven consecutive days. The first trial was an information trial, where the rat had to first locate the platform position for that day. Trials two through six were memory test trials, in which the location of the platform was repeatedly reinforced (Acosta et al., 2009b; Engler-Chiurazzi et al., 2011). For each trial (one-six), rats were dropped off in a

semi-randomly chosen start arm location, and were given a maximum of 90 sec to swim to the platform. If an animal did not locate the platform within the allotted time limit, it was gently guided to the platform using a black plastic rod. Once on the platform, the rat remained on it for 15 sec, followed by placement into a heated cage for a 30 sec inter-trial interval. An arm entry was counted when the tip of a rat's snout reached a mark delineated on the outside of the arm (11cm into the arm). Entry into an arm with no platform counted as an error, the dependent variable. As the rate of learning can change across the task and be impacted by treatment (Acosta et al., 2009a), and to gain insight into errors committed across different phases of task acquisition, we grouped the data into two three-day blocks (block one = days two to four; block two = days five to seven). As we have previously shown effects of Premarin on six-hour delayed memory retention (Engler-Chiurazzi et al., 2011), to test effects of E1 on extended memory retention after only one exposure to the platform location, on day eight rats were tested with a delayed inter-trial interval of six hours. Within treatment group comparisons of performance on trial two of baseline versus trial two of the delay day revealed that no group was impacted by the six-hour delay. Thus, on day nine, rats were tested with a longer delayed inter-trial interval of eight hours. Delayed inter-trial intervals were instituted between trial one (information trial) and trial two. Thus, on these days, animals were given trial one, given the appropriate delayed inter-trial interval, and then given trial two.

Open Field

The open field task evaluated locomotor activity and emotional reactivity in response to being placed in an empty open field (Denenberg, 1969). A black Plexiglas

box measuring 95.75 cm x 95.25 cm x 45.70 cm was utilized. The rat was placed in the apparatus facing the North wall. Each animal received a 10 min session whereby they were allowed to freely explore the box. Between each subject tested, the apparatus was thoroughly cleaned with 70% isopropyl alcohol. Each animal's activity was recorded using Ethovision (XT 5.1, Noldus Information Technology, Wageningenm, Netherlands) and the dependent variable was distance moved (cm). Using the computer system, the open field arena (9120.19 cm^2) was virtually divided into three concentric zones, including an outer (5790.19 cm^2), middle (3078.78 cm^2) and inner (251.63 cm^2) zone. Overall activity (total distance travelled in the box), as well as movement in each zone, were the dependent variables.

Morris Water Maze

The Morris water maze tested spatial reference memory and consisted of a round tub (188 cm in diameter) filled with room temperature water made opaque with black non-toxic paint. Briefly, the rat was placed in the maze from any of four locations (North, South, East, or West) and had 60 sec to locate the platform, which remained in a fixed location (Northeast quadrant). If an animal did not locate the platform within the allotted time limit, it was gently guided to the platform. After 15 sec on the platform, the rat was placed into its heated cage until the next trial. Animals were tested in squads (eight or nine rats in each squad) so that the first trial was completed for each rat in the group, then the second, etc., as done previously (Engler-Chiurazzi et al., 2011; Stavnezer et al., 2002). The testing procedure was based on the work of Markham and colleagues (2002) wherein beneficial effects of 17β-E2 have been noted in rats. Rats received six trials/day

for three days, with a 15 min delay instilled between trials three and four (Markham et al., 2002). There was approximately an eight to ten min inter-trial interval between all other trials. A video camera recorded each rat and a tracking system (Ethovision XT 5.1, Noldus) analyzed each rat's path. The dependent measure was swim distance (cm). To assess platform localization, a probe trial was given on trial seven of the last day of testing, whereby the platform was removed from the maze. For the probe, percent of total swim distance (cm) travelled in the target Northeast quadrant (i.e., quadrant that contained the platform) as compared to the opposite (Southwest) quadrant was the dependent measure (Stavnezer et al., 2002). Additional probe trial dependent variables included the frequency of crossing into the platform zone, the Northeast quadrant, and the Southwest quadrant.

Visible Platform

The visible platform task confirms that animals can perform the procedural components of a water escape task, including visual and motor competence. A rectangular tub (99 cm x 58.5 cm) was filled with clear water. A black platform (10 cm wide) was positioned approximately 3.75 cm above the water surface (Hunter et al., 2003). Opaque curtains covered obvious extramaze cues. Animals were given six trials in one day. The drop off location remained the same across trials, and the platform location for each trial varied semi-randomly. Each rat had 90 sec to locate the platform, where the rat then remained for 15 sec before being placed back into its heated cage. If an animal did not locate the platform within the allotted time limit, it was gently guided to the

platform. The inter-trial interval was approximately eight min. Latency (sec) to reach the platform was the dependent measure.

Tissue Collection, Hormone Assays, and Uterine Weights

The day following the completion of maze testing, all animals (15 months old at this time) were sacrificed on the same day, with researchers blinded to treatment group assignment. Rats were anesthetized with isoflurane, and rats were decapitated. Brains were rapidly removed and the anterior portion of the brain containing the basal forebrain was separated from the posterior portion of the brain. Uterine tissues were collected, trimmed of fat and connective tissue, and weighed as per previous methods (Acosta et al., 2009b). Wet uterine weight (g) was the dependent measure.

Basal Forebrain Choline Acetyltransferase-immunoreactive Neuron Counts

Each brain was post fixed in 4% paraformaldehyde in phosphate-buffer solution (PB, pH 7.4) for 48 hours, and then the tissues were transferred to phosphate buffer until sectioning. The basal forebrain region was sectioned (plates 1-25; Paxinos and Watson, 2005) on a Vibratome 3000 (Vibratome) in phosphate-buffered saline (pH 7.4) at 40 microns for immunohistochemistry (Granholm et al., 2002). Every fourth section through the basal forebrain was selected for the ChAT immunohistochemistry and incubated for 15 min in a 0.03% Triton (Triton X-100) in phosphate buffered saline to permeabilize the tissue. As done previously (Acosta et al., 2009b), the tissue was then blocked, by incubating tissues at room temperature for 30 min in a blocking solution containing 0.03% phosphate buffered saline with Triton and 0.03% heat inactivated horse serum

(Fischer Scientific, Pittsburg, PA). Three phosphate buffered saline washes (3 min each) were then done. The primary polyclonal antibody, goat Anti-Rat-ChAT (1:1000, Millipore, Billerica, MA), was added to each well, and sections were incubated overnight at 4°C on a Rocker II (Boekel Scientific, Feasterville, PA). Next, sections were washed in phosphate buffered saline three times (3 min each) followed by immersion in the secondary antibody solution (1: 200 biotinylated Donkey anti-Goat IgG, Vector) and blocking solution for 45 min on a Titer Plate Shaker (Barnstead International, Dubuque, IO) at room temperature. Sections were washed three times in phosphate buffered saline (3 min each), and then placed into an 11% methanol and 1% H_20_2 (Fischer) in phosphate buffered saline solution for 30 min on a Titer Plate Shaker to quench endogenous peroxidase activity. After three washes in phosphate buffered saline (3 min each), ABC reagent (Vector Laboratories, Burlingame, CA) was added to each well and incubated for 45 min at room temperature on a Titer Plate Shaker. Sections were washed three times in phosphate buffered saline (3 min each), and were then incubated with DAB Peroxidase Substrate (Vector). After the desired color was achieved (dark purple), brain sections were washed three times in phosphate buffered saline (3 min each), mounted on subbed slides, air dried, dehydrated and cover slipped with Permount (VWR, Randor, PA). Each group was equally represented in each round of staining, to avoid group inter-variability in staining. Further, control procedures were run excluding primary and secondary antibodies. Exclusion of the primary antibody resulted in no cell staining, and exclusion of secondary antibody resulted in a lack of DAB Peroxidase Substrate color development.

Basal Forebrain Choline Acetyltransferase Image Analysis

ChAT-IR neurons were quantified in the basal forebrain. Images were acquired using PictureFrame software (MicrobrightField, Burlington, VT) from a CX9000 camera (MicrobrightField) coupled to an Olympus BX51 microscope. A 4x objective was used to capture images (Olympus, Center Valley, PA). Captured images for each section were then manually counted using the "Point Picker" plugin from NIH ImageJ software (Rasband, 1997-2004). Three sections per animal within the range of plates 23-28 from Paxinos and Watson (2005) were quantified similar to prior publications (Gibbs, 1997). ChAT-IR neurons were counted in the MS, and the hDB/vDB, and counts from the three sections were averaged to yield one value per basal forebrain region per animal.

Since 17β-E2 impacts ChAT protein activity (Luine, 1985) and ChAT-IR neuron counts in the basal forebrain (Gibbs, 1997), a group of rats that had been administered continuous subcutaneous Vehicle (propylene glycol) or 17β-E2 were analyzed separately for basal forebrain quantifications to aid in interpretation of potential E1 effects found in the current study. These 15-16 month old rats were given Ovx, and 19 days later, administered an Alzet osmotic pump containing either propylene glycol or 4.0μg/day 17β-E2. The Ovx and pump insertion surgical procedures were similar to those used in the current study, and these animals were behaviorally tested on a cognitive maze battery (unpublished). 17β-E2 treatment was initiated 19±1 days after Ovx in the comparison study, which corresponds exactly to the E1 study whereby E1 treatment was administered 19±1 days after Ovx. For the 17β-E2 treated rats, treatment continued for approximately 50 days, until animals were sacrificed, brains removed, and sections processed via immunohistochemistry for ChAT identical to the methods described for the E1 study.

Statistical Analyses

To determine whether each treatment group showed learning of delayed match to sample and Morris water maze, we first assessed overall performance *within* each treatment group, with Days and/or Trials as the repeated measure. Our a priori interest was to determine the impact that each dose of E1 had on maze performance. Thus, to test the effects of each E1 dose, as compared to Vehicle (Vehicle vs. E1-Low, Vehicle vs. E1-Med, and Vehicle vs. E1-High), two-group planned comparisons were evaluated using an alpha level of 0.05, as Type I error correction is not necessary with orthogonal planned comparisons (Keppel and Wickens, 2004). For delayed match to sample, Morris water maze, and visible platform, data were analyzed using these two-group planned repeated measures ANOVAs with Treatment as the between factor, and Blocks of Days, Days, Trials, and/or Quadrants as the repeated measure, depending on the maze. For open field, data were analyzed using two-group planned comparisons with Treatment as the between factor and distance moved (cm) as the dependent variable. Uterine weight and ChAT-IR analyses were performed using planned t-tests for two-group comparisons. Because treatment with Premarin (Acosta et al., 2009b; Engler-Chiurazzi et al., 2011), 17β-E2 (Talboom et al., 2008), and E1 (Shiverick and Muther, 1982) has been previously shown to increase uterine weights, all analyses of uterine weights were one-tailed. ChAT-IR data in each distinct basal forebrain region from the comparison study using 17β-E2 were analyzed separately from the E1 study. Only significant interactions or main effects are reported.

Results

Vaginal Smears and Uterine Weights

After Ovx (before E1 administration), vaginal smears revealed that all animals exhibited diestrus-like smears, indicating a lack of uterine stimulation, as expected (Goldman et al., 2007). Five days after pump implantation, all Vehicle rats continued to exhibit diestrus-like smears, while E1-treated animals (all doses) alternated between estrus- and metestrus-like smears, with each smear showing numerous cornified cells, indicating uterine stimulation (Goldman et al., 2007). At sacrifice, pump inspection revealed that no pumps were cracked.

For uterine wet weights, as previously reported for E1 (Beyer et al., 1976), each dose of E1 increased uterine weights compared to the Vehicle group [Vehicle vs. E1-Low: $t(14)=11.28$; $p < 0.0001$; Vehicle vs. E1-Med: $t(15)=13.54$; $p < 0.0001$; Vehicle vs. E1-High: $t(15)=9.82$; $p < 0.0001$] (Chapter 5-Figure 1).

Delayed Match to Sample Water Maze

Testing with a 30-second inter-trial interval: Acquisition Effects: To evaluate learning, we analyzed performance within each treatment group from days one to seven (trials two to six), the repeated measure. This analysis revealed a main effect of Day for each group [Vehicle: $F(6,48)=4.81$; $p < 0.001$, E1-Low: $F(6,36)=2.63$; $p < 0.05$, E1-Med: $F(6,42)=3.68$; $p < 0.005$, E1-High: $F(6,42)=4.74$; $p < 0.001$], with errors decreasing as days progressed, indicating learning for each group (data not shown). There was also a main effect of Trial for each group [Vehicle: $F(4,32)=7.57$; $p < 0.0005$, E1-Low: $F(4,24)=8.70$; $p < 0.0005$, E1-Med: $F(4,28)=5.97$; $p < 0.005$, E1-High: $F(4,28)=6.27$; $p <$

0.001], with errors decreasing as trials progressed, indicating learning of the new platform location within a day for each group (data not shown).

Testing with a 30-second inter-trial interval: Treatment Effects: For all days (days one-seven) and trials (trials two-six), no E1 group differed from the Vehicle group. Since we have shown ovarian-hormone effects on this task that are specific to testing phase (Acosta et al., 2009a), we collapsed the data into three-day blocks. There were no significant differences between any E1-dosed group and the Vehicle group during the first testing block (days two to four). During the second testing block (days five to seven), the highest E1 dose impaired performance (Chapter 5-Figure 1a), with the E1-High group making more errors relative to the Vehicle group [Hormone Treatment main effect for Vehicle vs. E1-High: $F(1,15)=6.66$; $p < 0.05$].

Testing with a 6-hour inter-trial interval: No group showed a difference in errors committed on the post-delay test trial on day eight (the six-hour delay) versus that on day nine (the eight-hour delay); thus, we averaged the error scores across the two delays, into one overall delayed inter-trial interval measure. E1-High treatment impaired performance as compared to Vehicle [$F(1,15)=6.77$; $p < 0.05$], suggesting that the E1-High group was impaired on the post-delay trial, relative to the Vehicle group. As we found group differences between the Vehicle and the E1-High groups during the last three-day block of the delayed match to sample task, we then assessed performance on the last three-day block for trial two (baseline) as compared to the combined days of the delay challenge for trial two (the post-delay trial). For this assessment, a repeated measures ANOVA was used for each within group comparison, with Day as the repeated measures. The E1-High treated rats were impaired on the combined delay measure (Chapter 5-Figure 1b), making

more errors relative to their baseline performance on the post-delay trial during the last testing block [$F(1,7)=5.78$; $p < 0.05$]. Neither the Vehicle, E1-Low, or E1-Med groups were impaired by the delay as interpreted relative to their own baseline score (ps>0.50).

Open Field

To determine the impact of E1 on locomotor activity, we assessed distance moved (cm) in the whole open field arena, as well as each of the zones, with Zone as a repeated measure (inner, middle, and outer zones). There were no Hormone Treatment main effects or interactions for locomotor activity in any analyses (data not shown).

Morris Water Maze

Learning Effects: To evaluate learning, we analyzed performance across all days (days one to three), collapsed across trials (trials one to six). There was a main effect of Day for each treatment group [Vehicle: $F(2,16)=33.55$; $p < 0.0001$, E1-Low: $F(2, 12)=27.33$; $p < 0.0001$, E1-Med: $F(2,14)=47.15$; $p < 0.0001$, E1-High: $F(2,14)=29.69$; $p < 0.0001$], with swim distance decreasing as days progressed, indicating learning for each treatment group (data not shown).

Treatment Effects: No dose of E1 impacted overall performance as compared to Vehicle, for all days and trials (ps > 0.10; data not shown). Since we and others have shown that 17β-E2 (Markham et al., 2002; Talboom et al., 2008) and Premarin (Acosta et al., 2009b) can enhance retention of the platform location overnight, we assessed overnight retention here by comparing swim distance from the last trial on the first day (trial six on day one) to the first trial the next day (trial one on day two), as well as from

day two to three (trial six on day two, to trial one on day three). No group showed an increase in swim distance across either overnight interval. When we collapsed the data across the two overnight intervals, to increase power, still no Hormone Treatment main effects or interactions were observed for any E1 dose comparison to Vehicle.

Probe Trial Effects: For the probe trial, for each treatment group, there was a Quadrant effect, with a greater percent swim distance in the Northeast target, versus the Southwest opposite, quadrant [Vehicle: $F(1,8)=42.70$; $p < 0.0005$; E1-Low: $F(1,6)=38.60$; $p < 0.001$; E1-Med: $F(1,7)=94.76$; $p < 0.0001$; E1-High: $F(1,7)=38.03$; $p < 0.0005$]. Thus, all groups localized to the target quadrant (data not shown). There were no Hormone Treatment effects in the frequency of crossings in the Northeast target quadrant, or in the Southwest opposite quadrant, again suggesting that E1 did not affect spatial localization on this task.

Visible Platform

All animals located the platform within 20 sec during trial six confirming that all animals had the visual and motor competence to solve a swimming maze task. No E1 group differed from the Vehicle group on this task (data not shown).

Basal Forebrain Choline Acetyltransferase-immunoreactive Neuron Counts

Chapter 5-Figures 3a and 3b show mean ±SEM MS and hDB/vDB ChAT-IR neuron counts, and Chapter 5-Figures 3c-h are photomicrographs of the basal forebrain for each treatment group. For the MS, E1 did not impact the number of ChAT-IR neurons, as there were no significant pairwise comparisons between the Vehicle group

and any E1-dosed group. However, the comparison study showed that, replicating findings of others (Gibbs, 1997), 17β-E2 increased the number of ChAT-IR neurons in the MS relative to Vehicle treatment [$t(8)=2.34$; $p < 0.05$]. For the hDB/vDB, neither 17β-E2 nor E1 impacted the number of ChAT-IR neurons.

Chapter Summary and Discussion

In the current study, all E1 doses evaluated resulted in the peripheral estrogenic actions expected if estrogenic stimulation did indeed occur. These include increases in uterine weights (Westerlind, 1998) and cornified vaginal smears (Goldman et al., 2007). This confirms that E1 stimulated peripheral tissues, and was present in the E1-treated animals until the end of the experiment. No such effects were seen in Ovx animals given Vehicle treatment, as expected. These findings concur with work showing peripheral stimulation with Premarin treatment in middle-aged Ovx rats (Acosta et al., 2009b; Engler-Chiurazzi et al., 2011). Here, we found that the low and medium doses of E1 did not differ from Vehicle treatment in their impact on spatial memory performance. Yet, the high dose of E1 impaired both late acquisition, as well as retention, on the spatial working memory delayed match to sample task. The observed impairments of E1 therefore do not appear to be generalized to all memory types, as we found specific detriments on spatial working memory and delayed memory retention, with no impact on spatial reference memory, at least as measured on the Morris water maze. Further, in the current report while 17β-E2 increased basal forebrain ChAT-IR neuron counts, as expected based on prior studies (for review see Gibbs, 2010), no dose of E1 impacted this measure when compared to Vehicle treatment. Taken together, these findings extend

those of previous studies and indicate that E1, a primary circulating estrogen present after Premarin administration, impairs spatial working memory and delayed memory retention, and does not alter the number of cholinergic positive neurons in a brain region known to modulate memory, the basal forebrain, as does 17β-E2.

Behavioral findings regarding the impact of E1 on cognition are mixed. Administration of E1 directly into the hippocampus of adult mice post-training improved performance by decreasing the number of retention test trials needed to reach criterion on a T-maze footshock avoidance task (Farr et al., 2000). Behavioral effects may be dose specific, as Barha and colleagues (2009) noted that in adult rats, a subcutaneous E1 injection of 1.0μg impaired contextual fear conditioning, while 0.3μg or 10.0μg had no impact. It is important to note these outcomes reported previously may be mediated by a number of factors including the route of administration (intrahippocampal vs. subcutaneous), the timing of administration (post-training vs. before conditioning), the species (mouse vs. rat), or the specific paradigm wherein E1 is being studied. Further, since in our current study animals were tested on a cognitive maze battery, it is possible that the experience of being tested on multiple mazes influenced performance outcomes, especially on the latter cognitive tests. As such, further evaluation elucidating the potentially interactive effects of previous maze experience and estrogen treatments on memory is an interesting future direction of research. Systematic evaluations of each of these factors are important future directions in characterizing the potential cognitive impact of E1.

In women, Premarin can have beneficial effects on some memory measures; yet, some studies have found that Premarin has null or detrimental effects on memory (for

review see Hogervorst et al., 2000; Sherwin and Henry, 2008). Several factors, including socioeconomic status and age at time of HT treatment, as well as the temporal window of treatment after ovarian hormone loss, can impact the potential cognitive benefits of Premarin in human clinical studies (for review see Hogervorst et al., 2000; Rocca et al., 2010a). Studies using animal models, in which such issues are obviated or can be controlled, have reported beneficial cognitive effects of Premarin. For example, an subcutaneous Premarin injection regimen to middle-aged, Ovx rats benefitted memory retention for objects (Walf and Frye, 2008). We have shown that chronic subcutaneous Premarin injections enhanced spatial working memory, prevented scopolamine-induced amnesia, and improved overnight retention on the Morris water maze in middle-aged Ovx rats (Acosta et al., 2009b). We have also found that Premarin administered continuously via subcutaneous Alzet osmotic pumps to middle-aged, Ovx rats at medium (24μg daily Premarin) and high (36μg daily Premarin) doses enhanced working memory retention (Engler-Chiurazzi et al., 2011). However, the lowest dose (12μg daily Premarin) impaired learning on the delayed match to sample and Morris water maze tasks. We hypothesized that this dose-dependent effect of Premarin was related to the resulting circulating relative levels of E1 and 17β-E2. Indeed, in the same study, the lowest Premarin dose, which impaired memory scores, increased serum E1 but not 17β-E2 levels; whereas, the two higher doses each enhanced performance and concomitantly increased both E1 and 17β-E2. This suggested that elevated levels of E1 in the absence of sufficient 17β-E2, similar to the hormone profile of the postmenopausal woman (Gruber et al., 2002), impairs memory. In line with this, although the previously reported cognitive impacts of E1 are mixed (Barha et al., 2009; Farr et al., 2000), many studies

report enhanced spatial working memory (Bimonte and Denenberg, 1999; Daniel et al., 1997; Fader et al., 1999; Gibbs, 1999; Hruska and Dohanich, 2007) and reference memory (Bimonte-Nelson et al., 2006; Feng et al., 2004; Frick et al., 2004; Markham et al., 2002; Talboom et al., 2008) with subcutaneous 17β-E2 administration to Ovx rats, although this effect appears to depend on task, dose, age, and timing after surgical menopause (for review see Bimonte-Nelson et al., 2010; Daniel and Bohacek, 2010; Frick, 2009; Gibbs, 2010). The current findings build on this previous work, supporting the tenet that E1 can impair spatial memory.

Premarin is a complex mixture of at least 10 different estrogen moieties (Kuhl, 2005). It is therefore possible that some estrogens contained in Premarin, such as $\Delta^{8,9}$-dehydroestrone (Kuhl, 2005), are increased with higher Premarin doses, and, thus, contribute to Premarin's beneficial cognitive effects. Along these lines, we recently showed that middle-aged, Ovx rats treated with $\Delta^{8,9}$-dehydroestrone, but not the Premarin component equilin, enhanced learning on spatial working and reference memory (Talboom et al., 2010). As such, it appears that the issue is quite complex, and likely involves ratios of other steroid hormones. Indeed, E1 can be derived from the androgen precursor, androstenedione (Martini et al., 1993), which we have recently shown to correlate with memory impairment in the rodent at higher physiological levels (Acosta et al., 2010). Collectively, this suggests that cognitive benefits can be realized with estrogen-containing HTs given the "proper" parameters, including a hormone preparation with an optimal ratio of the various estrogens.

It has been established that estrogen and the basal forebrain cholinergic system are each intimately involved in learning and memory (for review see Gibbs, 2010). Basal

forebrain cholinergic neurons project to the hippocampus and surrounding cortical areas (Woolf, 1991), and basal forebrain lesion results in significant spatial memory impairments (Gibbs, 2002). Additionally, basal forebrain ChAT may be related to memory scores, as 2 month old female rats that were significantly impaired on the spatial working memory land radial-arm maze had less ChAT protein activity in the basal forebrain (Luine and Hearns, 1990). Premarin treatment in Ovx rats increased basal forebrain ChAT-IR neuron counts in the vDB, and concomitantly aided spatial working memory and Morris water maze overnight retention (Acosta et al., 2009b). Similarly, 17β-E2 treatment in Ovx rats increases ChAT-IR neuron counts (Gibbs, 1997) and ChAT protein activity (Luine, 1985) in the basal forebrain. Here, in the 17β-E2 comparison evaluation, continuous 17β-E2 treatment increased ChAT-IR cell counts in the MS, as expected. Yet, using the same quantifying procedures, E1 did not impact ChAT-IR cell counts in either the MS or the hDB/vDB regions, at least at the E1 doses tested. Although the duration of hormone treatment in the 17β-E2 comparison study was not identical to the hormone treatment used in the E1 study (treatment was 3 weeks longer in the 17β-E2 comparison study), for both the 17β-E2 ad E1 analyses, the sections were counted by the same experimenter, blind to the treatment group, using the same counting protocol. Thus, the finding that 17β-E2 increased ChAT-IR cell counts in the MS suggests that our counting procedure is effective in detecting significant treatment group differences and adds an important interpretative value for the lack of effects of E1 on this basal forebrain cholinergic system. Taken together, these findings suggest that E1 does not impact the basal forebrain cholinergic system as does 17β-E2 or Premarin. Thus, the negative impact of E1 on cognition may involve other estrogen sensitive neural systems

such as monoamines (Luine, 1998) and/or neurotrophins (Granholm, 2000).

In conclusion, this study demonstrates that the principal estrogen moiety E1, a primary circulating estrogen present after Premarin administration, can impair specific memory domains of spatial memory in middle-aged, surgically menopausal rats. E1 treatment at the doses tested in this study did not impact the number of ChAT-IR neurons in the MS or the hDB/vDB regions, whereas in a comparison study using the same quantification procedures, 17β-E2 increased the number of ChAT-IR neurons in the MS. That we have previously shown Premarin can enhance cognition and increase ChAT-IR basal forebrain neuron number, and now find that the primary circulating estrogen after Premarin treatment, E1, does not have these effects, suggests that previously observed beneficial effects of Premarin on these variables are not likely due to the E1 component alone. Findings from preclinical, interdisciplinary basic science studies can inform the design of specific combinations of estrogens that could be beneficial to the brain and cognition. The results shown here build on the findings of others and suggest that, for cognitive and brain health measures, E1 is not likely one of these key beneficial estrogens.

CHAPTER 6

A PUTATIVE MECHANISM OF ESTROGEN'S IMPACT ON SPATIAL MEMORY: RELATIONS WITH ESTROGEN RECEPTOR-ALPHA

Manuscript Status: In Preparation

Introduction

Estrogens, once thought to only to impact reproductive organs and associated sex behaviors, are now understood to impact substrates of the immune (Oertelt-Prigione, 2012; Zen et al., 2010), and central nervous (Bimonte-Nelson et al., 2010), systems. For instance, many studies report enhanced memory performance on tasks of spatial working memory (Bimonte and Denenberg, 1999; Daniel et al., 1997; Fader et al., 1999; Gibbs, 1999; Hruska and Dohanich, 2007) and spatial reference memory (Bimonte-Nelson et al., 2006; Feng et al., 2004; Frick et al., 2004; Markham et al., 2002; Talboom et al., 2008) following subcutaneous 17β-estradiol (17β-E2) administration to adult ovariectomized (Ovx) rats. However, cognitive responsiveness to estrogen stimulation seems to decline with age, especially on spatial reference memory tasks (Foster et al., 2003; Gresack et al., 2007; Talboom et al., 2008). For instance, the same dose of 17β-E2 treatment that effectively enhanced performance on the Morris water maze among 4 and 16 month old Ovx rats was generally ineffective in 24 month olds (Talboom et al., 2008). Given that estrogen-containing therapies are administered to middle-aged women for the treatment of menopausal symptoms, investigating the cognitive impacts of estrogen treatments and the mechanisms by which they impart these effects during middle-age is an important area of study.

A potential mechanism by which estrogens impact cognitive outcomes is via stimulation of the two nuclear estrogen receptor (ER) subtypes (Kuiper et al., 1996; Kuiper et al., 1997). Both ER subtypes, alpha (ERα) and beta (ERβ), are found in cognitive brain regions associated with learning and memory, such as the hippocampus and basal forebrain (Shughrue et al., 1997). Moreover, the neural expression and distribution of ERs is known to change during aging in humans and rodents (Adams et al., 2001; Ishunina et al., 2007; Mehra et al., 2005; Yamaguchi-Shima and Yuri, 2007), providing a potential mechanism by which cognitive responsiveness to estrogens declines with age.

Findings from studies using selective ER modulators (SERMs) as tools to clarify the mechanism of estrogen action at an individual ER subtype contribute to the complex cognitive role of ERα. In young adult, Ovx rats, acute injections of the ERα agonist, propylpyrazole triol (PPT), either enhanced novel object memory (Frye et al., 2007; Walf et al., 2006) or failed to impact performance on object recognition and object place recognition tasks (Jacome et al., 2010). For spatial memory, PPT also has unclear impacts. In young adult rats, PPT had no impact of the spatial reference memory Morris water maze (Rhodes and Frye, 2006) yet PPT enhanced spatial working memory on the delayed matching to place task (Hammond et al., 2009). Only one study to date has evaluated the impacts of SERMs in middle-aged rats, finding subtle impairments of chronic administration of PPT at the highest dose, 0.2 mg/kg/day, on the spatial working memory delayed alternation (Neese et al., 2010). Thus, the role of ERα in cognitive outcomes, especially during middle-age, remains unclear.

In the current study, we sought to yield further insight into the mechanism by which estrogens impact cognition in middle-aged, Ovx rats using PPT as a tool to preferentially stimulate ERα. Indeed, PPT binds with a 400-fold higher affinity for ERα than ERβ (Stauffer et al., 2000). We assessed memory using a battery of spatial memory tasks shown to be sensitive to aging (Frick et al., 1995; Talboom et al., 2008) and to estrogens (Acosta et al., 2009b; Bimonte-Nelson et al., 2006; Engler-Chiurazzi et al., 2011; Talboom et al., 2010; Talboom et al., 2008).

Materials and Methods

Subjects

Subjects were 23 middle-aged (13 month old) Fischer-344 female rats born and raised at the National Institute on Aging colony at Harlan Laboratories (Indianapolis, IN). Inbred rats were selected given that their low genetic and physiological variability allowed us to utilize a relatively small sample size to generalize observed cognitive impacts of our treatments to the general population (Nadon, 2004b). Animals were acclimated for several weeks at Arizona State University, had exposure to food and water *ad-libitum*, and were maintained on a 12-h light/dark cycle (7am/7pm) at 23°C. Experimental procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and adhered to Guidelines for the Care and Use of Laboratory Animals and National Institutes of Health standards.

Ovariectomy and Treatment

Approximately 28 days before behavioral testing began, under isoflurane anesthesia, all rats underwent Ovx surgery. For Ovx, dorsolateral incisions were made in the skin and peritoneum, and ovaries and tips of uterine horns were ligated and removed. Rats were then separated into the following groups: Ovx with Vehicle polyethylene glycol treatment (Vehicle, n=9), Ovx plus 125μg/day of PPT (PPT-Low, n=7) and Ovx plus 500μg/day of PPT (PPT-High, n=7). Polyethylene glycol was purchase from Sigma (St. Louis, MO), and PPT was purchased from Tocris Bioscience (Elliaville, MO). The doses of PPT were selected based on the 1.0mg/kg PPT dose used in the laboratory of Dr. Robert Handa, a dose shown to induce anxiety-like behaviors, as measured by increased rearing, in the open field test relative to Vehicle-treated animals (Weiser et al., 2009). To evaluate potential dose-related cognitive impacts of PPT, we used two doses of PPT, low and high; the PPT-Low dose was approximately half, and the PPT- High dose was double, the 1.0mg/kg PPT dose used in the previous study (Weiser, et al., 2009). Hormone treatment began approximately 19 days after Ovx, similar to prior studies testing cognitive effects of estrogens (Talboom et al., 2010). In parallel with other studies (Engler-Chiurazzi et al., 2011; Talboom et al., 2010), Vehicle and PPT treatments were administered continuously using Alzet osmotic pumps (Model 2004; Durect Corporation, Cupertino, CA). Briefly, the appropriate dose of PPT was dissolved in polyethylene glycol and inserted into the pumps as per the manufacturer's instructions; for the Vehicle group, pumps were filled with polyethylene glycol only. For pump insertion, under isoflurane anesthesia, a small incision was made in the dorsal scruff of the neck, and a

subcutaneous pocket was created. One pump filled with Vehicle or the appropriate dose of PPT was inserted into the pocket and the skin was stapled.

Verification of Peripheral Estrogenic Stimulation

Because both ER subtypes have been found in the uterus (Kuiper et al., 1997) and as it has been demonstrated that PPT stimulates rodent uterine tissue (Le Saux and Di Paolo, 2005; Morissette et al., 2008), we verified peripheral stimulation via evaluation of traditional markers of estrogenic action including vaginal smears (Goldman et al., 2007) and uterine weights (Westerlind et al. 1998). Smears were classified as indicative of a lack of, or a presence, of uterine stimulation. Eighteen days after Ovx surgeries, and one day before Vehicle or PPT administration via pump implantation, smears were taken to confirm lack of uterine stimulation and complete Ovx, indicated by the presence of leukocytes and a paucity of epithelial and cornified cells.

Delayed Match to Sample Water Maze

The delayed match to sample task assessed spatial working memory. The maze had four arms (each 38.1 cm long and 12.7 cm wide) in a plus configuration and was filled with room temperature water made opaque with black non-toxic paint. The maze had a hidden escape platform at the end of one arm. The platform location changed daily, but was fixed within a day. Each rat received six consecutive trials within a daily session for seven consecutive days. The first trial was the information trial where the rat was exposed to the platform location for that day. Trials two through six were memory test trials, in which the location of the platform was repeatedly reinforced (Engler-Chiurazzi

et al., 2011). Each rat was dropped off in a semi-randomly chosen start arm location and was given a maximum of 90 sec to locate the hidden platform. Once found, the rat remained on the platform for 15 sec and was then placed into a heated cage for a 30 sec inter-trial interval. An arm entry was counted when the tip of a rat's snout reached a mark delineated on the outside of the arm (11 cm into the arm). Entry into an arm with no platform was counted as an error, the dependent variable. To test memory retention across an extended inter-trial interval, rats were tested with a six-hour delay (day eight) and eight-hour delay (day nine). Since the working memory test trial (trial two) is the first trial to test recall of the updated information (working memory), the delays were given between trial one and trial two to determine whether the increased inter-trial interval impacted, specifically, memory retention after only one exposure to the platform location. Thus, on the delay days, animals were given the information trial (trial one), given the appropriate delayed inter-trial interval, and then given the post-delay working memory test trial (trial two) as done previously in our laboratory (Engler-Chiurazzi et al., 2011). On the post-delay trial, total errors were the dependent variable.

Open Field

On the day following the completion of delayed match to sample testing, each rat was tested on the open field task. The open field task evaluated locomotor activity and emotional reactivity levels (Denenberg, 1969). A black Plexiglas box measuring approximately 95.75 cm x 95.25 cm x 45.70 cm, was placed upon a plywood table. Eight desk lamps (60 watt each), four placed on the table and four placed on the floor, were used to provide indirect room lighting. All lamps were directed towards the walls and

away from the open field box. The rat was placed in the apparatus on the outside edge of the arena facing the North wall. Each animal received a ten min session whereby they were allowed to freely move within the box. Between each subject, the apparatus was thoroughly cleaned with 70% isopropyl alcohol. Each animal's locomotor activity was recorded using Ethovision (Noldus Information Technology, Wageningenm, Netherlands). Using the computer system, the open field arena (9120.19 cm^2) was virtually divided into three concentric zones including the outer frame (5790.19 cm^2), middle frame (3078.78 cm^2) and center zone (251.63 cm^2). The dependent variables were distance moved (cm) in the whole arena as well as in each of the zones, frequency of rearing, defined as a rat sitting up on its hind legs and sniffing (Weiser et al., 2009), and number of fecal boli excreted.

Morris Water Maze

The Morris water maze (Morris et al., 1982) tested spatial reference memory. This task consisted of a round tub (188 cm in diameter) filled with room temperature water made opaque with black non-toxic paint. Morris water maze testing began on the day following open field testing. For the testing procedure, rats received six trials/day for three days, with a 15 min delay instilled between trials three and four. This procedure was used in a previous study which reported beneficial effects of 17β-E2 (Markham et al., 2002). Briefly, for each trial, the rat was placed in the maze from any of four locations (North, South, East, or West) and was allowed 60 sec to locate the platform, which remained in a fixed location throughout all days and trials of testing (Northeast quadrant). If the rat failed to locate the platform during the allotted trial time, it was

gently led to the platform location. After 15 sec on the platform, the rat was placed into its heated cage until the next trial. Animals were tested in groups of eight to nine rats (all treatments was represented in each testing group) so that the first trial was completed for each rat in the testing group, then the second trial, etc., as done previously (Stavnezer et al., 2002). Thus, with the exception of the additional 15 min delay between trials three and four, there was approximately an eight to ten min inter-trial interval between all trials. A video camera recorded, and a tracking system (Ethovision XT 5.1, Noldus Information Technology, Wageningen, Netherlands) analyzed, each rat's path. The dependent measure was distance moved (cm). To assess platform localization, an additional trial whereby the platform was removed from the maze, referred to as a probe trial, was given on trial seven of the last day of testing. For the probe trial, percent of total distance (cm) moved in the target Northeast quadrant (i.e., quadrant that contained the platform) vs. the opposite Southwest quadrant was the dependent measure (Stavnezer et al., 2002). Additional probe trial dependent variables included the frequency of crossing into the platform zone, the Northeast quadrant, and the Southwest quadrant.

Visible Platform

This task confirmed that animals were able to perform the procedural components of water escape tasks by verifying rat visual and motor competence. A rectangular tub (99 x 58 cm) was filled with clear, room temperature water. A black platform (ten cm wide) was positioned approximately four cm above the water surface following previously published methods (Hunter et al., 2003). Opaque curtains covered all extramaze cues. Animals were tested on the day following the completion of Morris

water maze testing and were given six trials for one day. The drop off location remained the same across trials, and the platform location for each trial varied semi-randomly. Each rat had 90 sec to locate the platform. Once the platform was located, the rat remained on it for 15 sec before being placed back into a heated cage. The inter-trial interval was five to eight min. Latency (sec) to reach the platform was the dependent measure.

Tissue Collection and Uterine Weights

On the day following the end of maze testing, all subjects were sacrificed by researchers blinded to treatment group assignment. Rats were anesthetized with isoflurane, blood was taken via cardiocentesis, and rats were decapitated. Uterine tissues were collected, trimmed of fat and connective tissue, and weighed (g) per prior protocol (Acosta et al., 2009b; Ashby et al., 1997; Engler-Chiurazzi et al., 2011; Talboom et al., 2010). Wet uterine weight (g) was the dependent measure.

Statistical analyses

To test treatment group differences, our a priori interest was to determine the impact that each dose of PPT had on maze performance, as compared to Vehicle (Vehicle vs. PPT-Low and Vehicle vs. PPT-High). These two-group planned comparisons were evaluated using an alpha level of 0.05 as described previously (Keppel and Wickens, 2004). Maze data were analyzed with repeated measures ANOVAs with Treatment as the between variable and Blocks of Days, Days, Trials, and/or Quadrant as the repeated measure, unless otherwise noted. Because we expected an increase in uterine weights

following PPT treatment (Saux and Di Paolo, 2005), one-tailed analyses were performed for uterine weight two-group planned comparisons.

Results

Vaginal Smears and Uterine Weights

After Ovx but before PPT administration, vaginal smears showed that all animals were in diestrus, characterized by few leukocytes. At sacrifice, uterine horns were examined and lack of ovary was confirmed. For uterine weights (Chapter 6-Figure 1), both doses of PPT increased uterine weights relative to Vehicle [Vehicle vs. PPT-Low: $t(14)=3.25$; $p < 0.01$; Vehicle vs. PPT-High: $t(14)=2.20$; $p < 0.05$].

Delayed Match to Sample Water Maze

Testing with a 30-second inter-trial interval: There were no Treatment interactions with Days and/or Trials nor Treatment main effects for total errors on days one to seven, trials two to six. As we have shown ovarian hormone-induced effects specific to testing phase (Acosta et al., 2009a), we grouped the data into two three-day blocks. Planned comparisons of each dose of PPT versus Vehicle during each testing block revealed a Treatment main effect for Block 2 (days five to seven) of testing [Vehicle vs. PPT-Low: $F(1,14)=4.32$; $p < 0.06$; Vehicle vs. PPT-High: $F(1,14)=5.72$; $p < 0.05$] such that PPT-Low marginally, and PPT-High significantly, impaired performance (Chapter 6-Figure 2). When animals receiving both doses of PPT were combined, this PPT-treated group committed more errors than Vehicle-treated rats [$F(1,21)=6.21$, $p < 0.05$].

Testing with a 6-hour inter-trial interval: PPT did not impact delay performance. To determine if any treatment group was impacted by the extended inter-trial interval between the information and working memory trials, within each treatment group, we compared performance on the working memory trial of the last three-day block to performance on the working memory trial of the six- and the eight-hour delay, with Days as the repeated measure. As evidenced by non-significant effects of Days, no treatment group was impaired by either the six- (Vehicle: $p > 0.61$; PPT-Low: $p > 0.19$; PPT-High: $p > 0.38$) or the eight-hour (Vehicle: $p > 0.49$; PPT-Low: $p > 0.56$; PPT-High: $p > 0.85$) delay compared to the final testing block. Additionally, there were no main effects of Treatment for the working memory trial of the six-hour delay (mean±SEM = Vehicle: 0.67±0.29; PPT-Low: 0.57±0.43; PPT-High: 1.43±0.53) or the eight-hour delay (mean±SEM = Vehicle: 0.67±0.24; PPT-Low: 1.71±1.06; PPT-High: 0.86±0.26).

Open Field

Across the ten-min trial, Treatment did not impact overall locomotor activity as measured by distance moved (cm) in the arena (mean±SEM = Vehicle: 3001.63±252.65; PPT-Low: 2796.93±221.03; PPT-High: 2576.87±432.55). PPT-Low marginally increased the frequency of entry into the outer zone [$F(1,14)=3.51$; $p < 0.09$] (Chapter 6-Figure 3a). PPT treatment did not significantly impact the number of rears although both doses of PPT reduced rearing behavior (Chapter 6-Figure 3b), as predicted from findings of Weiser et al (2009),. As an additional measure of anxiety-like responsivity (Denenberg, 1969), we evaluated the number of fecal boli excreted while in the open field. PPT-Low increased fecal boli excreted [$F(1,14)=5.27$; $p < 0.05$] (Chapter 6-Figure 3c). Visual

inspection of the graph suggested that both doses of PPT increased the number of fecal boli. Thus, we assessed the impact of PPT (doses combined) on fecal boli excreted. This combination PPT analysis showed an increased number of fecal boli excreted in PPT-treated animals [$F(1,21)=5.55$; $p < 0.05$].

Morris Water Maze

Treatment with PPT imparted transient modest benefits for Morris water maze performance. We analyzed swim distance (cm) performance on days one to three, trials one to six, and no Treatment main effects or interactions with either Days of Trials were observed. For forgetting across the 15 min delay, we compared performance on trial three vs. trial four collapsed across all testing days (Chapter 6-Figure 4). There was a Treatment x Day x Trial interaction for the Vehicle versus PPT-Low comparison [$F(2,28)=4.14$; $p < 0.05$]. When we further probed this interaction by assessing performance across the 15 min delay on each testing day, there was a Treatment x Trial interaction on Day 1 [$F(1,14)=5.99$; $p < 0.05$]. For overnight forgetting, comparing performance on trial one vs. trial six of the previous day, Treatment did not interact with Days or Trials.

For the probe trial, there was a Quadrant main effect in the absence of Treatment main effects or interactions with Quadrant, with a greater percent swim distance in the Northeast (target) versus the Southwest (opposite) quadrant [Vehicle vs. PPT-Low: $F(1,14)=81.01$; $p < 0.0001$ Vehicle vs. PPT-High: $F(1,14)=119.51$; $p < 0.0001$]. This indicates that all rats swam a greater distance in the target quadrant regardless of treatment condition.

Visible Platform

When we assessed latency to reach the escape platform on trials one through six, there was a main effect of trials [Vehicle vs. PPT-Low: $F(5,70)=3.06$, $p < 0.05$; Vehicle vs. PPT-High: $F(5,70)=2.63$; $p < 0.05$] but there were no effects of Treatment, suggesting that all rats improved across trials. These data confirm that all animals had the visual and motor competence to solve a swimming maze task.

Chapter Summary and Discussion

Here, in middle-aged Ovx rats, we continuously administered PPT, the selective ERα modulator (Stauffer et al., 2000). We found that PPT treatment induced modest impairments of spatial working memory during the second testing block on the delayed match to sample task. That we found there were PPT-induced impairments during the second testing block, but not when memory was challenged by an extended delay between the information and working memory trials, suggests that impairments were specific to task learning. In addition, that PPT impaired performance only in the second testing block suggests that the PPT-high treatment did not influence initial task acquisition. Rather, data suggest that the PPT-High treatment specifically impaired later task learning as PPT-high treated animals failed to reach asymptotic performance levels. As treatment did not impact locomotor activity on the open field nor the rate of non-spatial visible platform task acquisition, the spatial working memory impairments of PPT are not likely due to motor or visual system changes with age or treatment. The PPT-induced spatial working memory delayed match to sample task impairment in middle-

aged rats shown here is noteworthy given that Hammond et al (2009) reported enhancements on the working memory delayed matching to position T-maze in young adult, Ovx rats using a similar method of PPT administration. Together, this suggests, that for spatial working memory, the cognitive effects of PPT may change during aging.

On the spatial reference memory Morris water maze, the PPT-Low treatment appeared to exert modest, very brief improvements of short-term retention of the platform location. The Treatment x Trial interaction was only found on the first testing day and did not persist throughout the remainder of testing. Thus, PPT seems to impart only limited, transient benefits to spatial reference memory in middle-aged Ovx rats after instillation of a short delay in inter-trial interval. Providing further support for the limited duration of these minor beneficial effects, on the probe trial, there were no differences in the extent of platform localization, suggesting that all rats, regardless of treatment group, learned the platform location. That here, in middle-aged Ovx rats, and elsewhere, in young adults (Rhodes and Frye, 2006), no impairments on the reference memory Morris water maze have been observed suggests that the PPT-induced memory impairments observed in the current study on the delayed match to sample task were memory type- and task-specific.

From our measures of peripheral estrogenic action and anxiety behavior, our findings suggest that PPT treatments were imparting effects. Given that ERα is present in uterus (Kuiper et al., 1997), it was not surprising that we found PPT-induced increases in uterine weights, as others have shown previously (Le Saux and Di Paolo, 2005; Morissette et al., 2008). This indicates that our PPT treatments induced peripheral estrogenic stimulation, which was present in PPT-treated animals at the completion of the study. PPT did not influence overall locomotor activity in the open field, indicating that

our observed behavioral findings were not likely due to changes in activity or exploration. The PPT-Low treatment tended to increase the frequency of outer zone entry, suggesting anxiogenic effects. As well, visual inspection of the graph revealed that our PPT treatments reduced number of rears, as expected given previous findings (Weiser et al., 2009). However, here, neither PPT group significantly differed from Vehicle in impact on rearing behavior in the open field. Thus, we failed to replicate the PPT-induced anxiogenic findings of Weiser and colleagues (2009), the study upon which our doses were based. However, evidence from increased fecal boli excretion indicates that PPT did induce anxiety-like behavior in the PPT-treated rats. Together, these trends suggest that the PPT-treatments we administered imparted expected physiological effects but that the PPT-induced deficits on spatial memory should be interpreted with caution given the lack of significant results on open field anxiety behavior.

Existing literature examining the roles of ERs on cognitive function suggests that ERα is has limited involvement with cognitive and memory performance in young adult rats. Both ERα and ERβ are found throughout the rat central nervous system (Österlund et al., 1998; Shughrue et al., 1997), with ERα more abundant in non-cognitive brain regions such as the hypothalamic nuclei and ERβ more abundant in cognitive brain regions such as the hippocampus. Studies using knockout mice have revealed that ERα knockout mice are able to learn spatial memory tasks such as the Morris water maze (Fugger et al., 1998), and that treatment with 17β-E2 can improve hippocampal-dependent Y-maze performance in ERα, but not ERβ, knockout animals (Liu et al., 2008). These findings suggest that ERα activation is not necessary for spatial learning

and memory nor for memory enhancements following 17β-E2 treatment. Yet, studies using PPT are inconsistent in findings regarding selective ERα stimulation. For instance, PPT also does not appear to influence inhibitory avoidance when given via a single subcutaneous post-training 10ug injection (Rhodes and Frye, 2006). Similarly, for object memory, PPT given either via an acute pre-training subcutaneous injection regimen (3 or 5mg/kg) to young adult rats had no impact on visual object recognition or place object recognition (Jacome et al., 2010). In the same study, PPT given as a single subcutaneous injection (1mg/kg) immediately following training also failed to enhance discrimination on these object tasks. Yet, Frye and colleagues (Walf et al., 2006, Frye et al., 2007) have found that a single post-training injection of PPT (0.9mg/kg) can enhance object recognition and place object recognition in young adult, Ovx. Further, for spatial memory, although post-training PPT (10ug injection) fails to impact spatial reference Morris water maze performance (Rhodes and Frye, 2006), another study noted that PPT, administered continuously via an osmotic pump (5μg per day) before training, enhanced spatial working memory on the delayed matching to position T-maze (Hammond et al., 2009), suggesting that the role of ERα in learning and memory may be task specific in young adult, Ovx rats. Taken together, in young adult animals, the impact of PPT, and the role of ERα, for cognition remains unclear.

Interestingly, the few studies testing PPT treatment in middle-aged animals suggest that ERα stimulation could impart memory impairments. Neese et al (2010) were the first to utilize a middle-aged, Ovx rodent model to evaluate the cognitive impacts of SERMs, finding that chronic subcutaneous injections of PPT resulted in subtle impairments on the operant spatial working memory delayed alternation (Neese et al

2010). Here, in middle-aged, Ovx rats, we found that continuous PPT impaired spatial working memory. Together, these collective findings suggest age could be an important factor when assessing the mnemonic impact of ERα stimulation via PPT. Yet, in addition to age, it is possible that dose of PPT impacted the realization of cognitive benefits among Ovx rats. In the current study, continuous PPT-High treatment administered via a subcutaneous osmotic pump (2.0 mg/kg/day; 500μg/day assuming a 250g rat) impaired spatial working memory on the delayed match to sample task. Yet, a lower dose of PPT administered continuously (5μg/day) to young adult, Ovx rats enhanced working memory on the delayed matching to position T-maze (Hammond et al., 2009). Future studies should evaluate the impact of lower doses of continuous PPT in the middle-aged rodent model to clarify this important issue.

In conclusion, the findings here indicate that continuous PPT, a selective ERα modulator, could impair late learning of the spatial working memory delayed match to sample task in middle-aged Ovx rats. However, further studies are necessary to better define these potential effects. Moreover, these spatial working memory impairments following PPT administration were found in middle-aged rats. Whereas other work has reported benefits on a similar task in younger animals, these findings suggest that the effects of ERα stimulation differ among unique age-groups. However, certain outcomes in the current experiment, namely the lack of open field anxiety replication, limit generalization of these findings. Additional studies isolating the factors that could have influenced the outcomes observed here, primarily subject age and treatment dosage, should be conducted to further clarify the role of ERα for cognition in middle-age.

CHAPTER 7

GENERAL SUMMARY AND DISCUSSION

The population of aging women in the United States is increasing (US Census, 2008). Each of these women will undergo the menopausal transition, accompanied by cognitive decline and other symptoms that reduce quality of life (Freedman, 2002; Sherwin and Henry 2008). Premarin is the most widely used hormone therapy (HT) for the treatment of menopausal symptoms in North America (Hersh et al., 2004). Clinical and preclinical research assessing its cognitive effects have yielded mixed results (Hogervorst et al., 2000; Sherwin and Henry, 2008). Elucidating factors that influence the cognitive and neurobiological effects of menopausal HT represents an important need relevant to every aging woman. To this end, the work contained in this dissertation has supported the hypothesis that multiple factors, including post-treatment circulating estrogen levels, experimental handling, type of estrogen treatment, and estrogen receptor (ER) activity, can impact the realization of cognitive benefits with Premarin. A summary of the research questions and findings from this dissertation are presented in Chapter 7-Table I. Information gathered from these studies can enable the development of future HTs in which these parameters are optimized.

It has been hypothesized that one of the many factors that influences the cognitive impact of Premarin-containing HT is route of administration (Sherwin and Henry, 2008). Prior studies evaluating Premarin have reported beneficial effects for object and spatial memory with either an acute (Walf and Frye, 2008) or chronic (Acosta et al., 2009b) subcutaneous injection regimen in middle-aged, ovariectomized (Ovx) rats. These beneficial findings in rats conflict with the null or detrimental cognitive effects of

Premarin-containing HT reported in the Women's Health Initiative Memory Study (Espeland et al., 2004; Shumaker et al., 2004; Shumaker et al., 2003) and indicate that Premarin could indeed result in benefits under optimal conditions. Extending this knowledge, findings from Chapter 2 of this dissertation noted dose-dependent spatial working memory enhancements of Premarin administered in a continuous subcutaneous regimen (Engler-Chiruazzi et al., 2011). Interestingly, memory benefits were only realized with the doses that resulted in elevated circulating 17β-estradiol (17β-E2) *and* estrone (E1) levels. Conversely, the only dose to impair memory performance was also the only dose to elevate circulating levels of E1 in the absence of elevated 17β-E2. Thus, data from this chapter indicate that non-optimal ratios of circulating estrogens impair spatial memory. Whether cognitive impairments could occur with a non-optimal, high E1 to 17β-E2 ratio resulting from a subcutaneous injection regimen like that used by Acosta and colleagues (2009b) is not known and is an important future direction in clarifying the cognitive role of circulating ratios of estrogens following HT.

Further clarifying the mnemonic impact of route of Premarin administration, in Chapter 3-Study 1, we assessed Premarin administered to middle-aged, Ovx rats via an oral route. The oral route of administration was the route used in the Women's Health Initiative Memory Study (via a daily pill), which reported null and detrimental cognitive effects of Premarin-containing treatments (Espeland et al., 2004; Shumaker et al., 2004; Shumaker et al., 2003). We found that orally administered Premarin impaired spatial working memory in middle-aged Ovx rats, supporting the hypothesis that route of administration was a crucial factor in determining the cognitive outcome of a Premarin-containing HT. However, experimental handling procedures can influence spatial

memory performance, independent of hormone treatment (Bohacek and Daniel, 2007). Thus, another factor that could have contributed to the detrimental memory findings in Chapter 3-Study 1 was the handling associated with oral treatment administration. Given that this handling differs from handling associated with subcutaneous injections (Acosta et al., 2009b) and the little handling required with subcutaneous continuous osmotic pumps (Engler-Chiurazzi et al., 2011), in Chapter 3-Study 2, we isolated the distinct memory impact of oral handling from that of Premarin treatment. To accomplish this, we methodically manipulated oral handling experience among rats given continuous subcutaneous Premarin using a method and dose we have previously found to benefit maze performance. We found that oral-associated handling impaired spatial working memory performance. As well, among Premarin-treated rats, oral-associated handling enhanced overnight retention of non-spatial information. Thus, the findings in Chapter 3-Study 2 suggest that the spatial working memory impairing effects of Premarin reported in Chapter 3-Study 1 were likely not due to the oral route of administration.

An additional methodological difference between the studies benefitting memory performance and those showing detrimental mnemonic effects following Premarin treatment is the difficulty of the memory task utilized. Indeed, the previously reported working memory enhancements of subcutaneous Premarin were found using a 4-arm version of the delayed match to sample task (Engler-Chiurazzi et al 2011). Yet, orally-administered Premarin (Chapter 3-Study 1) and oral handling (Chapter 3-Study 2) impaired performance on an 8-arm version of this same task. Thus, differences in the difficulty of the tasks used could also partly account for the conflicting outcomes of these studies. When we systematically evaluated the impact of handling and task difficulty in

Chapter 4, we found that among unhandled rats, 36μg subcutaneous Premarin, the dose with which we have shown benefits memory (Engler-Chiurazzi et al., 2011), enhanced working memory performance on the 4-arm, but impaired performance on the 8-arm, maze. Interestingly, these task-dependent effects of Premarin treatment were not observed among the acclimation and oral handled groups. In fact, both acclimation and oral handing obviated the beneficial effects of Premarin in the 4-arm task *and* obviated the detrimental effects of Premarin on the 8-arm task. Together, these data suggest that specific parameters regarding the handling experience and the difficulty of the cognitive task, but not route of administration, are necessary for the realization of Premarin-induced learning and memory benefits.

Another factor that may influence the cognitive outcome of Premarin is the unique cognitive and neurobiological actions of the specific estrogen types contained within it. Indeed, Premarin is over 50% E1-sulfate, but also is a complex mixture containing at least ten estrogens (Kuhl, 2005). Each of these estrogen subtypes differ in several ways including structure, ER binding affinity, and potency (Kuhl, 2005). These differences may partly explain the diversity of cognitive effects following treatment with estrogen-containing therapies reported in the literature. For instance, while exogenous 17β-E2 is used in most studies in middle-aged women and rodents that report cognitive benefits following HT, studies using Premarin have yielded conflicting findings (Bimonte-Nelson et al., 2010; Sherwin and Henry, 2008). In Chapter 5, we characterized the cognitive effects of E1. Although our laboratory has reported memory benefits with another Premarin component, $\Delta^{8,9}$-dehydroestrone (Talboom et al., 2010), E1 imparted spatial working memory impairments in middle-aged, Ovx rats. As well,

mechanistically, unlike other studies finding an increased number of basal forebrain choline acetyltransferase-immunoreactive (ChAT-IR) neurons following treatment with 17β-E2 (Gibbs, 1997) and Premarin (Acosta et al., 2009b), we found that E1 had no impact on the number of cholinergic ChAT-IR neurons in either sub-region of the basal forebrain. This is especially noteworthy given that following Premarin administration, and the removal of the bound sulfate group via hepatic metabolism (Kuhl, 2005), levels of E1 increase in serum (Acosta et al., 2009b; Yasui, 1999). As well, E1 is a component of the tri-estrogen bioidentical hormone therapy (BHT), Triest, a menopause treatment option that has experienced a recent surge in popularity despite a lack of objective evaluations of the long-term physiological and cognitive consequences of BHTs (Cirigliano, 2007). Thus, individual estrogens appear to exert unique impacts of cognitive performance and the brain. Findings from studies systematically evaluating the distinct contributions of these individual estrogens have meaningful implications for the composition of future HTs, informing the design of specific combinations of estrogens that could be beneficial to the brain and cognition. Importantly, these results suggest that, for cognitive and brain health measures, E1 is not likely one of these key beneficial estrogens and should be omitted from future HT options.

From a mechanistic approach, the diverse cognitive effects of estrogens and estrogen formulations are thought to be mediated by ligand interactions with the classical, nuclear ERs, alpha (ERα) and beta (ERβ). Both ER subtypes are localized to cognitive brain regions associated with learning and memory, such as the hippocampus and basal forebrain (Shughrue et al., 1997). Findings from some studies have indicated a role for ERβ in memory enhancements following estrogenic treatment. Specifically, Rissman

(2002) found that 17β-E2 impaired learning on the Morris water maze in ERβ knockout mice compared to estrogen-treated wild type controls. Similarly, ERβ knockout mice given 17β-E2 were impaired on the Y-maze, exhibiting a lower percentage of trials without an error than wild type and ERα knockout mice receiving the same treatment (Liu et al., 2010), further supporting the requirement of ERβ, and but not ERα, for 17β-E2-induced spatial memory enhancements. Interestingly, other findings highlight the importance of ERα in memory function. Foster and colleagues (2008) used a lentiviral vector to restore ERα expression in adult Ovx, ERα knockout mice, finding that increased ERα expression in these animals enhanced spatial reference memory Morris water maze performance compared to that of ERα knockout controls.

Studies using selective ER modulators (SERMS) as tools to evaluate the impact of ER stimulation in young adult rats have also imparted mixed mnemonic effects. For instance, there is disagreement regarding the impact of SERMS on object memory, with some studies reporting that both propylpyrazole triol (PPT) and diarylpropionitrile (DPN) enhance performance, and others reporting that either DPN or PPT, but not both, impart benefits (Frye et al., 2007; Jacome et al., 2010; Walf et al., 2006). As well, findings on spatial memory tasks are also inconsistent. For example, on the Morris water maze, DPN benefitted, while PPT failed to impact, spatial reference memory performance (Rhodes and Frye, 2005). Conversely, PPT, DPN and 17β-E2 each enhanced spatial working memory performance on the delayed matching to place task (Hammond et al., 2009). Given that estrogen-containing menopausal treatments are commonly prescribed to middle-aged women, it is clinically relevant to evaluate the cognitive impact of ER

stimulation in middle-age menopause models. Yet, the small, but growing literature regarding the cognitive impact of ER stimulation in middle-aged is conflicting. Recently, Foster and colleagues have proposed that changes in ER ratios during aging may account for these observed findings (Foster, 2012) and suggest that increasing ERα expression may ameliorate age-related cognitive decline. Initial support for this hypothesis is promising, given that transient 17β-E2 exposure in middle-age imparted long-lasting protection against age-related memory decline on the radial arm maze and increased hippocampal ERα expression (Rodgers et al., 2010). As well, directly increasing hippocampal ERα expression in middle-aged Ovx rats via a lentiviral vector improved spatial working memory on the radial arm maze (Witty et al., 2012). However, findings from studies using PPT suggest that memory impairments are associated with SERM-induced ERα stimulation in middle-aged, Ovx rats. Indeed, in Chapter 6, we found that stimulation of ERα using PPT is associated with spatial working memory impairments on the delayed match to sample task. These findings coincide with those from the only other study to assess the cognitive impacts of PPT-driven ERα modulation in middle-aged Ovx rats, which noted delayed alternation impairments (Neese et al., 2010). Thus, the conflicting findings from these studies indicate that the relationship between memory outcomes, ERs, ER ligand stimulation, and aging is complex and requires further investigation to uncover clinical applications for ER-targeted interventions.

Accumulating findings in translational neuroendocrinology have laid the groundwork for exciting future directions in the field of women's health. An overarching goal of this research is to develop menopausal HT options that are optimal for both the peripheral physiological symptoms, and the cognitive consequences, of menopause-

related hormone loss. To this end, an important research direction is to continue to isolate and assess the unique cognitive and neurobiological contributions of biologically relevant estrogens. Substantial work has evaluated the impact of 17β-E2 on memory performance and the aging brain (Acosta et al., 2013). As well, recent interest in the impact of E1 has identified this estrogen as a sub-optimal treatment option for the realization of memory benefits. Given the increasing popularity of BHT as an alternative to Premarin-containing formulations, there is a clinical need to evaluate the cognitive impact of exogenously administered estrogens that are found naturally in the female body. Estriol (E3), generally thought of as an estrogen of pregnancy (Gruber et al., 2002), is biologically weak due to its short interactions at the ER (Kuhl, 2005; Sitruk Ware, 2002). Although levels diminish during the menopausal transition, E3 is still present in circulation during aging (Gruber et al., 2002. Treatment with E3-containing formulations is reported to relieve peripheral menopausal symptoms such as hot flashes and vaginal dryness (Kuhl, 2005). However, little is known regarding the cognitive impacts of E3; E3 has yet to be methodically evaluated in the middle-aged, Ovx rat model. Thus, before clear interpretations can be made regarding the cognitive impacts of BHTs composed of several estrogens, the next step is to characterize the unique cognitive impacts of E3 and compare these outcomes with those of the more well-studied endogenous estrogens, 17β-E2 and E1. Further, the interactive cognitive effects of exogenous administration of several naturally-circulating estrogens, as done in BHT, is completely unexplored. Therefore, a parallel translational research direction will be to evaluate the mnemonic effects of clinically-used combinations of these hormones.

In conclusion, the study of women's health during aging is a clinically relevant and important research topic, yet options for menopausal HTs with optimal cognitive outcomes are limited. Data from studies here demonstrate that the most commonly utilized HT Premarin, has inconsistent impacts on cognition and memory performance that depend, at least partly, on the ratio of resulting circulating estrogens, the complexity of the task being assessed, and the handling associated with the experimental manipulations. Further, mechanistic findings suggest that each estrogen in circulation following Premarin treatment may distinctly impact memory performance. The unique mnemonic impacts of these individual estrogenic Premarin components may be in part due to the specific actions of each on ERα and ERβ. Taken together, data from this dissertation indicate that Premarin is not an ideal HT for the treatment of cognition-related menopausal symptoms. However, this work has also elucidated several important factors that impact the realization of memory benefits, and can inform the development of novel treatment options that are more optimal for cognitive outcomes. If optimal cognitive outcomes are a desired goal for treatment of menopausal symptoms, the data contained herein suggests that future HT options exclude estrogens or estrogenic compounds that impair memory, such as E1, or preferentially stimulate ERα, such as PPT.

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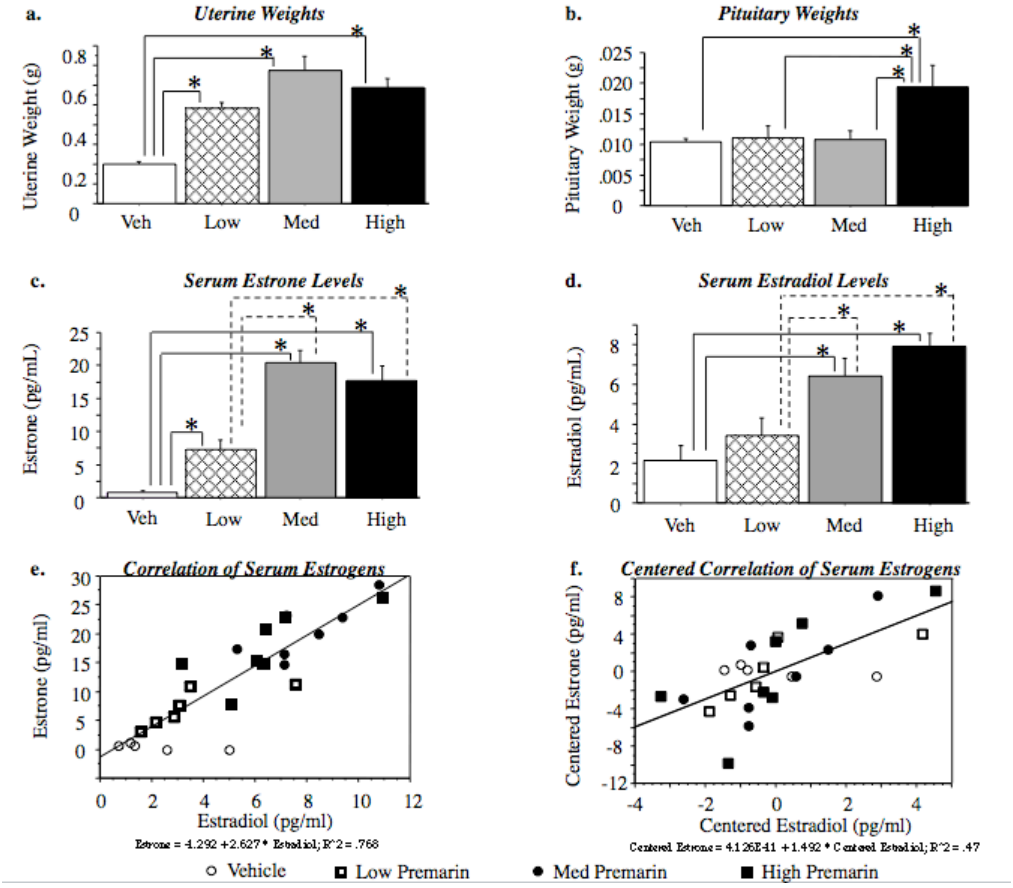
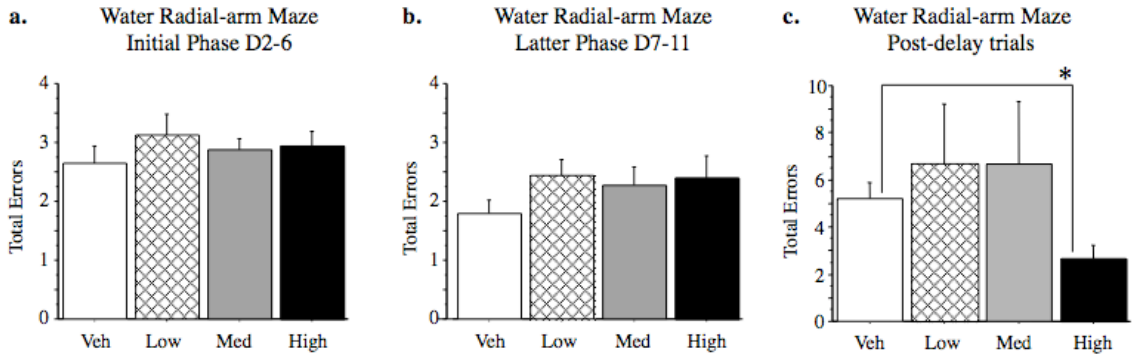
Chapter 2-Figure 1: Measures of Peripheral Estrogenic Stimulation



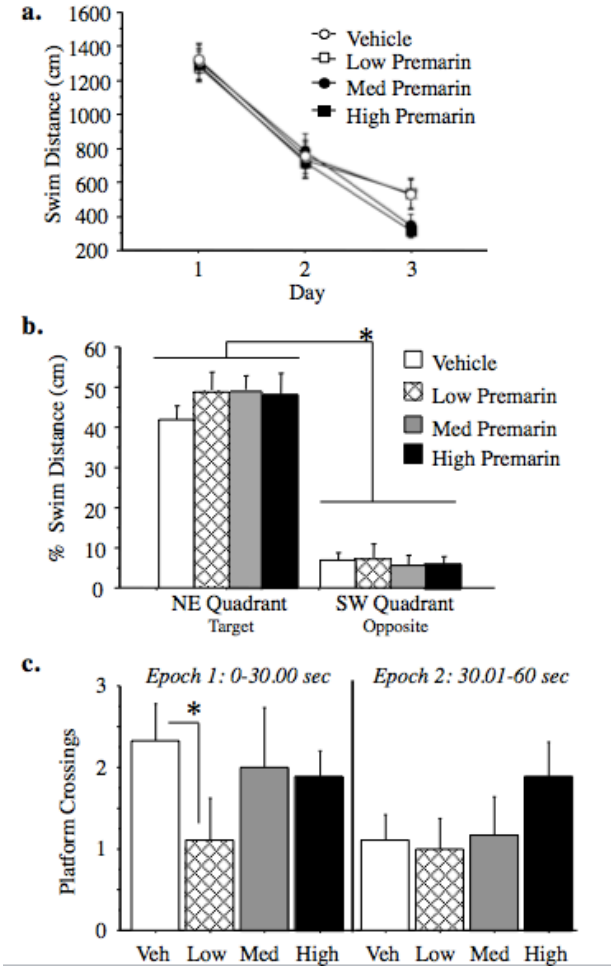
Chapter 2-Figure 1. a) Mean (±SEM) uterine weights (g). All doses of Premarin increased uterine weights relative to untreated controls. b) Mean (±SEM) pituitary weights (g). The highest dose of Premarin increased pituitary weights as compared to all other groups. c) Mean (±SEM) serum E1 (pg/ml). All doses of Premarin increased serum E1 levels relative to controls. Medium (dashed lines) and high (dashed lines) Premarin doses produced significantly higher E1 levels than the low dose. d) Mean (±SEM) serum 17β-E2 (pg/ml). Medium and high, but not low, dose Premarin increased serum 17β-E2 as compared to Vehicle controls. Medium (dashed lines: $p < 0.01$) and high (dashed lines) Premarin doses produced significantly higher 17β-E2 levels than the low dose. e) Scattergram of serum estrogens. 17β-E2 and E1 were significantly correlated ($r = 0.885$). f) Centered scattergram of serum estrogens. The correlation remained significant after mean group differences were removed, suggesting that the significant correlation was not due to group membership ($r = 0.710$). Sample size (N) for tissue weights- Vehicle = 7, Premarin-Low = 10, Premarin-Medium = 10, Premarin-High = 9. Sample size (N) for serum estrogens- Vehicle = 5, Premarin-Low = 6, Premarin-Medium = 7, Premarin-High = 7.

Chapter 2-Figure 2: Spatial Working and Reference Memory Water Radial Arm Maze



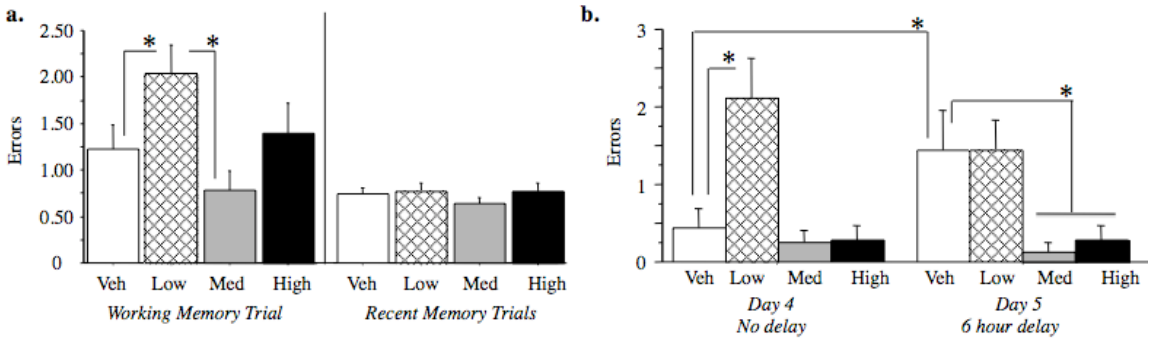
Chapter 2-Figure 2. a) Mean (±SEM) number of errors for total errors on the initial phase (D2-6) of the water radial arm maze. No dose of Premarin affected performance. b) Mean (±SEM) number of errors for total errors on the latter phase (D7-11) of the water radial arm maze. No dose of Premarin affected performance. c) Mean (±SEM) number of total errors on water radial arm maze post-delay trials, after a four-hour delay was imposed between trials two and three. High-dose Premarin animals made fewer errors than Vehicle-treated animals on the post-delay trials. Sample size (N)- Vehicle = 9, Premarin-Low = 9, Premarin-Medium = 9, Premarin-High = 9.

Chapter 2-Figure 3: Spatial Reference Memory Morris Water Maze



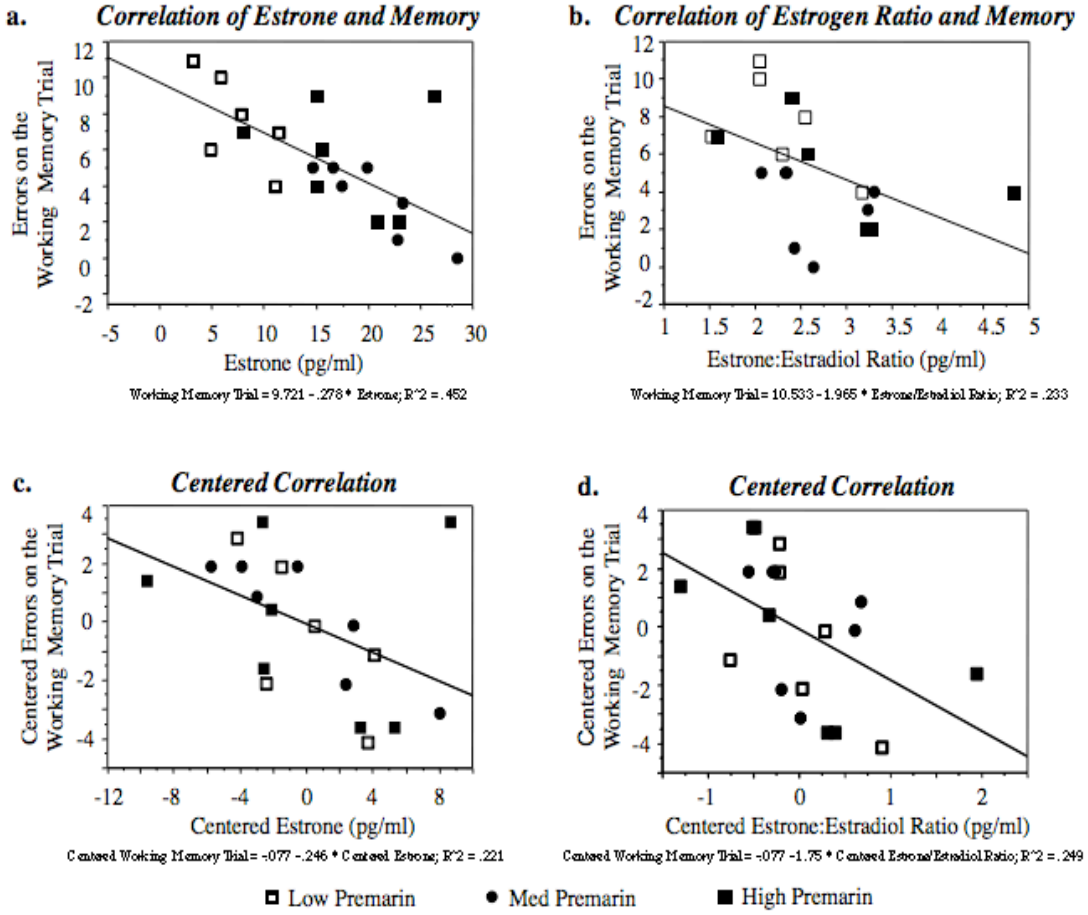
Chapter 2-Figure 3. a) Mean (±SEM) swim distance (cm) during Morris maze testing. There were no differences between Premarin- and vehicle- treated groups on learning across days. b) Mean (±SEM) probe trial percent distance in the target and opposite quadrants. All animals, regardless of treatment, swam a higher percent distance in the quadrant where the platform had been previously located, indicating that all animals localized the platform location. c) Mean (±SEM) platform crossings on the probe trial. During the first 30 sec of the probe trial, animals given low dose Premarin made fewer platform crossings than those given vehicle. During the second 30 sec, there were no group differences in number of platform crossings. Sample size (N)- Vehicle = 9, Premarin-Low = 8, Premarin-Medium = 7, Premarin-High = 9.

Chapter 2-Figure 4: Spatial Working Memory Delayed Match to Sample Water Maze



Chapter 2-Figure 4. a) Mean (±SEM) total errors during delayed match to sample testing. Animals given low dose Premarin made more working memory errors than those given Vehicle or medium dose Premarin (*p*s < 0.05). There were no group differences for recent memory. b) Mean (±SEM) errors on trial two of the baseline day (day 4) and on trial two after the six-hour delay (day 5). Animals given low dose Premarin were impaired on day 4 compared to those given Vehicle, and thus were not further impaired by the added challenge of a delay (day 4 compared to day 5). While all Premarin groups were unchanged from day 4 to day 5 suggested they were not affected by the delay, the Ovx-Vehicle group was significantly impaired after the delay. On the post-delay trials, the combined Medium and High group made significantly fewer errors compared to the Vehicle group. Sample size (N) - Vehicle = 9, Premarin-Low = 9, Premarin-Medium = 8, Premarin-High = 7.

Chapter 2-Figure 5: Serum Estrogen Correlations with Behavior



Chapter 2-Figure 5. a) E1 levels were negatively correlated with delayed match to sample errors on the working memory trial (r = -0.672, $p < 0.001$), indicating that animals with higher E1 levels tended to exhibit better working memory scores. b) The ratio of E1:17β-E2 was also negatively correlated with delayed match to sample performance on the working memory trial (r = -0.483, $p < 0.03$). c) Centered scattergram of E1 and memory. The correlation of E1 and total delayed match to sample errors committed on the working memory trial remained significant after mean group differences were removed, indicating that the significant correlation was not due to group differences in hormone levels or maze scores. (r = -0.470, $p < 0.05$). d) Centered scattergram of the estrogen ratio and memory. The correlation of the E1:17β-E2 ratio and total delayed match to sample errors committed on the working memory trial remained significant after mean group differences were removed, suggesting that the significant correlation was not due to group membership (r = -0.499, $p < 0.05$). Sample size (N) - Vehicle = 5, Premarin-Low = 6, Premarin-Medium = 7, Premarin-High = 7.

Chapter 2-Table I: Mean±SEM Neurotrophin Levels in Cognitive Brain Regions

	BDNF (pg/ml; mean±standard error)	**NGF** (pg/ml; mean±standard error)
Cingulate Gyrus	**Ovx-Vehicle = 1.24±0.15** **Ovx-Prem-Low = 1.70±0.13 *** **Ovx-Prem-Med= 1.79±0.18 *** **Ovx-Prem-High= 2.02±0.32 ***	**Ovx-Vehicle = 0.96± 0.04** Ovx-Prem-Low = 1.25±0.16 **Ovx-Prem-Med= 1.43± 0.12 **** **Ovx-Prem-High= 1.54±0.16 **
Frontal Cortex	Ovx-Vehicle = 0.97±0.10 Ovx-Prem-Low = 1.05±0.09 Ovx-Prem-Med= 1.01±0.09 Ovx-Prem-High= 0.98±0.11	Ovx-Vehicle = 0.72±0.06 Ovx-Prem-Low = 0.78± 0.05 Ovx-Prem-Med= 0.74±0.04 Ovx-Prem-High= 0.69±0.05
Entorhinal Cortex	Ovx-Vehicle = 1.88±0.23 Ovx-Prem-Low = 1.67±0.17 Ovx-Prem-Med= 2.17±0.39 Ovx-Prem-High= 1.80±0.23	Ovx-Vehicle = 0.89±0.15 Ovx-Prem-Low = 0.71± 0.06 Ovx-Prem-Med= 0.88±0.08 Ovx-Prem-High=0.73±0.08
Dorsal Hippo	Ovx-Vehicle = 4.93±0.85 Ovx-Prem-Low = 5.44±0.80 Ovx-Prem-Med= 5.26±0.56 Ovx-Prem-High= 5.26±0.83	Ovx-Vehicle = 2.50±0.27 Ovx-Prem-Low = 2.98±0.34 Ovx-Prem-Med= 3.11±0.32 Ovx-Prem-High= 2.71±0.31
Ventral Hippo	Ovx-Vehicle = 1.64±0.19 Ovx-Prem-Low = 1.94±0.12 Ovx-Prem-Med= 1.93±0.22 Ovx-Prem-High= 1.66±0.12	Ovx-Vehicle = 1.12±0.13 Ovx-Prem-Low = 1.44±0.14 Ovx-Prem-Med= 1.44±0.13 Ovx-Prem-High= 1.27±0.10
Perirhinal Cortex	**Ovx-Vehicle = 2.27±0.34** Ovx-Prem-Low = 1.97±0.30 Ovx-Prem-Med= 1.78±0.35 **Ovx-Prem-High= 1.39±0.11 ***	Ovx-Vehicle = 1.15±0.14 Ovx-Prem-Low = 1.13±0.22 Ovx-Prem-Med= 0.88±0.12 Ovx-Prem-High= 0.92±0.12
Temporal Cortex	Ovx-Vehicle = 0.34±0.11 Ovx-Prem-Low = 0.25±0.04 Ovx-Prem-Med= 0.26±0.02 Ovx-Prem-High= 0.25±0.04	Ovx-Vehicle = 0.58±0.06 Ovx-Prem-Low = 0.56±0.08 Ovx-Prem-Med= 0.54±0.05 Ovx-Prem-High= 0.57±0.05

* P < 0.05, significantly different from Ovx-Premarin-Vehicle

** P < 0.01, significantly different from Ovx-Premarin-Vehicle

Chapter 2-Table II: Significant Transcriptomic Changes in the Dorsal Hippocampi (Right Hemisphere) of Treatment Groups

Gene expression profiling was performed on dorsal hippocampi collected from the vehicle-treated group, low Premarin-dosed group, and high Premarin-dosed group. Comparative analyses led to the generation of multiple gene lists: (1) vehicle-treated group versus low Premarin-dosed group, (2) vehicle-treated group versus high Premarin-dosed group, The statistically significant genes (P<0.01) demonstrating the greatest fold changes are shown.

List 1: Vehicle-treated group versus low Premarin-dosed group

Probe Set ID	Gene Symbol	Gene Title	P-value	Fold
1370751_at	LOC257642	rRNA promoter binding protein	4.61E-03	19.21
1375422_at	---	----	5.48E-03	14.07
1398594_at	---	Transcribed locus	9.88E-03	12.10
1375212_at	Ankrd52_pred	ankyrin repeat domain 52 (predicted)	8.72E-03	8.75
1379101_at	Dhx36_pred	DEAH box polypeptide 36 (predicted)	2.87E-03	6.32
1376707_at	C1qtnf4_pred	C1q and tumor necrosis factor related protein 4 (predicted)	7.66E-05	-2.53
1368465_at	Accn1	amiloride-sensitive cation channel, neuronal (degenerin)	8.38E-03	-2.59
1368701_at	Atp1a3	ATPase, Na+/K+ transporting, alpha 3 polypeptide	4.71E-03	-3.22
1371376_at	RGD1565596	similar to Gene model 461 (predicted)	6.50E-03	-3.57
1385825_at	RGD1559930	similar to mKIAA0256 protein (predicted)	5.25E-03	-3.69

List 2: Vehicle-treated group versus high Premarin-dosed group

Probe Set ID	Gene Symbol	Gene Title	P-value	Fold
1377532_at	RGD1305020	similar to Hepatocellular carcinoma-associated antigen 58 homolog	7.08E-05	1.83
1370454_at	Homer1	homer homolog 1 (Drosophila)	3.95E-03	1.73
1392319_at	RGD1564983	similar to leucine rich repeat containing 10 (predicted)	5.36E-03	1.57
1386975_at	Pdk2	pyruvate dehydrogenase kinase, isoenzyme 2	8.82E-04	1.46
1390647_at	Phtf2_pred	putative homeodomain transcription factor 2 (predicted)	2.90E-03	1.36
1392613_at	---	Transcribed locus	5.98E-03	-1.29
1373092_at	---	Transcribed locus	5.84E-04	-1.31
1391605_at	---	Transcribed locus	5.06E-03	-1.34
1372702_at	PRP-2	proline-rich protein	9.12E-04	-1.36
1375122_at	LOC690262	similar to YY1-associated factor 2	4.52E-04	-1.40

Chapter 2-Table III: Genego Pathway Analysis of Significant Genes

Metacore Genego pathway analysis was performed on significant (P<0.01) genes from the following comparisons: vehicle-treated group versus low Premarin-dosed group & vehicle-treated group versus high Premarin-dosed group. The top ten processes identified for each comparison are listed. 'Ratio' represents the number of genes from the significant list compared to the total number of genes in the process database.

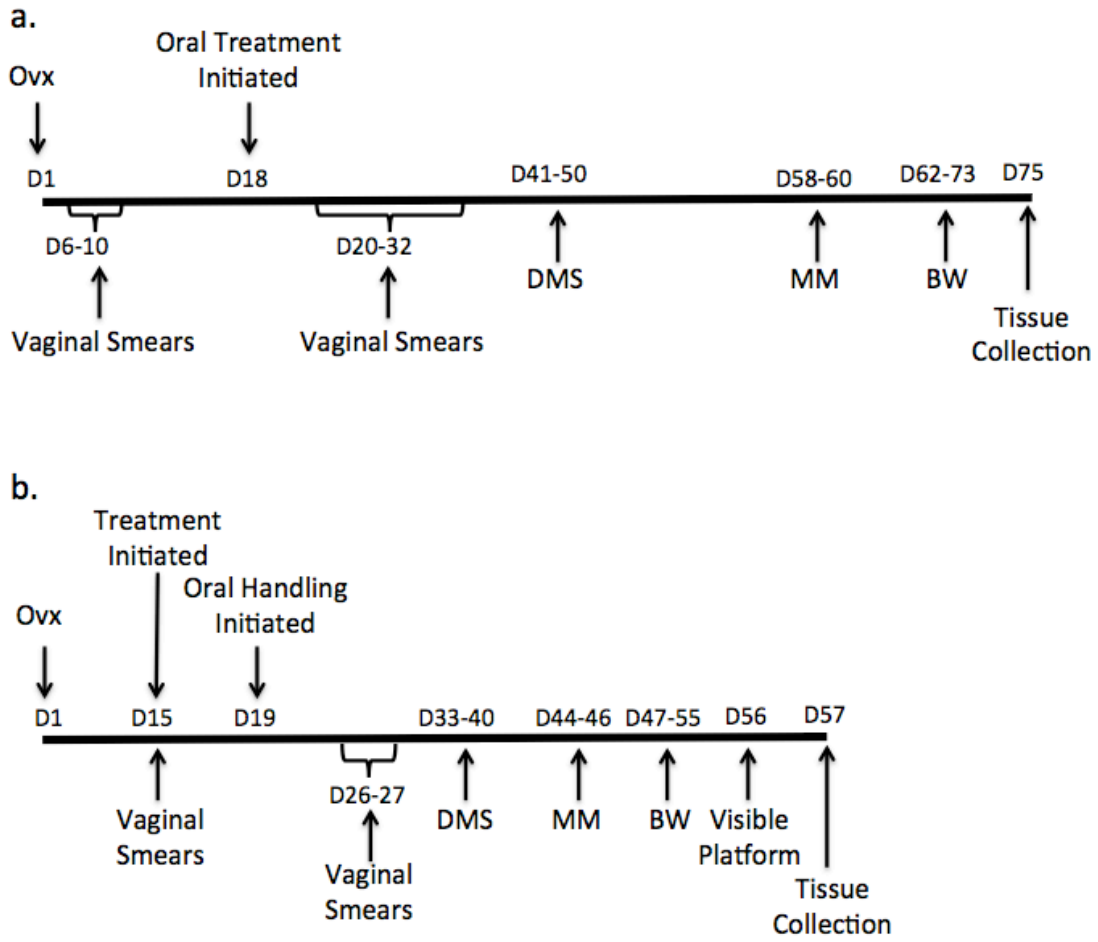
Vehicle-treated group versus low Premarin-dosed group

Process	Ratio	P-value
Cellular component organization & biogenesis	255/3593	5.50E-13
Protein metabolic process	271/3921	1.48E-12
Transport	220/3124	1.28E-10
Synaptic transmission	62/557	4.12E-10
Translation	64/595	8.69E-10
Establishment of localization	224/3262	8.82E-10
Cellular protein metabolic process	242/3602	1.04E-09
Macromolecule localization	97/1092	1.46E-09
Protein localization	94/1046	1.50E-09
Cellular macromolecule metabolic process	243/3660	3.01E-09

Vehicle-treated group versus high Premarin-dosed group

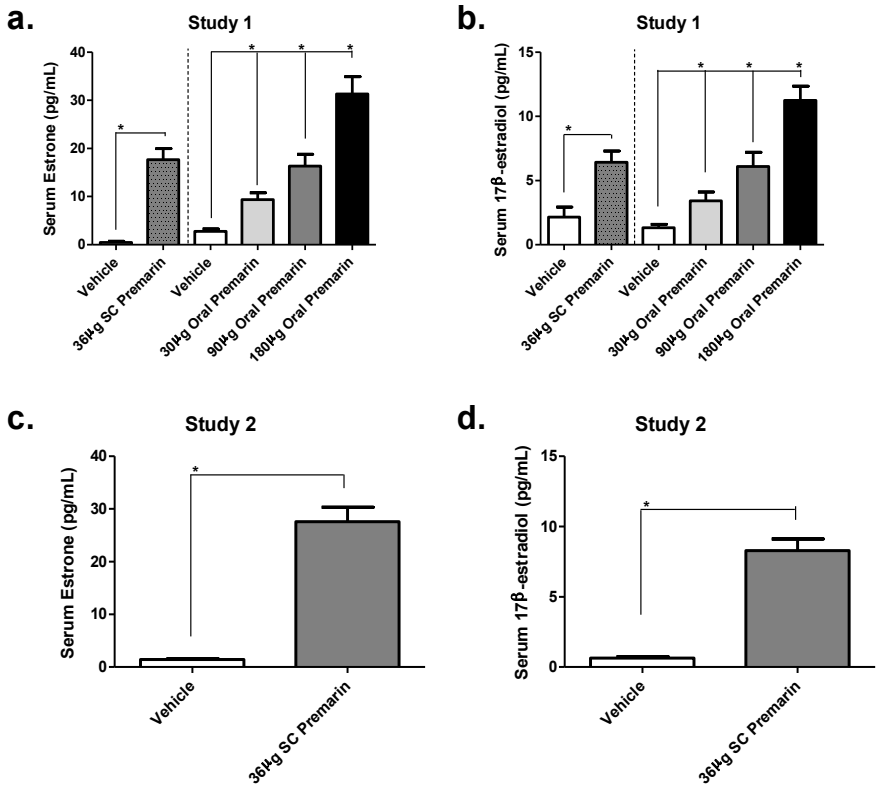
Process	Ratio	P-value
Behavioral fear response	4/25	1.05E-05
Behavioral defense response	4/25	1.05E-05
Detection of abiotic stimulus	6/93	1.31E-05
Fear response	4/31	2.54E-05
Negative regulation of glucose import	3/11	2.68E-05
Locomotory behavior	9/317	6.70E-05
Regulation of synaptic transmission	5/81	8.83E-05
Regulation of transmission of nerve impulse	5/86	1.17E-04
Intracellular signaling cascade	24/1966	1.28E-04
Negative regulation of protein catabolic process	3/20	1.79E-04

Chapter 3-Figure 1: Experimental Timelines



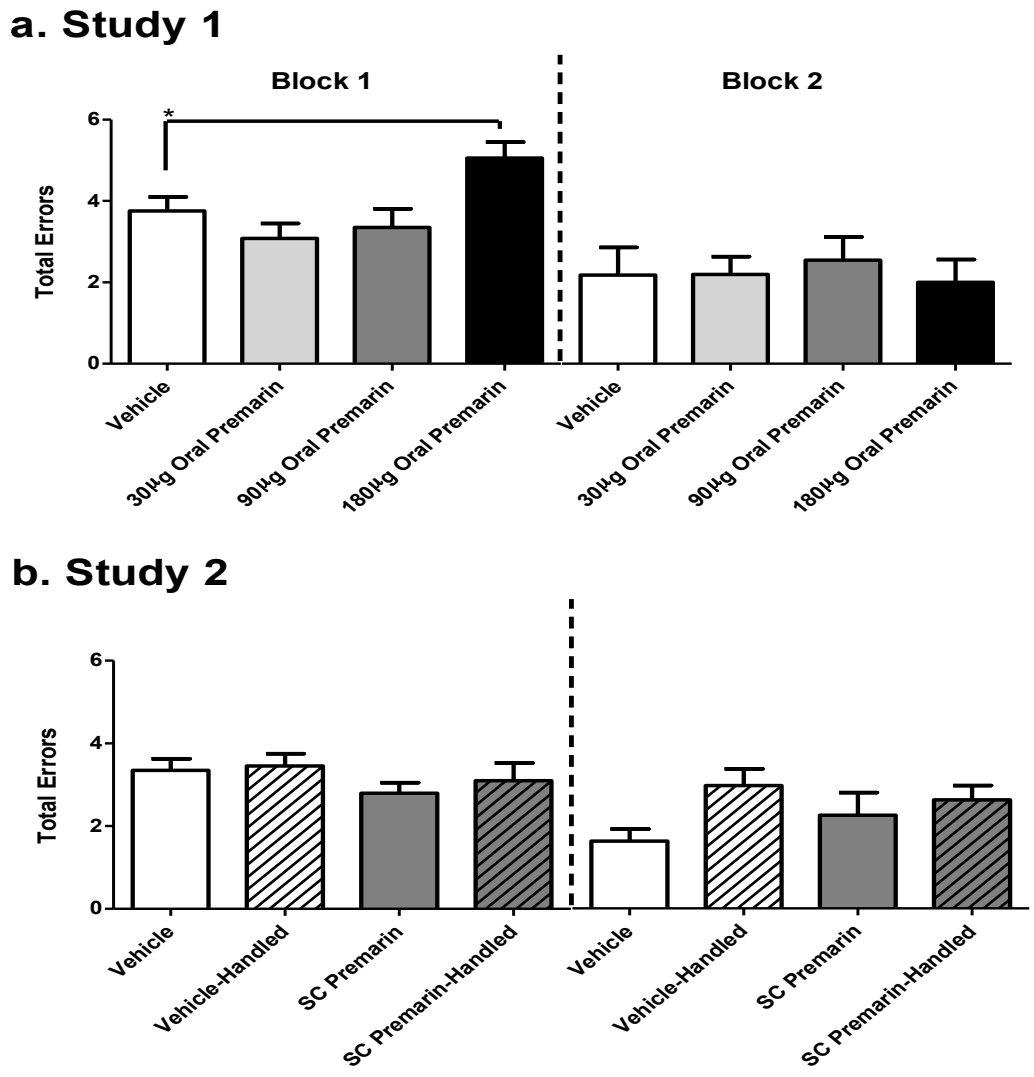
Chapter 3-Figure 1: a) Experimental timeline for Study 1. b) Experimental timeline for Study 2.

Chapter 3-Figure 2: Mean±SEM Serum Estrogen Levels



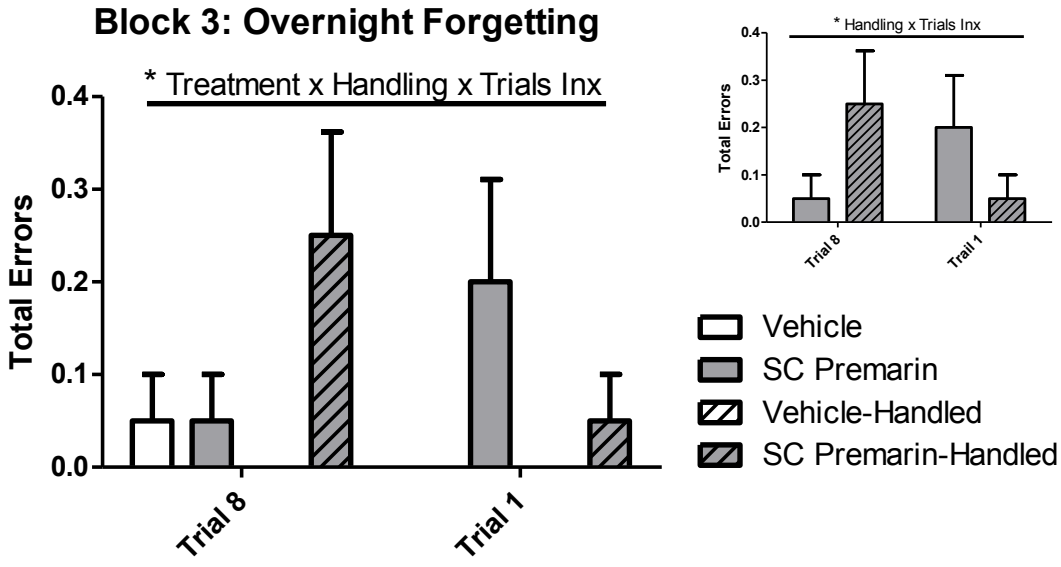
Chapter 3-Figure 2: a) Study 1 mean (±SEM) serum E1 levels (pg/ml). All doses of oral Premarin increased serum E1 levels (*p*s < 0.005). Comparison data from Engler-Chiurazzi et al (2011), revealed that the 90µg/day oral Premarin dose resulted in similar E1 levels to the 36µg/day dose of subcutaneously administered Premarin that enhanced working memory performance. Sample size (N)- Vehicle = 5, 36µg SC Premarin = 7, Vehicle = 7, 30µg Oral Premarin = 9, 90µg Oral Premarin = 10, 180µg Oral Premarin = 9. b) Study 1 mean (±SEM) serum 17β-E2 levels (pg/ml). All doses of oral Premarin increased serum 17β-E2 levels (*p*s < 0.05). The 90µg/day oral Premarin dose resulted in similar 17β-E2 levels to the 36µg/day dose of subcutaneously administered Premarin (Engler-Chiurazzi 2011). Sample size (N)- Vehicle = 5, 36µg SC Premarin = 7, Vehicle = 7, 30µg Oral Premarin = 9, 90µg Oral Premarin = 10, 180µg Oral Premarin = 9. c) Study 2 mean (±SEM) serum E1 levels (pg/ml). Subcutaneous Premarin (36µg/day) increased serum E1 levels ($p < 0.001$). Sample size (N)- Vehicle (Unhandled and Handled groups combined = 14, 36µg Premarin (Unhandled and Handled groups combined) = 20.
d) Study 2 mean (±SEM) serum 17β-E2 levels (g/ml). Subcutaneous Premarin (36µg/day) increased serum 17β-E2 levels ($p < 0.001$). Sample size (N)- Vehicle (Unhandled and Handled groups combined = 17, 36µg Premarin (Unhandled and Handled groups combined) = 20.

Chapter 3-Figure 3: Spatial Working Memory Delayed Match to Sample Water Maze



Chapter 3-Figure 3: a) Study 1 mean (±SEM) number of total errors. Ovx-Oral-180 rats made more total errors than Vehicle-treated rats during the first testing block ($p < 0.05$). Sample size (N)- Vehicle = 7, 30µg Premarin = 9, 90µg Premarin = 10, 180µg Premarin = 9. b) Study 2 mean (±SEM) number of total errors. There was a Treatment main effect ($p = 0.05$), such that oral handled rats made more total errors than unhandled rats. Sample size (N)- Vehicle-Unhandled = 10, Premarin-Unhandled = 10, Vehicle-Handled = 10, Premarin-Handled = 10.

Chapter 3-Figure 4: Non-spatial Reference Memory Black/White Discrimination Task



Chapter 3-Figure 4: Study 2 mean (±SEM) number of total errors during the overnight intervals of the third testing block. During the third testing block, there was a Treatment x Handling x Trials interaction. As indicated in the insert, there was a Handling x Trials interaction ($p < 0.05$), such that among Premarin-treated rats, handling enhanced overnight retention of the color of the platformed arm. Sample size (N): Vehicle-Unhandled = 10, Premarin-Unhandled = 10, Vehicle-Handled = 10, Premarin-Handled = 10.

Chapter 3- Table I: Mean±SEM Uterine and Pituitary Weights

	Treatment	Uterine Weight (g)	Pituitary Weight (g)
Study 1	Vehicle	0.212 ± 0.016	0.010 ±0.001
	Ovx-Oral-30	0.231 ± 0.024	0.012 ± 0.002
	Ovx-Oral-90	0.295 ± 0.017*	0.013 ± 0.001*
	Ovx-Oral-180	0.389 ± 0.035*	0.013 ± 0.001*
Study 2	Vehicle	0.142 ± 0.005	0.010 ± 0.0004
	Premarin	0.422 ± 0.057*	0.015 ± 0.001*

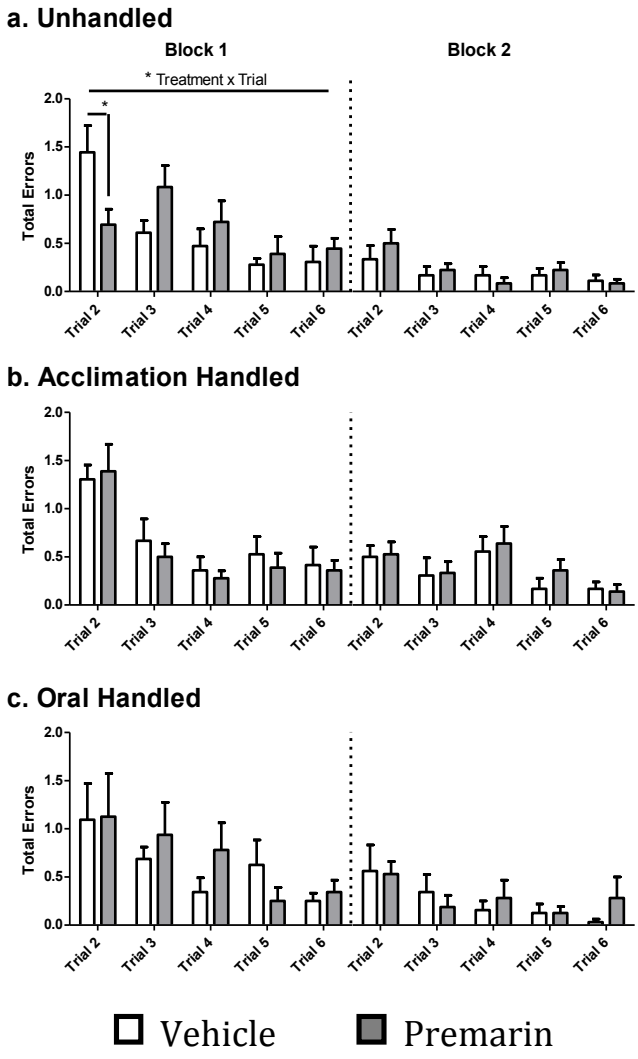
* = significantly different from Vehicle group, $p < 0.05$

Chapter 3-Table 2: Mean±SEM Neurotrophin Levels in Cognitive Brain Regions

	BDNF (pg/ml; mean±SEM)	NGF (pg/ml; mean±SEM)
Anterior Cingulate Cortex	Ovx-Oral-Vehicle = 1.24±0.19 Ovx-Oral-30 = 1.13±0.15 Ovx-Oral-90= 1.22±0.11 Ovx-Oral-180= 1.13±0.06	Ovx-Oral-Vehicle = 3.24± 0.29 Ovx-Oral-30 = 3.45±0.36 Ovx-Oral-90= 4.18± 0.39 Ovx-Oral-180= 3.62±0.27
Posterior Cingulate Cortex	Ovx-Oral-Vehicle = 1.98±0.34 Ovx-Oral-30 = 2.71±0.35 Ovx-Oral-90= 2.54±0.54 Ovx-Oral-180= 2.70±0.62	Ovx-Oral-Vehicle = 2.93±0.37 Ovx-Oral-30 = 3.56± 0.27 Ovx-Oral-90= 5.13±0.90 **Ovx-Oral-180= 3.97±0.36 ***
Frontal Cortex	Ovx-Oral-Vehicle = 1.97±0.51 Ovx-Oral-30 = 1.65±0.25 Ovx-Oral-90= 2.11±0.67 Ovx-Oral-180= 1.15±0.13	Ovx-Oral-Vehicle = 2.88±0.36 Ovx-Oral-30 = 2.98± 0.27 Ovx-Oral-90= 3.55±0.42 **Ovx-Oral-180=2.04±0.12 ***
Hippocampus	Ovx-Oral-Vehicle = 1.85±0.17 Ovx-Oral-30 = 1.91±0.29 Ovx-Oral-90= 1.92±0.29 Ovx-Oral-180= 1.94±0.30	Ovx-Oral-Vehicle = 2.80±0.32 Ovx-Oral-30 = 2.63±0.22 Ovx-Oral-90= 2.59±0.16 Ovx-Oral-180= 2.95±0.31
Entorhinal Cortex	Ovx-Oral-Vehicle = 2.16±0.66 Ovx-Oral-30 = 1.68±0.18 Ovx-Oral-90= 2.72±0.55 Ovx-Oral-180= 1.29±0.16	Ovx-Oral-Vehicle = 1.48±0.08 Ovx-Oral-30 = 1.65±0.14 Ovx-Oral-90= 1.70±0.19 Ovx-Oral-180= 1.28±0.11
Perirhinal Cortex	Ovx-Oral-Vehicle = 3.48±0.46 Ovx-Oral-30 = 4.84±0.99 Ovx-Oral-90= 4.13±0.49 Ovx-Oral-180= 3.98±0.35	Ovx-Oral-Vehicle = 1.79±0.19 Ovx-Oral-30 = 2.38±0.34 Ovx-Oral-90= 2.33±0.40 Ovx-Oral-180= 2.18±0.19
Pituitary	Ovx-Oral-Vehicle = 1.98±0.12 Ovx-Oral-30 = 1.77±0.18 Ovx-Oral-90= 1.78±0.18 Ovx-Oral-180=1.97±0.12	Ovx-Oral-Vehicle = 6.01±0.79 Ovx-Oral-30 = 4.71±0.92 Ovx-Oral-90= 4.60±0.80 Ovx-Oral-180= 7.05±1.27

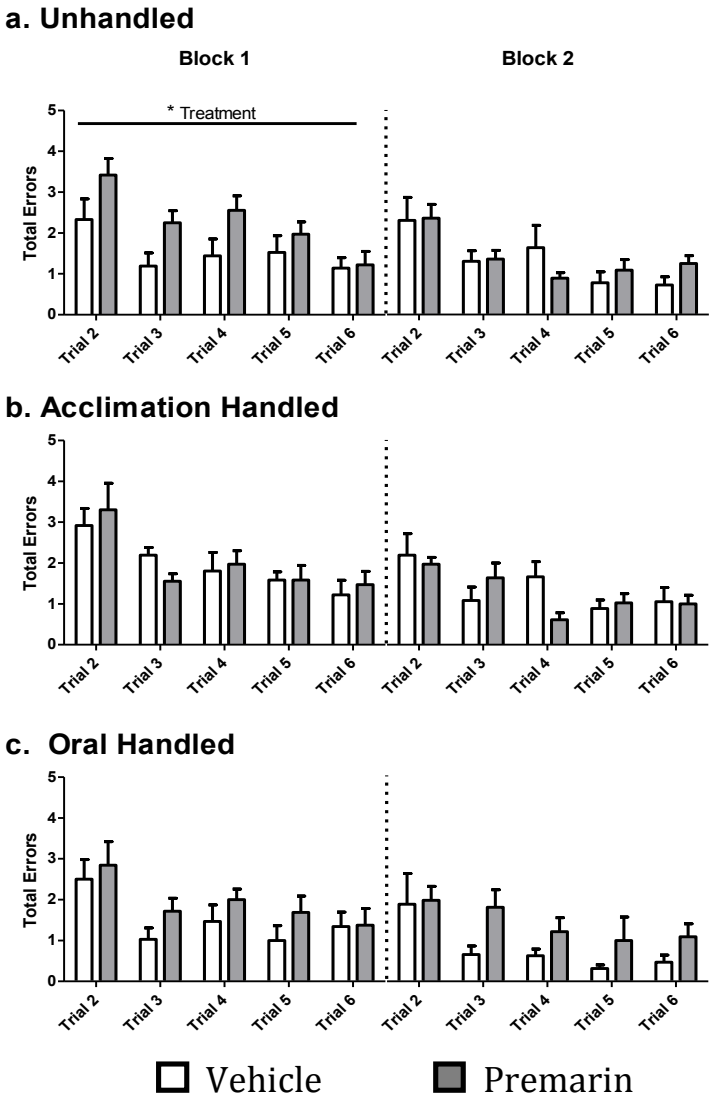
* = significantly different from Vehicle group, $p < 0.05$

Chapter 4-Figure 1: Spatial Working Memory Delayed Match to Sample Water Maze Performance on the 4-arm Task



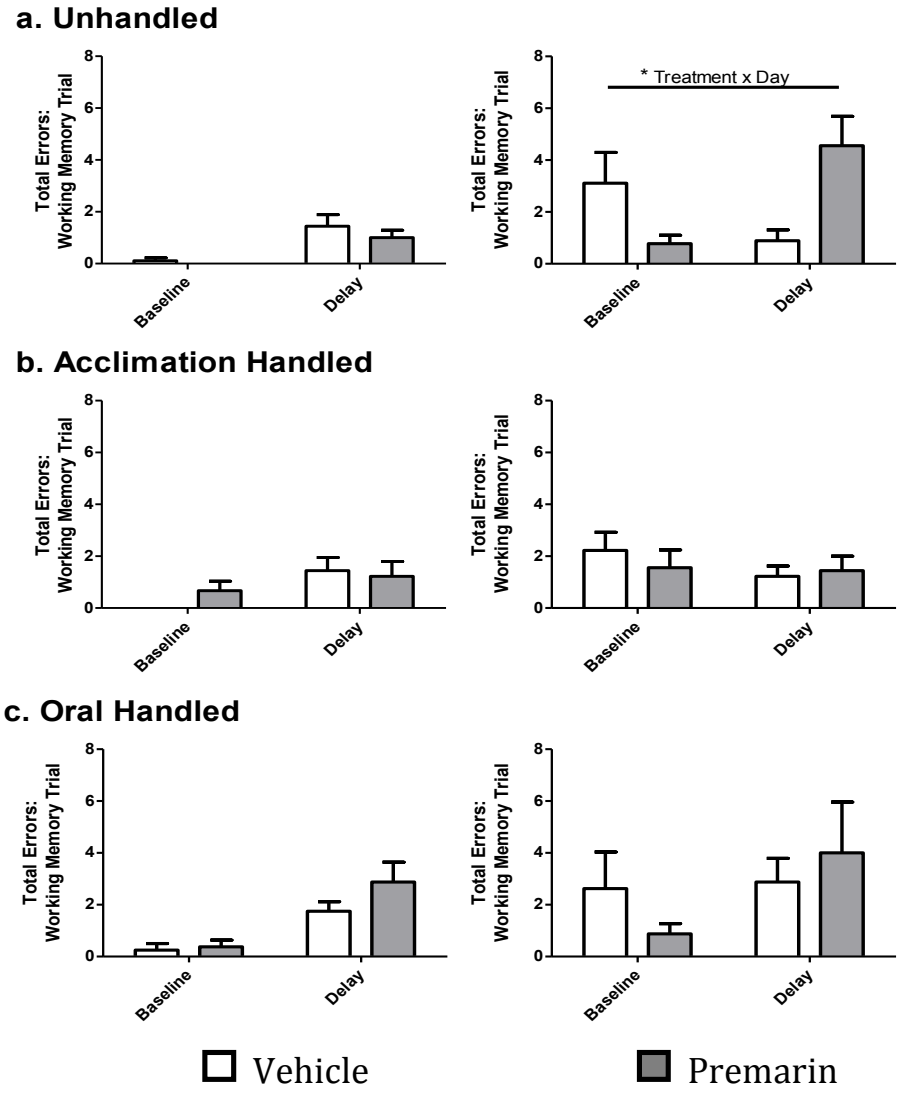
Chapter 4-Figure 1: a) Mean (±SEM) total errors among unhandled rats. During the first testing block, there was a Treatment x Trial interaction. Replicating previous findings, follow-up assessments revealed a main effect of Treatment for the working memory trial, such that Premarin enhanced performance ($p < 0.05$). There were no Treatment main effects nor interactions between Treatment and Trials for the second testing block. b) Mean (±SEM) total errors among acclimation handled rats. There were no Treatment main effects nor interactions between Treatment and any repeated measure. c) Mean (±SEM) total errors among oral handled rats. There were no Treatment main effects nor interactions between Treatment and any repeated measure. Sample size (N): Vehicle = 9, Premarin = 9, Vehicle-Acclimation = 9, Premarin-Acclimation = 9, Vehicle-Oral = 8, Premarin-Oral = 8.

Chapter 4-Figure 2: Spatial Working Memory Delayed Match to Sample Water Maze Performance on the 8-arm Task



Chapter 4-Figure 2: a) Mean (±SEM) total errors among Unhandled Rats. During the first testing block, there was a Treatment main effect, such that Premarin impaired performance ($p < 0.05$). There were no Treatment main effects nor interactions between Treatment and Trials for the second testing block. b) Mean (±SEM) total errors among acclimation handled rats. There were no Treatment main effects nor interactions between Treatment and any repeated measure. c) Mean (±SEM) total errors among oral handled rats. There were no Treatment main effects nor interactions between Treatment and any repeated measure. Sample size (N): Vehicle = 9, Premarin = 9, Vehicle-Acclimation = 9, Premarin-Acclimation = 9, Vehicle-Oral = 8, Premarin-Oral = 8.

Chapter 4-Figure 3: Spatial Working Memory Delayed Match to Sample Water Maze Performance on the 6-hour Delay Challenge



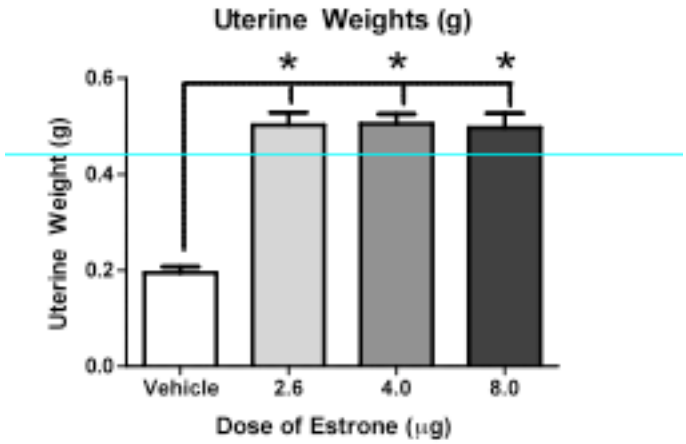
Chapter 4-Figure 3: a) Mean (±SEM) total errors among unhandled rats. For the 4-arm maze, there were no interactions between Treatment and Day. For the 8-arm maze, there was a Treatment x Day interaction (p <0.005), such that Premarin-treated rats outperformed Vehicle rats on baseline day 8 (30 sec inter-trial interval) but were impaired when memory was challenged on day 9 (six hour inter-trial interval). b) Mean (±SEM) total errors among acclimation handled rats. There were no Treatment x Day interactions for either the 4-arm nor the 8-arm mazes. c) Mean (±SEM) total errors among oral handled rats. There were no Treatment x Day interactions for either the 4-arm nor the 8-arm mazes. Sample size (N): Vehicle = 9, Premarin = 9, Vehicle-Acclimation = 9, Premarin-Acclimation = 9, Vehicle-Oral = 8, Premarin-Oral = 8.

Chapter 4-Table I: Mean±SEM Uterine and Pituitary Weights

	Treatment	Uterine Weight (g)	Pituitary Weight (g)
Engler-Chiurazzi et al., 2011	Vehicle	0.198 ± 0.014	0.010 ±0.001
	Low Premarin (12μg/day)	0.463 ± 0.033*	0.011 ± 0.002*
	Medium Premarin (24μg/day)	0.676 ± 0.068*	0.011 ± 0.001*
	High Premarin (36μg/day)	0.587 ± 0.047*	0.019 ± 0.004*
Current Study	Vehicle	0.175 ± 0.008	0.011 ± 0.001
	Premarin (36μg/day)	0.402 ± 0.018*	0.015 ± 0.002*

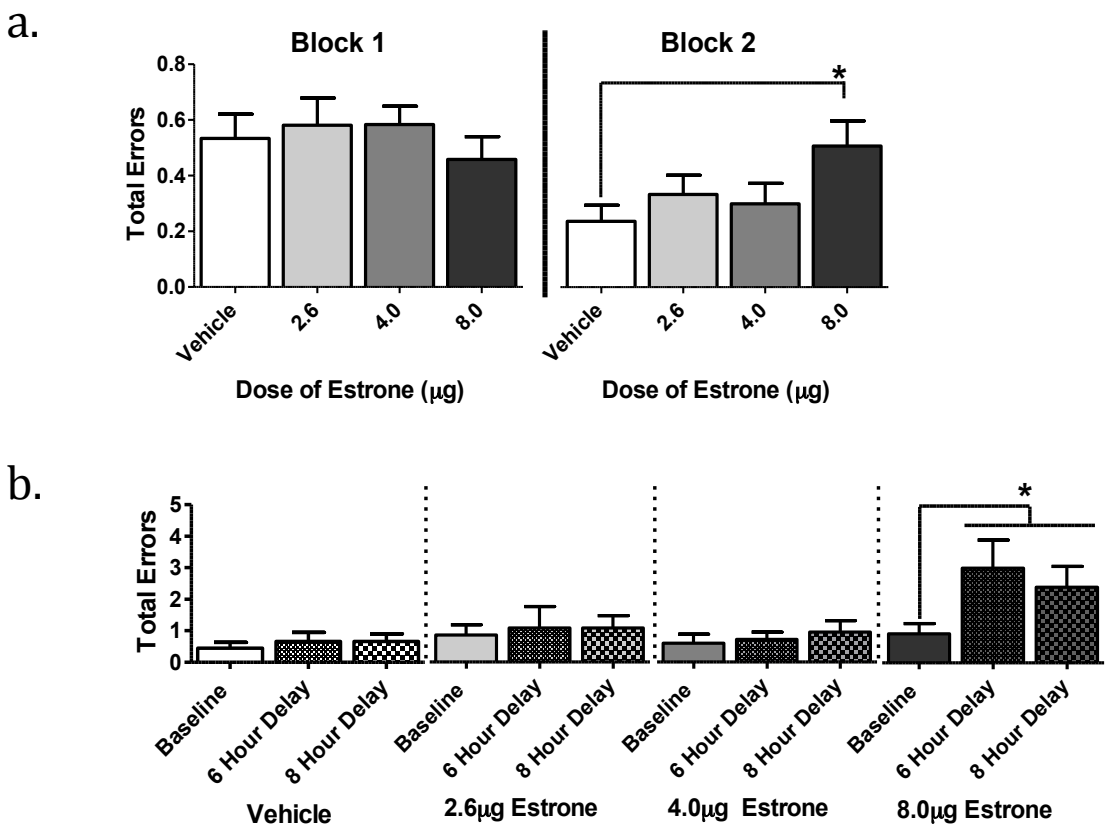
* = significantly different from Vehicle group in respective study, $p < 0.05$

Chapter 5-Figure 1: Mean±SEM Uterine Weights (g)



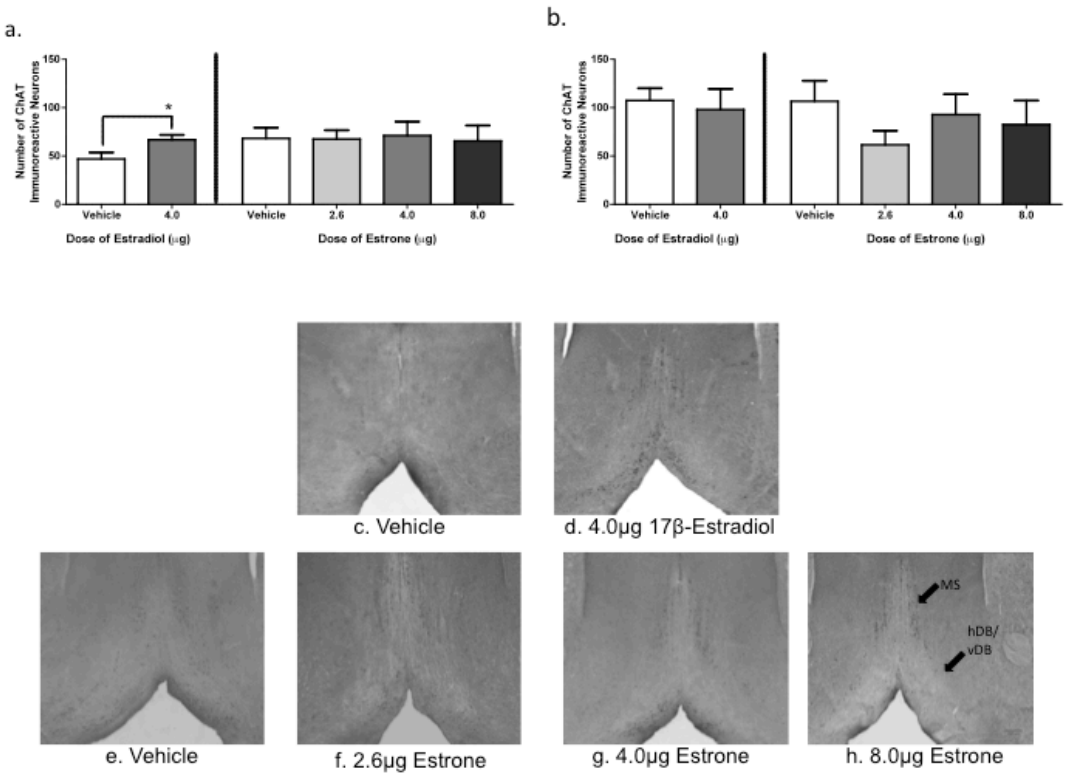
Chapter 5-Figure 1. All doses of E1 increased uterine weights relative to Vehicle rats. Sample size (N): Vehicle = 9, E1-Low = 7, E1-Medium = 8, E1-High = 8.

Chapter 5-Figure 2: Spatial Working Memory Delayed Match to Sample Water Maze



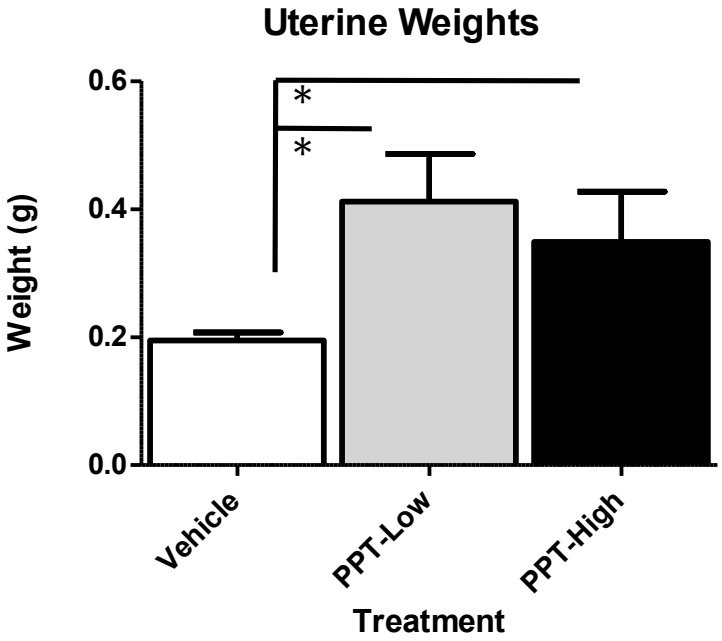
Chapter 5-Figure 2. a) Mean (±SEM) total errors during testing. During the second three-day testing block, the highest dose of E1 impaired performance, with -High rats making more total errors than Vehicle rats. b) Mean (±SEM) total errors during delay testing. Within subjects comparisons revealed that E1-High treated rats made more errors on the post-delay trial (trial two) of the combined delay measure, as compared to trial two of baseline. Sample size (N): Vehicle = 9, E1-Low = 7, E1-Medium = 8, E1-High = 8.

Chapter 5-Figure 3: Basal Forebrain Cholinergic System



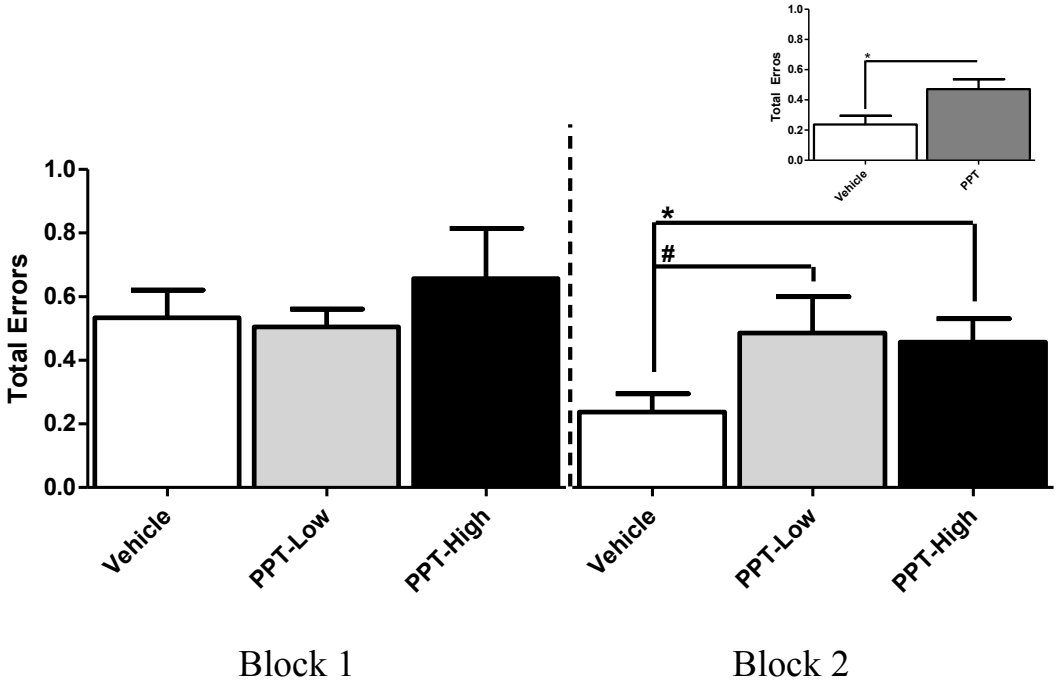
Chapter 5-Figure 3. a) Mean (±SEM) ChAT-IR neurons in the medial septum. As a positive control evaluation, a comparison group treated with 17β-E2, and corresponding Vehicle group, was used. Treatment with 4.0µg/day 17β-E2, but no dose of E1, increased ChAT-IR neurons (* $p < 0.05$). b) Mean (±SEM) ChAT-IR neurons in the hDB/vDB. Neither 17β-E2 nor any dose of E1 impacted the number of ChAT-IR neurons compared to Vehicle rats. Representative basal forebrain photomicrograph of the: c) Vehicle comparison group, d) 4.0µg/day 17β-E2 comparison group, e) Vehicle group, f) E1-Low group, g) E1-Med group, and h) of E1-High group. Sample size (N): Vehicle = 5, 4.0µg 17β-E2 = 5, Vehicle = 7, E1-Low = 7, E1-Medium = 6, E1-High = 5.

Chapter 6-Figure 1. Mean±SEM Uterine Weights (g)



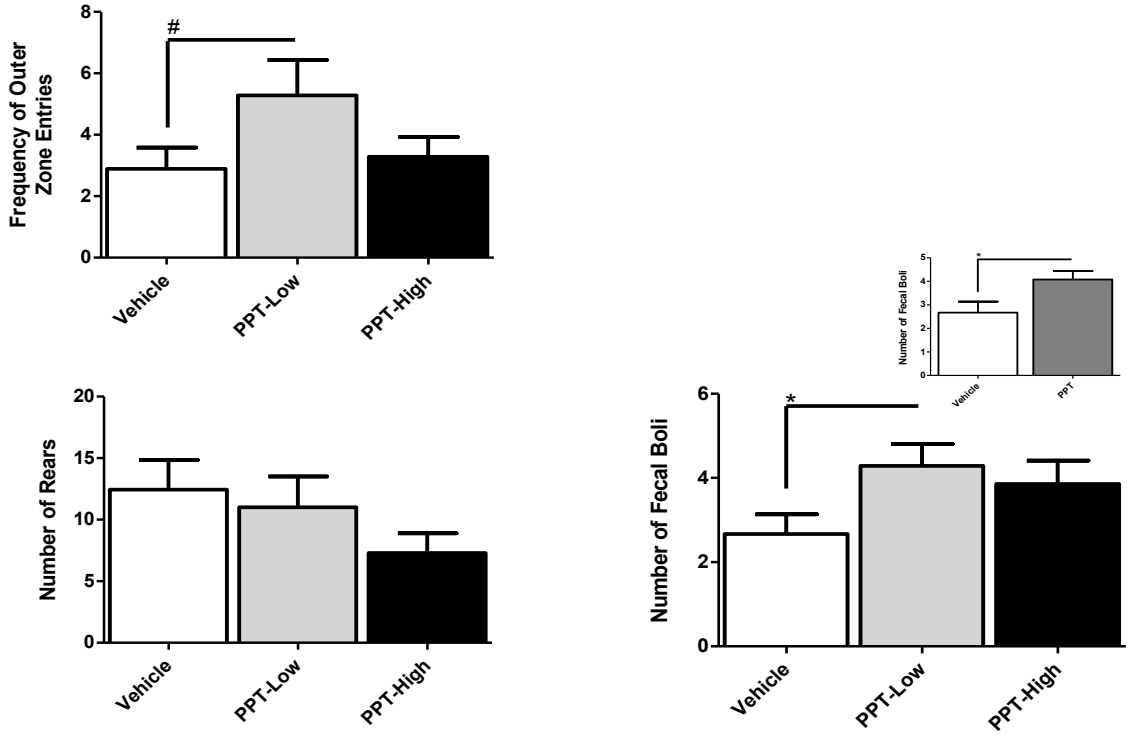
Chapter 6-Figure 1. Both doses of PPT increased uterine weights relative to Vehicle rats (* $p < 0.05$). Sample size (N): Vehicle = 9, PPT-Low = 7, PPT-High = 7.

Chapter 6-Figure 2: Spatial Working Memory Delayed Match to Sample Water Maze



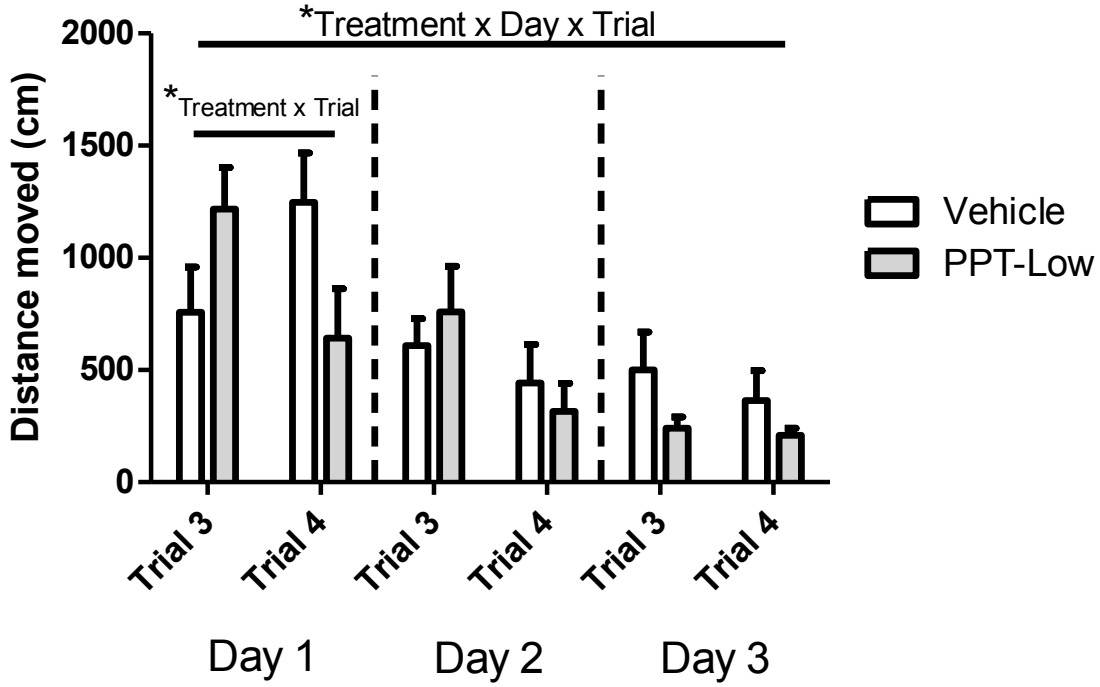
Chapter 6-Figure 2: a) Mean (±SEM) total errors during testing. During the second three-day testing block, PPT impaired performance, with PPT-Low (# $p < 0.06$) and PPT-High (* $p < 0.05$) rats making more total errors than Vehicle rats. The insert displays that when animals receiving both doses of PPT were combined, this PPT-treated group committed more errors than Vehicle-treated rats ($p < 0.05$). Sample size (N): Vehicle = 9, PPT-Low = 7, PPT-High = 7.

Chapter 6-Figure 3: Open Field



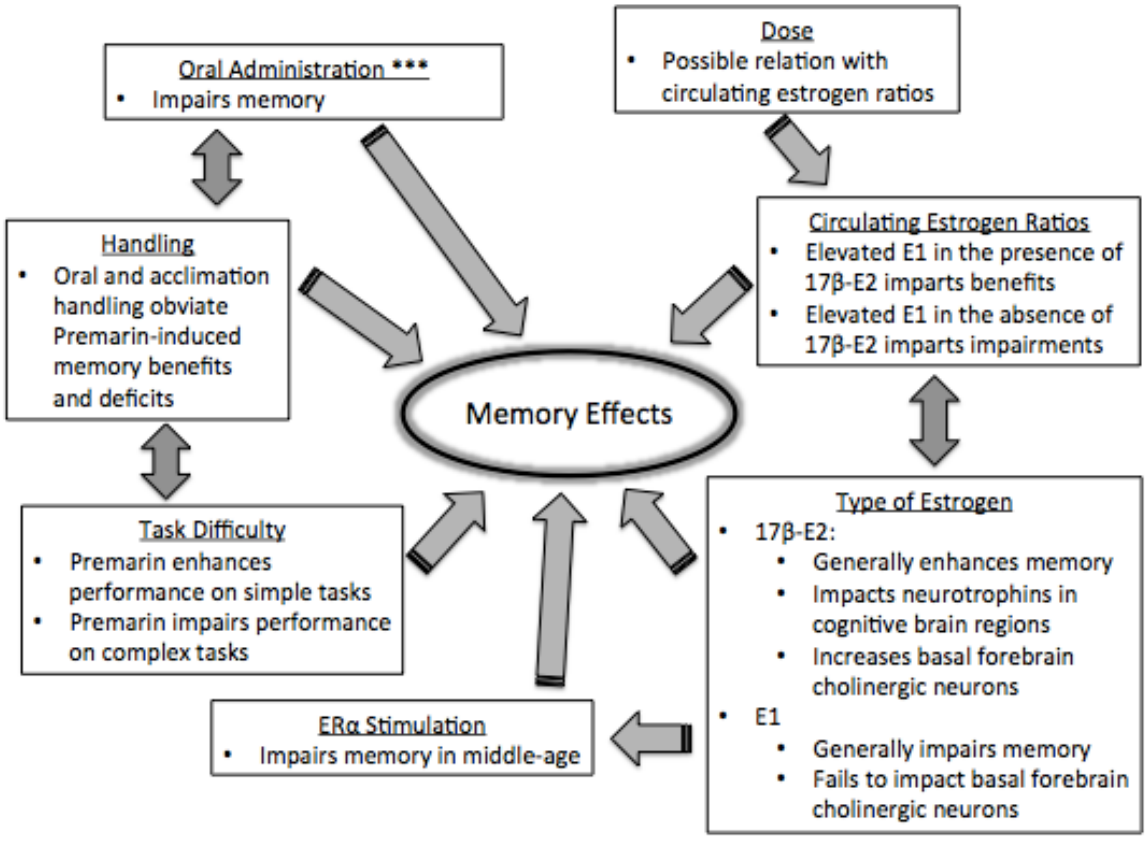
Chapter 6-Figure 3: a) Mean (±SEM) frequency of outer zone entry. PPT-Low marginally increased the frequency of outer zone entries ($p < 0.09$). b) Mean (±SEM) rears. PPT-treatment appeared to reduce the number of rears during the trial. c) Mean (±SEM) fecal boli excreted. PPT-Low increased the number of fecal boli excreted during the trial ($p < 0.05$). When PPT-treated groups were combined, PPT treatment increased the number of fecal boli excreted ($p < 0.05$). Sample size (N): Vehicle = 9, PPT-Low = 7, PPT-High = 7.

Chapter 6-Figure 4: Spatial Reference Memory Morris Water Maze



Chapter 6-Figure 4: Mean (±SEM) swim distance (cm) on trials 3 and 4 across all testing days. There was a Treatment x Day x Trial interaction for the Vehicle versus PPT-Low comparison (* $p < 0.05$). Further analyses indicated that this interaction was specific to Day 1 (* $p < 0.05$). Sample size (N): Vehicle = 9, PPT-Low = 7.

Chapter 7-Figure 1: Summary of Factors that Influence the Memory Effects of Premarin



Chapter 7-Figure 1: Several factors likely influence the realization of memory benefits with Premarin HT. Although evidence collected in this dissertation suggests that route of administration is not likely to influence the memory impact of Premarin, handling associated with a specific route can influence memory performance. Further, task complexity modulates Premarin's impact on memory such that Premarin enhances performance on simple tasks but impairs performance on complex tasks. It is also noted that handling and task difficulty interact to further influence Premarin's mnemonic impact. As well, rather than dose, the type of estrogen administered, and the circulating estrogen ratios, appear to account for observed benefits or detriments following Premarin treatment. Both these factors are important when considering that stimulation of ERα appears to impair memory in a middle-aged Ovx rat.

Chapter 7-Table I: Summary of Experimental Findings

Chapter		Research Question	Summary of Findings
2		Can continuous subcutaneous Premarin impact memory and neural outcomes?	• Premarin dose-dependently enhanced, and impaired, memory • Premarin dose-dependently increased neurotrophin levels in cingulate and perirhinal cortex • Premarin altered hippocampal gene expression of Homer1
3	Study 1	Does orally-administered Premarin impact memory and brain?	• The highest dose of oral Premarin impaired working memory • This same dose increased posterior cingulate, and decreased frontal cortex, nerve growth factor levels
	Study 2	Can handling procedures associated with oral administration account for Study 1 impairments?	• Oral handling impaired spatial working memory, regardless of treatment • Oral handling enhanced non-spatial memory in Premarin-treated rats
4		Does task difficulty impact Premarin's memory effects? Are these memory impacts influenced by oral or acclimation handling?	• Among unhandled rats, Premarin enhanced 4-arm, but impaired 8-arm, working memory tasks. • Acclimation and oral handling obviated these memory effects
5		How does the Premarin metabolite, E1, impact cognition and brain in middle age?	• Estrone impaired cognition • Unlike 17β-E2, E1 did not impact basal forebrain cholinergic neuron number
6		Does estrogen receptor alpha stimulation impact cognition in middle age?	• Propylpyrazole triol impaired working, and modestly enhanced short-term reference, memory

APPENDIX A

PASTA A LA CARBONARA

This recipe is one of my favorites; I ate a version of this dish the night I got engaged! It is sumptuous and comforting, yet refined and bright in flavor. My twist on a Roman classic, this meal will satisfy a craving for indulgence.

Ingredients:

- Splash of extra virgin olive oil
- 1 pound pancetta, diced
- freshly ground black pepper, to taste (for pancetta and for sauce)
- 6 eggs, room temperature
- 1/2 - 2/3 cup heavy whipping cream, room temperature
- at least 1 cup freshly grated Parmesan cheese
- juice of one lemon
- zest of one lemon, reserve some to garnish each plate
- 1 package arugula, rinsed
- a handful of salt (for pasta water)
- salt, to taste (for sauce)
- 1 pound dried spaghetti or other similar pasta

Directions:

In a large, flat sauté pan, over medium heat, add a splash of extra virgin olive oil to coat. Add in the pancetta and season with pepper. Cook until maillard reaction has taken place and the meat is nicely browned. Remove pan from heat and set aside.

Bring a large pot of water, with a generous helping of salt, to a rolling boil. Add spaghetti and continue boiling until just barely al dente (tender and slightly firm inside), about 7 minutes. Reserve at least 1 cup of cooking liquid.

Meanwhile, in a mixing bowl, combine eggs, cream, about 1/3 cheese, lemon juice, lemon zest, salt and pepper.

Put the sauté pan with the pancetta onto the stove over low heat. Add arugula and wilt briefly (just a few minutes). Just prior to adding the pasta, pour the egg mixture into the pan. When the pasta is ready, transfer while still hot to sauté pan and quickly toss with the pancetta and sauce using tongs. Work quickly to avoid curdling the egg. Toss with the remaining Parmesan cheese, add pasta water to loosen the sauce if necessary, and season with salt and pepper to taste. To serve, using tongs, grab a portion of pasta and lower into a bowl, rotating your wrist to create a bird’s nest shape. Garnish with lemon zest or twirls. Serve with tempranillo or other full-bodied red wine.