The Effects of Age, Hormone Loss, and Estrogen Treatment on Spatial Cognition in the Rat: Parameters and Putative Mechanisms

by

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ABSTRACT

Cognitive function is multidimensional and complex, and research indicates that it is impacted by age, lifetime experience, and ovarian hormone milieu. One particular domain of cognitive function that is susceptible to age-related decrements is spatial memory. Cognitive practice can affect spatial memory when aged in both males and females, and in females alone ovarian hormones have been found to alter spatial memory via modulating brain microstructure and function in many of the same brain areas affected by aging. The research in this dissertation has implications that promote an understanding of the effects of cognitive practice on aging memory, why males and females respond differently to cognitive practice, and the parameters and mechanisms underlying estrogen's effects on memory. This body of work suggests that cognitive practice can enhance memory when aged and that estrogen is a probable candidate facilitating the observed differences in the effects of cognitive practice depending on sex. This enhancement in cognitive practice effects via estrogen is supported by data demonstrating that estrogen enhances spatial memory and hippocampal synaptic plasticity. The estrogen-facilitated memory enhancements and alterations in hippocampal synaptic plasticity are at least partially facilitated via enhancements in cholinergic signaling from the basal forebrain. Finally, age, dose, and type of estrogen utilized are important factors to consider when evaluating estrogen's

effects on memory and its underlying mechanisms, since age alters the responsiveness to estrogen treatment and the dose of estrogen needed, and small alterations in the molecular structure of estrogen can have a profound impact on estrogen's efficacy on memory.

Collectively, this dissertation elucidates many parameters that dictate the outcome, and even the direction, of the effects that cognitive practice and estrogens have on cognition during aging. Indeed, many parameters including the ones described here are important considerations when designing future putative behavioral interventions, behavioral therapies, and hormone therapies. Ideally, the parameters described here will be used to help design the next generation of interventions, therapies, and nootropic agents that will allow individuals to maintain their cognitive capacity when aged, above and beyond what is currently possible, thus enacting lasting improvement in women's health and public health in general.

DEDICATION

I would like to dedicate my dissertation to a few special people who made it possible for me to achieve my dream of attaining a Ph.D. My first dedication goes to my incredible and loving mother Ellen A. Talboom, who is there whenever I need her and who imparted upon me a thirst for knowledge and a drive for excellence. I also want to recognize my brother Joseph R. Talboom, who has always been by my side as a family member, a friend, and a colleague. I dedicate my dissertation in loving memory of my dear friend Keley R. Schaefer, who left this life so young and did not get to realize her own dream of attaining a Ph.D. Lastly, my Ph.D. and dissertation would not have been possible without my wonderful and truly amazing graduate school mentor and committee chair Dr. Heather A. Bimonte-Nelson. Heather worked hard and looked past my many shortcomings to train me to be a respectable and successful scientist.

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TABLE OF CONTENTS

Page

CHAPTER

Page

CHAPTER

Page

CHAPTER

Page

CHAPTER

Page

CHAPTER

Page

CHAPTER

Page

CHAPTER

Page

CHAPTER

Page

CHAPTER

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

Figure Page

LIST OF SYMBOLS / NOMENCLATURE

Symbol Page

Symbol Page

CHAPTER 1

GENERAL INTRODUCTION

Cognitive Aging and the Risk of Alzheimer's Disease

According to U.S. census projections there will be an estimated 1.5 billion people over the age of 65 by the year 2050 (U.S. Census Bureau, 2007). In many individuals, learning and memory function deteriorates with age. Normally aged individuals require more time to learn novel information when compared to young individuals, and they are more susceptible to interference (Tulving and Craik, 2000). Age is the number one risk factor for developing Alzheimer's disease (AD), and there are more than 5 million people in the U.S. afflicted with AD (Alzheimer's Association, 2007). Currently, no treatments exist to reverse the pathology associated with AD. However, there may be non-pharmacological and hormonal treatments to protect individuals from memory loss and brain changes due to normal aging as well as AD.

Cognitive Activity

A non-pharmacological treatment to protect individuals from memory loss may be cognitive activity throughout life. This tenet is supported by evidence that education decreases risk of developing dementia and/or the symptoms of AD. Many studies report lower education as a risk factor in developing AD and other dementias (Bowirrat et al., 2002; De Ronchi et al., 1998; Fritsch et al., 2002; Gatz et al., 2001;

Letenneur et al., 1999; Ott et al., 1999). Additionally, aged individuals maintaining higher cognitive activity through leisure activities had a lower risk of developing dementia, mild cognitive impairment (MCI), and vascular cognitive impairment than those who were less active (Verghese et al., 2009; Verghese et al., 2006; Verghese et al., 2003). Corroborating the above studies in humans, work in animals has provided evidence that environmental stimulation enhances markers of neuronal health in aged animals (for review see Frick and Benoit, 2010; Swaab et al., 2002). Collectively, these findings indicate that age-related memory changes can be influenced by numerous experiential factors including prior education and cognitively-stimulating activities, and cognitive practice may lead to neurobiological changes that attenuate age-related memory decline.

Age-Related Changes in Rodent Memory

Similar to humans, rodents show age-related memory decline, specifically in reference memory (RM), a type of long-term memory where information remains constant, and working memory (WM), a type of short-term memory where information needs to be updated. Numerous studies have demonstrated RM and WM decline with age in rodents (Ando and Ohashi, 1991; Barnes et al., 1980; Bimonte et al., 2003; Chrobak et al., 1995; Frick et al., 1995; Matzel et al., 2011; Pitsikas and Algeri, 1992; Talboom et al., 2008; Wellman and Pelleymounter, 1999). Studies evaluating cognitive practice in animals have demonstrated that male rats

receiving practice on the spatial RM Morris maze for five days at 12 months of age performed better at 24 months of age on this practice task, as compared to age-matched controls that had received no practice. However, this enhancement in performance was not found on passive avoidance, suggesting the protective effects of cognitive practice may not transfer to another type of task (Pitsikas et al., 1991). In another study, male rats received practice on the RM T-Maze, RM Morris maze, and the WM radial-arm maze at 2-3 and 12-14 months of age, and were then tested on these same mazes at 25-27 months of age. The final assessment showed an attenuation of age-related memory change in comparison to naïve age-matched controls on only the RM T-maze and Morris maze (Dellu et al., 1997). Thus, cognitive practice on multiple WM and RM maze tasks may not protect against age-related WM decline, as tested in the radial-arm maze, for male rats. More recent longitudinal research found that extensive WM practice on a modified radial-arm maze in mice from 3 to 18 months of age conferred an attenuation of age-related learning and attentional deficits evaluated by a battery of tasks when the mice were aged (Matzel et al., 2011).

Unanswered Questions Regarding Cognitive Practice

Although pivotal in understanding the effects of cognitive practice, previous reports evaluating the ability of cognitive practice to alter the trajectory of age-related cognitive changes in animals left several

questions unanswered. The study described in Chapter 2 was designed to directly and methodically address these unanswered questions in the literature.

Ovarian Hormones and Cognition

An important consideration when evaluating cognitive aging in females is ovarian hormone status, as several basic and clinical studies have linked cognitive decline with age-related alterations in the ovarian hormone milieu (for review see Bimonte-Nelson et al., 2010; Sherwin and Henry, 2008). For example, ovarian hormone loss due to surgical or natural menopause has been associated with cognitive decline in women (Nappi et al., 1999; Phillips and Sherwin, 1992; Sherwin, 1988), and hormone therapy (HT) can attenuate some of these declines (Sherwin, 2006), although the parameters underlying the efficacy of this effect are controversial (see Craig et al., 2005; Sherwin, 2005). Estrogens are a class of hormones including 17β-estradiol (E2), estrone (E1), and estriol; for humans and rats E2 is the most potent naturally-circulating estrogen, followed by E1 and estriol, in order of receptor affinity (Kuhl, 2005; Sitruk-Ware, 2002). E2 is the primary export product from the human ovary (Turgeon et al., 2004). The majority of studies evaluating activational effects of estrogens in ovariectomized (Ovx) animals for spatial learning and memory have been performed using young rodents, with many works showing enhancements due to treatment (Bimonte and Denenberg, 1999;

Daniel et al., 1997; Daniel et al., 1999; Dohanich et al., 1994; Galea et al., 2001; Holmes et al., 2002; Luine et al., 2003; Luine et al., 1998b; Marriott and Korol, 2003; McLaughlin et al., 2008; Packard and Teather, 1997; Sandstrom and Williams, 2001; Singh et al., 1994). To summarize, these studies suggest that 1) ovarian hormone loss is detrimental, while HT is beneficial to cognition, 2) there are several estrogens naturally circulating in HTs with different potencies and receptor affinities, and 3) estrogen treatment enhances memory in young rodents. This work collectively supports the tenet that estrogen can help to attenuate age-related memory decline in females given the appropriate circumstances. However, what these appropriate circumstances are remain to be determined.

Estrogen Effects in Older Rodents

In some studies, E2 treatment has been shown to benefit memory in aged rodents (Aenlle et al., 2009; Foster et al., 2003; Frick et al., 2002; Gibbs, 2000b; Markham et al., 2002; Markowska and Savonenko, 2002a; Talboom et al., 2008); however, it is important to note that these effects depend on a multitude of factors, making the question of whether E2 can enhance memory in aged rodents more complex than it is for younger rodents. For example, two factors are the memory type evaluated and the demand placed on the memory system. WM enhancements due to tonic E2 treatment have been reported in aged Ovx rats, although this effect is more pronounced when memory demand is high (Gibbs, 2000b; Luine and

Rodriguez, 1994). My lab and others have shown E2-induced spatial WM improvements in young Ovx rats as well, an effect most pronounced when spatial WM demand is high (Bimonte and Denenberg, 1999), and is an effect that depends on administered E2 dose (Bimonte and Denenberg, 1999; Daniel et al., 1997; Holmes et al., 2002; Sandstrom and Williams, 2001). Furthermore, a higher supraphysiological E2 dose may be necessary to enhance spatial RM retention in rats approaching old age, as lower doses did not enhance RM retention (Foster et al., 2003).

Responsivity to Estrogen Treatment

A study has noted an age-related interaction with estrogen's effects on spatial memory retention when testing multiple ages after treatment with two separate doses of estradiol benzoate, and although serum levels of E2 were assayed, the correlation with memory performance was not evaluated (Foster et al., 2003). This study suggested that there may be an age-related alteration in the responsivity to estrogen treatment in relation to spatial memory, and a novel analysis would be to assess the relation between serum estrogen levels and memory performance. Chapter 3 evaluated the responsiveness of young, middle-aged, and aged Ovx rats to E2 treatment on the spatial RM Morris maze task. Chapter 3 also evaluated if circulating serum E2 levels correlated with spatial RM performance.

Estrogens Commonly Used in HT and their Composition

To date, E2 has been the primary estrogen used to investigate the cognitive effects of HT in the animal model, despite the fact that Premarin® (CEE) is the most commonly prescribed estrogen component of HT (Hersh et al., 2004). CEE contains the sulfates of more than 10 equine estrogens (Kuhl, 2005). In humans, CEE-containing therapy improves memory in females (Campbell and Whitehead, 1977a; Kantor et al., 1973; Ohkura et al., 1995), although recently the Women's Health Initiative (WHI) study found that CEE does not benefit cognition in women (for review see Coker et al., 2010). New evidence has demonstrated that both tonic and cyclic CEE, at doses relevant to what women take as HT, can enhance spatial memory and protect against cholinergic challenge on spatial tasks in middle-aged Ovx rats (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009). Although vital in understanding CEE's effects on memory, these previous animal studies did not evaluate E2, so a direct comparison between the effects of CEE and E2 on memory was not possible. Chapter 4 assessed the effects of E2 and CEE on memory in middle-aged Ovx female rats. While Chapter 4 and other previous studies identified an efficacious dose of CEE and E2 in female rats, a full evaluation of the dose response effects of E2 and CEE on spatial memory has not been evaluated. In fact, few researchers have employed more than two doses in a single study. It is conceivable that the dose response curve for each hormone follows a quadratic or perhaps cubic function,

which would be difficult to evaluate with two or fewer doses. Chapter 5 separately tested six tonic doses of E2 and CEE on the spatial WM Delay Match-to-Sample (DMS) Plus Maze task in middle-aged female Ovx rats.

Components of CEE

In the last decade, landmark basic science research from the Brinton laboratory has led to several discoveries regarding the neuroprotective properties of estrogens. This work demonstrated that some estrogen components of CEE enhanced markers of neuroprotection, while others showed little benefit (Brinton et al., 1997; Zhao and Brinton, 2006). Specifically, $\Delta^{8,9}$-dehydroestrone (Δ^{8}E1) and equilin, found naturally in horses but not in women or rats, were the two primary estrogenic CEE components that showed the most consistent and potent neuroprotective effects *in vitro* (Zhao and Brinton, 2006). Despite these exciting *in vitro* findings regarding Δ^{8}E1 and equilin, neither estrogen has been evaluated for their effects on memory or the cholinergic system. Chapter 6 assessed the effects of three separate doses of Δ^{8}E1 and equilin on spatial memory via the DMS Plus Maze and Morris maze.

Estrogen Prodrugs

Limited recent research has focused on developing prodrugs of E2 and E1. Prodrugs are inactive precursors of the therapeutic agents that are converted to the biologically active agents by enzymatic and/or chemical transformation *in vivo*–preferably at the site of action (Albert,

1958; Wermuth et al., 1998). Brain-targeted estrogen prodrugs conceivably have the ability to target the action of the hormone to only the brain, and thereby reduce the systemic effects and provide an exciting new potential therapeutic option as a HT (Estes et al., 1994; Prokai et al., 2003). My collaborators developed 10-hydroxyestra-1, 4-dien-3, 10-dione (E1-DHED) as a prodrug of E1, and indeed E1-DHED has been shown to protect against stroke-induced brain changes similar to the parent hormone E1 (see Prokai et al., 2003). More recently, 10,17β-dihydroxyestra-1,4-dien-3-one (DHED) has been implicated as a prodrug for E2 by that group as well. Chapter 4, in addition to testing E2 and CEE, also evaluated the effects of DHED on memory in middle-aged Ovx female rats.

The BF Cholinergic System, Estrogen, and Memory

Basal forebrain (BF) cholinergic neurons project to the hippocampus and surrounding cortical areas, and play an important role in learning and memory (Hasselmo, 2006). E2 enhances BF cholinergic function, as evidenced by expression of different cholinergic markers and pharmacological cholinergic challenges (e.g., Gibbs, 2000a; Markowska and Savonenko, 2002a; Packard and Teather, 1997). In fact, choline acetyltransferase (ChAT), the acetylcholine (ACh) synthesizing enzyme, increases in the BF even months after transient exposure to E2 (Bohacek et al., 2008; Gibbs, 1997; Rodgers et al., 2010). Cholinergic signaling is

directed by muscarinic ACh receptors (mAChR) and nicotinic ACh receptors (nAChR), which are both present in brain regions facilitating memory (Clarke et al., 1985; Court and Clementi, 1995; Tice et al., 1996; Vaucher et al., 2002), and are implicated in memory processing (Hasselmo, 2006; Konopacki et al., 1992; Rouse et al., 1999). Estrogens bind to the α4β2-nAChR, the most abundant nAChR subtype in the brain, and directly alter its function (i.e., alters the receptor's ion efflux) (Curtis et al., 2002; Paradiso et al., 2001). To address a potential mechanism of estrogen's effects on memory, Chapter 4 also evaluated the effects of E2, CEE, and DHED on BF ChAT positive cell counts in middle-aged Ovx female rats. Following simular lines of reasoning regarding the cholinergic system, Chapter 6 assessed the effects of Δ^8E1 and equilin treatments on nAChRs.

The Effects of Age and Estrogen on Synaptic Plasticity in Cognitive Brain Regions

Age-related cognitive decline likely involves neoplastic changes within the brain (Burke and Barnes, 2006), such as alterations in dendritic structure, spine number, and spine shape. Using various techniques, there is evidence that hippocampal and neocortical synapses decrease with normal aging (Bertoni-Freddari et al., 2003). Moreover, there are alterations in factors influencing synaptic transmission that occur with normal aging, including the reductions mentioned above in synaptic

density as well as dendritic regression in apical and basal neuron regions in brain areas that are known to be involved in memory processing (Esiri, 2001). Gonadal hormones, especially estrogen, have been shown to have marked effects on neuron morphology (for review see Woolley, 2007). Early work found that hormone fluctuations with the estrous cycle affect brain regions involved in cognition (Foy, 2001; Terasawa and Timiras, 1968). Subsequent studies have extended these findings to show that estrogens alter neuron morphology by increasing hippocampal CA1 spine density in Ovx rats (Gould et al., 1990; Silva et al., 2000; Woolley and McEwen, 1992; Woolley and McEwen, 1993). Dendritic spines have long been theorized to be a structural component of memory (Geinisman, 2000; Kasai et al., 2003; Moser, 1999; Nimchinsky et al., 2002; Sorra and Harris, 2000), leading to wide-reaching implications regarding estrogen's influence on cognitive function. Indeed, several studies have demonstrated that spatial WM and RM performance is enhanced after E2 injections (McLaughlin et al., 2008; Sandstrom and Williams, 2001; Sandstrom and Williams, 2004) within the timeframes corresponding to E2-induced increases in hippocampal dendritic spines (Gould et al., 1990; Silva et al., 2000; Woolley and McEwen, 1992; Woolley and McEwen, 1993) and other markers of synaptic plasticity (Foy, 2001). These studies suggest that age and ovarian hormones have a profound effect on hippocampal synaptic plasticity. Chapter 7 assessed how age and ovarian

hormone loss altered the distribution of excitatory and inhibitory putative synapses in several hippocampal lamina of female rats.

It is my hope that the work in this dissertation will maximize opportunities for the discovery of new hormonal and behavioral therapies and subsequent interventions so that individuals can maximize their potential for brain health as aging ensues. With improvements in cognition, the ever aging population would be able to maintain their autonomy and overall health longer, thus improving their quality of life and in turn the quality of life of their family and loved ones. Collectively, these enhancements in the life of aged individuals would translate to improvements in public health while decreasing the enormous cost that the detrimental effects of aging place on society.

CHAPTER 2

HOW LEARNING CAN HELP YOU REMEMBER: THE LONGITUDINAL EFFECTS OF LIFELONG REFERENCE MEMORY PRACTICE AND MEMORY TRANSFER IN MALE AND FEMALE RATS

Increasing age is the number one risk factor for developing AD, and there are more than 5 million people in the U.S. afflicted with AD (Alzheimer's Association, 2007). Currently, there are few treatments to alleviate the symptoms of AD, and no treatments exist to reverse the pathology associated with AD. However, there may be non-pharmacological that can help protect individuals from memory loss due to normal aging as well as AD. One non-pharmacological treatment to deter age-related or neurodegenerative memory changes may be remaining cognitively active, as evidence supports the idea that education decreases risk of developing dementia and/or the symptoms of AD (see Chapter 1). Indeed, college professors (with 21-23 years of education) maintained cognitive performance as they aged, with professors in their sixties performing as well as professors in their thirties on proactive interference and some WM tasks (Shimamura et al., 1995). While, greater mental ability as a child- measured on a global scale of cognitive functioning, decreases the risk of developing late-onset dementia (Whalley et al., 2000). Taken together, these suggest that cognitive practice may

attenuate age-related memory decline and perhaps protect against some of the symptoms of AD.

Rodent studies evaluating cognitive practice in addition to the ones mentioned earlier (see Chapter 1), found that cognitive practice is generally beneficial to aged cognition. Markowska & Savonenko (2002b) trained male rats on a battery of tests every six months, including RM and WM versions of the Morris Maze. When 24 months old, rats were assessed on the same battery of tests in which they had received practice on (Markowska and Savonenko, 2002b). Protection against age-related decline was found in both RM and WM Morris Maze tasks. Accurate spatial memory has also been found to be preserved after extensive testing for 10 months on the WM land radial-arm maze (Bierley et al., 1986). Rats were tested on the WM land radial-arm maze from 3-11 months of age; when 21.5 months of age, the experienced rats performed as well as young control animals (Bierley et al., 1986). More recent longitudinal research found that extensive WM cognitive practice on a modified radial-arm maze in mice from 3 to 18 months of age conferred an attenuation of age-related learning and attentional deficits evaluated by a battery of tasks when the mice were aged (Matzel et al., 2011).

Although vital, few to no previous inquiries into the effects of cognitive practice on age-related memory decline have used a different maze for training and final assessment. Hence, it is unclear whether

cognitive practice protects only within the practiced memory domain or whether it has a global protection that includes other memory types. Most previous reports used the same maze for training and final assessment. Thus, the question of whether cognitive practice protects against age-related memory changes on another non-practiced maze task has not been fully evaluated. These previous studies also did not control for the procedural aspects of maze testing. The procedural aspects of maze testing may provide protection against cognitive decline, insomuch as the act of maze testing involves aspects of enrichment that may enhance memory when aged (for review see Frick and Benoit, 2010). Finally, it is noteworthy that to the authors' knowledge only one study has evaluated the effects of cognitive practice on memory in females, and in this study only females were evaluated so relation to males could not be determined (Ando and Ohashi, 1991). Hence, it remains to be elucidated in a single study if sex differences exist in the response to cognitive practice.

BF cholinergic neurons project to the hippocampus and surrounding cortical areas, and play an important role in learning and memory (Hasselmo, 2006). Cholinergic neuron survival and maintenance is dependent on neurotrophins, which includes nerve growth factor (NGF) (Granholm, 2000; Levi-Montalcini, 1987; Woolf, 1991). Neurotrophin systems have been found to become aberrant with age (Mufson et al., 1995). My lab has reliably shown that neurotrophins correlate with

cognitive performance in aging rats, in that higher levels of NGF within the hippocampus and cortex related to worse memory performance in ovary-intact aged female rats (Bimonte et al., 2003; Bimonte-Nelson et al., 2003a). It remains to be evaluated if cognitive practice can alter neurotrophin levels in cognitive and motor brain regions and whether aged neurotrophin levels relate to memory performance when young in male and female rats.

Noting the several questions remaining to be addressed or fully evaluated in the literature, the current experiment was designed to directly and methodically address the following questions: a) Does RM cognitive practice prevent loss only the RM domain, or does it extend protection into the WM domain? b) Is memory protection from cognitive practice due to the procedural components of testing or the cognitive demand of the maze task? c) Is age-related cognitive protection due to cognitive practice only seen when the aged assessment task is the same as the cognitive practice task? d) Do males and females differ when comparing their memory and the effects of cognitive practice? e) Does cognitive practice alter cognitive and motor brain region specific levels of NGF? f) Is memory performance when young related to aged brain region specific levels of NGF? g) Is the rate at which animals learned the cognitive practice task when young related to how they perform when aged? The hypotheses of this study were: a) RM cognitive practice will extend a global protection

into the WM memory domain, b) cognitive demand will be necessary for protection of the memory system when aged (e.g. experience with only the procedural components will not be sufficient to enhance performance when aged), c) memory protection will extend to novel maze tasks, d) males and females will differ regarding how cognitive practice attenuates age-related memory decline, e) cognitive practice will alter specific brain region levels of NGF, f) memory performance when young will be related to aged NGF levels, and g) an animal's ability to learn the RM cognitive practice task when young will be related to its ability to perform when aged.

Methods

Subjects

Ninety 6 month old Fisher-344 rats (45 male, 45 female) were obtained from the National Institute on Aging colony at Harlan Laboratories and pair housed in the Arizona State University animal facility. Animals had exposure to food and water ad-lib, and were maintained on a 12-h light/dark cycle at 74 ºF (minimum 68 ºF / maximum 78 ºF). Procedures were approved by Arizona State University Institutional Animal Care and Use Committee (IACUC) and adhered to the Guide for the Care and Use of Laboratory Animals and NIH standards.

The age of initiation of the cognitive practice and swim only testing schedule for the present study was based on a previous study indicating

that cognitive practice beginning at 6 months of age exerts the strongest attenuation of age-related changes in male rats as compared to age-matched controls (Markowska and Savonenko, 2002b). Thus, maze practice period began when rats were 6 months of age. Practice continued for one year, with rats receiving their last practice session at 18 months of age. To keep the lifetime practice treatment conditions as constant and similar as possible, all lifetime practice groups were tested on a water-escape T-Maze. For final assessments, several additional groups were evaluated that did not have exposure to the water-escape T-Maze throughout life. Specifically, the following groups were evaluated: (a) aged naïve (Aged-Naïve), (b) aged swim only (Aged-Swim Only), and (c) aged cognitive practice (Aged-Cog Prac). The Aged-Swim Only and Aged-Cog Prac groups received maze testing from 6 to 18 months of age during the practice period, and testing was conducted in a water escape T-Maze once every three months.

All rats were divided into three testing waves with the same number of animals/treatment group per wave. This was done to allow the consecutive testing of a manageable number of animals. Thus, for the practice period, one wave of animals was tested every month; hence, each animal was tested once every three months. Put in other terms, there were three separate 6 month old cohorts of rats that started the cognitive practice or swim only task at different times (i.e. a new group

each month, with each group being tested every three months) to achieve the staggered three testing waves. For the final battery of tests, each wave was tested approximately three months after their last practice session. The final battery of testing also included an additional group of 6 month old young naïve animals (Young-Naïve). After behavioral testing all animals were sacrificed to collect brain tissue for NGF evaluations, as detailed below. For sacrifice, I included a final group of aged animals that were never tested (Aged-Never Test) and sat in their cages their entire life until sacrifice. The next section provides a detailed description of each treatment group, and Figure 1 displays the timeline of the experimental procedures.

Experimental Groups

Cognitive practice group (Aged-Cog Prac).

From 6 to 18 months of age, Aged-Cog Prac animals were tested every 3 months for 5 days with 6 trials per day on the RM T-Maze. This procedure is a modified protocol (Denenberg et al., 1991; Denenberg et al., 1990; Gibbs et al., 2004). The testing room had salient visual extra-maze cues that remained constant throughout testing. The black Plexiglass maze (each arm was 38.1cm x 12.7cm) was filled with water made opaque with black non-toxic paint, and had a hidden escape platform at the end of one of the two non-start arms. Each rat had an assigned exit location (East or West) for the entire duration of testing,

including when the RM T-Maze was evaluated on the final battery. Drop off locations varied between north and south arms to control for motoric/olfactory strategies for finding the platform. After a rat located the platform, it remained there for 5 seconds; the rat was then removed and placed in its heated cage for 30 seconds until the next trial. Between each animal the maze was cleaned with a net to further minimize the possibility of the animal using an olfactory strategy to solve the task. The dependent variable was the number of correct arm entries, quantified as the number of times an animal found the hidden escape platform within a day; this measure was capped at 6 since there were 6 trials per day. The Aged-Cog Prac group evaluated the effects of a lifetime of cognitive practice on aged cognitive performance.

Aged swim only group (Aged-Swim Only).

The Aged-Swim Only group received identical treatment conditions, in a T-Maze identical to the one used for the Aged-Cog Prac group, except the task was modified to minimize cognitive demand. For this group, exit arms were assigned randomly for each trial pair. With one arm blocked, the escape arm was the only other arm left open aside from the start arm; hence, Aged-Swim Only animals simply swam and turned once (i.e., a forced choice) to escape the maze and “win” each trial. The procedures minimized the cognitive demand placed on Aged-Swim Only animals. Drop off locations varied between north and south arms to maximize

swimming similarities to the Aged-Cog Prac group. The Aged-Swim Only group addressed whether the procedural aspects of maze testing throughout the practice period influenced age-related cognitive change. This group was used as the control group to evaluate the effects of cognitive practice, since they received the most similar lifetime experiences as compared to the Aged-Cog Prac group.

Aged naïve animals (Aged-Naïve).

The Aged-Naïve animals remained pair housed in their colony room cages for the entire practice period (i.e., from 6-21 months of age). Once the practice period was concluded, the Aged-Naïve animals were tested on the final battery. The Aged-Naïve group allowed me to make a comparison to the normally aged laboratory rat that did not receive any cognitive practice or the potentially enriching procedural components of testing.

Young naïve animals (Young-Naïve).

The Young-Naïve group consisted of eighteen (9 male & 9 female) young rats that were added to the study during the final battery of tests. This group was added to the study so that a comparison to the best of a rat's learning and memory ability could be made**.** It is important to note that these animals were 6 months of age when tested on the final battery, representing the age when cognitive practice started for the Aged-Cog Prac and Aged-Swim Only groups.

Aged never tested (Aged-Never Test).

The 18 (9 male & 9 female) Aged-Never Test animals remained in their colony room cages for the entire training period, and during the final battery testing period. Once the training and final battery testing period was concluded, the Aged-Never Test animals were sacrificed along with the other groups of animals. This group was included to evaluate neurotrophin levels in animals that simply sat in their cage their entire adult lives and represented normally aged laboratory rats not exposed to any behavioral testing.

Mazes Used During the Final Battery

The final battery of maze assessments was conducted when testing groups were 21 months of age (i.e., with the exception of the Young-Naïve group). The final battery of maze assessments included mazes tapping into both WM and RM. This was done so that an evaluation could be made of whether cognitive practice throughout life within the RM domain transferred to the WM domain once aged. Also, the final battery investigated whether training on the RM T-Maze benefited performance on other maze types. This was accomplished by testing rats on the WM and RM water radial-arm maze (WRAM), as well as the RM Morris maze; both mazes utilized novel RM and WM paradigms assessed in novel environments for all testing groups including the lifelong practiced Aged-Cog Prac and Aged-Swim Only groups.

RM Morris Maze.

The RM Morris Maze (Morris et al., 1982) consisted of a round tub (188 cm in diameter) filled with water made opaque with black, non-toxic paint. The rat was placed in the maze from any of four locations (i.e., North, South, East, or West) and had 60 seconds to locate a submerged hidden escape platform which remained in a fixed location (i.e., the target Northeast quadrant; NE) throughout testing. After 15 seconds on the platform, the rat was placed into its heated cage until the next trial; the inter-trial-interval was 5-8 minutes. For each rat, the testing session consisted of 4 trials/day for 5 days. A video camera recorded each rat, and a tracking system (EthoVision 3.1, Noldus Information Technology, Wageningen, Netherlands) analyzed each rat's path. The dependent measure was swim distance (cm), with less swim distance interpreted as better spatial RM performance. To assess platform localization, a probe trial was given on an additional trial (trial 5) on the last day of testing, whereby the escape platform was removed from the maze. The dependent measures for the probe trial were the number of crossings where the platform previously was located, as well as the percentage of total swim distance (cm) in the target NE quadrant (i.e., quadrant that contained the platform on the test trials) as compared to the opposite Southwest (SW) quadrant.

RM T-Maze.

The procedure for the RM T-Maze during the final battery was identical to that given to the Aged-Cog Prac group during their cognitive practice sessions from 6 to 18 months of age. All platform locations remained constant for an animal during cognitive practice training/testing but were semi-randomly selected for each animal. Hence, Aged-Cog Prac animals escaped from the maze in the same location in space throughout all practice sessions and during the final battery. Data were scored via assessing the number of correct arm entries made within a daily session, which served as the dependent variable.

WM and RM WRAM.

This win-shift radial 8-arm maze was constructed of black Plexiglass and filled with water (room temperature) made opaque via black non-toxic paint (e.g., Bimonte and Denenberg, 1999; Bimonte and Denenberg, 2000; Bimonte et al., 2000). It had hidden escape platforms with wire mesh tops (hidden about 1 cm below the water surface) placed in the ends of four of the 8 arms. Each subject had different platform locations that were semi-randomly determined and that remained fixed throughout testing.

A subject was released from the start arm and had 3 minutes to locate a platform. Once a platform was found, the animal remained on it for 15 seconds, and was then returned to its heated cage for a 30 s inter-trial interval (ITI) until its next trial. During the interval, the just-chosen

platform was removed from the maze. The animal was then placed again into the start alley and allowed to locate another platform. For each animal, a daily session consisted of four trials, with the number of platformed arms reduced by one on each subsequent trial. Thus, WM systems were increasingly taxed as trials progressed, allowing me to assess WM load. Each subject was given one session a day for 12 consecutive days.

The following quantification and blocking procedures are based upon previous studies using the WRAM (Bimonte-Nelson et al., 2003a; Bimonte and Denenberg, 1999; Bimonte and Denenberg, 2000; Bimonte et al., 2002; Bimonte et al., 2000; Bimonte et al., 2003; Hyde et al., 1998; Hyde et al., 2000). Behavioral testing took place between the hours of 0800 and 1700. An arm entry was counted when the tip of a rat's snout reached a mark delineated on the outside of the arm and not visible from the inside of the maze (11 cm into the arm). Errors were quantified using the orthogonal measures of WM and RM errors (Jarrard et al., 1984), as done previously in studies using the water escape radial-arm maze (Bimonte-Nelson et al., 2003a; Bimonte et al., 2002; Bimonte et al., 2000; Hyde et al., 2000). The last four days of testing were used to evaluate WM and RM competence, as this was after rules were learned and thereby evaluates a more pure measure of WM. WM correct (WMC) errors were the number of first and repeat entries into any arm from which a platform

had been removed during that day. RM errors (WRM) were the number of first entries into any arm that never contained a platform within a day. WM incorrect errors (WMI) were the number of repeat entries into an arm that never contained a platform within a day (i.e., within day repeat entries into a RM arm). The separate number of WMC, WMI, and WRM errors served as the dependent variables for statistical analysis.

Brain Dissection

At sacrifice, animals were anesthetized with isoflurane (Vetone, Meridian, Indiana) and decapitated according to NIH euthanasia guidelines, after which the brains were rapidly dissected. Using Paxinos and Watson (2005) as a reference, the right hemisphere, frontal cortex, parietal cortex, temporal cortex, striatum, and ventral hippocampus (CA1/2) were dissected for neurotrophin analyses. Dissected tissues were immediately placed in weighed microcentrifuge tubes and stored at -70 °C until tissue lysates were made.

NGF Quantification

NGF levels were assessed using commercially available assay kits from Promega (Madison, WI). Neurotrophin assay procedures were done as previously described (Bimonte-Nelson et al., 2008; Bimonte-Nelson et al., 2003a; Bimonte-Nelson et al., 2004a; Bimonte et al., 2003; Engler-Chiurazzi et al., 2009; French et al., 2006). Briefly, tissue lysates were made, and flat-bottom 96 well plates were coated with NGF capture

antibody. The captured NGF 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 a plate reader by measuring absorbance at 450 nm. Using these kits, 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%. The amount of NGF (pg/mg of tissue) present in the brain region of interest was the dependent variable used for statistical analysis.

Statistical Methods

Composite dependent variable construction.

Several composite dependent variables were created (see Table 1 for a summary of the dependent variable composites). Specifically, composite measures were created *for each maze* by summing the dependent variable(s) across: all days and trials (overall performance), as well as the last few days of testing with all trials, or only the last trial, depending on the specific maze type (asymptotic performance). Two additional composites evaluated overnight retention on the Morris maze as similarly performed in previous studies (Acosta et al., 2009b; Bimonte-Nelson et al., 2006; Markham et al., 2002), and young RM performance on

their first cognitive practice session at 6 months of age. Lastly, a global (i.e., multiple domains of memory) composite memory measure was created by using scores from all novel mazes (non-practiced tasks). For this composite, I converted the previously created RM Morris Maze distance composite (all days and trials) and RM and WM WRAM composite (WMC, WMI, & WRM errors) to z-scores and averaged them. This allowed evaluation of RM and WM performance during the final battery on tasks that were novel to the Aged-Cog Prac and Aged-Swim Only groups. I termed this composite measure the Aged Novel Global Memory Composite. The creation of these composites was based on previous animal work whereby analyses of variance (ANOVA) found effects on these tasks during similar blocks of days and trials (Bimonte and Denenberg, 1999; Daniel et al., 1997; Frick et al., 1995; Markham et al., 2002; Sandstrom and Williams, 2004; Talboom et al., 2010; Talboom et al., 2008), as well as previous clinical work in humans assessing global measures of memory (e.g., Huynh et al., 2011; Matzel et al., 2011).

Planned contrasts.

Since my *a priori* design was to evaluate several cognitive practice research questions not previously or fully addressed in animal literature, I employed planned contrasts to evaluate pairwise comparisons of groups, which assessed my primary research questions. Each question followed by the contrast name is as follows: 1) does cognitive practice enhance

memory when compared to similarly practice aged animals without cognitive demand? *Cognitive practice contrast*, Aged-Swim Only were compared to Aged-Cog Prac animals, 2) does cognitive practice obviate or attenuate age-related memory decline? *Aging with cognitive practice contrast*, Young-Naïve were compared to Age-Cog Prac animals, 3) is cognitive demand necessary to alter age-related cognitive decline? *Procedural component contrast*, Aged-Naïve were compared to the Aged-Swim Only group, and 4) does age alter performance on the final battery of mazes? *Age without practice contrast*, Young-Naïve animals were compared to Aged-Naïve animals. A final contrast evaluated the effects of age on brain NGF levels, independent of cognitive practice and behavioral testing; in this contrast Young-Naïve animals were compared to Aged-Never Test animals.

With specific *a priori* comparisons in mind, planned contrasts can aid in the interpretation of results and increase the statistical power in comparison to a less focused omnibus test (see section 8.5 in Cohen et al., 2003). Similar to a *t*-test, planned contrasts compare the means of two groups or a combination of groups; however, the error term (i.e., unexplained variance, Mean Square within-groups/error) is calculated from all the groups in the study (i.e., pooled within-group variance), not just the two groups being compared (see Rosenthal and Rosnow, 1985). Hence, statistical power can be increased by having a better estimate of

the unaccounted for variance (i.e., increase the *t*-value/ratio). Proc GLM was utilized in SAS (version 9.2, The SAS Institute Inc., Cary, NC) to evaluate each planned contrast by employing orthogonal contrast coding.

Linear growth modeling.

I utilized Mplus (version 5.21; Muthén & Muthén; Los Angeles, CA) to estimate a linear growth model that expressed the number of correct arm entries on the cognitive practice RM T-Maze (i.e., my outcome variable) as a function of Days 1-5 (i.e., my variable that captured the passage of time) during the first cognitive practice session when Cog Prac practice animals were 6 months of age (Enders, 2011). The unconditional linear growth model was estimated as follows: $\boldsymbol{NUMBER\ CORRECT} = \mathbf{B_0} + \mathbf{B_1}\ (\boldsymbol{DAY}) + \boldsymbol{b_0} + \boldsymbol{b_1}(\boldsymbol{DAY}) + \ \boldsymbol{\varepsilon}$, where $\boldsymbol{NUMBER\ CORRECT}$ was the number of correct arm entries made, $\boldsymbol{DAY}$ was the value of elapsed time in days since the onset of the cognitive practice session, $\mathbf{B_0}$ was the mean Intercept (i.e., mean initial performance), $\mathbf{B_1}$ was the mean Slope (i.e., mean change per day), $\boldsymbol{b_0}$ and $\boldsymbol{b_1}$ were residuals (i.e., random effects) that allowed the intercepts and the growth rates to vary from one rat to another, and $\boldsymbol{\varepsilon}$ was a time-specific residual that captured the difference between an animal's fitted linear trajectory and its observed data (Enders, 2011).

Growth models allow for the addition of predictors that can influence the Intercept, Slope, or both. As such, Sex as a binary predictor

(i.e., coded 0 for female and 1 for male) was added to the model in order to predict if males and females differed in their initial performance (i.e., the Intercept or test day 1) or the rate at which they learned (i.e., the Slope or change per day) the cognitive practice RM T-Maze at 6 months of age. Additionally, one of the strengths of Mplus is that the Intercept and the Slope can also serve as predictors of other outcomes. I capitalized on this tenet by adding to my model the Novel Global Memory Composite. This analysis was employed as I wanted to evaluate the question of whether how well an animal initially performed or the rate at which they learned the RM T-Maze cognitive practice task at 6 months of age was related to their overall cognitive performance when aged. An association between initial status/ability and aged cognitive ability has been previously suggested in the human literature (Whalley et al., 2000). Collectively, the expanded model was: $\boldsymbol{NUMBER\ CORRECT_{ti}} = \mathbf{B_0} + \mathbf{B_1}\ (\boldsymbol{DAY}) + \boldsymbol{B_2}(\boldsymbol{MALE}) + \boldsymbol{B_3}(\boldsymbol{DAY})(\boldsymbol{MALE}) + \boldsymbol{b_0} + \boldsymbol{b_1}(\boldsymbol{DAY}) + \boldsymbol{\varepsilon}$, where $\mathbf{B_0}$ and $\mathbf{B_1}$ were the initial performance and rates of change per day for female rats and $\mathbf{B_2}$ and $\mathbf{B_3}$ represent the amount by which values differed from females to males for initial performance and change per day, respectively. Figure 2a displays a plot of the linear growth model with the mean growth rate of Cog Prac animals at 6 months of age (sample) and the model estimated linear growth rate; 2b depicts a simplified path diagram of the full model with unstandardized path coefficients.

Results

Orthogonal Planned Contrasts

Effects of cognitive practice (Aged-Swim Only vs. Aged-Cog Prac, cognitive practice contrast).

There was a significant cognitive practice contrast for the RM T-Maze on the composite of the number correct arm entries for all days and trials (F[1,71] = 31.31, $p < 0.0001$) as well as for the last 3 days of testing for all test trials (F[1,71] = 21.54, $p < 0.0001$, Figure 3a), with the Aged-Cog Prac group outperforming the Aged-Swim Only group, suggesting that a lifetime of RM cognitive practice enhances RM performance in males and females on a *familiar RM task in a familiar environment*.

The cognitive practice contrast for the RM Morris Maze approached significance for the composite of the last 3 days of testing and all test trials (F[1,73] = 3.37, $p = 0.0564$, Figure 3b). I next evaluated the overnight retention contrast (test days 2-5 on trial 1 alone), which approached significance (F[1,73] = 3.51, $p = 0.0651$). Examination of the learning curves across days 2-5 from trial 4 of the previous day to trial 1 of the next day (Figure 3c) led me to contrast males and females separately. Aged-Cog Prac females performed better than Aged-Swim Only females (F[1,34]=6.26, $p = 0.0017$), an effect not found in males (F[1,39] = 1.03, $p = 0.7280$, Figure 3c). These RM Morris Maze results suggest that females, but not males, transferred the benefits of a lifetime of RM cognitive

practice to a *novel RM task in a novel environment*. The evaluation of the Morris maze probe trial revealed that each treatment group localized to the previously platformed quadrant, indicated by a higher percentage of total swim distance traveled in the target NE quadrant as compared to the opposite SW quadrant (F[1,158] = 374.49, $p < 0.0001$, Figure 3d). The Aged-Cog Prac group and the Aged-Swim Only group did not show different patterns of quadrant preference (NE quadrant F[1,73] = 0.50, p = 0.4838; SW quadrant F[1,73] = 1.22, p = 0.2724, Figure 3d), nor did they differ for number of platform crossings (F[1,73] = 0.11, p = 0.7461, Figure 3e). These results suggest that all animals were able to localize the platform quadrant and that cognitive practice did not alter this ability. For WM performance on the WM and RM WRAM, there was a significant cognitive practice contrast on the composite of WMI errors for the last 4 days of testing when WM load was the highest on trial 4 alone (F[1,75] = 4.93, p = 0.0294, Figure 3f). This indicates that both male and female RM cognitive practice animals were able to transfer the benefits of a lifetime of RM cognitive practice to a *novel WM task in a novel environment*.

The effects of cognitive practice to obviate or attenuate age-related memory decline (Young-Naïve vs. Aged-Cog Prac, age with cognitive practice contrast).

For the RM T-Maze, the age with cognitive practice contrast was significant for all days and trials, and for the last 3 days of testing for all

test trials. Aged-Cog Prac animals outperformed Young-Naïve animals (for all days $F[1,71] = 16.46$, $p < 0.0001$; for the last 3 days $F[1,71] = 12.57$, $p = 0.0007$, Figure 4a), suggesting that a lifetime of RM cognitive practice enhanced performance on the cognitive practice RM task.

Further analyses revealed that cognitive practice ***protected*** against age-related memory decline on the RM Morris Maze for overnight retention in females, as the young animals did not differ from aged animals that received cognitive practice (a significant contrast was not found ($F[1,34] = 1.19$, $p = 0.2823$, Figure 4b). For WRAM WMI errors, I found that cognitive practice ***attenuated*** age-related memory decline since Aged-Cog Prac animals outperformed the Aged-Swim Only group, but the Aged-Cog Prac group did not outperform the Young-Naïve group (summed across the last 4 days of testing on trial 4 alone ($F[1,75] = 6.86$, $p = 0.0107$, Figure 4c). When examining the probe trial on the Morris maze, I found the age with cognitive practice contrast was not significant when assessing percent swim distance in the NE and SW quadrants (NE $F[1,73] = 1.29$, $p = 0.2604$; SW $F[1,71] = 1.50$, $p = 0.2241$, Figure 3d). This indicated that the Young-Naïve and Aged-Cog Prac groups did not differ in their ability to localize the previously platformed quadrant. However, the age with cognitive practice contrast evaluating the number of platform crossings revealed that the Young-Naïve group made more crossings as compared to the Aged-Cog Prac group ($F[1,73] = 9.48$, $p =$

0.0029, Figure 3e). Collectively, these data suggest that although all animals were able to localize the general vicinity of platform location to the previously platformed quadrant of the maze, young animals were better able to localize to the *specific* previous platformed location.

The effects of the procedural components of cognitive practice on memory (Aged-Naïve vs. Aged-Swim Only; procedural component contrast).

An analysis of the WM and RM WRAM revealed a significant procedural component contrast on the composite created for WMC errors for all days and trials ($F[1,75] = 9.97$, $p = 0.0023$, Figure 4d); Aged-Swim Only animals committed more WMC errors as compared to the Aged-Naïve group. When evaluating the ability of Aged-Naïve and Aged-Swim Only animals to localize the platform on the probe trial for the RM-Morris maze, the Aged-Naïve group did not differ from the Aged-Swim Only group (NE quadrant percent distance $F[1,73] = 0.35$, $p = 0.5564$; SW quadrant percent distance $F[1,73] = 0.58$, $p = 0.4469$, Figure 3d; number of platform crossings $F[1,71] = 0.58$, $p = 0.4469$, Figure 3e). This indicates that that being exposed to the procedural components cognitive practice does not attenuate age-related changes in memory. In fact, exposure to the procedural components of cognitive practice may impair WM performance when aged.

The effects of age (Young-Naïve vs. Aged-Naïve; age without cognitive practice contrast).

The Young-Naïve group consistently outperformed the Aged-Naïve group on some measures of the RM Morris Maze (overnight retention in females $F[1,34] = 13.35$, $p = 0.0009$, Figure 5b; number of platform crossings $F[1,73] = 12.44$, $p = 0.0007$, Figure 3e) and the WRAM (WMI for all days and trials $F[1,75] = 6.89$, $p = 0.0105$; WRM for all days and trials $F[1,75]=16.70$, $p = 0.0001$; WMC across the last 4 days of testing for all test trials $F[1,75] = 9.62$, $p = 0.0027$, Figure 5c; WMI across the last 4 days of testing for all test trials $F[1,75] = 11.23$, $p = 0.0013$; WRM across the last 4 days of testing for all test trials $F[1,75] = 23.29$, $p < 0.0001$; WMI across the last 4 days of testing for trial 4 alone $F[1,75] = 9.77$, $p = 0.0025$, Figure 5d). The age without cognitive practice contrast evaluating the composites of the RM T-Maze was not significant (for all days and trials $F[1,71] = 0.54$, $p = 0.4669$; for the last 3 days of testing and all trials $F[1,71]=1.35$, $p = 0.2490$, Figure 5a), indicating a lack of an age effect on this task. I also did not find a significant age without cognitive practice contrast for RM Morris Maze platform localization via my quadrant data, indicating that young and aged animals all learned the quadrant that contained the platform (NE quadrant percent distance $F[1,73] = 1.75$, $p = 0.1902$; SW quadrant percent distance $F[1,71] = 2.58$, $p = 0.1123$, Figure 3d).

Neurotrophin Contrast Analyses

For the cognitive practice contrast comparing Young-Naïve animals to Aged-Cog Prac animals, the Aged-Cog Prac group had significantly higher levels of NGF levels as compared to the Young-Naïve group in the: striatum (F[1,83] = 9.40, p = 0.0029), frontal cortex (F[1,84] = 6.71, p = 0.0113), parietal cortex (F[1,84] = 4.49, p = 0.0370), and temporal cortex (F[1,84] = 4.59, p = 0.0351) (Figure 6a). Since these NGF level increases were found in the Aged-Cog Prac group without concomitant significant contrasts between the Young-Naïve and Aged-Naïve groups, or the Young-Naïve and Aged-Never Test groups, it appears that age and cognitive practice act together to increase NGF levels in the striatum, frontal cortex, and temporal cortex of rats. I found a significant age without cognitive practice contrast (Young-Naïve vs. Aged-Naïve) for parietal cortex NGF levels (F[1,84] = 4.67, p = 0.0335, Figure 6b), and another significant contrast for parietal cortex NGF levels between the Young-Naïve and Aged-Never Test groups (F[1,84] = 7.11, p = 0.0092, Figure 6c). This suggests that in the parietal cortex, age and not cognitive practice likely results in the observed NGF level increase in this brain region, since aged animals who received cognitive practice, aged animals that never received cognitive practice/testing, and aged animals that were only tested on the final battery all had increased parietal cortex NGF levels in comparison to young animals. Interestingly, the collected data indicate

that cognitive demand was necessary for the NGF level increase observed in aged animals within the striatum, frontal cortex, and temporal cortex.

Regression Analyses: Relationships Between Memory Performance When Young and NGF Levels When Aged

I conducted primary regression analyses relating ventral hippocampal and frontal cortex NGF levels to RM T-Maze performance during the Cog Prac group's first cognitive practice session when they were 6 months of age. Specifically, RM T-Maze correct entries summed across all days and trials was the predictor variable, and NGF levels in the ventral hippocampus (CA1/2) and frontal cortex were the outcome variables. For male and female Aged-Cog Prac animals, there was a positive relationship between the number of correct arm entries made at 6 months of age and ventral hippocampal NGF levels assessed when aged (b = 0.10, r = 0.59, z[19] = 2.72, p = 0.0065, Figure 7a); this association was not found in the frontal cortex. Since one of my primary research questions was to evaluate sex differences, I conducted these same regression analyses in males and females separately. For ***female*** Aged-Cog Prac animals, there was a positive relationship between the number of correct arm entries made at 6 months of age and the measure of aged ventral hippocampal NGF levels (pg/mg) (b = 0.13, r = .79 z[8] = 2.39, p = 0.0169, Figure 7b), as well as aged frontal cortex NGF levels (pg/mg) (b = 0.26, r = .71 z[8] = 2.00, p = 0.0457, Figure 7c). These relationships were

not found when male Aged-Cog Prac animals were evaluated alone, suggesting that *females* were responsible for the association between young RM performance and aged hippocampal NGF levels. Taken together, these associations suggest that in females, better RM performance when young is related to higher levels of hippocampal and frontal cortex NGF when aged.

Linear Growth Modeling

An assessment of RM T-Maze performance across the first RM practice session via linear growth modeling revealed that 6 month old Cog Prac males and females made approximately ***four*** correct arm choices on the first day of RM T-Maze practice (Intercept/initial performance; B_0 = 4.03, $p < .000$). This analysis also revealed that 6 month old Cog Prac males and females learned the RM T-Maze at the rate of ***one-half*** of a correct arm entry per day (Slope/change per day; B_1 = 0.45, $p < 0.000$, Figure 2a). Further analyses indicated that 6 month old female Cog Prac animals on their own across the first RM cognitive practice session made approximately ***three and a half*** correct arm entries on the first day of RM T-Maze practice (Intercept/initial performance; B_0 = 3.56, $p = 0.000$, Figure 2b) and learned at the rate of roughly ***one-half*** of an additional correct arm entry per day (Slope/change per day; B_1 = 0.58, $p = 0.000$, Figure 2b).

When comparing 6 month old male and female Cog Prac animals on their performance during the first day of RM T-Maze practice as well as

the rate at which they learned the RM T-Maze across the first RM practice session (i.e., intercept/initial performance and slope/change per day, respectively), the linear growth model revealed that 6 month old male Cog Prac animals made ***one*** additional correct arm entry on the first day of RM T-Maze practice as compared to 6 month old Cog Prac females (B_2 = 0.96, p = .003). In contrast, 6 month old male Cog Prac animals learned the RM T-Maze practice task at the rate of ***a third*** of a correct arm entry per day less across the first RM practice session when compared to 6 month old Cog Prac females (B_3 = -0.27, p = 0.001). This suggests that 6 month old Cog Prac males performed better than 6 month old Cog Prac females on the first day of RM T-Maze practice; however, 6 month old Cog Prac females learned the RM T-Maze practice task during the first practice session at a faster rate when compared to 6 month old Cog Prac males.

Lastly, when relating the rate at which 6 month old Cog Prac males and females learned the RM T-Maze across the first RM practice session (i.e., slope/change per day) to the Novel Aged Global Memory Composite, I found a positive relationship between how fast Cog Prac males and females learned the RM T-Maze when young and their novel global memory performance when aged (i.e., a positive relationship between slope/change per day to the Novel Aged Global Memory Composite). Specifically, Cog Prac males and females that learned the RM T-Maze at a faster rate when young (i.e., 6 months of age) had enhanced WM and

RM assessed on novel tasks during the final battery when aged (i.e., 21 months of age) (B = 3.37, p ≤ .05, Figure 2b). The relationship between rate of learning when young (i.e., slope/change per day) and novel global memory performance when aged (i.e., the Novel Aged Global Memory Composite) was strong by conventional standards (see Cohen, 1988), as evidenced by a high Pearson *r* value (*r* = 0.83, *p* = 0.003). Collectively, these data suggest that young 6 month old male and female animals differed in their RM performance on the first day of RM T-Maze practice as well as the rate at which they learned the RM T-Maze task across the first RM practice session. These data also highlighted an exciting positive relationship between RM learning rate when young and global memory performance when aged.

Discussion

The primary findings in this study were that: a) cognitive practice enhanced RM performance for males and females on the cognitive practice task, b) cognitive practice attenuated age-related WM changes on a novel WM task for males and females, c) cognitive practice protected against age-related memory changes for overnight retention on a novel RM task for females only, e) cognitive demand was necessary for protection against age-related memory changes, f) age and cognitive practice acted together to alter NGF levels in the striatum, frontal cortex, and temporal cortex, g) better RM performance when young was related

to higher levels of hippocampal and frontal cortex NGF when aged in only females, and h) the faster an animal learned the cognitive practice task when young the better its global memory performance when aged. As outlined in the Introduction, several questions were specifically addressed in this study. The question and corresponding answer, as addressed by the current study, follow.

1. Does RM Cognitive Practice Prevent Loss Only the RM Domain, or Does it Extend Protection into the WM Domain?

RM cognitive practice appears to protect in its own domain, as well as extending global protection into the WM domain. The results suggest that RM cognitive practice helped both aged male and female animals to handle an increasing WM load (i.e., effect found for trial 4 alone on the WRAM) on a novel WM task, and attenuated the age-related WM decline found on the WRAM in this and other studies (Bimonte-Nelson et al., 2003c; Bimonte et al., 2002; Bimonte et al., 2003). Thus, the results indicate that RM cognitive practice exerts a global protection, including to WM systems. Since the Aged-Cog Prac males and females performed better on the RM T-Maze cognitive practice task during the final battery when compared to Aged-Swim Only and Young-Naïve animals, for both sexes RM cognitive practice protected in its own memory domain as well. However, this is not entirely clear as I did not find an age effect on the RM T-Maze during the final battery (i.e., Young-Naïve vs. Aged-Naïve

animals). This lack of an age effect corroborates previous research where no age effects were found on a land T-Maze except in the type of strategy that young and aged animals were more likely to use (i.e., aged = response and young = place) (Barnes et al., 1980). In females only, RM cognitive practice protected within its own domain on a novel RM task; on the RM Morris Maze for overnight retention, there was an age effect: Aged-Cog Prac females performed better than Aged-Swim Only females, and Aged-Cog Prac females did not differ from Young-Naïve females. Collectively, RM cognitive practice initiates a global protection into the WM domain, an effect that was observed on a novel WM task. Cognitive practice likely protected against age-related memory changes on the cognitive practice RM task and attenuated age-related WM deficits on a novel task. Interestingly, only females showed a cognitive practice-facilitated enhancement in novel RM task performance that was protected against age-related memory changes.

2. Is Age-Related Cognitive Protection Due to Cognitive Practice Only Seen When the Aged Assessment Task Is the Same as the Cognitive Practice Task?

The present study found that cognitive practice can protect against age-related decline on novel tasks. Results revealed that a lifetime of cognitive practice protects memory on a novel WM task (i.e., WRAM) for males and females and a novel RM task (i.e., Morris Maze) for females

only. Since these effects were observed on mazes novel to the cognitive practice groups, the effects could not simply be due to familiarity or prior experience with the task. The enhanced ability of the Aged-Cog Prac group as compared to the Aged-Swim Only group on the RM T-Maze during the final battery is most likely due to retention and not relearning of the platform location since their last cognitive practice session, as scores were essentially asymptotic after the first cognitive practice session and persisted to the last session at 18 months. Hence, cognitive practice benefits are not just seen on the cognitive practice task, but transfer to novel tasks as well, an effect that differs by sex.

3. Is Memory Protection From Cognitive Practice Due to the Procedural Components of Testing or the Cognitive Demand of the Maze Task?

This study found that rats receiving only the procedural aspects of the task exhibited no protection against age-related memory decline. Indeed, in the Aged-Swim Only group, animals had to swim to find the platform without having to decide the correct arm in the water escape T-Maze; thus, cognitive demand was minimized. Across all measures, Aged-Swim Only animals did not outperform Aged-Naïve animals. In fact, Aged-Swim Only animals demonstrated worse WM performed in comparison to the Aged-Naïve group on the WM and RM WRAM task (i.e., WMC errors). I decided to use the Aged-Swim Only group as the reference group to

analyze the effects of a lifetime of cognitive practice, as these animals had a similar non-cognitive experience to the Aged-Cog Prac group (i.e.,. stress, handling, and possible enrichment); thus, I thought it was safe to assume that the only difference between the Aged-Swim Only and Aged-Cog Prac groups was cognitive demand. Taken together, this suggests that cognitive demand is necessary for a lifetime of practice to enhance aged memory performance or attenuate/obviate age-related memory changes.

4. Do Males and Females Differ When Comparing Their Memory and the Effects of Cognitive Practice?

I noted several differences between male and female animals. Specifically, the linear growth model revealed that male and female animals differed in their initial performance and how fast they acquired the RM T-Maze when young. However, all Aged-Cog Prac animals reached and maintained near asymptotic performance by the end of their first cognitive practice session, thereby limiting the possibility that the sex differences in relation to cognitive practice were due to differential learning of the cognitive practice task. Furthermore, aged females were the only group to transfer the benefits of cognitive practice to a novel RM task (i.e., RM Morris Maze overnight retention), and females were the ones to show the relationship between young RM performance and aged NGF levels. Taken together, this suggests that the ovary intact female is more

behaviorally plastic than their male counterparts, an effect perhaps related to their hormonal milieu and/or trophic systems.

5. Does Cognitive Practice Alter Cognitive and Motor Brain Region Specific Levels of NGF?

I found that that age and cognitive practice likely act together to increase NGF levels in the striatum, frontal cortex, and temporal cortex of male and female rats, while only age likely results in the observed NGF increase in the parietal cortex. Neurotrophins may be one mechanism of how cognitive practice enhances aged performance or attenuates age-related cognitive changes. Survival and maintenance of cholinergic neurons are dependent upon neurotrophins, including NGF, and NGF has been associated with cognitive function (Bimonte-Nelson et al., 2008; Bimonte et al., 2003; Granholm, 2000; Levi-Montalcini, 1987; Siegel and Chauhan, 2000; Woolf, 1991). Thus, cognitive practice likely alters NGF to enhance synaptic efficacy or cholinergic activity, both of which have positive effects on memory and may explain why cognitive practice enhanced performance in the aged males and females.

6. Is Memory Performance When Young Related to Aged Brain Region Specific Levels of NGF?

Via regression analyses I noted sex-specific associations between RM performance when young and NGF levels when aged in cognitive brain regions. Specifically, the relationship between better RM

performance when young and higher levels of hippocampal and frontal cortex NGF levels when aged was found only in the female Aged-Cog Prac group. My lab and others have found associations between brain region NGF levels and memory (Albeck et al., 2005; Bimonte et al., 2003), and my lab has previously noted associations between frontal cortex brain-derived neurotrophic factor (BDNF) and memory in Ts65Dn mice and female rats (Bimonte-Nelson et al., 2003a; unpublished observations). Collectively, this suggests that memory performance when young is related to NGF levels when aged, an effect seen in females only.

7. Is the Rate at Which Animals Learned the Cognitive Practice Task When Young Related to How They Perform When Aged?

Indeed, I found a positive relationship between aged memory performance and “rate of change/learning” (i.e., slope of the growth model) when young. This finding is corroborated in part by another study where a negative relationship was found between late-onset dementia and “level” of mental ability when young (Whalley et al., 2000). Collectively, these data suggest that cognitive faculty measured early in life can predict aspects of aged memory in both rats and humans.

General Discussion

To my knowledge, the current study is the first single study to specifically address and fully evaluate several gaps in our understanding of the effects of cognitive practice on male and female rats. Overall, the

results show that spatial WM and RM decline with age, and that some of these age-related effects can be attenuated or obviated by cognitive practice. The finding that cognitive practice attenuates or obviates age-related changes in RM corresponds to other studies finding this same effect in males (Dellu et al., 1997; Markowska and Savonenko, 2002b; Pitsikas et al., 1991). The current study extends these works by showing that it is specifically the cognitive demand that is protective. Additionally, it is specifically RM cognitive practice that enhanced RM in both sexes. As previously shown in other studies on the WM and RM WRAM (Bimonte-Nelson et al., 2003c; Bimonte et al., 2002; Bimonte et al., 2003), I found that Aged-Naïve male and female rats showed an age-related memory decline in comparison to their Young-Naïve counterparts. This finding is also apparent on the RM Morris Maze in females, where Aged-Naïve animals exhibited poorer performance when compared to the Young-Naïve group. These findings are consistent with others showing age-associated memory decline on the RM Morris Maze (Markowska and Savonenko, 2002b; Pitsikas et al., 1991). Studies report that both RM and WM cognitive practice given to the same animals did not attenuate age-related WM decline (Dellu et al., 1997). Yet, others found that extensive WM cognitive practice (Bierley et al., 1986), or WM and RM cognitive practice (Markowska and Savonenko, 2002b), attenuated or reversed age-related WM decline. Thus, this study extends the previous findings by

showing that in both males and females, RM cognitive practice attenuated age-related WM changes. In addition, given that the Aged-Swim Only group did not show protection against age-related memory changes on any measure, the cognitive demand placed on the animals by the cognitive practice task was likely the reason why this study found age-related protection due to maze testing throughout life. The effects were not due to familiarity with the task or the handling by the experimenter that coincides with maze testing.

I would be remiss if I did not note the possibility that exercise played a role in the ability of cognitive practice to attenuate age-related memory changes. Studies have reported in rodents that exercise on a treadmill or running wheel enhances spatial memory on the radial-arm maze (Anderson et al., 2000), Morris maze (Liu et al., 2011), and the T- and Y-Mazes (Pang et al., 2006). However, the possibility that exercise was responsible for the effects of cognitive practice in the current study is diminished by the fact that exposure to the procedural components of testing (i.e., the Aged-Swim Only group), which included all the motoric and swimming (i.e., exercise) components of cognitive practice with minimal cognitive demand, did not enhance performance. This suggests that RM cognitive practice can attenuate age-related memory changes, an effect that is not linked solely to exercise. The next step is to fully elucidate the associated neurobiological changes, and how these effects are

modulated by sex. Spatial memory is intimately linked to hippocampal functioning, and researchers have suggested that learning may influence, and be dependent on, cell proliferation in the dente gyrus of the hippocampus (Klempin and Kempermann, 2007). Recently, in male rats who had the best performance on the RM Morris Maze, Drapeau et al. (2007) found that cells in the dentate gyrus generated before learning had occurred had an increased rate of survival, while those that were generated after the initial learning phase had a decreased rate of survival. The tenet that the integration of new neurons into existing neural networks may confer plasticity and thus enhance ability for learning and memory when aged is an exciting explanation for the present results. Adult neuronal neogenesis facilitated via learning and memory processes supports the hypothesis that you “use it or lose it”, as well as supporting the hypothesis that there is a mechanism for developing a “cognitive reserve” (Katzman et al., 1988). It may be that the demand of storing information long-term (i.e., RM), which increases the survival rate and integration of neurons from the dentate gyrus, enhances spatial memory in aged animals who have had a lifetime of cognitive demand.

Aside from newly generated neurons, another theory is that the biological underpinnings of memory may also be influenced by cognitive practice. Synaptic plasticity, thought to be the neurobiological correlate of memory, has been extensively studied under the paradigm of long-term

potentiation (LTP) and long-term depression (LTD) (see Bliss et al., 2003). LTP and LTD paradigms reveal that correctly timed presynaptic and postsynaptic high frequency bursts of current can alter many proteins in the area of the synapse (i.e., synaptic plasticity), leading to an alteration in the amplitude of excitatory postsynaptic currents, with the summed alteration in excitatory postsynaptic currents of numerous synapses being the hypothesized engram (see Bliss et al., 2003). LTP paradigms have also been shown to increase dendritic spine formation (Harris et al., 2003), and previous literature has found that memory decline in aged rats is related to decreased dendritic spine density (Wallace et al., 2007), but not the postsynaptic protein spinophilin (Calhoun et al., 2008). Taken together, it is conceivable to think that the neural activity generated by cognitive practice may be similar to the neural activity necessary to experimentally generate LTP and LTD, in turn enhancing synaptic plasticity and synaptic spine number. Collectively, this suggests that cognitive practice may lead to enhancements in neural activity, synaptic plasticity, and synaptic spine number, which may be responsible for the presently observed effects of cognitive practice.

One possibility for the sex differences found in this study is related to the work showing that ovarian hormones have a profound effect on neurotrophin systems. E2 is most potent activator of the classical genomic pathway and the primary export product of a woman's ovary (Prokai et al.,

2003). Neurotrophin and neurotrophin receptor mRNA levels have been found to fluctuate across the estrus cycle (Gibbs, 1998). E2 treatment significantly impacts neurotrophins in young and aged Ovx rats (i.e., rats with their endogenous source of E2 removed) by increasing neurotrophin protein mRNA as well as neurotrophin receptor (e.g., TrkA) mRNA levels in the BF, frontal cortex, and hippocampus (Gibbs, 1998; McMillan et al., 1996; Pan et al., 1999), and elevating NGF protein levels in cognitive brain regions (Bimonte-Nelson et al., 2004a). Taken together, this suggests that E2 alters the neurotrophic system (i.e., neurotrophins and neurotrophin receptor levels) in cognitive brain regions; this may be one reason why females appeared more behaviorally plastic, which allowed them to transfer a lifetime of RM cognitive practice to the novel RM Morris Maze. Indeed, this tenet is supported by the fact that females appeared to be responsible for the association between young RM performance and aged hippocampal and frontal cortex NGF levels, two brain regions necessary for RM and WM performance (Balota et al., 2000; Eichenbaum, 2000; O'Keefe and Nadel, 1978). It is conceivable that E2 facilitates female alterations in trophic systems that may enhance cognitive brain regions in a manner that facilitates behavioral plasticity and a better cognitive aging profile in comparison to males.

In conclusion, males and females differed in their initial performance on the cognitive practice task when young. When aged,

males and females benefited from cognitive practice on the cognitive practice RM task and a novel WM task, but only females transferred this benefit to a novel RM task. Furthermore, aged NGF levels were altered by cognitive practice and age, and were related to young memory performance only in females. Lastly, the faster an animal learned the cognitive practice task when young the better his or her aged memory performance, suggesting a link between genes and/or early life experience and the formation of a “cognitive reserve” that ultimately alters the trajectory of age-related cognitive changes. Future research using both males and females could be conducted in order to assess the effects that cognitive practice has on cholinergic tone, excitatory synapses, and the effects and interactions that neurotrophins have on these systems. Specifically, this could be done by evaluating if neurotrophins and cognitive practice alter ACh levels and the number of glutamate receptors in cognitive brain regions. Finally, since dendritic spines are thought to be the functional neuroanatomical units of plasticity whereby learning and memory occur, a direct evaluation of the changes in dendritic spines as a result of a lifetime of cognitive practice may yield exciting information. Collectively, this information could one day lead to new treatments and therapies to prevent, attenuate, or reverse, age and disease related memory decline, thereby reducing the strain that aging and dementia places on individuals, their families, and the public health system.

CHAPTER 3

HIGHER LEVELS OF ESTRADIOL REPLACEMENT CORRELATE WITH BETTER SPATIAL MEMORY IN SURGICALLY MENOPAUSAL YOUNG AND MIDDLE-AGED RATS

There is abundant clinical and basic science evidence that estrogens impact cognitive function (Dohanich, 2002). Since the first controlled clinical evaluation showing that estrogen injections given to 75 year-old women enhanced memory (Caldwell and Watson, 1952), there have been numerous studies showing cognitive decline after ovarian hormone loss, and enhancement after estrogen treatment, in menopausal women (Sherwin, 2006). However, several newer clinical studies, including the large, placebo-controlled, multi-center Women's Health Initiative Memory Study (WHIMS), have indicated that certain regimens of hormone treatment (e.g., use of CEE with or without medroxyprogesterone acetate [MPA]) can have no effect on, or even be detrimental to, cognition and dementia risk in women (for review see Coker et al., 2010).

A majority of the animal studies evaluating the effects of estrogens on learning and memory have been performed in young rodents (See Chapter 1). While there has been a recent increase in the number of studies evaluating estrogen effects in middle-aged or older female rodents, most using the most potent estrogen, E2 (Bimonte-Nelson et al.,

2006; Foster et al., 2003; Frick et al., 2002; Gibbs, 2000b; Luine and Rodriguez, 1994; Markham et al., 2002; Markowska and Savonenko, 2002a; Savonenko and Markowska, 2003; Ziegler and Gallagher, 2005), there is only limited work comparing estrogen treatment effects in Ovx animals at different ages within the same study (Foster et al., 2003). Hence, it is still a question whether age changes responsiveness to ovarian hormone replacement. This is a clinically important question, as some menopausal women begin HT at a younger age, while some start later in life. Women in the WHIMS were 65 years of age and over, which may have played a critical role in the lack of efficacy of estrogen treatment since age may impact responsiveness (Craig et al., 2005).

Animal work that has been done testing E2 treatment at multiple ages showed that administered E2 dose impacted spatial memory retention, and that these effects acted together with age in Ovx rodents (Foster et al., 2003). My Lab and others have also noted divergent cognitive effects depending on administered estradiol dose in mice and rats (Bimonte-Nelson et al., 2006; Bimonte and Denenberg, 1999; El-Bakri et al., 2004; Frick et al., 2002; Gresack and Frick, 2004; Holmes et al., 2002; Packard and Teather, 1997; Rissanen et al., 1999). In these studies, however, serum hormone levels were estimated based on prior studies or manufacturer reports (Bimonte-Nelson et al., 2006; Bimonte and Denenberg, 1999; El-Bakri et al., 2004; Frick et al., 2002; Gresack

and Frick, 2004; Holmes et al., 2002; Packard and Teather, 1997; Rissanen et al., 1999), or circulating E2 levels were determined but statistical correlations with performance were not reported (Foster et al., 2003). In fact, in no animal study testing estrogen treatment have serum levels within individual animals been correlated with memory scores. There may be a positive correlation between circulating estrogen level and memory, as seen with endogenous levels in healthy menopausal women (Phillips and Sherwin, 1992; Wolf and Kirschbaum, 2002) , and after exogenous estradiol administration to postmenopausal women with probable mild to moderate AD (Asthana et al., 1999). Alternatively, the relation between serum estrogen level and memory could hold to an inverted U-shaped function, whereby very low or very high values result in the poorest cognitive function. Many biological systems fit this quadratic function (Bimonte et al., 2002), and low, but not high, estradiol levels have been associated with better cognitive performance in older women (Barrett-Connor and Goodman-Gruen, 1999). Hence, while some studies suggest that estrogen treatment influences cognition in women and the rodent model, the relation between circulating level of estrogen and memory performance is unclear.

My Lab previously showed that Ovx did not influence spatial RM performance in middle-aged female rats (Bimonte-Nelson et al., 2006). Yet, in this age group E2 treatment resulted in marked enhancements in

spatial RM performance (Bimonte-Nelson et al., 2006). These data suggest that (1) naturally circulating, endogenous estrogen in an ovary-intact individual may relate to cognition in a different way than exogenously administered estrogen due to replacement after Ovx, and (2) sensitivity to ovarian hormone loss does not predict sensitivity to estrogen replacement for spatial RM. My Lab and others have shown, in young rats, WM deficiencies after Ovx and enhancements after estradiol treatment (Bimonte and Denenberg, 1999; Daniel et al., 1997; Daniel et al., 1999; Holmes et al., 2002; Sandstrom and Williams, 2001). Additionally, in middle-aged rats no RM deficiencies were seen after Ovx, but benefits of estradiol treatment were observed (Bimonte-Nelson et al., 2006). While suggestive of differences in response to ovarian hormone loss and treatment depending on age, these separate studies could have been due to memory type and not age.

The current studies examined the spatial RM effects of Ovx and E2 treatment in young, middle-aged and aged rats. my lab and others have found significant variability in circulating E2 levels after treatment with the same manufacture-labeled dose of E2 pellets from Innovative Research of America (unpublished observations; Diel et al., 2005). Here I capitalized on this range of serum E2 levels to investigate relation with memory scores. Circulating E2 levels were determined in all animals with the intention of correlating these values with maze performance. I performed

correlation analyses in animals that were ovary-intact to determine associations between *endogenous* E2 levels and performance, and within animals that had received Ovx plus E2 treatment to determine relations between *exogenous* E2 levels and performance. The relation between circulating E2 and cognition may be affected by whether the E2 is endogenous (via an intact ovary) or exogenous (experimentally administered) due to presence of progesterone in ovary-intact animals (Bimonte-Nelson et al., 2006; Bimonte-Nelson et al., 2004a; Bimonte-Nelson et al., 2004b; Johansson et al., 2002; Nilsen and Brinton, 2002a; Nilsen and Brinton, 2002b; Woolley and McEwen, 1992), as well as the cyclicity of E2 exposure (e.g. cyclic from the ovary vs. a tonic regimen of treatment; Bimonte-Nelson et al., 2006; Bimonte-Nelson et al., 2004b; Gibbs, 2000b; Johansson et al., 2002; Markowska and Savonenko, 2002a; Nilsen and Brinton, 2002a; Nilsen and Brinton, 2002b; Woolley and McEwen, 1992).

Methods

Subjects and Treatment Procedures

For Study 1, subjects were 34 young (4 months old at test) and 33 middle-aged (16 months old at test) Fischer-344 female rats born and raised at the National Institute on Aging at Harlan Laboratories (Indianapolis, IN). After arrival, animals were pair housed, had exposure to food and water *ad lib*, and were maintained on a 12-h light/dark cycle. All

procedures were approved by IACUC and adhered to NIH standards. Each young and middle-aged group contained the following treatment groups: ovary-intact sham (Sham), Ovx with no hormone treatment (Ovx), Ovx plus a 0.25 mg/60 day release E2 pellet and Ovx plus a 0.50 mg/60 day release E2 pellet. Thus, there were a total of eight groups: Young-Sham (n=8), Young-Ovx (n=9), Young-Ovx + 0.25 E2 pellet (n=8), Young Ovx + 0.50 E2 pellet (n=9), Middle-aged-Sham (n=8), Middle-aged-Ovx (n=9), Middle-aged Ovx + 0.25 E2 pellet (n=8), and Middle-aged Ovx + 0.50 E2 pellet (n=8). Ovx surgery was performed two months before testing at 2 and 14 months of age for young and middle-aged groups, respectively. Rats were anesthetized with an intraperitoneal injection of 70 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA, USA)/and 6 mg/kg xylazine (Lloyd Laboratories, Shenandoah, IA, USA). For 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. Sham surgery consisted of skin incision and staple in the same fashion.

E2 treatment via pellets (Innovative Research of America, Sarasota, Florida) was administered one month after Ovx, one month before testing ensued, at 3 months old for young rats, and 15 months old for middle-aged rats. Since pellets released hormone for 60 days, animals with the pellets received E2 for the entire study, including during

behavioral testing and through sacrifice. Under Ketamine/Xylazine anesthesia, a small incision was made in the scruff of the neck, and a subcutaneous pocket was created. For animals receiving E2, one pellet of the appropriate dose was inserted and the skin stapled. The groups not receiving E2 treatment (Sham and Ovx groups) received a sham pellet surgery, which included identical procedures except the pocket was left empty.

For Study 2, subjects were 6 young (4 months old at test) and 21 aged (24 months old at test) Fischer-344 female rats born and raised at the aging colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN). There were a total of four groups in Study 2: Young-Sham (n=6), Aged-Sham (n=6), Aged-Ovx (n=7), and Aged-Ovx-E (n=7). Just one type of E2 pellet was chosen for this study (0.25 mg pellet) since the variability and mean levels were comparable between the 0.25 and 0.50 doses in Study 1. The timeframes and surgical procedures of Study 2 were identical to those of Study 1.

Assessment of Serum Hormone Levels

For both studies, after behavioral testing rats were anesthetized with Halothane anesthesia and decapitated. Blood was collected from the trunk (Vacutainer 367986, Becton Dickinson and Company, Franklin Lakes, NJ), was allowed to clot at 4º C and serum was collected after centrifugation (3220xG, 20 min). Serum was stored at -20º C until E2

assays were performed by the Core Endocrinology Laboratory at Pennsylvania State University College of Medicine using a kit from Diagnostic Products Corporation, Los Angeles, CA (Coat-A-Count E2 kit, product number TKE21). E2 was determined in serum by a solid-phase radioimmunoassay based on E2-specific antibodies that are immobilized to the wall of polypropylene tubes and ^{125}I -labeled E2 as the tracer following extraction with diethyl ether. Serum (2.4. ml) was extracted and the ether portion collected and evaporated to dryness. The sample was reconstituted in assay buffer and a competitive radioimmunoassay was performed using ^{125}I E2 with high specific activity and a high affinity, highly specific antibody. Separation of bound from free was achieved with activated charcoal and the data reduction was performed with the use of a five point standard curve and purified E2 standards. The functional sensitivity of the assay was 5 pg/ml. The interassay precision at a concentration of 35 pg/ml was 8%.

Spatial RM Morris Maze Testing

Rats in both studies received identical testing on the Morris maze, which consisted of a round water-filled tub with a hidden platform. The rat was placed in the maze from any of four locations (North, South, East, or West) and had 60s to locate the platform which remained in a fixed location throughout testing. After 15s on the platform, the rat was removed from the maze and placed into its heated cage until the next trial. Rats

were given 3 trials a day for 5 days. After the last trial on Day 5, each rat was given a 60s probe trial whereby the platform was removed. A video camera suspended on the ceiling above the maze tracked the rat's path and a tracking system (SMART system, San Diego Instruments) was used to analyze each rat's tracing.

Dependent Variables and Statistical Methods

Analyses for study 1.

Using StatView (version 5.01, The SAS Institute Inc., Cary, NC), performance was assessed by swim path distance (centimeters) to the platform. I also analyzed swim speed (distance/time) since it can be affected by age, cognitive demand, and estradiol treatment; this measure may aid interpretation of my findings (Bimonte-Nelson et al., 2006; Foster et al., 2003; Markowska and Savonenko, 2002a; Markowska and Savonenko, 2002b). Studies 1 and 2 were analyzed separately, as were Distance and Speed measures. For Study 1, the E2 groups (Ovx + 0.25 pellet and Ovx + 0.50 pellet) were combined into one group (Ovx-E; Table 2). To analyze Study 1, I first performed an omnibus 3 x 5 x 3 mixed model ANOVA within each age with Treatment (Sham, Ovx, Ovx-E) as the between-subjects factor, and Days (1-5) and Trials (1-3) as repeated measures. Specific a priori, planned contrasts were run to further evaluate significant omnibus ANOVA main effects or interactions with Treatment. Unless otherwise specified, these analyses were run as described above

but with one between-subject factor (Treatment) comparing two groups (Sham vs. Ovx to determine Ovx effects, and Ovx vs. Ovx-E to ascertain E2 treatment effects). Post hoc comparisons across ages were used selectively to aid interpretation of E2-related effects. To evaluate effects of age, I used the same mixed model ANOVA, except Age (young vs. middle-aged) was the between-subject variable and the comparison was limited to the Sham groups.

To assess whether rats localized the platform to the spatial location and to confirm that animals were not utilizing a motoric strategy to solve the task, the platform was removed after all testing trials were completed and animals' swim distance was quantified (day 5, trial 4 was the probe trial). The maze was virtually divided into four equal quadrants (Bimonte-Nelson et al., 2006). Percent of total distance in the platform quadrant (NE, or target) was compared to the quadrant diagonally opposite (SW). Rats that learned the platform location and were not using a motoric strategy were expected to spend the greatest percent of total distance in the quadrant that had contained the platform. Probe trial data were first analyzed using a 2 x 2 mixed model ANOVA within each age, with Treatment as the between-subjects variable and maze Quadrant as the within-subjects variable (Bimonte-Nelson et al., 2006). Unless otherwise specified, follow-up two group comparisons and age effects were analyzed as above, except Treatment or Age were the between variable. E2 levels

were run similar to the above procedures; post-hoc evaluations were run using t-tests.

To evaluate potential relations between maze scores and E2 levels, multiple regression analyses and Pearson correlations were run to analyze E2's association with five performance variables: distance score on the final test trial (day 5, trial 3), distance score averaged across all five days, percent of total distance in the target (NE) quadrant on the probe trial, speed on the final test trial, and speed averaged across all five testing days. I also evaluated the correlation between speed and distance, and speed and percent distance swum for the target (NE) quadrant on the probe trial. Each of these were run as follows: average of days 1-5 distance was correlated with the average of days 1-5 speed, distance on the final test trial was correlated with speed on the final test trial, and the average of days 1-5 speed was correlated with percent distance in the target (NE) quadrant for the probe trial. Unless otherwise specified, multiple regression analysis and Pearson correlations were run within ovary-intact groups, within Ovx groups and within E2 treatment groups collapsed across young and middle-aged animals. Next, Pearson r correlations *within* each young and each middle-aged group were run (thus, ages run separately), for these groups.

Analyses for study 2.

For Study 2, using StatView (version 5.01, The SAS Institute Inc., Cary, NC), distance scores, speed, and probe trial data were analyzed via an omnibus mixed model ANOVA within the three Aged groups (Aged-Sham, Aged-Ovx and Aged-Ovx-E) and repeated measures as described above. For E2 levels, an omnibus ANOVA was run within the three Aged groups. Age effects and follow up two-group comparisons were run as for Study 1. Multiple regression analyses and correlations including E2 were not run for Study 2 since two serum samples from the E2 group were of low quality and could not be assayed for E2, limiting the sample size to five for the E2 treatment group. For Study 2, I evaluated the correlation between speed and distance, and speed and percent distance swum for the target (NE) quadrant on the probe trial as described for Study 1 above. Correlations were run first with all subjects included, then within ovary-intact groups collapsed across young and aged groups, and then within all aged groups combined.

Results

Study 1: Effects of Ovx and E2 Treatment in Young and Middle-Aged Animals

Study 1: serum E2 levels.

The top half of Table 2 shows the mean ±*SE*, range and median serum levels of E2 for each group of young and middle-aged rats. An omnibus one-way ANOVA [with the two dosage levels combined for one

E2 treatment group] for young animals revealed a Treatment main effect (F[2,29] = 35.17, p < 0.0001), as seen for middle-aged animals also (F[2,27] = 16.13, p < 0.0001). As expected, Ovx animals exhibited lower E2 levels than Sham animals for young (t[15] = 2.23, p < 0.05) and middle-aged (t[15] = 4.97, p < 0.0002) groups, and Ovx-E animals had higher levels than Ovx animals for young (t[22] = 6.83, p < 0.0001) and middle-aged groups (t[20] = 3.81, p < 0.001). Of importance, there was great variability within the E2-treated rats, as anticipated and detailed in Table 2. There were no mean differences in E2 level between any of the E2-treated groups, within and across ages (ps > 0.36).

Study 1: maze performance.

Figure 8 depicts the learning curves across days for each treatment group of a) young 4 month olds and b) middle-aged 16 month olds, and the inset graphs show the mean ± SE distance scores for each group collapsed across all testing days. The omnibus mixed model ANOVA in young animals revealed a main effect of Treatment (F[2,29] = 3.91, p < 0.05), and a Day x Treatment interaction (F[8,116] = 3.74, p < 0.001); there was no main effects of interaction with Trial. Distance scores of Young-Ovx rats were higher than Young-Sham rats, although this main effect only approached significance (F[1,15] = 4.276, p = 0.06). For this analysis, there was a significant Day x Ovx interaction (F[4,12] = 3.137, p < 0.05), with Young-Ovx rats swimming a shorter distance to the platform

on day 1, but a greater distance on days 2-5 as compared to Young-Sham rats (Figure 8a). A post-hoc test comparing these two groups on day 1 showed that Ovx did not influence performance on this first testing day. However, a post-hoc repeated measures ANOVA revealed that Young-Ovx animals swam a greater distance than Young-Sham animals for days 2-5 ($F[1,15] = 7.83$, $p < 0.05$). Young-Ovx-E animals showed lower distance scores than Young-Ovx animals across all testing days (E2 Treatment main effect: $F[1,22] = 6.39$, $p < 0.05$, Figure 8a).

For the omnibus ANOVA with middle-aged animals, there was a Day x Treatment interaction ($F[8,120] = 2.41$, $p < 0.05$), and a marginal Trial x Treatment interaction ($F[4,60] = 2.34$, $p = 0.06$), in the absence of a Treatment main effect. Distance scores of Middle-Aged-Sham and Middle-Aged-Ovx rats did not differ, nor did Trial or Days interact with Ovx. Middle-Aged-Ovx rats showed enhancements due to E2 treatment, exhibiting faster learning of the platform location (Day x E2 Treatment interaction: $F[4,92] = 2.66$, $p < 0.05$, Figure 8b); a post-hoc assessment confirmed that E2 treatment enhanced performance on the initial two testing days in middle-aged animals (E2 Treatment main effect for days 1 and 2: $F[1,23] = 16.45$, $p < 0.001$).

There was a Trial x E2 Treatment interaction for middle-aged animals only, with E2-treated rats exhibiting lower distance scores on trial 1 as compared to Ovx rats ($F[2,46] = 3.64$, $p < 0.05$, Figure 8c). Given

previous findings that E2 aids overnight retention of the platform location in middle-aged rats (Bimonte-Nelson et al., 2006; Markham et al., 2002) I further evaluated this effect by removing day 1 since there was no prior information about this maze to the animal on trial 1, day 1. E2 aided in remembering the platform location overnight in middle-aged rats, as shown by the Trial x E2 Treatment interaction for days 2-5 (F[2,46] = 3.21, $p < 0.05$). E2 did not influence overnight forgetting in young animals, as determined by the same analysis in young animals (Trial x E2 Treatment interaction, $p > .86$), nor did Ovx influence overnight forgetting in young or middle-aged animals (separate repeated measures ANOVAs within each age, Trial x Ovx interaction ps > .89).

There was a main effect of Age for Distance scores. Young-Sham rats exhibited better performance than Middle-Aged-Sham rats (F[1,14] = 14.50, $p < 0.005$). Post-hoc comparisons determined that Ovx in young rats impaired performance to the extent that the Young-Ovx group did not differ from the Middle-Aged-Ovx group or the Middle-Aged-Sham group for overall performance across the five testing days. However, Middle-Aged-Ovx-E animals exhibited poorer overall performance than the Young-Sham rats (F[1,22] = 6.48, $p < 0.05$), indicating that estrogen treatment did not reverse age-related performance deficiencies.

Figure 9 shows the mean swim speed ±SE for each treatment group for a) young animals and b) middle-aged animals. While the

omnibus ANOVA for speed for young animals was not significant, for middle-aged animals there was a main effect of Treatment (F[2,30] = 18.88, $p < 0.0001$). In middle-aged animals Ovx did not affect swim speed and E2 treatment decreased swim speed (F[1,23] = 16.56, $p = 0.0005$). Age did not influence swim speed (F[1,14] = 0.68, $p = .42$).

Figure 8d depicts the mean percent distance ±SE spent in the target and opposite quadrants for the probe trial for each treatment group within each age. All groups localized to the platform location. As revealed by the omnibus ANOVA, all young animals spent a greater percent distance in the target versus the opposite quadrant (Quadrant main effect: F[1,28] = 156.16, $p < 0.0001$), regardless of treatment (Nonsignificant Treatment x Quadrant interaction). Identical findings were shown for middle-aged animals (Quadrant main effect: F[1,29] = 62.38, $p < 0.0001$; Treatment x Quadrant interaction not significant). For the analysis of Age, there was a Quadrant main effect (F[1,14] = 132.78, $p < 0.0001$) with a higher percent distance in the target quadrant, and a Treatment x Quadrant interaction (F[1,14] = 4.89, $p < 0.05$). A post-hoc t-test determined that Young-Shams spent a greater percent distance in the target quadrant than Middle-Aged-Shams (t[29] = 7.90, $p < 0.05$).

Study 1: multiple regression and correlations.

I conducted two primary regression analyses relating serum E2 levels to performance measures in the young and middle-aged Ovx rats

with E2 treatment. Serum E2 level was related to maze performance on the last trial of testing (b = -2.11, r = -.38, t[26] = -20.09, p = 0.046, Figure 10a) indicating that for each pg/ml increase in serum E2 level, there was a 2.11 cm decrease in swimming distance. My collaborator used several procedures described in Cook and Weisberg (1999) to probe for possible curvilinear effects; none approached statistical significance. The Cook and Weisberg (1983) test could not reject the null hypothesis of constant variance and the results of an alternative regression analysis that used the log of swimming distance as the dependent variable to stabilize the variance did not differ from the first (t[26] = -20.09, p = 0.046). Finally, my collaborator found no difference in the slopes of the young and middle-aged Ovx rats. These results show that the relationship approximates linearity over the range of serum E2 studied, which ranged from low physiological to supraphysiological (Butcher et al., 1974).

Serum E2 level was also related to better spatial localization of the platform location during the probe trial in the young and middle-aged Ovx rats. Higher serum E2 levels were related to greater percent distance in the target quadrant (b = 0.257, r = 0.59, t[26] = 3.71, p = 0.001, Figure 10b). Once again, my collaborator detected no evidence of curvilinearity, nonconstant variance, or differences in the slopes of Young-Ovx-E and Middle-Aged-Ovx-E groups. There were no significant E2-percent distance

relationships in the ovary-intact Young-Sham or Middle-Aged-Sham analyses when groups were analyzed together or separately.

Speed did not significantly correlate with E2 levels for any assessment when young and middle-aged animals were analyzed together, or when they were analyzed separately. Moreover, speed did not significantly correlate with distance scores during testing, or with percent distance in the target quadrant on the probe trial, for Sham, Ovx or Ovx-E animals. This was true when young and middle-aged animals were analyzed together, as well as separately.

Study 2: Effects of Ovx and E2 Treatment in Aged Animals

Study 2 was performed to assess whether Ovx had an impact on performance in aged 24 month old animals, and whether this E2 treatment regimen was still effective in aged animals. Young 4 month old ovary-intact sham animals were also evaluated to confirm age-related performance deterioration, and as a relative performance assessment for the E2-treated group.

Study 2: serum E2 levels.

The bottom half of Table 2 shows the mean ±*SE*, range and median serum levels of E2 for young and each group of aged rats in Study 2. The omnibus ANOVA with all aged groups revealed a main effect of Treatment ($F[2,15] = 21.91$, $p < 0.0001$). Aged-Ovx animals exhibited lower E2 levels than Aged-Sham rats ($t[10] = 2.65$, $p < 0.05$), and Aged-

Ovx-E rats had higher levels than Aged-Ovx rats (t[11] = 5.28, p < 0.0005). Age did not influence E2 levels (p = 0.67).

Study 2: maze performance.

Figure 11a depicts the learning curves across days for each treatment group of aged of 21 month olds. Once again, an omnibus mixed model ANOVA including all three aged groups revealed no main effect or Day or Trial interactions with Treatment for distance scores. It was noted, however, that overall performance collapsed across all five testing days of the Aged-Ovx-E group appeared better than the Aged-Sham and Aged-Ovx groups (Figure 11a). As a post-hoc assessment, repeated measures ANOVAs were used to test two-group comparisons. These analyses confirmed that the Aged-Ovx-E group did not significantly differ from the Aged-Ovx or the Aged-Sham group across the five testing days; this was true for both main effects and interactions with Treatment (ps > 0.12).

Age influenced performance; Young-Sham rats exhibited lower distance scores collapsed across all days as compared to Aged-Sham rats (Age main effect: F[1,10] = 4.96, p = 0.05). There was also a Day x Age interaction (F[4,40] = 3.75, p < 0.05), with distance scores comparable on day 1 but markedly lower for days 2 through 5 for the Young-Sham group (Figure 11a).

Figure 11b shows the mean swim speed ±SE for each aged group for each test day. The omnibus ANOVA for all aged groups revealed that

treatment affected swim speed as days progressed (Day x Treatment interaction: $F[4,12] = 2.38$, $p < 0.05$). There was no Ovx effect on swim speed. E2 treatment decreased swim speed (Treatment main effect: $F[1,13] = 6.56$, $p < 0.05$). Age influenced swim speed as days progressed, with Young-Sham animals swimming slower than Aged-Sham animals toward the end of testing (Day x Age interaction: $F[4,40] = 4.16$, $p < 0.05$, Figure 11b). A post-hoc repeated measures ANOVA (Day and Trial as repeated measures) for each sham group alone revealed that Young-Sham animals did not change swim speed across days ($p = 0.31$), while Aged-Sham animals increased speed in the initial portion of testing ($F[4,20] = -6.50$, $p < 0.05$, Figure 11b).

All aged rats localized to the target location. For the ANOVA with all aged groups included, there was a main effect of Quadrant, with rats spending a greater percent distance in the target versus the opposite quadrant suggesting platform localization ($F[1,17] = 39.62$, $p < 0.0001$, Figure 11d). The nonsignificant Group x Quadrant interaction indicated that all groups showed this pattern of spending a greater percent distance in the target versus opposite quadrant. To further detail potential E2 treatment effects in aged animals, I performed a post-hoc analysis comparing the Aged-Ovx-E and Aged-Ovx groups on the probe trial. The repeated measures ANOVA (with E2 Treatment as the between variable and Quadrant as the repeated measures) revealed a Treatment x

Quadrant interaction (F[1,12] = 59.61, p < 0.05, Figure 11c). A post-hoc t-test showed that aged Ovx animals receiving E2 spent a higher percent distance in the target quadrant as compared to aged Ovx animals that did not (t[12] = 3.11, p < 0.01). Young rats localized to the platform location, as shown on the probe trial with a greater percent distance spent in the target quadrant (main effect of Quadrant: F[1,5] = 7.50, p <0.05, Figure 11d).

Study 2: multiple regression and correlation.

Speed did not significantly correlate with distance scores during testing, nor percent distance in the target quadrant on the probe trial, for any evaluation.

Discussion

The current findings show for the first time that higher serum levels of E2 treatment correlate with better maze performance in young and middle-aged Ovx animals. I found that higher exogenous E2 treatment levels were related to better spatial RM performance after surgical ovarian hormone loss, while endogenous E2 levels in ovary-intact animals did not relate to spatial RM performance in either age (Study 1). This report replicates my lab's previous findings for spatial RM in middle-aged female rats, whereby Ovx does not affect performance, yet estradiol treatment enhances performance after Ovx (Bimonte-Nelson et al., 2006). I now extend these findings, demonstrating that in contrast to no effect of Ovx in

middle-aged females, Ovx impacts young females' spatial RM performance. Young animals were responsive to *both* ovarian hormone removal and treatment, and middle-aged animals were not responsive to ovarian hormone removal but were responsive to estrogen treatment. Furthermore, at the current timing of ovarian hormone loss and regimen of E2 treatment, aged animals were not responsive to ovarian hormone removal or treatment for the test trials on the spatial RM Morris maze (Study 2).

While Ovx did not influence spatial RM performance in middle-aged animals, in young animals Ovx compromised performance relative to young ovary-intact sham animals on days 2-5, the latter portion of testing. Ovx in young animals impaired performance to the point that this group did not differ from middle-aged Ovx or Sham animals. This negative effect of Ovx corresponds with findings of others that Ovx in young rats impairs Morris maze performance, specifically, at the end of a five-day testing period (Feng et al., 2004). Whether Ovx animals would have eventually attained performance comparable to that of young ovary-intact sham animals had testing continued remains to be determined. It is noteworthy that by the last trial on the last day of testing, however, Young-Ovx animals did learn the platform location. This is revealed by the probe trial data showing that all groups spent a greater percent distance in the platform versus the opposite quadrant. These findings, combined with the

lack of a Quadrant x Treatment interaction, suggest that regardless of ovarian hormone status all young and middle-aged groups learned the platform location by the end of testing.

My observation that E2 enhances spatial RM on the Morris maze in young and middle-aged female rats is consistent with other studies using young to middle-aged rodents tested on the Morris maze (El-Bakri et al., 2004; Feng et al., 2004; Markham et al., 2002; McLaughlin et al., 2008). It is noteworthy that I found E2-induced enhancements on the first two days of testing in middle-aged animals, indicating faster platform acquisition, yet the middle-aged Ovx group given E2 still performed worse than the young sham group. These findings indicate that while E2 does enhance learning of spatial RM on the Morris maze, it does not completely reverse age-related performance deficiencies.

In contrast to the current findings that E2 did not improve spatial learning in aged Ovx rats; some studies have shown that aged female rodents exhibit cognitive enhancements in response to E2 treatment. For example, estradiol injections enhanced spatial RM in 27-28 month old ovary-intact mice (Frick et al., 2002). The difference in results may relate to the type of estradiol administration as cyclic versus tonic; cyclic administration via daily injection was used in the Frick study (Frick et al., 2002) described above, while tonic administration was used in the current studies. Providing further support for an influence of administration

method on cognitive effectiveness in aged rodents, priming with cyclic estradiol enhanced responsiveness to tonic estradiol in older Ovx rats (Markowska and Savonenko, 2002a). Whether estrogen treatment improves performance of aged animals may also relate to memory type. WM enhancements due to tonic estradiol treatment have been reported in aged Ovx rats (Gibbs, 2000b; Luine and Rodriguez, 1994). My lab and others have shown estradiol-induced WM improvements in young Ovx rats as well, an effect reported to depend on administered estradiol dose, although dose-dependent differences in circulating estradiol levels were not confirmed (Bimonte and Denenberg, 1999; Daniel et al., 1997; Holmes et al., 2002; Sandstrom and Williams, 2001). Accordingly, in young rats high physiological estradiol levels have been shown to enhance learning a place strategy and impair learning a response strategy on a land Plus Maze (Korol and Kolo, 2002). Further, while Foster et al. (2003) found that tonic estradiol treatment had no influence on acquisition of the Morris maze in 6, 12 or 17 month old Ovx rats, physiological-dose estradiol treatment influenced 24hr retention in the 12 month old rats only, and the supraphysiological dose influenced retention in the 17 month old rats only. Thus, a higher supraphysiological estradiol dose may be necessary to enhance spatial RM retention in rats approaching old age.

Age impacted whether swim speed changed across days, an effect seen in Study 2 comparing Young-Sham and Aged-Sham animals (Figure

11b). Young-Sham animals showed no change in swim speed across days, and Aged-Sham animals increased swim speed across the initial testing sessions and stabilized by the end of testing. This increase in swim speed after day 2 in aged animals may be influenced by maze acclimation after an initial stress response due to the swimming component of the task, an effect which may not be seen in younger animals (Kramer and Bodnar, 1986). Swim speed in both middle-aged and aged animals decreased with E2 treatment (Studies 1 and 2), an effect observed previously in my and other laboratories (Bimonte-Nelson et al., 2006; Foster et al., 2003; Markowska and Savonenko, 2002a). Middle-aged and aged animals may be particularly sensitive to the motor effects of E2 treatment. Indeed, E2 treatment did not influence swim speed in young animals in this study. Interestingly, swim speed did not correlate with distance to the escape platform nor probe trial performance, indicating that swim speed does not relate to a more- or less- direct search path to the platform. This was true when correlations were run within and across ages, and within and across treatment groups for Studies 1 and 2. Distances to the platform and probe trial measures have been asserted to be most reflective of task acquisition (e.g., Foster et al., 2003). The currently reported lack of correlation between distance and probe trial measures with speed, as well as other research showing that decreased motor function with age does not correlate with Morris maze performance

(Shukitt-Hale et al., 1998), support the tenet that there is a disconnect between cognitive status and sensory/motor performance variables such as latency and speed on this test. Yet, it is also tempting to speculate that a slower swim speed may be reflective of solving strategy wherein the animal is encoding or attending to spatial stimuli, with this process not necessarily related to final performance scores as operationally defined by distance swum. Further evaluations are necessary to decipher the complex age and E2 interactive effects on swim speed, as well as whether swim speed relates to encoding or utilization of spatial stimuli.

It is noteworthy that the range of E2 levels using the pellets from Innovative Research of America was quite large. This has been discussed in prior work from other laboratories, and my lab has noted this in prior studies within as well (unpublished observations; Diel et al., 2005). While for the current experiments capitalized on this variability to address possible relationships between E2 treatment levels and memory scores within individuals, the lack of difference in the mean serum E2 level for the 0.25 mg and 0.50 mg pellets is disconcerting, as is the similarity in range and median values. Importantly, sacrifice and blood collection for E2 assays occurred within the 60 day timeframe of pellet release. Collectively, these findings suggest that serum E2 levels should be determined after using these pellets, and potentially other forms of

estrogen treatment as well, to ascertain an accurate estimate of estrogen levels and allow appropriate interpretation of findings.

Performance on the hidden platform version of the Morris maze, as used in the current report, is known to be influenced by integrity of the hippocampus (e.g., Morris et al., 1982; Schenk and Morris, 1985). The E2-induced spatial memory enhancement seen in the current and other reports coincides with evidence that estrogen influences the physiology, connectivity, and function of the hippocampus, including increases in dendritic spine density and reductions in GABA neurotransmission (Bimonte-Nelson et al., 2006; Gould et al., 1990; Murphy et al., 1998; Woolley and McEwen, 1992). While it is not currently known why variables such as specific estrogen regimen and age may influence efficacy of estrogen treatment for cognition, data from neurobiological experiments yield insight and suggest that such interactions are biologically plausible. It has been well-documented that age-related changes occur in neural systems known to influence learning and memory, and many of these neural systems are also influenced by estrogen. For example, short-term estrogen treatment via injection enhanced place-learning-induced hippocampal ACh release (Marriott and Korol, 2003). There are interactions between the cholinergic system, estrogen treatment and age; susceptibility to cholinergic challenge differed in middle-aged versus aged rodents given estradiol, with middle-aged, but not aged, animals showing

protection due to estradiol treatment (Markowska and Savonenko, 2002a; Savonenko and Markowska, 2003). In addition, there are age-related alterations in the number and activity of estrogen receptors (ER) which could also influence responsivity as aging ensues (Chakraborty and Gore, 2004). Newer data suggest an estrogen-receptor dependent mechanism of estrogen-induced benefits on spatial memory, as the benefits of systemic estrogen treatment on place learning were reversed with hippocampal estrogen-receptor antagonism using ICI 182,780 (Zurkovsky et al., 2006). Thus, changes in ERs may relate to age-related responsiveness to estrogen for spatial memory.

In summary, the current report indicates that for spatial RM, the effects of surgical ovarian hormone loss and E2 treatment change with age, at least for the temporal and dose parameters utilized in these studies. The data indicate that young and middle-aged animals that have undergone Ovx benefit from E2 treatment, and that higher exogenous E2 levels relate to better spatial RM in both ages. Indeed, higher levels of E2 treatment correlated with better maze performance during testing and enhanced platform localization as demonstrated during the probe trial. No relationships between endogenous E2 level and maze scores were seen in young and middle-aged sham animals that were ovary-intact. By old age, the E2 treatment regimen beneficial at earlier ages was no longer effective during testing, and had only minor benefits for platform

localization as assessed on the probe trial. My findings demonstrate a distinction between sensitivity to surgical ovarian hormone loss and responsiveness to E2 treatment after surgical ovarian hormone loss.

CHAPTER 4

A PRODRUG OF 17β-ESTRADIOL ENHANCES SPATIAL COGNITION AND ALTERS THE CHOLINERGIC SYSTEM: COMPARISON TO 17β-ESTRADIOL AND PREMARIN®

Ovarian hormone output ends typically in the fifth decade of a women's life in what is commonly termed as menopause; menopause and the postmenopausal state results in very low circulating levels of estrogen and progesterone (Timaras et al., 1995). It is estimated that by the year 2050 there will be 45 million postmenopausal women in the U.S. alone (U.S. Census Bureau, Bureau, 2007). This, taken with the fact that menopause has been linked to a multitude of negative effects, including cognitive deficits (Nappi et al., 1999; Phillips and Sherwin, 1992; Sherwin, 1988), which can diminish a women's quality of life (Klein and Berlin, 1996), makes the treatment and/or proper management of menopause an important public health concern. Estrogen-containing hormone therapies (HT) have been shown to obviate the vasomotor symptoms of menopause (Campbell and Whitehead, 1977b); and improve some menopause-related cognitive deficits (Campbell and Whitehead, 1977b; Duka et al., 2000; Kantor et al., 1973; Ohkura et al., 1995; Phillips and Sherwin, 1992; Sherwin, 1988; Wolf et al., 1999). However, conflicting reports in the literature suggest that the risk to benefit ratio for replacing ovarian hormones is currently unresolved.

CEE, a combination of estrogens derived from the urine of pregnant mares, is the most widely used HT in the U.S. (Stefanick, 2005). CEE has been found to enhance memory in menopausal women via self-report (Campbell and Whitehead, 1977a), case studies (Ohkura et al., 1995) and randomized psychometric evaluations (Kantor et al., 1973). In rats, CEE enhanced working and RM and hippocampal independent object recognition memory (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009; Walf and Frye, 2008).Yet, findings from the WHIMS, the largest placebo-controlled clinical trial performed to date, found no or detrimental effects on cognition and probable dementia risk when CEE was taken alone or in combination with MPA (Espeland et al., 2004; Shumaker et al., 2004; Shumaker et al., 2003), and recently, the WHIMS-MRI study found that CEE use with or without MPA was associated with small but measurable atrophy in the frontal cortex and hippocampus (Resnick et al., 2009). While noting many factors likely affected the WHIMS' findings including the global nature of the cognitive measure, older age of participants, and duration of hormone deprivation before treatment initiation (see Sherwin, 2005), these studies suggest that while the absence of ovarian hormones may be detrimental to a women's health, so to may the currently prescribed HTs. Collectively, highlighting the necessity to develop novel HTs that maximize benefits while minimizing or obviating the risks normally associate with HT.

One of the risks linked to HT, and specifically to estrogen therapy (ET) alone, is the increased risk for developing endometrial hyperplasia. Classically ET is opposed with a progestin to combat the increased risk of endometrial hyperplasia (Smith et al., 1975; Ziel and Finkle, 1975). However, opposed ET comes with its own risks. In the WHIMS, CEE+ MPA treatment increased the risk of probable dementia (Shumaker et al., 2004). Similarly, my lab has found in Ovx rats that progesterone treatment abolishes the beneficial cognitive and neurotrophin effects of E2 treatment (Bimonte-Nelson et al., 2006; Bimonte-Nelson et al., 2004a). Furthermore, my lab have found that MPA treatment is detrimental to cognitive performance in aged female Ovx rats, an effect comparable to progesterone (Braden et al., 2010). Together, this suggests that the necessity of opposing ET with a progestin may obviate the beneficial cognitive effects of ET. One method to sidestep the necessity of opposing ET with a progestin is to keep systemic estrogen stimulation at a minimum by restricting estrogen's actions to a certain target site of action, for example, the brain. Prodrugs are inactive precursors of the therapeutic agents that are converted to the biologically active agents by enzymatic and/or chemical transformation in vivo–preferably at the site of action (Albert, 1958; Wermuth et al., 1998). Hence, brain targeted estrogen prodrugs conceivably have the ability to target the action of the hormone to only the brain, and thereby reduce the systemic effects (Estes et al.,

1994; Prokai et al., 2003). Considering that 10-hydroxyestra-1,4-dien-3,10-dione (E1-DHED) has been shown to be a prodrug for E1 (Prokai et al., 2003), My collaborators have implicated 10,17β-dihydroxyestra-1,4-dien-3-one (DHED) as a prodrug for E2. As E2 is one of the most potent activators of the classical genomic pathway and the primary export product of a woman's ovary (Prokai et al., 2003), the current study set out evaluate DHED's *in vitro* conversion to E2 in as well as its effects on cognition in vivo. DHED has the possibility to revolutionize the field of HT, whereby, one day, there may be minimal risks that a woman needs to consider before initiating ET.

The cognitive effects of estrogens are thought to be directed in part by enhancements in the cholinergic system (for review see: Gibbs and Aggarwal, 1998). The hippocampus and entorhinal cortex receive cholinergic innervation from the BF, which supports memory function (for review see Hasselmo, 2006). In animals, E2 and CEE enhance BF integrity (Acosta et al., 2009b; Gibbs, 1997). E2 potentiates increases in hippocampal ACh levels during maze learning relative to vehicle treatment in Ovx rats (Marriott and Korol, 2003), and E2-induced memory enhancements are present in animals with intact BF cholinergic neurons, but not in animals with BF cholinergic lesions (Gibbs, 2002). Treatment with E2 and CEE increases the number of ChAT immunoreactive (ChAT-IR) positive cells in BF (Acosta et al., 2009b; Gibbs, 1997), and CEE and

E2 also protect against muscarinic cholinergic receptor challenges (i.e., scopolamine-induced amnesia) in rats (Acosta et al., 2009b; Packard and Teather, 1997; Rodgers et al., 2010; Savonenko and Markowska, 2003). Taken together, this suggests that E2 and CEE may enhance memory via the BF. However, no single study has compared CEE's and E2's ability to enhance BF health, nor a prodrug of E2. Research along this path is critical for the elucidation of estrogen-facilitated cognitive enhancement and for the development of novel optimal HTs benefiting cognition.

The female rodent model can provide insight into the cognitive effects of hormones, allowing evaluative changes in cognition due to hormone withdrawal and treatment, while enabling experimental control not possible in clinical research. Utilizing this tenet, the current study was designed to confirm that DHED is converted to E2 in brain. Furthermore, I wished to evaluate the cognitive and cholinergic health effects of the prodrug DHED, with reference and comparison to, the most potent and frequently used estrogen in basic science research (E2), and the most commonly prescribe HT in women (CEE). Specifically, in study 1, I evaluated the conversion of DHED to bioactive E2 in brain tissue. In study 2, utilizing a tonic dosing regimen, I evaluated the cognitive effects of DHED, E2 and CEE and on a battery of water-escape mazes designed to test spatial working and RM. Furthermore, I evaluated several peripheral markers that are routinely noted to change E2 and CEE, including vaginal

cytology, uterine weights, and serum blood levels of E2 and E1. At the conclusion of the study, brains were harvested, processed and the number of BF ChAT-IR positive neurons were counted. Lastly, as per prior studies in my lab (Engler-Chiurazzi et al., 2009; Talboom et al., 2008), I evaluated relations between serum E2 and E1 levels, memory measures and ChAT-IR neuron counts.

Methods

Study 1: In Vitro Prodrug Activation

Compounds.

E2 was obtained from Steraloids Inc. (Newport, RI). All other chemicals and reagents were purchased from Sigma (St. Louis, MO), unless otherwise specified. DHED (10,17β-dihydroxyestra-1,4-dien-3-one) was prepared by a facile microwave-assisted organic synthesis reported earlier (Prokai-Tatrai et al., 2007). Briefly, E2 was oxidized with Pb(OAc)4 in AcOH under microwave irradiation in a CEM (Matthews, NC) Discover monomode microwave apparatus at 40 °C for 15 min. The obtained DHED acetate was subsequently converted in 5 min to DHED by microwave-assisted hydrolysis with NaOMe. Product isolation was done through column chromatographic purification.

Animal subjects and surgeries.

Tissue for in vitro biotransformation experiments were obtained from Ovx female rats housed at the animal facility of the University of

North Texas Health Science Center. All rats in the in vitro experiment were Ovx. For Ovx, bilateral dorsolateral incisions were made in the skin and peritoneum, and the ovaries and tips of uterine horns were ligated and removed. The muscle was then sutured and the skin stapled.

In vitro prodrug activation.

Rats were euthanized with CO2 asphyxiation approximately 2 weeks after Ovx surgery. They were decapitated; the brain was carefully removed and transferred to a 15-mL glass, Potter-Elvehjem tissue grinder vessel (Wheaton, Millville, NJ) placed into an ice bath. After adding ice-cold phosphate-buffered saline (PBS; the volume in ml was equivalent to 4-times the weight of the tissue in grams), the homogenate (20%, w/v) was prepared by rotating the ball-shaped Teflon® pestle of the grinder, with the steel shaft connected to a motor-driven overhead stirrer (Wheaton), for 80 s at 1,500 rpm. Protein content of the brain homogenates was determined by a dye-binding assay using bovine serum albumin (BSA) as a reference (Lowry et al., 1951). Ten µl of DHED solution (100 µM, in ethanol) was added to 0.99 mL of tissue homogenate and aliquots (3 x 200 µL) were withdrawn from the mixture after 2.5 min of incubation at 37 °C in a shaking water bath. The aliquots were added respectively to glacial acetic acid (50 µL) containing 1 µM of d5-E2 (C/D/N Isotopes, Pointe-Claire, Quebec, Canada) as an internal standard in 1.5-mL polypropylene centrifuge tubes. Ethyl acetate (3 x 500 µL) was added,

and the mixtures were vortexed for 1 min and centrifuged (10,000 rpm, 5 min). From each centrifuge tube, the organic layers were removed, combined, and dried under a nitrogen stream to afford three samples for subsequent analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

E2 assay.

The dried residue was re-dissolved in 50 µL of sodium bicarbonate buffer (100 mM, pH 10.5) and an equal volume of 1 mg/mL dansyl chloride in acetonitrile was added, vortexed for 1m, followed by incubation in a heating block at 60° C for 10 min. Analyses were performed on an LCQ ion-trap mass spectrometer (Thermo Fisher, San Jose, CA, USA) equipped with the manufacturer's ESI source and operated in positive ion mode. The mobile phase (80% acetonitrile, 19% water and 1% acetic acid, v/v) was delivered by a PM-80 pump (BAS, West Lafayette, IN, USA) for LC separations on a 50 x 2.1 mm i.d. Discovery HS C18 column packed with 5 µm particles (Supelco, Bellefonte, PA, USA) at 250 µL/min flow rate. Samples were injected manually using a Rheodyne (Cotati, CA, USA) 7125 injection valve equipped with a 20 µL loop. The effluent was diverted to waste for 1 min after injection to reduce the amount of salt entering the ESI source in which nitrogen was used as both the sheath and auxiliary gas at a pressure of 40 and 10 units, respectively. The spray voltage was set at 4.0 kV and the capillary temperature was 200 °C.

Product-ion MS/MS spectra were obtained by collision-induced dissociation (with helium as target gas) and collected using 1.0 Th parent ion isolation width and 35% relative collision energy. The parent ions of dansylated E2 (m/z 506) and dansylated d5-E2 (m/z 511) were mass-selected for MS/MS. Data acquisition and processing were performed by the manufacturer's XCalibur (version 1.4) software.

Study 2: In Vivo Behavior Testing and ChAT-IR Effects After Tonic Treatment in Middle-Aged Ovx Animals

Subjects.

Subjects were 42 middle-age (15-16 months old at test) Fischer-344 female rats born and raised at the National Institute on Aging at Harlan Laboratories (Indianapolis, IN). After arrival, animals were pair housed, had exposure to food and water *ad lib*, and were maintained on a 12-h light/dark cycle. Procedures were approved by the Arizona State University IACUC, and adhered to the Guide for the Care and Use of Laboratory Animals and NIH standards.

Hormone treatment procedures.

Middle-aged rats were separated into the following six groups: Ovx with only vehicle treatment (Ovx-Veh; *n*=8), Ovx plus a 4 µg/day of E2 (Ovx-E2; *n*=7), Ovx plus a 12 µg/day of CEE (Ovx-CEE-Low; *n*=8), Ovx plus a 24 µg/day of CEE (Ovx-CEE-High; *n*=6), Ovx plus a 4 µg/day of DHED (Ovx-DHED-Low; *n*=7) and Ovx plus a 8 µg/day of DHED (Ovx-

DHED-High; *n*=6). Ovx surgery was then performed approximately 40 days before behavioral testing began at 13 months of age. For Ovx, rats were anesthetized with vaporized isoflurane inhalant, bilateral dorsolateral incisions were made in the skin and peritoneum, and the ovaries and tips of uterine horns were ligated and removed. The peritoneum and skin was then sutured. CEE, E2 and DHED treatment was initiated via Alzet® pumps (Model 2ML4; Durect Corporation, Cupertino, California). Appropriate doses of prodrug or hormone were suspended in propylene glycol (PG); calculated to deliver the single and/or low and high dose described above for each hormone per day; this solution was then placed into the pump. For Ovx-Veh rats, pumps were only filled with the PG. Pumps were prepared the day before surgery and allowed to equilibrate in physiological saline at 37 °C overnight. Hormone treatment began approximately 20 days after Ovx, 20 days before testing ensued (Bimonte-Nelson et al., 2006). For pump insertion, under vaporized isoflurane inhalant anesthesia, a small incision was made on the left side of the neck scruff, and a subcutaneous pocket was created. For each animal, one pump of the appropriate prodrug, hormone or vehicle was inserted into the pocket and the skin was stapled. Recovery from anesthesia occurred under a heat lamp with monitoring of body temperature. Since the pumps released hormone or vehicle for 28 days, animals received a pump replacement surgery 3 days before testing ensued; this ensured that all

animals had hormone or the prodrug on board for the duration of behavioral testing and through sacrifice.

Morris maze: spatial RM.

The Morris maze (Morris et al., 1982) consisted of a round tub (188 cm in diameter) filled with water made opaque with black non-toxic paint. Each subject had 60 seconds to locate the hidden escape platform with a wire mesh top (hidden about 1 cm below the water surface) that remained in a fixed location (in the NE quadrant) throughout testing. If the rat did not find the platform in 60 seconds, it was gently guided to it. The trial was terminated when the rat found the platform. After fifteen seconds on the platform, the rat was gently removed from the maze and placed into its heated cage until the next trial. The rats were tested for five days, with four trials per day. The drop off location for each trial varied semi-randomly. A video camera suspended on the ceiling above the maze tracked the rat's path during each trial and a tracking system (Ethovision 3.1, Noldus Information Technology, Wageningen, Netherlands) was used to analyze each rat's swim path. The dependent measure was swim distance (cm) collapsed across all days and trials and a separate analysis for day three trial one. To assess platform localization, a probe trial was given on trial 5 of the last day of testing (i.e., day 5), whereby the platform was removed from the maze. For the probe, percent of total distance swum (cm) in the target NE quadrant (i.e., quadrant that contained the

platform) as compared to the opposite SW quadrant was the dependent measure.

DMS Plus Maze: spatial WM.

I tested spatial WM and short-term memory retention using a win-stay DMS Plus Maze task, with the protocol based on studies showing E2-induced improvements (Gibbs, 2000b; Korol and Kolo, 2002; Sandstrom and Williams, 2004), and CEE-induced improvements (Acosta et al., 2009b) in Ovx rats. The black Plexiglass 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 four arms. Start location varied across trials, and the platform location changed every day. Rats received either 2 or 6 trials within a daily session, for 13 days. Rats were given 90 seconds to locate the platform. Once on the platform, the rat remained on it for 15 seconds, followed by placement into a heated cage for a 30s 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 and not visible from the inside of the maze (11 cm into the arm). Entry into any non-platformed arm was counted as an error. The dependent variables were the number of errors committed during a trial. Trial 1 was the information trial informing the animal where the platform was for that day's session, trial 2 was the WM test trial and trials 3-6 six were recent memory test trials (Frick et al., 1995).

After 8 days of acquisition testing (6 consecutive trials), rats were tested with delays to increase memory demand. First, on Day 9, a 4hr delay was initiated between test trials 1 and 2 to assess retention of recent memory. Since this delay did not influence performance, on Day 10, a 6h delay between test trials 1 and 2 was given. For delay testing, after the initial pre-delay trial/s, rats were taken to the colony room and were left in their testing cages until the end of the delay, at which time they were brought back into the testing room and tested on the post-delay trial/s. On day 12, following an additional acquisition/baseline day (day 11), where each animal received 6 consecutive trials, animals were then immediately taken in to an adjacent room containing a small rectangular tub (99.1 cm x 58.4 cm) filled with black opaque water. Each subject had 2m to locate a hidden escape platform that remained in a fixed location. If the rat did not find the platform it was gently guided to it. The trial was terminated when the rat found the platform. After fifteen seconds on the platform, the rat was gently removed from the maze and placed into its heated cage for 30s. The drop off location for each trial varied semi-randomly. After 5 interference trials, rats were taken back into the DMS Plus Maze room to assess performance on the WM trial (trial 2). On day 13, rats received a 0.2 mg/kg intraperitoneal injection of scopolamine (Sigma-Aldrich Inc., St Louis, MO) dissolved in physiological saline, 20m prior to testing on the DMS Plus Maze (6 consecutive trials).

The dependent measure for acquisition was the number of errors committed on the trials 2-6 on days 1-5 (i.e., initial acquisition of the task) (Acosta et al., 2009b). To assess performances after delay challenges, the dependent measure was the number of errors committed on trial 2 across the 4 and 6hr delays. To evaluate which groups were impaired by the delay and interference challenges, the dependent measure was the number of errors committed on trial 2 across the last baseline day and the number of errors committed on trial 2 during the specific challenge day. Lastly, I evaluated if groups were impaired by the scopolamine challenge, by analyzing the number of errors committed on trials 2-6 across the last baseline day to the number of errors committed on trials 2-6 during the scopolamine challenge.

ChAT immunohistochemistry.

Techniques were performed using previously published methods (Acosta et al., 2009b). At dissection brains were blocked. The anterior portion of the brain was post fixed in 4% paraformaldehyde in phosphate-buffer solution (PB; pH 7.4). After 48 hours the brains were moved to PB until sectioning. Brains were sectioned (plates 1-25: Paxinos and Watson, 2005) on a Vibratome (Vibratome 3000) in PBS (pH 7.4) at 40 µm throughout the BF and collected for immunohistochemistry (Granholm et al., 2002). Every 2nd section through the BF was selected for ChAT antibody stain and incubated for 15 minutes in a 0.03% Triton (Triton

100X) in phosphate-buffered saline tween (PBST) to permeabilize the tissue. The tissue was then blocked, by incubating tissues at room temperature (RT) for 30m in a blocking solution (BKS) containing 0.03% PBST and 0.03% heat inactivated horse serum (Fischer). Three PBS washes (3m each) were then done. The primary antibody, goat Anti-ChAT (1:1000, Chemicon), 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 PBS three times (3m each) followed by immersion in the secondary antibody solution (1: 200 biotinylated Donkey anti-Goat IgG, Vector) and BKS for 45 min on a Titer Plate Shaker (Barnstead International, Dubuque, IO) at RT. Sections were washed three times in PBS (3m each), and then placed into an 11% methanol and 1% H^2O^2 (Fischer) in PBS solution for 30m on a Titer Plate Shaker to quench endogenous peroxidase activity. After three washes in PBS (3m each), ABC reagent (Vector) was added to each well and incubated for 45m at RT on a Titer Plate Shaker. Sections were washed three times in PBS (3m each), and were then incubated with DAB solution (Vector). After the desired color was achieved (dark purple), brain sections were washed three times in PBS (3m each), mounted on subbed slides, air dried, dehydrated and cover slipped with Permount. 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 lack of DAB color development.

ChAT stereological cell counts.

Quantitative estimates of the total number of ChAT-IR neurons in the BF were determined using an unbiased, stereological cell counting method as described in previous published work (Granholm et al., 2002). Briefly, the optical fractionator system consisted of a computer assisted image analysis system including a Olympus BX-51 microscope hard-coupled to a MAC 5000 computer controlled x–y–z motorized stage, an mbf CX9000 video camera, a Dell Precision 390 computer and StereoInvestigator 8.31 stereological software (MicroBrightField, Colchester, VT). The region of interest was outlined under a low magnification (4x), and the outlined region was measured with a systematic random design of disector counting frames (the size of the counting frames was 100x100 mm). A 40x objective lens with a 0.75 numerical aperture was used to count and measure individual cells within the counting frames. The first section in the rostral portion of the BF was selected randomly, thereafter every second section was evaluated with ChAT immunohistochemistry and total cell counts were generated. The following landmarks were used: the rostral border consisted of the medial orbital cortex (at the level of the midline fusion of the corpus callosum), the

caudal border consisted of the midline fusion of the anterior commissure, and the lateral borders consisted of the nucleus accumbens shell. At least 150 sites were counted in each brain. The counting brick was approximately 20-mm thick (18-21 mm). The analysis rendered a mean coefficient of error of .16, as calculated according to Gundersen (i.e., m = 1 on SteroInvestigator), with most coefficient errors in the range of 0.11-0.21. An upper and lower guard zone of 2 mm was excluded from counting. Data were available from 7 Ovx-Veh, 5 Ovx-E2, 7 Ovx-CEE-Low, 6 Ovx-CEE-High, 5 Ovx-DHED-Low and 4 Ovx-DHED-High animals. Counts and region area measurements were then exported to Excel 2007(Microsoft, Redmond, WA). Three or more sections per animal were quantified, corresponding to plates 13-17 from Paxinos and Watson (2005), similar to prior publications (Acosta et al., 2009b). The number of ChAT-IR positive cells counted in the medial septum (MS) was the dependent measure.

Serum E2 and E1 assays.

E2 and E1 levels were determined in animals. Rats were anesthetized with vaporized isoflurane inhalant and decapitated. Blood was collected from the trunk at the time of decapitation into a serum separator tube (Vacutainer 367986, Becton Dickinson and Company, Franklin Lakes, NJ). Blood was allowed to clot at 4º C and serum was collected after centrifugation (3220xG, 20m). Serum was stored at -20 ºC

until assays were performed. E2 and E1 hormone assays were performed by the Core Endocrinology Laboratory at Pennsylvania State University College of Medicine using a kit from Diagnostic Products Corporation, Los Angeles, CA (Coat-A-Count E2 kit, product number TKE21). E2 and E1 was determined in serum by a solid-phase radioimmunoassay based on E2- and E1-specific antibodies that are immobilized to the wall of polypropylene tubes and ^{125}I -labeled E2 or E1 as the tracer following extraction with diethyl ether. Serum (2.4. ml) was extracted and the ether portion collected and evaporated to dryness. The sample was reconstituted in assay buffer and a competitive radioimmunoassay was performed using ^{125}I E2 or E1 with high specific activity and a high affinity, highly specific antibody. Separation of bound from free was achieved with activated charcoal and the data reduction was performed with the use of a five point standard curve and purified E2 or E1 standards. The functional sensitivity of the assay was 5 pg/ml. The interassay precision at a concentration of 35 pg/ml was 8%. The dependent measure was the amount of E1 or E2 in pg/ml of serum.

Vaginal smears and uterine weights.

To evaluate uterine stimulation, vaginal smears were taken before the first pump insertion, the day of the second pump insertion as well as the morning on the day of sacrifice. Smears were classified as proestrus, estrous, metestrus or diestrus (Goldman et al., 2007). For collection of

uterine tissues, after sacrifice, a ventral incision was made in the abdominal region, and the uterus was cut above the junction with the cervix and on the uterine horn below the ligature remaining from Ovx (Acosta et al., 2009b; Ashby et al., 1997a; Engler-Chiurazzi et al., 2009). The wet weight (g) of uteri, trimmed of fat and connective tissue, was the dependent measure.

Statistical methods.

In study 2, since my interest was to determine whether each hormone or prodrug dose enhanced performance relative to the Ovx-Veh group, all of my follow-up two-group comparisons for each hormone type employed Fisher's Protected Least Significant Difference (PLDS) post hoc tests when a significant omnibus ANOVA was found, noting that Type 1 error correction is not necessary with orthogonal planned comparisons (Keppel and Wickens, 2004). Alpha was set at 0.05 for all tests. For behavior assessments and biological measurements, data were analyzed separately for each maze or measurement, first with an omnibus one–way or mixed model ANOVA with Treatment as the between variable and Days and/or Trials as the within variable, as appropriate for the specific maze test or biological measure. Due to my lab's previous findings whereby CEE enhanced the learning on the DMS Plus Maze task and overnight retention on the Morris maze (Acosta et al., 2009b), acquisition on the DMS Plus Maze and day 3 trial 1 on the Morris maze was assessed via a

priori planned comparisons using t-tests; alpha was set at 0.05, two-tailed. StatView (version 5.01, The SAS Institute Inc., Cary, NC) was used for all analyses.

Results

Study 1: *In Vitro* Prodrug Activation

As shown in Figure 12a, there was a facile DHED to E2 (prodrug to drug/hormone) biotransformation in the brain. Even within 2.5 min incubation of 100 μM DHED, the formation of approximately 10 μM E2 was detected in this tissue homogenate (20% w/v), thus, the initial rate of E2 formation was approximately 200 nmol· min-1 · (mg protein)-1. At the same time, as shown in Figure 12b, in a control experiment where no brain tissue was present (PBS only), My collaborators did not detect the formation of E2 from its DHED precursor.

Study 2: *In Vivo* Behavior Testing and ChAT-IR Effects After Tonic Treatment in Middle-Aged Ovx Animals

Morris maze: spatial RM.

Figure 13 depicts a) the learning curves (i.e., mean swim distance ±*SE*) for all days and all trials for each group, the inset graph depicts swim distance from day 2 trial 4 to day 3 trial 1, and b) the percent distance swam in the target as compared to the opposite quadrant on the probe trial. No main effects or interactions were found for swim distance across days. The graphical representation of the data (Figure 13a) revealed that

the Ovx-CEE-High group showed markedly better performance on day 3, I evaluated performance for the first trial on day three; the Ovx-CEE-High group had decreased distance as compared to the Ovx-Veh group (F[1,14] = 7.48, p = 0.0161). The probe trial revealed a main effect of Quadrant (F[1,43] = 302.71, p < 0.0001), in the absence of a main effect of Treatment (F[5,43] = 2.16, p = 0.0758), and a Treatment x Quadrant interaction (F[5,43] = 1.38, p = 0.2506). This indicated that rats swam significantly more in the quadrant that once contained the platform as compared to the opposite quadrant during the probe trial, a pattern that was consistent for all groups. This indicated that each group of animals was able to localize to the hidden escape platform regardless of treatment.

DMS Plus Maze: spatial WM.

In figure 14, bars represent mean number ±*SE* of errors for a) acquisition b) after delay challenges. Line graphs depict the mean number ±*SE* errors from baseline day 11 to c) interference challenge (day 12) and d) scopolamine challenge (day 13). The Ovx-CEE-High group displayed superior acquisition during the initial learning phase (t[13] = 2.34, p = 0.0357). No other estrogen impacted performance during acquisition or initial learning. After the acquisition, I increased the memory demand of the task to test retention by instituting delay challenges. There was a Treatment main effect (F[5,38] = 2.74, p = 0.0331]. Each hormone group made fewer errors than the Ovx-Veh group (ps < 0.0442). A further

analysis of the delay challenges revealed that only the Ovx-Veh group increased the number of errors they committed from baseline day 8 to the delay challenges (F[1,7] = 5.9, p = 0.0452). The delay had no impact on the estrogen- or prodrug-treated group performance for this measure. All groups increased errors after the interference challenge [main effect of Day, (F[1,37] = 12.32, p = 0.0012, post-hoc ps < 0.05; nonsignificant Hormone Treatment x Day interaction). Pharmacological challenge via administration of the M1 muscarinic antagonist scopolamine also impaired the performance of all groups [main effect of Day, (F[1,36] = 11.19, p = 0.0019, post-hoc ps < 0.05; nonsignificant Hormone Treatment x Day interaction).

BF ChAT neuron counts.

Figure 15 represent the mean ±*SE* stereological ChAT-IR neuron counts in the MS of the BF. There was a main effect of Hormone Treatment (F[5,28] = 3.23, p < 0.0200). The Ovx-E2, Ovx-CEE-Low and Ovx-DHED-Low group had the highest number of ChAT-IR neurons, which differed from the Ovx-Veh group (post-hoc ps < 0.0438).

Serum E2 and E1 levels.

In figure 16, bars depict the mean serum hormone levels ±*SE* [pg/ml] for a) E2 b) E1. There was a main effect of Hormone Treatment (E2 F[5,23] = 85.89, p < 0.0001; E1 F[5,23] = 8.25, p = 0.0001). The Ovx-E2 group had the highest serum E2 and E1 levels, which differed from the

Ovx-Veh group (post-hoc *p*s < 0.0001). Due to the high variability within the Ovx-E2 group (E2: Mean Square with the Ovx-E2 group = 2332.840 vs. Mean Square without the Ovx-E2 group = 42.107; E1: Mean Square with the Ovx-E2 group = 139.469 vs. Mean Square without the Ovx-E2 group = 105.576), a second set of analyses were performed in order to elucidate further group differences between the Ovx-Veh compared to the non-E2 hormone-treated groups. The analysis excluding the Ovx-E2 group, revealed a main effect of Hormone Treatment (E2 $F[4,20] = 6.73$, $p = 0.0013$; E1 $F[4,20] = 9.74$, $p = 0.0002$). Both doses of CEE increased serum E2 and E1 levels as compared to Ovx-Veh animals (post-hoc *p*s ≤ .05). Importantly, both DHED doses did not increase serums levels of E2 or E1, as both DHED groups did not differ from the Ovx-Veh group (post-hoc *p*s > 0.32).

Uterine weights.

The bars in figure 17 represent the mean uterine weight ±*SE* for each group. A main effect of Hormone Treatment was found ($F[5,34] = 5.12$, $p = 0.0013$). E2 treatment had the most profound effect for increasing uterine weight when compared to the Ovx-Veh group (post-hoc $p = 0.0001$). However, due to the high variability within the Ovx-E2 group (Mean Square with the Ovx-E2 group = 1.442 vs. Mean Square without the Ovx-E2 group = 0.075), a second analysis was performed in order to elucidate further group differences between the Ovx-Veh compared the

other hormone-treated groups. For this second analysis, excluding the Ovx-E2 group, revealed a main effect of Hormone Treatment (F[4,28] = 7.87, p = 0.0002). Both CEE doses increased uterine weight (post-hoc ps < 0.0007) as compared to the Ovx-Veh group. Neither DHED dose increased uterine weight (post-hoc ps >.1577), suggesting the DHED did not stimulate the uterus like E2 and CEE.

Vaginal smears.

Initial vaginal smears after Ovx confirmed that each animal was in an anestrous state. After pump insertion and at sacrifice, vaginal smears confirmed that all Ovx-Veh rats consistently maintained an anestrous state (i.e., no uterine stimulation), while Ovx-CEE-High, Ovx-CEE-Low and Ovx-E2-treated rats demonstrated consistent estrus smears indicative of uterine stimulation. However, every rat in the Ovx-DHED-Low and Ovx-DHED-High groups presented a diestrus smears which looked identical to those of Ovx-Veh rats, thereby indicating no uterine stimulation (data not shown).

Regression: relations between serum E2, E1 ChAT-IR neuron counts and memory.

Since I and others in my lab have previously found that the serum levels of E2 and E1 are related to memory performance (Engler-Chiurazzi et al., 2009; Talboom et al., 2008); I conducted a primary regression analysis relating serum E2 and E1 levels to performance on each maze

and to the number of ChAT-IR neurons in the MS. There were a total of six separate analyses: (α was set to 0.05 for each separate effect). Specifically, serum E2, serum E1 or MS ChAT-IR were separate predictor variables in the model, and Morris maze distance scores collapsed across all days, the mean win-stay DMS Plus Maze WM errors collapsed across all days, or MS ChAT-IR counts were the outcome variables. To ensure that significant regression models were not attributable to group differences in my outcome or predictor variables, due to the experimental manipulations, I centered the data by subtracting the mean score of the treatment group [from which the animal belonged] from each animal's score (for detailed methods and rationale see Enders and Tofighi, 2007; Hallahan and Rosenthal, 2000). Figure 18a and 18b depicts scatterplots of serum E2 and E1 levels respectively and the number of MS ChAT-IR neurons for the Ovx-E2, Ovx-CEE-Low and Ovx-CEE-High groups (i.e., groups with detectable hormone levels). Serum E2 and E1 were negatively related to the number of MS ChAT-IR neurons (E2 b = -8.31, r = -.64, z[17] = -2.83, p = .0046; E1 b = -3.13, r = -.56, z[17] = -2.38, p = .0174). Figure 18c and 18d depicts scatterplots of serum E2 and E1 levels respectively and Morris maze distance scores collapsed across all days for the Ovx-E2, Ovx-CEE-Low and Ovx-CEE-High groups. Serum E2 and E1 were negatively related to the mean distance swam on the Morris maze (E2 b = -8.31, r = -.54, z[17] = -2.28, p = .0228; E1 b = -3.13, r = -

.57, $z[17] = -2.40$, $p = .0163$). Serum E2 and E1 levels were not related to DMS Plus Maze errors. As my lab has found previously, ChAT-IR counts were not related to memory outcome variables (data not shown Acosta et al., 2009b).

Discussion

In the current two studies, I evaluated if DHED, a prodrug of E2, was converted to E2 in brain and compared the cognitive and peripheral effects of DHED to its parent compound E2 (i.e., the most commonly studied hormone in preclinical research), and CEE (i.e., the number one prescribed HT in the U.S.; Hersh et al., 2004). In study 1, I found that DHED was converted to E2 in brain tissue. In study 2, this conversion presumably led to bioactive E2 being present in brain, which in turn enhanced WM on the DMS Plus Maze, as well as increased in the number of ChAT-IR neurons in the MS, similar to the effects of E2 alone. My data also suggest that CEE may be the most beneficial hormone preparation for cognition under the current experimental paradigm. CEE enhanced overnight retention on the Morris maze, enhanced the acquisition (i.e. learning) of the DMS Plus Maze and protected against the delay challenges (i.e. retention) and increased the number of ChAT-IR positive neurons in the MS. As expected, treatment with CEE and E2 stimulated the uterus, as demonstrated by consistent estrus vaginal cytology and an increase in uterine weight. CEE's and E2's uterine stimulation was

accompanied by raised serum E2 and E1 levels. Importantly, DHED did not affect any peripheral marker of estrogenic action nor raise serum levels of E2 or E1. Taken together, my data suggest that tonic CEE is beneficial to memory over and above E2 under the current paradigm. Furthermore, DHED is converted into bioactive E2 in the brain, but not the periphery, where it exerts its effects on memory in the same manner as E2. Earlier experiments have indicated that NAD(P)H is a coenzyme involved in the conversion from DHED to E2, although the full mechanism of this conversion remains to be elucidated (Prokai-Tatrai et al., 2008; Prokai et al., 2003; Rivera-Portalatin et al., 2007).

The present CEE data corroborate previous findings from my lab and others whereby CEE enhances memory (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009; Walf and Frye, 2008). My lab has previously noted modest enhancements on the Morris maze with cyclic CEE treatment (i.e., overnight retention) (Acosta et al., 2009b); indeed, here again, I found that the high dose of CEE enhanced overnight retention between days two and three on the Morris maze. Furthermore, my lab has have previously found that CEE treatment enhances acquisition (Acosta et al., 2009b), and protects against memory challenges on the DMS Plus Maze (Engler-Chiurazzi et al., 2009), here again I noted these same CEE-facilitated WM enhancements. In study 2, DHED was similar to E2, whereby both did not affect Morris maze performance, but did protect against the delay

challenges on the DMS Plus Maze. Specifically, both DHED and E2 did not enhance spatial RM, but did enhance spatial WM retention after a delay was imposed to increase memory demand. This finding is not surprising, as many studies report no effect or modest effects regarding E2's effects on RM performance (for review see Dohanich, 2002), but do find reliable WM enhancements after E2 treatment (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). Despite the fact that no treatment effects were found on the Morris maze, serum E2 levels were related to Morris maze performance. Higher circulating serum E2 and E1 levels were related to less distance swam and better performance, an effect I have found previous for E2 (Talboom et al., 2008).

E2 has been found to have a profound impact on numerous measures of cholinergic system integrity (Gibbs, 1997; Gibbs et al., 1994; Luine, 1985), thought to support memory processing (see Hasselmo, 2006). Thus, cholinergic system modulation is one hypothesized mechanism for estrogen's effects on memory (for review see: Gibbs and Aggarwal, 1998). In addition to my current finding where E2 treatment increased the number of ChAT-IR positive neurons in the MS, E2 has also been found to alter ChAT in the MS of young Ovx animals (Gibbs, 1996; Gibbs, 1997; Gibbs and Pfaff, 1992; Gibbs et al., 1994), and my lab has

found that CEE treatment increases the number of ChAT-IR positive neurons vertical diagonal band in middle-aged rats (Acosta et al., 2009b). Moreover, E2 treatment has been found to alter multiple markers of ChAT in middle-aged and aged animals, which appears to differ depending on duration of ovarian hormone deprivation before treatment as well as specific brain region and BF nuclei evaluated (Bohacek et al., 2008; Kompoliti et al., 2004; Singer et al., 1998). In young adult Ovx rats, delay match-to-position acquisition correlated with increased ChAT activity in the hippocampus and frontal cortex (Gibbs, 2000a), two targets of BF cholinergic innervation where high affinity choline uptake was also increased (O'Malley et al., 1987; Singh et al., 1994). It is likely that the current DHED-spatial memory enhancements are also directed by the cholinergic system, as DHED increased the number of ChAT-IR positive neurons in the MS. Collectively, CEE and DHED, similar to the effects of E2, likely enhance the BF cholinergic system leading to cognitive enhancement.

Some of the results of study 2 may appear counter-intuitive at first. Specifically, the Ovx-CEE-High and Ovx-DHED-High groups exhibited superior memory performance as compared to Ovx animals, but did not demonstrate elevated numbers of ChAT-IR neurons in the MS, similar to their low dose counterparts, when compared to Ovx animals. An explanation for this discrepancy may lie in the fact that Gibbs (1997),

found that estrogens effect on the BF varied as a function of dose. Higher doses of E2 induced a more rapid increase in the number of ChAT-IR neurons in the BF, which quickly diminished, as compared to a lower dose of E2 (Gibbs, 1997). Hence, in the current study, higher estrogenic stimulation over a long time frame (i.e., 30 days) may have initially led to an increase in ChAT which diminished by the time the brains were harvested. In addition to the dose of the hormones used, properties of CEE and DHED may have caused these compounds to produce higher levels of estrogenic stimulation as compared to E2. CEE is made up of several estrogenic components, and many of these component estrogens, on different measures, have been found to exert greater biological activity than E2 (Bhavnani, 2003; Bhavnani et al., 2008). If fact, recently I have shown that one such CEE component Δ^8E1, had a marked impact on spatial RM, over and above the modest effects generally found with E2 (Talboom et al., 2010). Regarding DHED, due to the structural modification of E2 to create the prodrug, DHED is likely not bound by sex hormone binding globulin, and since my data support the fact that DHED is not converted into E2 within peripheral tissues (study 1), nor found naturally in the body; DHED likely rapidly pulled into the brain via diffusion. Both of these factors would lead to high levels of DHED in the brain which in turn produce high levels of E2 in brain. Taken together, my current findings are not surprising, as the higher doses of CEE and DHED,

producing higher levels of estrogenic stimulation, may have initially and rapidly increased the number of ChAT-IR cells in the MS, this effect likely diminished upon completion of the study when the brains were harvested for ChAT analysis. These data also suggest that estrogen-induced alterations in the number of ChAT-IR neurons may not necessarily correspond to actually cholinergic function, as it has been found that E2-facilitated increases in potassium-evoked ACh release are still present (i.e., 12-13 weeks) (Gabor et al., 2003), long after the number ChAT-IR neurons have been found to decrease (i.e., 4 weeks) (Gibbs, 1997). This suggests a dissociation between ChAT-IR positive cells, cholinergic function, and memory, a tenet further supported by my data in study 2, whereby serum E2 and E1 levels were inversely related to the number of ChAT-IR neurons in the MS and spatial memory in the Morris maze (i.e., less distance equals better performance). In future experiments I wish to evaluate brain region specific levels of E2 and the amount of ACh released from the BF utilizing the same dosing and treatment regimens used in the present study.

In the current report, CEE treatment produced a beneficial memory profile that exceeded E2's and DHED's; this effect was not expected. An explanation may lie in the composition of CEE; since CEE is made up of several estrogenic components, the actions of some unique components such as Δ^8E1, may under the current experimental paradigm, create a

more favorable environment for brain health and memory enhancement than E2 alone (for discussion see Bhavnani, 2003; Utian et al., 2006). Furthermore, like many biological systems, it has been noted that estrogen's effects on multiple systems follows a nonmonotonic inverted-U or quadratic function, whereby low or high levels of estrogens produce minimal effects and a more moderate doses produce a larger effect (Alyea and Watson, 2009; Welshons et al., 2003). Taking into account the high E2 levels in my E2-treated animals (i.e., around high proestrus levels; Butcher et al., 1974), which has been noted to impair spatial memory (Warren and Juraska, 1997); perhaps E2 was at the high end of the inverted-U, and CEE, producing lower levels of E2, fell closer to the middle. Lastly, estrogenic effects may be also directed by membrane bound G-protein coupled receptors (i.e., ER-X and/or GPR30) (see: Prossnitz et al., 2008b; Toran-Allerand, 2005), whereby different estrogens, including component estrogens of CEE, may bind with various affinities and ultimately differentially modulate memory and endocrine responses through these receptors. Further studies are necessary; evaluating dose, regimen and the region specific genomic and/or proteomic profile of CEE, DHED and E2. Studies of this nature will begin to elucidate the mechanistic differences between these hormones that lead to differing cognitive and brain health profiles.

I and others in my lab have previously found RM enhancement after tonic treatment with E2 (Bimonte-Nelson et al., 2006; Talboom et al., 2008). An explanation for the differences in effects between my lab's previous reports and the ones discussed currently may lie in the fact that in previous studies conducted in my lab had purchased premade hormone pellets from Innovative Research of America instead of employing Alzet® pumps. I noted that E2 treatment with the Innovative Research of America pellets produced substantial variability in the circulating E2 levels (Talboom et al., 2008), whereas the Alzet® pumps used in the present study did not. Specifically, although the current mean E2 levels produced by the Alzet® pumps were close to those found in Talboom et al., (2008) (≈ 56 pg/ml compared to ≈ 54 pg/ml respectively), the range of serum E2 levels were vastly different (56.7-87.4 pg/ml as compared to 15-121 pg/ml respectively). Conceivably, my disparate RM effects could be due to this variation in the range of serum levels. Indeed, RM performance has been found to fluctuate with physiological levels of E2 (Warren and Juraska, 1997), although an exception has been noted (Berry et al., 1997). In this view, study 2's consistent proestrus levels of E2 (Butcher et al., 1974), may have been too high for RM enhancement, an effect that has been found in another lab (Warren and Juraska, 1997).

I would be remiss if I did not note the fact that the current report is somewhat in opposition to the findings in a recently published paper from

my lab (Engler-Chiurazzi et al., 2009). The previous publication from my lab found that the 12 µg tonic dose of CEE was detrimental to performance on the Morris maze and DMS Plus Maze. Specifically, this low 12 µg tonic dose decreased the number of platform crossings during the probe trial on the Morris maze, indicating that this group was not able to localize to the previously platformed location as well as the Ovx group. Furthermore, the 12 µg tonic dose increased the number of total errors committed on the DMS Plus Maze during all acquisition days and on day 4 alone before a delay was instituted. In fact, since animals receiving 12 µg were already impaired on the final acquisition day, no further impairment was observed on the following day when a 6hr delay was instituted between trial 1 and 2. In the current report, I observed no such impairment, and in fact I observed enhancement on the DMS Plus Maze and an increase in the number if ChAT-IR neurons with the group receiving 12 µg CEE via the same tonic dosing regimen (i.e., Alzet® pumps). I think that there are two primary reasons for this discrepancy; the first is that both doses of CEE in the current report raised both serum levels of E2 (*M* = 4.82) as well as E1 (*M* = 7.02) which differed significantly from the Ovx-Veh group. It was noted in my lab's previous report that the 12 µg dose impaired performance, but only under the conditions where serum E1 (*M* = 7.32) levels were raised not in the presence of raised serum E2 levels (*M* = 3.43) (i.e., levels of E2 which did not differ from the

Ovx-Veh group) (Engler-Chiurazzi et al., 2009). This hypothesis is further supported by the fact that in the current report higher levels of E1 were related to better performance on the Morris maze, whereas higher levels of E1 in my lab's previous report were related to worse performance on the Morris maze (Engler-Chiurazzi et al., 2009). Hence, the 12 µg dose may have been beneficial in the current report due to the levels of circulating E1 in the presence of higher levels of E2. Further research should be directed towards an evaluation of CEE's and DHED's ability to stimulate brain derived ERs (both classic and newly discovered) as well as the effect that an animal's E1 to E2 ratio has on cognition.

In summary, with the staggering number of menopausal women in the next 41 years who will have to make the choice of weather to use HT or not (U.S. Census Bureau, Bureau, 2007), current evidence from the WHI and ancillary WHIMS studies, suggests that CEE-containing HT should not be used (Shumaker et al., 2004). While noting that the WHIMS' results were likely affected by factors other than just CEE treatment (see Sherwin, 2005); a new option for these women may be estrogen prodrugs. Prodrugs are inactive precursors of the therapeutic agents that are converted to the biologically active agents by enzymatic and/or chemical transformation in vivo–preferably at the site of action (Albert, 1958; Wermuth et al., 1998). The application of prodrugs to overcome barriers to a drug's usefulness and improve its therapeutic index is an established

method (Ettmayer et al., 2004; Prokai and Prokai-Tatrai, 1999). Although previous efforts to utilize this approach to improve targeting of E2 into the brain have shown increased brain-uptake of E2 and reduced systemic burden (for review see: Prokai et al., 2000), further developments are needed to realize the potential of the DHED as a new class of HT. I believe the current report is an important step in realizing this approach leading to the synthesis a more optimal HT.

In the current study I found, in middle-aged Ovx rats receiving a tonic regimen, CEE enhanced spatial working and RM, and protected against WM challenges. Tonic DHED and E2 enhanced WM and protected against delay challenges. Unlike CEE and E2, treatment with DHED did not affect peripheral markers of estrogenic actions. Within the parameters of the current study, the data suggests that tonic CEE is more cognitively beneficial than DHED and E2. Furthermore, CEE, DHED and E2 treatment resulted in an increase in the number of ChAT-IR positive neurons in the BF, an indicator of cholinergic modulation by these hormones. Serum E2 and E1 levels were related to spatial memory and the number of ChAT-IR neurons in the MS. Hence, these hormone treatments likely provide cognitive benefits via alterations of the cholinergic system. Further inquiry is necessary evaluating CEE's and DHED's mechanistic effects on cognition in comparison to E2; however, the results in the current study support the tenet that DHED is a promising

drug candidate for a different class of future HTs, which maximize the beneficial cognitive effects while minimizing unwanted peripheral effects.

CHAPTER 5

THE RIGHT DOSE: 17β-ESTRADIOL AND PREMARIN'S® DOSE RESPONSE EFFECTS ON SPATIAL WORKING MEMORY

By the year 2050, an estimated 45 million postmenopausal women in the United States will have to make the choice of whether to utilize HT (U.S. Census Bureau, Bureau, 2007). Ovarian hormone loss due to surgical or natural menopause has been associated with cognitive decline in women (Nappi et al., 1999; Phillips and Sherwin, 1992; Sherwin, 1988); however, the question of how HT impacts cognition is unclear. CEE, a complex preparation synthesized from the urine of pregnant mares, has been given since 1942 and is the most widely used estrogen component of HT in the United States (Hersh et al., 2004; Stefanick, 2005). Some studies in women demonstrate that CEE-containing HT improved memory (Campbell and Whitehead, 1977a; Kantor et al., 1973; Ohkura et al., 1995). However, WHI study found that CEE does not benefit cognition in women (for review see Coker et al., 2010). While noting many factors likely affected the WHI findings, including the global nature of the cognitive measure, older age of participants, and duration of hormone deprivation before treatment initiation (for discussion see Sherwin, 2005), the collected findings suggest that although the absence of ovarian hormones is not optimal for cognition, neither is the most commonly utilized HT.

To date, E2 has been the primary estrogen used to investigate the cognitive effects of HT in the animal model, despite the fact that CEE is the most commonly prescribed estrogen component of HT (Hersh et al., 2004). CEE contains the sulfates of more than 10 equine estrogens (Bhavnani, 2003; Bhavnani et al., 1998; Mayer et al., 2008; Sitruk-Ware, 2002). New evidence has demonstrated that both tonic and cyclic CEE, at doses relevant to what women take as HT, can enhance spatial memory and protect against cholinergic challenge on spatial tasks in middle-aged Ovx rats (Chapter 4; Acosta et al., 2009b; Engler-Chiurazzi et al., 2009). Although vital in understanding CEE's effects on memory, these previous animal studies did not evaluate E2, so a direct comparison between the effects of E2 on memory (the most common estrogen employed in animal models of cognition) to CEE (the most commonly perscribed estrogen component of HT) was not possible.

Unpublished regression analyses from studies previously conducted in my lab revealed that serum levels of E1 and E2 were positively rank-ordered according to the dose of CEE given (Engler-Chiurazzi et al., 2009). That is, the low dose of CEE produced lower circulating levels of E1 and E2, whereas the higher dose of CEE produced higher levels of E1 and E2. Using these data, I performed further analyses, which revealed that serum E1 and E2 levels have a linear or nonlinear relationship depending on the day and trial block composite

measure of WM and recent memory performance. Furthermore, several studies have noted that cognition is related to circulating ovarian hormone levels in humans and animals (Asthana et al., 1999; Engler-Chiurazzi et al., 2009; Lebrun et al., 2005; Phillips and Sherwin, 1992; Ryan et al., 2010; Talboom et al., 2008; Wolf and Kirschbaum, 2002). Despite these findings, no single study to my knowledge has assessed memory performance across a range of E2 and CEE doses, whereby there were more than three doses given. It is conceivable that the dose response curve for each hormone may follow a linear, quadratic or even a cubic relationship with spatial memory; relationship is difficult to evaluate with three or fewer doses. Indeed, nonlinear effects of E2 and CEE on memory would be of particular importance to elucidate in that future studies could establish an *a priori* range of doses with a reduced chance of getting unexpected effects due to an unknown curvilinear relationship at lower or higher doses. To that aim, in middle-aged female Ovx rats, I separately tested a 0 dose (i.e., Ovx animals receiving only vehicle) and five tonic doses of E2 and CEE for their effects on spatial working/recent memory assayed via the DMS Plus Maze task. The hypothesis was that E2 and CEE would follow a quadratic relationship (i.e., across the tested dose range) with working/recent memory performance.

Methods

Subjects

Subjects were 36 middle-aged (12-13 month old) Fischer-344 female rats born and raised at the National Institute on Aging colony at Harlan Laboratories (Indianapolis, IN). Animals were acclimated for several weeks and pair housed with an identical treatment assigned cage-mate in the Arizona State University animal facility. All animals had exposure to food and water *ad-lib*, and were maintained on a 12-h light/dark cycle at 23 °C. Procedures were approved by Arizona State University IACUC and adhered to the Guide for the Care and Use of Laboratory Animals and NIH standards.

Ovx Surgery and Hormone Treatments

At 12 months of age, approximately 28 days before behavioral testing ensued, all rats underwent Ovx surgery. Under isoflurane anesthesia, dorsolateral incisions were made in the skin and peritoneum, and ovaries and tips of uterine horns were ligated and removed. Ovx rats were then separated into the following groups to estimate a dose response curve of tonic subcutaneous CEE and E2: Ovx plus vehicle polyethylene glycol (PEG; Ovx-Veh, n = 4), Ovx plus 6 µg/day of CEE (Ovx-n = 4), Ovx plus 12 µg/day of CEE (n = 4), Ovx plus 36 µg/day of CEE (n = 3), Ovx plus 60 µg/day of CEE (n = 4), Ovx plus 72 µg/day of CEE (n = 4), Ovx plus 0.75 µg/day of E2 (n = 4), Ovx plus 1.5 µg/day of E2 (n = 4), Ovx plus 2.25 µg/day of E2 (n = 4), Ovx plus 3 µg/day of E2 (n = 4), and Ovx plus 3.75 µg/day of E2 (n = 4). CEE was manufactured by

Wyeth (Philadelphia, PA) but purchased from a commercial pharmacy via veterinary prescription. I chose the 36µg/day as the medium dose of CEE as it approximated a rat equivalent of the daily 0.625mg CEE dose that was evaluated in the WHIMS and is the dose commonly prescribed to women (see Shumaker et al., 1998; Stefanick, 2005). Moreover, the 36µg/day dose of CEE is also the approximate dose of CEE or its component estrogen Δ^8E1 that my and my lab's previous studies have found to enhance working and/or recent memory on the DMS Plus Maze (Chapter 4; Acosta et al., 2009b; Engler-Chiurazzi et al., 2009; Talboom et al., 2010). The highest dose of E2 (Sigma) was based on a previous study from my laboratory where a 4µg/day tonic dose of E2 delivered via Alzet® pumps enhanced WM with minimal negative physiological effects (i.e., higher doses of E2 were detrimental to the animals' health and performance; unpublished observations; Chapter 4).

For CEE, two doses below the middle 36µg dose (6 & 12µg/day) and two doses above (60 & 72µg/day) were tested; for E2, four doses of E2 below the high 3.75µg (0.75, 1.5, 2.25, and 3.0µg/day) were also tested to expand the dose range for the sake of an evaluation of the dose response curve. Hormone treatment began approximately 14 days after Ovx, and treatments (vehicle PEG or PEG + CEE) were administered via Alzet® osmotic pumps (Model 2004; Durect Corporation, Cupertino, California). CEE was suspended in PEG (Sigma), and the solution was

placed into pumps before insertion. For pump insertion, under isoflurane anesthesia, a small incision was made in the dorsal scruff of the neck, and a subcutaneous pocket was created. For each animal, one pump of the appropriate hormone dose was inserted into the pocket and the skin was stapled. Animals were treated with their appropriate hormone dose or vehicle 12 days before behavioral testing began. All animals had hormone exposure for the duration of behavioral testing through sacrifice.

Vaginal smears and uterine weights

To confirm Ovx, E2, and CEE treatment, vaginal smears were taken at three times: 13 days following Ovx surgeries, 11 days following pump insertion and just prior to sacrifice (26 days after pump insertion). Smears were classified as either: proestrus, estrous, metestrus, or diestrus (Goldman et al., 2007). To examine the effects of Ovx and subcutaneous E2 and CEE on stimulation of uterine tissues, after maze testing animals were anesthetized with isoflurane and sacrificed. A ventral incision was made in the abdominal region, and the uterus was cut above the junction with the cervix and on the uterine horn below the ligature remaining from Ovx (Ashby et al., 1997b). Uteri were trimmed of all visible fat and were immediately weighed (wet weight). This weight in grams (g) served as the dependent variable for statistical analysis.

Working/Recent Memory DMS Plus Maze

The testing procedure has been previously published (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009; Talboom et al., 2010). This task assesses spatial working/recent memory. 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. Entry into an arm with no platform counted as an error. WM performance was evaluated by assessing the ability to navigate to the new platform location on trial 2. Hence, trial 1 was the information trial, trial 2 was the WM test trial, and trials 3-6 six were recent memory test trials (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009). Rats were dropped off in a semi-randomly chosen start arm location, and were given a maximum of 90 s to swim to the platform. Once on the platform, the rat remained on it for 15 s, followed by placement into a heated cage for a 30 s inter-trial interval (ITI). 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). Animals completed six trials/day for seven days. The number of total errors (i.e., initial and repeat errors) and repeat errors (i.e., repeat entries into arms that did not contain the platform) committed on trials 2-6 across the first 5 days of testing (i.e., days 1-5 baseline learning), as well as errors committed on trials 2-6 across all days of testing (i.e., days 1-7, overall performance) were the

four dependent measure composites created for statistical analysis, with one for each error type (see Table 3). These composites were created based on previous research where CEE and a component estrogen of CEE benefitted performance on this task across similar day and trial blocks (Chapter 4; Acosta et al., 2009b; Talboom et al., 2010).

Statistical Methods

To evaluate uterine weights, using StatView (version 5.01, The SAS Institute Inc., Cary, NC), I performed a one-way analysis of variance (ANOVA) separately in 1) the Ovx-Veh group compared to all E2 treated groups, and 2) the Ovx-Veh groups compared to all CEE treated groups. Since my *a priori* interest was to determine whether each hormone dose increased uterine weight relative to the Ovx-Veh group, all follow-up comparisons employed Fisher's PLSD post-hoc tests when a significant omnibus ANOVA was found, noting that Type 1 error correction is not necessary with orthogonal planned comparisons (Keppel and Wickens, 2004).

Using StatView (version 5.01, The SAS Institute Inc., Cary, NC), I performed an one-way analysis of variance (ANOVA) separately in 1) the Ovx-Veh group compared to all E2 treated groups, and 2) the Ovx-Veh groups compared to all CEE treated groups; this was conducted to determine differences between Ovx-Veh and E2 as well as Ovx-Veh and CEE treatments on uterine weights. Since my *a priori* interest was to

determine whether each hormone dose increased uterine weight relative to the Ovx-Veh group, all follow-up comparisons employed Fisher's PLSD post-hoc tests when a significant omnibus ANOVA was found, noting that Type 1 error correction is not necessary with orthogonal planned comparisons (Keppel and Wickens, 2004).

The primary goal of this study was to estimate the dose response curve for E2 and CEE's effect on spatial working/recent memory, and to this aim I used multiple regression analyses to describe the form of the relationship between dose and working/recent memory errors (i.e., summed composite measure). The baseline linear regression model was as follows: $\boldsymbol{Y = B_0 + B_1(DOSE)}$**,** where B_0 was the intercept and B_1 was the linear coefficient. The quadratic and cubic regression models were $\boldsymbol{Y = B_0 + B_1(DOSE) + B_2\ (DOSE^2)}$ and $\boldsymbol{Y = B_0 + B_1(DOSE) + B_2(DOSE^2) + B_3(DOSE^3)}$**, respectively,** where B_0 was the intercept, B_1 was the linear coefficient, B_2 was the quadratic coefficient, and B_3 was the cubic coefficient. Collectively, the regression coefficients defined a function/relationship that allowed the outcome variable means to vary in a linear or nonlinear fashion depending on dose.

Curve estimation was performed and partial R^2 values were calculated using SPSS (version 18.0; IBM Corporation; Somers, NY) after the data were graphed, a 48 segment cubic spline (i.e., a smooth line/curve through the set of data points) was fit to the data to visualize

trends, and outliers were identified and removed via the polynomial regression equation that best fit the cubic spline using GraphPad Prism (version 5.03, GraphPad Software, La Jolla, CA). Lastly, gain in prediction *F*-tests were used to evaluate whether or not there was a significant increase in the increment of prediction from a linear, a quadratic, or a cubic model to a full model with 5 terms (i.e., G [the number of groups] -1 = 5). The software DataFit was used to estimate the model with 5 terms (i.e., $\boldsymbol{Y = B_0 + B_1(DOSE) + B_2(DOSE^2) + B_3(DOSE^3) + B_4(DOSE^4) + B_5(DOSE^5)}$; version 9, Oakdale Engineering, Oakdale, PA) (see Cohen et al., 2003).

Results

Peripheral Markers of Estrogenic Action

After Ovx, but before CEE administration, vaginal smears showed that all animals were in diestrus, indicating a lack of estrogenic stimulation (Goldman et al., 2007). After pump insertion, all Ovx-Veh rats exhibited continuous diestrus smears with few cells, and all Ovx-E2 and Ovx-CEE treated rats, regardless of dose, exhibited vaginal smears with many cornified cells. The presence of many cornified cells indicated estrogenic stimulation of uterine/vaginal tissues (Goldman et al., 2007). An evaluation of uterine weights in the Ovx-Veh and all Ovx-E2 groups as well as the Ovx-Veh and all Ovx-CEE groups revealed a significant treatment main effect for both analyses (E2 $F[5,13] = 4.07$, $p = 0.0192$; CEE ($F[5,15] =$

24.88, $p < 0.0001$). Post-hoc analyses revealed that each E2 and CEE treated group had higher uterine weights when compared to the Ovx-Veh group (ps < 0.0305, Figure 19). Collectively, these peripheral markers of estrogenic action indicate that Ovx-Veh animals likely had little to no circulating estrogen present, while all Ovx-E2 and Ovx-CEE treated animals had hormone on board at the initiation of treatment through sacrifice.

Linear and Nonlinear Regression: Dose Response Effects of E2 and CEE on Working and Recent Memory

Ovx-Veh and E2 treated animals.

I used a regression model to describe the form of the relationship between *E2 dose and repeat errors committed during the baseline learning phase* on the DMS Plus Maze (i.e., trials 2-6 across days 1-5). I found a significant omnibus F statistic for the linear regression model (F[1, 22] = 5.19, $p = 0.0328$). This indicated that a straight line described the relationship between E2 dose and repeat errors committed during the baseline learning phase, an effect that was in line with my previous research (Talboom et al., 2008). The partial R^2 statistic from the linear model was 0.19, suggesting that the linear relationship explained 19% of the variation in repeat errors. The magnitude of the partial R^2 statistic was considered a medium effect by convention (see Cohen, 1988). The intercept and linear coefficients from the regression model were $B_0 = 2.71$

and B_1 = -0.58 respectively. Collectively, these coefficients describe the regression line in Figure 20a where repeat working/recent errors decreased in a linear fashion as the tonic dose of E2 increased. It is important to note that the quadratic regression model was significant (F[2, 21] = 4.54, p = 0.0229, partial R^2 = 0.14); however, a nonsignificant gain in prediction F-test (F[4,18] = 0.81, p = 0.5833) suggested that the full model with 5 terms did not enhance the increment in prediction over and above the linear model. This indicated that the linear model fit the data appropriately.

The regression model evaluating the relationship between *E2 dose and repeat errors committed across all test days* (i.e., overall performance) on the DMS Plus Maze (i.e., trials 2-6 across days 1-7), revealed a significant omnibus F statistic for the linear regression model (F[1, 22] = 6.32, p = 0.0197, Figure 20b), indicating a straight line described the relationship between tonic E2 dose and repeat errors committed across all test days and trials. The partial R^2 statistic was 0.22 and considered a medium effect by convention. The intercept and linear coefficients from the regression model were B_0 = 3.14 *and* B_1 = -0.68, respectively. Here again the quadratic regression model was significant (F[2, 21] = 3.76, p = 0.0403, partial R^2 = 0.05), but the gain in prediction F-test was not significant (F[4,18] = 0.65, p = 0.6561), indicating that the linear model was an appropriate fit to the data.

Collectively, these two models suggest that the tonic dose of E2 (within the current dose range tested) had a negative linear relationship with repeat working/recent memory errors committed during the learning phase and across all days on the DMS Plus Maze. Specifically, increasing from 0µg/day of E2 (i.e., Ovx-Veh rats) to 3.75µg/day of E2, the higher the dose, the better an animal's working/recent memory performance was. Nonsignificant regression models were found when attempting to describe the form of the relationship between E2 dose and total errors committed during the baseline learning phase or total errors for overall performance.

Ovx-Veh and CEE treated animals.

When describing the relationship between *CEE dose and total errors committed across all test days* on the DMS Plus Maze (i.e., overall performance, trials 2-6 across days 1-7), the linear regression model was not significant ($F[1,19] = 1.72$, $p = 0.2048$, partial $R^2 = 0.08$), which indicated that a linear model did not accurately fit the data. Although my previous research on CEE and one of its component estrogens did not lead me to expect a linear relationship (Chapter 4; Talboom et al., 2010), the linear model provided a baseline for comparing a non-linear dose-error curve. Next, I fit two polynomial regression models: one that included a linear and a quadratic term (i.e., dose & dose squared), and another that included a linear, a quadratic, and a cubic term (i.e., dose, dose squared, & dose cubed); these terms served as explanatory variables. The

quadratic regression model was significant (F[2,18] = 5.51, p = 0.0136, partial R^2 = 0.32); however, the cubic regression model was also significant (F[3,17] = 5.44, p = 0.0083), along with the gain in prediction F-test for the full model with 5 predictors against the quadratic model (F[3,15] = 3.39, p = 0.0379), indicating that the cubic and not the quadratic model was a more appropriate fit to the data. An evaluation of the regression coefficient from the cubic model revealed that the cubic coefficient was not significant (B_3 = 0.000, t = 1.92, p = 0.0720, Figure 20c). Interestingly, the quadratic coefficient was significant (B_2 = 0.008, t = 2.93, p = 0.0089), collectively suggesting that the relationship between CEE dose and total working/recent errors committed during testing did not have extensive cubic but did have substantial quadratic curvature. The partial R^2 statistic from the cubic model was 0.18, suggesting that the cubic relationship explained 18% of the variation in total errors; this was considered a medium effect by convention (Cohen, 1988). The intercept, linear, and quadratic coefficients from the cubic model were B_0 = 16.36, B_1 = 0.20, and B_2 = -0.19, respectively. I would be remiss if I did not note the possibility of a 4th or higher order model being able to describe this relationship; however, the gain in prediction F-test from the cubic model to the full model with 5 predictors was not significant (F[2,15] = 2.85, p = 0.0649), indicating that the cubic model was appropriate, not a model containing additional terms (i.e., 4th order).

An evaluation of the relationship between *CEE dose and repeat errors committed during the baseline learning phase* on the DMS Plus Maze (i.e., trials 2-6 across days 1-5), revealed a nonsignificant omnibus F statistic for the linear model ($F[1,19] = 0.67$, $p = 0.4214$, partial $R^2 = 0.03$). However, the quadratic regression model was significant ($F[2,18] = 8.30$, $p = 0.0028$, Figure 20d), as was the test for the quadratic coefficient ($B_2 = 0.003$, $t = 3.93$, $p = 0.0009$), indicating that the relationship between dose and repeat working/recent errors committed during the baseline learning phase had substantial curvature. The partial R^2 for the quadratic model was 0.46 and considered a large effect by convention. The intercept and linear coefficients from the regression model were $B_0 = 3.43$ and $B_1 = -0.20$ respectively. The cubic regression model was significant ($F[3,17] = 5.95$, $p = 0.0057$, partial $R^2 = 0.06$), but the gain in prediction F-test was not significant for the full model ($F[3,15] = 1.62$, $p = 0.2217$), suggesting that a quadratic model fit the data appropriately.

Taken together, these two models suggest that tonic CEE dose had a curvilinear or U-shaped relationship with total working/recent memory errors committed across all days and repeat working/recent memory errors committed during the learning phase. Specifically, the no 0µg/day (Ovx-Veh), low 6µg/day, high 60µg/day, and high 72µg/day dose of CEE had more errors relative to the middle 36µg/day dose of CEE. Nonsignificant regression models were found when attempting to describe

the form of the relationship between CEE dose and total errors committed during the baseline learning phase or repeat errors for overall performance. Table 3 summarizes the form of the relationship between each composite dependent variable and E2 or CEE dose with Ovx-Veh animals serving as a 0μg/day dose.

Discussion

To the author's knowledge, the present study is the first to evaluate more than three doses of E2 and CEE on cognition in a single study. In middle-aged surgically menopausal rats, the peripheral markers of estrogenic action suggested that Ovx ablated the endogenous source of estrogens before experimental exogenous treatment began, and that all Ovx-E2 and Ovx-CEE, but not Ovx-Veh treated animals, had hormone on board for the duration of the study. The dose response results suggest that as the tonic dose of E2 increased from 0μg/day in Ovx-Veh animals to 3.75μg/day in E2 high dose animals, the number of repeat working/recent memory errors decreased in a linear fashion during learning (i.e., days 1-5) and across all days (i.e., 1-7). In CEE treated animals, as the tonic dose of CEE increased from 0μg/day in Ovx-Veh animals to 72μg/day in the CEE high group, the number of total working/recent memory errors committed across all test days followed a curvilinear cubic relationship. A quadratic U-shaped relationship in CEE treated animals was found for repeat working/recent memory errors committed during the learning phase

(i.e., days 1-5), with the optimal middle dose being approximately 36µg/day (Table 3).

I did not find my hypothesized U-shaped relationship with working/recent memory in E2-treated animals. This is likely due to my use of lower tonic doses of E2 in comparison to most reports using 10µg and above (see Dohanich, 2002). I specifically chose lower tonic E2 doses in this study because my lab and I have found that higher long-term (i.e., ≥ 28 days) tonic doses of E2 administered via Alzet® pumps (Durect Corporation, Cupertino, California) or E2 pellets (Innovative Research of America, Sarasota, Florida) without the coadministration of progesterone caused health problems and increased mortality in my lab's previous studies (Chapter 4; Bimonte-Nelson et al., 2006). Still, these present E2 linear relationships with spatial working/recent memory corroborate previous findings whereby E2 benefited spatial WM in Ovx rats (Chapter 4; Bimonte and Denenberg, 1999; Daniel et al., 1997; Daniel et al., 2005; Gibbs, 1999; Gibbs, 2000b; Gibbs, 2002; Gibbs, 2007; Gibbs and Johnson, 2008; Luine et al., 1998a; Rodgers et al., 2010). Interestingly, my results here evaluating working/recent memory on the DMS Plus Maze correspond with my prior findings testing RM on the Morris maze, indicating that this may relationship may exist within other memory domains (Talboom et al., 2008). Indeed, higher levels of circulating E2 were linearly related to better spatial RM performance.

Linear relationships between E2 and memory have been noted in several other reports. Asthana and colleagues (1999) found that plasma E2 levels, after exogenous E2 treatment via a transdermal patch, were positively related in a linear fashion to verbal memory in aged women diagnosed with probable AD. Others have also noted linear relationships between semantic and verbal memory and E2 in middle-aged women (Phillips and Sherwin, 1992; Ryan et al., 2010); however, it is important to note that other studies have not found these relationships in aged women (e.g., Almeida et al., 2005; Barrett-Connor and Goodman-Gruen, 1999). Interestingly, my previous study also noted that linear relationships between E2 and memory were present in young and middle-aged, but not aged rats (Talboom et al., 2008), suggesting that age may attenuate, mask, or abolish relationships between E2 and memory in both rodent and human females. Collectively, my data suggest that E2 has a positive linear relationship with regard to working/recent memory performance (i.e., higher doses had fewer errors and better performance), and this relationship is supported in part by other reports in both the human and rat literature.

The current curvilinear/U-shaped relationship between CEE dose and spatial working/recent memory is in part corroborated by previous findings where higher doses of CEE, or a separate component estrogen of CEE, Δ^8E1 (still below 60µg/day), benefited spatial working and/or recent

memory in Ovx rats (Chapter 4; Acosta et al., 2009b; Engler-Chiurazzi et al., 2009; Talboom et al., 2010). Specifically, in the current study, CEE dose exhibited a U-shaped relationship with total errors across all days (i.e., 1-7) and repeat working/recent memory errors during learning (i.e., 1-5), where previous studies evaluating CEE's effects on the DMS Plus Maze noted CEE- facilitated enhancements to working/recent memory assessed via total errors committed during the learning phase (i.e., days 1-5) (Chapter 4; Acosta et al., 2009b). Of particular interest is the fact that my previous studies suggested a U-shaped or linear relationship between CEE/Δ^8E1and WM errors, since lower doses had no effect or impaired performance, and the ≈ 36μg dose enhanced performance relative to Ovx animals (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009; Talboom et al., 2010). I must note that there is some disparity between the error types and day blocks in which I noted CEE effects on working/recent memory between the current study and lab's previous reports. Specifically, in my current study I did not find a relationship between CEE dose and total errors committed across the learning phase. However, the design and conducted analyses between studies were conceptualized differently since I set out to describe relationships via multiple regression in the current study (using more groups with fewer subjects), while previous studies were designed to evaluate pairwise comparisons via ANOVA (using fewer groups with more subjects). Nonetheless, even with this caveat in mind,

the current results are in line with previous reports whereby CEE can be beneficial to working and/or recent memory with the appropriate treatment dose.

While the gain in prediction test indicated that a quadratic model was not an ideal model to describe the relationship between CEE dose and total working/recent memory errors committed during all days of testing (i.e., Days 1-7 Trials 1-6), the medium to large effect size (i.e., quadratic partial R^2 = 0.32) and the quadratic coefficient (i.e., B_2 = 0.008) suggest that the model was a decent fit. In fact, the quadratic model, in respect to the partial R^2 and quadratic curvature, appeared to be a better fit than the cubic model (i.e., cubic partial R^2 = 0.18 & nonsignificant cubic term). Collectively, this suggests that some interpretations can be made from the quadratic model in this instance and that another model aside from cubic should be used to evaluate these data. With that in mind, the U-shaped relationship between CEE dose and working/recent memory is particularly exciting because it identifies the 36µg/day as the optimal dose for working/recent memory performance. The U-shaped relationship also suggests that CEE's effects on memory systems may follow the Yerkes-Dodson law (Yerkes and Dodson, 1908). The Yerkes-Dodson law states that low and high levels of arousal are detrimental to performance but a midlevel of arousal is beneficial, outlining the quadratic or inverted-U shaped relationship (Yerkes and Dodson, 1908). Of interest is the fact that

the Yerkes-Dodson law seems to explain relationships between memory performance and circulating levels of glucocorticoids (i.e., cortisol, a stress hormone) in humans as well as glucocorticoid levels (i.e., corticosterone) and LTP in rats (i.e., a hypothesized neurobiological underpinning of memory) (for review see Lupien et al., 2007). Together, this adds validity to my CEE findings, since other hormone systems also demonstrate a U-shaped relationship with regard to memory.

It is not surprising that animals receiving the 6μg/day dose exhibited more errors in relation to the 36μg/day dose; CEE is comprised of ≈ 50% E1 which has a lower binding affinity to the ERα and ERβ (Kuhl, 2005; Sitruk-Ware, 2002). The reason for the 12μg/day, 60μg/day, and 72μg/day doses of CEE increasing the number of errors in relation to the 36μg/day dose may be due in part to the rat pharmacokinetics of CEE, specifically, in the circulating ratio of E1 to E2. My lab has noted that a 12μg/day dose of CEE impaired WM and RM and produced serum levels of E1 that differed from Ovx animals, but serum E2 levels that did not differ from Ovx animals (Engler-Chiurazzi et al., 2009). In this study serum E1, and the ratio of serum E1 to E2, also correlated with WM performance (Engler-Chiurazzi et al., 2009). This suggests a possibility that the current 12μg/day, 60μg/day, and 72μg/day CEE doses may have produced high levels of E1 in the presence of low levels of E2, causing decrements in WM performance that may be related to weaker estrogens such as E1

acting as a competitive ligand to E2 and its effects. Collectively, the quadratic U-shaped relationship between CEE and memory is not surprising since quadratic relationships exist between other hormones and memory. Furthermore, the CEE data indicated that the 36μg/day is optimal for working/recent memory where other doses are not, perhaps due to weaker estrogenic action and the pharmacokinetics of CEE producing higher levels of E1 and lower levels of E2.

Higher doses of E2 and the 36μg/day dose CEE likely facilitated better performance on the DMS Plus Maze by predisposing animals to use a place strategy (allocentric) as opposed a response strategy (egocentric) to locate the escape platform (Korol and Kolo, 2002). In my DMS Plus Maze task, animals using a response strategy (i.e., turn left or turn right) would have been inefficient at locating the platform. I speculate that the use of a place strategy allowed higher dose Ovx-E2 and medium dose Ovx-CEE animals to accumulate fewer errors by creating a more stable cognitive map, in part by having a better ability to hold the spatial location of the platform in WM (Barnes et al., 1997; Wilson et al., 2004). Indeed, this cognitive map was likely successively enhanced as the tonic dose of E2 increased, and was therefore maximally enhanced in CEE treated animals at the tonic 36 μg/day dose. WM and RM performance is reliant on integrity of the hippocampus and surrounding cortices, including the entorhinal cortex (for review see Morris et al., 1982; Zola-Morgan et al.,

1994), and WM is also reliant on the prefrontal cortex (for review see Jones, 2002). The hippocampus, entorhinal cortex, and prefrontal cortex receive cholinergic innervation from the BF, which supports memory function (Hasselmo, 2006; Henny and Jones, 2008). E2 potentiates increases in hippocampal ACh levels during maze learning relative to vehicle treatment in Ovx rats (Marriott and Korol, 2003), and E2-induced memory enhancements are present in animals with intact BF cholinergic neurons, but not in animals with BF cholinergic lesions (Gibbs, 2002). Treatment with E2 and CEE increases the number of ChAT-IR positive neurons in the BF (Acosta et al., 2009b; Gibbs, 1997), and E2 and CEE treatment also protect against muscarinic challenges (i.e., scopolamine-induced amnesia) in rats (Acosta et al., 2009b; Savonenko and Markowska, 2003). Similarly, the dose response relationship between E2 and CEE could reflect enhanced cholinergic neuronal function in the BF and elevated ACh tone in the hippocampus.

In conclusion, Ovx abolished or decreased peripheral markers of estrogenic action, while E2 and CEE treatment increased peripheral markers, suggesting that Ovx surgery and hormone treatments were successful. My dose response results suggested that as the tonic dose of E2 increased, the number of repeat working/recent errors decreased in a linear fashion. In CEE treated animals, as the tonic dose of CEE increased, the number of total and repeat working/recent errors followed a

quadratic U-shaped relationship, identifying the 36μg/day dose as optimal for performance. Future studies could expand the dose range of E2 in order to elucidate a possible U-shaped relationship with regard to WM performance as well as evaluate an alternative to the cubic model. Another future direction is to look for these same linear and quadratic relationships after E2 and CEE treatment in measures assessing the biological underpinnings of memory. For example, future studies could assess the dose response effects of E2 and CEE on BF and hippocampal ACh levels as well as hippocampal and prefrontal cortex LTP. It is my hope that these findings will aid in the planning of future evaluations of the effects of these and other hormones on memory, ultimately helping to elucidate the many parameters that dictate whether HT is detrimental or beneficial to the ever expanding aging population.

CHAPTER 6

A COMPONENT OF PREMARIN® ENHANCES MULTIPLE COGNITIVE FUNCTIONS AND INFLUENCES NICOTINIC RECEPTOR EXPRESSION

CEE contains the sulfates of more than 10 estrogens, is over 50% E1, 20-25% equilin, 3.5% Δ^8E1, and contains only trace amounts of E2 (the most potent naturally circulating estrogen in women and rats); after metabolism, the resulting biologically active circulating hormones are primarily E1 and equilin, and after E1's conversion, E2 (Bhavnani, 2003; Bhavnani et al., 1998; Mayer et al., 2008; Sitruk-Ware, 2002). In the last decade, landmark basic science research from the Brinton laboratory has led to several discoveries regarding the neuroprotective properties of estrogens. This work demonstrated that some components of CEE enhanced markers of neuroprotection, while others showed little benefit (Brinton et al., 1997; Zhao and Brinton, 2006). The estrogens Δ^8E1 and equilin, found naturally in horses but not in women or rats, were the two primary CEE components that showed the most consistent and potent neuroprotective effects in vitro (Zhao and Brinton, 2006). Δ^8E1- and equilin-facilitated neuroprotection in vitro could potentially translate to improved function of neural networks and brain regions directing cognitive function, resulting in memory enhancements in vivo.

BF cholinergic neurons project to the hippocampus and surrounding cortical areas, and play an important role in learning and

memory (Hasselmo, 2006). E2 enhances BF cholinergic function, as evidenced by expression of different cholinergic markers and pharmacological cholinergic challenges (e.g., Gibbs, 2000a; Markowska and Savonenko, 2002a; Packard and Teather, 1997). In fact, ChAT increases in the BF months after transient exposure to E2, noting effects are sensitive to several variables including timing and dose (Bohacek et al., 2008; Gibbs, 1997; Rodgers et al., 2010). My laboratory has demonstrated CEE-induced benefits to the cholinergic system, whereby CEE treatment prevented scopolamine-induced amnesia and increased number of ChAT positive neurons in the vertical diagonal band of the BF in Ovx rats (Acosta et al., 2009b). Cholinergic signaling is directed by muscarinic mAChR and nAChR, which are present in brain regions known to be responsible for memory processing (Clarke et al., 1985; Court and Clementi, 1995; Tice et al., 1996; Vaucher et al., 2002) and are implicated in memory processing (Hasselmo, 2006; Konopacki et al., 1992; Rouse et al., 1999). Nicotine administration improves memory in humans (Buccafusco et al., 2005), and my lab and others have noted nicotine-induced memory enhancement in rats across multiple memory domains (Arendash et al., 1995; French et al., 2006; Riekkinen and Riekkinen, 1997; Socci et al., 1995). E2 and ethinyl β-estradiol bind to the α4β2-nAChR, the most abundant nAChR subtype in the brain, and directly potentiate the function of human α4β2-nAChRs (hα4β2-nAChR); ethinyl β-

estradiol, but not E2, potentiates rat α4β2-nAChRs (rα4β2-nAChR) (Curtis et al., 2002; Paradiso et al., 2001). Recently, in vivo research has demonstrated that nicotine co-administered with estradiol potentiates visual spatial memory in Ovx rats, beyond that of either compound administered alone (Taylor and Maloney, 2010). Collectively, these studies suggest a link between estrogens, nAChRs, and memory.

Numerous studies have demonstrated that E2 benefits spatial working and RM in young Ovx rats (Bimonte and Denenberg, 1999; Daniel et al., 1997; Daniel et al., 2005; El-Bakri et al., 2004; Fader et al., 1998; Feng et al., 2004; Gibbs, 2007; Hruska and Dohanich, 2007; Korol and Kolo, 2002; Luine and Rodriguez, 1994), as well as in middle-aged Ovx rats (Bimonte-Nelson et al., 2006; Foster et al., 2003; Markham et al., 2002; Talboom et al., 2008). CEE can also benefit cognition in middle-aged Ovx rats, although these effects are dose and task specific (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009; Walf and Frye, 2008). The current study utilized a similar model, the middle-aged Ovx rat, to test the impact of Δ^8E1 and equilin on memory and rα4β2-nAChR expression and function. I used a battery of water-escape mazes previously shown to be influenced by age as well as ovarian hormone loss and estrogen treatment (Acosta et al., 2009a; Acosta et al., 2009b; Bimonte-Nelson et al., 2006; Engler-Chiurazzi et al., 2009; Talboom et al., 2008). These mazes evaluate WM, which is information that needs to be updated and is

pertinent for a short time, and RM, which is information that remains constant over time (see Jarrard et al., 1984; Jones, 2002). ^{125}I-labeled epibatidine (I-epi) radioligand binding assays were used to evaluate rα4β2-nAChR expression levels in the hippocampus and entorhinal cortex, and cell culture was used to evaluate whether Δ^8E1 and equilin directly altered hα4β2-nAChR function via $^{86}Rb^+$ efflux experiments. Lastly, several peripheral markers routinely noted to change with E2 treatment, including serum luteinizing hormone (LH) levels, vaginal smears, and uterine weights, were assessed.

Methods

Subjects

Subjects were 50 middle-aged (12-13 month old) Fischer-344 female rats born and raised at the National Institute on Aging colony at Harlan Laboratories (Indianapolis, IN). Animals were acclimated for several weeks to the vivarium at Arizona State University, had exposure to food and water ad-lib, and were maintained on a 12-h light/dark cycle at 23 °C. Procedures were approved by Arizona State University IACUC and adhered to the Guide for the Care and Use of Laboratory Animals and NIH standards.

Hormone Manipulation

Ovx, group assignment and hormone dosing.

Thirty days after arrival, all rats received Ovx under isoflurane anesthesia. Dorsolateral incisions were made in the skin and peritoneum, and ovaries and tips of uterine horns were ligated and removed. Rats were randomly assigned into a control group receiving vehicle only or groups receiving one of three doses of hormone delivered via osmotic mini pump. Specifically, the groups were: Ovx plus vehicle (Ovx-Veh, n=8), Ovx plus 2.6 µg/day of Δ^8E1 (Ovx-Δ^8E1-Low, n=7), Ovx plus 17.5 µg/day of Δ^8E1 (Ovx-Δ^8E1-Med, n=7), Ovx plus 35 µg/day of Δ^8E1 (Ovx-Δ^8E1-High, n=7), Ovx plus 2.6 µg/day of equilin (Ovx-Equilin-Low, n=7), Ovx plus 6.25 µg/day of equilin (Ovx-Equilin-Med, n=7), and Ovx plus 12.5 µg/day of equilin (Ovx-Equilin-High, n=7). All hormones were purchased from Steraloids Inc. (Newport, RI). Low doses of Δ^8E1 and equilin were derived from studies demonstrating cognitive enhancement using 3.6 µg of CEE. Doses were adjusted to account for Δ^8E1 and equilin being in its unconjugated form[1], and to approximate the rat body weight equivalent of the 0.625 mg dose, which was used in the WHIMS and is what women are most commonly prescribed (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009). The high doses for Δ^8E1 and equilin were based on the only other available reports in the rat model using these hormones, wherein Δ^8E1 decreased depolarization-induced cardiac synapse norepinephrine release, and equilin elevated vascular reactivity in the mesenteric vascular

bed (Eskin et al., 2003; Mark et al., 2007). The medium doses of Δ^8E1 and equilin were half of the high doses.

[1] The 20-36 µg of CEE powder that my lab has shown to enhance cognition was actually 2.0-3.6 µg of estrogens, respectively, since it was ≈ 10% hormone and ≈ 90% filler (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009). CEE is a mixture of estrogens conjugated to sulfate; the hormones must be deconjugated by the liver to become bioactive (Bhavnani et al., 1998). I used bioactive, unconjugated Δ^8E1 and equilin in this study. To attain an approximately equivalent amount of estrogen molecules between my current Δ^8E1 and equilin low dose, and my previous CEE dose, I had to account for the fact that estrogens in CEE are conjugated, and conjugated estrogens weigh more. Since the molecular weights of Δ^8E1 sulfate and equilin sulfate (the conjugated estrogens) are each 370.08 (as monosodium salts), while that of Δ^8E1 and equilin (as unconjugated estrogens) are each 268.35, the final concentrations for the low doses were reduced by 28% (i.e., 3.6 µg/day - 28% ≈ 2.6 µg/day).

Hormone treatment procedure.

Hormone treatment began approximately 16 days after Ovx (Bimonte-Nelson et al., 2006), 12 days before behavior testing began. Vehicle only (PEG, average molecular weight of 300, Sigma-Aldrich, St. Louis, Missouri), or Δ^8E1 or equilin in vehicle, was administered via Alzet® pumps (Model 2004; Durect Corporation, Cupertino, California). The concentration of Δ^8E1 or equilin, suspended in PEG, in the pumps was calculated to deliver the low, medium or high dose for each hormone per day. Pumps were prepared the day before surgery and allowed to equilibrate in physiological saline at 37 °C overnight. For pump insertion, animals were anesthetized under vaporized isoflurane anesthesia, an incision was made in the dorsal scruff of the neck, the pump was inserted, and the skin was stapled. Pumps remained in the subjects throughout

behavioral testing and until sacrifice (approximately 4 weeks), which provided consistent exposure to their assigned treatment for the duration of behavioral testing, through sacrifice.

Behavior Testing

DMS Plus Maze (spatial working and recent memory).

I used the DMS Plus Maze task to test spatial working and recent memory. The protocol was based on studies showing E2- and CEE-induced improvements in Ovx rats (Acosta et al., 2009b; Gibbs, 2000b; Korol and Kolo, 2002; Sandstrom and Williams, 2004). The black Plexiglass maze (each arm was 38.1cm x 12.7cm) was filled with water made opaque with black non-toxic paint. The submerged platform was hidden at the end of one of the four arms. The start location varied across trials, and the platform location stayed in the same arm within a day, but semi-randomly changed arms across days. This a place learning task, whereby animals must learn to navigate to a hidden escape platform by using distal spatial cues (Restle, 1957). Since the platform changed position relative to space each day, this task is differentiated from other match-to-sample or match-to-position tasks where animals solve the task using response learning (i.e., making the same turn regardless of the spatial location) (Restle, 1957). Rats received 6 consecutive trials within a daily session, for 7 days, and were given 90 seconds to locate the platform. Once on the platform, the rat remained on it for 15 seconds,

followed by placement into a heated cage for a 30s 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 any non-platformed arm was counted as an error, and fewer errors are indicative of better performance. At the start of an animal's daily testing session, it had no information regarding the platform location. Hence, on trial 1, the rat swam until it located the platform. The rat then needed to remember this platform location and return to it on subsequent trials. WM performance was evaluated by assessing the ability to navigate to the new platform location on trial 2. Hence, trial 1 was the information trial, trial 2 was the WM test trial, and trials 3-6 six were recent memory test trials (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009). The dependent variables were the number of initial and repeat errors committed during trial 2 (as a measure of WM) or trials 3-6 (as a measure of recent memory). Day 1 was considered the training day, as no prior information regarding task demands or platform location was available to the animals. My lab routinely treats day 1 as a training day on WM tasks (Acosta et al., 2009a; Bimonte-Nelson et al., 2003a; Bimonte-Nelson et al., 2003b; Bimonte-Nelson et al., 2004b; Bimonte and Denenberg, 1999; Bimonte et al., 2003; Huentelman et al., 2009).

Morris maze (spatial RM).

Using a previously published protocol (Markham et al., 2002), three days after DMS Plus Maze testing concluded, I tested spatial RM via the Morris maze, as I and others have shown enhancements due to E2 treatment in Ovx rats on this task (Bimonte-Nelson et al., 2006; Markham et al., 2002; Talboom et al., 2008). Similar to the DMS Plus Maze, the Morris maze task assesses place learning; however, in the Morris maze the platform remains in the same spatial location for all days and trials, making it a spatial RM task. This can be contrasted with the DMS Plus Maze, which is a spatial WM task since the platform is moved to a new spatial location every day. The Morris maze (Morris et al., 1982) consisted of a round tub (188 cm in diameter) filled with water made opaque with black, non-toxic paint. The rat was placed in the maze from any of four locations (North, South, East, or West) and had 60 seconds to locate a submerged hidden escape platform which remained in a fixed location (NE quadrant) throughout testing. After 15 seconds on the platform, the rat was placed into its heated cage until the next trial; the inter-trial-interval was 5-8 minutes. For each rat, the testing session consisted of 6 trials/day for 3 days. A video camera recorded each rat, and a tracking system (Ethovision XT 5.1, Noldus Information Technology, Wageningen, Netherlands) analyzed each rat's path. The dependent measure was swim distance (cm), with less swim distance interpreted as better spatial RM performance. To assess possible differences in the learning rates among

my treatment groups, data were collapsed into 3-trial blocks so that Treatment x Block interactions could be tested. To assess platform localization, a probe trial was given on an additional trial (trial 7) on the last day of testing, whereby the platform was removed from the maze. For the probe trial, percent of total swim distance (cm) in the target NE quadrant (i.e., quadrant that contained the platform on the test trials) as compared the opposite SW quadrant, was the dependent measure.

Visible Platform (motoric and visual competence).

One day after Morris maze testing, I evaluated motoric and visual competence using the visible platform task. A rectangular tub (39 x 23 in) was filled with clear room temperature water. A black platform (10 cm wide) was positioned 1.5” above the water surface following previously published methods (Hunter et al., 2003). Opaque curtains covered extramaze cues. Animals were given 6 trials in 1 day. The drop off location remained the same across trials, and the platform location for each trial varied semi-randomly. Each rat had 90 seconds to locate the platform, where it remained for 15 seconds before being placed back into its heated cage awaiting the next trial. The inter-trial-interval was 5-8 minutes. Latency (seconds) to reach the platform was the dependent measure.

Evaluation of Vaginal Smears, Uteri, and Serum LH

I evaluated the effects of Δ^8E1 or equilin treatments on several peripheral markers that are routinely noted to change with E2 treatment.

To confirm Ovx as well as Δ^8E1 and equilin treatments, vaginal smears were taken prior to Ovx and pump surgery, immediately before behavior testing, and the day before sacrifice. Vaginal cytology via smears was classified as proestrus, estrous, metestrus or diestrus (Goldman et al., 2007). Final vaginal smears were stained with eosin Y and hematoxylin (Sigma) for further brightfield microscopic examination. At sacrifice, animals were anesthetized with isoflurane. I confirmed complete Ovx via visual inspection of uterine horns, and I evaluated uterine weights as conducted in previous studies (Acosta et al., 2009b). A ventral incision was made in the abdominal region, and the uterus was cut above the junction with the cervix and on the uterine horn below the ligature remaining from Ovx (Ashby et al., 1997b). Uteri were trimmed of all visible fat and were immediately weighed to obtain wet weight, which was the dependent measure. Also, at this time, blood was collected via cardiocentesis. Five subjects per treatment group were randomly chosen to obtain serum LH levels. LH was determined at the Core Endocrine Laboratory at Pennsylvania State University College of Medicine by competitive radioimmunoassay using reagents obtained from ALPCO Diagnostics (Salem, NH), based on prior protocols (Roman et al., 2003). The assay uses ^{125}I-labeled rat LH tracer for binding to a highly specific rabbit polyclonal antibody and separation of bound from free trace with an anti-rabbit IgG precipitating antibody. Results are expressed in ng/ml.

Lower limit of quantification for the assay is 0.5 ng/ml and within run and between run accuracy averaged 7% and 9% respectively at a concentration of 4.2 ng/ml.

Brain Dissection and nAChR Agonist Binding

Using radioligand binding assays, I investigated whether treatment with Δ^8E1 or equilin affected rα4β2-nAChR expression levels. I-epi binding was used to estimate number of β2*- and β4*-nAChRs in the hippocampus and entorhinal cortex (Whiteaker et al., 2000; Whiteaker et al., 2008). At sacrifice, brains were rapidly dissected; one subject was excluded due to technical error. Each brain was cut on the coronal plane. Next, the entorhinal cortex (taking a 2- to 3- mm sample ventral to the hippocampus) and the CA1/CA2 region of the hippocampus (dentate gyrus and alveus excluded) were dissected. Dissected tissues were immediately placed in microcentrifuge tubes, frozen on dry ice, and stored at -70 °C until analysis. Hippocampal and entorhinal cortex tissues were separately homogenized in ice-cold 1x Ringer's buffer with sodium azide (in mM : 144 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 50 Tris-base; and 0.02 % (w/v) NaN_3; pH 7.4; binding buffer). Homogenates were washed three times by centrifugation (12,000 x g; 15 minutes; 4 °C) and rehomogenized into 1x binding buffer and stored, pelleted, at 4 °C. I-epi binding was assessed at 1 nM. Non-specific binding was assessed in the presence of 300 mM carbamylcholine. Specific binding was defined as the difference

between total and non-specific binding as fmol per mg of protein; this served as the dependent variable for statistical analyses. Since spatial memory processing is dependent on both the hippocampus and entorhinal cortex, and the functioning of these regions is intimately linked (for review see Jarrard, 1993; Zola-Morgan et al., 1994), specific I-epi binding data in the hippocampus and entorhinal cortex were analyzed together creating a single measure; the combined hippocampal and entorhinal cortex data will now be referred to as the hippocampus+entorhinal cortex. In the hippocampus/entorhinal cortex the contribution of β4*-nAChR was minimal (data not shown). Accordingly, I-epi binding populations in the hippocampus/entorhinal cortex are considered to represent β2 subunit-containing nAChR, likely α4β2-nAChR (Whiteaker et al., 2000), as such, specific I-epi binding will be referred to as rα4β2-nAChR expression.

$^{86}Rb^{+}$ Efflux For Δ^{8}E1 and Equilin Effects on hα4β2-nAChR Function

Routine culture of SH-EP1 cells.

To ascertain whether hormone effects could be due to direct actions on receptor function, I evaluated whether Δ^{8}E1 or equilin exposure directly affected function of heterologously expressed, hα4β2-nAChR assessed using $^{86}Rb^{+}$ efflux experiments. SH-EP1 cells stably expressing hα4β2-nAChR were maintained as previously described (Eaton et al., 2003). Briefly, cells were grown in Dulbecco's modified Eagle's medium (high glucose, bicarbonate-buffered, with 1 mM sodium pyruvate and 8

mM L-glutamine) supplemented with 10% horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (all from Invitrogen, Carlsbad, CA) plus 5% fetal bovine serum (Hyclone, Logan, UT) on 100-mm diameter plates in a humidified atmosphere containing 5% CO2 in air. Positive selection for the human α4 and β2 subunits (in pcDNA3.1/zeo and pcDNA3.1/Hygro, respectively; also from Invitrogen) was maintained by further supplementation of the growth medium with 0.25 mg/ml zeocin (Invitrogen), and 0.4 mg/ml hygromycin B (Calbiochem, San Diego, CA). Cells were maintained at low passage numbers (1-26 from frozen stocks), and passaged weekly by splitting confluent cultures 1/20 – 1/40 to maintain cells in proliferative growth.

$^{86}Rb^{+}$ efflux assays in the presence of acute hormone.

Cells were harvested at confluence from 100-mm plates by mild trypsinization before being resuspended in growth medium and evenly seeded at a density of one confluent 100-mm plate per 24-well plate. Cells were allowed to adhere for a minimum of 6hrs. Medium was removed and replaced with 250 µl/well of medium supplemented with approximately 300,000 cpm of $^{86}Rb^{+}$ (PerkinElmer; counted at 40 % efficiency using Cerenkov counting, PerkinElmer Tricarb 1900 LSC). After 4hrs of loading, $^{86}Rb^{+}$ efflux was measured using the flip-plate technique (Lukas et al., 2002). Following aspiration of the bulk of $^{86}Rb^{+}$ loading medium from each well of the cell plate, each well containing cells was rinsed three times with

2 ml of fresh efflux buffer (130 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, and 50 mM HEPES, pH 7.4) to remove extracellular $^{86}Rb^{+}$. After removal of residual rinse buffer by aspiration, the flip-plate technique was used again to simultaneously introduce fresh efflux buffer containing drugs of choice at indicated final concentrations from a 24-well "efflux/drug plate" into the wells of the cell plate. In the case of the acute hormone effect experiments, the drugs used consisted of a test agonist (carbamylcholine at 100 µM) in the presence of Δ^8E1, equilin, and PEG (95 fM-1 µM), representing concentrations below, at, or above physiological relevant brain levels of E2 (see Woolley, 2007). After a 5m incubation, the solution was "flipped" back into the efflux/ drug plate, any remaining medium was removed by aspiration. Suspensions in each well were then subjected to Cerenkov counting (PerkinElmer Wallac Micobeta Trilux 1450; 25% efficiency) after placement of inserts (PerkinElmerWallac 1450-109) into each well to minimize cross-talk between wells. Specific $^{86}Rb^{+}$ efflux was calculated as efflux above the no-agonist control and expressed as a percentage of efflux evoked by a maximally-effective carbamylcholine (1 mM) control stimulation. Increases or decreases in specific agonist-induced efflux (i.e. efflux above non-specific levels) would be interpreted as direct, nAChR- facilitated, acute allosteric agonist or antagonist (respectively) effects. Intrinsic agonist activity of Δ^8E1, equilin or PEG was calculated in samples containing the same concentrations tested in the

presence of agonist. The amount of specific $^{86}Rb^{+}$ efflux, expressed as % of control, in response to log dilutions of Δ^{8}E1, equilin, and PEG served as the dependent measure of hα4β2-nAChR receptor function.

$^{86}Rb^{+}$ efflux assays after chronic hormone treatment.

Cell plating for chronic hormone studies were the same as described previously for assessment of acute hormone effects, with the exception that cells were seeded more thinly into the 24-well plates (one half of a confluent 100-mm plate per six 24-well plates), allowing them to be grown for 48hrs in the presence of either Δ^{8}E1 or equilin (9 aM - 1 µM) or 0.1% PEG and to reach confluence at the end of this period. Effects of chronic hormone exposure on $^{86}Rb^{+}$ efflux were assessed as previously described for measurement of acute effects with minor exceptions. Briefly, after the 48hr hormone pretreatment period, media containing the hormone was removed and replaced fresh media containing $^{86}Rb^{+}$ but devoid of hormone for the 4hr isotope loading period. Cells were rinsed free of extracellular isotope, and hormones at desired concentrations or 0.1% PEG (matching conditions used in the preincubation period) were then simultaneously introduced in fresh efflux buffer with or without carbamylcholine from a 24-well “efflux/drug plate” into the wells of the cell plate. After a 5m incubation, the solution was “flipped” back into the efflux/drug plate, any remaining medium was removed by aspiration. The dependent measure was identical to the acute experiment (see above).

Statistical Methods

For behavior assessments, I used StatView (version 5.01, The SAS Institute Inc., Cary, NC) to analyze data using an omnibus mixed model ANOVA with Treatment as the between variable, and Days, Block, and/or Trials as the within variable(s), as appropriate for the specific maze test. rα4β2-nAChR expression data were analyzed using an omnibus factorial ANOVA with Treatment and Brain region as between variables. Uterine weight and LH levels were analyzed using an omnibus one-way ANOVA with Treatment as between variable. In all ANOVAs conducted, each hormone type was analyzed separately, so each omnibus ANOVA included the Ovx-Veh group as well as the Low, Med, and High group of either Δ^8E1 or equilin. Since my interest was to determine whether each hormone dose enhanced performance relative to the Ovx-Veh group, all follow-up comparisons employed Fisher's PLSD post hoc tests when a significant omnibus ANOVA was found, noting that Type 1 error correction is not necessary with orthogonal planned comparisons (Keppel and Wickens, 2004). For efflux experiments, I used GraphPad Prism (version 5.03, GraphPad Software, La Jolla, CA) to perform multiple regression analyses that evaluated if the slope of the "line of best fit" differed from 0, followed by a Wald runs test for randomness to determine if the data deviated from linearity (Wald and Wolfowitz, 1940).

Results

DMS Plus Maze: Spatial Working and Recent Memory

Spatial WM was assessed using the win-stay DMS Plus Maze task. Analysis of repeat errors committed on trial 2 alone (the WM trial) for Δ^8E1 revealed a significant main effect of Treatment (F[3,25] = 3.02, p = 0.0405). The Ovx-Δ^8E1-Med and Ovx-Δ^8E1-High groups made fewer repeat errors on trial 2 as compared to the Ovx-Veh group (ps < 0.0107, Figure 21a). The analysis of repeat errors for trials 3-6 (recent memory trials) revealed a marginal Treatment main effect for Δ^8E1 (F[3,25] = 2.45, p = 0.0867). When all DMS test trials were combined (trials 2-6, working and recent memory trials) there was a Treatment main effect for Δ^8E1 (F[3,25] = 3.61, p = 0.0272). The Ovx-Δ^8E1-Med and Ovx-Δ^8E1-High groups made fewer repeat errors for all test trials combined as compared to the Ovx-Veh group (ps < 0.0107). There were no main effects or interactions for repeat errors for equilin (Figure 21b), nor were there effects for initial errors for either hormone.

Morris Maze: Spatial RM

Animals were tested on the Morris maze to evaluate spatial RM. There was a main effect of Treatment (F[3,26] = 6.64, p = 0.0018), in the absence of a Block x Treatment interaction (F[3,26] = 0.21, p = 0.8921). Post-hoc analyses revealed that Ovx-Δ^8E1-Low, Ovx-Δ^8E1-Med, and Ovx-Δ^8E1-High groups each swam less distance than the Ovx-Veh group (post-hoc ps < 0.0027, Figure 22a). For equilin, there were no significant

main effects or interactions (Figure 22b). For the probe trial, each treatment group localized to the NE quadrant which previously contained the hidden escape platform, with a higher percent distance in the NE quadrant (Quadrant main effect: Δ^8E1 $F[1,26] = 181.21$, $p < 0.0001$, Figure 22c; Equilin $F[1,26] = 116.19$, $p < 0.0001$, Figure 22d). This was in the absence of a Treatment x Quadrant interaction for either hormone, indicating that all groups localized to the previously platformed quadrant.

Visible Platform Task: Motoric and Visual Competence

Motor and visual competence was assessed using a visible platform task. There were no main effects of Treatment for Δ^8E1 or equilin, thereby indicating that animals, regardless of hormone status, were able to locate the visible platform (Δ^8E1 $F[3,26] = 0.83$, $p = 0.4908$, Figure 23a; Equilin $F[3,26] = 0.59$, $p < 0.6302$, Figure 23b). All animals found the platform within 10 seconds on the last two trials, thereby indicating that all animals were able to perform the procedural components of a swim maze task.

nAChR Agonist Binding: rα4β2-nAChR Expression Levels

I investigated whether treatment with Δ^8E1 or equilin affected nAChR expression levels using a radioligand binding assay. Analysis of Δ^8E1 in the hippocampus+entorhinal cortex revealed a significant main effect of Treatment ($F[3,48] = 4.33$, $p = 0.0089$), with each Δ^8E1 group showing decreased rα4β2-nAChR expression as compared to the Ovx-

Veh group (*p*s ≤ .05, Figure 24a). Moreover, when Ovx-Δ^8E1-Low, Ovx-Δ^8E1-Med and Ovx-Δ^8E1-High groups were combined into one Δ^8E1 group, there was a Treatment main effect (F[1,54] = 5.25, p = 0.0259, Figure 24a). There were also greater rα4β2-nAChR levels in the entorhinal cortex compared to the hippocampus (Brain Region main effect: (F[1,48] = 59.81, p < 0.0001, Figure 24a), and there was a nonsignificant Treatment x Brain Region interaction indicating this pattern was shown for all Δ^8E1 all doses. In the equilin analysis, there was no significant Treatment main effect (F[3,50] = 1.84, p = 0.1517); however, as seen in the Δ^8E1 omnibus ANOVA, there were greater rα4β2-nAChR levels in the entorhinal cortex compared to the hippocampus (Brain Region main effect: (F[1,50] = 41.63, p < 0.0001, Figure 24b), in the absence of a Treatment x Brain Region interaction.

Regression: Relation between Nicotinic Receptor Agonist Binding and Memory

Since a decrease in rα4β2-nAChR expression was noted with Δ^8E1 treatment in the hippocampus+entorhinal cortex, I conducted primary regression analyses relating rα4β2-nAChR expression in the hippocampus+entorhinal cortex to maze performance. Specifically, rα4β2-nAChR expression in the hippocampus+entorhinal cortex was the predictor variable in the model, and Morris maze swim distance collapsed across all days, repeat DMS Plus Maze errors on trial 2 alone (collapsed

across days 2-7), or repeat DMS Plus Maze errors on trials 2-6 (collapsed across days 2-7), was the outcome variable. There was a positive relationship between rα4β2-nAChR expression in the hippocampus+entorhinal cortex and Morris maze swim distance (b = 0.03, r = 0.64, z[28] = 3.75, p = 0.0002, Figure 25). Since greater swim distance indicates poorer performance, better spatial RM performance was associated with less rα4β2-nAChR expression in the hippocampus+entorhinal cortex. When the data were centered to control for group membership (for more detailed methods and rationale see Enders and Tofighi, 2007; Hallahan and Rosenthal, 2000), the regression analysis remained significant (b = 7.71, r = 0.44, z[28] = 2.36, p = 0.0182). There were no significant regression analyses relating rα4β2-nAChR expression in the hippocampus+entorhinal cortex to working or recent memory repeat errors on the win-stay DMS Plus Maze (trial 2: b = 0.001, r = 0.06, z[28] = 0.26, p = 0.7829; trials 2-6: b = 0.001, r = 0.15, z[28] = 0.78, p = 0.4365).

Efflux Experiments: Evaluation of Direct Estrogenic Actions on hα4β2-nAChR Function

I evaluated whether Δ^8E1 or equilin exposure directly affected the function of hα4β2-nAChRs assessed using $^{86}Rb^+$ efflux experiments in vitro. For either acute or chronic administration, regression analyses revealed that slopes of the best fit line did not differ from zero (ps > 0.05),

and the data did not deviate from linearity (Wald runs test for randomness, all *p*s > 0.05, Figures 26a, 26b and 26c). PEG did not affect or act together with Δ^8E1 or equilin in altering $^{86}Rb^+$ efflux during acute, or after chronic exposure. This indicates that neither acute nor chronic effects of hormone or vehicle on hα4β2-nAChR function occur at the concentrations tested.

Vaginal Smears, Uterine Weights and Serum LH: Classic Estrogenic Actions

I tested peripheral markers that are routinely noted to change with E2 treatment. Before Ovx surgeries, all animals demonstrated normal cyclicity. After Ovx (but before hormone treatment), all rats consistently exhibited leukocytic, diestrus smears, as expected. Two days prior to behavior testing, after assigned vehicle or hormone had been administered, all Ovx-Veh-treated rats presented consistent diestrus smears, whereas hormone-treated rats showed constant cornified, estrous smears demonstrating uterine stimulation. These cytological profiles remained stable through sacrifice (Figure 27). Furthermore, each Δ^8E1 group, and each Equilin group, had increased uterine weight compared to the Ovx-Veh group (Δ^8E1 $F[3,25] = 13.51.$, $p < 0.0001$, Figure 28a; Equilin $F[3,25] = 34.96$, $p < 0.0001$, Figure 28b, post-hoc *p*s < 0.0028), and lower LH levels as compared to the Ovx-Veh group (Δ^8E1 $F[3,15] = 4.95$, $p =$

0.0139, Figure 28c; Equilin $F[3,15] = 8.67$, $p = 0.0014$, Figure 28d, post-hoc ps < 0.0143).

Discussion

The present study is the first to evaluate whether Δ^8E1 and equilin, two primary components of CEE shown to have the most neuroprotective effects in vitro (Brinton et al., 1997; Zemlyak et al., 2002; Zhao and Brinton, 2006), influence cognition. In middle-aged surgically menopausal rats, Δ^8E1 treatment enhanced spatial reference, working, and recent memory. In these same animals, Δ^8E1 treatment decreased rα4β2-nAChR expression (i.e., specific I-epi binding) in the hippocampus and entorhinal cortex, which was related to better spatial RM performance on the Morris maze. Equilin treatment did not impact spatial memory or rα4β2-nAChR expression. There were no direct effects on hα4β2-nAChR function with acute or prolonged exposure to Δ^8E1, equilin or PEG (vehicle).

Δ^8E1 And Equilin Effects on Spatial Working and Recent Memory

The Δ^8E1 medium and high doses enhanced working and recent memory performance on the DMS Plus Maze task, while equilin did not. These Δ^8E1-induced spatial WM enhancements corroborate previous findings whereby E2, and higher doses of CEE, benefited spatial WM in Ovx rats (Acosta et al., 2009b; Bimonte and Denenberg, 1999). It is conceivable that the Δ^8E1 medium and high doses facilitated better performance on this task by predisposing these animals to use a place

strategy, as this is seen with E2 treatment (Korol and Kolo, 2002). In my DMS Plus Maze task, animals using a response strategy (i.e., turn left or turn right) would have been inefficient at locating the platform, as the start location was varied and the goal arm remained in the same place in space within a day, leading to more errors. I speculate that the use of a place strategy allowed Δ^8E1 medium and high dose treated animals to accumulate fewer errors by creating a more stable cognitive map, in part by having a better ability to hold the spatial location of the platform in WM(MacLusky et al., 2006; Wilson et al., 2004).

Δ^8E1 and Equilin Effects on Spatial RM

I found that all doses of Δ^8E1 enhanced spatial RM performance. This was evidenced by less overall Morris maze swim distance in the Δ^8E1 treated groups as compared to the Vehicle group. Equilin treatment did not affect Morris maze performance. Tonic subcutaneous Δ^8E1-induced spatial RM enhancements are in accordance with Morris maze improvements shown by others after tonic subcutaneous E2 treatment (Bimonte-Nelson et al., 2006; Foster et al., 2003; Talboom et al., 2008), and after cyclic subcutaneous CEE treatment (Acosta et al., 2009b), in middle-aged Ovx animals. Interestingly, Δ^8E1's spatial RM enhancement did not vary within the dose range studied, and the effect was robust. In fact, Δ^8E1's effects noted here are relatively more pronounced as compared to other reports using E2 or CEE, where effects were specific to

aiding overnight retention or performance during the first 6 test trials (Acosta et al., 2009b; Markham et al., 2002; Talboom et al., 2008).

It is noteworthy that the Morris maze learning curves and probe trial data suggest that by the last testing day all groups learned the task and localized to the platform quadrant. Further, all animals readily learned the visible platform task with no group differences. That all animals could learn these two tasks suggests that my observed Treatment effects were not likely due to differences in visual competence or motoric ability.

Δ^8E1 and Equilin Effects on $\alpha4\beta2$-nAChR Expression and Function

All doses of Δ^8E1 decreased rα4β2-nAChR expression in the hippocampus and entorhinal cortex, while equilin did not alter rα4β2-nAChR expression in any region. My hippocampus and entorhinal cortex nAChR data are in agreement with other studies demonstrating that E2 treatment decreased the number of nAChRs and mAChRs in the central nervous system of Ovx rodents (Cardoso et al., 2004; El-Bakri et al., 2002), although not all studies show this effect (see Centeno et al., 2006; Lapchak et al., 1990). The current results also indicate that hippocampus+entorhinal cortex r$\alpha4\beta2$-nAChR expression has implications for cognition. Indeed, a decrease in r$\alpha4\beta2$-nAChR expression in the hippocampus+entorhinal cortex was associated with better spatial RM performance (i.e., $r = 0.64$), representing a large effect size as defined by Cohen (Chakraborty and Gore, 2004).

I performed a follow-up study to assess possible direct actions of Δ^8E1and equilin on hα4β2-nAChR, as ethinyl β-estradiol has been found to directly potentiate both hα4β2- and rα4β2-nAChR function (i.e., ion efflux) in vitro (Paradiso et al., 2001). I found that exposure to Δ^8E1 or equilin, below, at, or above physiologically relevant brain concentrations of E2 (95 fM-1 μM for my acute experiments, and 9 aM-1 μM for my chronic experiments) (see Woolley, 2007), had no detectable effect on function of hα4β2-nAChRs. Previous in vitro studies reporting potentiation of α4β2-nAChRs by estrogens found that in some cases, rα4β2-nAChRs and hα4β2-nAChR do not show comparable responses to the same estrogens, and the concentrations of estrogens used were higher in comparison to the current evaluations (Paradiso et al., 2001). These higher concentrations may not be physiologically relevant in the context of endogenous estrogen levels (Paradiso et al., 2001). This previous report also suggests that the lack of a hydroxyl group at the 17th position on Δ^8E1 may prevent it from potentiating hα4β2-nAChR (Paradiso et al., 2001). Taken together with my data indicating the absence of direct Δ^8E1 effects on hα4β2-nAChR, I hypothesize that Δ^8E1-facilitated alterations in rα4β2-nAChRs expression reflect an adaptation to Δ^8E1 directed changes to other aspects of the cholinergic system. It is possible that, similar to E2 (Marriott and Korol, 2003), Δ^8E1 treatment leads to enhanced ACh release

in the hippocampus and entorhinal cortex which may, in turn, result in reduced expression of α4β2-nAChRs as a compensatory mechanism.

A Potential Source of Δ^8E1's Effects and Future Directions

Δ^8E1 and equilin bind to, and produce biological activity via, both ER subtypes (Bhavnani et al., 2008). It is likely that Δ^8E1-facilitated spatial memory enhancements are primarily, but not solely, directed by ER activation, as E2's place learning enhancement is influenced by hippocampal ER activation (Zurkovsky et al., 2006). Recent evidence also suggests that estrogenic effects are influenced by the putative G-protein coupled membrane-bound ER (ER-X; reviewed in Toran-Allerand, 2005) and/or GPR30, a G-protein coupled receptor that responds rapidly to estrogen (reviewed in Prossnitz et al., 2008a). It is possible that Δ^8E1's cognitive effects are directed in part by these non-classic ERs. In fact, recently, Gibbs and colleagues found that the GPR30 agonist G1 enhanced delayed-match to position learning (Hammond et al., 2009). It is also noted that Δ^8E1-induced memory enhancements could be related to non-genomic mechanisms as well, as estrogens impart robust non-genomic effects (Prokai and Simpkins, 2007)

My current experimental design did not allow me to decipher the effects of behavior testing from the effects of hormone treatment on rα4β2-nAChRs expression. Indeed, rα4β2-nAChR expression may have been influenced by behavior testing alone, in a similar manner as that

previously noted for hippocampal dendritic spines (see Frick et al., 2004). In addition, the regulation of nicotinic receptor expression by ligands occurs by diverse mechanisms, and these are conserved across different systems (for review see Beato et al., 1996; Morris et al., 1986; Singh et al., 1996). However, the use of a model system allowed me to study hormone effects on a single, well-defined subtype. In my in vitro model system, whole-animal pharmacokinetics and the effects of brain-region differences were not an issue. Due to the lack of effect in the model system (where there is no synaptic function), in contrast to the effects in vivo, I conclude that my in vivo effects on 4β2-nAChRs likely reflect a functional adaptation in response to the Δ^8E1 treatment, and not a direct effect of the hormones on the receptors themselves. Further studies are necessary to detail the specific contributions of Δ^8E1 on cholinergic function and rα4β2-nAChR expression, independent of behavioral testing effects, as well as to fully identify the mechanisms of Δ^8E1-facilitated memory enhancements both at the cellular and the systems level.

Conclusions

There is increasing evidence that Δ^8E1 demonstrates unique properties compared to more widely-studied estrogens such as 17α- and E2 (Baracat et al., 1999; Bhavnani, 1998). Data from other laboratories have demonstrated that Δ^8E1 has an attenuated toxic potential compared to other CEE components (Zhang et al., 2001), shows distinct tissue and

cell-specific estrogenic properties in relation to E1 (Baracat et al., 1999), and appears to be converted to only one metabolite in women, giving Δ^8E1 a unique pharmacokinetic profile (Bhavnani, 1998). Two studies have evaluated Δ^8E1 in women. These works found that Δ^8E1 exerted an overall beneficial profile for health-related concerns associated with menopause (Baracat et al., 1999; Bhavnani et al., 1998; for review see Utian et al., 2006). Together with the data presented here, the collected evidence suggests that Δ^8E1 is a uniquely beneficial estrogen that warrants further study as a potential nootropic therapy. In summary, Δ^8E1 enhanced multiple domains of learning and memory, with a link to the cholinergic system as shown by changes in nicotinic receptor expression. Equilin did not affect spatial memory and had no effects on nicotinic receptor expression. Select components of CEE may offer promising new HT options that positively impact brain health during aging. An exciting new direction might be to further define the individual components of CEE. This would allow composition of a novel combined ET, with the ultimate goal to optimize the potential benefits, and obviate the risks, of currently used estrogen therapies.

CHAPTER 7

AGE AND OVARIAN HORMONE STATUS ALTERS THE NUMBER OF PUTATIVE SYNAPSES IN SPECIFIC LAMINA OF THE FEMALE RAT HIPPOCAMPUS

Novel memory formation allows adaptability in an organism. It allows acquisition and updating of knowledge and skills within different and overlapping neurobiological domains (Kausler, 1994). As individuals age, it is well documented that some memory loss occurs (Erickson and Barnes, 2003; Tulving and Craik, 2000), and age-related cognitive decline likely involves changes in neuroplasticity and factors influencing synaptic transmission within the brain (see Chapter 1). In the United States, the proportion of the population that is over 65 is increasing. Today, about 12% of the population is over 65, and this percentage is expected to substantially increase to 20% by the year 2020 due to aging of the “baby boomer” generation (U.S. Census Bureau, 2007).

Several basic and clinical studies have linked age-related cognitive decline with age-related alterations in the ovarian hormone milieu (for review see Bimonte-Nelson et al., 2010; Sherwin and Henry, 2008). For example, ovarian hormone loss due to surgical or natural menopause has been associated with cognitive decline in women (Nappi et al., 1999; Phillips and Sherwin, 1992; Sherwin, 1988). In animals, previous studies have found that Ovx in young female rats is detrimental to spatial WM

performance (Bimonte and Denenberg, 1999), while Ovx in aged female rats facilitates spatial WM performance (Bimonte-Nelson et al., 2003b). However, it is important to note that slight differences existed in how spatial WM was assessed on the WRAM task between these two previous studies (i.e., 4 arms were baited instead of 7). Despite this small discrepancy, the collected human and animal findings suggest that ovarian hormone loss alters memory; thus, similar to age-related cognitive decline, hormone loss-facilitated cognitive alterations likely involve changes in measures of neuroplasticity within the brain (see Woolley, 2007).

In young female rats, E2 treatment in Ovx animals increased hippocampal spine density, an effect dependent on the duration of ovarian hormone deprivation (i.e., Ovx), dose of E2, and the addition of progesterone (Gould et al., 1990; McLaughlin et al., 2008; Woolley, 1998; Woolley and McEwen, 1992). E2 supplementation reversed the age-related decreases in hippocampal MAP2 staining in a mouse model of Down Syndrome, suggesting an increase in dendritic density due to E2 (Granholm et al., 2003). Further, chronic E2 and β-Estradiol-3-benzoate treatment increased hippocampal synaptophysin protein levels in middle-aged female mice in a dose-specific manner, an effect that corresponded with enhanced object recognition performance (Fernandez and Frick, 2004; Frick et al., 2002). Although not a highly investigated area, research

conducted thus far suggests that progesterone, when given alone, increases hippocampal synaptophysin levels (Choi et al., 2003). These studies strongly support the hypothesis that estrogen and progesterone influence synaptic plasticity. Yet, the work done to date has primarily tested Ovx animals with and without hormone treatment, leaving the question of effects of ovarian hormone loss as yet unanswered. For example, the only research evaluating Ovx-induced changes in dendritic spine density in hippocampus has been done in young animals, with decreases seen in CA1, but not CA3 (Wallace et al., 2006; for review see Woolley, 1998). There has been no published study evaluating Ovx effects in aged rats.

The current study assessed how age and ovarian hormone loss altered the distribution of excitatory and inhibitory putative synapses in several hippocampal laminas of female rats. Many, but not all, studies assessing age- and hormone- related changes in synaptic density have been limited to non-specific markers such as synaptophysin and synapsin. An inherent weakness of evaluating general markers of synaptic density using SNARE proteins (e.g. synaptophysin, synapsin) is that the functional subtypes of synapses are not revealed (excitatory vs. inhibitory). Therefore, I assessed specific markers of excitatory and inhibitory synapses to estimate the balance of excitation and inhibition in hippocampal circuits. The hypothesis was that age and ovarian hormone

loss via Ovx would act together to alter the number of excitatory and inhibitory putative synapses within distinct lamina of the hippocampus in female rats. Specifically, using immunohistochemical (IHC) staining techniques and fluorescent confocal microscopy, this experiment evaluated the number excitatory putative synapses, assayed via a primary antibody targeting the NR1 subunit of the NMDA receptor followed by a fluorescent secondary antibody, and inhibitory putative synapses, assayed via a primary antibody targeting the vesicular GABA transporter (vGAT) followed by a fluorescent secondary antibody. ICH methods of this nature combined with fluorescent confocal microscopy reveal points or spots of NR1 or vGAT protein aggregation staining termed “punctate”, and are thought to represent putative synapses (Jelks et al., 2007; Khan et al., 2010). In fact these studies have used punctate staining to identify putative synapses using both NR1 and vGAT (Jelks et al., 2007; Khan et al., 2010).

Methods

Subjects

Subjects were female Fisher-344 rats aged 5 (young), 12 (early middle-aged), 18 (late middle-aged), or 20 (aged) months at the time of testing. Each age consisted of ovary-intact Sham and Ovx groups as follows: 5 month Sham (*n* = 4), 5 month Ovx (*n* = 4), 12 month Sham (*n* = 4), 12 month Ovx (*n* = 4), 18 month Sham (*n* = 4), 18 month Ovx (*n* = 4),

20 month Sham (*n* = 3), and 20 month Ovx (*n* = 4). Rats were pair-housed with a same-treatment cage mate in the animal facility of the Psychology department at Arizona State University, were given food and water *ad libitum* and maintained on a 12hr light-dark cycle. All procedures were approved by the local Institutional Animal Care and Use Committee and adhered to National Institute of Health (NIH) standards.

Surgical Procedures and Vaginal Smears

All rats received surgery approximately 6 weeks before behavioral testing and 9 weeks before sacrifice. Rats were anesthetized with an intraperitoneal injection of a cocktail of 70 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA, USA) and 6 mg/kg xylazine (Lloyd Laboratories, Shenandoah, IA, USA). Rats of each age received either Sham surgery (5 month Sham, 12 month Sham, 18 month Sham, 20 month Sham) or Ovx (5 month Ovx, 12 month Ovx, 18 month Ovx, & 20 month Ovx). For Ovx, dorsolateral incisions were made in the skin and peritoneum, and the ovaries and tips of uterine horns were ligated and removed. The muscle was then sutured and the skin stapled. Sham surgery consisted of skin incision and staple in the same manner (Talboom et al., 2008).

Vaginal smears were performed before and after behavioral testing to confirm hormone status (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009; Talboom et al., 2010). A cotton swab was dipped in saline and the

vaginal lumen was gently swabbed. Vaginal smears were viewed under a light microscope and classified as proestrus (round nucleated epithelial cells), estrous (cornified cells), metestrus (epithelial, cornified and leukocytic cells) or diestrus (primarily leukocytes) (Goldman et al., 2007). As expected, all Ovx animals exhibited leukocytic acyclic smears. Five and 12 month old Sham animals showed smears reflective of normal cycles, while 18 and 20 month old Sham animals showed persistent diestrous smears.

Sacrifice, Brain Dissection, Post Fixation, and NR1 and vGAT Fluorescence IHC

Approximately 3±1 days after all animals were tested on a battery of water-escape mazes assessing working and RM (data now shown here), animals were anesthetized with isoflurane (Vetone, Meridian, Indiana), decapitated (i.e., according to NIH euthanasia guidelines), and their brains dissected promptly. Each blocked posterior half of the brain was post fixed in 4% paraformaldehyde in PB (pH 7.4) for 48 hours, and then the tissues were transferred to PB until sectioning. Blocked brains were sectioned (plates 55-68 in the Rat Brain Atlas 5th Edition Paxinos and Watson, 2005) on a Vibratome (Model 3000, Vibratome, Bannockburn, IL) in PBS (pH 7.4) at 75 μm throughout the dorsal hippocampus (i.e., anterior portion containing CA1, CA2, CA3 and dentate

gyrus) and collected for IHC (similar to Chapter 4; Acosta et al., 2009b; Granholm et al., 2002).

Four nonconsecutive sections through the dorsal hippocampus (free-floating section) from each animal were selected for NR1 and vGAT antibody stain, placed in a 24 well tissue culture plate (Evergreen) and incubated at RT for 15 min in PBS (pH 7.4) and 0.03% Triton (Triton 100X) to permeabilize the tissue. To increase the punctate signal, each tissue slice was incubated at 37 C° for 30 min with pepsin solution (25% 1N HCl and 0.375 (w/v) Pepsin [Sigma]) followed by one PBS wash (5 min). Next, in order to evaluate the number putative excitatory synapses, I used a primary polyclonal antibody targeting the NR1 subunit of the NMDA receptor, while putative inhibitory synapses were assessed via a primary monoclonal antibody targeting the vesicular GABA transporter. Sections were incubated with primary antibodies, rabbit anti-NR1 (1:500, Millipore) and mouse anti-vGAT (1:500, Synaptic Systems) in BKS (10 % heat inactivated horse serum, 2% BSA and 0.25% Triton X100 in PBS), overnight at 4 °C on a Rocker II plate rocker (Boekel Scientific, Feasterville, PA). Sections were washed four times in PBS for 20 min (5 min each) followed by immersion in the secondary antibody (1:500 Alexa Fluor 488 goat anti-Rabbit IgG and Alexa Fluor 633 goat anti-mouse IgG (Invitrogen) in PBS for 20 min on a Titer Plate Shaker (Barnstead International, Dubuque, IO) at RT. Sections were then washed four times

in PBS for 80 min (20 min for each wash). Brain sections were mounted on Superfrost Slides (Fisher) and excess moisture was aspirated via a vacuum line. Slides were coverslipped with Vectorshield (Vector Laboratories), and then sealed with clear nail polish.

Slides were then stored in the dark at 4 °C. These methods were similar to ones previously published (see Tyler and Pozzo-Miller, 2003). Each group was equally represented in each round of staining to avoid group inter-variability in staining. Due to variations in antibody lot, one round of staining was excluded from analysis since values that were not concordant with the other rounds. As a control for the IHC methods, tissue sections were evaluated whereby the primary antibodies were omitted; this resulted in no distinct punctate staining for either NR1 or vGAT, as expected.

Hippocampal Lamina Image Capture and Analysis

Images were acquired using FluoView software (5.0 Olympus America, Inc., Center Valley, PA) from a FV-300 laser-scanning confocal microscope (Olympus America, Inc., Center Valley, PA). Excitation of Alexa Fluor 488 (i.e., NR1 secondary) was achieved using the 488 nm argon laser, and Alexa Fluor 633 (i.e., vGAT secondary) was excited using a 633 nm HeNe laser. A ×60 (1.41 NA; Olympus; digital 2X) PlanApo oil immersion lens was used to capture images. Confocal microscopy image z-stacks with an effective lateral (x-y axis) pixel resolution of 0.09 µm

pixel^{-1} (calculated via reconstructing fluorescent microspheres) and a step-size of ≈ 7-10μm between optical planes were acquired, yielding 6-8 images per stack that were collected at randomly selected sites within stratum oriens (dorsal to CA1), CA1 stratum pyramidale, the polymorphic layer of the dentate gyrus, and in stratum granulosum (i.e., the ventral blade of the dentate gyrus). Figure 29a illustrates the lamina where images were acquired in the hippocampus and Figure 29b shows two representative fluorescent micrographs for NR1 and vGAT in the polymorphic layer and stratum granulosum dentate of the gyrus, respectively.

Captured images for each lamina were then semi-automatically counted using NIH ImageJ software (Rasband, 1997-2004). Raw FluoView (Olympus America, Inc., Center Valley, PA) tiff image z-stacks were first split between the two channels (i.e., one for 488 NR1 and one for 633 vGAT) into separate image z-stacks. The first and the last images were deleted so that 4 images remained in a z-stack. For the stratum pyramidale and stratum granulosum, a region of interest (ROI) was traced around the cell bodies with the “Polygon Selections” tool, and only putative synapses within that ROI were counted (i.e., only those that were located on the cell bodies). Next, a spot detector based on a “3D LoG” filter, with a “sigma X” of 2 and a “sigma Y” of 2, was applied to the four images to reduce noise and enhance the punctate (Figure 30a) (Sage et al., 2005).

Images were thresholded via “Mixture Modeling” (Figure 30b) (Mei and Dauphin, 2003), and the ImageJ “Analyze Particles” tool was used to automatically quantify the number of punctate in each of the four images with a size (pixel^2) of 13-150 and a circularity of 0.00-1.00 (Figure 30c). Raw data were then exported pasted into Excel 2007 (Microsoft, Redmond, WA) and saved for analysis. Steps used to quantify putative synapses were identical for both NR1 and vGAT, and the number of NR1 and vGAT punctate in each separate hippocampal lamina were the dependent variable used for statistical analysis. This method of counting yielded accurate putative synapse counts when compared to manual counting in a small subset of the images (Figure 30d) as well as what I consider unbiased counts, since I did not use different threshold values for each image/z-stack (i.e., the ImageJ “Mixture Modeling” plugin did not have parameters to adjust) (see discussion in Hiscock et al., 2000).

Statistical Methods

Using StatView (version 5.01, The SAS Institute Inc., Cary, NC) for each hippocampal lamina imaged, I first performed an omnibus mixed model ANOVA within each age (5, 12, 18, & 20) and treatment (Sham & Ovx) group. Age and Treatment were the between-subjects factor while Section (1–4) and Image (1–4) were the repeated measures. For all pairwise ANOVAs (i.e., Ovx vs. Sham), Cohen’s d was employed as a measure of effect size that assessed the difference between two group

means represented in standard deviation units (Cohen, 1988). Lastly, for linear trend analyses of age in relation to putative synapse numbers, I evaluated polynomial contrasts under the general linear model using SPSS (version 18.0; IBM Corporation; Somers, NY). Linear trends were assessed from 5 to 20 months of age to assess linear effects of age on putative synapses numbers. I also assess linear trends from 12 to 20 months of age, since estropause begins by 12 months of age (Dudley, 1982) and may be the age range where previous studies in my lab found that Ovx in young female rats is detrimental to spatial WM performance (Bimonte and Denenberg, 1999), but facilitates spatial WM performance in aged Ovx female rats (Bimonte-Nelson et al., 2003b).

To evaluate effects of age I performed planned contrasts with specific *a priori* comparisons in mind to aid in the interpretation of results and increase the statistical power in comparison to a less focused omnibus test (see section 8.5 in Cohen et al., 2003). Similar to a *t*-test, planned contrasts compare the means of two groups or a combination of groups; however, the error term (i.e., unexplained variance, MS within-groups/error) is calculated from all the groups in the study (i.e., pooled within-group variance), not just the two groups being compared (see Rosenthal and Rosnow, 1985). Hence, statistical power can be increased by having a better estimate of the unaccounted for variance (i.e., increase the *t*-value/ratio). This study was designed *a priori* to evaluate the effects

of age and ovarian hormone loss on putative synapse numbers, while paying particular attention to changes that may account for the previously observed transition where Ovx is detrimental to WM when young (Bimonte and Denenberg, 1999), but Ovx facilitates WM when aged (Bimonte-Nelson et al., 2003b). To this aim, two contrasts were used to evaluate pairwise effects of age as follows: 1) 5 month old as compared to 20 month old animals (i.e., young verses aged contrast) and 2) 12 month old (i.e., estropause begins by 12 months of age; Dudley, 1982) as compared to 20 month old animals (i.e., early middle-aged verses aged contrast).

Results

Treatment Effects: Ovx Compared to Sham Animals

When evaluating the effects of Ovx or Sham surgery, there was a significant main effect of Treatment for NR1 in stratum granulosum ($F[1,20] = 4.34$, $p \leq 0.05$, $d = 0.93$, Figure 31), indicating that Ovx animals had fewer putative excitatory synapses in comparison to ovary-intact Sham animals. The Cohen's *d* statistic was considered a large effect by convention (Cohen, 1988). This suggests that the loss of ovarian hormones decreased the number of putative excitatory synapses on stratum granulosum, the granular cells of the ventral blade of the dentate gyrus. There were no other main effects of Ovx for the discrete region analyses.

Trend Analyses: Lineal Relationships between Age and Putative Synapse Numbers

An evaluation of age via linear trend analyses revealed that the number putative inhibitory synapses in stratum pyramidale decreased from 5 to 20 months of age in Ovx and Sham animals (vGAT $F[1,19] = 5.94$, $p = 0.0248$). When I analyzed Ovx and Sham groups separately, Ovx (vGAT $F[1,8] = 6.84$, $p = 0.0308$, Figure 32a) but not Sham (vGAT $F[1,7] = 0.38$, $p = 0.5547$) animals, showed this effect. The of number of putative inhibitory synapses decreased from 12 to 20 months of age in the polymorphic layer of both Ovx and Sham animals (vGAT $F[1,14] = 6.58$, $p = 0.0224$, Figure 32b). This effect was not seen when the Ovx (vGAT $F[1,5] = 2.91$, $p = 0.1489$) or Sham (vGAT $F[1,6] = 3.18$, $p = 0.1247$) groups were analyzed alone. Together, these trend analyses suggest that age and ovarian hormone loss act together to decrease the number of putative inhibitory synapses on the pyramidal cells of CA1 (from 5-20 months of age), while age alone (from 12-20 months of age) may be the factor that decreases the number of putative inhibitory synapses along the axons of the granular cells in the polymorphic layer of the dentate gyrus.

Planned Contrasts: Pairwise Comparisons between 5 and 20, as well as 12 and 20, Month Old Animals

When evaluating pairwise differences via planned contrasts, I found a significant young verses aged contrast in stratum pyramidale of Ovx and

Sham animals (vGAT $F[1,19] = 6.02$, $p = 0.0239$), as well as Ovx (vGAT $F[1,8] = 6.48$, $p = 0.0344$, Figure 33a) but not Sham (vGAT $F[1,7] = 0.52$, $p = 0.4935$) animals when evaluating the treatment groups alone, indicating that putative inhibitory synapses decreased from 5 to 20 months of age on the pyramidal cells of CA1 primarily in Ovx animals. A significant young verses aged contrast was found in stratum oriens of Ovx and Sham animals (vGAT $F[1,27] = 5.20$, $p = 0.0306$). Showing the same pattern as in the stratum pyramidale, when the treatments were evaluated separately, the age effect was significant for the Ovx animals (vGAT $F[1,12] = 4.69$, $p \leq 0.05$, Figure 33b), but not Sham (vGAT $F[1,11] = 1.02$, $p = 0.3332$) animals, suggesting that Ovx animals were responsible for the decreased number of putative inhibitory synapses from 5 to 20 months of age on the dendrites of CA1 pyramidal cells. I also noted that in the polymorphic layer, the number of putative inhibitory synapses decreased from 12 to 20 months of age in Ovx and Sham animals (significant early middle-aged verses aged contrast vGAT $F[1,17] = 6.34$, $p = 0.0221$, Figure 33c), but not in Ovx (vGAT $F[1,6] = 2.32$, $p = 0.1785$) or Sham (vGAT $F[1,7] = 3.38$, $p = 0.1087$) animals alone. This indicated that age and not ovarian hormone loss is likely responsible for the decreased number of putative inhibitory synapses along the axons of the granular cells in the dentate gyrus. Collectively, aging and ovarian hormone loss does appear to act together to decrease the number of inhibitory synapses

in the stratum pyramidale and stratum oriens between 5 and 20 months of age. Furthermore, as aging ensues from 12 to 20 months of age, inhibitory synapses decrease in the polymorphic layer of the dentate gyrus of female rats, an effect that is likely facilitated by age and not ovarian hormone loss. Table 4 summarizes the effects of age and ovarian hormone loss on putative synapse numbers.

Discussion

Here, utilizing fluorescent confocal microscopy and methods similar to previous studies identifying putative synapses (Jelks et al., 2007; Khan et al., 2010), I capitalized on the modular features of ImageJ to automate aspects of counting discrete punctate (i.e., putative synapses) (Rasband, 1997-2004). I found that Ovx decreased the number of excitatory putative synapses on the granular cell bodies in the ventral blade of the dentate gyrus, as compared to the Sham group. Trend analyses revealed that putative inhibitory synapse counts decreased in a linear fashion from 5 to 20 months of age primarily in Ovx animals on the CA1 pyramidal cell bodies, while putative inhibitory synapse counts decreased in a linear fashion from 12 to 20 months of age only when Ovx and Sham animals were evaluated together in the polymorphic layer of the dentate gyrus. Lastly, contrast analyses revealed that the number of putative inhibitory synapse counts decreased between 5 and 20 months of age mainly in Ovx animals on the CA1 pyramidal cell bodies and in the stratum oriens

dorsal to CA1. Paralleling the trend analysis, there was a decrease in the number of putative inhibitory synapse counts between 12 and 20 month old in Ovx and Sham animals in the polymorphic layer of the denate gyrus. Collectively, these data suggest that ovarian hormone loss decreases excitatory synapse numbers on the granular cells of the dentate gyrus, while age and ovarian hormone loss may act together to decrease inhibitory synapse numbers on the pyramidal cells of CA1 and in the stratum oriens dorsal to CA1. The data suggested the age and ovarian hormone loss act together because I found that the age-related decreases in putative inhibitory synapses were primarily found in Ovx, but not Sham, animals. Notably, these data identified ovarian hormone loss as a principal factor that decreased putative synapse numbers in the female rat hippocampus.

Effects of Ovarian Hormone Loss on Synapses

Although to my knowledge no single study has assessed the effects of age and ovarian hormone loss on putative synapse numbers in the hippocampus, my findings that ovarian hormone loss decreased putative synapse numbers are in accordance with several studies assessing the effects of E2 treatment in systems devoid of E2. Specifically, previous research has noted that in young Ovx animals or in cultured hippocampal neurons grown in sera devoid of ovarian hormones, E2 treatment increases excitatory and decreases inhibitory neurotransmission in the

hippocampus or hippocampal cells of rats. In young Ovx rats, E2 increases NR1 immunofluorescence on the soma and dendrites of CA1 cells and on the soma of granular cells in the dentate gyrus (Gazzaley et al., 1996b). Others have found *in vitro* that E2 treatment (10 nm) for 48 hours increases NR1 and vGLUT colocalization along dendrites; they also noted that ER were located along the NR1 containing dendrites (Jelks et al., 2007). In regard to *in vitro* inhibitory neural transmission, ERs colocalize with neurons expressing glutamate decarboxylase (GAD, the enzyme that synthesized GABA), and a 24hr E2 exposure decreases GAD content by up to 80% and GAD cell population to 12%. In this same study, E2 treatment was found to decrease GABAergic miniature inhibitory postsynaptic currents in size and frequency, while miniature excitatory postsynaptic currents increased (Murphy et al., 1998). Furthermore, E2 treatment increases GAD mRNA levels in GABAergic neurons in CA1 but not in the stratum oriens; progesterone was found to abolish this effect (Weiland, 1992). Conceivably, this could be a compensatory mechanism to the fast E2-facilitated suppression of inhibitory neurotransmission (Murphy et al., 1998). Collectively, these studies suggest that E2 treatment after ovarian hormone removal alters markers of the GABAergic system and enhances markers of the glutamatergic system. These effects, while noting some important differences (e.g., no comparison to sham animals, *in vivo* vs. *in vitro* methods, & not all studies evaluated

progesterone), essentially parallel the present findings. When E2 treatment conditions were compared to Ovx animals or neuronal cultures that were devoid of E2, there were decreases in excitatory markers of synaptic transmission and alterations in markers of the GABAergic system. This suggests that the Ovx-facilitated changes in excitatory and inhibitory markers/neurotransmission during aging may well be, or result in, my observed decrease in excitatory and inhibitory putative synapses.

I hypothesize that if E2 treatment after Ovx increases excitatory transmission and decreases inhibitory transmission, then E2's removal for long periods of time may imbalance excitatory verses inhibitory neurotransmission resulting in declines in excitatory synaptic numbers. It is also noted that another laboratory has found results that appear in direct opposition to my own. Specifically, rats with short-term Ovx (1 mo) have substantially higher NMDA receptor subunit mRNA levels than animals with long-term Ovx (6 mo); however, the most dramatic increases in NR1 mRNA were seen during aging in the ventral hippocampus (Adams et al., 2001). With this said, it important to note two caveats to this study in comparison to my own: 1) Adams and colleagues (2001) only found effects in the ventral (i.e., not dorsal) hippocampus; I noted all of my effects in the dorsal hippocampus, and 2) the NR1 antibody targets NR1 protein and not mRNA; hence, my IHC methods targeted NR1 that had

already been translated. Indeed, increases in mRNA are not always concomitant with increases in the translated protein.

Effects of Aging on Synapses

A previous study using male and female *Macaca mulatta* monkeys found age-related declines in synaptic markers that are in line with the current study. Specifically, in aged monkeys, as compared to adult monkeys, there was a 30% decrease in NR1 immunofluorescence on the granule cells of the dentate gyrus (Gazzaley et al., 1996a). In addition to non-human primate work, previous studies using rodents have noted similar effects; however, an important caveat is that only males were evaluated in these studies. A comparison of 4 to 24 month old rats showed that NR1 protein levels decreased in the CA1 region of the ventral hippocampus (Liu et al., 2008). Recently, a comparison between young (2 month old) and aged rats found that markers of the synapses synaptophysin (between 18-24 months of age) and SNAP-25 (between 18-24 months of age), markers of the glutamatergic terminals vGluT1 (between 12-18 months of age) and vGluT2 (between 12-18 months of age), markers of cholinergic terminals vAChT (between 12-18 months of age), and vGAT (at 24 months of age) all decreased with age (Canas et al., 2009). The authors concluded that aging causes an imbalance of excitatory versus inhibitory markers primarily leading to a decrease in inhibitory mechanisms (Canas et al., 2009), which is notably a similar and

independent conclusion to the present findings. Indeed, aside from vGAT differences not being evident until 24 months of age (perhaps due to strain or sex differences), these results map almost directly on to the ones presented here. Furthermore, the findings of Canas and colleagues (2009) are particularly exciting and relevant to the current discussion because they suggest a mechanism within a similar age range where my lab previously found that Ovx in young female rats is detrimental to WM (Bimonte and Denenberg, 1999), while Ovx in aged female rats facilitates WM (Bimonte-Nelson et al., 2003b). Indeed, changes in cholinergic function, plasticity, and glutamatergic transmission outside the realm of synaptic number and the scope of the present study may well underlie this transition. In summary, these collective results corroborate my current findings and suggest that as aging ensues there is a decrease in markers of both excitatory and inhibitory synapses.

It is noteworthy that the majority of studies evaluating synaptic alterations (for example, synaptic density as revealed via synaptophysin evaluations) in healthy individuals have shown an age-related decline, resulting in a potentially interesting dissociation between synapse loss and neuron loss (Hof and Morrison, 2004; Terry, 2006). In fact, in corroboration with my findings, there is evidence using updated stereology techniques that number of hippocampal synapses decreases with the progression from young adulthood to normal aging, and that synapse

numbers in AD hippocampi are fewer than in those of normally aged (Bertoni-Freddari et al., 2003). It has been well established that there are profound age-related changes in the strength of synaptic connections in the hippocampus (Erickson and Barnes, 2003; Rosenzweig and Barnes, 2003). Interestingly, there is also evidence of a link between hippocampal synaptic density and cognition during aging. For example, a recent report indicates that a decrease in CA1 stratum radiatum perforated postsynaptic density is related to age-related learning impairment (Nicholson et al., 2004). Earlier work has shown a similar relationship in the dentate gyrus (Geinisman et al., 1986). Here, I demonstrate that there is likely a decrease in the number of actual synapses and that age and ovarian hormone loss differentially affect excitatory and inhibitory synapse loss within the female rat hippocampus.

Broader Interpretations

Interpretations of the lamina specific decreases in putative synapse numbers have specific implications in relation to memory. A straight forward interpretation of the decrease in putative excitatory synapses on the granular cells of the dentate gyrus is that the decrease may be the primary drive of age- and ovarian hormone loss-facilitated spatial memory impairment. In fact, this may indeed be a principal factor in why Ovx has been found to be detrimental to spatial memory (reviewed in Bimonte-Nelson et al., 2010). Specifically, glutamatergic neurotransmission is

critical for memory processing (see Bliss et al., 2003; Izquierdo et al., 1993), the granular cells of the dentate gyrus are principally important for the relay of information between the hippocampus and entorhinal cortex (i.e., the perforant pathway) (see Andersen, 2007), and the hippocampus and entorhinal cortex as well as their interactions are critical for spatial memory processing (Morris et al., 1982; Pappas et al., 2005; Zola-Morgan et al., 1994). Collectively this suggests that disruptions in excitatory transmission in the granular cells, and thus along the perforant pathway, may drive spatial memory impairment in Ovx animals.

Interestingly, a majority of my age effects were found to be primarily in Ovx animals and always in measures of inhibitory synapses (i.e., vGAT punctate). Specifically, I found age related declines in putative inhibitory synapses found on the cell bodies of CA1, as well as in the stratum oriens dorsal to CA1. Analogous to disruptions in excitatory neurotransmission, here a disruption of inhibitory transmission may cause memory impairment since CA1 cells and GABAergic neurotransmission are known to be critical in memory processing (Andersen, 2007; Daumas et al., 2005; Izquierdo et al., 1993). Furthermore, stratum oriens contains the dendrites of CA1 cells receiving inputs from other pyramidal cells, cholinergic basal septum fibers, and connections with the contralateral hippocampus (see Andersen, 2007), which are all factors that are necessary to properly process memory (see Andersen, 2007; Hasselmo, 2006). Thus, memory

would more likely than not be impaired if inhibitory synaptic transmission were disrupted. This suggests that the current age- and Ovx-facilitated decrease in putative inhibitory synapse numbers may underlie in part the previously noted age- and Ovx-facilitated alterations in spatial memory (see Bimonte-Nelson et al., 2010).

My data also indicated that age and perhaps not ovarian hormone loss is primarily responsible for the decrease in putative inhibitory synapses from 12 to 20 months of age in the polymorphic layer of the dentate gyrus, since age effects were only found when Ovx and Sham animals were evaluated together. The ploymorphic layer shows aged related declines in synaptic markers (Gazzaley et al., 1996a), and contains several interneurons, which may explain why I observed a decrease in putative inhibitory synapses since many interneurons are GABAergic (see Ji and Dani, 2000). Furthermore, the axons of the dentate gyrus granular cells pass through this layer on their way to CA3 (see Andersen, 2007), underscoring the importance of correct synaptic transmission since this is a part of the perforant pathway and is critical for spatial memory processing (Morris et al., 1982; Pappas et al., 2005; Zola-Morgan et al., 1994). Collectively, the results suggest that the polymorphic layer of the dentate gyrus is vulnerable to age, and alterations in neurotransmission with it, (i.e., specifically inhibitory) may in part underlie

the age-related changes in memory seen in rodents (see Bimonte-Nelson et al., 2010).

In conclusion, my data suggest that Ovx decreases the number of excitatory synapses on the granular cell bodies of the dentate gyrus. Aging and ovarian hormone loss likely act together to decrease the number of inhibitory synapses in CA1 stratum oriens and on CA1 pyramidal cell bodies, whereas age alone may have a greater effect on the observed decrease in the number of putative inhibitory synapses in the polymorphic layer of the dentate gyrus. Future evaluations could assess the effects of age and ovarian hormone loss, as well as subsequent treatment, on synapse number using cholinergic, glutamatergic, and GABAergic markers using electron microscopy. Indeed, more studies are necessary to evaluate the effects that ovarian hormone loss has on the molecular and neurobiological correlates of memory. Future studies could also use measures of the numbers of specific synapses and synaptic markers to assess relationships with memory. For example, excitatory and inhibitory synapse numbers could be used to predict spatial memory outcome measures assessed on mazes via regression analyses. Evaluations of this nature as well as the data from the current study are critical in order to fully elucidate the mechanism of how age and ovarian hormone loss alter memory in both humans and rodents. The translation of these findings to humans has exciting and far reaching potential;

indeed, such studies may lead to novel therapies and pharmaceuticals that can attenuate or ameliorate the effects of age and ovarian hormone loss on the brain with a concomitant improvement in memory, thus taking a substantial burden off of the ever-aging population.

CHAPTER 8

GENERAL DISCUSSION

The research in this dissertation has implications that promote an understanding of the effects of cognitive practice on aging memory, why males and females respond differently to cognitive practice, and the parameters and mechanisms underlying estrogen's effects on memory. This body of work suggests that cognitive practice can enhance memory when aged and that estrogen is a probable candidate that facilitates the observed differences in the effects of cognitive practice depending on sex. The effects of estrogen on memory appear to be dependent on several factors such as age, memory domain, dose, and the type of estrogen. Lastly, the present data indicates that the cholinergic system and hippocampal synapses are likely two non-mutually exclusive mechanisms involved in the effects of estrogen on memory.

In Chapter 2, I found that RM cognitive practice enhanced memory in both aged male and female rats on the RM cognitive practice task, and attenuated age related memory decline on a novel WM task. A notable finding of this study was that aged males and females differed in their response to RM cognitive practice on a novel RM task, and in the relationship between RM performance when young and aged neurotrophin levels in cognitive brain regions. Females in this study transferred the benefits of RM cognitive practice to a novel RM task when aged (i.e.,

Morris maze overnight retention), whereas males did not. Females also appeared to be responsible for the relationship between better RM performance when young and higher levels of hippocampal and frontal cortex NGF levels when aged. Collectively, Chapter 2 suggested that ovary intact females are more cognitively plastic than their male counterparts, an effect may be related to the interaction between estrogens and NGF (Engler-Chiurazzi et al., 2009; Fernandez and Frick, 2004). This enhanced cognitive plasticity in females was intriguing and led to studies that helped to elucidate some of the parameters and mechanisms of this observed enhanced cognitive plasticity. A logical place to start searching for this sex difference was to examine the major difference in sex hormone milieu between males and females (see Tarnopolsky, 1999). Decades of accumulating evidence has demonstrated that E2 enhances memory, influences neuronal plasticity and growth factor levels in cognitive brain regions, and attenuates neurotoxicity (for review see Bimonte-Nelson et al., 2010). For this reason, estrogens became a primary research target to elucidate the parameters and mechanisms of this female specific enhancement in cognitive plasticity.

In Chapter 3, I evaluated E2's effects on RM in young, middle-aged, and aged animals, and determined whether or not circulating serum E2 levels were related to RM performance. The results from Chapter 3 suggested that E2 loses its ability to enhance RM in aged female rats, and

that better RM performance was related to higher circulating E2 levels in young and middle-aged female rats. This age-related alteration in the ability of estrogen to enhance RM performance has been corroborated by others (Foster et al., 2003). Of interest is the fact that some studies have shown that aged female rodents exhibit RM enhancements in response to estrogen treatment (Frick et al., 2002; Markowska and Savonenko, 2002a); however, this may be due to the dose administered because only high supraphysiological doses of estrogen were able to influence RM retention in 17 month old rats (Foster et al., 2003). Thus, a higher E2 dose may be necessary to enhance RM in rats approaching old age. Notably, Chapter 3 supported the concept that E2 can enhance RM performance, with higher circulating E2 levels relating to better RM performance.

Important as the findings were in Chapter 3, they did not address a possible mechanism for E2's effects on memory or cognitive plasticity. Chapter 4 was designed to evaluate E2's effects on both WM and RM and to determine a possible mechanism underlying E2's enhancement of memory and cognitive plasticity. Chapter 4 also directly compared the effects of E2 to the complex hormone preparation CEE as well as DHED, the prodrug of E2, on spatial memory and putative mechanism of cognitive plasticity. I evaluated these additional compounds in comparison to E2 because CEE is the estrogenic component of HT that is most commonly prescribed to women, and DHED is conceivably E2 that only becomes

bioactive in the brain (Hersh et al., 2004; Prokai et al., 2003). Specifically, I wanted to examine the WM, RM, and ChAT-IR positive BF neuron profile of the estrogen most commonly evaluated in basic research (i.e., E2), as compared to the estrogen that women are commonly prescribed for HT (i.e., CEE; Hersh et al., 2004) and a prodrug of E2 which has the potential to become a novel HT (i.e., DHED, see Prokai et al., 2003). Middle-aged rats were chosen for Chapters 4-7 since this is the approximate age when ovarian hormone production is altered and E2 responsiveness starts to decline in both female rats and humans (see Bimonte-Nelson et al., 2010; Dudley, 1982). In Chapter 4 I found that DHED is readily converted to E2 in a brain homogenate, but not in an inert buffer. Surprisingly, the results also indicated that a higher tonic CEE dose was more beneficial to memory than a tonic E2 or tonic DHED dose; that is, the 24µg/day dose of CEE enhanced overnight retention on the Morris maze, as well as acquisition and retention on the DMS Plus Maze, whereas E2 and DHED only enhanced retention on the DMS Plus Maze. Corroborating earlier findings regarding CEE (Acosta et al., 2009b) and E2 (Gibbs, 1997), I found that E2, CEE and DHED all increased the number of BF ChAT-IR neurons, suggesting that all three compounds altered the cholinergic system. Importantly, the increased number of BF ChAT-IR positive neurons represented a putative mechanism of estrogen's enhancement of memory (see Gibbs and Aggarwal, 1998; Hasselmo, 2006).

One explanation for the disparate effects of E2 and CEE on WM and RM performance found in Chapter 4 is that a more optimal dose of CEE and a less optimal dose of E2 were evaluated. Although all hormone dose(s) in Chapter 4 were based off of previous studies (Acosta et al., 2009b; unpublished observations; Talboom et al., 2008), the idea of differential effects being determined by the dose and estrogen type highlighted the fact that the dose response relationship between E2 and memory, as well as CEE and memory, is not fully understood. Indeed, an evaluation of the relationship that E2 and CEE have on memory had not been conducted, since no studies including the study in Chapter 4 employed a large enough dose range (i.e., greater than 3) to correctly evaluate and interpret dose response relationships.

Chapter 5 was designed to assess the relationship that a baseline Ovx (i.e., a “0” dose) and a range of 5 separate tonic doses of E2 and CEE had on WM performance. Using linear and nonlinear regression models, Chapter 5 revealed that E2 from 0µg/day to 3.75µg/day exhibited a linear dose response relationship to working and recent memory performance, with higher doses being related to better performance. CEE exhibited a quadratic or inverted U shape dose response relationship to working and recent memory performance, with the middle 36 µg/day dose representing the “optimal” dose. While noting some differences in the memory domains that were evaluated, the linear relationship between E2

memory performance corroborated other linear relationships found between E2 and memory in previous studies (Chapter 3; Asthana et al., 1999; Phillips and Sherwin, 1992; Ryan et al., 2010). Chapter 5 also identified the 36μg/day dose of CEE as optimal dose for working and recent memory performance, which is corroborated by previous studies assessing CEE or one of its component estrogens (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009; Talboom et al., 2010). Collectively, Chapter 5 indicated that the dose response relationship of E2 and CEE to memory did differ, further suggesting that the dose and type of estrogen evaluated are important factors that dictate whether estrogen is beneficial or detrimental to memory.

In examining the idea that different estrogens have different effects on memory independent of other factors, another explanation for the disparate memory effects found in Chapter 4 and the differing relationships to memory found in Chapter 5, when comparing E2 to CEE, is revealed. Unlike E2, which is a single estrogen with a consistent molecular structure, CEE is a complex formulation containing several different estrogenic components all having slightly different molecular structures (see Bhavnani, 1998). Since CEE is comprised of so many different estrogenic components, it is conceivable to think that some component estrogens may be more efficacious to cognition than others, a hypothesis supported by the fact that many of the component estrogens

vary in their ER binding affinities as well as their pharmacokinetic and pharmacodynamic profiles (Bhavnani, 1998; Bhavnani, 2003; Bhavnani et al., 2008). Indeed, this tenet was realized and capitalized on by Dr. Brinton and colleagues (1997; 2006) in research where they found that certain select estrogenic components of CEE were more efficacious than others in enhancing markers of neuronal health *in vitro*. Chapter 6 extended these findings by testing Δ^8E1 and equilin, the top two neuroprotective estrogenic components of CEE (Zhao and Brinton, 2006), *in vivo* for their effects on learning and memory and on markers of the cholinergic system. Chapter 6 found that Δ^8E1 benefited spatial WM and RM and that it altered markers of the cholinergic system, while equilin did not. Taken together with the disparate CEE and E2 effects in Chapters 4 and 5, Chapter 6 suggests that different estrogens with slightly different molecular compositions and receptor affinities can have radically different behavioral and neurobiological effects (for discussion see Bhavnani et al., 2008).

Now with many parameters of estrogen's effects on memory elucidated, Chapter 7 examined a potential mechanism behind estrogen's ability to enhance plasticity and cognitive performance. Chapter 7 addressed the effects of age and ovarian hormone loss on hippocampal putative excitatory and inhibitory synapse counts. This measure and mechanism was chose as synapses are thought to be the structural

component of memory, are influenced by estrogen, and are altered during aging in cognitive brain regions (see Bliss et al., 2003; Geinisman et al., 1986; Nicholson et al., 2004; Woolley, 2007). I found that putative excitatory synapses were decreased in the dentate gyrus by ovarian hormone loss, an effect that is in line with, but not directly comparable to, previous literature reporting E2's effects on dendritic spines after Ovx (see Woolley, 2007). Ovarian hormone loss was also found to act together with age and affect putative inhibitory synapse counts in the dorsal cell layer of CA1, with putative inhibitory synapses numbers decreasing with age in Ovx animals. Taken together with the findings from previous literature (see Andersen, 2007; Bliss et al., 2003; Woolley, 2007) and the memory results from Chapters 2 through 6, the data from Chapter 7 suggest that estrogen's enhancement of spatial memory is likely directed via alterations in synaptic plasticity in the hippocampus and perhaps surrounding cortex. This ovarian hormone loss effect on hippocampal putative synapses may be an intrinsic effect in the hippocampus (Woolley et al., 1997), initiated by alterations in cholinergic signaling and NGF levels (see Granholm, 2000; Hasselmo, 2006), or comprise an interaction of all three.

Collectively, this body of work suggests that cognitive practice can benefit memory when aged in both males and females. In females, estrogen may enhance the effects of cognitive practice as it has been found to enhance memory and hippocampal synaptic plasticity. The

memory enhancements and alterations in hippocampal synaptic plasticity are at least partially directed by enhancements in cholinergic signaling from the BF. Lastly, age, dose, and type of estrogen utilized are important factors to consider when evaluating estrogen's effects on memory and its underlying mechanisms. For example, age, dose, and the type of estrogen utilized are important factors to consider when evaluating estrogen's effects on memory and its underlying mechanisms, since age alters the responsiveness to estrogen treatment and the dose of estrogen as well as small alterations in the molecular structure of estrogen can have a profound impact on estrogen's efficacy on memory. Many parameters including the ones described here become important considerations when designing future behavioral interventions/therapies and HTs, and this body of work helps to determine a few of those parameters. Ideally, this work will aid in understanding of the underlying mechanism that is responsible for estrogen's ability to enhance memory, and plasticity. These properties could then be used in turn to help design the next generation of interventions, therapies, and nootropic agents that will hopefully enact lasting improvements in public health.

REFERENCES

Acosta J.I., Mayer L., Talboom J.S., Tsang C.W., Smith C.J., Enders C.K., Bimonte-Nelson H.A. (2009a) Transitional versus surgical menopause in a rodent model: etiology of ovarian hormone loss impacts memory and the acetylcholine system. Endocrinology 150:4248-59. DOI: en.2008-1802 [pii]10.1210/en.2008-1802.

Acosta J.I., Mayer L., Talboom J.S., Zay C., Scheldrup M., Castillo J., Demers L.M., Enders C.K., Bimonte-Nelson H.A. (2009b) Premarin improves memory, prevents scopolamine-induced amnesia and increases number of basal forebrain choline acetyltransferase positive cells in middle-aged surgically menopausal rats. Horm Behav 55:454-64. DOI: S0018-506X(08)00315-2 [pii]10.1016/j.yhbeh.2008.11.008.

Adams M.M., Oung T., Morrison J.H., Gore A.C. (2001) Length of postovariectomy interval and age, but not estrogen replacement, regulate N-methyl-D-aspartate receptor mRNA levels in the hippocampus of female rats. Exp Neurol 170:345-56. DOI: 10.1006/exnr.2001.7716.

Aenlle K.K., Kumar A., Cui L., Jackson T.C., Foster T.C. (2009) Estrogen effects on cognition and hippocampal transcription in middle-aged mice. Neurobiology of Aging 30:932-945. DOI: S0197-4580(07)00378-8 [pii]10.1016/j.neurobiolaging.2007.09.004.

Albeck D.S., Beck K.D., Kung L.H., Sano K., Brennan F.X. (2005) Leverpress escape/avoidance training increases neurotrophin levels in rat brain. Integr Physiol Behav Sci 40:28-34.

Albert A. (1958) Chemical aspects of selective toxicity. Nature 182:421-2.

Almeida O.P., Lautenschlager N., Vasikaram S., Leedman P., Flicker L. (2005) Association between physiological serum concentration of estrogen and the mental health of community-dwelling postmenopausal women age 70 years and over. Am J Geriatr Psychiatry 13:142-9. DOI: 13/2/142 [pii]10.1176/appi.ajgp.13.2.142.

Alyea R.A., Watson C.S. (2009) Differential regulation of dopamine transporter function and location by low concentrations of environmental estrogens and 17beta-estradiol. Environ Health Perspect 117:778-83. DOI: 10.1289/ehp.0800026.

Alzheimer's Association. (2007) Alzheimer's disease facts and figures Retrieved on April 21. 2007, from http://www.alz.org/national/documents/Report_2007 FactsAndFigures.pdf.

Andersen P. (2007) The hippocampus book Oxford University Press, Oxford ; New York.

Anderson B.J., Rapp D.N., Baek D.H., McCloskey D.P., Coburn-Litvak P.S., Robinson J.K. (2000) Exercise influences spatial learning in the radial arm maze. Physiol Behav 70:425-9.

Ando S., Ohashi Y. (1991) Longitudinal study on age-related changes of working and reference memory in the rat. Neurosci Lett 128:17-20. DOI: 0304-3940(91)90750-N [pii].

Arendash G.W., Sanberg P.R., Sengstock G.J. (1995) Nicotine enhances the learning and memory of aged rats. Pharmacol Biochem Behav 52:517-23. DOI: 009130579500119H [pii].

Ashby J., Fletcher K., Williams C., Odum J., Tinwell H. (1997a) Lack of activity of estradiol in rodent bone marrow micronucleus assays. Mutat Res 395:83-8.

Ashby J., Odum J., Foster J.R. (1997b) Activity of raloxifene in immature and ovariectomized rat uterotrophic assays. Regul Toxicol Pharmacol 25:226-31. DOI: S0273-2300(97)91108-9 [pii]10.1006/rtph.1997.1108.

Asthana S., Craft S., Baker L.D., Raskind M.A., Birnbaum R.S., Lofgreen C.P., Veith R.C., Plymate S.R. (1999) Cognitive and neuroendocrine response to transdermal estrogen in postmenopausal women with Alzheimer's disease: results of a placebo-controlled, double-blind, pilot study. Psychoneuroendocrinology 24:657-77. DOI: S0306-4530(99)00020-7 [pii].

Balota D.A., Dolan P.O., Duchek J.M. (2000) Memory changes in healthy young and older adults, in: E. Tulving and F. I. M. Craik (Eds.), Handbook of Memory, Oxford University Press. pp. 395-410.

Baracat E., Haidar M., Lopez F.J., Pickar J., Dey M., Negro-Vilar A. (1999) Estrogen activity and novel tissue selectivity of delta8,9-dehydroestrone sulfate in postmenopausal women. J Clin Endocrinol Metab 84:2020-7.

Barnes C.A., Nadel L., Honig W.K. (1980) Spatial memory deficit in senescent rats. Can J Psychol 34:29-39.

Barrett-Connor E., Goodman-Gruen D. (1999) Cognitive function and endogenous sex hormones in older women. J Am Geriatr Soc 47:1289-93.

Beato M., Chavez S., Truss M. (1996) Transcriptional regulation by steroid hormones. Steroids 61:240-51. DOI: 0039-128X(96)00030-X [pii].

Berry B., McMahan R., Gallagher M. (1997) Spatial learning and memory at defined points of the estrous cycle: effects on performance of a hippocampal-dependent task. Behav Neurosci 111:267-74.

Bertoni-Freddari C., Fattoretti P., Solazzi M., Giorgetti B., Di Stefano G., Casoli T., Meier-Ruge W. (2003) Neuronal death versus synaptic pathology in Alzheimer's disease. Annals New York Academy of Sciences 1010:635-638.

Bhavnani B.R. (1998) Pharmacokinetics and pharmacodynamics of conjugated equine estrogens: chemistry and metabolism. Proc Soc Exp Biol Med 217:6-16.

Bhavnani B.R. (2003) Estrogens and menopause: pharmacology of conjugated equine estrogens and their potential role in the prevention of neurodegenerative diseases such as Alzheimer's. J Steroid Biochem Mol Biol 85:473-82. DOI: S0960076003002206 [pii].

Bhavnani B.R., Cecutti A., Dey M.S. (1998) Biologic effects of delta-8-estrone sulfate in postmenopausal women. J Soc Gynecol Investig 5:156-60. DOI: S1071-5576(98)00003-3 [pii].

Bhavnani B.R., Tam S.P., Lu X. (2008) Structure activity relationships and differential interactions and functional activity of various equine estrogens mediated via estrogen receptors (ERs) ERalpha and ERbeta. Endocrinology 149:4857-70. DOI: en.2008-0304 [pii]10.1210/en.2008-0304.

Bierley R.A., Rixen G.J., Troster A.I., Beatty W.W. (1986) Preserved spatial memory in old rats survives 10 months without training. Behav Neural Biol 45:223-9.

Bimonte-Nelson H.A., Acosta J.I., Talboom J.S. (2010) Neuroscientists as Cartographers: Mapping the Crossroads of Gonadal Hormones,

Memory and Age Using Animal Models. Molecules 15:6050-6105. DOI: 10.3390/molecules15096050.

Bimonte-Nelson H.A., Francis K.R., Umphlet C.D., Granholm A.C. (2006) Progesterone reverses the spatial memory enhancements initiated by tonic and cyclic oestrogen therapy in middle-aged ovariectomized female rats. Eur J Neurosci 24:229-42. DOI: EJN4867 [pii]10.1111/j.1460-9568.2006.04867.x.

Bimonte-Nelson H.A., Granholm A.C., Nelson M.E., Moore A.B. (2008) Patterns of neurotrophin protein levels in male and female Fischer 344 rats from adulthood to senescence: how young is "young" and how old is "old"? Exp Aging Res 34:13-26. DOI: 789519642 [pii]10.1080/03610730701761908.

Bimonte-Nelson H.A., Hunter C.L., Nelson M.E., Granholm A.-C.E. (2003a) Frontal cortex BDNF levels correlate with working memory in an animal model of Down syndrome. Behavioural Brain Research 139:47-57.

Bimonte-Nelson H.A., Nelson M.E., Granholm A.C. (2004a) Progesterone counteracts estrogen-induced increases in neurotrophins in the aged female rat brain. Neuroreport 15:2659-63. DOI: 00001756-200412030-00021 [pii].

Bimonte-Nelson H.A., Singleton R.S., Hunter C.L., Price K.L., Moore A.B., Granholm A.C. (2003b) Ovarian hormones and cognition in the aged female rat: I. Long-term, but not short-term, ovariectomy enhances spatial performance. Behav Neurosci 117:1395-406. DOI: 10.1037/0735-7044.117.6.13952003-10460-025 [pii].

Bimonte-Nelson H.A., Singleton R.S., Nelson M.E., Eckman C.B., Barber J., Scott T.Y., Granholm A.C. (2003c) Testosterone, but not nonaromatizable dihydrotestosterone, improves working memory and alters nerve growth factor levels in aged male rats. Exp Neurol 181:301-12. DOI: S001448860300061X [pii].

Bimonte-Nelson H.A., Singleton R.S., Williams B.J., Granholm A.C. (2004b) Ovarian hormones and cognition in the aged female rat: II. progesterone supplementation reverses the cognitive enhancing effects of ovariectomy. Behav Neurosci 118:707-14. DOI: 10.1037/0735-7044.118.4.7072004-16908-005 [pii].

Bimonte H.A., Denenberg V.H. (1999) Estradiol facilitates performance as working memory load increases. Psychoneuroendocrinology 24:161-73. DOI: S0306-4530(98)00068-7 [pii].

Bimonte H.A., Denenberg V.H. (2000) Sex differences in vicarious trial-and-error behavior during radial arm maze learning. Physiol Behav 68:495-9. DOI: S0031-9384(99)00201-2 [pii].

Bimonte H.A., Granholm A.C., Seo H., Isacson O. (2002) Spatial memory testing decreases hippocampal amyloid precursor protein in young, but not aged, female rats. Neurosci Lett 328:50-4. DOI: S0304394002004421 [pii].

Bimonte H.A., Hyde L.A., Hoplight B.J., Denenberg V.H. (2000) In two species, females exhibit superior working memory and inferior reference memory on the water radial-arm maze. Physiol Behav 70:311-7. DOI: S0031-9384(00)00259-6 [pii].

Bimonte H.A., Nelson M.E., Granholm A.C. (2003) Age-related deficits as working memory load increases: relationships with growth factors. Neurobiol Aging 24:37-48. DOI: S0197458002000155 [pii].

Bliss T., Collingridge C., Morris R. (2003) Long-term Potentiation: Enhancing neuroscience for 30 years Oxford University Press, Oxford.

Bohacek J., Bearl A.M., Daniel J.M. (2008) Long-term ovarian hormone deprivation alters the ability of subsequent oestradiol replacement to regulate choline acetyltransferase protein levels in the hippocampus and prefrontal cortex of middle-aged rats. J Neuroendocrinol 20:1023-7. DOI: JNE1752 [pii]10.1111/j.1365-2826.2008.01752.x.

Bowirrat A., Friedland R.P., Farrer L., Baldwin C., Korczyn A. (2002) Genetic and environmental risk factors for Alzheimer's disease in Israeli Arabs. J Mol Neurosci 19:239-45. DOI: JMN:19:1-2:239 [pii]10.1007/s12031-002-0040-4.

Braden B.B., Talboom J.S., Crain I.D., Simard A.R., Lukas R.J., Prokai L., Scheldrup M.R., Bowman B.L., Bimonte-Nelson H.A. (2010) Medroxyprogesterone acetate impairs memory and alters the GABAergic system in aged surgically menopausal rats. Neurobiol Learn Mem 93:444-53. DOI: S1074-7427(10)00003-1 [pii]10.1016/j.nlm.2010.01.002.

Brinton R.D., Proffitt P., Tran J., Luu R. (1997) Equilin, a principal component of the estrogen replacement therapy premarin, increases the growth of cortical neurons via an NMDA receptor-dependent mechanism. Exp Neurol 147:211-20. DOI: S0014-4886(97)96619-X [pii]10.1006/exnr.1997.6619.

Buccafusco J.J., Letchworth S.R., Bencherif M., Lippiello P.M. (2005) Long-lasting cognitive improvement with nicotinic receptor agonists: mechanisms of pharmacokinetic-pharmacodynamic discordance. Trends Pharmacol Sci 26:352-60. DOI: S0165-6147(05)00128-8 [pii]10.1016/j.tips.2005.05.007.

Bureau U.S.C. (2007) U.S. Census Bureau Word population information, http://www.census.gov/ipc/www/world.html.

Burke S.N., Barnes C.A. (2006) Neural plasticity in the ageing brain. Nat Rev Neurosci 7:30-40. DOI: nrn1809 [pii]10.1038/nrn1809.

Butcher R.L., Collins W.E., Fugo N.W. (1974) Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17beta throughout the 4-day estrous cycle of the rat. Endocrinology 94:1704-8.

Caldwell B.M., Watson R.I. (1952) An evaluation of psychologic effects of sex hormone administration in aged women. I. Results of therapy after six months. J Gerontol 7:228-44.

Calhoun M.E., Fletcher B.R., Yi S., Zentko D.C., Gallagher M., Rapp P.R. (2008) Age-related spatial learning impairment is unrelated to spinophilin immunoreactive spine number and protein levels in rat hippocampus. Neurobiol Aging 29:1256-64. DOI: S0197-4580(07)00053-X [pii]10.1016/j.neurobiolaging.2007.02.013.

Campbell S., Whitehead M. (1977a) Oestrogen therapy and the menopausal syndrome. Clin Obstet Gynaecol 4:31-47.

Campbell S., Whitehead M. (1977b) Oestrogens for menopausal flushing. Br Med J 1:104-5.

Canas P.M., Duarte J.M., Rodrigues R.J., Kofalvi A., Cunha R.A. (2009) Modification upon aging of the density of presynaptic modulation systems in the hippocampus. Neurobiology of aging 30:1877-84. DOI: 10.1016/j.neurobiolaging.2008.01.003.

Cardoso C.C., Pereira R.T., Koyama C.A., Porto C.S., Abdalla F.M. (2004) Effects of estrogen on muscarinic acetylcholine receptors in the rat

hippocampus. Neuroendocrinology 80:379-86. DOI: NEN2004080006379 [pii]10.1159/000084202.

Centeno M.L., Henderson J.A., Pau K.Y., Bethea C.L. (2006) Estradiol increases alpha7 nicotinic receptor in serotonergic dorsal raphe and noradrenergic locus coeruleus neurons of macaques. J Comp Neurol 497:489-501. DOI: 10.1002/cne.21026.

Chakraborty T.R., Gore A.C. (2004) Aging-related changes in ovarian hormones, their receptors, and neuroendocrine function. Exp Biol Med (Maywood) 229:977-87. DOI: 229/10/977 [pii].

Choi J.M., Romeo R.D., Brake W.G., Bethea C.L., Rosenwaks Z., McEwen B.S. (2003) Estradiol increases pre- and post-synaptic proteins in the CA1 region of the hippocampus in female rhesus macaques (Macaca mulatta). Endocrinology 144:4734-8. DOI: 10.1210/en.2003-0216.

Chrobak J., Hanin I., Lorens S., Napier C. (1995) Within subject decline in delayed-non-match-to-sample radial-arm maze performance in aging Sprague-Dawley rats. Behavioral Neuroscience 109:241-245.

Clarke P.B., Schwartz R.D., Paul S.M., Pert C.B., Pert A. (1985) Nicotinic binding in rat brain: autoradiographic comparison of [3H]acetylcholine, [3H]nicotine, and [125I]-alpha-bungarotoxin. J Neurosci 5:1307-15.

Cohen J. (1988) Statistical power analysis for the behavioral sciences. 2nd ed. L. Erlbaum Associates, Hillsdale, N.J.

Cohen J., Cohen P., West S.G., Aiken L.S. (2003) Applied multiple regression/correlation analysis for the behavioral sciences. 3rd ed. L. Erlbaum Associates, Mahwah, N.J.

Coker L.H., Espeland M.A., Rapp S.R., Legault C., Resnick S.M., Hogan P., Gaussoin S., Dailey M., Shumaker S.A. (2010) Postmenopausal hormone therapy and cognitive outcomes: the Women's Health Initiative Memory Study (WHIMS). The Journal of steroid biochemistry and molecular biology 118:304-10. DOI: 10.1016/j.jsbmb.2009.11.007.

Cook R., Weisberg S. (1983) Diagnostics for heteroscedasticity in regression. Biometrika 70:1-10.

Cook R., Weisberg S. (1999) Applied regression including computing and graphics Wiley, New York, NY.

Court J., Clementi F. (1995) Distribution of nicotinic subtypes in human brain. Alzheimer Dis Assoc Disord 9 Suppl 2:6-14.

Craig M.C., Maki P.M., Murphy D.G. (2005) The Women's Health Initiative Memory Study: findings and implications for treatment. Lancet Neurol 4:190-4. DOI: S1474442205010161 [pii]10.1016/S1474-4422(05)01016-1.

Curtis L., Buisson B., Bertrand S., Bertrand D. (2002) Potentiation of human alpha4beta2 neuronal nicotinic acetylcholine receptor by estradiol. Mol Pharmacol 61:127-35.

Daniel J.M., Fader A.J., Spencer A.L., Dohanich G.P. (1997) Estrogen enhances performance of female rats during acquisition of a radial arm maze. Horm Behav 32:217-25. DOI: S0018-506X(97)91433-1 [pii]10.1006/hbeh.1997.1433.

Daniel J.M., Hulst J.L., Lee C.D. (2005) Role of hippocampal M2 muscarinic receptors in the estrogen-induced enhancement of working memory. Neuroscience 132:57-64. DOI: S0306-4522(05)00097-7 [pii]10.1016/j.neuroscience.2005.01.002.

Daniel J.M., Roberts S.L., Dohanich G.P. (1999) Effects of ovarian hormones and environment on radial maze and water maze performance of female rats. Physiol Behav 66:11-20. DOI: S0031-9384(98)00272-8 [pii].

Daumas S., Halley H., Frances B., Lassalle J.M. (2005) Encoding, consolidation, and retrieval of contextual memory: differential involvement of dorsal CA3 and CA1 hippocampal subregions. Learning & memory 12:375-82. DOI: 10.1101/lm.81905.

De Ronchi D., Fratiglioni L., Rucci P., Paternico A., Graziani S., Dalmonte E. (1998) The effect of education on dementia occurrence in an Italian population with middle to high socioeconomic status. Neurology 50:1231-8.

Dellu F., Mayo W., Vallee M., Le Moal M., Simon H. (1997) Facilitation of cognitive performance in aged rats by past experience depends on the type of information processing involved: a combined cross-sectional and longitudinal study. Neurobiol Learn Mem 67:121-8. DOI: S1074742796937508 [pii].

Denenberg V.H., Sherman G.F., Schrott L.M., Rosen G.D., Galaburda A.M. (1991) Spatial learning, discrimination learning, paw preference and neocortical ectopias in two autoimmune strains of mice. Brain Res 562:98-104. DOI: 0006-8993(91)91192-4 [pii].

Denenberg V.H., Talgo N.W., Schrott L.M., Kenner G.H. (1990) A computer-aided procedure for measuring discrimination learning. Physiol Behav 47:1031-4. DOI: 0031-9384(90)90031-X [pii].

Diel P., Laudenbach-Leschowsky U., Friedel A., Voss A., Roussel J. (2005) Pulsed estradiol exposure has a limited ability to induce uterine proliferation in ovariectomised female Wistar rats. Mol Cell Endocrinol 230:7-15. DOI: S0303-7207(04)00424-1 [pii]10.1016/j.mce.2004.11.007.

Dohanich G.P. (2002) Gonadal steroids, learning and memory, in: D. W. Pfaf, et al. (Eds.), Hormones, brain and behavior, Academic Press, San Diego. pp. 267-327.

Dohanich G.P., Fader A.J., Javorsky D.J. (1994) Estrogen and estrogen-progesterone treatments counteract the effect of scopolamine on reinforced T-maze alternation in female rats. Behav Neurosci 108:988-92.

Drapeau E., Montaron M.F., Aguerre S., Abrous D.N. (2007) Learning-induced survival of new neurons depends on the cognitive status of aged rats. J Neurosci 27:6037-44. DOI: 27/22/6037 [pii]10.1523/JNEUROSCI.1031-07.2007.

Dudley S.D. (1982) Responsiveness to estradiol in central nervous system of aging female rats. Neurosci Biobehav Rev 6:39-45.

Duka T., Tasker R., McGowan J.F. (2000) The effects of 3-week estrogen hormone replacement on cognition in elderly healthy females. Psychopharmacology (Berl) 149:129-39.

Eaton J.B., Peng J.H., Schroeder K.M., George A.A., Fryer J.D., Krishnan C., Buhlman L., Kuo Y.P., Steinlein O., Lukas R.J. (2003) Characterization of human alpha 4 beta 2-nicotinic acetylcholine receptors stably and heterologously expressed in native nicotinic receptor-null SH-EP1 human epithelial cells. Mol Pharmacol 64:1283-94. DOI: 10.1124/mol.64.6.128364/6/1283 [pii].

Eichenbaum H. (2000) A cortical-hippocampal system for declarative memory. Nature Review Neuroscience 1:41-50.

El-Bakri N.K., Adem A., Suliman I.A., Mulugeta E., Karlsson E., Lindgren J.U., Winblad B., Islam A. (2002) Estrogen and progesterone treatment: effects on muscarinic M(4) receptor subtype in the rat brain. Brain Res 948:131-7. DOI: S0006899302029621 [pii].

El-Bakri N.K., Islam A., Zhu S., Elhassan A., Mohammed A., Winblad B., Adem A. (2004) Effects of estrogen and progesterone treatment on rat hippocampal NMDA receptors: relationship to Morris water maze performance. J Cell Mol Med 8:537-44. DOI: 008.004.13 [pii].

Enders C.K. (2011) Missing not at random models for latent growth curve analyses. Psychological methods 16:1-16. DOI: 10.1037/a0022640.

Enders C.K., Tofighi D. (2007) Centering predictor variables in cross-sectional multilevel models: a new look at an old issue. Psychol Methods 12:121-38. DOI: 2007-07830-001 [pii]10.1037/1082-989X.12.2.121.

Engler-Chiurazzi E., Tsang C., Nonnenmacher S., Liang W.S., Corneveaux J.J., Prokai L., Huentelman M.J., Bimonte-Nelson H.A. (2009) Tonic Premarin dose-dependently enhances memory, affects neurotrophin protein levels and alters gene expression in middle-aged rats. Neurobiol Aging. DOI: S0197-4580(09)00311-X [pii]10.1016/j.neurobiolaging.2009.09.005.

Erickson C.A., Barnes C.A. (2003) The neurobiology of memory changes in normal aging. Experimental Gerontology 38:61-69. DOI: S0531556502001602 [pii].

Esiri M. (2001) The neuropathology of Alzheimer's disease, in: D. Dawbarn and S. Allen (Eds.), Neurobiology of Alzheimer's Disease, Oxford University Press, New York, NY.

Eskin B.A., Snyder D.L., Roberts J., Aloyo V.J. (2003) Cardiac norepinephrine release: modulation by ovariectomy and estrogen. Exp Biol Med (Maywood) 228:194-9.

Espeland M.A., Rapp S.R., Shumaker S.A., Brunner R., Manson J.E., Sherwin B.B., Hsia J., Margolis K.L., Hogan P.E., Wallace R., Dailey M., Freeman R., Hays J. (2004) Conjugated equine estrogens and global cognitive function in postmenopausal women: Women's Health Initiative Memory Study. JAMA 291:2959-68. DOI: 10.1001/jama.291.24.2959291/24/2959 [pii].

Estes K.S., Brewster M.E., Bodor N. (1994) of an estradiol chemical delivery system (CDS) designed to provide enhanced and sustained hormone levels in the brain. Advanced Drug Delivery Reviews 16:167-175.

Ettmayer P., Amidon G.L., Clement B., Testa B. (2004) Lessons learned from marketed and investigational prodrugs. J Med Chem 47:2393-404. DOI: 10.1021/jm0303812.

Fader A.J., Hendricson A.W., Dohanich G.P. (1998) Estrogen improves performance of reinforced T-maze alternation and prevents the amnestic effects of scopolamine administered systemically or intrahippocampally. Neurobiol Learn Mem 69:225-40. DOI: S1074-7427(98)93820-5 [pii]10.1006/nlme.1998.3820.

Feng Z., Cheng Y., Zhang J.T. (2004) Long-term effects of melatonin or 17 beta-estradiol on improving spatial memory performance in cognitively impaired, ovariectomized adult rats. J Pineal Res 37:198-206. DOI: 10.1111/j.1600-079X.2004.00158.xJPI158 [pii].

Fernandez S.M., Frick K.M. (2004) Chronic oral estrogen affects memory and neurochemistry in middle-aged female mice. Behav Neurosci 118:1340-51. DOI: 2004-21410-020 [pii]10.1037/0735-7044.118.6.1340.

Foster T.C., Sharrow K.M., Kumar A., Masse J. (2003) Interaction of age and chronic estradiol replacement on memory and markers of brain aging. Neurobiol Aging 24:839-52. DOI: S0197458003000149 [pii].

Foy M.R. (2001) 17β-estradiol: effect on CA1 hippocampal synaptic plasticity. Neurobiology of Learning and Memory 76:239-252. DOI: 10.1006/nlme.2001.4018S1074-7427(01)94018-3 [pii].

French K.L., Granholm A.C., Moore A.B., Nelson M.E., Bimonte-Nelson H.A. (2006) Chronic nicotine improves working and reference memory performance and reduces hippocampal NGF in aged female rats. Behav Brain Res 169:256-62. DOI: S0166-4328(06)00046-5 [pii]10.1016/j.bbr.2006.01.008.

Frick K.M., Baxter M.G., Markowska A.L., Olton D.S., Price D.L. (1995) Age-related spatial reference and working memory deficits assessed in the water maze. Neurobiol Aging 16:149-60. DOI: 0197458094001553 [pii].

Frick K.M., Benoit J.D. (2010) Use it or lose it: environmental enrichment as a means to promote successful cognitive aging. ScientificWorldJournal 10:1129-41. DOI: 10.1100/tsw.2010.111.

Frick K.M., Fernandez S.M., Bennett J.C., Prange-Kiel J., MacLusky N.J., Leranth C. (2004) Behavioral training interferes with the ability of gonadal hormones to increase CA1 spine synapse density in ovariectomized female rats. Eur J Neurosci 19:3026-32. DOI: 10.1111/j.0953-816X.2004.03427.xEJN3427 [pii].

Frick K.M., Fernandez S.M., Bulinski S.C. (2002) Estrogen replacement improves spatial reference memory and increases hippocampal synaptophysin in aged female mice. Neuroscience 115:547-58. DOI: S0306452202003779 [pii].

Fritsch T., McClendon M.J., Smyth K.A., Ogrocki P.K. (2002) Effects of educational attainment and occupational status on cognitive and functional decline in persons with Alzheimer-type dementia. Int Psychogeriatr 14:347-63.

Gabor R., Nagle R., Johnson D.A., Gibbs R.B. (2003) Estrogen enhances potassium-stimulated acetylcholine release in the rat hippocampus. Brain Res 962:244-7. DOI: S0006899302040532 [pii].

Galea L.A., Wide J.K., Paine T.A., Holmes M.M., Ormerod B.K., Floresco S.B. (2001) High levels of estradiol disrupt conditioned place preference learning, stimulus response learning and reference memory but have limited effects on working memory. Behav Brain Res 126:115-26. DOI: S0166432801002558 [pii].

Gatz M., Svedberg P., Pedersen N.L., Mortimer J.A., Berg S., Johansson B. (2001) Education and the risk of Alzheimer's disease: findings from the study of dementia in Swedish twins. J Gerontol B Psychol Sci Soc Sci 56:P292-300.

Gazzaley A.H., Siegel S.J., Kordower J.H., Mufson E.J., Morrison J.H. (1996a) Circuit-specific alterations of N-methyl-D-aspartate receptor subunit 1 in the dentate gyrus of aged monkeys. Proc Natl Acad Sci U S A 93:3121-5.

Gazzaley A.H., Weiland N.G., McEwen B.S., Morrison J.H. (1996b) Differential regulation of NMDAR1 mRNA and protein by estradiol in the rat hippocampus. J Neurosci 16:6830-8.

Geinisman Y. (2000) Structural synaptic modifications associated with hippocampal LTP and behavioral learning. Cerebral Cortex 10:952-962.

Geinisman Y., de Toledo-Morrell L., Morrell F. (1986) Loss of perforated synapses in the dentate gyrus: morphological substrate of memory deficit in aged rats. Proc Natl Acad Sci U S A 83:3027-31.

Gibbs R.B. (1996) Expression of estrogen receptor-like immunoreactivity by different subgroups of basal forebrain cholinergic neurons in gonadectomized male and female rats. Brain Res 720:61-8. DOI: 0006-8993(96)00106-0 [pii]10.1016/0006-8993(96)00106-0.

Gibbs R.B. (1997) Effects of estrogen on basal forebrain cholinergic neurons vary as a function of dose and duration of treatment. Brain Res 757:10-6. DOI: S0006-8993(96)01432-1 [pii].

Gibbs R.B. (1998) Levels of trkA and BDNF mRNA, but not NGF mRNA, fluctuate across the estrous cycle and increase in response to acute hormone replacement. Brain Res 810:294.

Gibbs R.B. (1999) Estrogen replacement enhances acquisition of a spatial memory task and reduces deficits associated with hippocampal muscarinic receptor inhibition. Horm Behav 36:222-33. DOI: 10.1006/hbeh.1999.1541S0018-506X(99)91541-6 [pii].

Gibbs R.B. (2000a) Effects of gonadal hormone replacement on measures of basal forebrain cholinergic function. Neuroscience 101:931-8. DOI: S0306-4522(00)00433-4 [pii].

Gibbs R.B. (2000b) Long-term treatment with estrogen and progesterone enhances acquisition of a spatial memory task by ovariectomized aged rats. Neurobiol Aging 21:107-16. DOI: S0197-4580(00)00103-2 [pii].

Gibbs R.B. (2002) Basal forebrain cholinergic neurons are necessary for estrogen to enhance acquisition of a delayed matching-to-position T-maze task. Horm Behav 42:245-57. DOI: S0018506X02918258 [pii].

Gibbs R.B. (2007) Estradiol enhances DMP acquisition via a mechanism not mediated by turning strategy but which requires intact basal forebrain cholinergic projections. Horm Behav 52:352-9. DOI: S0018-506X(07)00131-6 [pii]10.1016/j.yhbeh.2007.05.011.

Gibbs R.B., Aggarwal P. (1998) Estrogen and basal forebrain cholinergic neurons: implications for brain aging and Alzheimer's disease-related cognitive decline. Horm Behav 34:98-111. DOI: S0018-506X(98)91451-9 [pii]10.1006/hbeh.1998.1451.

Gibbs R.B., Gabor R., Cox T., Johnson D.A. (2004) Effects of raloxifene and estradiol on hippocampal acetylcholine release and spatial learning in the rat. Psychoneuroendocrinology 29:741-8. DOI: 10.1016/S0306-4530(03)00118-5S0306453003001185 [pii].

Gibbs R.B., Johnson D.A. (2008) Sex-specific effects of gonadectomy and hormone treatment on acquisition of a 12-arm radial maze task by Sprague Dawley rats. Endocrinology 149:3176-83. DOI: en.2007-1645 [pii]10.1210/en.2007-1645.

Gibbs R.B., Pfaff D.W. (1992) Effects of estrogen and fimbria/fornix transection on p75NGFR and ChAT expression in the medial septum and diagonal band of Broca. Exp Neurol 116:23-39. DOI: 0014-4886(92)90173-N [pii].

Gibbs R.B., Wu D., Hersh L.B., Pfaff D.W. (1994) Effects of estrogen replacement on the relative levels of choline acetyltransferase, trkA, and nerve growth factor messenger RNAs in the basal forebrain and hippocampal formation of adult rats. Exp Neurol 129:70-80. DOI: S0014-4886(84)71148-4 [pii]10.1006/exnr.1994.1148.

Goldman J.M., Murr A.S., Cooper R.L. (2007) The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. Birth Defects Res B Dev Reprod Toxicol 80:84-97. DOI: 10.1002/bdrb.20106.

Gould E., Woolley C.S., Frankfurt M., McEwen B.S. (1990) Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. J Neurosci 10:1286-91.

Granholm A.C. (2000) Oestrogen and nerve growth factor - neuroprotection and repair in Alzheimer's disease. Expert Opin Investig Drugs 9:685-94. DOI: 10.1517/13543784.9.4.685.

Granholm A.C., Ford K.A., Hyde L.A., Bimonte H.A., Hunter C.L., Nelson M., Albeck D., Sanders L.A., Mufson E.J., Crnic L.S. (2002) Estrogen restores cognition and cholinergic phenotype in an animal model of Down syndrome. Physiol Behav 77:371-85. DOI: S0031938402008843 [pii].

Granholm A.C., Sanders L., Seo H., Lin L., Ford K., Isacson O. (2003) Estrogen alters amyloid precursor protein as well as dendritic and cholinergic markers in a mouse model of Down syndrome. Hippocampus 13:905-14. DOI: 10.1002/hipo.10130.

Gresack J.E., Frick K.M. (2004) Environmental enrichment reduces the mnemonic and neural benefits of estrogen. Neuroscience 128:459-71. DOI: 10.1016/j.neuroscience.2004.06.011.

Hallahan M., Rosenthal R. (2000) Interpreting and reporting results, in: H. E. A. Tinsley and S. D. Brown (Eds.), Handbook of multivariate statistics and mathematical modeling, Academic Press, San Diego, CA. pp. 183-208.

Hammond R., Mauk R., Ninaci D., Nelson D., Gibbs R.B. (2009) Chronic treatment with estrogen receptor agonists restores acquisition of a spatial learning task in young ovariectomized rats. Horm Behav 56:309-14. DOI: S0018-506X(09)00138-X [pii]10.1016/j.yhbeh.2009.06.008.

Harris K.M., Fiala J.C., Ostroff L. (2003) Structural changes at dendritic spine synapses during long-term potentiation. Philos Trans R Soc Lond B Biol Sci 358:745-8. DOI: 10.1098/rstb.2002.1254.

Hasselmo M.E. (2006) The role of acetylcholine in learning and memory. Curr Opin Neurobiol 16:710-5. DOI: S0959-4388(06)00122-X [pii]10.1016/j.conb.2006.09.002.

Henny P., Jones B.E. (2008) Projections from basal forebrain to prefrontal cortex comprise cholinergic, GABAergic and glutamatergic inputs to pyramidal cells or interneurons. Eur J Neurosci 27:654-70. DOI: 10.1111/j.1460-9568.2008.06029.x.

Hersh A.L., Stefanick M.L., Stafford R.S. (2004) National use of postmenopausal hormone therapy: annual trends and response to recent evidence. JAMA 291:47-53. DOI: 10.1001/jama.291.1.47291/1/47 [pii].

Hiscock J.J., Murphy S., Willoughby J.O. (2000) Confocal microscopic estimation of GABAergic nerve terminals in the central nervous system. Journal of neuroscience methods 95:1-11.

Hof P.R., Morrison J.H. (2004) The aging brain: Morphomolecular senescence of cortical circuits. Trends in Neurosciences 27:607-613.

Holmes M.M., Wide J.K., Galea L.A. (2002) Low levels of estradiol facilitate, whereas high levels of estradiol impair, working memory performance on the radial arm maze. Behav Neurosci 116:928-34.

Hruska Z., Dohanich G.P. (2007) The effects of chronic estradiol treatment on working memory deficits induced by combined infusion of beta-amyloid (1-42) and ibotenic acid. Horm Behav 52:297-306. DOI: S0018-506X(07)00110-9 [pii]10.1016/j.yhbeh.2007.05.010.

Huentelman M.J., Stephan D.A., Talboom J., Corneveaux J.J., Reiman D.M., Gerber J.D., Barnes C.A., Alexander G.E., Reiman E.M., Bimonte-Nelson H.A. (2009) Peripheral delivery of a ROCK inhibitor improves learning and working memory. Behav Neurosci 123:218-23. DOI: 2009-00623-015 [pii]10.1037/a0014260.

Hunter C.L., Bimonte H.A., Granholm A.C. (2003) Behavioral comparison of 4 and 6 month-old Ts65Dn mice: age-related impairments in working and reference memory. Behav Brain Res 138:121-31. DOI: S0166432802002759 [pii].

Huynh T.N., Krigbaum A.M., Hanna J.J., Conrad C.D. (2011) Sex differences and phase of light cycle modify chronic stress effects on anxiety and depressive-like behavior. Behav Brain Res 222:212-22. DOI: 10.1016/j.bbr.2011.03.038.

Hyde L.A., Hoplight B.J., Denenberg V.H. (1998) Water version of the radial-arm maze: learning in three inbred strains of mice. Brain Res 785:236-44. DOI: S0006-8993(97)01417-0 [pii].

Hyde L.A., Sherman G.F., Hoplight B.J., Denenberg V.H. (2000) Working memory deficits in BXSB mice with neocortical ectopias. Physiol Behav 70:1-5. DOI: S0031-9384(00)00239-0 [pii].

Izquierdo I., Medina J.H., Bianchin M., Walz R., Zanatta M.S., Da Silva R.C., Bueno e Silva M., Ruschel A.C., Paczko N. (1993) Memory processing by the limbic system: role of specific neurotransmitter systems. Behav Brain Res 58:91-8.

Jarrard L.E. (1993) On the role of the hippocampus in learning and memory in the rat. Behav Neural Biol 60:9-26.

Jarrard L.E., Okaichi H., Steward O., Goldschmidt R.B. (1984) On the role of hippocampal connections in the performance of place and cue tasks: comparisons with damage to hippocampus. Behav Neurosci 98:946-54.

Jelks K.B., Wylie R., Floyd C.L., McAllister A.K., Wise P. (2007) Estradiol targets synaptic proteins to induce glutamatergic synapse formation in cultured hippocampal neurons: critical role of estrogen receptor-alpha. J Neurosci 27:6903-13. DOI: 10.1523/JNEUROSCI.0909-07.2007.

Ji D., Dani J.A. (2000) Inhibition and disinhibition of pyramidal neurons by activation of nicotinic receptors on hippocampal interneurons. J Neurophysiol 83:2682-90.

Johansson I.M., Birzniece V., Lindblad C., Olsson T., Backstrom T. (2002) Allopregnanolone inhibits learning in the Morris water maze. Brain Res 934:125-31. DOI: S0006899302024149 [pii].

Jones M.W. (2002) A comparative review of rodent prefrontal cortex and working memory. Curr Mol Med 2:639-47.

Kantor H.I., Michael C.M., Shore H. (1973) Estrogen for older women. Am J Obstet Gynecol 116:115-8.

Kasai H., Matsuzaki M., Noguchi J., Yasumatsu N., Nakahara H. (2003) Structure-stability-function relationships of dendritic spines. Trends in the Neurosciences 26:360-368.

Katzman R., Terry R., DeTeresa R., Brown T., Davies P., Fuld P., Renbing X., Peck A. (1988) Clinical, pathological, and neurochemical changes in dementia: a subgroup with preserved mental status and numerous neocortical plaques. Ann Neurol 23:138-44. DOI: 10.1002/ana.410230206.

Kausler D.H. (1994) Learning and Memory in Normal Aging Academic Press, New York, NY.

Keppel G., Wickens T.D. (2004) Design and analysis : a researcher's handbook. 4th ed. Pearson Prentice Hall, Upper Saddle River, N.J.

Khan M., De Sevilla L., Mahesh V.B., Brann D.W. (2010) Enhanced glutamatergic and decreased GABAergic synaptic appositions to GnRH neurons on proestrus in the rat: modulatory effect of aging. PLoS One 5:e10172. DOI: 10.1371/journal.pone.0010172.

Klein R., Berlin L. (1996) Benefits and risks of hormone replacement therapy. In: Pavlik E. J., (Ed). Estrogens, Progestins and Their Antagonists. . Birkha¨user, Boston pp. 3–50.

Klempin F., Kempermann G. (2007) Adult hippocampal neurogenesis and aging. Eur Arch Psychiatry Clin Neurosci 257:271-80. DOI: 10.1007/s00406-007-0731-5.

Kompoliti K., Chu Y., Polish A., Roberts J., McKay H., Mufson E.J., Leurgans S., Morrison J.H., Kordower J.H. (2004) Effects of estrogen replacement therapy on cholinergic basal forebrain neurons and cortical cholinergic innervation in young and aged ovariectomized rhesus monkeys. J Comp Neurol 472:193-207. DOI: 10.1002/cne.20050.

Konopacki J., Golebiewski H., Eckersdorf B. (1992) Carbachol-induced theta-like activity in entorhinal cortex slices. Brain Res 572:76-80. DOI: 0006-8993(92)90453-G [pii].

Korol D.L., Kolo L.L. (2002) Estrogen-induced changes in place and response learning in young adult female rats. Behav Neurosci 116:411-20.

Kramer E., Bodnar R.J. (1986) Age-related decrements in the analgesic response to cold-water swims. Physiol Behav 36:875-80.

Kuhl H. (2005) Pharmacology of estrogens and progestogens: influence of different routes of administration. Climacteric 8 Suppl 1:3-63. DOI: R1P31542521JL4X6 [pii]10.1080/13697130500148875.

Lapchak P.A., Araujo D.M., Quirion R., Beaudet A. (1990) Chronic estradiol treatment alters central cholinergic function in the female rat: effect on choline acetyltransferase activity, acetylcholine content, and nicotinic autoreceptor function. Brain Res 525:249-55. DOI: 0006-8993(90)90871-8 [pii].

Lebrun C.E., van der Schouw Y.T., de Jong F.H., Pols H.A., Grobbee D.E., Lamberts S.W. (2005) Endogenous oestrogens are related to cognition in healthy elderly women. Clin Endocrinol (Oxf) 63:50-5. DOI: CEN2297 [pii]10.1111/j.1365-2265.2005.02297.x.

Letenneur L., Gilleron V., Commenges D., Helmer C., Orgogozo J.M., Dartigues J.F. (1999) Are sex and educational level independent predictors of dementia and Alzheimer's disease? Incidence data from the PAQUID project. J Neurol Neurosurg Psychiatry 66:177-83.

Levi-Montalcini R. (1987) The nerve growth factor 35 years later. Science 237:1154-62.

Liu H.L., Zhao G., Cai K., Zhao H.H., Shi L.D. (2011) Treadmill exercise prevents decline in spatial learning and memory in APP/PS1 transgenic mice through improvement of hippocampal long-term potentiation. Behav Brain Res 218:308-14. DOI: 10.1016/j.bbr.2010.12.030.

Liu P., Smith P.F., Darlington C.L. (2008) Glutamate receptor subunits expression in memory-associated brain structures: regional variations and effects of aging. Synapse 62:834-41. DOI: 10.1002/syn.20563.

Luine V., Rodriguez M. (1994) Effects of estradiol on radial arm maze performance of young and aged rats. Behav Neural Biol 62:230-6.

Luine V.N. (1985) Estradiol increases choline acetyltransferase activity in specific basal forebrain nuclei and projection areas of female rats. Exp Neurol 89:484-90. DOI: 0014-4886(85)90108-6 [pii].

Luine V.N., Jacome L.F., Maclusky N.J. (2003) Rapid enhancement of visual and place memory by estrogens in rats. Endocrinology 144:2836-44.

Luine V.N., Richards S.T., Wu V.Y., Beck K.D. (1998a) Estradiol enhances learning and memory in a spatial memory task and effects levels of monoaminergic neurotransmitters. Horm Behav 34:149-62. DOI: 10.1006/hbeh.1998.1473.

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

Lukas R.J., Fryer J.D., Eaton J.B., Gentry C.L. (2002) Some methods for studies of nicotinic acetylcholine receptor pharmacology, in: E. D. Levin (Ed.), Nicotinic Receptors and the Nervous System, CRC Press, Boca Raton. pp. 3–27.

Lupien S.J., Maheu F., Tu M., Fiocco A., Schramek T.E. (2007) The effects of stress and stress hormones on human cognition: Implications for the field of brain and cognition. Brain and cognition 65:209-37. DOI: 10.1016/j.bandc.2007.02.007.

MacLusky N.J., Hajszan T., Prange-Kiel J., Leranth C. (2006) Androgen modulation of hippocampal synaptic plasticity. Neuroscience

138:957-65. DOI: S0306-4522(06)00041-8 [pii]10.1016/j.neuroscience.2005.12.054.

Mark C.J., Tatchum-Talom R., Martin D.S., Eyster K.M. (2007) Effects of estrogens and selective estrogen receptor modulators on vascular reactivity in the perfused mesenteric vascular bed. Am J Physiol Regul Integr Comp Physiol 293:R1969-75. DOI: 00260.2007 [pii]10.1152/ajpregu.00260.2007.

Markham J.A., Pych J.C., Juraska J.M. (2002) Ovarian hormone replacement to aged ovariectomized female rats benefits acquisition of the morris water maze. Horm Behav 42:284-93. DOI: S0018506X02918192 [pii].

Markowska A.L., Savonenko A.V. (2002a) Effectiveness of estrogen replacement in restoration of cognitive function after long-term estrogen withdrawal in aging rats. J Neurosci 22:10985-95. DOI: 22/24/10985 [pii].

Markowska A.L., Savonenko A.V. (2002b) Protective effect of practice on cognition during aging: implications for predictive characteristics of performance and efficacy of practice. Neurobiol Learn Mem 78:294-320. DOI: S1074742702940645 [pii].

Marriott L.K., Korol D.L. (2003) Short-term estrogen treatment in ovariectomized rats augments hippocampal acetylcholine release during place learning. Neurobiol Learn Mem 80:315-22. DOI: S107474270300090X [pii].

Matzel L.D., Light K.R., Wass C., Colas-Zelin D., Denman-Brice A., Waddel A.C., Kolata S. (2011) Longitudinal attentional engagement rescues mice from age-related cognitive declines and cognitive inflexibility. Learn Mem 18:345-56. DOI: 10.1101/lm.2034711.

Mayer P., Tse S., Sendi M., Bourg D., Morrison D. (2008) Steady-state pharmacokinetics of conjugated equine estrogens in healthy, postmenopausal women. J Reprod Med 53:97-101.

McLaughlin K.J., Bimonte-Nelson H., Neisewander J.L., Conrad C.D. (2008) Assessment of estradiol influence on spatial tasks and hippocampal CA1 spines: evidence that the duration of hormone deprivation after ovariectomy compromises 17beta-estradiol effectiveness in altering CA1 spines. Horm Behav 54:386-95. DOI: 10.1016/j.yhbeh.2008.04.010.

McMillan P.J., Singer C.A., Dorsa D.M. (1996) The effects of ovariectomy and estrogen replacement on trkA and choline acetyltransferase mRNA expression in the basal forebrain of the adult female Sprague-Dawley rat. J Neurosci 16:1860-5.

Mei C., Dauphin M. (2003) Mixture Modeling, ImageJ Image Processing and Analysis in Java, National Institutes of Health Washington D.C.

Morris R.G., Anderson E., Lynch G.S., Baudry M. (1986) Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. Nature 319:774-6. DOI: 10.1038/319774a0.

Morris R.G., Garrud P., Rawlins J.N., O'Keefe J. (1982) Place navigation impaired in rats with hippocampal lesions. Nature 297:681-3.

Moser M.B. (1999) Making more synapses: a way to store information? Cellular and Molecular Life Science 55:593-600.

Mufson E.J., Conner J.M., Kordower J.H. (1995) Nerve growth factor in Alzheimer's disease: defective retrograde transport to nucleus basalis. Neuroreport 6:1063-6.

Murphy D.D., Cole N.B., Greenberger V., Segal M. (1998) Estradiol increases dendritic spine density by reducing GABA neurotransmission in hippocampal neurons. J Neurosci 18:2550-9.

Nappi R.E., Sinforiani E., Mauri M., Bono G., Polatti F., Nappi G. (1999) Memory functioning at menopause: impact of age in ovariectomized women. Gynecol Obstet Invest 47:29-36. DOI: goi47029 [pii].

Nicholson D.A., Yoshida R., Berry R.W., Gallagher M., Geinisman Y. (2004) Reduction in size of perforated postsynaptic densities in hippocampal axospinous synapses and age-related spatial learning impairments. J Neurosci 24:7648-53. DOI: 10.1523/JNEUROSCI.1725-04.2004.

Nilsen J., Brinton R.D. (2002a) Impact of progestins on estradiol potentiation of the glutamate calcium response. Neuroreport 13:825-30.

Nilsen J., Brinton R.D. (2002b) Impact of progestins on estrogen-induced neuroprotection: synergy by progesterone and 19-norprogesterone and antagonism by medroxyprogesterone acetate. Endocrinology 143:205-12.

Nimchinsky E.A., Sabatini B.L., Svoboda K. (2002) Structure and function of dendritic spines. Annu Rev Physiol 64:313-353.

O'Keefe J., Nadel L. (1978) The Hippocampus as a Cognitive Map Clarendon Press, Oxford, England.

O'Malley C.A., Hautamaki R.D., Kelley M., Meyer E.M. (1987) Effects of ovariectomy and estradiol benzoate on high affinity choline uptake, ACh synthesis, and release from rat cerebral cortical synaptosomes. Brain Res 403:389-92.

Ohkura T., Isse K., Akazawa K., Hamamoto M., Yaoi Y., Hagino N. (1995) Long-term estrogen replacement therapy in female patients with dementia of the Alzheimer type: 7 case reports. Dementia 6:99-107.

Ott A., van Rossum C.T., van Harskamp F., van de Mheen H., Hofman A., Breteler M.M. (1999) Education and the incidence of dementia in a large population-based study: the Rotterdam Study. Neurology 52:663-6.

Packard M.G., Teather L.A. (1997) Posttraining estradiol injections enhance memory in ovariectomized rats: cholinergic blockade and synergism. Neurobiol Learn Mem 68:172-88. DOI: S1074-7427(97)93785-0 [pii]10.1006/nlme.1997.3785.

Pan Y., Anthony M., Clarkson T.B. (1999) Effect of estradiol and soy phytoestrogens on choline acetyltransferase and nerve growth factor mRNAs in the frontal cortex and hippocampus of female rats. Proc Soc Exp Biol Med 221:118-25.

Pang T.Y., Stam N.C., Nithianantharajah J., Howard M.L., Hannan A.J. (2006) Differential effects of voluntary physical exercise on behavioral and brain-derived neurotrophic factor expression deficits in Huntington's disease transgenic mice. Neuroscience 141:569-84. DOI: 10.1016/j.neuroscience.2006.04.013.

Pappas B.A., Payne K.B., Fortin T., Sherren N. (2005) Neonatal lesion of forebrain cholinergic neurons: further characterization of behavioral effects and permanency. Neuroscience 133:485-92. DOI: S0306-4522(05)00250-2 [pii]10.1016/j.neuroscience.2005.02.040.

Paradiso K., Zhang J., Steinbach J.H. (2001) The C terminus of the human nicotinic alpha4beta2 receptor forms a binding site required

for potentiation by an estrogenic steroid. J Neurosci 21:6561-8. DOI: 21/17/6561 [pii].

Paxinos G., Watson C. (2005) The Rat Brain in Stereotaxic Coordinates, 5 ed. 5 ed. Elsevier Academic Press, New York.

Phillips S.M., Sherwin B.B. (1992) Effects of estrogen on memory function in surgically menopausal women. Psychoneuroendocrinology 17:485-95.

Pitsikas N., Algeri S. (1992) Deterioration of spatial and nonspatial reference and working memory in aged rats: protective effect of life-long calorie restriction. Neurobiol Aging 13:369-73.

Pitsikas N., Biagini L., Algeri S. (1991) Previous experience facilitates preservation of spatial memory in the senescent rat. Physiol Behav 49:823-5. DOI: 0031-9384(91)90325-I [pii].

Prokai-Tatrai K., Perjesi P., Rivera-Portalatin N.M., Simpkins J.W., Prokai L. (2008) Mechanistic investigations on the antioxidant action of a neuroprotective estrogen derivative. Steroids 73:280-8. DOI: S0039-128X(07)00209-7 [pii]10.1016/j.steroids.2007.10.011.

Prokai-Tatrai K., Rivera-Portalatin N., Rauniyar N., Prokai L. (2007) A facile microwave-assisted synthesis of p-Quinols by lead(IV) acetate oxidation. Letters in Organic Chemistry 4:3.

Prokai L., Prokai-Tatrai K. (1999) Metabolism-based drug design and drug targeting. Pharm Sci Technolo Today 2:457-462. DOI: S1461534799002084 [pii].

Prokai L., Prokai-Tatrai K., Bodor N. (2000) Targeting drugs to the brain by redox chemical delivery systems. Med Res Rev 20:367-416. DOI: 10.1002/1098-1128(200009)20:5<367::AID-MED3>3.0.CO;2-P [pii].

Prokai L., Prokai-Tatrai K., Perjesi P., Zharikova A.D., Perez E.J., Liu R., Simpkins J.W. (2003) Quinol-based cyclic antioxidant mechanism in estrogen neuroprotection. Proc Natl Acad Sci U S A 100:11741-6. DOI: 10.1073/pnas.2032621100 [pii].

Prokai L., Simpkins J.W. (2007) Structure-nongenomic neuroprotection relationship of estrogens and estrogen-derived compounds. Pharmacol Ther 114:1-12. DOI: S0163-7258(07)00019-8 [pii]10.1016/j.pharmthera.2007.01.006.

Prossnitz E.R., Arterburn J.B., Smith H.O., Oprea T.I., Sklar L.A., Hathaway H.J. (2008a) Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. Annu Rev Physiol 70:165-90. DOI: 10.1146/annurev.physiol.70.113006.100518.

Prossnitz E.R., Sklar L.A., Oprea T.I., Arterburn J.B. (2008b) GPR30: a novel therapeutic target in estrogen-related disease. Trends Pharmacol Sci 29:116-23. DOI: S0165-6147(08)00024-2 [pii]10.1016/j.tips.2008.01.001.

Rasband W. (1997-2004) ImageJ National Institute of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/.

Resnick S.M., Espeland M.A., Jaramillo S.A., Hirsch C., Stefanick M.L., Murray A.M., Ockene J., Davatzikos C. (2009) Postmenopausal hormone therapy and regional brain volumes: the WHIMS-MRI Study. Neurology 72:135-42. DOI: 72/2/135 [pii]10.1212/01.wnl.0000339037.76336.cf.

Restle F. (1957) Discrimination of cues in mazes: a resolution of the place-vs.-response question. Psychol Rev 64:217-28.

Riekkinen M., Riekkinen P., Jr. (1997) Nicotine and D-cycloserine enhance acquisition of water maze spatial navigation in aged rats. Neuroreport 8:699-703.

Rissanen A., Puolivali J., van Groen T., Riekkinen P., Jr. (1999) In mice tonic estrogen replacement therapy improves non-spatial and spatial memory in a water maze task. Neuroreport 10:1369-72.

Rivera-Portalatin N.M., Vera-Serrano J.L., Prokai-Tatrai K., Prokai L. (2007) Comparison of estrogen-derived ortho-quinone and para-quinol concerning induction of oxidative stress. J Steroid Biochem Mol Biol 105:71-5. DOI: S0960-0760(07)00104-5 [pii]10.1016/j.jsbmb.2006.11.025.

Rodgers S.P., Bohacek J., Daniel J.M. (2010) Transient estradiol exposure during middle age in ovariectomized rats exerts lasting effects on cognitive function and the hippocampus. Endocrinology 151:1194-203. DOI: en.2009-1245 [pii]10.1210/en.2009-1245.

Roman O., Seres J., Herichova I., Zeman M., Jurcovicova J. (2003) Daily profiles of plasma prolactin (PRL), growth hormone (GH), insulin-like growth factor-1 (IGF-1), luteinizing hormone (LH), testosterone,

and melatonin, and of pituitary PRL mRNA and GH mRNA in male Long Evans rats in acute phase of adjuvant arthritis. Chronobiol Int 20:823-36.

Rosenthal R., Rosnow R.L. (1985) Contrast analysis : focused comparisons in the analysis of variance Cambridge University Press, Cambridge Cambridgeshire ; New York.

Rosenzweig E.S., Barnes C.A. (2003) Impact of aging on hippocampal function: plasticity, network dynamics, and cognition. Prog Neurobiol 69:143-79.

Rouse S.T., Marino M.J., Potter L.T., Conn P.J., Levey A.I. (1999) Muscarinic receptor subtypes involved in hippocampal circuits. Life Sci 64:501-9.

Ryan J., Stanczyk F.Z., Dennerstein L., Mack W.J., Clark M.S., Szoeke C., Kildea D., Henderson V.W. (2010) Hormone levels and cognitive function in postmenopausal midlife women. Neurobiol Aging. DOI: 10.1016/j.neurobiolaging.2010.07.014.

Sage D., Neumann F.R., Hediger F., Gasser S.M., Unser M. (2005) Automatic tracking of individual fluorescence particles: application to the study of chromosome dynamics. IEEE Trans Image Process 14:1372-83.

Sandstrom N.J., Williams C.L. (2001) Memory retention is modulated by acute estradiol and progesterone replacement. Behav Neurosci 115:384-93.

Sandstrom N.J., Williams C.L. (2004) Spatial memory retention is enhanced by acute and continuous estradiol replacement. Horm Behav 45:128-35. DOI: 10.1016/j.yhbeh.2003.09.010S0018506X0300237X [pii].

Savonenko A.V., Markowska A.L. (2003) The cognitive effects of ovariectomy and estrogen replacement are modulated by aging. Neuroscience 119:821-30. DOI: S0306452203002136 [pii].

Schenk F., Morris R.G. (1985) Dissociation between components of spatial memory in rats after recovery from the effects of retrohippocampal lesions. Exp Brain Res 58:11-28.

Sherwin B.B. (1988) Estrogen and/or androgen replacement therapy and cognitive functioning in surgically menopausal women.

Psychoneuroendocrinology 13:345-57. DOI: 0306-4530(88)90060-1 [pii].

Sherwin B.B. (2005) Estrogen and memory in women: how can we reconcile the findings? Horm Behav 47:371-5. DOI: S0018-506X(04)00268-5 [pii]10.1016/j.yhbeh.2004.12.002.

Sherwin B.B. (2006) Estrogen and cognitive aging in women. Neuroscience 138:1021-6. DOI: S0306-4522(05)00842-0 [pii]10.1016/j.neuroscience.2005.07.051.

Sherwin B.B., Henry J.F. (2008) Brain aging modulates the neuroprotective effects of estrogen on selective aspects of cognition in women: a critical review. Front Neuroendocrinol 29:88-113. DOI: S0091-3022(07)00050-7 [pii]10.1016/j.yfrne.2007.08.002.

Shimamura A., Berry J., Mangels J., Rustig C. (1995) Memory and Cognitive Abilities in University Professors. Psychological Science 6:271-277.

Shukitt-Hale B., Mouzakis G., Joseph J.A. (1998) Psychomotor and spatial memory performance in aging male Fischer 344 rats. Exp Gerontol 33:615-24. DOI: S0531556598000242 [pii].

Shumaker S.A., Legault C., Kuller L., Rapp S.R., Thal L., Lane D.S., Fillit H., Stefanick M.L., Hendrix S.L., Lewis C.E., Masaki K., Coker L.H. (2004) Conjugated equine estrogens and incidence of probable dementia and mild cognitive impairment in postmenopausal women: Women's Health Initiative Memory Study. JAMA 291:2947-58. DOI: 10.1001/jama.291.24.2947291/24/2947 [pii].

Shumaker S.A., Legault C., Rapp S.R., Thal L., Wallace R.B., Ockene J.K., Hendrix S.L., Jones B.N., 3rd, Assaf A.R., Jackson R.D., Kotchen J.M., Wassertheil-Smoller S., Wactawski-Wende J. (2003) Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. JAMA 289:2651-62. DOI: 10.1001/jama.289.20.2651289/20/2651 [pii].

Shumaker S.A., Reboussin B.A., Espeland M.A., Rapp S.R., McBee W.L., Dailey M., Bowen D., Terrell T., Jones B.N. (1998) The Women's Health Initiative Memory Study (WHIMS): a trial of the effect of estrogen therapy in preventing and slowing the progression of

dementia. Control Clin Trials 19:604-21. DOI: S0197245698000385 [pii].

Siegel G.J., Chauhan N.B. (2000) Neurotrophic factors in Alzheimer's and Parkinson's disease brain. Brain Res Brain Res Rev 33:199-227. DOI: S0165017300000308 [pii].

Silva I., Mello L.E., Freymuller E., Haidar M.A., Baracat E.C. (2000) Estrogen, progestogen and tamoxifen increase synaptic density of the hippocampus of ovariectomized rats. Neuroscience Letters 291:183-186.

Singer C.A., McMillan P.J., Dobie D.J., Dorsa D.M. (1998) Effects of estrogen replacement on choline acetyltransferase and trkA mRNA expression in the basal forebrain of aged rats. Brain Res 789:343-6. DOI: S0006-8993(98)00142-5 [pii].

Singh G.K., Kochanek K.D., MacDorman M.F. (1996) Advance report of final mortality statistics, 1994. Monthly Vital Statistics Report 45:1–80.

Singh M., Meyer E.M., Millard W.J., Simpkins J.W. (1994) Ovarian steroid deprivation results in a reversible learning impairment and compromised cholinergic function in female Sprague-Dawley rats. Brain Res 644:305-12. DOI: 0006-8993(94)91694-2 [pii].

Sitruk-Ware R. (2002) Hormonal replacement therapy. Rev Endocr Metab Disord 3:243-56.

Smith D.C., Prentice R., Thompson D.J., Herrmann W.L. (1975) Association of exogenous estrogen and endometrial carcinoma. N Engl J Med 293:1164-7.

Socci D.J., Sanberg P.R., Arendash G.W. (1995) Nicotine enhances Morris water maze performance of young and aged rats. Neurobiol Aging 16:857-60. DOI: 019745809500091R [pii].

Sorra K.E., Harris K.M. (2000) Overview on the structure, composition, function, development, and plasticity of hippocampal dendritic spines. Hippocampus 10:501-511.

Stefanick M.L. (2005) Estrogens and progestins: background and history, trends in use, and guidelines and regimens approved by the US Food and Drug Administration. Am J Med 118 Suppl 12B:64-73. DOI: S0002-9343(05)00919-8 [pii]10.1016/j.amjmed.2005.09.059.

Swaab D.F., Dubelaar E.J., Hofman M.A., Scherder E.J., van Someren E.J., Verwer R.W. (2002) Brain aging and Alzheimer's disease; use it or lose it. Prog Brain Res 138:343-73.

Talboom J.S., Engler-Chiurazzi E.B., Whiteaker P., Simard A.R., Lukas R., Acosta J.I., Prokai L., Bimonte-Nelson H.A. (2010) A component of Premarin((R)) enhances multiple cognitive functions and influences nicotinic receptor expression. Horm Behav. DOI: S0018-506X(10)00243-6 [pii]10.1016/j.yhbeh.2010.09.002.

Talboom J.S., Williams B.J., Baxley E.R., West S.G., Bimonte-Nelson H.A. (2008) Higher levels of estradiol replacement correlate with better spatial memory in surgically menopausal young and middle-aged rats. Neurobiol Learn Mem 90:155-63. DOI: S1074-7427(08)00061-0 [pii]10.1016/j.nlm.2008.04.002.

Tarnopolsky M. (1999) Gender differences in metabolism : practical and nutritional implications CRC Press, Boca Raton.

Taylor G.T., Maloney S. (2010) Gender differences and the role of estrogen in cognitive enhancements with nicotine in rats. Pharmacol Biochem Behav 95:139-45. DOI: S0091-3057(10)00002-X [pii]10.1016/j.pbb.2009.12.018.

Terasawa E., Timiras P.S. (1968) Electrical activity during the estrous cycle of the rat: cyclic changes in limbic structures. Endocrinology 83:207-216.

Terry R.D. (2006) Alzheimer's disease and the aging brain. Journal of Geriatric Psychiatry and Neurology 19:125-128. DOI: 19/3/125 [pii]10.1177/0891988706291079.

Tice M.A., Hashemi T., Taylor L.A., McQuade R.D. (1996) Distribution of muscarinic receptor subtypes in rat brain from postnatal to old age. Brain Res Dev Brain Res 92:70-6. DOI: 0165380695015159 [pii].

Timaras P., Quay W., vernadakis A. (Eds.). (1995) Hormones and aging, CRC Press, Boca Raton, NY, London, Tokyo.

Toran-Allerand C.D. (2005) Estrogen and the brain: beyond ER-alpha, ER-beta, and 17beta-estradiol. Ann N Y Acad Sci 1052:136-44. DOI: 1052/1/136 [pii]10.1196/annals.1347.009.

Tulving E., Craik F.I.M. (Eds.). (2000) Oxford Handbook of Memory, Oxford, NY.

Turgeon J.L., McDonnell D.P., Martin K.A., Wise P.M. (2004) Hormone therapy: physiological complexity belies therapeutic simplicity. Science 304:1269-73. DOI: 10.1126/science.1096725304/5675/1269 [pii].

Tyler W.J., Pozzo-Miller L. (2003) Miniature synaptic transmission and BDNF modulate dendritic spine growth and form in rat CA1 neurones. J Physiol 553:497-509. DOI: 10.1113/jphysiol.2003.052639.

Utian W.H., Ravnikar V.A., Sarrel P.M., Simon J. (2006) The Clinical Significance of Δ8,9-Dehydroestrone Sulfate in Treating Menopausal Vasomotor Symptoms. A supplement to OBG Management:8.

Vaucher E., Reymond I., Najaffe R., Kar S., Quirion R., Miller M.M., Franklin K.B. (2002) Estrogen effects on object memory and cholinergic receptors in young and old female mice. Neurobiol Aging 23:87-95. DOI: S0197458001002500 [pii].

Verghese J., Cuiling W., Katz M.J., Sanders A., Lipton R.B. (2009) Leisure activities and risk of vascular cognitive impairment in older adults. J Geriatr Psychiatry Neurol 22:110-8. DOI: 0891988709332938 [pii]10.1177/0891988709332938.

Verghese J., LeValley A., Derby C., Kuslansky G., Katz M., Hall C., Buschke H., Lipton R.B. (2006) Leisure activities and the risk of amnestic mild cognitive impairment in the elderly. Neurology 66:821-7. DOI: 01.wnl.0000202520.68987.48 [pii]10.1212/01.wnl.0000202520.68987.48.

Verghese J., Lipton R.B., Katz M.J., Hall C.B., Derby C.A., Kuslansky G., Ambrose A.F., Sliwinski M., Buschke H. (2003) Leisure activities and the risk of dementia in the elderly. N Engl J Med 348:2508-16. DOI: 10.1056/NEJMoa022252348/25/2508 [pii].

Wald A., Wolfowitz J. (1940) On a test whether two samples are from the same population. The Annals of Mathematical Statistics 11:147-162.

Walf A.A., Frye C.A. (2008) Conjugated equine estrogen enhances rats' cognitive, anxiety, and social behavior. Neuroreport 19:789-92. DOI: 10.1097/WNR.0b013e3282fe209c00001756-200805070-00016 [pii].

Wallace M., Frankfurt M., Arellanos A., Inagaki T., Luine V. (2007) Impaired recognition memory and decreased prefrontal cortex spine density in aged female rats. Ann N Y Acad Sci 1097:54-7. DOI: 1097/1/54 [pii]10.1196/annals.1379.026.

Wallace M., Luine V., Arellanos A., Frankfurt M. (2006) Ovariectomized rats show decreased recognition memory and spine density in the hippocampus and prefrontal cortex. Brain Res 1126:176-82. DOI: S0006-8993(06)02191-3 [pii]10.1016/j.brainres.2006.07.064.

Warren S.G., Juraska J.M. (1997) Spatial and nonspatial learning across the rat estrous cycle. Behav Neurosci 111:259-66.

Weiland N.G. (1992) Glutamic acid decarboxylase messenger ribonucleic acid is regulated by estradiol and progesterone in the hippocampus. Endocrinology 131:2697-702.

Wellman C.L., Pelleymounter M.A. (1999) Differential effects of nucleus basalis lesions in young adult and aging rats. Neurobiol Aging 20:381-93. DOI: S0197-4580(99)00025-1 [pii].

Welshons W.V., Thayer K.A., Judy B.M., Taylor J.A., Curran E.M., vom Saal F.S. (2003) Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environ Health Perspect 111:994-1006. DOI: sc271_5_1835 [pii].

Wermuth C.G., Ganellin C.R., Lindberg P., Mitscher L.A. (1998) Glossary of terms used in medicinal chemistry. Pure and Applied Chemistry 70:1129-1143.

Whalley L.J., Starr J.M., Athawes R., Hunter D., Pattie A., Deary I.J. (2000) Childhood mental ability and dementia. Neurology 55:1455-9.

Whiteaker P., Jimenez M., McIntosh J.M., Collins A.C., Marks M.J. (2000) Identification of a novel nicotinic binding site in mouse brain using [(125)I]-epibatidine. Br J Pharmacol 131:729-39. DOI: 10.1038/sj.bjp.0703616.

Whiteaker P., Marks M.J., Christensen S., Dowell C., Collins A.C., McIntosh J.M. (2008) Synthesis and characterization of 125I-alpha-conotoxin ArIB[V11L;V16A], a selective alpha7 nicotinic acetylcholine receptor antagonist. J Pharmacol Exp Ther 325:910-9. DOI: jpet.108.136895 [pii]10.1124/jpet.108.136895.

Wilson I.A., Ikonen S., Gureviciene I., McMahan R.W., Gallagher M., Eichenbaum H., Tanila H. (2004) Cognitive aging and the hippocampus: how old rats represent new environments. J Neurosci 24:3870-8. DOI: 10.1523/JNEUROSCI.5205-03.200424/15/3870 [pii].

Wolf O.T., Kirschbaum C. (2002) Endogenous estradiol and testosterone levels are associated with cognitive performance in older women and men. Horm Behav 41:259-66. DOI: 10.1006/hbeh.2002.1770S0018506X02917708 [pii].

Wolf O.T., Kudielka B.M., Hellhammer D.H., Torber S., McEwen B.S., Kirschbaum C. (1999) Two weeks of transdermal estradiol treatment in postmenopausal elderly women and its effect on memory and mood: verbal memory changes are associated with the treatment induced estradiol levels. Psychoneuroendocrinology 24:727-41. DOI: S0306-4530(99)00025-6 [pii].

Woolf N.J. (1991) Cholinergic systems in mammalian brain and spinal cord. Prog Neurobiol 37:475-524. DOI: 0301-0082(91)90006-M [pii].

Woolley C.S. (1998) Estrogen-mediated structural and functional synaptic plasticity in the female rat hippocampus. Hormones and behavior 34:140-8. DOI: 10.1006/hbeh.1998.1466.

Woolley C.S. (2007) Acute effects of estrogen on neuronal physiology. Annu Rev Pharmacol Toxicol 47:657-80. DOI: 10.1146/annurev.pharmtox.47.120505.105219.

Woolley C.S., McEwen B.S. (1992) Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. J Neurosci 12:2549-54.

Woolley C.S., McEwen B.S. (1993) Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. J Comp Neurol 336:293-306. DOI: 10.1002/cne.903360210.

Woolley C.S., Weiland N.G., McEwen B.S., Schwartzkroin P.A. (1997) Estradiol increases the sensitivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input: correlation with dendritic spine density. J Neurosci 17:1848-59.

Yerkes R.M., Dodson J.D. (1908) The relation of strength of stimulus to rapidity of habit-formation. Journal of Comparative Neurology and Psychology 18:459-482.

Zemlyak I., Brooke S.M., Sapolsky R.M. (2002) Protection against gp120-induced neurotoxicity by an array of estrogenic steroids. Brain Res 958:272-6. DOI: S0006899302035588 [pii].

Zhang F., Yao D., Hua Y., van Breemen R.B., Bolton J.L. (2001) Synthesis and reactivity of the catechol metabolites from the equine estrogen, 8,9-dehydroestrone. Chem Res Toxicol 14:754-63. DOI: tx010049y [pii].

Zhao L., Brinton R.D. (2006) Select estrogens within the complex formulation of conjugated equine estrogens (Premarin) are protective against neurodegenerative insults: implications for a composition of estrogen therapy to promote neuronal function and prevent Alzheimer's disease. BMC Neurosci 7:24. DOI: 1471-2202-7-24 [pii]10.1186/1471-2202-7-24.

Ziegler D.R., Gallagher M. (2005) Spatial memory in middle-aged female rats: Assessment of estrogen replacement after ovariectomy. Brain Research 1052:163-173.

Ziel H.K., Finkle W.D. (1975) Increased risk of endometrial carcinoma among users of conjugated estrogens. N Engl J Med 293:1167-70.

Zola-Morgan S., Squire L.R., Ramus S.J. (1994) Severity of memory impairment in monkeys as a function of locus and extent of damage within the medial temporal lobe memory system. Hippocampus 4:483-95. DOI: 10.1002/hipo.450040410.

Zurkovsky L., Brown S.L., Korol D.L. (2006) Estrogen modulates place learning through estrogen receptors in the hippocampus. Neurobiol Learn Mem 86:336-43. DOI: S1074-7427(06)00089-X [pii]10.1016/j.nlm.2006.07.008.

Table 1

Summary of Dependent Variable Composites

Composite	Memory Type	Day and Trial Blocks
1. RM T Maze		
All days and trials 1st practice session at 6 months of age	RM	Days 1-5 Trials 1-6
All days and trials	RM	Days 1-5 Trials 1-6
Last 3 days all trials	RM	Days 3-5 Trials 1-6
2. RM Morris Maze		
All days and trials	RM	Days 1-5 Trials 1-6
Last 3 days all trials	RM	Days 3-5 Trials 1-6
Overnight retention	RM Retention	Days 2-5 Trial 1
3. WM and RM WRAM		
WMC all days and trials	WM	Days 1-12 Trials 2-4
WMC last 4 days all trials	WM	Days 9-12 Trials 2-4
WMI all days and trials	WM	Days 1-12 Trials 1-4
WMI last 4 days all trials	WM	Days 9-12 Trials 1-4
RMW all days and trials	RM	Days 1-12 Trials 1-4
RMW last 4 days all trials	RM	Days 9-12 Trials 1-4
WMC all days trial 4 alone	WM Load	Days 1-12 Trial 4
WMC last 4 days trial 4 alone	WM Load	Days 9-12 Trial 4
WMI all days trial 4 alone	WM Load	Days 1-12 Trial 4
WMI last 4 days trial 4 alone	WM Load	Days 9-12 Trial 4
RMW all days trial 4 alone	RM	Days 1-12 Trial 4
RMW last 4 days trial 4 alone	RM	Days 9-12 Trial 4
4. Novel Global Memory Composite		
Mean z-score of all days and all trials for tasks novel to the Aged-Cog Prac group: RM T Maze, RM Morris Maze, and WM RM WRAM	Global Novel WM and RM	All Test Days all Trials

Table 2

Mean ±SE, median, and range of E2 levels for each group

Group	Mean E2 level ±*SE*	Range of E2 levels	Median E2 level
1. Study 1			
Young Sham	13.50±4.11	Undetectable-26	17.5
Young Ovx	3.78 ±1.91	Undetectable-13	0
Young Ovx + 0.25 E	65.88 ±10.75	56-98	77
Young Ovx + 0.50 E	61.75 ±12.30	22-114	49.5
Middle-aged Sham	21.88 ±1.89	13-29	23
Middle-aged Ovx	5.11 ±2.70	Undetectable-21	0
Middle-aged Ovx + 0.25 E	54.80 ±6.19	32-68	57
Middle-aged Ovx + 0.50 E	53.38 ±13.24	15-121	44
2. Study 2			
Young Sham	16.83±4.56	Undetectable-34	18.5
Aged Sham	19.20 ±2.06	12-24	20
Aged Ovx	11.00±2.15	Undetectable-19	11
Aged Ovx + 0.25 E	112.50 ±20.79	55-180	104

Table 3

Summary of Relationships between Tonic E2 or CEE Dose and Working/Recent Memory

Groups and Composite	Form of the Relationship with Dose
1. Ovx-Veh group and all E2 doses	
Ovx-E2 and Ovx-Veh *Total Errors* Baseline Learning (Days 1-5 Trials 1-6)	Form Not Described
Ovx-E2 and Ovx-Veh *Total Errors* Overall Performance (Days 1-7 Trials 1-6)	Form Not Described
Ovx-E2 and Ovx-Veh *Repeat Errors* Baseline Learning (Days 1-5 Trials 1-6)	Negative Linear
Ovx-E2 and Ovx-Veh *Repeat Errors* Overall Performance (Days 1-7 Trials 1-6)	Negative Linear
2. Ovx-Veh group and all CEE doses	
Ovx-CEE and Ovx-Veh *Total Errors* Baseline Learning (Days 1-5 Trials 1-6)	Form Not Described
Ovx-CEE and Ovx-Veh *Total Errors* Overall Performance (Days 1-7 Trials 1-6)	Cubic
Ovx-CEE and Ovx-Veh *Repeat Errors* Baseline Learning (Days 1-5 Trials 1-6)	Quadratic/U-Shaped
Ovx-CEE and Ovx-Veh *Repeat Errors* Overall Performance (Days 1-7 Trials 1-6)	Form Not Described

Table 4

Summary of the Effects of Age and Ovarian Hormone Loss on Putative Synapse Numbers

Analysis	Putative Synapse Type	Hippocampal Lamina	Group(s)	Effect
1. Pairwise ANOVA: Ovx vs. Sham				
Significant ANOVA	Excitatory(NR1)	Stratum Granulosum	Ovx	↓**Decrease**↓
2. Age Linear Trends				
Significant Trend from 5 to 20 mo	Inhibitory(VGAT)	Stratum Pyramidale	Ovx & Sham	↓**Decrease**↓
Significant Trend from 5 to 20 mo	Inhibitory(VGAT)	Stratum Pyramidale	Ovx	↓**Decrease**↓
Significant Trend from 12 to 20 mo	Inhibitory(VGAT)	Polymorphic Layer	Ovx & Sham	↓**Decrease**↓
3. Pairwise Age Contrasts				
Significant Contrast: 5 vs. 20 mo	Inhibitory(VGAT)	Stratum Pyramidale	Ovx & Sham	↓**Decrease**↓
Significant Contrast: 5 vs. 20 mo	Inhibitory(VGAT)	Stratum Pyramidale	Ovx	↓**Decrease**↓
Significant Contrast: 5 vs. 20 mo	Inhibitory(VGAT)	Stratum Oriens	Ovx & Sham	↓**Decrease**↓
Significant Contrast: 5 vs. 20 mo	Inhibitory(VGAT)	Stratum Oriens	Ovx	↓**Decrease**↓
Significant Contrast: 12 vs. 20 mo	Inhibitory(VGAT)	Polymorphic Layer	Ovx & Sham	↓**Decrease**↓

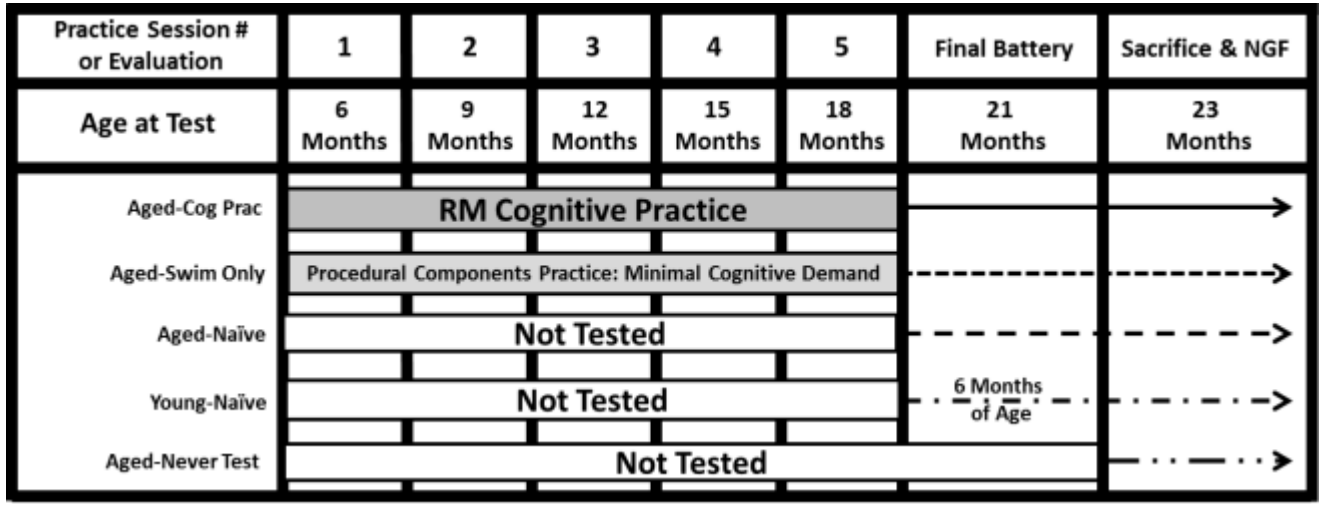

Practice Session # or Evaluation
1
2
3
4
5
Final Battery
Sacrifice & NGF
Age at Test
6 Months
9 Months
12 Months
15 Months
18 Months
21 Months
23 Months
Aged-Cog Prac
RM Cognitive Practice
Aged-Swim Only
Procedural Components Practice: Minimal Cognitive Demand
Aged-Naïve
Not Tested
Young-Naïve
Not Tested
6 Months of Age
Aged-Never Test
Not Tested

Figure 1. A time line of experiment 1 listing each group in the study and summarizing the sequence of cognitive practice and/or the length of practice/testing absence that each group received through the final battery, ending with sacrifice and an evaluation of NGF levels.

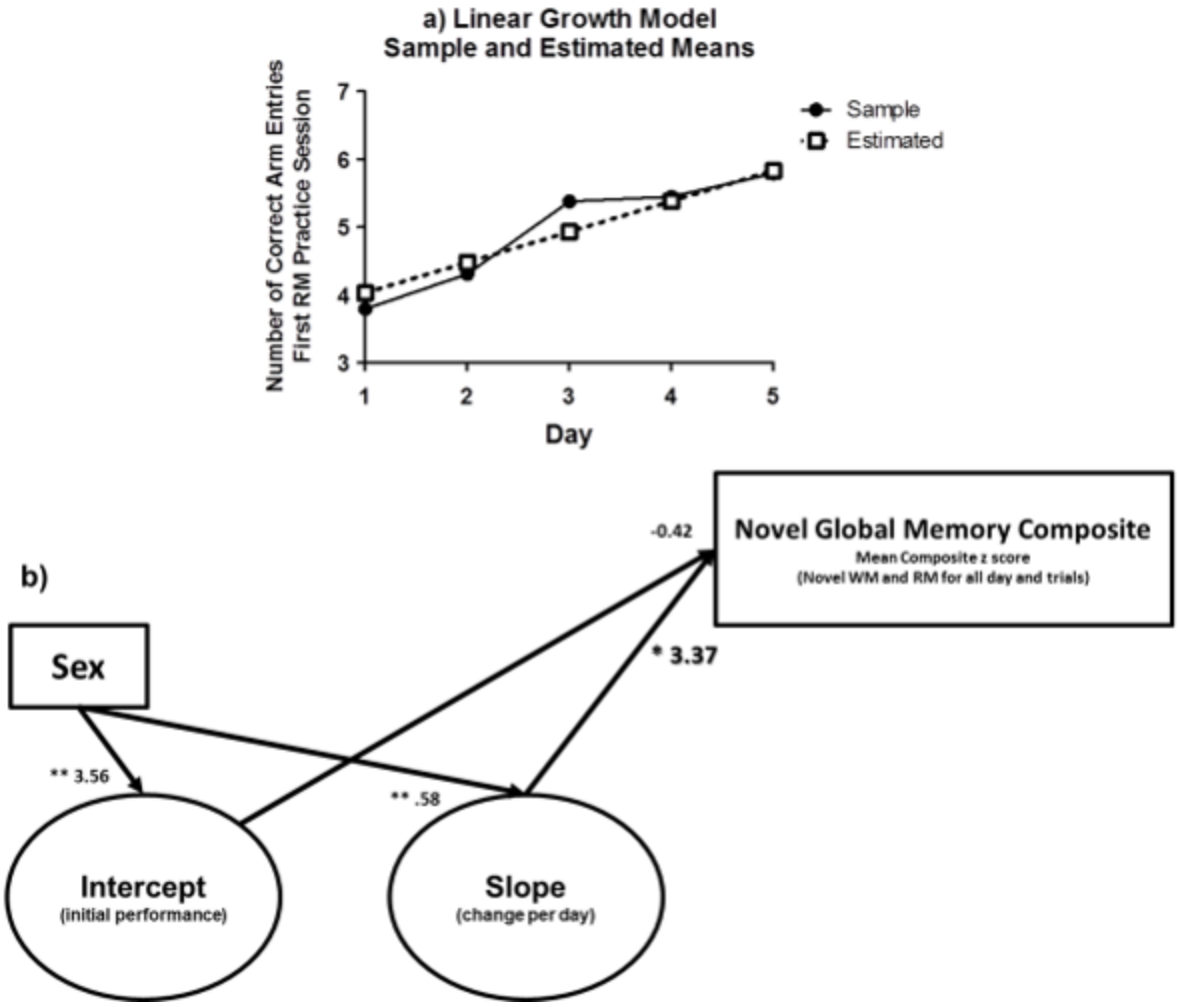

a) Linear Growth Model
Sample and Estimated Means
Number of Correct Arm Entries
First RM Practice Session
7
6
5
4
3
1
2
3
4
5
Day
Sample
Estimated
b)
Sex
** 3.56
Intercept
(initial performance)
** .58
Slope
(change per day)
-0.42
* 3.37
Novel Global Memory Composite
Mean Composite z score
(Novel WM and RM for all day and trials)

Figure 2. a) Plot of the linear growth model displaying the mean growth rate of Cog Prac animals during the first cognitive practice session at 6 months of age (sample) and the model estimated linear growth rate. b) Simplified path diagram depicting the linear growth model estimating the number of correct arm entries made on day 1 (i.e., initial performance or Intercept) and the change in the number of correct arm entries made per day (i.e., change per day or Slope) for Cog Prac male and female rats during their first cognitive practice session at 6 months of age. a) The mean growth rate of Cog Prac animals at 6 months of age closely followed the linear trajectory estimated by the model. b) The full growth model was constructed by adding Sex as a binary predictor of the Intercept and Slope as well as the Slope of young animals predicting the Aged Novel Global Memory Composite. Numbers represent the unstandardized path coefficients estimated by maximum likelihood. * $p \leq .05$ ** $p < .0001$.

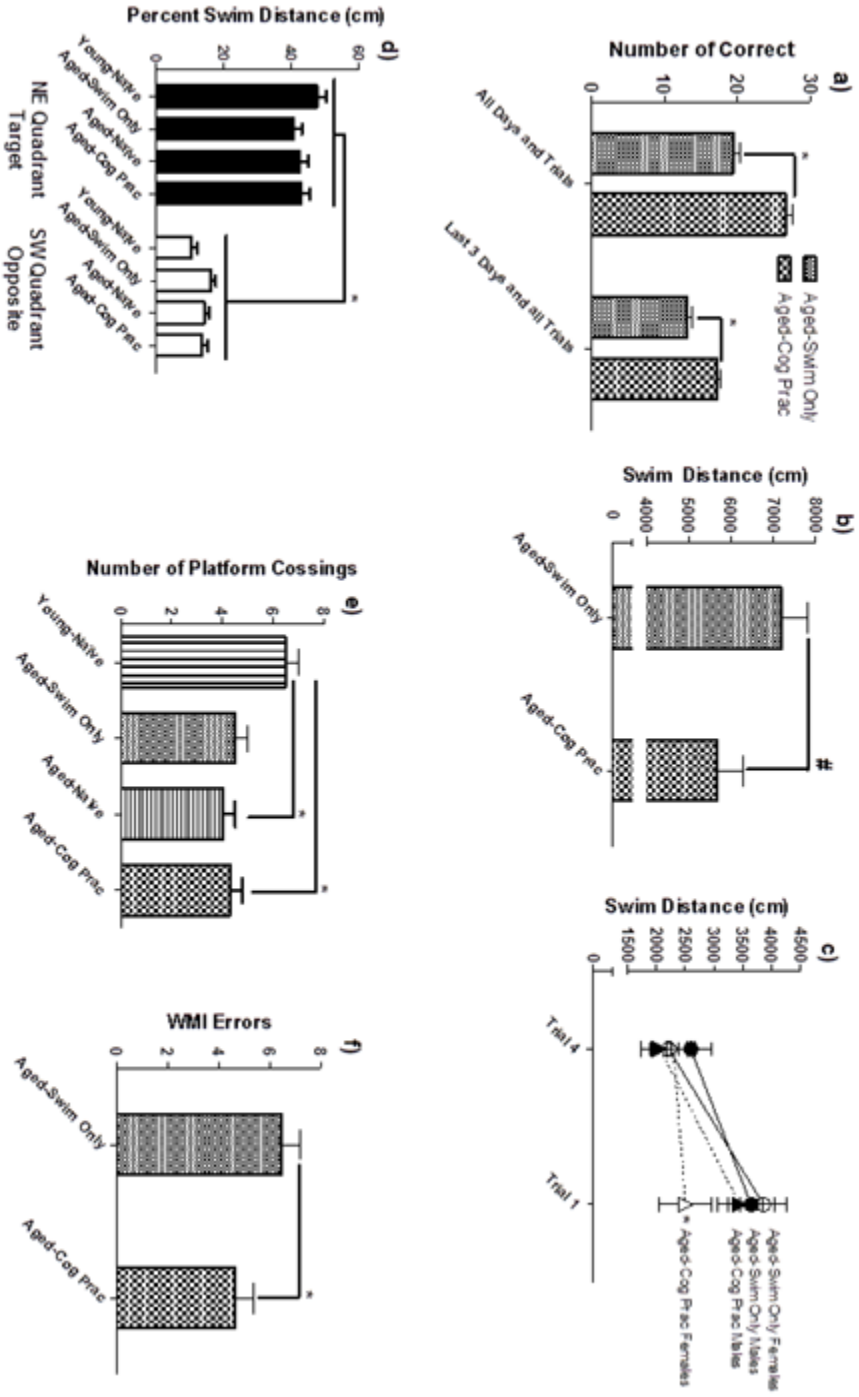

a)
Number of Correct
All Days and Trials
Last 3 Days and all Trials
Aged-Swim Only
Aged-Cog Prac
b)
Swim Distance (cm)
AgedSwim Only
Aged-Cog Prac
c)
Swim Distance (cm)
Trial 4
Trial 1
Aged-Swim Only Females
Aged-Swim Only Males
Aged-Cog Prac Males
Aged-Cog Prac Females
d)
Percent Swim Distance (cm)
NE Quadrant Target
SW Quadrant Opposite
Young-Native
Aged-Swim Only
Aged-Native
Aged-Cog Prac
e)
Number of Platform Cossings
Young-Native
Aged-Swim Only
Aged-Native
Aged-Cog Prac
f)
WMI Errors
Aged-Swim Only
Aged-Cog Prac

Figure 3. Effects of cognitive practice. a) Mean ±average SE number correct arm entries made on two separate composite measures summed across all days and trials and the last 3 days of testing with all test trials on the RM T-Maze. b) Mean ±average SE for a composite measure of swim distance (cm) summed across the last 3 days of testing and all test trials on the RM Morris Maze. c) Mean ±average SE RM Morris Maze swim distance (cm) summed across days 2-5 from trial 4 of the previous day to trial 1 of the next day. d) Mean ±*SE* RM Morris Maze percent of total swim distance in the target NE and opposite SW quadrants during the probe trial. e) RM Morris Maze mean ±average SE total number of platform crossings made during the probe trial. f) Mean ± average SE WMI errors committed on the WM and RM WRAM for a composite measure of WMI errors summed across the last 4 days of testing for trial 4 alone. * $p < .05$. # $p < .10$.

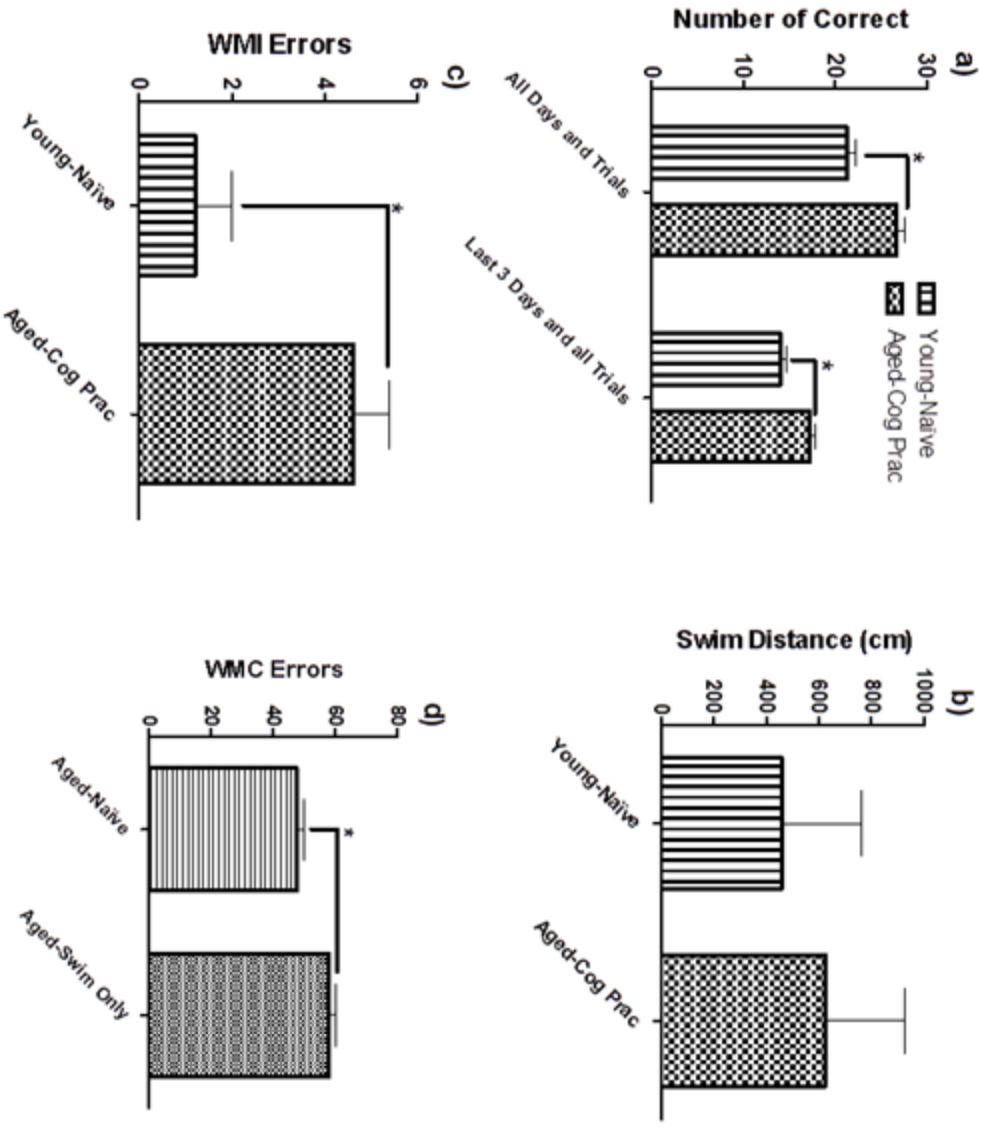

a)
Number of Correct
0
10
20
30
All Days and Trials
Last 3 Days and all Trials
*
*
Young-Naïve
Aged-Cog Prac
c)
WMI Errors
0
2
4
6
Young-Naïve
Aged-Cog Prac
*
b)
Swim Distance (cm)
0
200
400
600
800
1000
Young-Naïve
Aged-Cog Prac
d)
WMC Errors
0
20
40
60
80
Aged-Naïve
Aged-Swim Only
*

Figure 4. The effects of cognitive practice to attenuate or obviate age-related memory decline and the effects of the procedural components of testing on memory. a) Mean ±average SE number correct arm entries made on two separate composite measures summed across all days and trials and the last 3 days of testing for all test trials on the RM T-Maze. b) Mean ±average SE female swim distance (cm) during days 2-5 for trial 1 on the RM Morris Maze. c) Mean ±average SE WMI errors committed on the WM and RM WRAM from a composite measure of WMI errors summed across the last 4 days of testing on trial 4 alone. d) Mean ±average SE WMC errors committed on the WM and RM WRAM from a composite measure of WMC errors summed across all days and trials. a) For both composites, the Aged-Cog Prac group outperformed the Young-Naïve group during the final battery on the cognitive practice RM task. b) Young-Naïve females did not differ from Aged-Cog Prac females on the RM Morris Maze. c) Young-Naïve animals committed fewer WMI errors in comparison to the Aged-Cog Prac group. d) Summed across all days and trials, Aged-Naïve animals committed less WMC errors when compared to the Aged-Swim Only group. * $p < .05$.

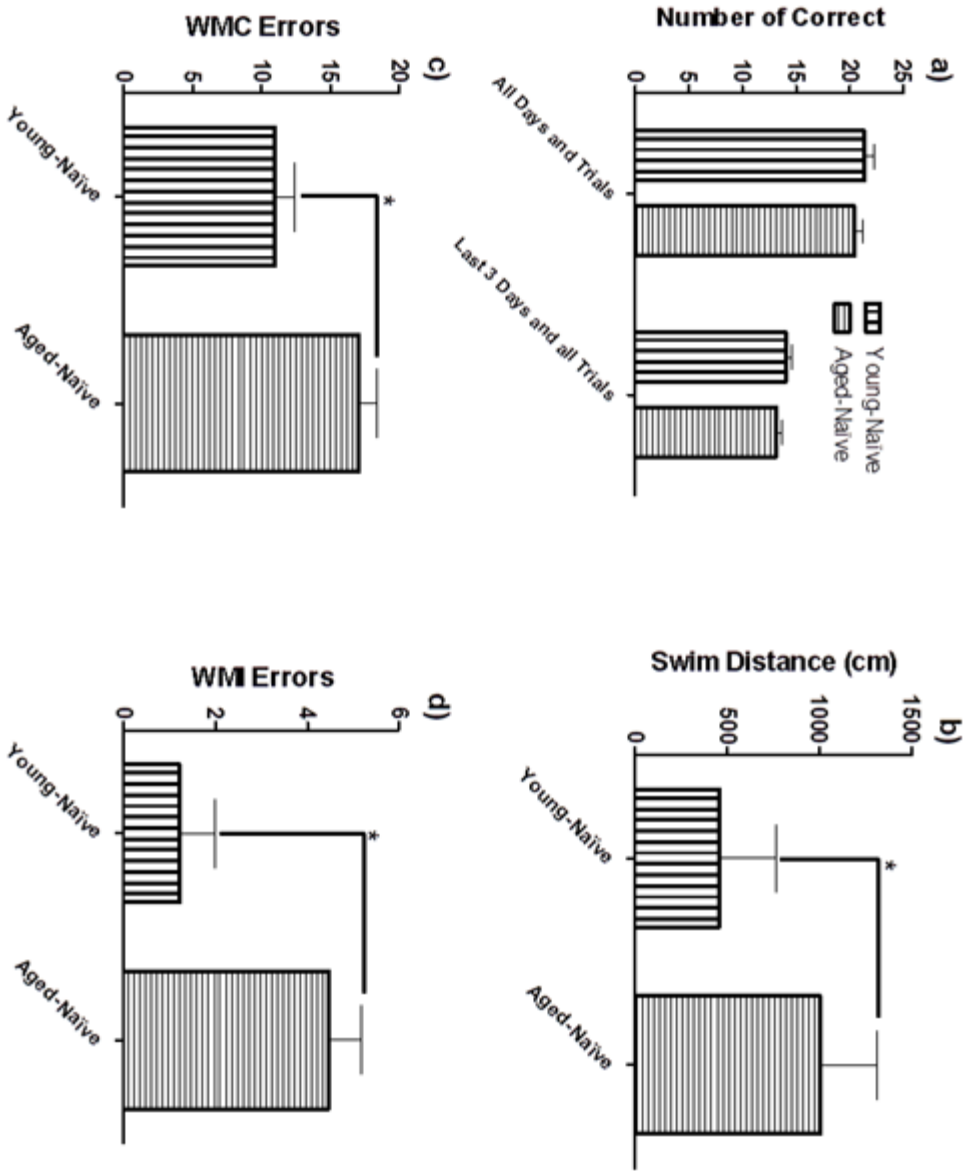

a)
Number of Correct
0
5
10
15
20
25
All Days and Trials
Last 3 Days and all Trials
Young-Naïve
Aged-Naïve
b)
Swim Distance (cm)
0
500
1000
1500
Young-Naïve
Aged-Naïve
*
c)
WMC Errors
0
5
10
15
20
Young-Naïve
Aged-Naïve
*
d)
WMI Errors
0
2
4
6
Young-Naïve
Aged-Naïve
*

Figure 5. The effects of age. a) Mean ±average SE number correct arm entries made on two separate composite measures summed across all days and trials and for the last 3 days of testing for all test trials on the RM T-Maze. b) Mean ±average SE female swim distance (cm) during days 2-5 on trial 1 alone on the RM Morris Maze. c) Mean ±average SE WMC errors committed on the WM and RM WRAM from a composite measure of WMC errors summed across the last 4 days of testing for all test trials. d) Mean ±average SE WMI errors committed on the WM and RM WRAM from a composite measure of WMI errors summed across the last 4 days of testing on trial 4 alone. a) The Young-Naïve group did not differ from the Aged-Naïve group on both composite measures of RM T-Maze performance. b) Young-Naïve females demonstrated superior RM Morris Maze performance in comparison to Aged-Naïve females. c) Young-Naïve animals committed fewer WMC errors when compared to the Aged-Naïve group during the last 4 days of testing. d) For the composite of WMI errors, the Young-Naïve group committed fewer errors in comparison to Aged-Naïve animals. * $p < .05$.

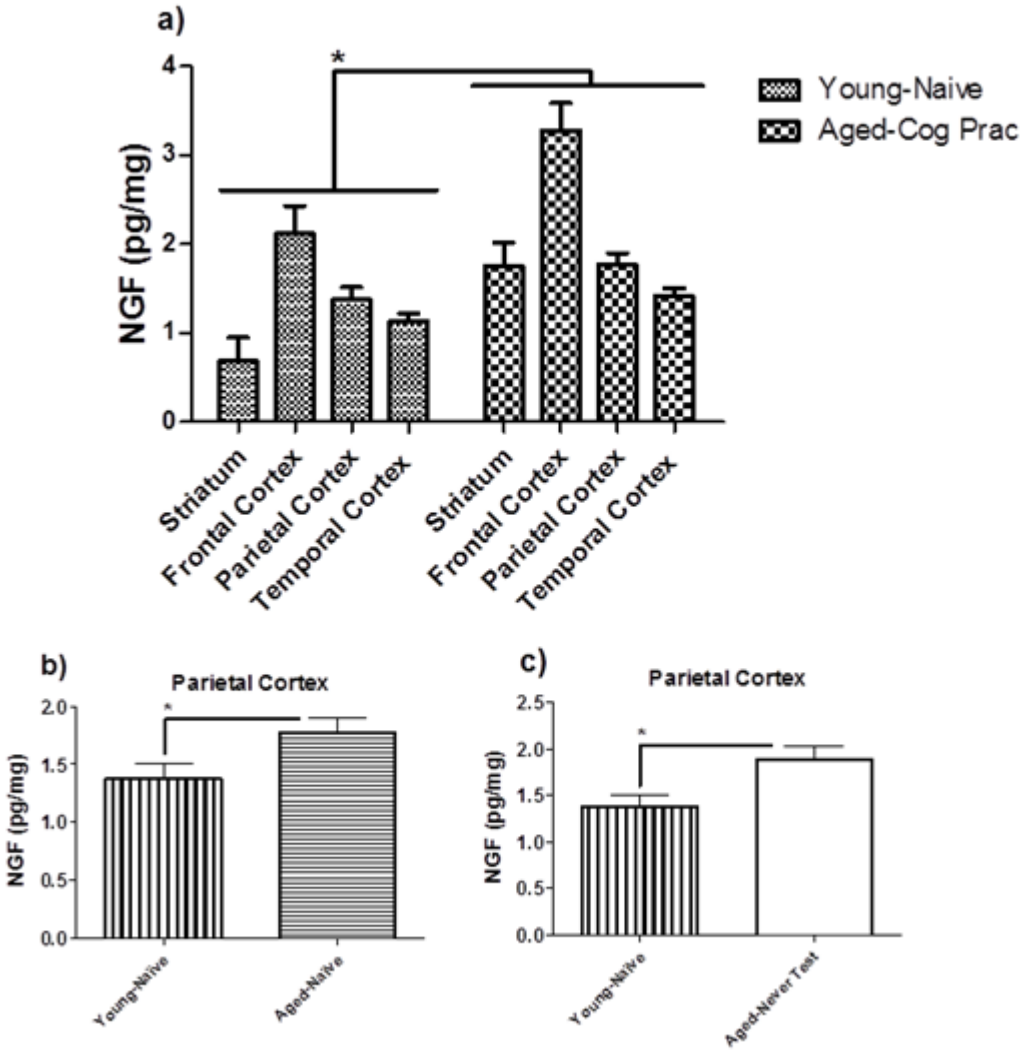
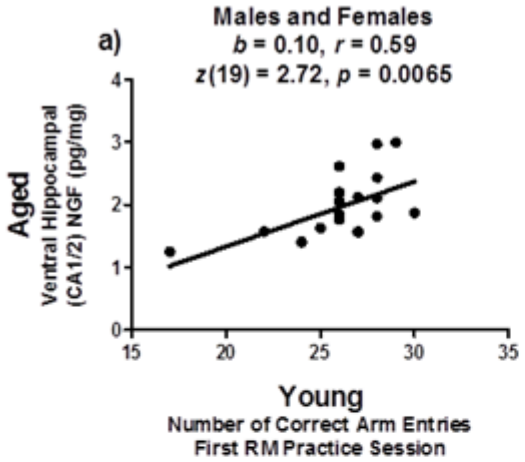
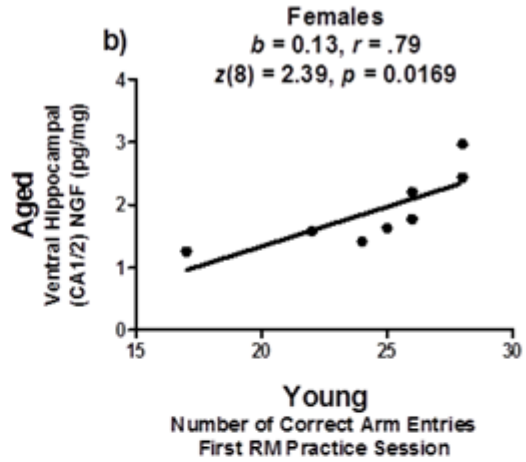

a)
NGF (pg/mg)
0
1
2
3
4
*
Young-Naive
Aged-Cog Prac
Striatum
Frontal Cortex
Parietal Cortex
Temporal Cortex
Striatum
Frontal Cortex
Parietal Cortex
Temporal Cortex
b)
Parietal Cortex
NGF (pg/mg)
0.0
0.5
1.0
1.5
2.0
*
Young-Naive
Aged-Naive
c)
Parietal Cortex
NGF (pg/mg)
0.0
0.5
1.0
1.5
2.0
2.5
*
Young-Naive
Aged-Never Test

Figure 6. NGF Contrast Analyses. a) Mean ±average SE NGF levels (pg/mg) in the striatum, frontal cortex, parietal cortex and temporal cortex of Young-Naïve and Aged-Cog Prac animals. b) Mean ±average SE NGF levels (pg/mg) in the parietal cortex of Young-Naïve and Aged-Naïve animals. c) Mean ±average SE NGF levels (pg/mg) in the parietal cortex of Young-Naïve and Aged-Never Test animals. a) The Aged-Cog Prac group had increased NGF levels in the striatum, frontal cortex, parietal cortex, and temporal cortex as compared to Young-Naïve animals. b) and c) Both the Aged-Naïve and Aged-Never Test groups had increased NGF levels in the parietal cortex as compared to the Young-Naïve group.


a)
Males and Females
b = 0.10, r = 0.59
z(19) = 2.72, p = 0.0065
Aged
Ventral Hippocampal (CA1/2) NGF (pg/mg)
0
1
2
3
4
15
20
25
30
35
Young
Number of Correct Arm Entries
First RM Practice Session


b)
Females
b = 0.13, r = .79
z(8) = 2.39, p = 0.0169
Aged
Ventral Hippocampal (CA1/2) NGF (pg/mg)
0
1
2
3
4
15
20
25
30
Young
Number of Correct Arm Entries
First RM Practice Session

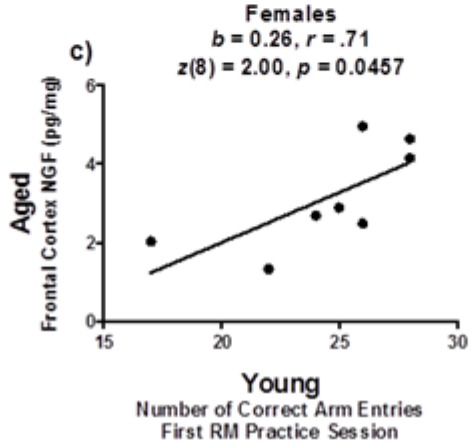

c)
Females
b = 0.26, r = .71
z(8) = 2.00, p = 0.0457
Aged
Frontal Cortex NGF (pg/mg)
0
2
4
6
15
20
25
30
Young
Number of Correct Arm Entries
First RM Practice Session

Figure 7. Primary regression analyses. Scatterplots representing the relationship between RM T-Maze number of correct arm entries made during the first cognitive practice session when animals were 6 months of age (Y axis) and aged NGF levels (X axis): a) in the ventral hippocampus (CA1/2) of male and female Aged-Cog Prac animals, b) in the ventral hippocampus (CA1/2) of female Aged-Cog Prac animals, and c) in the frontal cortex of female Aged-Cog Prac animals. The line represents the linear regression analysis of best fit. a) In both males and females, young RM performance was positively related to aged ventral hippocampal NGF levels. b) and c) In females alone, young RM performance was positively related to aged ventral hippocampal and frontal cortex NGF levels.

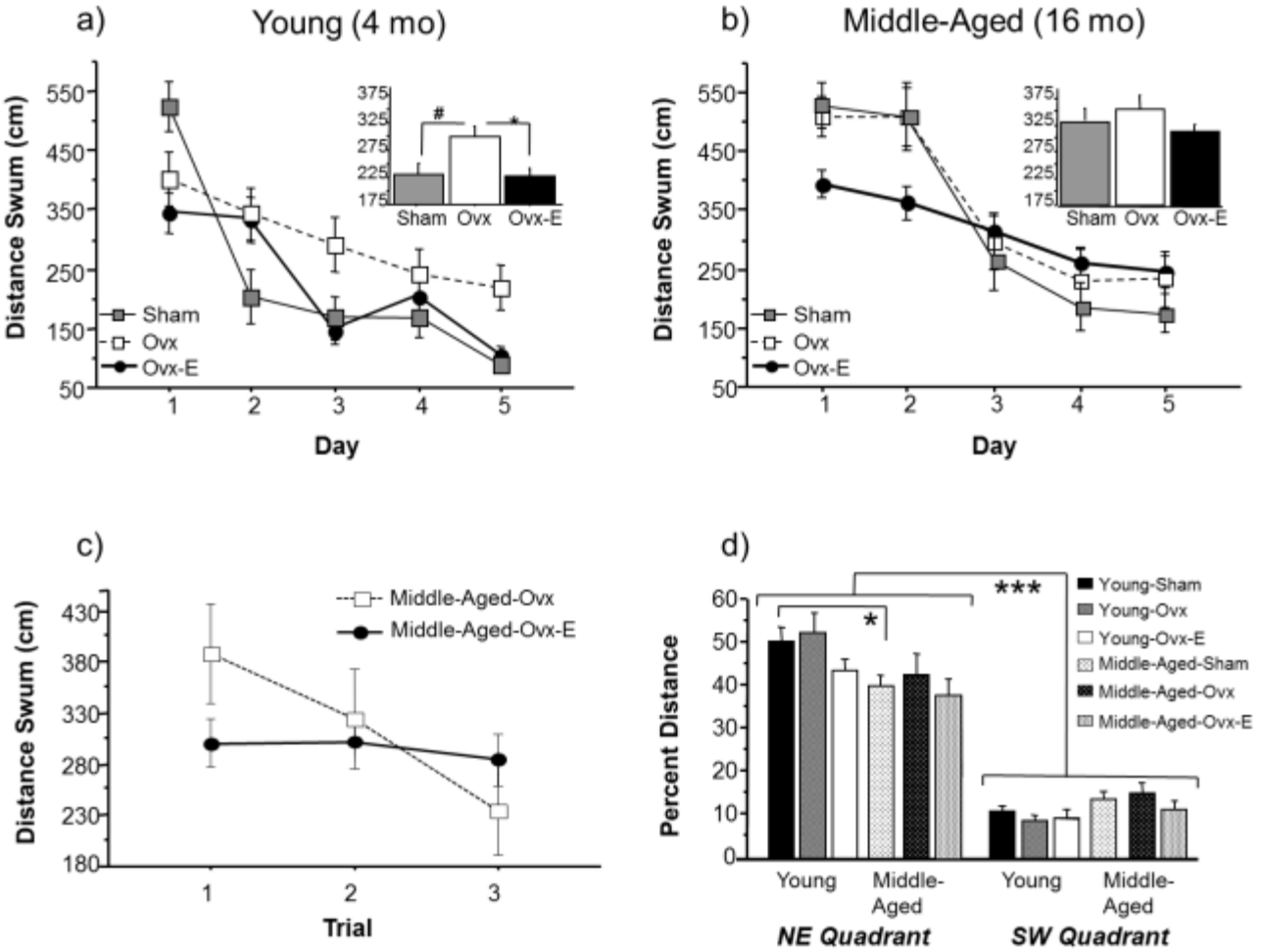

a)
Young (4 mo)
Distance Swum (cm)
550
450
350
250
150
50
1
2
3
4
5
Day
Sham
Ovx
Ovx-E
375
325
275
225
175
#
*
Sham Ovx Ovx-E
b)
Middle-Aged (16 mo)
Distance Swum (cm)
550
450
350
250
150
50
1
2
3
4
5
Day
Sham
Ovx
Ovx-E
375
325
275
225
175
Sham Ovx Ovx-E
c)
Distance Swum (cm)
430
380
330
280
230
180
1
2
3
Trial
Middle-Aged-Ovx
Middle-Aged-Ovx-E
d)
Percent Distance
60
50
40
30
20
10
0
*
***
Young
Middle-Aged
Young
Middle-Aged
NE Quadrant
SW Quadrant
Young-Sham
Young-Ovx
Young-Ovx-E
Middle-Aged-Sham
Middle-Aged-Ovx
Middle-Aged-Ovx-E

Figure 8. For Study 1, mean± SE distance scores (cm) across days of testing on the Morris maze for a) Young Sham, Ovx and Ovx-E groups and b) Middle-Aged Sham, Ovx and Ovx-E groups; the inset graphs represent the data collapsed across days. For Young animals, Ovx impaired performance on the latter portion of testing, as represented by the Ovx x Day interaction. There was no effect of Ovx in middle-aged animals. E2 treatment enhanced performance in both age groups. Thus, there was a dissociation between sensitivity to Ovx and responsiveness to E2 treatment. c) Mean ±*SE* distance scores for each trial for days 2-5 for Middle-Aged-Ovx and Middle-Aged-Ovx-E groups for Study 1. There was a significant Trial x E2 Treatment interaction, indicating that E2 aided in remembering the platform location overnight in middle-aged rats. d) Mean percent distance ±*SE* spent in the target (NE) and opposite (SW) quadrant for each treatment group within each age for Study 1. Neither Ovx nor E2 treatment influenced performance on the probe trial. All groups localized to the platform location, spending a greater percent distance in the target versus the opposite quadrant. * $p < .05$, # $p = .06$, *** $p < .0001$.

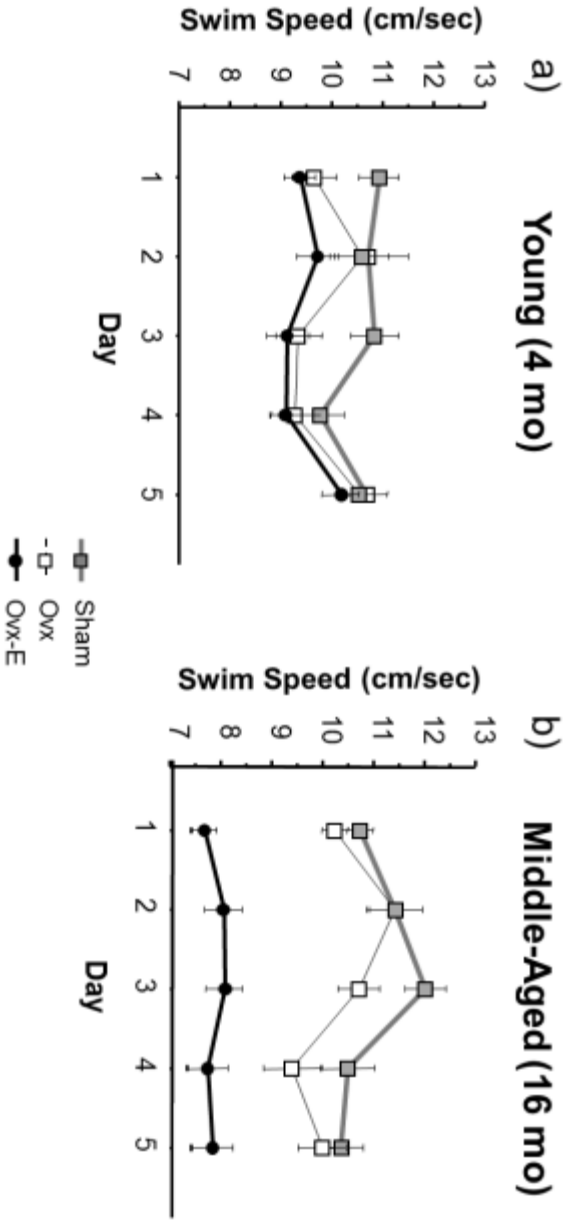

a)
Young (4 mo)
Swim Speed (cm/sec)
7
8
9
10
11
12
13
1
2
3
4
5
Day
b)
Middle-Aged (16 mo)
Swim Speed (cm/sec)
Day
Sham
Ovx
Ovx-E

Figure 9. Mean ±*SE* swim speed for each treatment group for a) young animals and b) middle-aged animals for Study 1. While there was no effect of swim speed in young animals, middle-aged animals showed a main effect of Treatment. Follow-up analyses showed that Ovx did not influence swim speed in middle-aged animals, and E2 treatment decreased swim speed.

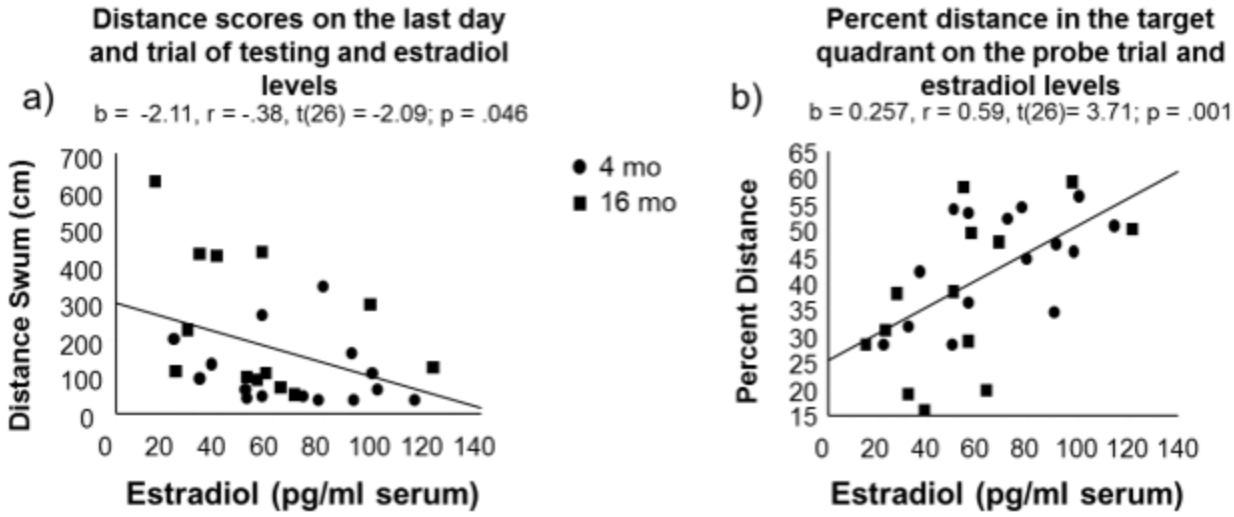

a)
Distance scores on the last day and trial of testing and estradiol levels
b = -2.11, r = -.38, t(26) = -2.09; p = .046
Distance Swum (cm)
700
600
500
400
300
200
100
0
0 20 40 60 80 100 120 140
Estradiol (pg/ml serum)
4 mo
16 mo
b)
Percent distance in the target quadrant on the probe trial and estradiol levels
b = 0.257, r = 0.59, t(26)= 3.71; p = .001
Percent Distance
65
60
55
50
45
40
35
30
25
20
15
0 20 40 60 80 100 120 140
Estradiol (pg/ml serum)

Figure 10. For Study 1, scatterplots for serum E2 levels and a) the last trial on the last day of day of testing with the platform in the maze, and b) percent distance in the previously platformed quadrant on the probe trial. Higher levels of E2 treatment, as determined by serum E2 levels, correlated with better performance (lower distance) on the last test trial as well as a higher percent distance in the previously platformed quadrant, representing superior platform localization.

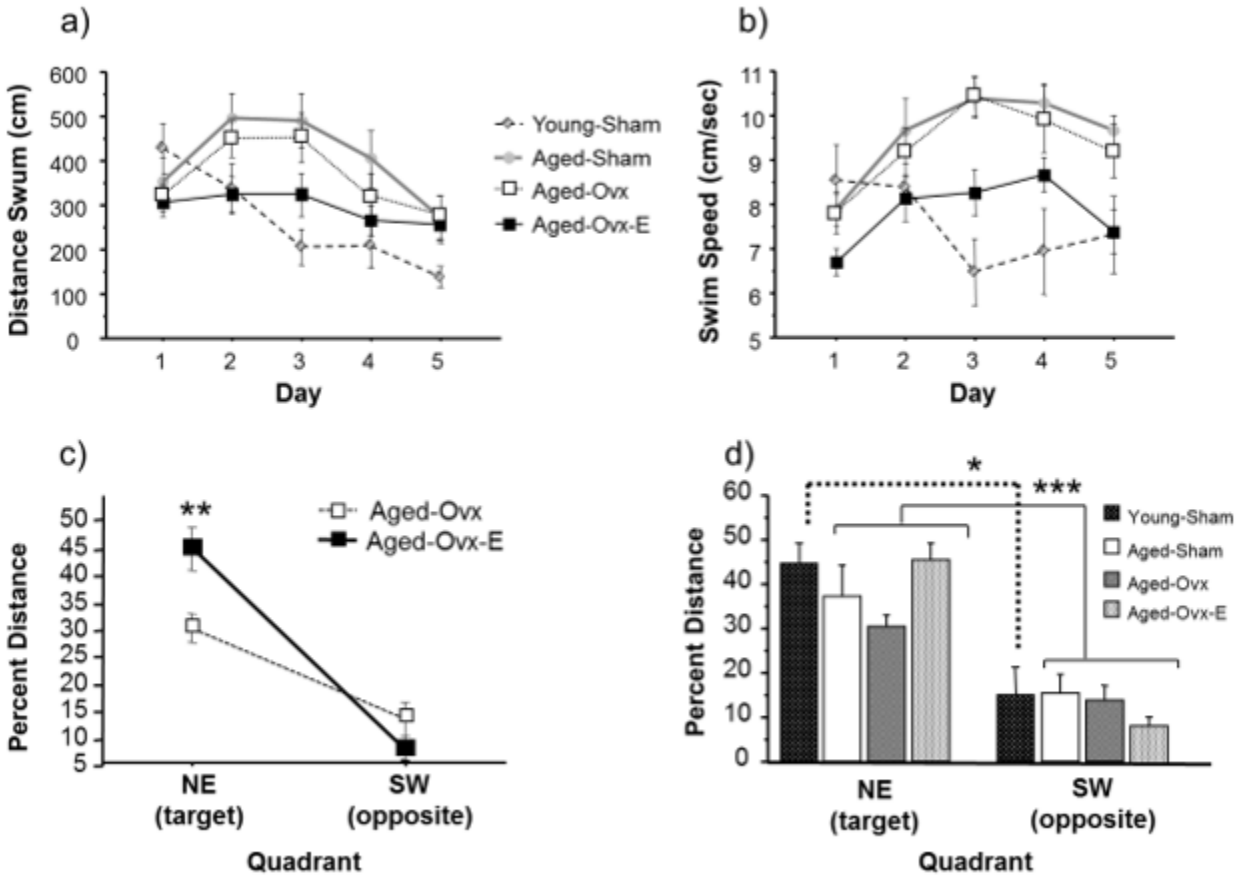

a)
Distance Swum (cm)
600
500
400
300
200
100
0
1
2
3
4
5
Day
Young-Sham
Aged-Sham
Aged-Ovx
Aged-Ovx-E
b)
Swim Speed (cm/sec)
11
10
9
8
7
6
5
1
2
3
4
5
Day
c)
Percent Distance
50
45
40
35
30
25
20
15
10
5
**
Aged-Ovx
Aged-Ovx-E
NE
(target)
SW
(opposite)
Quadrant
d)
*
***
Percent Distance
60
50
40
30
20
10
0
Young-Sham
Aged-Sham
Aged-Ovx
Aged-Ovx-E
NE
(target)
SW
(opposite)
Quadrant

Figure 11. a) Mean ±*SE* distance scores (cm) across day of testing on the Morris maze for Aged-Sham, Aged-Ovx, Aged-Ovx-E and Young groups for Study 2. There were no significant main effects or interactions for distance scores due to Ovx or E2 treatment in aged animals. b) mean ±*SE* swim speed for each aged group for each test day. The omnibus ANOVA revealed that Treatment affected swim speed as days progressed, with a significant Day x Treatment interaction. As observed in middle-aged animals, Ovx did not influence swim speed and E2 treatment decreased swim speed. c) Mean ±*SE* percent distance in the target (NE) and opposite (SW) quadrants on the probe trial for Aged-Ovx-E and Aged-Ovx groups for Study 2. There was a Quadrant x Treatment interaction, and a post-hoc t-test showed that aged Ovx animals receiving E2 spent a higher percent distance in the target quadrant as compared to aged Ovx animals that did not, suggesting better platform acquisition in aged Ovx animals given E2 treatment. d) Mean ±*SE* percent distance in the target (NE) and opposite (SW) quadrants on the probe trial for Aged-Ovx and Aged-Ovx-E groups for Study 2. There was a Quadrant x Treatment interaction, and a post-hoc t-test showed that aged Ovx animals receiving E2 spent a higher percent distance in the target quadrant as compared to aged Ovx animals that did not, suggesting better platform acquisition in aged Ovx animals given estradiol treatment. ** $p < .01$ * $p < .05$, *** $p < .0001$.

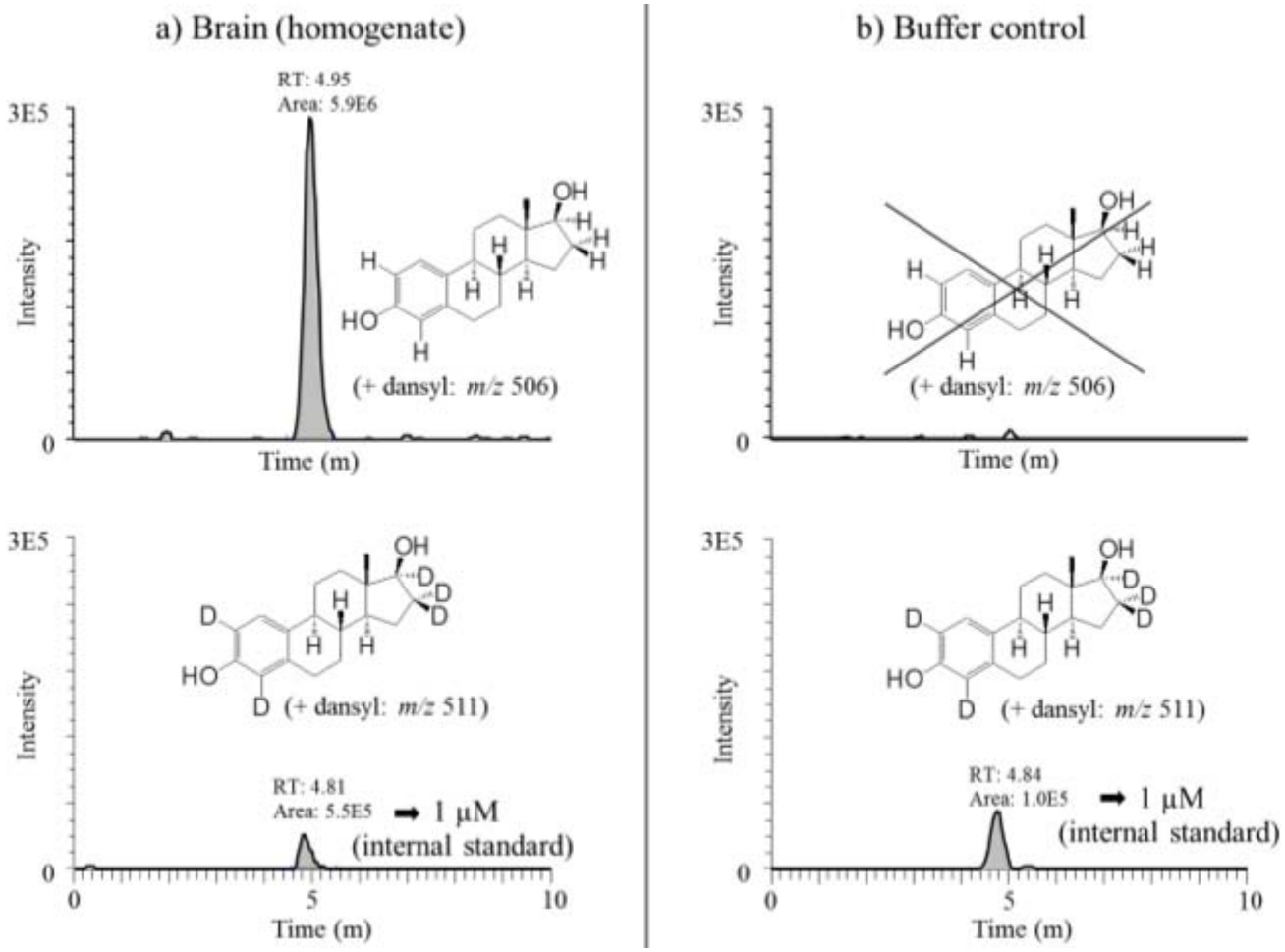

a) Brain (homogenate)
b) Buffer control
RT: 4.95
Area: 5.9E6
3E5
Intensity
0
Time (m)
(+ dansyl: m/z 506)
(+ dansyl: m/z 511)
RT: 4.81
Area: 5.5E5
1 μM
(internal standard)
RT: 4.84
Area: 1.0E5
1 μM
(internal standard)
0
5
10

Figure 12. a) DHED converts to E2 in brain tissue, as shown by LC-MS/MS analysis of product formed from 100 μM of DHED after 2.5-min incubation in rat-brain homogenate (20% w/v) with an initial rate of E2 formation corresponding to approximately 200 nmol min-1 (mg protein)-1. b) The absence of E2 formation was observed after incubation in buffer only (i.e., the control).

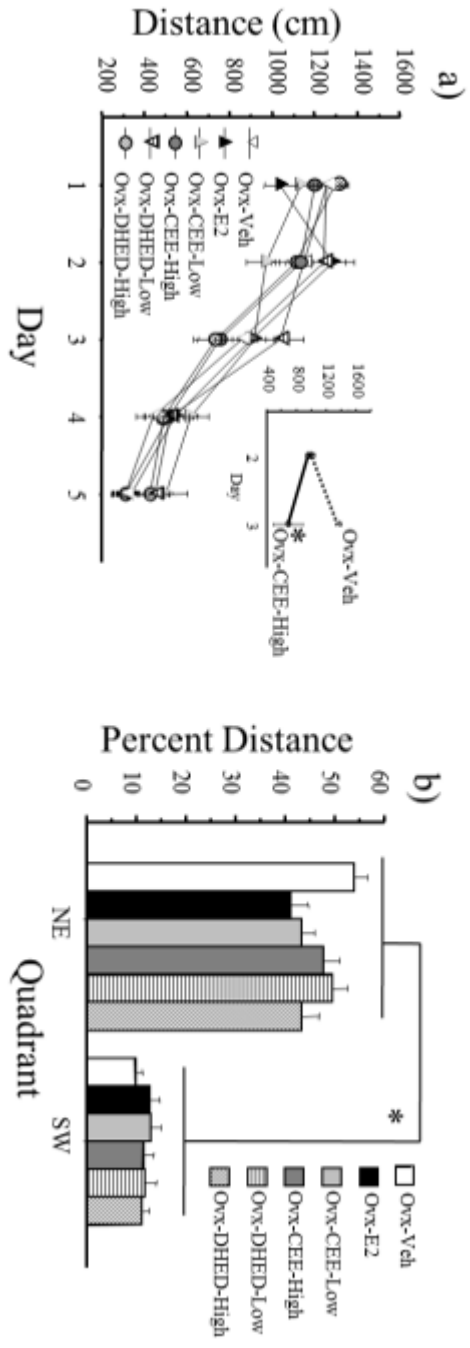

a)
Distance (cm)
1600
1400
1200
1000
800
600
400
200
1
2
3
4
5
Day
Ovx-Veh
Ovx-E2
Ovx-CEE-Low
Ovx-CEE-High
Ovx-DHED-Low
Ovx-DHED-High
1600
1200
800
400
2
3
Day
Ovx-Veh
*
Ovx-CEE-High
b)
Percent Distance
60
50
40
30
20
10
0
NE
SW
Quadrant
*
Ovx-Veh
Ovx-E2
Ovx-CEE-Low
Ovx-CEE-High
Ovx-DHED-Low
Ovx-DHED-High

Figure 13. Mean ±*SE* swim distance (cm) across days of testing on the Morris maze for (a) Ovx-Veh and each group and; the inset graph represents overnight forgetting data from trial 4 day 2 to trial 1 day 3. b) depicts the Mean ±*SE* percent distance swam in the target and opposite quadrants on the probe trial for each experimental group. There were no treatment effects across, however the analysis of overnight forgetting reveled that only the Ovx-CEE-High group had enhanced retention from day 2 trial 4 to day 3 trial 1. On the probe trial, all animals localized to the quadrant that previously contained the platform (target, NE) as compared to the opposite quadrant (SW). * $p < 0.05$.

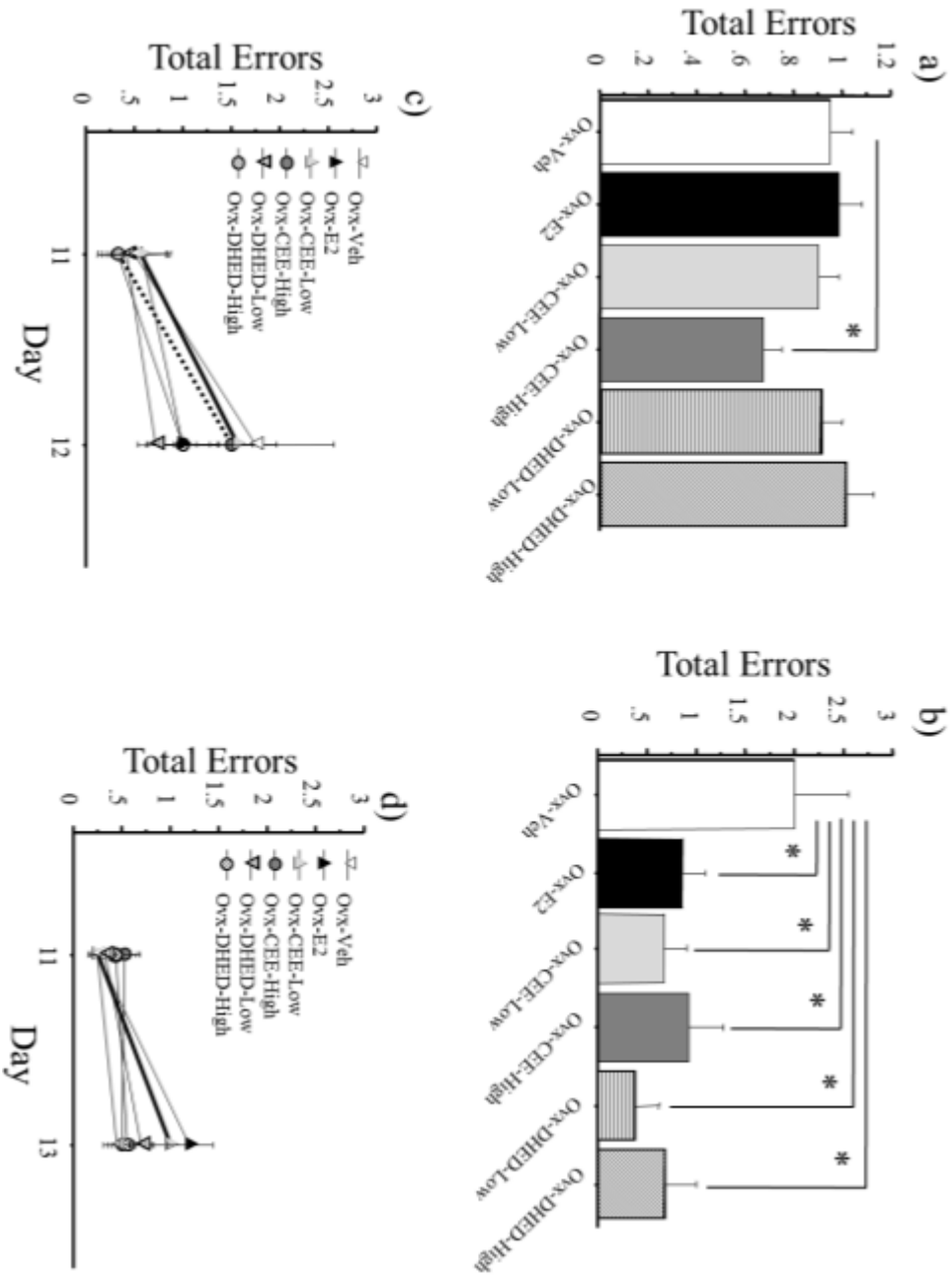

a)
Total Errors
Ovx-Veh
Ovx-E2
Ovx-CEE-Low
Ovx-CEE-High
Ovx-DHED-Low
Ovx-DHED-High
b)
Total Errors
c)
Total Errors
Day
d)
Total Errors
Day

Figure 14. a) Mean number ±*SE* of total errors across test trials for acquisition days 1-5 on the DMS Plus Maze for each group. b) Mean number ±*SE* of total errors committed across both delay challenges for each group. c) Mean number ±*SE* of total errors committed from the last day of baseline (day 11) to the interference challenge (day 12) for each group. d) Mean number ±*SE* of total errors committed from the last day of baseline (day 11) to the scopolamine challenge (day 13) for each group. a) The Ovx-CEE-High group displayed superior acquisition during the initial learning phase. No other estrogen impacted performance during acquisition or initial learning. b) After the acquisition, I increased the memory demand of the task to test retention by instituting a 4hr and a 6hr delay challenges. Each hormone group made fewer errors than the Ovx-Ovx-Veh group. In figures c) and d) all animals were impaired with the delay and interference challenge as evidenced by the increase from baseline. * $p < 0.05$.

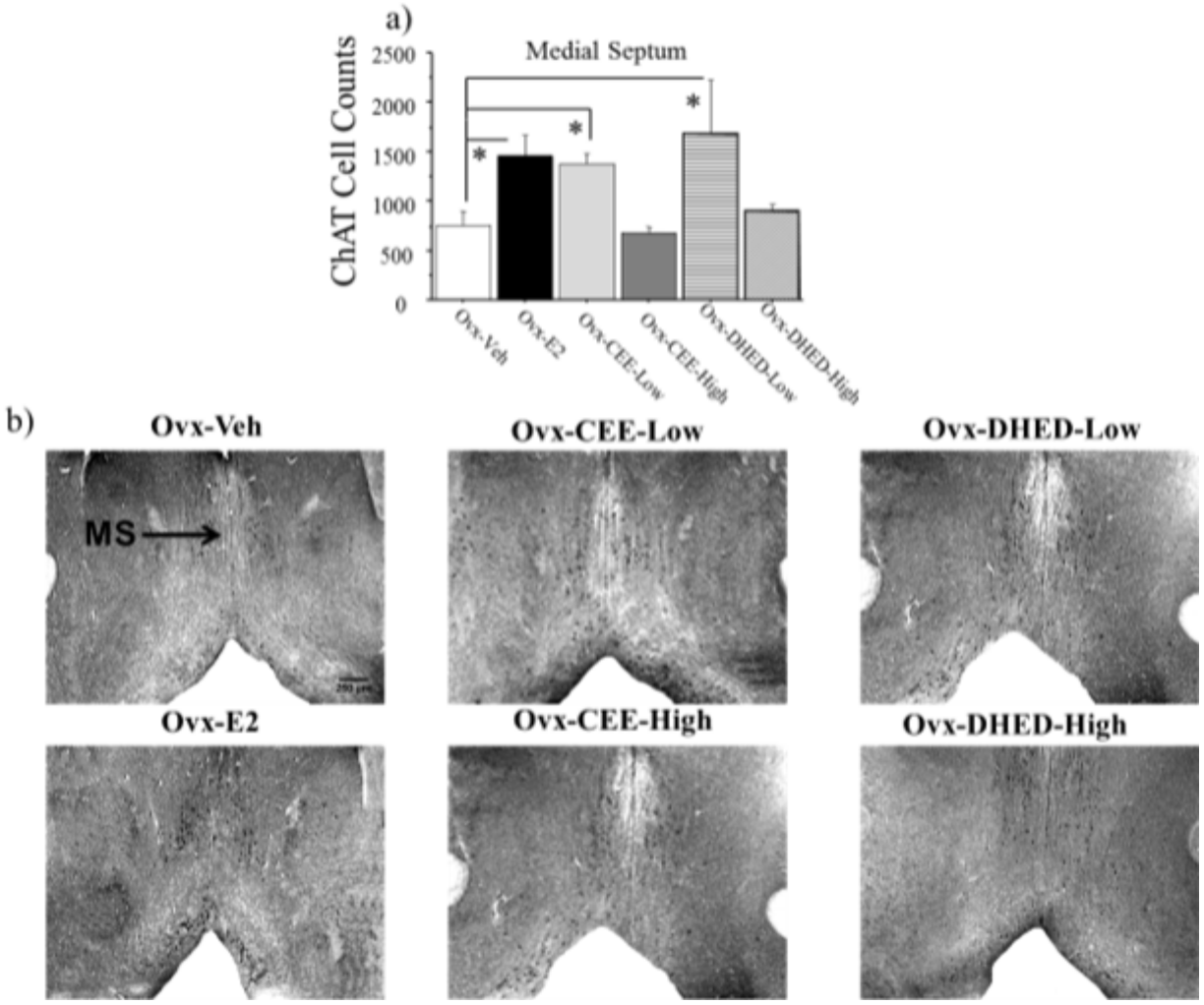

a)
Medial Septum
ChAT Cell Counts
2500
2000
1500
1000
500
0
*
*
*
Ovx-Veh
Ovx-E2
Ovx-CEE-Low
Ovx-CEE-High
Ovx-DHED-Low
Ovx-DHED-High
b)
Ovx-Veh
MS
Ovx-CEE-Low
Ovx-DHED-Low
Ovx-E2
Ovx-CEE-High
Ovx-DHED-High

Figure 15. a) Mean ±*SE* stereological ChAT-IR neuron counts in MS of the BF of each group. The Ovx-E2, Ovx-CEE-Low and Ovx-DHED-Low groups all had a higher number of ChAT-IR neuron count in the MS, as compared to the Ovx-Veh group. b) Representative photomicrographs show ChAT-IR neurons in sections through the MS of the BF of each group. * $p < 0.05$.

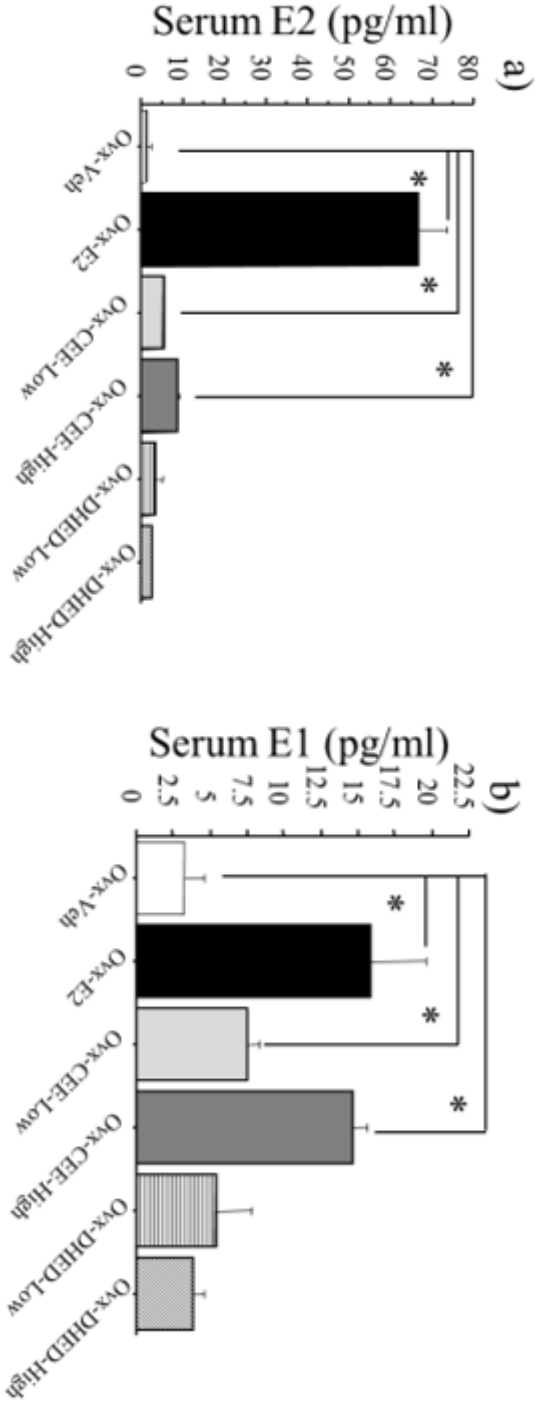

Serum E2 (pg/ml)
a)
0
10
20
30
40
50
60
70
80
Ovx-Veh
Ovx-E2
Ovx-CEE-Low
Ovx-CEE-High
Ovx-DHED-Low
Ovx-DHED-High
*
*
*
Serum E1 (pg/ml)
b)
0
2.5
5
7.5
10
12.5
15
17.5
20
22.5
Ovx-Veh
Ovx-E2
Ovx-CEE-Low
Ovx-CEE-High
Ovx-DHED-Low
Ovx-DHED-High
*
*
*

Figure 16. Bars represent mean serum hormone levels ±*SE* (pg/ml) for a) E2 b) E1. For both E2 and E1 the Ovx-E2 group exhibited the highest serum levels. Due to the high variability of the E2 group, analyses without the Ovx-E2 group reveled that both CEE doses increased serum E2 and E1 levels. Importantly, both DHED doses did not produce levels of E2 or E1 which differed from the Ovx-Veh group. * $p < 0.05$.

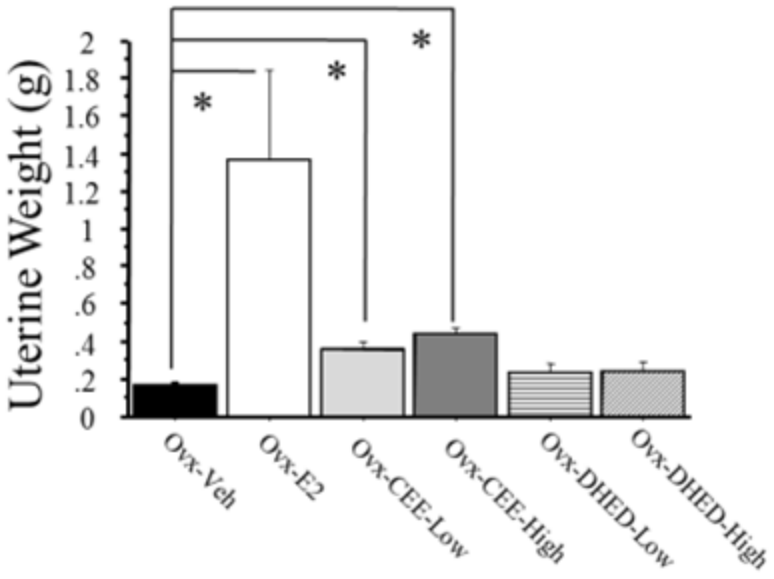

Uterine Weight (g)
2
1.8
1.6
1.4
1.2
1
.8
.6
.4
.2
0
*
*
*
Ovx-Veh
Ovx-E2
Ovx-CEE-Low
Ovx-CEE-High
Ovx-DHED-Low
Ovx-DHED-High

Figure 17. Bars depict the mean uterine weight ±*SE* for each group. The Ovx-E2 group exhibited the highest uterine weights. Due to the high variability of the E2 group, analyses without the Ovx-E2 group reveled that both CEE doses increased uterine weights. Importantly, both DHED doses did not produce uterine weights which differed from Ovx animals. * $p < 0.05$.

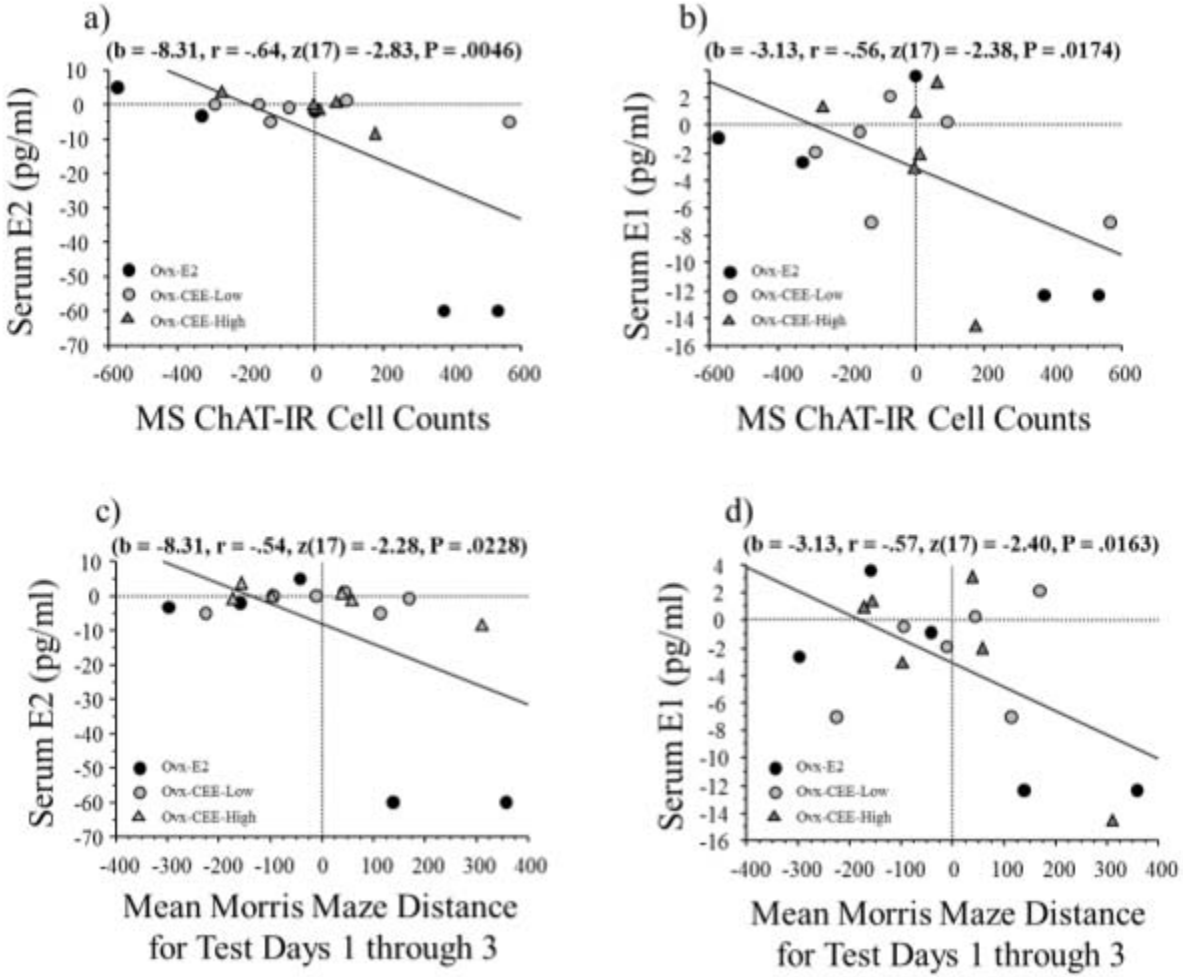

a)
(b = -8.31, r = -.64, z(17) = -2.83, P = .0046)
Serum E2 (pg/ml)
MS ChAT-IR Cell Counts
Ovx-E2
Ovx-CEE-Low
Ovx-CEE-High
b)
(b = -3.13, r = -.56, z(17) = -2.38, P = .0174)
Serum E1 (pg/ml)
MS ChAT-IR Cell Counts
c)
(b = -8.31, r = -.54, z(17) = -2.28, P = .0228)
Serum E2 (pg/ml)
Mean Morris Maze Distance
for Test Days 1 through 3
d)
(b = -3.13, r = -.57, z(17) = -2.40, P = .0163)
Serum E1 (pg/ml)
Mean Morris Maze Distance
for Test Days 1 through 3

Figure 18. Figure a) and b) centered scatterplots of serum E2 and E1 levels respectively and the number of MS ChAT-IR neurons for the Ovx-E2, Ovx-CEE-Low and Ovx-CEE-High groups. Higher serum E2 and E1 levels were related to less ChAT-IR positive cells in the MS. Fig. 5 c) and d) scatterplots of centered data for serum E2 and E1 levels respectively and Morris maze distance scores collapsed across all days for the Ovx-E2, Ovx-CEE-Low and Ovx-CEE-High groups. Higher serum E2 and E1 levels were related to less better performance (lower distance) across Morris maze testing.

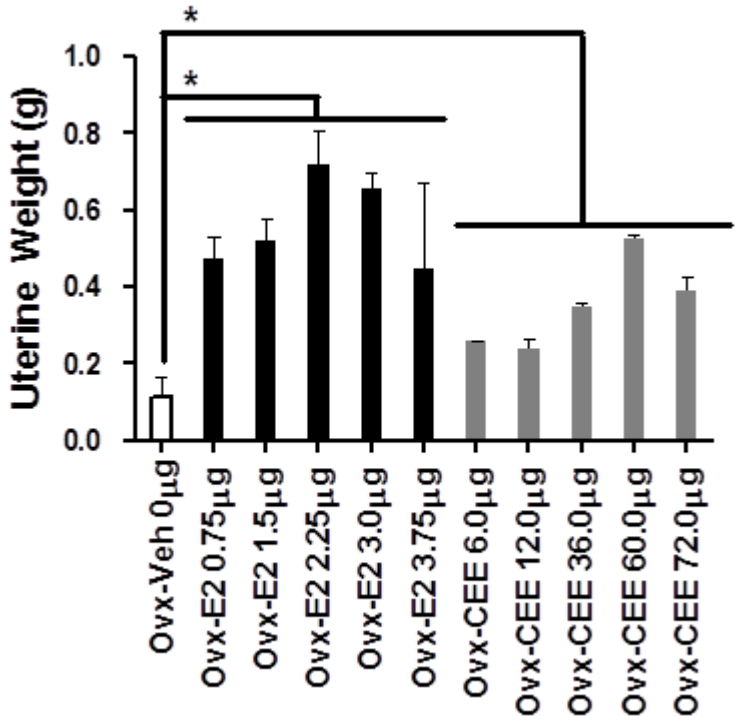
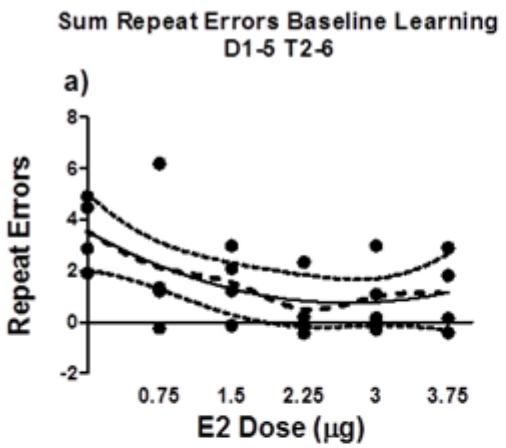
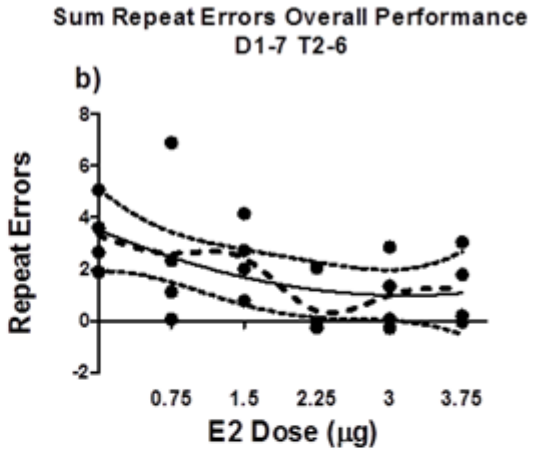
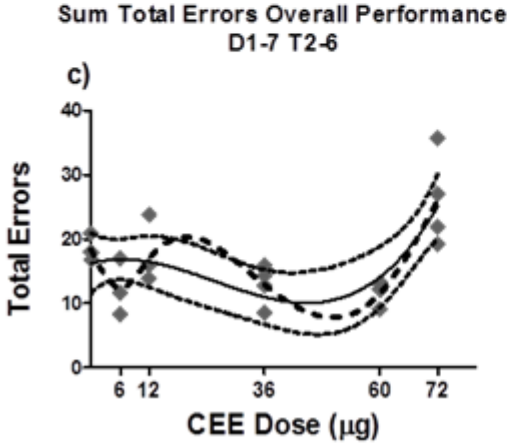

Uterine Weight (g)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Ovx-Veh 0µg
Ovx-E2 0.75µg
Ovx-E2 1.5µg
Ovx-E2 2.25µg
Ovx-E2 3.0µg
Ovx-E2 3.75µg
Ovx-CEE 6.0µg
Ovx-CEE 12.0µg
Ovx-CEE 36.0µg
Ovx-CEE 60.0µg
Ovx-CEE 72.0µg

Figure 19. Mean ±*SE* uterine weights (g) per group. At sacrifice, each tonic E2 and CEE treated group had higher uterine weights in comparison to the Ovx-Veh group. * $p < .05$.


Sum Repeat Errors Baseline Learning
D1-5 T2-6
a)
Repeat Errors
8
6
4
2
0
-2
0.75
1.5
2.25
3
3.75
E2 Dose (µg)


Sum Repeat Errors Overall Performance
D1-7 T2-6
b)
Repeat Errors
8
6
4
2
0
-2
0.75
1.5
2.25
3
3.75
E2 Dose (µg)


Sum Total Errors Overall Performance
D1-7 T2-6
c)
Total Errors
40
30
20
10
0
6
12
36
60
72
CEE Dose (µg)

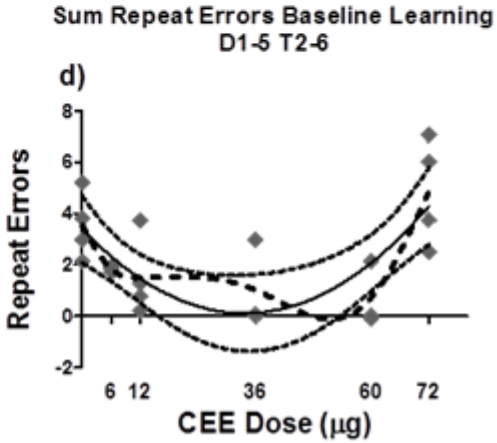

Sum Repeat Errors Baseline Learning
D1-5 T2-6
d)
Repeat Errors
8
6
4
2
0
-2
6
12
36
60
72
CEE Dose (µg)

Figure 20. Scatterplots of E2 or CEE dose (μg/day) and the sum of working/recent memory errors (i.e., trials 2-6) committed on the DMS Plus Maze. a) Repeat errors committed across the baseline learning phase (days 1-5) by the Ovx-Veh and all Ovx-E2 groups, b) repeat errors committed across all test days (days 1-7 overall performance) by the Ovx-Veh and all Ovx- E2 groups, c) total errors committed across all test days (days 1-7 overall performance) by the Ovx-Veh and all Ovx-CEE groups, and d) repeat errors committed across the baseline learning phase (days 1-5) by the Ovx-Veh and all Ovx-CEE groups.

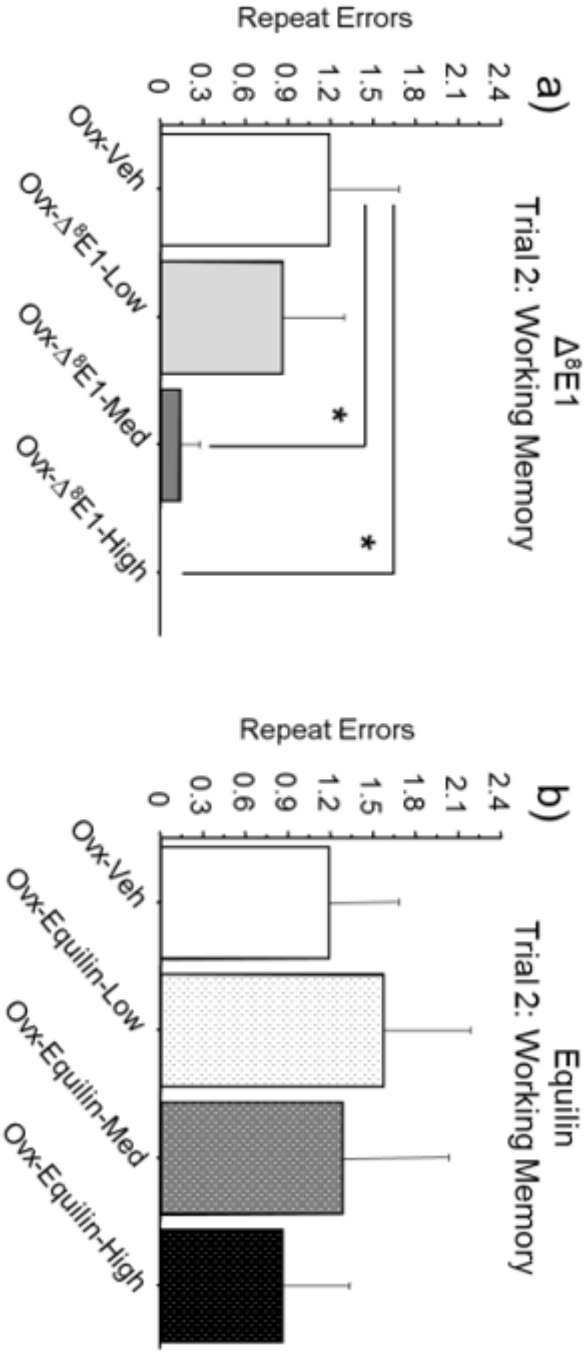

a)
Δ8E1
Trial 2: Working Memory
Repeat Errors
0
0.3
0.6
0.9
1.2
1.5
1.8
2.1
2.4
Ovx-Veh
Ovx-Δ8E1-Low
Ovx-Δ8E1-Med
Ovx-Δ8E1-High
*
*
b)
Equilin
Trial 2: Working Memory
Repeat Errors
0
0.3
0.6
0.9
1.2
1.5
1.8
2.1
2.4
Ovx-Veh
Ovx-Equilin-Low
Ovx-Equilin-Med
Ovx-Equilin-High

Figure 21. DMS Plus Maze. Mean number ±*SE* of repeat errors committed on the WM trial across testing days 2-7 for: a) Ovx-Veh and each Δ^8E1 group, and b) Ovx-Veh and each Equilin group. On the WM trial, the medium and high doses of Δ^8E1 decreased the number of repeat errors committed relative to the Ovx-Veh group. Equilin treatment did not yield significant effects. * $p < 0.05$.

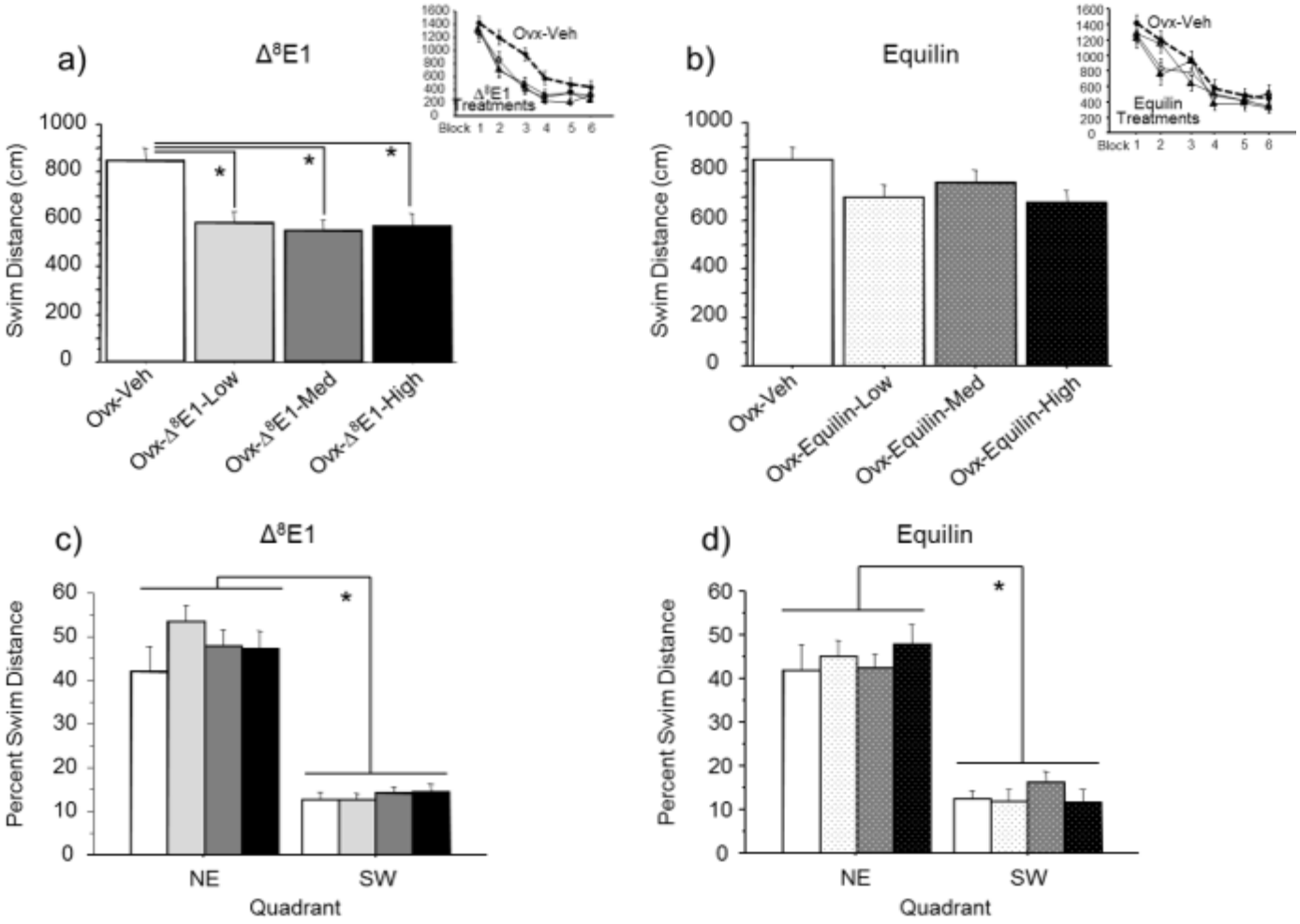

a)
Δ8E1
Swim Distance (cm)
1000
800
600
400
200
0
Ovx-Veh
Ovx-Δ8E1-Low
Ovx-Δ8E1-Med
Ovx-Δ8E1-High
Ovx-Veh
Δ8E1 Treatments
Block 1 2 3 4 5 6
b)
Equilin
Swim Distance (cm)
Ovx-Veh
Ovx-Equilin-Low
Ovx-Equilin-Med
Ovx-Equilin-High
Ovx-Veh
Equilin Treatments
c)
Δ8E1
Percent Swim Distance
60
50
40
30
20
10
0
NE
SW
Quadrant
d)
Equilin
Percent Swim Distance
NE
SW
Quadrant

Figure 22. Morris maze. Mean ±*SE* swim distance (cm) collapsed across days for: (a) Ovx-Veh and each Δ^8E1 group, and b) Ovx-Veh and each Equilin group; the inset graphs represent swim distance over the 6 blocks of testing. c) and d) depict mean ±*SE* percent swim distance in the target and opposite quadrants on the probe trial for the Ovx-Veh and each Δ^8E1 group, and the Ovx-Veh and each Equilin group, respectively. Each Δ^8E1 dose enhanced spatial RM relative to the Ovx-Veh group, collapsed across all days. Equilin did not impact spatial RM performance. For the probe trial, all groups localized to the quadrant that previously contained the platform (target, NE) as compared to the opposite quadrant (SW). * $p < 0.05$.

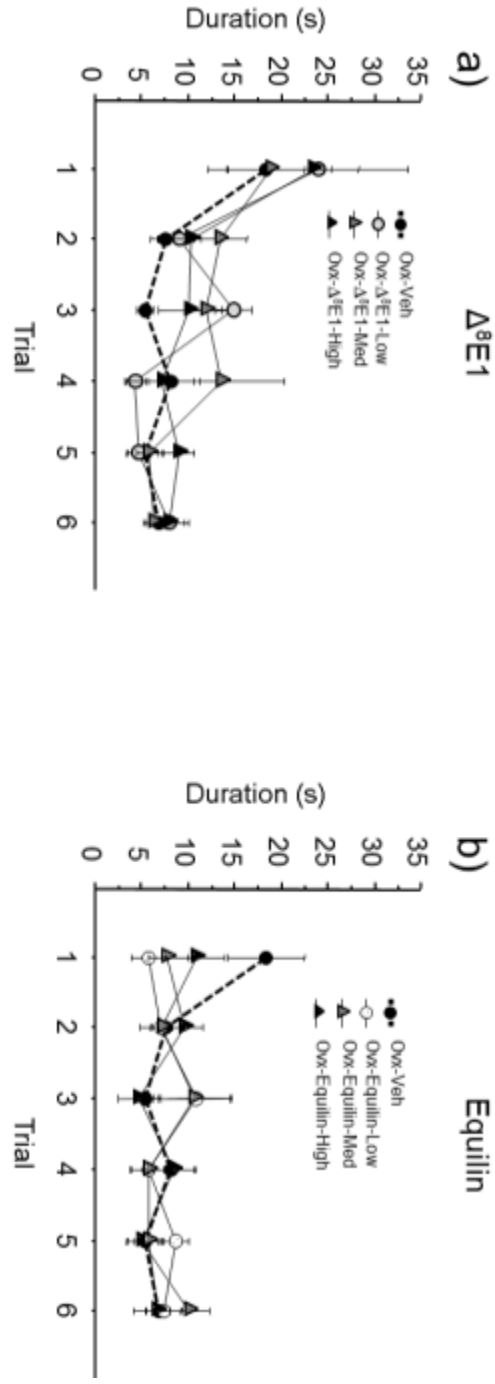

a)
Δ8E1
Duration (s)
0
5
10
15
20
25
30
35
1
2
3
4
5
6
Trial
Ovx-Veh
Ovx-Δ8E1-Low
Ovx-Δ8E1-Med
Ovx-Δ8E1-High
b)
Equilin
Duration (s)
0
5
10
15
20
25
30
35
1
2
3
4
5
6
Trial
Ovx-Veh
Ovx-Equilin-Low
Ovx-Equilin-Med
Ovx-Equilin-High

Figure 23. Visible Platform. Mean ±*SE* latency (seconds) to reach the platform on the visible platform task for: a) Ovx-Veh and each Δ^8E1 group, and b) Ovx-Veh and each Equilin group. There were no Treatment effects, and all groups readily located the visible platform within 10 seconds. These data confirm visual and motor competence by all subjects for platform search and localization.

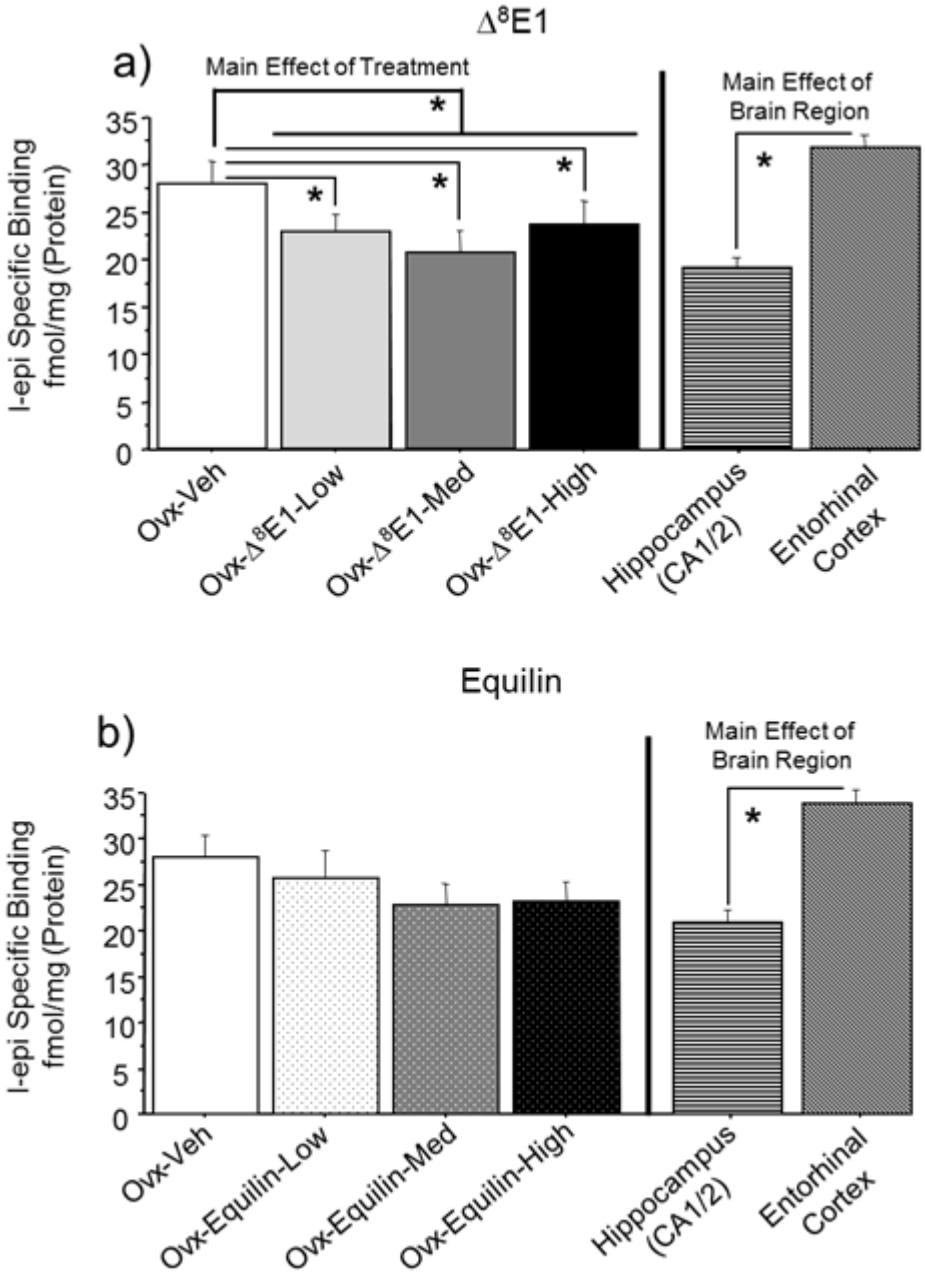

Δ8E1
a)
Main Effect of Treatment
Main Effect of Brain Region
I-epi Specific Binding fmol/mg (Protein)
35
30
25
20
15
10
5
0
*
Ovx-Veh
Ovx-Δ8E1-Low
Ovx-Δ8E1-Med
Ovx-Δ8E1-High
Hippocampus (CA1/2)
Entorhinal Cortex
Equilin
b)
Main Effect of Brain Region
I-epi Specific Binding fmol/mg (Protein)
Ovx-Veh
Ovx-Equilin-Low
Ovx-Equilin-Med
Ovx-Equilin-High
Hippocampus (CA1/2)
Entorhinal Cortex

Figure 24. Mean ±*SE* rα4β2-nAChR expression in the hippocampus and entorhinal cortex for: a) Ovx-Veh and each Δ^8E1 group, and b) Ovx-Veh and each Equilin group. As represented in the left panel, for the hippocampus+entorhinal cortex (regions combined), all Δ^8E1 groups had decreased $\alpha4\beta2$-nAChR expression as compared to the Ovx-Veh group. Equilin treatment did not alter r$\alpha4\beta2$-nAChR expression. As represented in the right panel, both the Δ^8E1 and equilin analyses, more $\alpha4\beta2$-nAChRs were present in the entorhinal cortex as compared to the hippocampus. * $p < 0.05$.

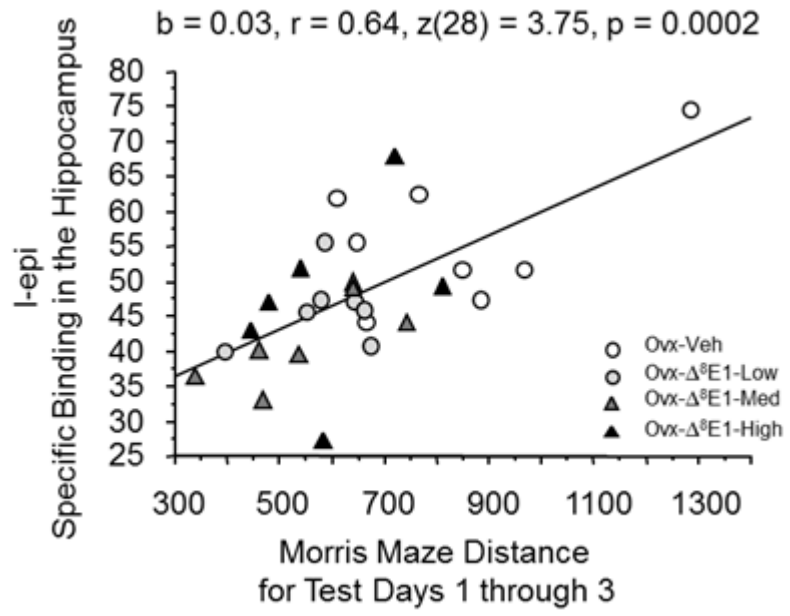

b = 0.03, r = 0.64, z(28) = 3.75, p = 0.0002
I-epi
Specific Binding in the Hippocampus
80
75
70
65
60
55
50
45
40
35
30
25
300
500
700
900
1100
1300
Morris Maze Distance
for Test Days 1 through 3
Ovx-Veh
Ovx-Δ8E1-Low
Ovx-Δ8E1-Med
Ovx-Δ8E1-High

Figure 25. Scatterplot representing the relation between rα4β2-nAChR expression levels in the hippocampus+entorhinal cortex and Morris maze swim distance across all days and trials for all Δ^8E1 groups and the Ovx-Veh group. The line represents the linear regression analysis of best fit. Better RM performance (lower swim distance) was related to lower rα4β2-nAChR expression.

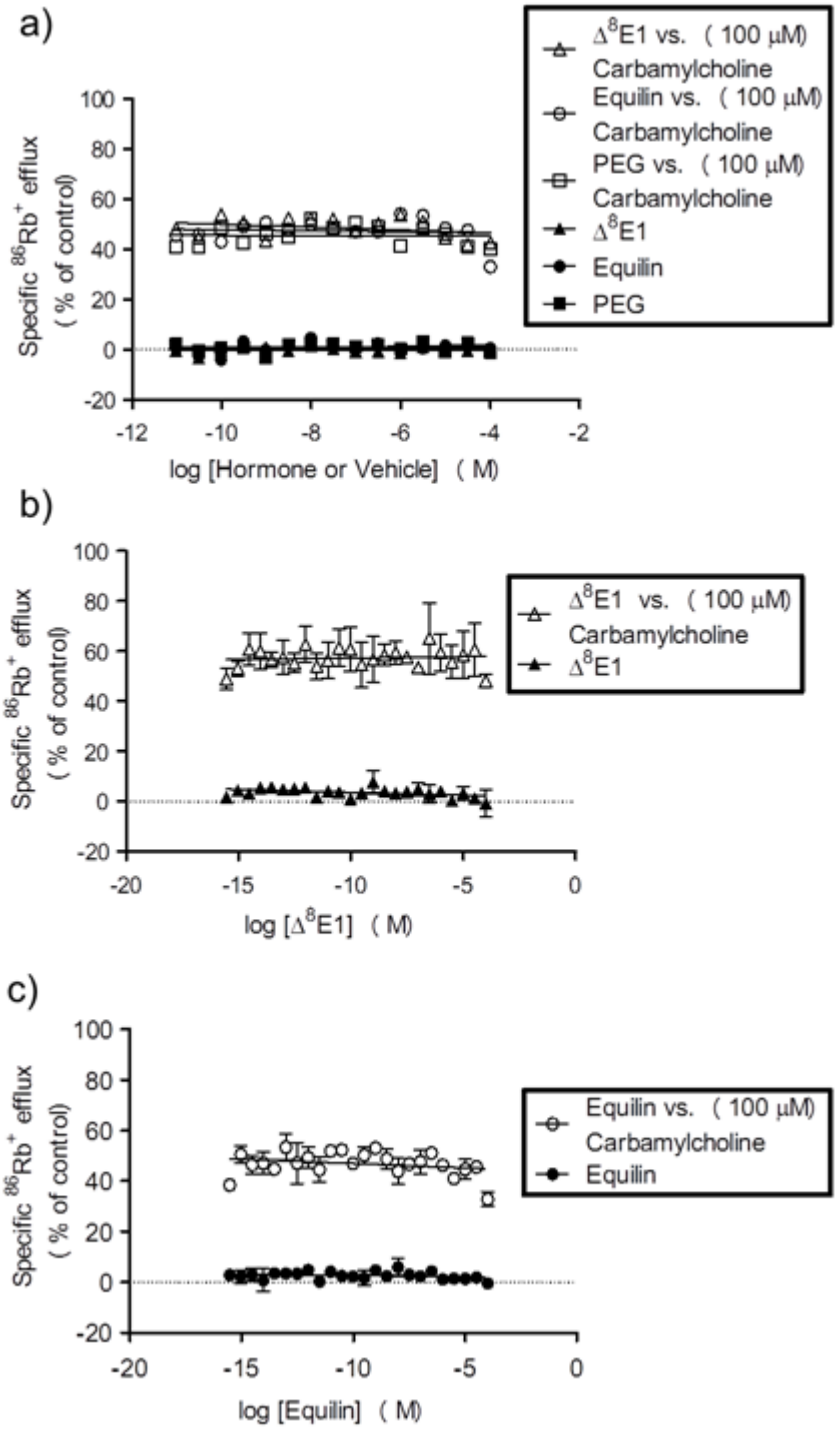

a)
Specific $^{86}Rb^{+}$ efflux
(% of control)
100
80
60
40
20
0
-20
-12
-10
-8
-6
-4
-2
log [Hormone or Vehicle] (M)
Δ^8E1 vs. (100 μM) Carbamylcholine
Equilin vs. (100 μM) Carbamylcholine
PEG vs. (100 μM) Carbamylcholine
Δ^8E1
Equilin
PEG
b)
Specific $^{86}Rb^{+}$ efflux
(% of control)
100
80
60
40
20
0
-20
-20
-15
-10
-5
0
log [Δ^8E1] (M)
Δ^8E1 vs. (100 μM) Carbamylcholine
Δ^8E1
c)
Specific $^{86}Rb^{+}$ efflux
(% of control)
100
80
60
40
20
0
-20
-20
-15
-10
-5
0
log [Equilin] (M)
Equilin vs. (100 μM) Carbamylcholine
Equilin

Figure 26. Concentration-response profiles showing effects on hα4β2-nAChR function (i.e., ion efflux) following: a) acute treatment with Δ^8E1, equilin or vehicle PEG, b) 48hr treatment with Δ^8E1 and c) 48hr treatment with equilin. The lines on each graph represent the linear regression analysis of best fit. No systematic effect of acute or 48hr hormone or vehicle exposure on specific $^{86}Rb^+$ efflux was observed over the range of hormone or vehicle concentrations tested.

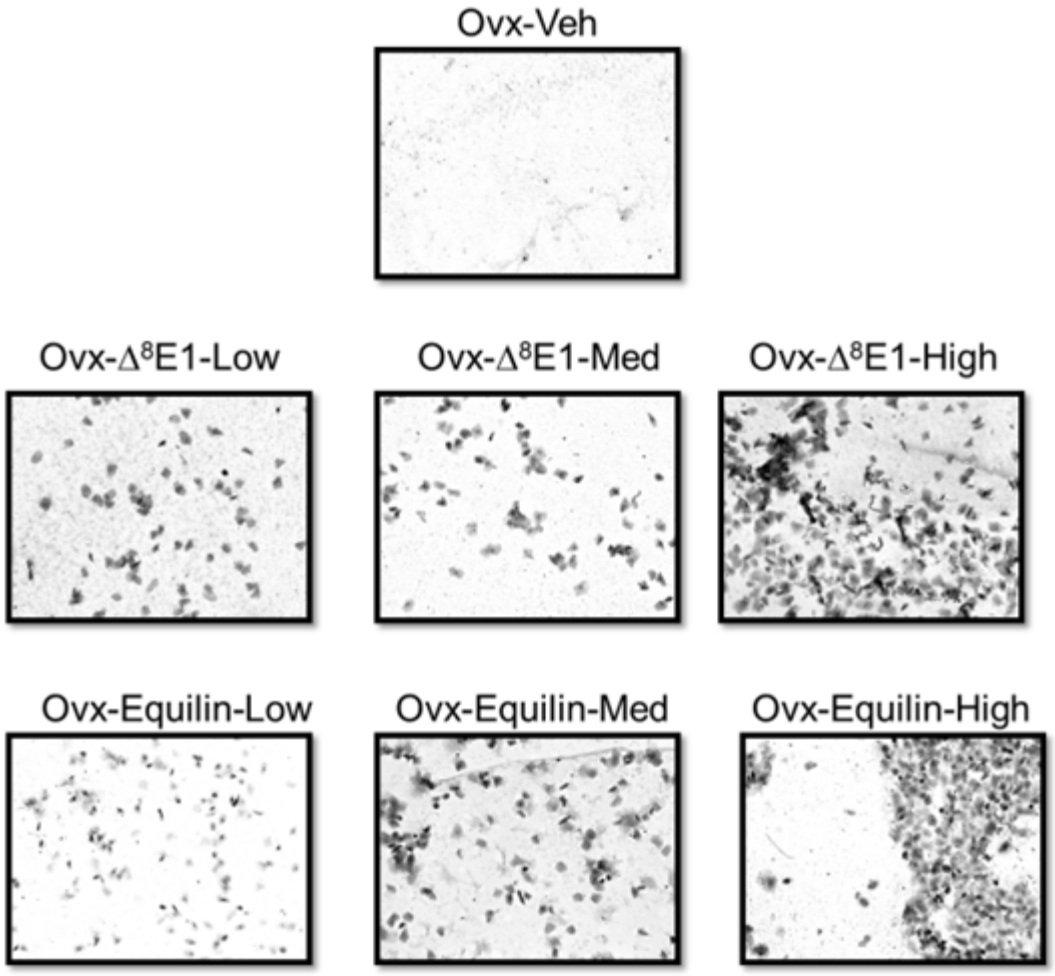

Ovx-Veh
Ovx-Δ8E1-Low
Ovx-Δ8E1-Med
Ovx-Δ8E1-High
Ovx-Equilin-Low
Ovx-Equilin-Med
Ovx-Equilin-High

Figure 27. Representative brightfield eosinY- and hematoxylin- stained vaginal cytology pictures (10X) from Ovx-Veh, Δ^8E1, and Equilin groups, taken the day before sacrifice. Ovx-Veh animals displayed few cells, consistent with diestrus and no uterine stimulation. Each group receiving Δ^8E1 or equilin had numerous stained cornified cells, indicative of estrous and uterine stimulation.

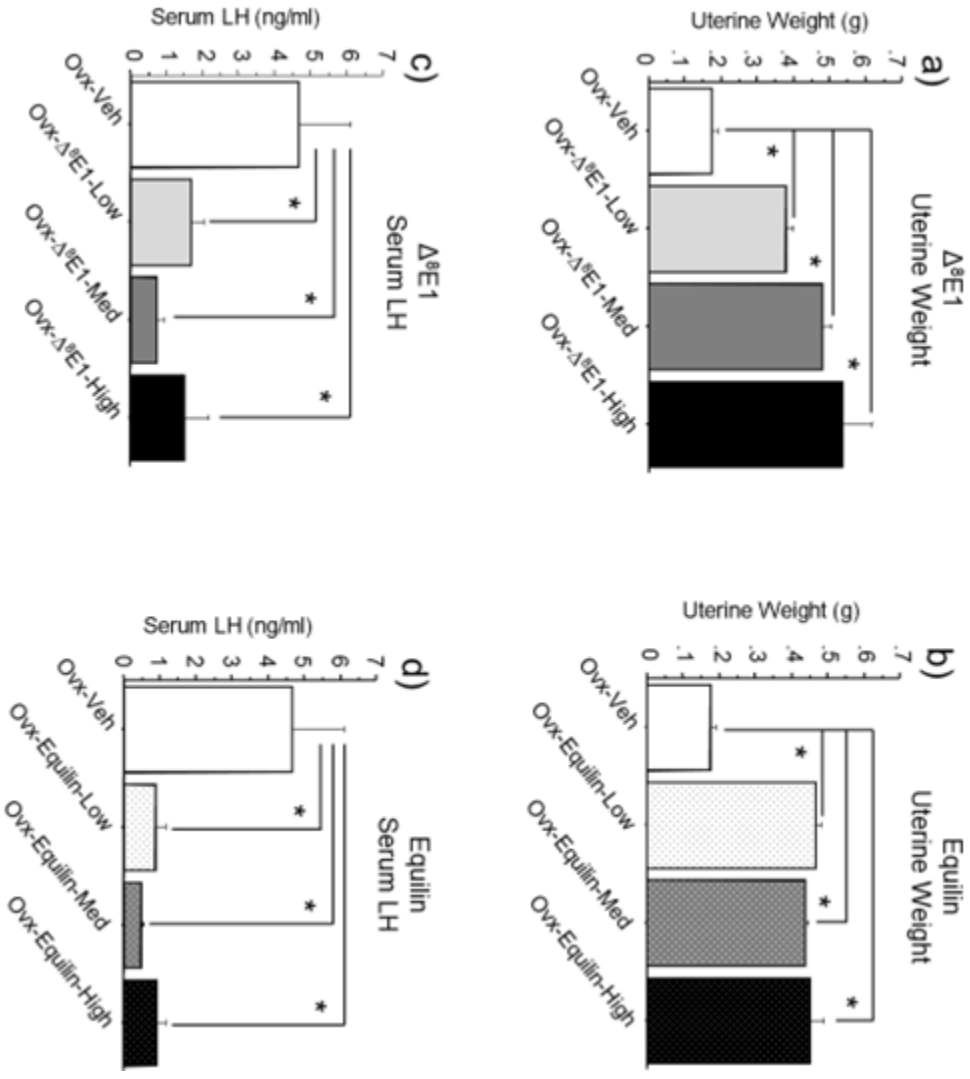
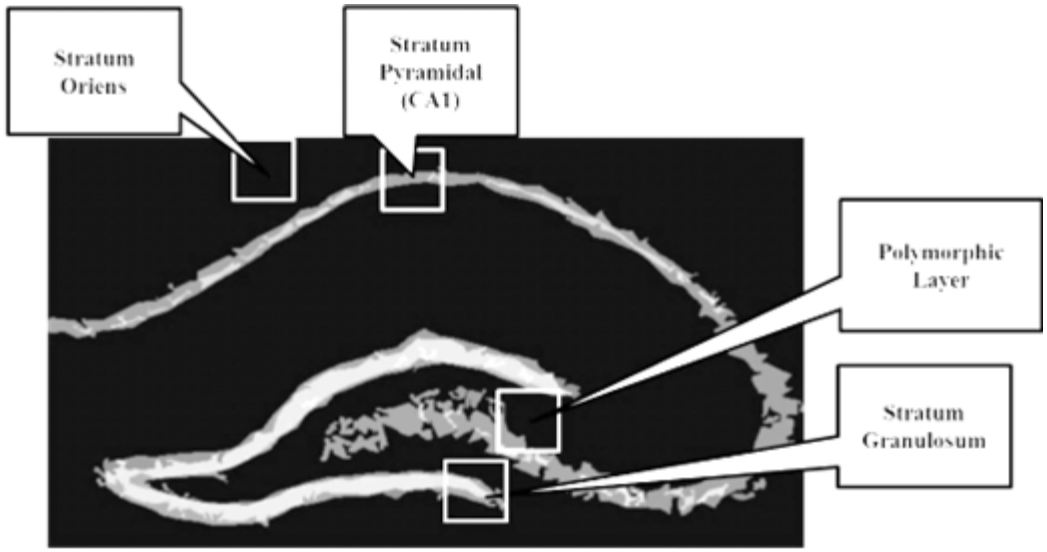

a)
Δ8E1
Uterine Weight
Uterine Weight (g)
0
.1
.2
.3
.4
.5
.6
.7
Ovx-Veh
Ovx-Δ8E1-Low
Ovx-Δ8E1-Med
Ovx-Δ8E1-High
*
b)
Equilin
Uterine Weight
Uterine Weight (g)
Ovx-Veh
Ovx-Equilin-Low
Ovx-Equilin-Med
Ovx-Equilin-High
c)
Δ8E1
Serum LH
Serum LH (ng/ml)
0
1
2
3
4
5
6
7
d)
Equilin
Serum LH
Serum LH (ng/ml)

Figure 28. Mean ±*SE* uterine weight (grams) for: a) Ovx-Veh and each Δ^8E1 group, and b) Ovx-Veh and each Equilin group. Mean ±*SE* serum LH levels (ng/ml) for: c) Ovx-Veh and each Δ^8E1 group, and d) Ovx-Veh and each Equilin group. Each dose of Δ^8E1 and equilin increased uterine weights and decreased serum LH levels as compared to the Ovx-Veh group. * $p < 0.05$.

a)


Stratum
Oriens
Stratum
Pyramidal
(CA1)
Polymorphic
Layer
Stratum
Granulosum

b)

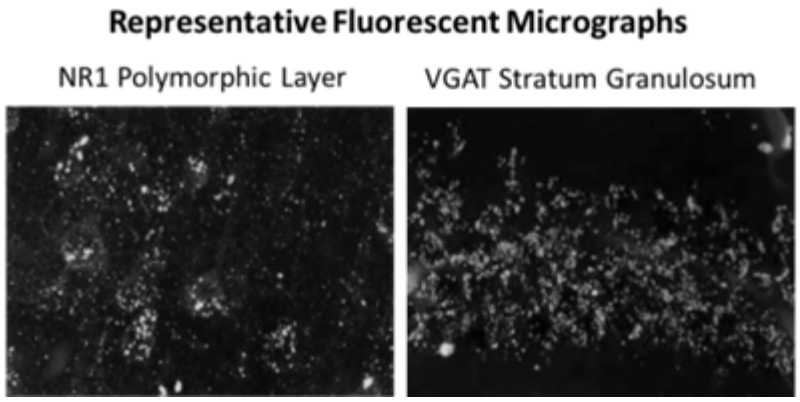

Representative Fluorescent Micrographs
NR1 Polymorphic Layer
VGAT Stratum Granulosum

Figure 29. a) Illustration detailing where each image stack was acquired in order to assess putative synapse numbers throughout the female rat hippocampus. Four images/z-stack were acquired and quantified in CA1 stratum oriens and CA1 stratum pyramidal as well as the polymorphic layer and granular cell layer of the dentate gyrus. b) Representative fluorescent micrographs of NR1 and vGAT punctate staining in the hippocampus of female rats. Both NR1 and vGAT IHC procedures produced distinct punctate staining revealed via laser scanning confocal microscopy throughout the female rat hippocampus.

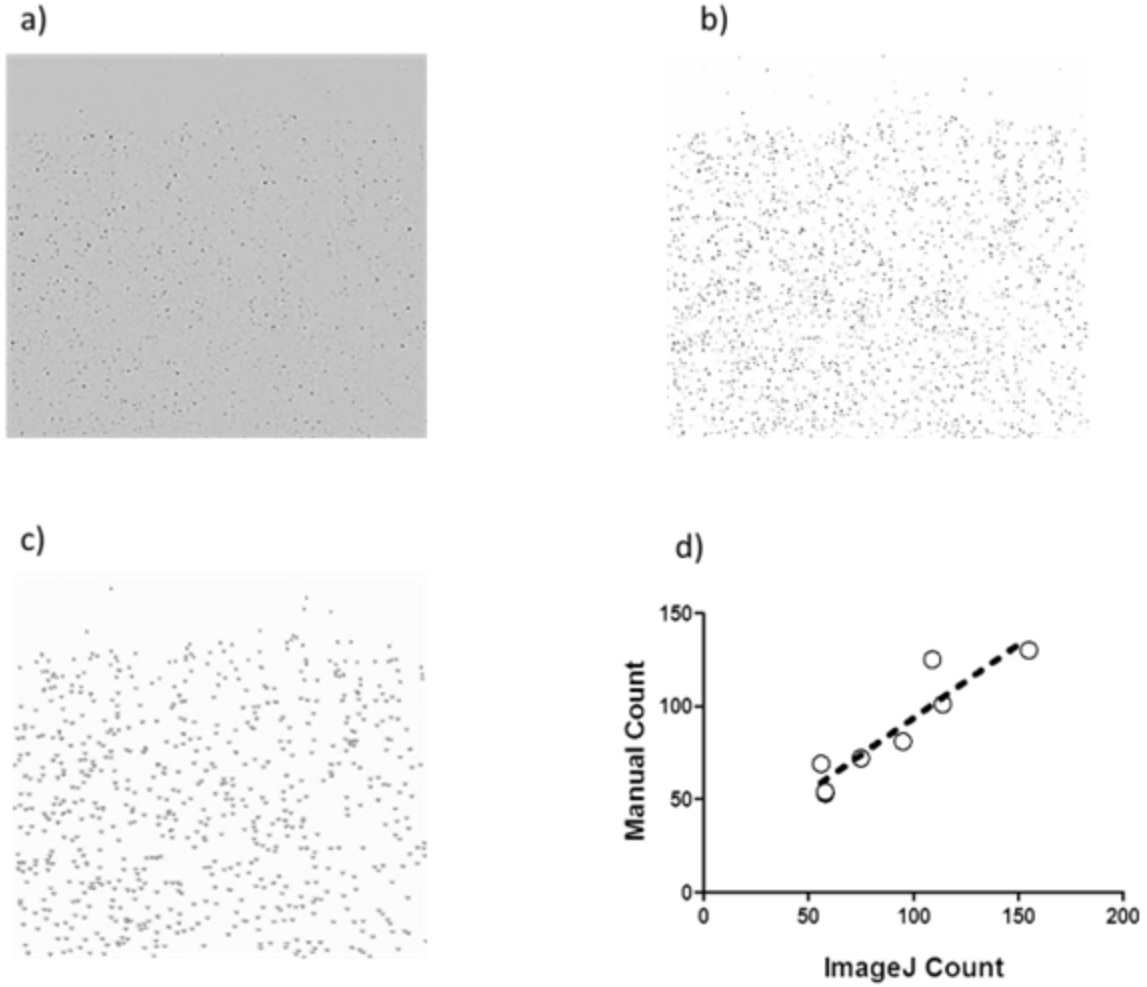

a)
b)
c)
d)
150
100
50
0
0
50
100
150
200
Manual Count
ImageJ Count

Figure 30. a) through c) represents a step by step presentation of the ImageJ procedures used to quantify the number of punctate staining in each of the four images in a z-stack. d) Scatter plot of the number of punctate counted via the ImageJ semi-automatic method related to the number of punctate counted manually in the same image sections; the dotted line represents the linear regression line of best fit. Separately for both NR1 and vGAT z-stacks, after the first and last images were deleted, the raw FluoView tiff 4 image z-stacks had a: a) spot detector based on a "3D LoG" filter applied to each image to decrease background and enhance the punctate staining, next b) each image was thresholded via "Mixture Modeling," and c) the ImageJ "Analyze Particles" tool was used to automatically count each punctate across all 4 images in a z-stack. d) Primary regression analysis revealed that there was a strong linear relationship ($R^2 = 0.85$) between the number of punctate that the semi-automatic and the manual method counted.

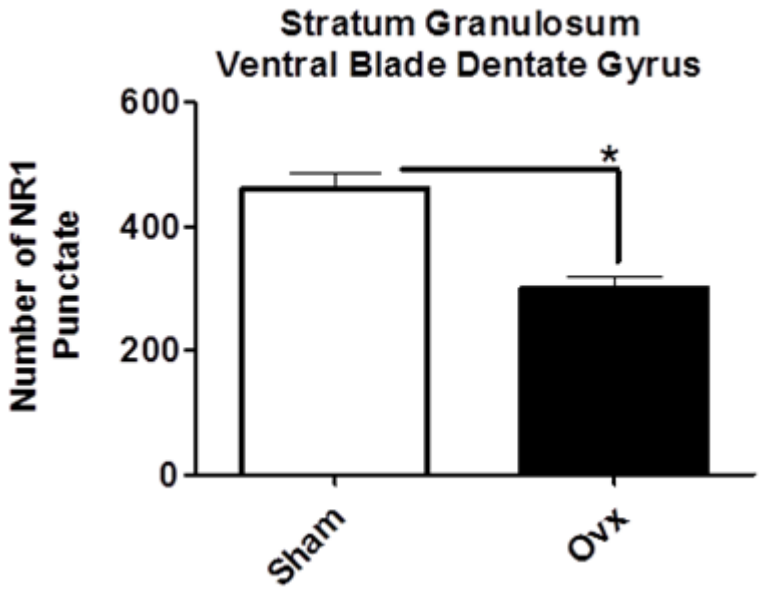

Stratum Granulosum
Ventral Blade Dentate Gyrus
Number of NR1 Punctate
600
400
200
0
*
Sham
Ovx

Figure 31. Mean ±*SE* number of NR1 punctate counted in the stratum granulosum of the dentate gyrus. Ovx animals in comparison to Sham ovary intact animals had a decreased in the number of putative excitatory synapses found on ventral blade granular cells of the dentate gyrus. * $p \leq 0.05$.

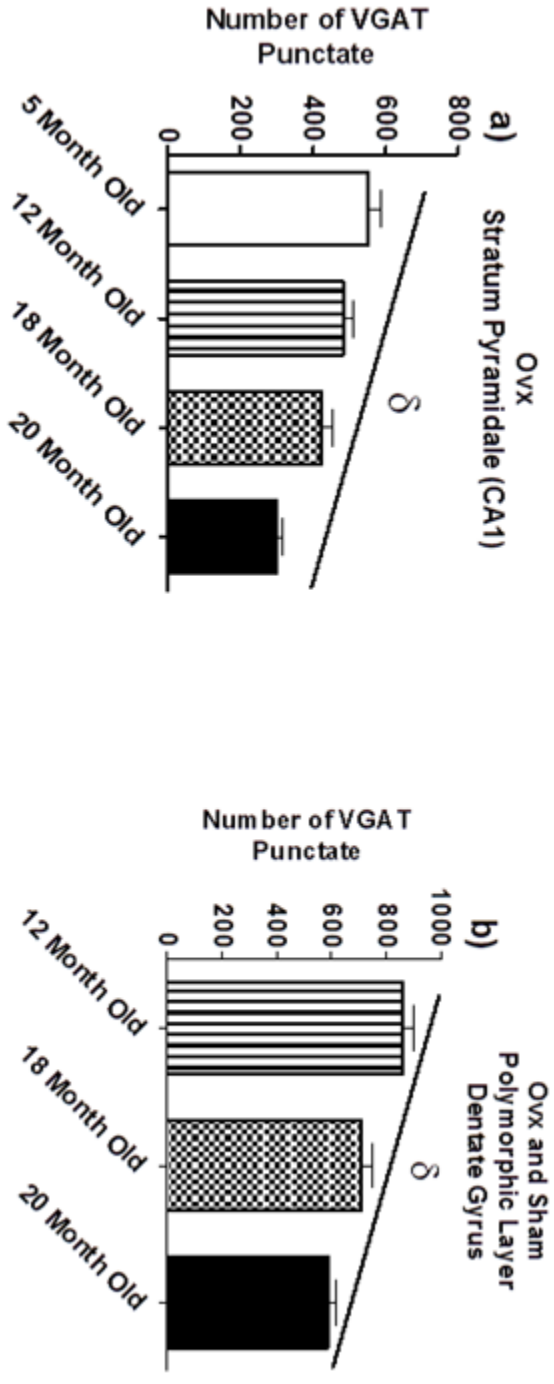

a)
Ovx
Stratum Pyramidale (CA1)
Number of VGAT Punctate
0
200
400
600
800
5 Month Old
12 Month Old
18 Month Old
20 Month Old
δ
b)
Ovx and Sham
Polymorphic Layer
Dentate Gyrus
Number of VGAT Punctate
0
200
400
600
800
1000
12 Month Old
18 Month Old
20 Month Old
δ

Figure 32. Mean ±*SE* number of vGAT punctate in separate age groups counted in a) CA1 stratum pyramidale and b) the polymorphic layer of the dentate gyrus; solid lines represent linear trends (i.e., linear contrasts) across the mean punctate count for each age group. a) The number of putative inhibitory synapses decreased in linear fashion from 5 to 20 months of age on the pyramidal cells of CA1 in Ovx animals. b) Putative inhibitory synapse numbers decreased in linear fashion from 12 to 20 months of age in the polymorphic layer of the dentate gyrus when Ovx and Sham animals were evaluated together. δ linear trend $p \leq 0.05$.

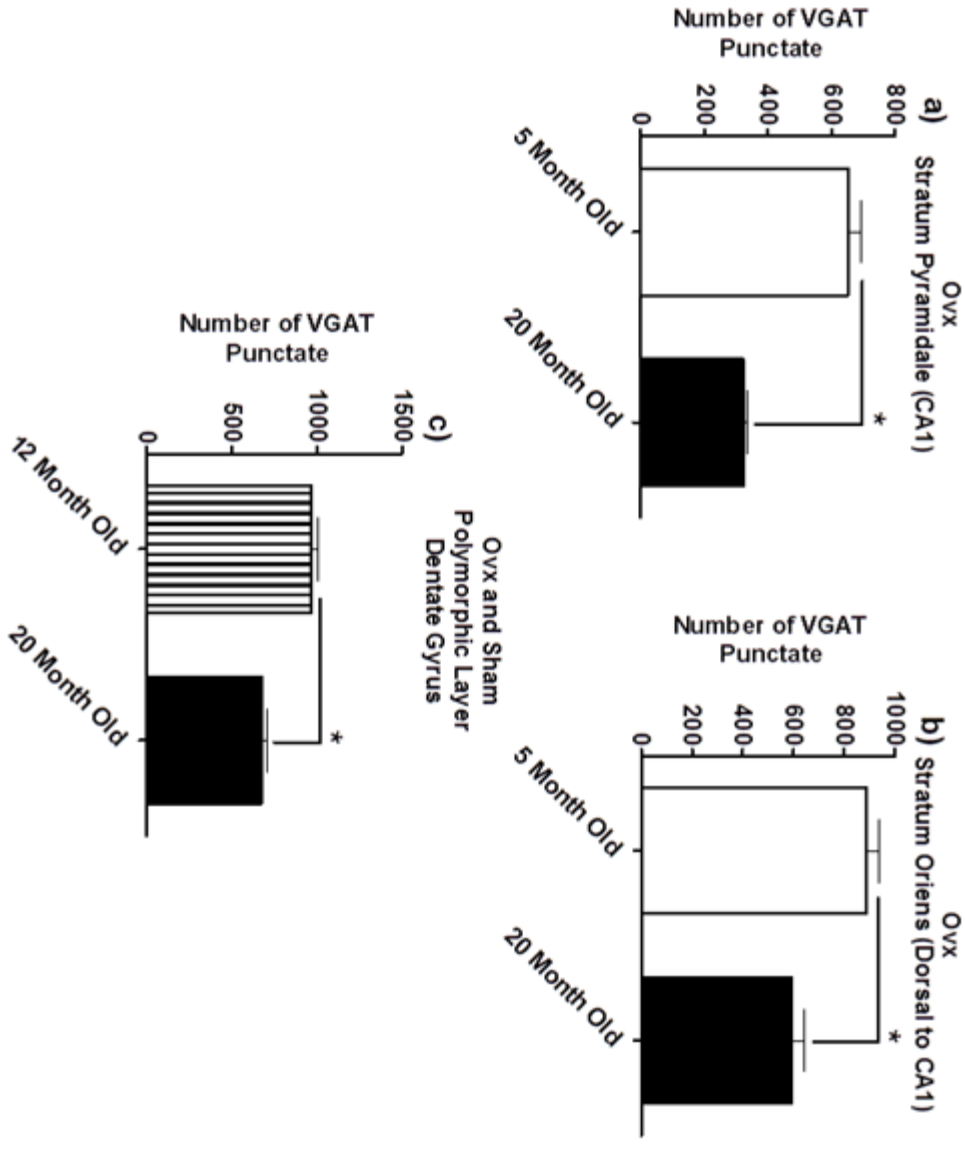

a)
Ovx
Stratum Pyramidale (CA1)
Number of VGAT Punctate
0
200
400
600
800
5 Month Old
20 Month Old
*
b)
Ovx
Stratum Oriens (Dorsal to CA1)
Number of VGAT Punctate
0
200
400
600
800
1000
5 Month Old
20 Month Old
*
c)
Ovx and Sham
Polymorphic Layer
Dentate Gyrus
Number of VGAT Punctate
0
500
1000
1500
12 Month Old
20 Month Old
*

Figure 33. Mean ±*SE* number of vGAT punctate between 5 and 20 as well as 12 and 20 month old age groups counted in a) CA1 stratum pyramidale, b) stratum oriens, and c) the polymorphic layer of the dentate gyrus. a) The numbers of putative inhibitory synapses on the pyramidal cells of CA1 in Ovx animals were decreased in the 20 month old group when compared to the 5 month old group. b) In Ovx animals, the numbers of putative inhibitory synapses in stratum oriens dorsal to the CA1 cell layer were decreased in the 20 month old group when compared to the 5 month old group. c) Putative inhibitory synapse numbers decreased in the 20 month old group when compared to the 12 month old group in the polymorphic layer of the dentate gyrus when Ovx and Sham animals were evaluated together. * $p \leq 0.05$.

APPENDIX A

SECURED PERMISSION TO INCLUDE PUBLISHED RESEARCH

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

APPENDIX B

DIRTY VODKA MARTINI

Ingredients

6 fluid ounces vodka

1 dash dry vermouth

1 fluid ounce brine from olive jar

4 stuffed green olives

Directions

In a mixing glass, combine vodka, dry vermouth, brine and olives. Pour into a glass over ice and stir gently. Either drink on the rocks, or strain into a chilled cocktail glass.