EFFECTS OF ESTROGEN ON HIPPOCAMPAL FUNCTION IN YOUNG ADULT AND AGED FEMALE RATS

by

LINDSEY C. VEDDER

LORI L. MCMAHON, PhD, COMMITTEE CHAIR
J. MICHAEL WYSS, PhD
CANDACE FLOYD, PhD
J. DAVID SWEATT, PhD
LINDA OVERSTREET WADICHE, PhD

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ABSTRACT

In rodents, the ovarian estrogen 17β–estradiol (E2) is a potent modulator of hippocampal function and the hippocampus is critical for many types of learning and memory. Specifically, E2 increase the magnitude of long-term potentiation (LTP) at CA3-CA1 synapses, the density of dendritic spines in CA1 pyramidal cells and current mediated by NR2B-containing NMDA receptors. The E2-induced increase in LTP further requires NR2B-containing NMDARs. While considered a cellular correlate of learning and memory, enhanced LTP does not always predict enhanced learning. The first goal of this dissertation was to investigate the relationship between E2-enhanced LTP and learning and memory in young adult female rats and to determine whether E2-enhanced learning and memory requires NR2B-containing NMDARs. The results in the first part of this dissertation mechanistically link E2-enhanced learning and memory with E2-enhanced magnitude of LTP.

The Women's Health Initiative Memory Study reported that estrogen replacement therapy does not protect against dementia and cognitive decline in post-menopausal women. The critical period hypothesis could explain this lack of benefit,

stating that a window of time post-menopause may exist during which E2 must be replaced to remain beneficial to cognition. The second goal of this dissertation was to directly test whether such a critical period exists during which time E2 is able to enhance hippocampal physiology, morphology and learning and memory in female rats. It was also determined whether chronic replacement with E2 at physiological levels is sufficient to protect against the loss of E2-enhanced hippocampal function directly caused by long-term ovarian hormone loss. Results from this work are in strong support of the critical period hypothesis as E2 enhances hippocampal function using precisely the same mechanisms in young and aged female rats until 15 months of ovarian hormones loss, being absent after 19 months of ovarian hormone loss. Chronic E2 replacement is not sufficient to completely protect against the loss of E2-effectiveness and thus a more cycling replacement schedule of E2, or E2 along with progesterone to mimic ovarian hormones in ovary intact female rats may be required for maximal protection of hippocampal function.

DEDICATION

To my loving and supportive husband Alan as I could not have accomplished all I have without him, and to my son Owen for bringing me endless joy.

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First I would like to thank Dr. Lori McMahon for allowing me to join her lab and work on this amazing project. Dr. McMahon provided the perfect balance of freedom and guidance. She allowed me to take this project in new directions, while making sure I never strayed too far off course. On top of being an outstanding mentor, Dr. McMahon also collected the whole-cell data in the aged ovary intact and chronic estrogen replacement studies and I thoroughly enjoyed our weekends at the rigs. Dr. McMahon has shown me what it takes to be a great mentor, scientist and teacher. I am grateful for having been given the opportunity to be trained in her lab and a part of the McMahon lab family.

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I often wonder if I would have gone to graduate school without my experiences in the McNair Scholars Program while at SUNY Buffalo. This program provided me with the skills I needed to get into graduate school, and the support from the staff as well as other students in the program gave me the drive to push through the application process. I know that without the advice from Cecil Walters, the program's director, I would have taken a year off after undergraduate school and who knows where I would be today without his coaching.

I would also like to mention that my inspiration for entering the field of learning and memory came from my Pap-pap, who struggled with Alzheimer's disease for years before his death a year ago. I have very dear memories while I was growing up spending weekends with him and my Nana at their country home in Pennsylvania. I hope that future studies will lessen the burdens experienced by people like my Pap-pap, as well as people like my Nana, who care for a person they love inflicted with this disease.

I cannot thank my family enough for their endless love and support, especially during the past 5 years, and I am very grateful for how close we all are. My mother is the most patient and thoughtful person I know and is always there for me when I need her, and I needed her a lot during graduate school. My father has always put a lot of emphasis on education and he has encouraged my accomplishments as far back as I can remember. My sister, Tiffany, has become my best friend and while I am the first person in my family to earn this degree I know that she will be close behind me and I can't wait to share this experience with her. I also want to thank my husband's family for their love and support, and thank them for including me in their family as if I were always apart of it. I'm looking forward to moving closer to home and "stealing time" with all of them.

Finally, I will be forever indebted to my husband, Alan. I thank him for his sacrifices, as I know how hard it was for him to be so far from his family, especially with the loss of his mother and grandmother. Alan also put up with me working many late hours and weekends, playing the single parent while I finished my degree. I am so lucky to have found him and have him in my life. He is an amazing father to our son Owen and I know he is as excited as I am to embark on this next chapter in our life together.

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LIST OF ABBREVIATIONS

AMPAR	2-amino-3-proponoic acid receptor
APV	amino-5-phosphonovaleric acid
DNQX	6,7-dinitroquinoxaline-2,3-dione
E2	17β-estradiol
ERT	estrogen replacement therapy
ER	estrogen receptor
ERα	estrogen receptor
ERβ	estrogen receptor
LTP	long-term potentiation
NMDAR	N-methyl D-aspartate receptor
NOR	novel object recognition
ORTHO	orthovanadate
OVX	ovariectomy
mPOVX	months post ovariectomy
RO	Ro25-6981
Sal	saline
V	vehicle

INTRODUCTION

Clinical significance

In post-menopausal women, a sharp decline in circulating levels of the ovarian estrogen 17β-estradiol (E2) (Longcope, 1971) occurs simultaneously with deficits in cognition (Sherwin, 1988; Phillips and Sherwin, 1992a; Zec and Trivedi, 2002). E2 replacement therapy has been shown to alleviate cognitive decline in post-menopausal women (Phillips and Sherwin, 1992a; Zec and Trivedi, 2002) suggesting that the loss of E2 in menopause may be causal to this reduction in cognition. This strong clinical support for the beneficial effects of E2 replacement to alleviate hormone related cognitive decline lead to the Women's Health Initiative Memory Study (WHIMS), a multi-institutional placebo-controlled trial to investigate the effects of estrogen or estrogen plus progesterone replacement on cognition in post-menopausal women (Shumaker et al., 2004). Findings from this study contradicted earlier reports that hormone replacement therapy benefited cognition in post-menopausal women. In fact, the WHIMS reported that hormone replacement can even cause negative effects on cognition in post-menopausal women and the authors recommended against hormone replacement therapy in women over the age of 65 for the treatment of deficits in cognition (Shumaker et al., 2003; Shumaker et al., 2004). This major discrepancy to previously accepted scientific evidence necessitates that the mechanisms and effectiveness of E2-enhanced cognition in aging and periods of long-term ovarian hormone loss be determined. In this dissertation these concerns were directly addressed using a rodent model. The goal of this work was

to first determine mechanisms of E2-enhanced learning and memory in young adult ovariectomized (OVX) rats and second to determine whether these mechanisms persist during aging, long-term ovarian hormone loss and chronic E2 replacement.

Why 17β-estradiol?

There are three types of estrogen produced by the mammalian reproductive system, estrone (E1), estradiol (E2) and estriol (E3). Each of theses estrogens dominate during different reproductive stages, with levels of E2 being highest during non-pregnant, reproductive years, E3 levels being highest during pregnancy and E1 levels being highest after menopause. These estrogens also differ in their affinity for estrogen receptors (ERs), with E2 having the highest affinity, followed by E3 and E1 having the lowest affinity (Blair et al., 2000). In women, E2 is the most predominant estrogen during the course of a women's lifetime. Most importantly, the levels of E2 during the menstrual cycle are correlated with learning in young adult women (Phillips and Sherwin, 1992b). It is the dramatic decrease in the levels of E2 specifically that is correlated with cognitive decline post-menopause (Sherwin, 1997). While it is interesting to understand how each estrogen type modulates synaptic function and cognition, the predominance of E2 during a women's lifetime, correlation of E2 levels with cognitive function and high affinity of E2 for ERs provide clear reasons to begin with E2 to establish the role of estrogen in modulation of cognitive function.

Learning and memory

Learning is as an alteration in behavioral response due to a previous experience and memory is the stored representation of that previous experience. The ability to learn thus necessitates a memory and this requirement leads to the reciprocal dependence of memory for learning to occur. In humans, memory can be defined as either nondeclarative (implicit) or declarative (explicit) (Milner et al., 1998). Nondeclarative memories are those one has no conscious awareness of and includes classical conditioning, non associative learning, priming and procedural skills. Of particular interest to the research undertaken in this dissertation is declarative memory, of which one is consciously aware and includes episodic memories of past events in space oriented in time allowing a sort of "mental time travel" (Tulving, 2002), semantic memory of general knowledge and facts, autobiographical memory of one's self and prospective memory, or the memory to do something in the future (Glisky, 2007). Each type of memory can be either short-term (STM) or long-term (LTM). STM is defined as fleeting, lasting seconds to minutes. Separate from STM but on a similar time scale is working memory, which further involves manipulation of a STM, i.e. reciting a phone number backwards (Baddeley, 1974). LTM lasts from hours to a lifetime, requires persistent network and cellular changes, and depends upon the synthesis of new proteins (Castellucci et al., 1989). In humans, an increased understanding in the mechanisms of normal learning and memory is important to further determine how these mechanisms change during normal aging and in neurodegenerative diseases associated with aging. As humans are living longer than ever before, research in these areas is of critical clinical importance.

Learning and memory in aging

Cognitive decline is a common complaint in aging, and is even considered normal to some degree. Normal decline in learning and memory caused by aging is only seen within particular types of learning and memory. STM seems relatively intact in aged compared to young adults (Nilsson, 2003), however working memory and episodic memories dramatically decline with age (Salthouse et al., 1989; Nilsson, 2003). Interestingly, episodic memory also exhibits a sex difference, where women tend to perform better on these tests (Nilsson, 2003).

Rodent models

Age-related deficits in learning and memory have also been exhibited in rodent models, a model that is useful to determine the molecular and physiological changes during aging that alter learning and memory. In the Barnes maze, a tall circular platform with holes around the edge where one hole contains an escape box beneath it, aged rodents consistently have trouble remembering the location of the escape hole (Barnes et al., 1980; Bach et al., 1999). When the escape hole is marked with a visual cue, aged rodents are just as competent as young rodents in escaping the maze (Bach et al., 1999). Therefore, this deficit is due to spatial memory and not some other physical component of aging such as decreased motor skills. Other spatial memory tasks also show a deficit in learning and memory in aged rodents including T-maze (Barnes et al., 1980), 8 arm radial maze (Barnes et al., 1980; Wallace et al., 1980), and Morris water maze (Gage et al., 1984). These studies in rodents support a decline in function of spatial memory during aging specifically.

Estrogen effects on learning and memory.

Women

In young adult cycling women, learning and memory is positively correlated to serum estrogen levels and women in a state of lower estrogen (menstrual phase) have decreased memory (Phillips and Sherwin, 1992b). This effect of E2 on learning and memory is task specific, with enhancements in verbal and visual episodic memory (Phillips and Sherwin, 1992b). Studies in naturally and surgically post-menopausal women have also shown deficits in verbal memory correlated with the acute decline in circulating ovarian hormones after menopause (Sherwin, 1988; Phillips and Sherwin, 1992a). Given the benefit of E2 on episodic memory in young adult women, it seems reasonable that E2 replacement would benefit cognition in post-menopausal women. However, as mentioned above, while some studies have reported beneficial effects of E2 replacement therapy in post-menopausal women (Sherwin, 1988; Phillips and Sherwin, 1992a; Abraham and Williams, 2003; Joffe et al., 2006; Maki and Sundermann, 2009; Henderson and Greicius), the WHIMS found no benefit of E2 replacement on preventing the occurrence of mild cognitive decline and dementia (Shumaker et al., 2003; Shumaker et al., 2004). This influential study resulted in the recommendation to not prescribe hormone replacement therapy for the treatment of cognitive decline in post-menopausal women 65 years of age and older. One potential explanation for the results of the WHIMS failing to produce cognitive benefit with HRT in post-menopausal women is the length of time the participants in the study experienced between menopause and treatment. Therefore, a "window of opportunity" may exist during which time E2 replacement after menopause could still be beneficial and the window may have been

missed in the WHIMS study (Sherwin, 2005; Sherwin, 2006, 2007). It has been hypothesized that after this period of time, changes resulting from deprivation of ovarian hormone, aging processes, or both could result in the inability of E2 to exert positive effects on cognition. In support of this hypothesis, E2 administration to surgically menopausal women immediately after surgery prevents deficits in learning and memory using a paragraph test of verbal recall (Sherwin, 1988; Phillips and Sherwin, 1992a). In the United States, the average age of the onset of menopause is approximately 51 years (Bromberger et al., 1997; Lamberts et al., 1997), and being that the average age of women in the WHIMS at the onset of treatment was 69 years of age (Shumaker et al., 2003; Shumaker et al., 2004), the majority of women in the WHIMS were more than a decade post-menopause. Whether this duration of time post-menopause is a critical determinant in the effectiveness of E2 is of current debate.

Nonhuman primates

Studies in female nonhuman primates, which undergo similar changes in ovarian hormone levels during ovarian senescence as women, suggest that that E2 replacement is also beneficial for learning and memory. In OVX monkeys, E2 replacement administered cyclically once per month reverses a deficit in a test of spatial working memory and object recognition (Rapp et al., 2003), replicating studies in women with decreased visual memory during states of low E2 (Phillips and Sherwin, 1992b). To date, few studies have been performed in aged intact monkeys and none of these studies investigated the effects of E2-replacement on cognitive function when monkeys are aged ovary intact. This is mostly due to the late stage during which monkeys enter

menopause. The average lifespan of a rhesus monkey is 30 years, with the age of menopause averaging at 25 years of age (Tigges, 1988). The proportion of time post-menopause in a monkey is therefore less than what is experienced in humans, where approximately a third of women's life is spent in a post-menopausal state compared to $1/6^{th}$ in monkeys. One study in post-menopausal rhesus monkeys found cognitive decline in a delayed response task, a test of working memory, compared to young adult monkeys, but effectiveness of E2 replacement was not investigated (Roberts et al., 1997). More studies have been conducted in OVX monkeys where immediate E2 replacement post-OVX enhanced spatial learning compared to vehicle(V)-treated controls (Rapp et al., 2003; Hao et al., 2006). Studies of E2 replacement after long-term ovarian hormone deprivation in monkeys show that monkeys remain responsive to E2 after 12 years post-OVX with E2 enhancing spatial working memory (Lacreuse et al., 2002). This benefit of E2 is task dependent in monkeys, as this same treatment did not benefit executive function in these animals (Lacreuse et al., 2004). Unlike humans, monkeys may be resistant to mechanisms rendering E2 ineffective during periods of long-term ovarian hormone loss and aging.

Rodents

Just as in humans and nonhuman primates, E2 facilitation of learning and memory in female rodent models is also task specific, suggesting that particular learning and memory processes are sensitive to the serum levels of E2. Specifically, performance in working memory tasks including Morris water maze, T-Maze (Fader et al., 1998; Gibbs, 1999) and radial arm maze (Daniel et al., 1997; Luine et al., 1998; Bimonte and

Denenberg, 1999) are enhanced by high levels of endogenous or exogenous E2. Alternatively, in tasks testing reference memory, where information is not trial dependent but requires information from multiple trials, E2 is not beneficial and can even be impaired in rats with high E2 levels (Frye, 1995; Warren and Juraska, 1997; Daniel et al., 1999). The task dependent effects of E2 on learning and memory indicate that E2 action in the brain is region specific.

The effectiveness of E2 replacement in rodents is in strong support of the critical window hypothesis. In 2000, Dr. Robert Gibbs showed that in rats OVX at 13 months of age, initiation of E2 replacement within 3 months, but not after 10 months post OVX significantly decreased the amount of errors in a delayed-matching to position task, supporting that there is a defined period of time post-OVX during which E2 can be replaced and remain beneficial to cognitive function (Gibbs, 2000). In another study, Dr. Gibbs showed that in rats that are OVX during young adulthood (3 months of age), E2 remains able to enhance this same delayed-matching to position task for 10-13 months post-OVX, and after this time E2 is no longer beneficial (Gibbs et al., 2009). Another study of animals OVX during middle age (13 months) reported that E2 facilitated learning only if given before 10 months post-OVX (Markowska and Savonenko, 2002). Again in support of this critical period, Daniel et al showed that middle aged OVX rats (12 months) replaced with E2 immediately after OVX, but not 5 months after OVX, enhanced working memory in an 8 arm radial maze (Daniel et al., 2006). Together, these data in rats suggest that this window of opportunity for E2 to remain beneficial for learning and memory depends upon the age of the animal when ovarian hormone loss is

initiated as well as the length of hormone deprivation. From these behavioral studies, the length of this window is dynamic and likely decreases in length with age.

Hippocampus

E2's effects on learning and memory support E2 may target hippocampus, a brain region within the temporal lobe that is most studied because of its role in learning and memory processes (Scoville, 1957; Milner, 1972). Much of what we now know about the role of hippocampal function in learning and memory came from an unfortunate side effect of a temporal lobe biopsy performed to reduce symptoms of temporal seizures (Scoville, 1957). This patient, Henry Gustav Molaison, famously known as patient H.M., had most of his hippocampus, hippocampal gyrus and amygdala removed. He experienced anterograde amnesia of episodic and semantic memory but had intact short-term memory and procedural memory (Corkin, 2002). Studies of H.M., as well as other patients with similar procedures, defined a role of hippocampus in learning and memory, but also assisted in the understanding multiple memory systems, where each system requires select brain regions (Milner et al., 1998). While other regions of the brain have been shown to be critical for learning and memory processes, the hippocampus remains in the spotlight experimentally as it has the advantage of being a large region with a laminar cellular architecture that is easily investigated using a variety of scientific techniques.

Estrogen effects on hippocampus dependent behavioral tasks

E2 is able to enhance many different tasks of spatial memory, implying a role for the hippocampus in the ability of E2 to enhance learning and memory in rodents. Specifically, proestrus or proestrus-like levels of E2 are beneficial to Morris water maze (Warren and Juraska, 1997), novel object placement (Frye et al., 2007) and spatial versions of the delayed matching to position T-maze (Gibbs, 1999; Sandstrom and Williams, 2001). Proestrus cycling levels of E2 and replacement of E2 in OVX rats that mimic proestrus also enhances novel object recognition (NOR), a nonspatial learning and memory task (Frye, 2006; Michael C. Lewis, 2008). These results from behavioral tasks support that E2 targets hippocampus to enhance learning and memory in rodents.

Cellular mechanisms of learning and memory

Neurons are specialized cells within the nervous system designed to transmit information either through direct connection with another neuron (electrical synapses) or through release of a neurotransmitter in close proximity to an adjacent neuron (chemical synapses). Through Hebb's postulate, repeated or persistent activity between a pre and postsynaptic neuron causes a change in one or both neurons resulting in a higher response of the post-synaptic cell to the pre-synaptic cell (Hebb, 1949). This theory provides a mechanism through which neurons can alter their level of response to a particular input and thus create cellular memory. It is widely thought that changes in the strength of these synaptic connections, or plasticity, directly results in learning and memory in behaving animals (Malenka and Bear, 2004).

Dendritic spines

Dendritic spines are protrusions along the dendrite of neurons and were first described 120 years ago by Ramón y Cajal using golgi impregnation (Cajal S, 1891). Today, there is still much debate about the function of these structures and their role in cognition. Some studies show increased dendritic spine density correlates to increases in learning and memory (Leuner et al., 2003; Leuner and Shors, 2004; Smith and McMahon, 2005). However, pathological increases in dendritic spine density have also been shown in neurodevelopmental disorders such as fragile-X syndrome, where cognition is impaired (Comery et al., 1997; Irwin et al., 2000). These results support that the association of increased dendritic spine density with enhanced cognitive function cannot be generalized. In excitatory synapses, the location of glutamate receptors that are crucial for learning and memory are restricted to the dendritic spine on the post-synaptic cell (Nusser et al., 1998). Spines are also very plastic, growing, retracting and changing shape in response to activity providing an attractive mechanism for learning and memory storage at the cellular level (Matsuzaki et al., 2004). Dendritic spine head shape is of particular interest as it may predict changes in electrophysiological properties and glutamate receptor content (Matsuzaki et al., 2001; Noguchi et al., 2005). Stimulation of a single spine initiates enlargement of the spine head, which is dependent on the N-methyl D-aspartic acid (NMDA) glutamate receptor for induction and requires protein synthesis for maintenance (Harvey and Svoboda, 2007; Yang et al., 2008a). However, this rapid increase in spine head shape with activity is not always observed (Nevian and Sakmann, 2006) and may depend upon additional glutamate receptors inserted into the spine head (Kopec et al., 2007). Therefore, not much can be interpreted when an increase

in dendritic spine density is observed in isolation. It should also be considered whether these spines are accompanied by presynaptic input, if there is an increase in a specific spine shape, and what types of receptors are located within the spines to better predict the consequence of the increased spine density.

Estrogen effects on dendritic spines

The first modulation of hippocampus by E2, found more than 20 years ago, was that E2 induces an increase in the dendritic spine density in CA1 pyramidal cells. This data was reported in back to back Journal of Neuroscience papers from lab of Dr. Bruce McEwen. The first report used a model of OVX female rats where E2 was replaced with two daily E2 injections (10μg) (Gould et al., 1990). E2 treatment was found to cause a 40% increase in the density of dendritic spines in the apical dendrites of dorsal CA1 pyramidal cells. This effect was not seen in the basal dendrites of CA1 pyramidal cells, the apical or basal dendrites of CA3 pyramidal cells or the proximal and distal dendrites of dentate gyrus (DG) granule cells. The subsequent study from the lab of Dr. McEwen asked whether dendritic spine density fluctuates during the estrous cycle using an intact rat model (Woolley et al., 1990). In cycling rats, the low levels of ovarian hormones during estrus are sufficient to decrease the density of dendritic spines in CA1 pyramidal cells. This result shows that the decrease in dendritic spine density does not require a complete loss of ovarian hormones, but later studies from McEwen's group found that there is a significant decrease in the density of dendritic spines in OVX rats compared to cycling rats that bottom out after 5 days post OVX (Woolley and McEwen, 1993). E2-enhanced dendritic spine density in OVX rats treated with two daily E2 injections is

increased for up to 72 hours after E2-treatment (Smith and McMahon, 2005). These spines are also associated with presynaptic input as shown by electron microscopy studies, supporting that E2 increases the number of functional synapses (Woolley et al., 1996; Adams et al., 2001b). E2 does not increase the number of presynaptic boutons, but rather increases the proportion of multiple synapse boutons (Woolley et al., 1996; Adams et al., 2001b). Later studies determined that this E2-induced increase in dendritic spine density is not specific to hippocampus as E2 increases dendritic spine density of pyramidal cells in prefrontal cortex as well (Hao et al., 2006). Initially, this increase in spine density was thought to require a time course of hours to days to occur, however, increases in dendritic spine density with E2 or with agonists of ERs can increase dendritic spine density within 30 minutes *in vitro* and *in vivo* (MacLusky et al., 2005; Phan et al., 2011). Collectively, these results support that E2 is a rapid and reversible modulator of synapse number in specific regions of the brain and could potentially facilitate synaptic responses within these regions. Whether or not the E2-induced increase in dendritic spine density is critical for E2-enhanced learning and memory is yet to be unveiled.

Long-term potentiation and long term-depression

One of the most important properties of neurons is the ability to alter their function as a consequence of experience. This plasticity allows neurons to alter output depending on the strength of input and provides a cellular mechanism for learning and memory (Malenka and Bear, 2004). Long-term potentiation (LTP) is a sustained increase in synaptic strength (Bliss and Lomo, 1973). LTP, just like forms of memory, can be either short or long-lasting. Early-LTP, like STM, lasts for minutes, while late-LTP, like

LTM can lasts for days, and likely a lifetime (Stanton and Sarvey, 1984). Late-LTP also shares the requirement of new protein synthesis with LTM (Stanton and Sarvey, 1984). While not directly investigated in this dissertation, it should not be neglected that what goes up, must come down, or else saturation of cellular mechanisms involved in LTP would result in a ceiling of synaptic strength, which has been shown to prevent learning and memory (Barnes et al., 1994). Therefore, long-term depression (LTD), or the sustained weakening of synaptic strength, is critically important for normal synaptic function and has also likely critical for normal learning and memory.

Influx of calcium through NMDARs (described in detail below) is required for the induction of many types of plasticity (Malenka and Nicoll, 1993). As NMDARs require synaptic activity of both the pre and post-synaptic cell, LTP and LTD dependent on NMDARs by definition are activity dependent (Bliss and Lomo, 1993; Abraham, 2003 #3819; Malenka and Bear, 2004). The time course and magnitude of this Ca^{2+} influx determines whether LTD or LTP will occur. If there is a robust, but brief increase in Ca^{2+}, then activation of protein kinases leads to the insertion of glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors into the synapse resulting in expression of LTP (Bear and Malenka, 1994). If there is a small and enduring influx of calcium, then activation of phosphatases (Mulkey et al., 1993; Winder and Sweatt, 2001) will initiate endocytosis of AMPARs and thus weaken the synaptic response upon future activation, leading to LTD (Bear and Malenka, 1994; Selig et al., 1995). Ca^{2+} then activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which is also a key modulator in the induction of LTP (Lisman et al., 2002), acting as an on switch to activate signaling cascades leading to the expression of LTP.

If LTP is the cellular correlate of learning and memory, and memories can last up to an entire lifetime, mechanisms must exist that maintain LTP for a lifetime. Maintenance of LTP is dependent on new gene transcription and protein synthesis (Abraham and Williams, 2003) depending on the ERK and calcium/calmodulin kinase IV (CaMKIV) pathways that activate key transcription factors such as CREB and CREB binding protein (CBP). This new protein synthesis could then lead to structural changes in the neuron such as new dendritic spines, leading to long-lasting increases in synaptic efficacy.

Estrogen effects on LTP

The magnitude of LTP at CA3-CA1 synapses is increased in naturally cycling rats at proestrus (Warren et al., 1995) and in OVX rats treated with exogenous E2 at proestrus levels *in vivo* and *in vitro* (Cordoba Montoya and Carrer, 1997; Bi et al., 2001; Smith and McMahon, 2005). This enhancement in the magnitude of LTP is time dependent and occurs 24 (E24) and 48 (E48) hours after E2 treatment (Smith and McMahon, 2005) and dissipates over time, being strongest at E24 and no longer significantly increased at 72 hours (E72) after E2 treatment (Smith and McMahon, 2005). This enhancement of LTP is dependent on E2 activation of estrogen receptors as it is prevented by the ER antagonist tamoxifen (Smith and McMahon, 2005), a similar dependence to the E2-induced increase in dendritic spine density (Murphy and Segal, 1996). Whether or not the ability of E2 to enhance LTP causes the E2-induced increase in learning is yet to be determined.

Glutamate receptors

Glutamate is the main excitatory neurotransmitter in the brain and receptors for glutamate are involved in both synaptic plasticity (Abraham and Mason, 1988; Bliss and Collingridge, 1993) and learning and memory (Tsien et al., 1996; Xu et al., 2003). Glutamate receptors are either ligand-gated ion channels, including; NMDA , AMPA and Kainate receptors or G-protein coupled receptors known as metabotropic glutamate receptors (mGluRs) (Hollmann and Heinemann, 1994). AMPARs and NMDARs have well-studied, crucial roles in LTP and LTD. Both of these receptors are nonselective cation channels, with NMDARs having a stronger permeability to Ca^{2+} (Mayer et al., 1987). Glutamate released from the presynaptic cell binds both AMPARs and NMDARs with NMDARs also requiring binding of the coagonist glycine or D-serine (Wolosker, 2006). While AMPARs immediately open allowing influx of primarily sodium ions (Na^{+}), the hyperpolarized state of the post-synaptic cell results in a flickering block of NMDARs with magnesium ions (Mg^{2+})(Mayer et al., 1984). The influx of Na^{+} through AMPARs depolarizes the post-synaptic cell, allows for removal of the Mg^{2+} block at NMDARs and leads to an increase in intracellular Ca^{2+}(Cormier et al., 2001). This requirement of activity dependence at both the pre-synaptic and post-synaptic cell for NMDAR activation allows for NMDARs to act as coincident detectors. The coincidence detection of NMDARs is critical for association of activity at the synaptic level, and lead to the overall connectivity of circuits allowing for associations at the behavioral level.

NMDA Receptor subunits.

The NMDA receptor (NMDAR) consists of four subunits (Laube et al., 1998), containing two obligatory NR1 subunits which bind to glycine, or D-serine and two NR2 subunits which bind to glutamate (Wisden and Seeburg, 1993; Kumar and Huguenard, 2003; Liu et al., 2004). There are two main NR2 subtypes dominant in the hippocampus (NR2A and NR2B)(Monyer et al., 1994). The channel dynamics of NMDARs containing NR2B subunits have a longer open time, due to a slower rate of inactivation, than NMDARs containing NR2A subunits (Monyer et al., 1994). During development, the hippocampus expresses more NR2B-containing NMDARs than NR2A-containing NMDARs (Portera-Cailliau et al., 1996). In early post-natal development, this ratio of NR2 subunits undergoes a developmental switch after which, the NR2A subunit predominates and less NR2B-containing NMDARs contribute to excitatory transmission (Monyer et al., 1994; Portera-Cailliau et al., 1996). This switch in NR2 subunit composition of NMDARs is associated with an increase in the threshold for LTP induction (Portera-Cailliau et al., 1996), as NMDARs containing NR2A subunits have a much more rapid inactivation rate (Monyer et al., 1994). Indeed, overexpression of NR2B subunits in a transgenic mouse or rat decreases the threshold for LTP, resulting in enhanced magnitude of LTP in these overexpressing rodents (Tang, 1999; Wang et al., 2009). NR2B-overexpression also results in enhanced learning and memory in both rat and mouse (Tang, 1999; Wang et al., 2009).

NR2B subunits can also undergo phosphorylation which increases their function and binding to the post-synaptic density (Cheung and Gurd, 2001). Phosphorylation of the NR2B subunit also increases after LTP induction (Cheung and Gurd, 2001). NR2B phosphorylation occurs on the cytoplasmic tail by Src family kinases, including Src and

Fyn kinases (Cheung and Gurd, 2001). Fyn knockout mice have a significant decrease in tyrosine phosphorylation of NR2B subunits and also exhibit learning and memory deficits as well as decreases in dendritic spine density (Rosenblum et al., 1996; Cheung and Gurd, 2001; Babus et al., 2011).

Effects of estrogen on glutamate receptors

Glutamate receptors are critical for LTP as mentioned earlier and E2-induced enhancement in the function of these receptors directly increases LTP magnitude (Smith and McMahon, 2006). Proestrus-like levels of E2 increases the maximal binding of NMDARs suggesting an increase in density of these receptors compared to V-treated controls (Woolley et al., 1997). Functional studies also find an increase in synaptic NMDAR current in E2-treated rats (Woolley et al., 1997; Smith and McMahon, 2005) and the ratio of current mediated by NMDARs compared to AMPAR currents (NMDAR/AMPAR) is increased only at time points where E2-induces enhanced magnitude of LTP (Smith and McMahon, 2005). This increase in NMDAR/AMPAR is caused by a selective increase in the function of NMDARs containing NR2B subunits (Smith and McMahon, 2006) and blocking NR2B-containing NMDARs prevents the E2-induced increase in LTP magnitude (Smith and McMahon, 2006). These results demonstrate that the E2-induced increase in LTP magnitude requires an increase in the ratio of NMDARs, caused specifically by an increase in NR2B-containing NMDAR function. It is currently unclear whether E2 increases the function of NR2B-containing NMDARs by increase the density of these receptors, the activity of these receptors through increased phosphorylation, or both.

Effects of aging on synaptic plasticity

Experiments investigating alterations in plasticity in aging suggest that aged rats have normal LTP induction only in robust induction protocols (Landfield and Lynch, 1977; Landfield et al., 1978) as weaker induction protocols show LTP deficits (Landfield et al., 1978). LTD is intact in aged rats, and in aged animals that experience learning deficits, there is a lower threshold of LTD induction and LTP reversal in comparison to aged animals without learning deficits (Foster and Kumar, 2007).

A potential cause for these deficits in plasticity is an increase in cell death, however, neurodegeneration of hippocampal pyramidal cells does not occur in the course of normal aging in rodents (Rapp and Gallagher, 1996), monkeys (Keuker et al., 2003) or humans(West et al., 1994), a key difference between aged onset of neurodegenerative diseases such as Alzheimer's Disease (West et al., 1994). Normal aging processes may instead result in deficits at the level of the synapse, small changes that over time cause notable decreases in plasticity in hippocampus (Burke and Barnes, 2010). In the middle and inner molecular layers of the DG, a decrease in axospinous contacts made of granule cells has been identified using electron microscopy (Geinisman et al., 1992). However, no loss of synaptic contacts are seen in the Schaffer collateral (Smith et al., 2000) or temporoammonic pathways in CA1 (Smith et al., 2000), supporting that aging may alter synaptic function in a synapse specific manner. In area CA1, synaptic efficacy may decline due to the larger number and reduced size of perforated post-synaptic densities (Nicholson et al., 2004). This decreased size of perforated synapses could indicate an increase in nonfunctional synapses, resulting in decreased synaptic response presented in

CA1 fEPSPs (Landfield et al., 1986; Barnes et al., 1992). Another possibility is that there are changes in the glutamate receptor content in synapses supported by an increase in AMPARs and a decrease in NMDARs observed in DG (Yang et al., 2008b). An increase is L-type calcium channels also occurs during aging which leads to an increase in intracellular calcium (Thibault and Landfield, 1996). This increase in L-type calcium channels may shift during aging toward utilizing non-NMDAR dependent types of plasticity, which may not be as effective at inducing LTP. In support of this, blocking these calcium channels can reverse age-related decreases in LTP (Norris et al., 1998). Also, increases in L-type calcium channels in aging are associated with cognitive decline and blocking these channels prevents this decrease in cognition (Thibault et al., 2001; Veng et al., 2003).

Effectiveness of estrogen to enhanced synaptic plasticity in aging.

The interaction of aging and ovarian loss on hippocampal plasticity is less explored. It has been found that the age induced increase in susceptibility to LTD induction is ameliorated with E2 (Vouimba et al., 2000). Exogenous acute *in vivo* E2 treatment also enhances LTP in aged intact transgenic mice, which carry Alzheimer's genes (apoE4)(Sung Hwan Yun, 2007). The ability of E2 to enhance dendritic spine density in aging has been examined. Adams et al show that chronic E2 replacement in aged rats did not increase the number of synapses, but did increase the number of NR2B-containing NMDARs within the synapse compared to cholesterol replaced rats (Adams et al., 2001b; Adams et al., 2004). In another study, McLaughlin et al showed that 10 weeks of ovarian hormone deprivation decreased the efficacy of acute E2 to increase

dendritic spine density (McLaughlin et al., 2008). These studies support that the E2-induced increases in dendritic spine density is dependent on both aging and long-term ovarian hormone loss, but it is yet to be determined which factor, age or duration of ovarian hormone loss, is more critical for E2 effectiveness. The effects of age and long-term ovarian hormone loss on hippocampal plasticity will be directly examined in this dissertation.

Mechanisms of E2 enhanced plasticity

Ruling out GABAergic inhibition as a mechanism of E2-induced increase in magnitude of LTP.

One potential explanation for the increase in LTP following E2 treatment is that there is a decrease in activity at GABAergic interneurons leading to a decrease in inhibition of CA1 pyramidal cells, which then increases NMDAR activation and thus enhances the magnitude of LTP. In support of a role for disinhibition in E2-enhanced hippocampal function, decreased inhibition is required for the E2-induced increase in dendritic spine density (Murphy et al., 1998) and a single 10μg E2 injection causes disinhibition of CA1 pyramidal cells through decreased GABAergic response 24 hours after treatment (Rudick and Woolley, 2001). However, E2 does not increase the magnitude of LTP 24 hours after a single 10μg injection, under the same conditions that result in decreased GABAergic inhibition (Smith and McMahon, 2005). Also blocking $GABA_A$Rs pharmacologically with 100μM picrotoxin does not mimic E2-treatment in slices from V-treated rats, as would be expected if E2 increases LTP magnitude through

GABAergic disinhibition (Smith and McMahon, 2005). Therefore, disinhibition is not required for E2-induced increase in the magnitude of LTP.

Silent synapses

How does E2-increase LTP? Does the increased dendritic spine density in CA1 pyramidal cells matter? The dendritic spine density remains elevated even after E2 no longer increases LTP, showing that the increase in spine density alone does not account for the E2 induced changes in synaptic efficacy. The increase in LTP magnitude with E2 treatment is NMDAR dependent, and does coincide well with an increased ratio of NMDARs/AMPARs (Smith and McMahon, 2005, 2006). Even though total glutamate transmission remains elevated, i.e at E72, there is a loss of the increased NMDAR/AMPAR, which occurs simultaneously with the loss of E2-enhanced LTP. The return of the E2-induced increase in NMDAR/AMPAR is caused by an increase in AMPAR current, supporting the hypothesis that E2 increases silent synapses. These synapses contain only NMDARs and are therefore "silent" until depolarization of the spine occurs, likely by overflow of depolarization that infiltrates the spine from activity at nearby synapses (Isaac et al., 1995). Once the silent synapses are depolarized sufficiently to remove the Mg^{2+} block from NMDARs, Ca^{2+} influx through NMDARs can then lead to the insertion of AMPARs into the membrane and lead to activation of the synapse. Therefore, upon consecutive stimulation, these once silent synapses are now active, resulting in LTP. If E2 increases silent synapses, then activation of these synapse through an increase in AMPAR insertion into these synapses causing a decrease in the NMDAR/AMPAR back to vehicle levels, therefore no longer enhancing the magnitude of

LTP. Therefore at E72, when dendritic spine density is still significantly increased, the lack of increased LTP magnitude could be explained by conversion of these new synapses from silent to active. This is supported by an increase in AMPAR transmission which occurs gradually after E2-treatment and reverses the increased NMDAR/AMPAR ratio precisely at E72, when LTP is no longer enhanced (Smith and McMahon, 2005).

Estrogen receptors

Estrogen binds to either traditional genomic estrogen receptors (ERα or ERβ) to initiate gene transcription leading to expression of new proteins, or a membrane bound estrogen receptor such as the more recently identified G-protein coupled ER (GPER) (Carmeci et al., 1997), activating more immediate intracellular signaling cascades. While ERα is 97% homologous to ERβ, activation of ERα can differ from activation of ERβ in the associative binding of specific coactivators or other transcription factors, where ERa activation is more effective to induced ERE-linked transcription (ref). ERα activation has been shown to promote cell survival during calcium excitotoxicity in neuronal cultures by altering calpain expression {Gamerdinger, 2006 #3830}, while ERβ activation is more effective at attenuating pro-inflammatory cytokines in both microglia and astrocytes {Lewis, 2008 #3831}. GPER and PI3K….

Deciphering which receptor E2 engages to illicit an enhancement of hippocampal function is an extraordinary challenge as currently there are no selective antagonists for the separate ER types. Most of the studies investigating the role of ERs therefore use either agonists that cannot distinguish between membrane bound versus cytosolic ERs as both genomic receptors have been colocalized to the membrane in hippocampal neurons

(Milner et al., 2001; Milner et al., 2005), or transgenic knockout mice in which compensatory mechanism may effect results. In rats, studies support a more critical role for ERα in the ability of E2 to enhance learning and memory (Rodgers et al., 2010), however a role for ERβ cannot be excluded (Hammond et al., 2009). ERα expression levels decrease in rats during aging and during periods of long-term ovarian hormone deprivation (Adams et al., 2002; Mehra et al., 2005). There is a trend for this similar effect to occur in aging humans (Tohgi et al., 1995). In contrast, monkeys show little to no expression of ERα in hippocampus suggesting a prominent role for ERβ in E2 modulation of hippocampal function (Gundlah et al., 2000). Studies in mice, however, support that E2 uses ERβ to enhance learning and memory (Liu et al., 2008), however the contribution of receptor subtype may be task specific as agonists for ERα have been shown to enhance novel object recognition and placement and also to enhance dendritic spine density (Phan et al., 2011). These contradictions in results may be related to the species-specific difference in the concentration of these receptors and the ratio of ERα to ERβ may be the more crucial factor determining E2s effects on hippocampal function (Foster, 2011).

Hypotheses

E2 increases hippocampus dependent learning and memory and synaptic physiology using a common mechanism, NR2B-containing NMDARs

While E2 enhances learning and memory as well as synaptic physiology, it is unknown whether a direct causal relationship exists between E2-enhanced LTP magnitude and learning and memory. If E2-enhanced magnitude of LTP causes the E2-enhanced learning and memory, then both should occur over the same time course and

through the same mechanism. My first hypothesis is that E2 increases hippocampus dependent learning and memory and LTP using a common mechanism, NR2B-containing NMDARs. To test this hypothesis, I first investigated whether E2 enhances learning and memory in a rodent model over the same time course that E2 was previously shown to enhance the NR2B-mediated current. As NR2B-mediated current is increased only when E2-increases LTP, if the E2-induced increase in LTP causes enhanced learning, then E2 will enhance learning and memory only when NR2B-mediated current are increased. To further test this hypothesis, if E2-increased learning requires NR2B containing NMDARs, then blocking these receptors in hippocampus should block the E2-induced increase in learning. I therefore investigated whether E2-enhanced learning when young adult OVX rats were treated with an antagonist of NR2B-containing NMDARs.

E2-enhanced hippocampal function is critically dependent on the length of time post-OVX

The ability of E2-treatment to enhance hippocampal function is thought to be dependent on the duration of time after loss of ovarian function in aging. If a time dependent loss of E2 effectiveness exists, then there should also be a critical period of time during which E2 can be replaced and remain beneficial. While empirical data support that there is a critical period during which E2 remains able to enhance learning and memory, no one has investigated whether there also exists a critical period of time during which E2 remains able to enhance hippocampal LTP, NMDAR transmission and spine density. Also, it has not been has systematically tested whether increases in synaptic physiology in aging and periods of long-term ovarian hormone loss is associated with the ability of E2 to facilitate learning and memory. I used a long-term OVX model

in rats to test the hypothesis that E2-enhanced hippocampal function is critically dependent on the length of time post-OVX.

Loss of E2-enhanced hippocampal function caused by long-term ovarian hormone loss can be prevented with E2 replacement

In women, the leading hypothesis for the ineffectiveness of E2 replacement during the WHIMS is that too much time has lapsed between menopause and treatment. It is not understood whether this window is dynamic, being molded by mechanisms initiated by age, duration of ovarian hormone loss, or both. Behavioral studies have shown that chronic E2 replacement can protect against deficits in learning and memory caused by long-term ovarian hormone loss, but whether these treatments can enhance hippocampal physiology is unexplored. Using a chronic low dose E2 replacement initiated prior to the loss of E2-enhanced hippocampal function, I tested the hypothesis that E2 replacement could prevent the loss in E2-induced hippocampal function caused by long-term ovariectomy.

Experimental animal models

Of the many behavioral tasks which test learning and memory in rodents, I chose to use novel object recognition (NOR) because this task is reliably enhanced by E2 in various labs (Luine et al., 2003; Walf et al., 2006; Frye et al., 2007; Fernandez et al., 2008; Harburger et al., 2009; Frick et al., 2011) and this E2-induced enhancement requires hippocampal NMDARs (Michael C. Lewis, 2008). Also, this task can be preformed in a short period of time, ensuring that E2 levels remain constant during training and testing, allowing for E2 to benefit both acquisition and memory. To test

learning and memory in NOR, I chose to OVX rats and replace them with E2 to directly compare my results to previous electrophysiology results from our lab. This model removes any circulating ovarian hormones, and through exogenous E2-treatment with acute subcutaneous injections, I could regulate the E2 serum levels across animals. In my aging study, even though 20 month old rats are likely acyclic, they could still have elevated levels of E2 due to being in states of either persistent diestrus or constant estrus and through removal of endogenous ovarian hormones, I could then better control E2 levels between rats.

ESTRADIOL-INDUCED INCREASE IN NOVEL OBJECT RECOGNITION LEARNING REQUIRES HIPPOCAMPAL NR2B- CONTAINING NMDA RECEPTORS

by

LINDSEY C. VEDDER, CAROLINE C. SMITH AND LORI L. MCMAHON

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LINDSEY C. VEDDER, CAROLINE COFER SMITH AND LORI L. MCMAHON

ABSTRACT

17β-estradiol (E2) enhances learning and memory, making it a clinical treatment for hormone-related cognitive decline. Previously we reported that increasing plasma E2 in ovariectomized rats enhances the magnitude of long-term potentiation (LTP) at CA3-CA1 synapses, which is caused by an increase in current mediated by NR2B-containing NMDARs and an increase in the NMDAR/AMPAR ratio. Whether this enhanced hippocampal plasticity is causally related to the ability of E2 to enhance learning and memory has yet to be tested. Here, we find that E2 enhances novel object recognition (NOR) learning with the same time course we previously showed E2 enhances the LTP magnitude, linking the increase in LTP to the enhanced learning. Furthermore, using the selective NR2B subunit antagonist Ro25-6981, we find that the E2-enhanced NOR, like the enhanced LTP, requires hippocampal NR2B-containing NMDARs, specifically in area CA1. Finally, using whole-cell recordings and the phosphatase inhibitor orthovanadate, we investigated whether the E2-induced increase in NMDAR current is caused by an increase in the density of synaptic NMDARs or subunit phosphorylation and suggest that both mechanisms participate to enhance NMDAR current in E2-treated rats. These results show that the E2-enhanced learning requires a functional increase in

NR2B-containing NMDARs, a requirement shared with E2-enhanced LTP at CA3-CA1 synapses, supporting the hypothesis that the increased in LTP directly enhances learning following an increase in plasma E2 levels.

INTRODUCTION

Elevated plasma levels of the ovarian hormone 17β-estradiol (E2) are associated with heightened hippocampal dependent learning and memory in women and in animal models (Rosenberg and Park, 2002; Rhodes and Frye, 2004; Frye et al., 2007). At the synaptic level, elevated plasma E2 increases dendritic spine density on hippocampal CA1 pyramidal cells in female rats, transmission mediated by NR2B-containing NMDARs which increases the NMDAR/AMPAR ratio (Woolley et al., 1997; Smith and McMahon, 2005, 2006; Snyder et al., 2010), and the magnitude of LTP at CA3-CA1 synapses (Smith and McMahon, 2005), a cellular correlate of learning and memory (Malenka and Bear, 2004). However, because a mechanistic link has yet to be demonstrated, it remains unknown whether the E2-induced increase in LTP is causally related to the enhanced learning. It is assumed this is the case, but an enhanced LTP magnitude can occur independent of an increase in learning, and vice versa (Migaud et al., 1998; Dutar et al., 2002).

Using ovariectomized (OVX) rats, we previously reported that elevated plasma E2 increases the LTP magnitude precisely when transmission mediated by NR2B-containing NMDARs and the NMDAR/AMPAR ratio are also increased (Smith and McMahon, 2005). In fact, the increase in NR2B-mediated transmission is causal to the increase in LTP (Smith and McMahon, 2006; Snyder et al., 2010). In transgenic mice, over-expression of NR2B subunits enhances both learning and hippocampal LTP (Tang,

1999), suggesting that the increase in NR2B function stimulated by E2 may be responsible for the E2-induced increase in hippocampal learning.

Here, we used novel object recognition, a hippocampal dependent behavioral task that is enhanced by increased E2 levels (Luine et al., 2003; Frye et al., 2007; Fernandez et al., 2008; Michael C. Lewis, 2008), to test the hypothesis that E2 increases learning only at time points when E2 increases NR2B transmission and LTP, and that like LTP, E2-enhanced learning also requires NR2B-containing NMDARs. We also determined whether E2 increases receptor density, phosphorylation of NMDAR subunits, or both, to functionally increase NR2B transmission.

METHODS

All experiments were performed in accordance with the IACUC of the University of Alabama at Birmingham and the National Institutes of Health.

Animals

Sprague-Dawley female rats (Charles River Laboratories), on a 12hr light/dark cycle and given food and water ad libitum, were 8-12 weeks of age for all experiments except cannula experiments where animals were 16-20 weeks old.

Surgery

All surgeries were performed under 2.5% isoflurane anesthesia in 100% oxygen using aseptic conditions. Animals were given either Tylenol in drinking water or a single carprofen (10mg) subcutaneous injection for analgesia.

Ovariectomy

Performed as described previously (Smith and McMahon 2005).

Intra-hippocampal Cannula Placement. After at least 7 days post-OVX, 26 gage bilateral hippocampal guide cannulae (Plastics One) were inserted into area CA1 using the following coordinates (from Bregma, AP -4.2mm, ML -2.5mm,+2.5mm, DV -2.3mm). Cannulae were secured using dental cement.

Drugs

E2 treatment. Two daily injections of 10μg 17β-estradiol (E2, Sigma) in 0.1cc cotton seed oil or cotton seed oil vehicle (V) were given subcutaneously (Smith and McMahon, 2005). Uterine weights were measured to verify hormone treatment and successful ovary removal. Animals were tested in behavior and sacrificed either 24(E24), 48(E48) or 72(E72) hours after E2 treatment.

Ro25-6981 (Sigma). Systemic: RO was dissolved in saline (2mg/ml) and injected intraperitoneally (5mg/kg) (Figure 1A). *IntraCA1 Infusion:* RO was infused manually by depressing a 10μL Hamilton syringe 0.2μL every 40s until 1μl was dispensed. Cannulae were left in place for one minute to allow for diffusion of drug. Correct cannula placement was verified with cresyl violet. Only animals with cannula tips in area CA1 were included for analysis (Figure 2C).

Novel object recognition (NOR)

Rats were handled for 1 minute and habituated to the empty NOR box (40cm x 40cm x 60cm, black plexi-glass) for 10 minutes. The next day, rats were placed in the NOR box and trained with 2 identical objects for 3 minutes, returned to their home cage for 2 hours and returned to the NOR Box for 3 minutes where one of the training objects (familiar) was replaced by a novel object. All sessions were videotaped and analyzed by blind observers. Investigative behaviors were defined as the rat's nose coming in direct

contact or within 1cm of the object. Training objects and novel object locations during testing were counter balanced to avoid an effect of object or place preferences. Animals were excluded if they did not have at least 5 total seconds of investigative behavior or spend at least 1 second investigating both objects during the testing session.

Electrophysiology

Whole cell patch clamp recordings were obtained from CA1 pyramidal cells as done previously (Smith and McMahon, 2006). The non-selective phosphatase inhibitor orthovanadate (100μM) was added to the pipet solution to saturate phosphorylation by preventing de-phosphorylation. A stimulating electrode was placed in stratum radiatum of area CA1 to evoke glutamate transmission at CA3-CA1 synapses. Picrotoxin (100μM) and DNQX (10μM) were bath applied to isolate NMDAR currents and cells were held at -20mV to relieve the voltage-dependent Mg^{2+} block. From each animal, recordings were performed with and without orthovandate to allow for within animal comparison and V and E2-treated animals were interleaved.

Statistics

NOR: A significant increase compared to chance (50%) in percent time spent on the novel object out of total time spent investigating during the testing session was considered as learning using a one-sample t-test. Percent time spent on the left object during training was compared to chance to rule out side preference. Differences between groups were compared using a student t-test or One-way ANOVA with Tukey post-hoc analysis.

Electrophysiology: Data were compared using repeated measures two-way ANOVA using Tukey for post-hoc comparison. Statistical significance was declared if $p<0.05$.

RESULTS

E2-induced enhancement in novel object recognition is time dependent

If the E2-induced increase in LTP is directly related to the mnemonic enhancing effects of E2, then the increase in learning should occur with an identical time course as the enhanced LTP we published previously (Smith and McMahon, 2005). To test this idea, OVX female rats were treated with E2 or vehicle (V) and working memory was assessed using novel object recognition (NOR) at 24(E24), 48(E48), or 72(E72) hours after E2 treatment. For all treatment groups the percent time spent with the novel object was significant compared to chance, demonstrating learning (Figures 1B-D, Right, E24; V, $t(8)=1.87$, $p<0.05$, E2, $t(6)=5.86$, $p<0.001$, E48; V, $t(7)=2.18$, $p<0.05$, E2, $t(8)=4.78$, $p<0.001$, E72; V, $t(8)=2.04$, $p<0.05$, E2, $t(8)=2.93$, $p<0.01$). Importantly, we find that E2 induces an increase in NOR beyond that measured in V-treated animals at E24 (Figure 1B, Right, $t(14)=1.97$, $p<0.05$) and E48 (Figure 1C, Right, $t(15)=1.78$, $p<0.05$), time points when E2 also enhances LTP (Smith and McMahon 2005). However, at E72, learning is no longer enhanced (Figure 1D, Right, $t(16)=0.84$, $p=0.42$), and neither is LTP (Smith and McMahon 2005). The E2-enhanced NOR cannot be explained by an increase in investigative behavior, as the total time spent investigating during the training session with identical objects was not significantly different between V and E2-treated rats (Figures 1B-D, Left, E24; $t(14)=0.292$, $p=0.77$, E48; $t(15)=1.73$, $p=0.11$, E72;

t(16)=0.286, p=0.79). This alleviates the concern that E2 is increasing investigation or motivation, which could non-mnemonically increase percent time spent with the novel object.

E2-enhanced novel object recognition requires NR2B-containing NMDARs.

If the E2-induced increase in LTP and learning are mechanistically tied together as suggested by the time course data above, then E2-enhanced NOR should require NR2B-containing NMDARs similarly to the E2-enhanced LTP (Smith and McMahon 2006). To test this, we first blocked NR2B-containing NMDARs systemically using the selective NR2B subunit antagonist Ro25-6981 (RO;5mg/kg,ip) injected 30 minutes prior to NOR training at E24 (Figure 2A1). There was no effect of RO on investigative behavior between groups during the training session (Figure 2A2, $F(3,52)=0.33$, $p=0.80$). All groups except E2+RO showed significant NOR learning (Figure 2A3, V+Sal, $t(16)=3.37$, $p<0.001$, V+RO, $t(11)=2.07$,$p<0.05$, E2+Sal, $t(17)=6.28$, $p<5.0\text{x}10^{-6}$, E2+RO, $t(8)=0.14$, $p=0.45$). The increase in variance in E2+RO-treated rats indicates that NOR was disrupted, as these animals prefer objects at random, and did not spend significantly more time than chance on the novel object (Figure 2A3). This effect was not seen in V+RO-treated rats, suggesting that NOR is more heavily dependent on NR2B-containing NMDARs in E2-treated rats.

To further define a role for hippocampal NR2B-containing NMDARs, we next asked if blocking these receptors only in hippocampus prevents the E2-induced increase in learning. RO (0.375μg; 1μL) or saline was infused directly into area CA1 of V or E2-treated rats 15 min prior to training (Figure 2A1-2A3). In all animal groups, significantly more time was spent with the novel object compared to chance, demonstrating NOR

learning (Figure 2A3, V+Sal;t(8)=3.47, p<0.005, E2+Sal;t(9)=9.74, p<0.000005, V+RO;t(6)=2.51, p<0.05, E2;+RO;t(9)=5.47, p<0.0005). As predicted given their role in the increased LTP magnitude (Smith and McMahon, 2006), the targeted block of NR2B-containing NMDARs eliminated the enhanced NOR observed in E2-treated rats (Figure 2A3, F(3, 32,)=8.39, P<0.0005), decreasing it to a level not different from that measured in V-treated animals. As before, investigative behavior did not differ between treatment groups during training (Figure 2A2, F(3,33)=2.16, p=0.11). It is important to note that while infusion of RO (0.375μg) into CA1 had no effect on NOR in V-treated control rats, a 10 fold higher concentration of RO(3.75μg) disrupts NOR as denoted by the increase in variance (Figure 2D, V+RO, t(11)=0.50, p=0.63), demonstrating that NR2B containing receptors participate in normal learning, but their role is heightened in E2-treated rats when NOR is increased.

E2-enhanced current through NR2B-containing NMDARs is due to both increased receptor number and phosphorylation.

Although elevated E2 increases NR2B-mediated NMDAR current (Smith and McMahon, 2006; Snyder et al., 2010), biochemical assays have been unable to determine whether the increase in current amplitude results from an increase in receptor density, subunit phosphorylation, or both (Snyder et al., 2010). In an attempt to distinguish between these mechanisms, we included the phosphatase inhibitor orthovanadate in the pipet solution during whole-cell voltage clamp recordings to saturate NMDAR subunit phosphorylation, a strategy used previously (Wang and Salter, 1994)(See Figure 3A-C for experimental model) and measured the input/output relationship of pharmacologically isolated NMDAR currents from V- and E2-treated rats. We reasoned that if E2 increases phosphorylation to increase the NMDAR current, then orthovanadate should be unable to

increase the maximum current amplitude in cells from E2-treated rats while increasing the maximum current in cells from V-treated rats to the same maximum as in E2-treated rats (Figure 3A). Alternatively, if E2 primarily increases receptor density, enhancing phosphorylation with orthovanadate should increase the maximum NMDAR current in cells from both animal groups in parallel, and the NMDAR current amplitude in V-treated will not reach the same maximum as in E2-treated rats (Figure 3B). Lastly, it is possible that the increased NMDAR current in cells from E2-treated rats results from an increase in phosphorylation and an increase in receptor density. In this case, the maximum current amplitude in E2-treated rats will be unaffected by orthovanadate as suggested in the first paradigm; however, orthovanadate will induce an increase in NMDAR current in cells from V-treated rats that will remain significantly smaller than NMDAR current in cells from E2-treated rats (Figure 3C).

In interleaved recordings with and without orthovanadate, we find that the maximum NMDAR current amplitude in cells from V-treated rats was significantly greater with orthovanadate, demonstrating that NMDAR subunit phosphorylation is not saturated under control conditions ($t(6)=1.50$, $P<0.05$, Figure 3D). In contrast, the maximum NMDAR current amplitude was not significantly different with or without orthovanadate in cells from E2-treated rats, suggesting that phosphorylation is saturated by E2 (Figure 3D, $t(5)=0.059$, $p=0.48$). Importantly, the maximum NMDAR current with orthovanadate remains significantly less in cells from V-treated rats than the maximum current in cells from E2-treated rats, suggesting that E2 also increases the density of synaptically located NMDARs ($F(1,11)= 25.19$, $p<0.0005$).

DISCUSSION

These results support a mechanistic link between the E2-enhanced hippocampal dependent learning and memory and the E2-enhanced LTP at CA3-CA1 synapses reported previously (Smith and McMahon, 2005). The shared requirement of NR2B-containing NMDARs, along with a similar time course of enhanced learning after E2 treatment suggests that the E2-enhanced LTP may cause the E2-enhanced learning.

NOR exploits the natural tendency of rodents to explore a novel object (Ennaceur and Delacour, 1988). This task is sensitive to hormonal fluctuations and is enhanced in cycling female rats at proestrus as well as in OVX rats treated with exogenous E2 (Luine et al., 2003; Walf et al., 2006; Fernandez et al., 2008; Michael C. Lewis, 2008; Harburger et al., 2009). While many brain regions contribute to this task, (Buckley, 2005; Dere et al., 2007; Ennaceur, 2010), the E2-enhanced performance in NOR is specifically due to hippocampal NMDARs (Michael C. Lewis, 2008). We have extended this observation by showing that the E2-enhanced NOR is prevented by selective blockade of NR2B-containing NMDARs in area CA1 of hippocampus (Figure 2B3). Interestingly, in contrast to the prevention of NOR in E2-treated rats when RO was administered systemically (Figure 2A3), intraCA1 RO infusion only prevented the E2-enhanced learning, rendering NOR in E2+RO treated animals not different from V-treated rats with or without RO (Figure 2B3). This blockade of only the enhanced learning by intraCA1 RO is reminiscent of our previous LTP results showing that bath application of RO to acutely prepared hippocampal slices only prevents the E2-induced increase in LTP magnitude, with the resulting LTP magnitude not different from that measured in slices from V-treated rats with or without RO (Smith and McMahon, 2006). The differential

effect of systemic versus intraCA1 RO administration on NOR in E2-treated rats is likely explained by the systemic treatment causing a more complete blockade of NR2B in hippocampus, as well as other brain regions. Importantly, the loss of NOR in E2-treated rats, while V-treated rats are unaffected by systemic RO administration indicates that NOR is more highly dependent upon NR2B containing NMDARs following elevated E2. This idea is further supported by a 10 fold higher RO concentration (although still selective for NR2B subunits) needed to prevent NOR in V-treated rats, demonstrating that normal learning is dependent upon NR2B-containing NMDARs, but to a lesser degree than in E2-treated animals (Figure 2D).

Biochemical and anatomical studies have thus far been unable to determine the mechanism causing the increase in NR2B current measured using electrophysiology (Adams et al., 2004; Snyder et al., 2010). Our results are consistent with the interpretation that E2-treatment increases both the number and phosphorylation of synaptic NMDARs (Figure 3D). Importantly, because the E2-induced increase in NMDAR current is entirely due to an increase in NR2B-containing receptors (Smith and McMahon, 2006), we can conclude that the increase in current with orthovanadate is due to phosphorylation of NR2B-containing NMDARs. Our data show clear evidence for an increase in the number of NR2B-containing NMDARs in synapses of cells from E2-treated rats, yet it is still unclear whether these receptors are exocytosed or translocated into the synapse from extra synaptic locations. It also is not known whether these NR2B-containing NMDARs are located within silent synapses (Smith et al., 2009). The increase in dendritic spine density with E2-treatment (Gould et al., 1990; Woolley et al., 1990; Murphy and Segal, 1996; Smith and McMahon, 2005) synchronized with a

selective increase in NMDA transmission (Woolley et al., 1997; Smith and McMahon, 2005) strongly supports the concept that an increase in activation of silent synapses may cause the increase in LTP magnitude, which we show here is associated with enhanced learning.

Estrogen could use a traditional genomic estrogen receptor (ERα or ERβ) and initiate gene transcription which requires hours to days, or E2 could engage a membrane bound ER resulting in the rapid activation of signaling cascades to enhance hippocampal LTP, NR2B-mediated current, spines and learning and memory. We have previously shown that LTP at CA3-CA1 synapses is not enhanced 24 hours after a single E2 injection (10μg)(Smith and McMahon, 2005), suggesting a genomic mechanism is required for the synaptic changes we observe. However, E2 can enhance NMDAR-mediated responses within minutes of bath application of 1nM E2 in recordings from acute hippocampal slices (Foy et al., 1999). Also, intrahippocampal or intracerebroventricular E2 infusion within 30 min after exposure to objects in the NOR task enhances consolidation, due to activation of a membrane bound estrogen receptor which stimulates PKA and ERK activation (Fernandez et al., 2008; Michael C. Lewis, 2008). Recent data further supports that the ERα agonists PPT can increase dendritic spine density and learning within 40 minutes of a systemic injection (Phan et al., 2011). It is most likely that the effects we observe here are caused by a combination of rapid and genomic effects of E2 that occur in parallel, using separate mechanisms to enhance hippocampal function.

E2 may also act indirectly through cholinergic input into hippocampus to enhance potentiation at CA3-CA1 synapses and learning. E2 enhanced cholinergic function in

hippocampus (Luine, 1985; Gibbs, 2000) is critical for E2-enhanced learning and memory (Gibbs, 2002; Daniel et al., 2005; Gibbs, 2007) and increased NMDARs in binding assays (Daniel and Dohanich, 2001). New studies are required to determine a direct linkage between cholinergic innervation into hippocampus and E2-enhanced magnitude of LTP and to further determine whether these effects are causal to the ability of E2 to enhance learning.

Our findings that E2 requires NR2B-containing NMDARs to enhance hippocampal dependent learning and memory are integral for the development of targeted treatments for hormone-related cognitive decline during menopause. We have previously reported that the E2-enhanced magnitude of LTP at CA3-CA1 synapses is lost after long periods of hormone deprivation (Smith et al., 2010). This loss in E2-enhanced LTP is prevented in aged matched ovary intact rats, suggesting that the loss in enhanced plasticity is not solely age dependent, but depend on duration of hormone deprivation. These findings support the theory that a window of opportunity exists after hormone loss during which time E2 can enhance hippocampal LTP. Future studies are required to determine whether E2-enhanced cognition is maintained in aging and through periods of long-term ovarian hormone loss and whether NR2B-containing NMDARS are also critical for these enhancements should they occur.

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FIGURES AND LEGENDS

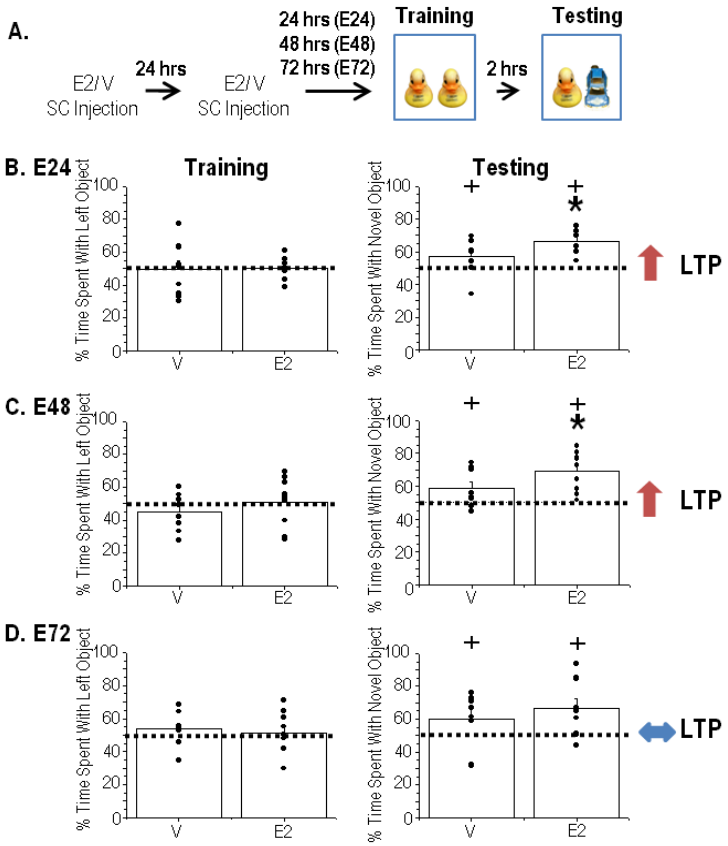

Figure 1. E2-enhanced NOR occurs at 24 and 48, but not at 72 hours post E2 treatment. A. Experimental timeline. B-D, Left, The average percent time spent investigating the left object during training demonstrates no object preference in E2 and V-treated rats at E24, E48 and E72. B-C, Right, Percent time spent with the novel object at E24 and E48 was significantly greater in E2-treated compared to V-treated rats. D. Right, Percent time spent with novel object at E72 was not different between E2 and V-treated rats. Far right arrows represent LTP at CA3-CA1 synapses for respective time points as reported in Smith and McMahon, 2005. + indicates significance from chance at $p<0.05$. * indicates significance of hormone treatment at $p<0.05$. Closed circles represent individual animals.

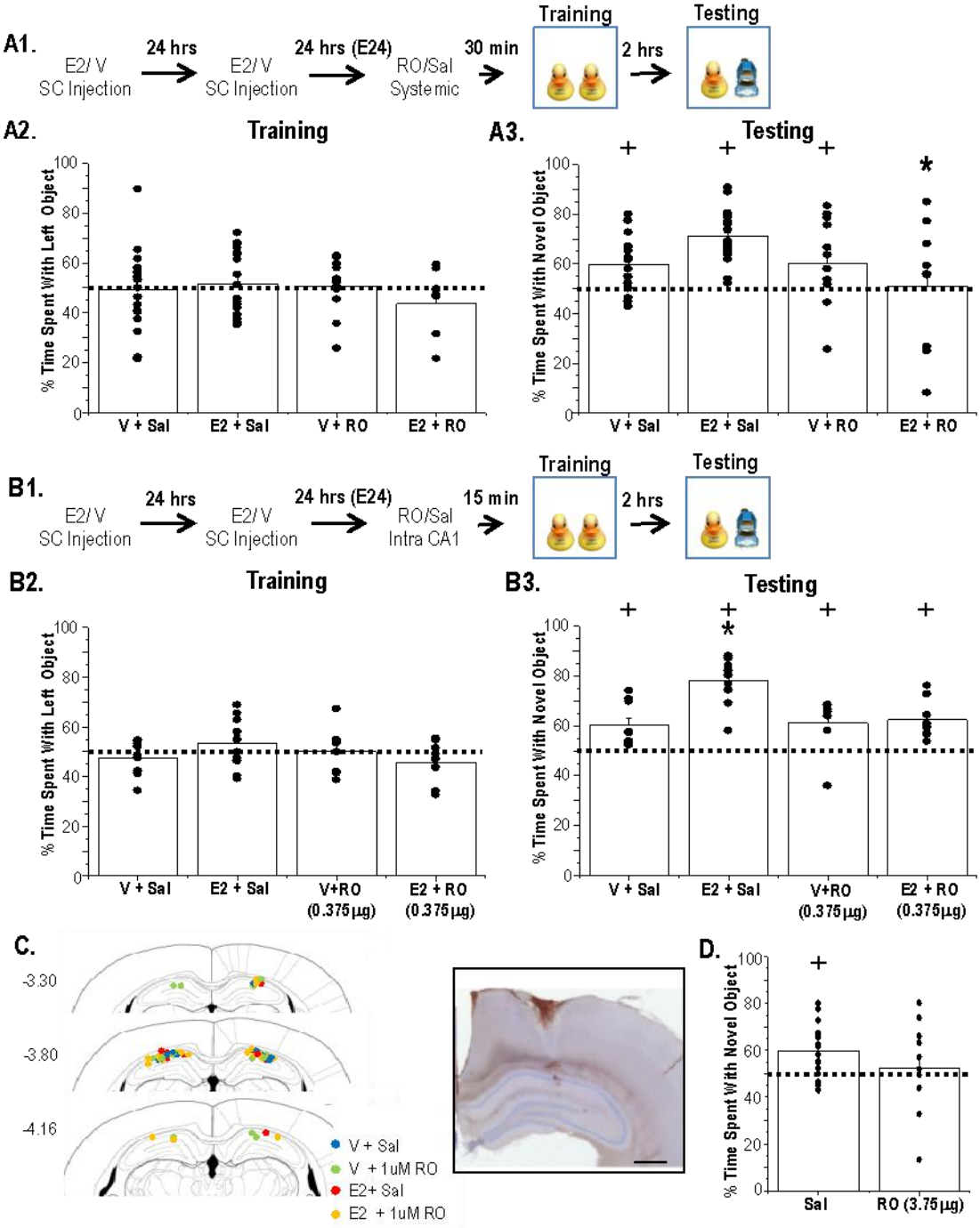
A1.
E2/ V
SC Injection
24 hrs
E2/ V
SC Injection
24 hrs (E24)
RO/Sal
Systemic
30 min
Training
2 hrs
Testing
A2.
Training
% Time Spent With Left Object
V + Sal
E2 + Sal
V + RO
E2 + RO
A3.
Testing
% Time Spent With Novel Object
B1.
E2/ V
SC Injection
24 hrs
E2/ V
SC Injection
24 hrs (E24)
RO/Sal
Intra CA1
15 min
Training
2 hrs
Testing
B2.
Training
% Time Spent With Left Object
V + Sal
E2 + Sal
V+RO
(0.375µg)
E2 + RO
(0.375µg)
B3.
Testing
% Time Spent With Novel Object
C.
-3.30
-3.80
-4.16
V + Sal
V + 1uM RO
E2+ Sal
E2 + 1uM RO
D.
% Time Spent With Novel Object
Sal
RO (3.75µg)

Figure 2. E2-enhanced NOR is prevented by blocking NR2B-containing NMDARs.

A1. Experimental timeline. A2. No object preference during training. A3. Systemic RO (5mg/Kg) blocks learning in E2-treated animals only. B1. Experimental timeline for intraCA1 infusion of RO. B2. No object preference during training. B3. IntraCA1 RO (0.375μg/μL) infusion blocks E2-enhanced learning. Importantly, V-treated animals still spend significantly more time on the novel object compared to chance. C. Cannula tip locations for each animal included in behavioral experiments. Inset: Cresyl violet stained hippocampal brain slice showing representative cannula tip placement. Scale bar=300μM. D. Infusion of 3.75μg/1μL RO blocks NOR in V-treated animals. + indicates percent time spent on novel object is significant from chance. *$p<0.05$ comparing E2 Sal and E2 RO. Closed circles represent individual animals.

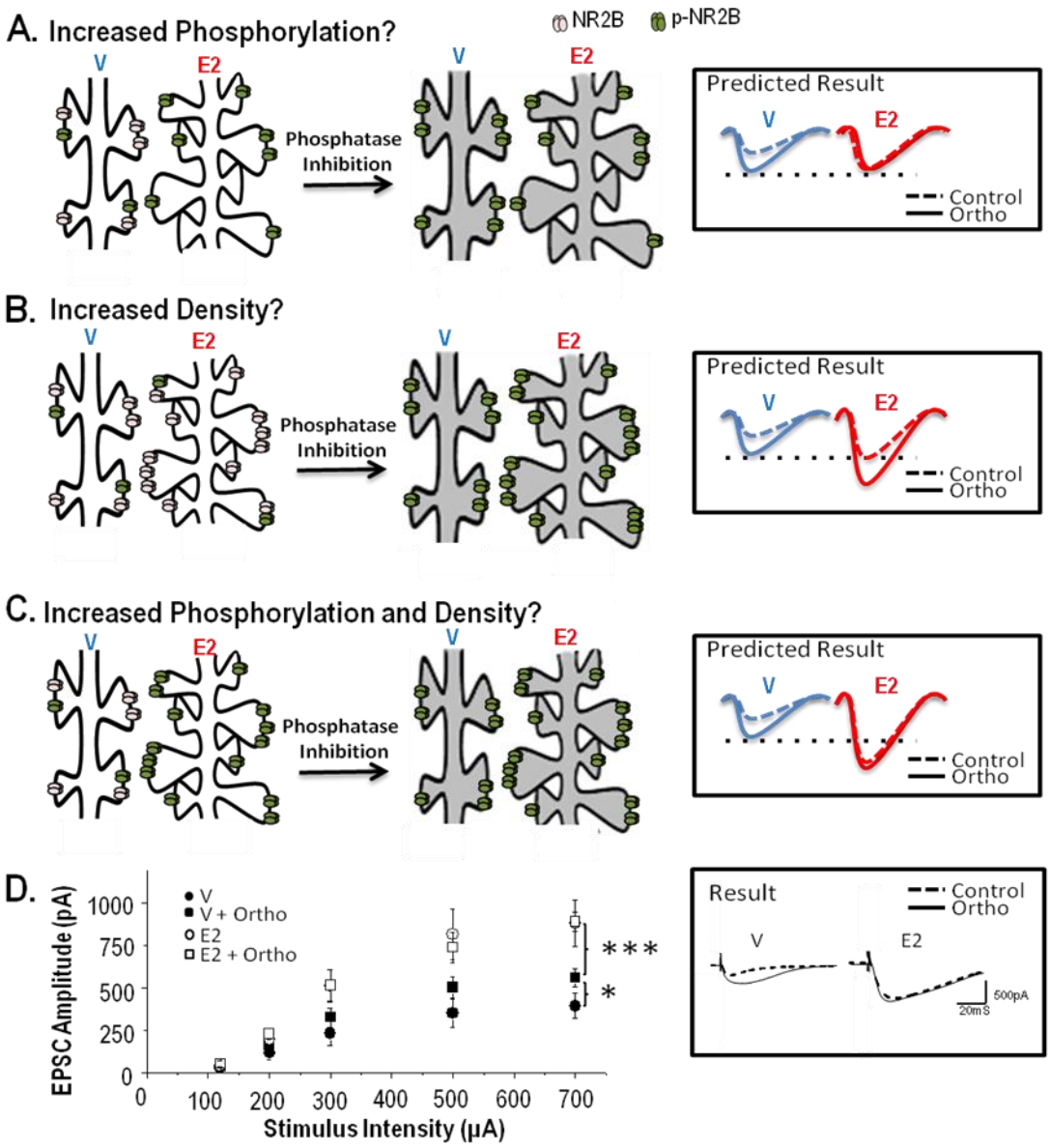
A. Increased Phosphorylation?
NR2B
p-NR2B
V
E2
Phosphatase Inhibition
Predicted Result
Control
Ortho
B. Increased Density?
C. Increased Phosphorylation and Density?
D.
EPSC Amplitude (pA)
Stimulus Intensity (μA)
V
V + Ortho
E2
E2 + Ortho
***
*
Result
500pA
20mS

Figure 3. E2 enhances both phosphorylation of NR2B-containing NMDARs and total NR2B-containing NMDARs in the synapse.

Experimental models with predicted results if E2 treatment enhances synaptic NR2B-containing NMDAR phosphorylation (A), density (B) or both (C). D. Whole-cell recordings of evoked NMDAR EPCSs at increasing stimulus intensities from V-treated animals with (closed squares) or without (closed circles) orthovanadate in the pipet solution and E2-treated animals with (open squares) or without (open circles) orthovanadate. The maximum evoked NMDAR current measured in cells from E2-treated animals is significantly increased above that measured in cells from V-treated rats. Orthovanadate has no effect in cells from E2-treated animals, but significantly enhances the maximum current elicited in cells from V-treated animals. ***$p<0.0005$, *$p<0.05$.

DURATION OF ESTROGEN DEPRIVATION, NOT CHRONOLOGICAL AGE, PREVENTS ESTROGEN'S ABILITY TO ENHANCE HIPPOCAMPAL SYNAPTIC PLASTICITY

by

CAROLINE C. SMITH*, LINDSEY C. VEDDER*, AMY R. NELSON, TERUKO M. BREDEMANN, LORI L. MCMAHON

*CCS and LCV contributed equally to this work

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CAROLINE C. SMITH*, LINDSEY C. VEDDER*, AMY R. NELSON, TERUKO M. BREDEMANN, LORI L. MCMAHON

ABSTRACT

Whether estrogen replacement is beneficial to cognitive health is controversial. Some studies show estrogen replacement therapy relieves memory impairment associated with menopause in women, while others suggest that not only is estrogen incapable of providing benefit, but it can be detrimental. One explanation for this discrepancy could be the duration of time following menopause prior to commencing estrogen replacement. It has been proposed that a critical period exists during which estrogen replacement must be administered in order to enhance cognitive function. However, this notion has yet to be directly tested using functional synaptic studies. Here we investigated whether prolonged hormone deprivation caused by ovariectomy (OVX) in young adult rats prevents the ability of estrogen replacement to increase synaptic function in hippocampus necessary for the estrogen-induced improvement in learning and memory. Remarkably, estrogen replacement increases long-term potentiation (LTP), current mediated by NR2B-containing NMDA receptors, and dendritic spine density at CA3-CA1 synapses up to 15 months post-OVX. However, by 19 months post-OVX, the same estrogen replacement is unable to induce these changes. Importantly, this loss of estrogen's effectiveness is a consequence of the duration of deprivation because, in female rats aged with their ovaries

intact and examined at the same chronological age as the 19 month-post-OVX group, estrogen replacement significantly increases synaptic function and spine density. These data clearly demonstrate that a critical period of time exists in which estrogen replacement must be administered and once this time is exceeded, the beneficial effects are lost.

INTRODUCTION

Cognitive function fluctuates across the menstrual cycle in women, with increased learning occurring when plasma estrogen levels are highest (1, 2). As such, loss of endogenous estrogen production after menopause, a consequence of normal aging, correlates with cognitive deficits (3). Treatment with estrogen replacement therapy (ERT) is not always successful in alleviating this hormone related cognitive decline (4, 5). This lack of benefit of ERT may be explained by the critical period hypothesis which proposes that there is a critical period following reproductive senescence where estrogen is capable of increasing hippocampal function necessary to enhance memory processing. After this time, ERT may become ineffective and possibly detrimental (6, 7).

During proestrus in cycling rats, hippocampal learning is increased, and this can be mimicked in young adult ovariectomized (OVX) rats treated with the ovarian estrogen 17β-estradiol (E2) (8-11). Similar to women, rats that experience long-term ovarian hormone deprivation no longer benefit from the enhancing effects of E2 on hippocampal learning (12-15). The role of ovarian hormone senescence in the effectiveness of E2 replacement in enhancing learning is difficult to distinguish from normal aging processes, as aging alone leads to learning deficits in rats (16, 17). However, recent studies suggest that the length of ovarian hormone deprivation, not just chronological age, is a key factor that limits the ability of E2 replacement to enhance learning (13, 14). Unfortunately, the precise cellular mechanisms contributing to the loss of the beneficial effects of E2 replacement remain incompletely understood.

Long-term potentiation (LTP) is considered a cellular correlate of learning and memory (18). Estrogenic conditions that enhance learning in rats, including proestrus and treatment of OVX rats with exogenous E2, enhance LTP and dendritic spine density in CA3-CA1 synapses (19-24). E2 also increases phosphorylation of NR2 NMDAR subunits (25), and selectively increases NMDAR current mediated by NR2B-containing receptors, which increases the NMDAR/AMPAR ratio and causes the increase in LTP magnitude (26, 27). In various studies of hippocampal function, changes such as these have been correlated to increased learning, likely because they underlie enhanced synaptic plasticity (28, 29). Currently, there is a complete lack of functional studies at the synaptic level investigating the impact of prolonged hormone loss on the E2-induced increase in hippocampal synaptic function.

Here we investigated whether the beneficial effects of E2 replacement on hippocampal synaptic function and morphology are lost as a consequence of long-term E2 deprivation. We hypothesize that there exists a point following prolonged ovarian E2 deprivation that E2 replacement becomes ineffective at increasing synaptic function and spine density, and that chronological age at the time of replacement is not the major factor in determining E2's effectiveness. Finding this critical period is a necessary step in understanding why some studies do not observe a cognitive benefit with ERT (4, 5). Clearly, this issue is of clinical importance because as life expectancy lengthens, women will experience a larger percentage of their lives negatively impacted by the loss of E2.

RESULTS

The E2-induced increase in LTP magnitude is lost following prolonged hormone deprivation.

We first tested the impact of prolonged hormone deprivation on the ability of E2 replacement to increase the LTP magnitude. Surprisingly, we find in rats 9 and 15 months post-OVX, E2 replacement remains able to induce a significant increase in the magnitude of LTP (Fig. 1*A-B*; 9 months post-OVX, $t(8)=2.82$, $p<0.05$; 15 months post-OVX, $t(7)=2.36$, $p<0.05$). However, by 19 months post-OVX, the magnitude of LTP in slices from E2 treated rats is no longer different from vehicle treated controls (Fig. 1*C*; $t(11)= -0.15$, $p=0.44$). The ability of the same E2 replacement regimen to increase the LTP magnitude at 9 and 15, but not at 19 months post-OVX, supports the critical period hypothesis.

E2 replacement increases NR2B current, the NMDAR/AMPAR ratio, and spine density in animals experiencing prolonged hormone deprivation only when the LTP magnitude is also increased.

In young adult OVX rats we reported that the increase in LTP magnitude only occurs when transmission mediated by NR2B-containing NMDARs, the NMDAR/AMPAR ratio, and spine density are simultaneously increased (23, 26). Therefore, the effects of prolonged E2 deprivation on the LTP magnitude in E2 treated animals predicts that NR2B transmission, the NMDAR/AMPAR ratio, and spine density will be increased at 15 but not at 19 months post-OVX. As expected, at 15 months post-OVX, E2 significantly increases the fraction of current mediated by NR2B-containing NMDARs (Fig. 2*B*; $t(10)=1.91$, $p<0.05$) and this increase in NR2B current is responsible for significantly increasing the NMDAR/AMPAR ratio (Fig. 2*C*; $F(3,20)=12.83$, $p<0.005$). Along with the increase in synaptic function, spine density is also significantly increased in E2 treated 15 month post-OVX animals (Fig. 2*D-E*; $t(8)=3.62$, $p<0.005$). These results

mimic our previous findings in young adult OVX rats that were treated with this same E2 regimen 2 weeks post-OVX (23, 26).

However, by 19 months post-OVX, current mediated by NR2B-containing receptors is not significantly increased (Fig. 3*B*; $t(18)=1.08$, $p=0.15$). Accordingly, the NMDAR/AMPAR ratio is not increased, nor is there an increase in spine density (Fig. 3*C*-*E*; NMDAR/AMPAR, $F(3,34)=2.64$, $p=0.07$; spines, $t(14)=2.15$, $p=0.41$).

The E2-induced increase in LTP, NR2B current, NMDAR/AMPAR ratio, and spine density still occurs in female rats aged with intact ovaries.

The lack of E2 benefit in the 19 month post-OVX group could simply be a consequence of the chronological age of the animals, rendering them unresponsive to E2 using the replacement protocol that enhances synaptic function and spine density in younger rats. In an attempt to distinguish between hormone deprivation and age, the LTP magnitude was measured in animals of the same chronological age as the 19 month post-OVX group (21 months old at time of experimentation). Ovaries were left intact until 20 months of age; E2 was replaced one month later and was administered as in the other groups (supplemental Fig 1). Remarkably, the LTP magnitude was significantly increased in the E2 treated group compared to vehicle (Fig. 4*A*; $t(10)=1.81$, $p<0.05$). As would be predicted from the enhancing effect on LTP, E2 increased current mediated by NR2B-containing NMDARs (Fig. 4*C;* $t(15)=2.83$, $p<0.01$), the NMDAR/AMPAR ratio (Fig.4D; $F(3,28)=12.51$, $p<0.00005$), and dendritic spine density (Fig 4*E*-*F* $t(13)=2.15$, $p<0.05$). Collectively, these findings strongly suggest that the period of hormone deprivation rather than chronological age is primarily responsible for the loss of effectiveness of E2 replacement in enhancing hippocampal synaptic function and spine density.

DISCUSSION

This is the first report to investigate the impact of prolonged E2 deprivation on the ability of E2 replacement to enhance hippocampal synaptic transmission and plasticity. Our findings clearly support the critical period hypothesis since E2 replacement in female rats up to 15, but not at 19, months post-OVX remains able to increase synaptic function and spine density, mimicking the effects of E2 in young adult OVX rats following a 2 week deprivation (23, 26). The preserved beneficial effects of E2 replacement in 21 month old rats (same chronological age as the 19 month post-OVX group) measured 1 month post-OVX strongly indicates that the period of E2 deprivation, rather than chronological age, is the limiting factor in determining whether E2 replacement remains beneficial for hippocampal function.

In concert with our synaptic physiological studies reported here, behavioral studies find that E2 replacement can be beneficial for hippocampal dependent learning in aged OVX rats when administered within a critical time frame, after which E2 is no longer effective (12-15, 30). Of particular relevance to the current study is a recent report showing that E2 replacement administered at ≥19 months post-OVX no longer enhances delayed matching to position acquisition (13), which is in direct agreement with our finding that E2 no longer enhances LTP in the 19 month post-OVX group. Also consistent with our findings from the aged ovary intact group where E2 replacement increases the LTP magnitude, E2 replacement remains effective in enhancing hippocampal dependent learning in aged animals with a short term (3 month) E2

deprivation (12). We speculate that because aged rats enter a state of constant estrus or diestrus (31), in contrast to what occurs in women during menopause, ovarian E2 released throughout the rat's lifespan likely protects hippocampal function. Collectively, results from both animal behavioral studies and our synaptic physiology studies demonstrate that a critical window exists during which E2 replacement is beneficial for hippocampal function. However, the duration of the window is variable and depends on the chronological age at the time of ovariectomy and the duration of hormone deprivation (32-34). Therefore, to precisely define the duration of the critical period, investigations are needed that simultaneously combine measures of synaptic physiology, morphology and behavior within the same cohort of ovariectomized animals at various chronological ages and durations of hormone deprivation. These studies will be particularly important in order to more accurately define the critical window in women, who experience a rapid loss of circulating E2 after menopause, while aged rats experience estrus or diestrus levels of circulating E2 after reproductive senescence which may lengthen this window.

In intact cycling rats, LTP at CA3-CA1 synapses and phosphorylation of NMDAR NR2 subunits are increased at proestrus, which is also when hippocampal learning is enhanced, linking the increase in synaptic function with learning (11, 19-21, 23, 35). We speculate that the increase in NR2B current and NMDAR/AMPAR ratio we observe in E2 treated OVX rats is directly linked with heightened hippocampal dependent learning and memory because the LTP magnitude is only increased when NR2B current and the NMDAR/AMPAR ratio are increased (26). This idea is directly supported by the increase in NMDAR transmission and LTP at CA3-CA1 synapses and heightened hippocampal learning in NR2B over-expressing transgenic mice (28). Therefore, we propose that the

inability of E2 replacement to increase NR2B current, NMDAR/AMPAR ratio, and LTP provides a mechanistic explanation for the loss of cognitive benefit of E2 following prolonged ovarian E2 depletion.

Our results suggest that the duration of E2 deprivation in the 19 month post-OVX group is responsible for the lack of increase in the LTP magnitude in E2 treated animals. Alternatively, an increase in NR2B current in vehicle treated animals, compared to vehicle treated animals in other groups, may cause a ceiling effect on the LTP magnitude, preventing a further increase in E2 treated animals. However, in our studies in young adult OVX rats, the increase in LTP magnitude occurs only when spine density, the NMDAR/AMPAR ratio, and transmission mediated by NR2B-containing NMDARs are simultaneously increased (23, 26). This coincident increase in spine density, NMDAR/AMPAR ratio, and NR2B transmission is consistent with the hypothesis that E2 increases the density of silent synapses, which likely contributes to the increase in LTP (37). Therefore, because there is only an increase in NR2B current in vehicle treated 19 month post-OVX animals, without a simultaneous increase in spine density and NMDAR/AMPAR ratio, the lack of an increase in the LTP magnitude is not likely a result of a ceiling effect.

Remarkably, E2 replacement remains able to increase current mediated by NR2B-containing receptors, the NMDAR/AMPAR ratio, and dendritic spine density in rats aged with their ovaries intact. This increase in NR2B current we observe is consistent with results from 23-24 month old rats where E2 stimulates lateral movement of NR2B-containing NMDARs into the synapse (38). A recent study by Snyder et al confirms our original finding that E2 increases synaptic NR2B-mediated current, and because they do

not find an increase in NR2B expression, they suggest that the increase in current is due to lateral movement of NR2B-containing receptors into the synapse (27). However, in contrast to our findings and those of Snyder and colleagues, Adams et al. show no increase in NR2B-containing receptors in synapses of young animals treated with E2 (38). Also, some studies show no increase in axospinous synapse or dendritic spine densities in aged animals (39, 40). The reasons for these discrepancies are not clear, but may be related to the sensitivity of measuring NR2B-containing receptors using anatomical versus physiological approaches, the amount of enrichment experienced by the animals through exposure to behavioral tasks, and the methodological differences in measuring spine density. Importantly, our data from this current study are in agreement with our previously reported data strongly linking the E2-induced increase in LTP magnitude, with an increase in NR2B current, NMDAR/AMPAR ratio, and dendritic spine density (23).

The inability of E2 to increase synaptic plasticity and spine density reported here, as well as hippocampal learning reported by others following prolonged E2 deprivation, could be explained by decreased hippocampal cholinergic function. Cholinergic innervation is required for the E2-induced increase in NMDAR binding (41) and an extensive literature documents a key role of cholinergic innervation in mediating the beneficial effects of E2 on spatial learning (see (42) for review). Ovariectomy leads to a significant decrease in ChAT mRNA in basal forebrain cholinergic neurons and acetylcholine release in hippocampus (43, 44) beyond that of normal aging (45-47). Thus, a threshold may exist at which cholinergic function is so severely depleted that E2 effects on synaptic function and learning cannot be elicited. In support of this idea, a recent

elegant study (13) showed that pharmacological inhibition of acetylcholinesterase *in vivo* rescues the ability of E2 to enhance cognitive function in aged rats 19-24 months post-OVX. Because E2 is neuroprotective for cholinergic neurons (46-48), the normal decline of endogenous E2 production, as occurs during menopause, renders cholinergic neurons at greater risk of degenerating. Accordingly, declining E2 in postmenopausal women is linked to increased risk of Alzheimer's disease (AD) and E2 replacement decreases this risk (46, 49). Thus, the critical period of E2's effectiveness will likely depend upon the integrity of the cholinergic system.

In addition to acetylcholine, BDNF may mediate the effects of E2 in hippocampus (50) because blocking TrkB receptors prevents the E2-induced increase in spine density (51), and BDNF increases NMDAR current (52) and learning (53). At proestrus, BDNF expression is highest (54), therefore, the decrease in endogenous E2 in aging will lead to a decrease in BDNF expression and contribute to the loss of E2's beneficial effects. Additional factors include the impact of ovarian E2 depletion on local estrogen synthesis in hippocampus, which recently has been shown to be critical for hippocampal function (56)

This study has yielded invaluable information regarding the effectiveness of E2 replacement on hippocampal synaptic function, which is directly determined by the length of time after ovarian hormone deprivation, not chronological age at the time of E2 replacement. This work is crucial because these functional changes, at the level of the synapse, are likely the cause of the E2-induced learning enhancement. Further studies uncovering the mechanisms by which E2 enhances synaptic plasticity, synaptic morphology, and learning in the context of long-term ovarian deprivation will provide

evidence for the effective use of estrogen replacement therapy in post-menopausal women.

METHODS

Animals

All experimental procedures have been approved by the UAB IACUC in accordance with NIH experimental guidelines.

Surgical Procedures

Four groups of female Sprague-Dawley rats were used (supplemental Fig. 1). Three groups underwent ovariectomy (OVX) at 2 months of age (using 2.5% isoflurane and aseptic conditions) and were housed for 9, 15, or 19 months post-OVX prior to experimentation. The 4th group was aged with ovaries intact to 20 months old prior to undergoing OVX. At the time of experimentation this group was the same chronological age as the 19 month post-OVX group. Prior to OVX, the aged ovary intact animals were cycled for 5 days using saline vaginal lavage and cells were imaged using bright field microscopy at 40X. Stages of the estrous cycle were determined based on cells present or absent within vaginal smears based on criteria from The Laboratory Rat, 2nd edition (supplemental table 1) (57, 58). Whether the cycles were regular or irregular is unknown; collection of vaginal smears was limited to 5 days to prevent potential occlusion of the E2 effects in hippocampus, as enrichment from excessive handling can block the benefits of E2 replacement on cognition (59).

Estradiol Replacement

OVX rats received a subcutaneous injection of either E2 (10μg/250g in cotton seed oil) or oil vehicle alone on 2 consecutive days, and were sacrificed for experiments 24 hours

following the final injection, as done previously (23, 26). Although we did not measure plasma E2 levels, this injection protocol, adopted from Woolley and McEwen, 1993, yields approximate proestrus plasma levels (80-120 pg/ml) (60). Dry uterine weights were obtained to confirm success of the E2 injections (61). Uterine weights in all E2 treated animals were at least double that in vehicle treated OVX rats, $t(72)=5.27$, $p<0.000001$.

Electrophysiology

Rats were transcardially perfused with a high sucrose, low NaCl aCSF (mM): 85 NaCl, 2.5 KCl, 4 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 glucose, 75 sucrose, and 0.5 ascorbate; saturated with 95%O_2:5%CO_2 (pH 7.4) and rapidly decapitated. Coronal dorsal hippocampal slices (400μm) were cut in high sucrose solution and stored in a submersion chamber containing normal aCSF (mM): 119 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 10 glucose, saturated with 95%O_2:5%CO_2, at room temperature. For patch recording, slices were stored for an additional 30 minutes in high sucrose solution with 2μM kynurenic acid before being transferred to normal aCSF. Recordings were performed in a submersion chamber perfused with aCSF (26-28^{o}C) and saturated with 95%O_2:5%CO_2.

Extracellular LTP Recordings. Schaffer collaterals were stimulated (0.1 Hz, 100μs duration) to elicit 0.6-0.7 mV field excitatory post-synaptic potentials (fEPSPs; ~ 50% of maximum) at CA3-CA1 synapses. Following a 20 minute baseline, LTP was induced using high frequency stimulation (hfs; 100 Hz, 0.5s duration, 4 times, 20s interval, at 1.5x baseline intensity) (23, 26). Experiments were excluded if baseline transmission varied by more than 8%.

Patch-Clamp Recordings. Whole cell recordings were obtained from CA1 pyramidal cells using the blind patch technique (R_{input}: 100-160mΩ; R_{series}: 15-25mΩ). Experiments were omitted if R_{input} or R_{series} varied by more than 10%. Electrodes (3-6 MΩ) were filled with (mM): 117 cesium gluconate, 0.6 EGTA, 2.8 NaCl, 5 $MgCl_2$, 2 ATP, 0.3 GTP, 20 HEPES, 5 QX-314, and 0.4% biocytin. $GABA_A$Rs were blocked with picrotoxin (100 μM) and L-type Ca^{2+} channels were blocked with nifedipine (10 μM) to prevent their activation at depolarized potentials. Glutamate currents were evoked using a stimulating intensity that generated a 100-200pA EPSC. Cells were voltage clamped at -20mV to maximize the contribution of NMDARs to the glutamate current. The contribution of NR2B-containing NMDARs to the total NMDAR current and the NMDAR/AMPAR ratio was determined using the selective antagonist RO25-6981 (RO; 0.5μM). Next, the remaining NMDAR current was pharmacologically blocked using D,L APV (100μM) and AMPARs were subsequently blocked with DNQX (10 μM). The contribution of each current was calculated by subtraction as we have done previously (26).

Axoclamp 2A and Axopatch 1D amplifiers (Molecular Devices, Sunnyvale CA) were used to record fEPSP and whole-cell currents, respectively, and data were acquired and analyzed using custom made Labview software (Dr. Richard Mooney, Duke University).

Histology. At the conclusion of each recording, slices were fixed in 4% paraformaldehyde (PFA) in PBS *overnight. Incubation with fluorescent streptavidin was used to visualize biocytin filled cells.* Confocal imaging and spine counts were performed with the experimenter blind to treatment group and electrophysiological results. Tertiary dendritic segments from the apical arbor in stratum radiatum were imaged (63x at 2.0 digital zoom) with 3-5 segments analyzed per animal (n=at least 4 animals per

experimental group). Protrusions were counted as spines only if the length was < 3.0μm from the dendrite (62). Dendritic segments (10 μM) were chosen for analysis if they were completely filled, clearly visible, and continuous with the dendrite. Dendrites were imaged (Leica Application Suite Advanced) by acquiring Z-stacks (1μM thick); spines were counted by scanning through individual focal planes in the Z-stack prior to collapsing all of the images into a maximum 3D projection.

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FIGURES AND LEGENDS

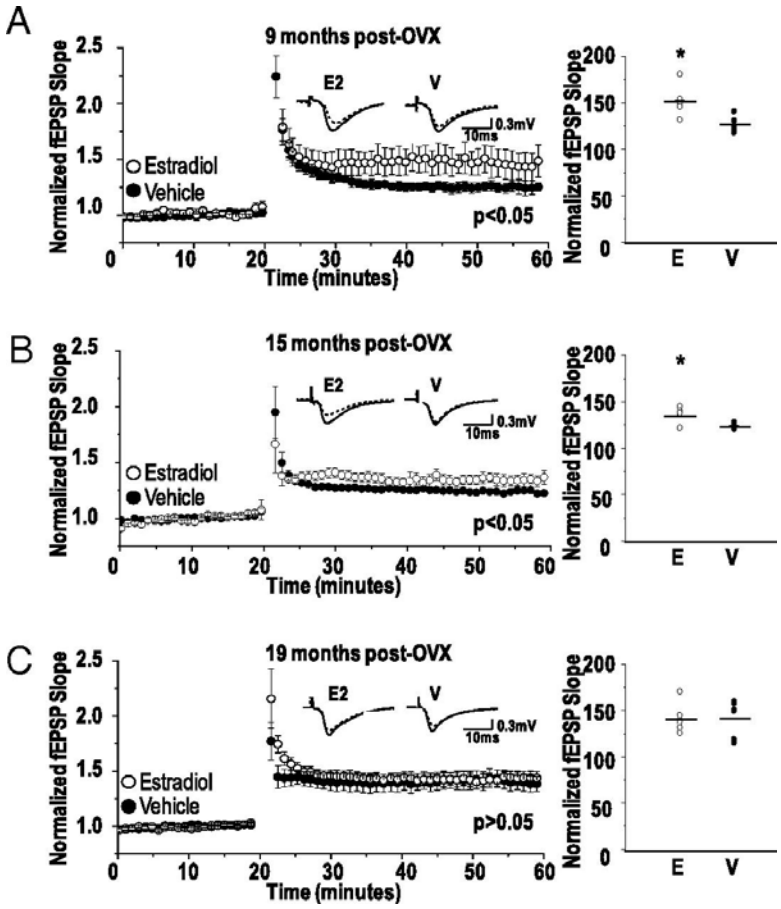


Figure 1: Long-term E2 deprivation prevents E2-enhanced LTP at CA3-CA1 synapses. A) E2 increases the magnitude of LTP at 9 months post-OVX compared to vehicle treated control OVX animals (E2: 152±8%, N=5, V: 127±4%, N=5). B) At 15 months post-OVX, E2 is still capable of increasing the magnitude of LTP (E2: 136±4%, N=4, V: 124±1%, N=5). C) By 19 months post-OVX, E2 is no longer capable of increasing the LTP magnitude (E2: 141±6%, N=7, V: 142±8%, N=6). fEPSP waveforms are averages of 10 events during baseline (dashed line) and 30 minutes post tetanus (solid line). Graphs on the right show the LTP magnitude from individual experiments that were included in group averages, * $p<0.05$. Error bars indicate SEM.

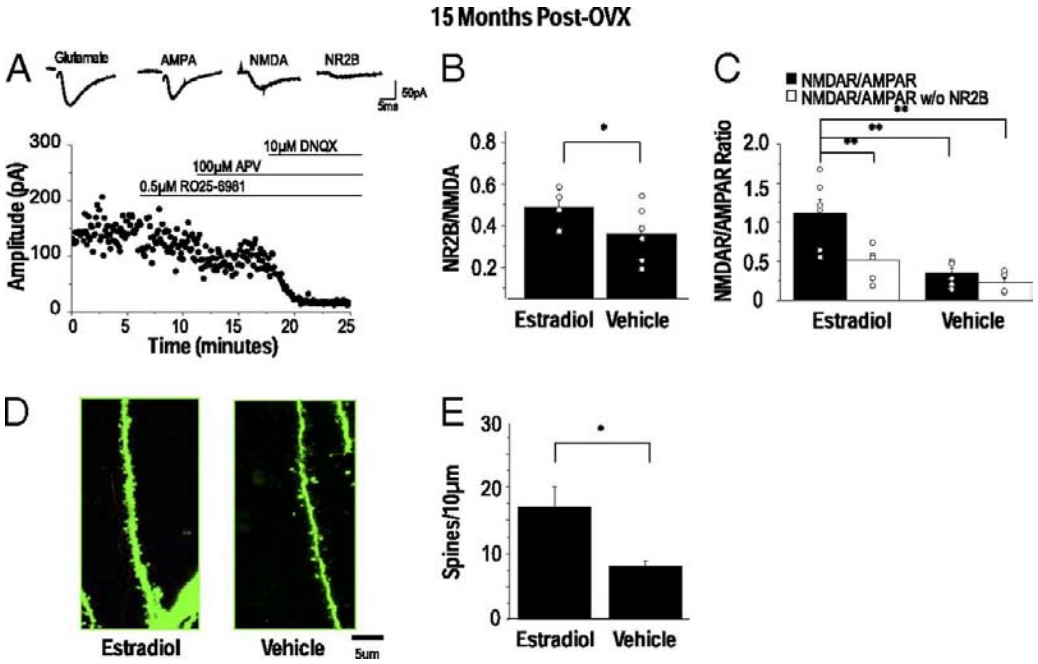


Figure 2*:* E2 replacement increases current mediated by NR2B-containing NMDARs, the NMDAR/AMPAR ratio, and spine density at 15 months post-OVX. A) A representative whole cell recording from a vehicle treated animal showing total glutamate, AMPA-only, NMDA-only and NR2B-mediated NMDA currents isolated pharmacologically. B) E2 increases the fraction of the NMDA current carried by NR2B-containing NMDARs (E2: 0.49±0.06 N=11 cells/6 animals, V: 0.36±0.04 N=9 cells/6 animals). C) E2 increases the NMDAR/AMPAR ratio, which is blocked by the NR2B antagonist RO25-6981 (NMDAR/AMPAR; E2: 1.11±0.2 N=11 cells/6 animals, V: 0.35±0.06 N=9 cells/6 animals; NMDAR/AMPAR without NR2B-containing NMDARs; E2: 0.51±0.1 N=11 cells/6 animals, V: 0.23±0.05 N=9 cells/6 animals). D) Representative confocal images of a tertiary CA1 dendrite from an E2 and a vehicle treated animal. E) E2 increases the density of dendritic spines on CA1 pyramidal cells (E2: 17.1±2.9/10μm of dendrite N=4 animals, V: 8.0±0.8/10μm of dendrite N=6 animals). *$p<0.05$,**$p<0.005$. Error bars indicate SEM.

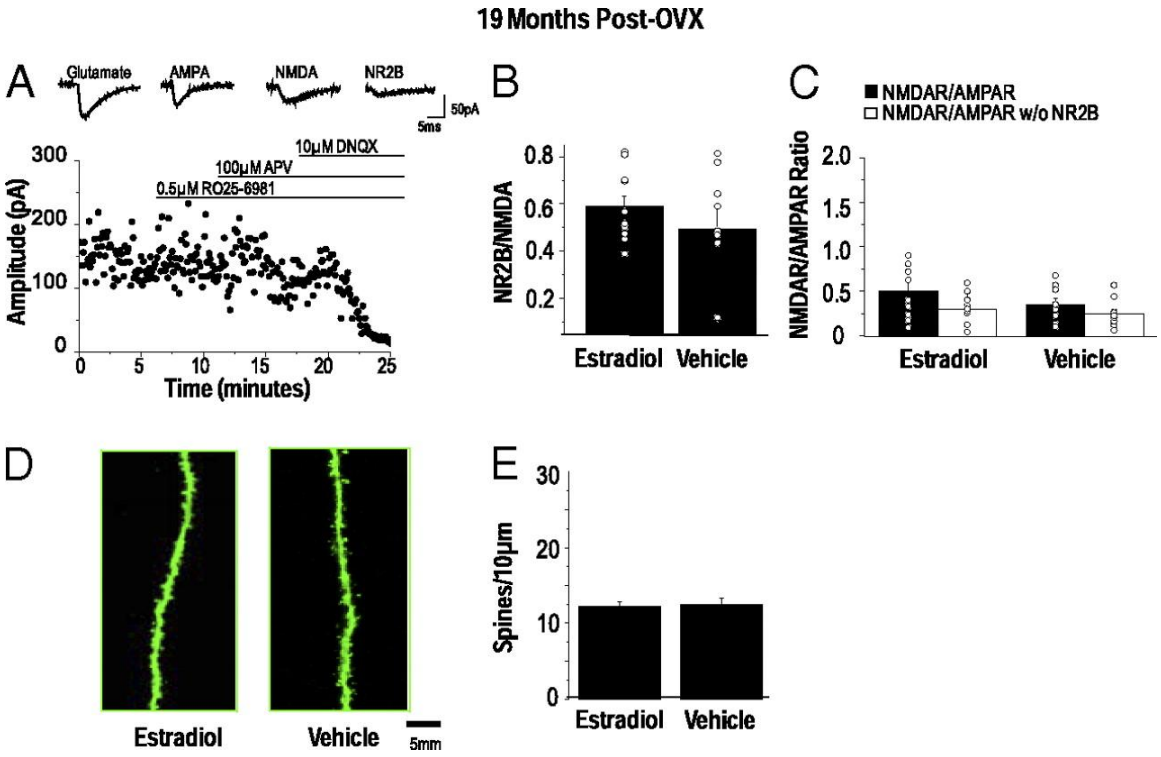


Figure 3: E2 replacement does not increase the NR2B-mediated NMDAR current, the NMDAR/AMPAR ratio or spine density at 19 months post-OVX. A) A representative whole cell recording from a vehicle treated animal showing total glutamate, AMPA-only, NMDA-only, and NR2B- mediated NMDA currents isolated pharmacologically. B, C) E2 does not increase the fraction of current mediated by NR2B-containing NMDARs (E2: 0.59±0.04 N=11 cells/10 animals, V:0.49±0.08 N=9 cells/9 animals) or the NMDAR/AMPAR ratio (NMDAR/AMPAR, E2: 0.50±0.09 N=11 cells/10 animals, V: 0.35±0.04 N=9 cells/9 animals; NMDAR/AMPAR without NR2B-containing NMDARs, E24: 0.30±0.03 N=15 cells/10 animals, V: 0.25±0.05 N=9 cells/9 animals.) D) A representative confocal image of a tertiary CA1 dendrite from an E2 and a vehicle treated animal. E) E2 does not significantly increase spine density (E2: 12.3±0.6/10μm of dendrite N=10 animals, V: 12.5±0.8/10μm of dendrite N=6 animals). Error bars indicate SEM.

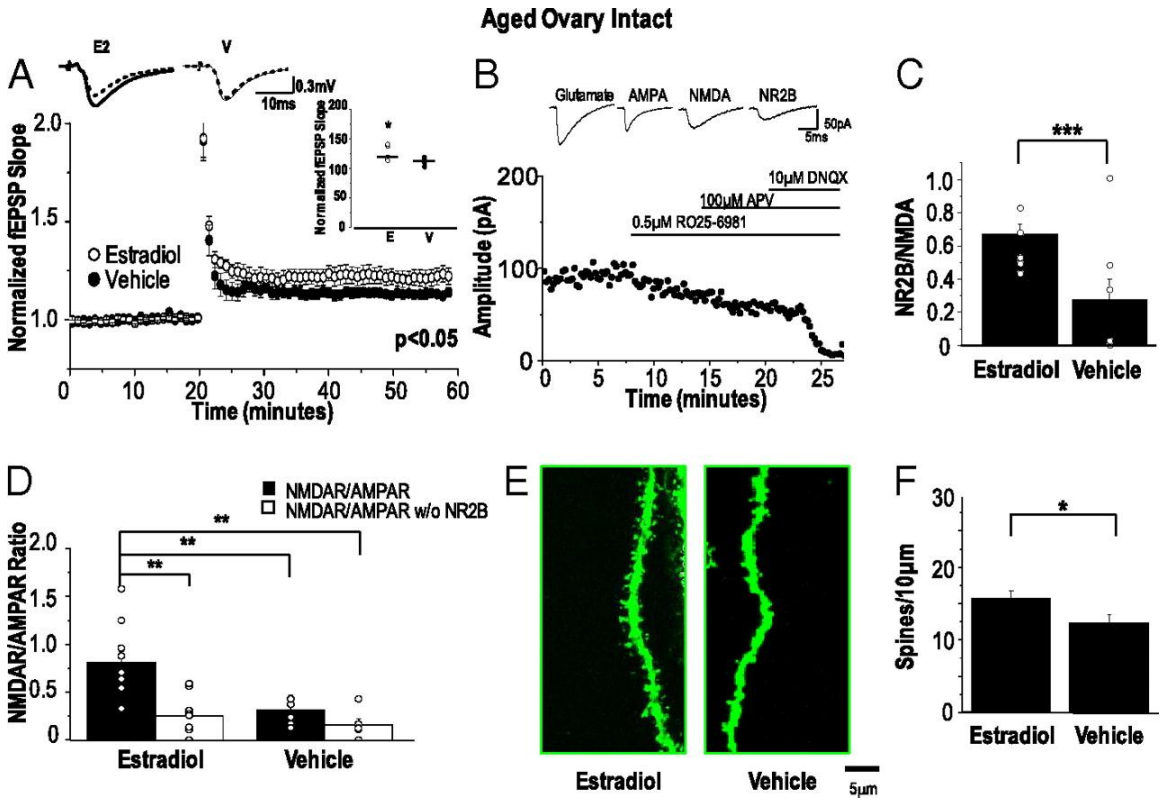


Figure 4: E2 increases the LTP magnitude, NR2B-mediated NMDAR current, the NMDAR/AMPAR ratio, and spine density in 21 month old rats aged with intact ovaries. A) E2 significantly increases the LTP magnitude compared to vehicle treated animals (E24: 121%±4, N=8, V: 113±2, N=8). fEPSP waveforms are averages of 10 events during baseline (dashed line) and 30 minutes post-tetanus (solid line). Inset graph shows the LTP magnitude from individual experiments that were included in group averages B) A representative whole cell recording from an E2 treated animal showing total glutamate, AMPA-only, NMDA-only and NR2B-mediated NMDA currents isolated pharmacologically. C,D) E2 increases the fraction of the NMDA current mediated by NR2B-containing receptors (E2: 0.67+ 0.06 N=10 cells/7 animals, V: 0.28±0.13 N=7 cells/6 animals) and the NMDAR/AMPAR ratio (NMDAR:AMPAR; E24: 0.81 ±0.1

n=10 cells/7 animals, V: 0.31±0.15 n=7 cells/6 animals, NMDAR:AMPAR without NR2B-containing NMDARs; E24: 0.25±0.05 n=10 cells/7 animals, V: 0.16±0.06 n=7 cells/6 animals). E) Representative confocal images of a tertiary CA1 dendrite from an E2 and a vehicle treated animal. F) E2 significantly increases spine density (E2: 15.8±1.1/10µm of dendrite N= 8 animals, V: 12.4±1.3/10µm of dendrite N=6 animals) p*<0.05, p**<0.005, ***p<0.001. Error bars indicate SEM.

DETAILED SUPPLEMENTAL METHODS

Animals

All experimental procedures have been approved by the UAB IACUC in accordance with NIH experimental guidelines.

Surgical Procedures

Four groups of female Sprague-Dawley rats were used (supplemental Fig. 1). Three groups underwent ovariectomy (OVX) at 2 months of age (using 2.5% isoflurane and aseptic conditions) and were housed for 9, 15, or 19 months post-OVX prior to experimentation. The 4th group was aged with ovaries intact to 20 months old prior to undergoing OVX. At the time of experimentation this group was the same chronological age as the 19 month post-OVX group. Prior to OVX, the aged ovary intact animals were cycled for 5 days using saline vaginal lavage and cells were imaged using bright field microscopy at 40X. Stages of the estrous cycle were determined based on cells present or absent within vaginal smears based on criteria from The Laboratory Rat, 2nd edition (supplemental table 1) (57, 58). Whether the cycles were regular or irregular is unknown; collection of vaginal smears was limited to 5 days to prevent potential occlusion of the E2 effects in hippocampus, as enrichment from excessive handling can block the benefits of E2 replacement on cognition (59).

Estradiol Replacement

OVX rats received a subcutaneous injection of either E2 (10μg/250g in cotton seed oil) or oil vehicle alone on 2 consecutive days, and were sacrificed for experiments 24 hours following the final injection, as done previously (23, 26). Although we did not measure plasma E2 levels, this injection protocol, adopted from Woolley and McEwen, 1993,

yields approximate proestrus plasma levels (80-120 pg/ml) (60). Dry uterine weights were obtained to confirm success of the E2 injections (61). Uterine weights in all E2 treated animals were at least double that in vehicle treated OVX rats, $t(72)=5.27$, $p<0.000001$.

Electrophysiology

Rats were transcardially perfused with a high sucrose, low NaCl aCSF (mM): 85 NaCl, 2.5 KCl, 4 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 glucose, 75 sucrose, and 0.5 ascorbate; saturated with 95%O_2:5%CO_2 (pH 7.4) and rapidly decapitated. Coronal dorsal hippocampal slices (400μm) were cut in high sucrose solution and stored in a submersion chamber containing normal aCSF (mM): 119 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 10 glucose, saturated with 95%O_2:5%CO_2, at room temperature. For patch recording, slices were stored for an additional 30 minutes in high sucrose solution with 2μM kynurenic acid before being transferred to normal aCSF. Recordings were performed in a submersion chamber perfused with aCSF (26-28^oC) and saturated with 95%O_2:5%CO_2.

Extracellular LTP Recordings. Schaffer collaterals were stimulated (0.1 Hz, 100μs duration) to elicit 0.6-0.7 mV field excitatory post-synaptic potentials (fEPSPs; ~ 50% of maximum) at CA3-CA1 synapses. Following a 20 minute baseline, LTP was induced using high frequency stimulation (hfs; 100 Hz, 0.5s duration, 4 times, 20s interval, at 1.5x baseline intensity) (23, 26). Experiments were excluded if baseline transmission varied by more than 8%.

Patch-Clamp Recordings. Whole cell recordings were obtained from CA1 pyramidal cells using the blind patch technique (R_{input}: 100-160mΩ; R_{series}: 15-25mΩ)).

Experiments were omitted if R_{input} or R_{series} varied by more than 10%. Electrodes (3-6 MΩ) were filled with (mM): 117 cesium gluconate, 0.6 EGTA, 2.8 NaCl, 5 $MgCl_2$, 2 ATP, 0.3 GTP, 20 HEPES, 5 QX-314, and 0.4% biocytin. $GABA_A$Rs were blocked with picrotoxin (100 μM) and L-type Ca^{2+} channels were blocked with nifedipine (10 μM) to prevent their activation at depolarized potentials. Glutamate currents were evoked using a stimulating intensity that generated a 100-200pA EPSC. Cells were voltage clamped at -20mV to maximize the contribution of NMDARs to the glutamate current. The contribution of NR2B-containing NMDARs to the total NMDAR current and the NMDAR/AMPAR ratio was determined using the selective antagonist RO25-6981 (RO; 0.5μM). Next, the remaining NMDAR current was pharmacologically blocked using D,L APV (100μM) and AMPARs were subsequently blocked with DNQX (10 μM). The contribution of each current was calculated by subtraction as we have done previously (26).

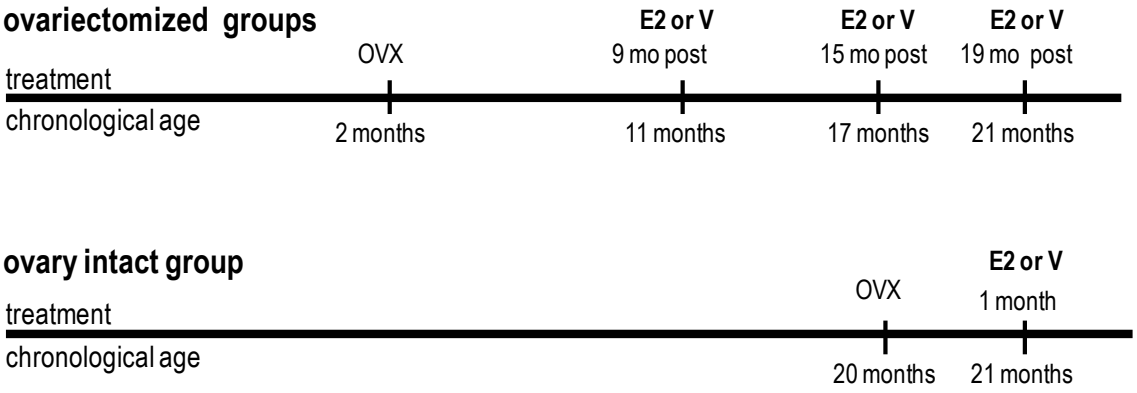
Axoclamp 2A and Axopatch 1D amplifiers (Molecular Devices, Sunnyvale CA) were used to record fEPSP and whole-cell currents, respectively, and data were acquired and analyzed using custom made Labview software (Dr. Richard Mooney, Duke University).

Histology

At the conclusion of each recording, slices were fixed in 4% paraformaldehyde (PFA) in PBS overnight. Incubation with fluorescent streptavidin was used to visualize biocytin filled cells. Confocal imaging and spine counts were performed with the experimenter blind to treatment group and electrophysiological results. Tertiary dendritic segments from the apical arbor in stratum radiatum were imaged (63x at 2.0 digital zoom) with 3-5

segments analyzed per animal (n=at least 4 animals per experimental group). Protrusions were counted as spines only if the length was < 3.0μm from the dendrite (62). Dendritic segments (10 μM) were chosen for analysis if they were completely filled, clearly visible, and continuous with the dendrite. Dendrites were imaged (Leica Application Suite Advanced) by acquiring Z-stacks (1μM thick); spines were counted by scanning through individual focal planes in the Z-stack prior to collapsing all of the images into a maximum 3D projection.

SUPPLEMENTAL FIGURES AND LEGENDS



Supplemental Figure 1. Timeline showing experimental design and animal groups

SUPPLEMENTAL TABLES

Table S1. Criteria used for determining different stages of the estrous cycle based on population of cells present in the vaginal smear

Estrous Cycle Stage	Cells Present
Proestrus	Primarily Nucleated Epitheilial Cells, No Leukocytes
Estrus	Cornified Cells and Kertonized Cells, Few Leukocytes
Metestrus	Mixture Cornified Cells, Leukocytes and Nulceated epithelial cells
Diestrus	Primarily leukocytes

Table S2. Number of animals with vaginal smears that contained cells representing different stages of the estrous cycle during 5 days of sampling

Representative Smears Present Over 5 Days	Number of Animals
Proestrus (one day)	2
Estrus Only	1
Estrus and Diestrus	2
Metestrus and Diestrus	4
Diestrus Only	8

Proestrus animals had a day during the sampling period during which vaginal cytology was consistent with proestrus. Estrus-only animals had 5 d of vaginal cytology consistent with estrus. Estrus and diestrus animals had both days of estrus and days diestrus during the sampling period, and diestrus and metestrus animals similarly had days of both diestrus and metestrus smears. Finally, diestrus-only animals had 5 d of vaginal smears containing cells representative of diestrus.

CHRONIC REPLACEMENT WITH 17β-ESTRADIOL PREVENTS THE LOSS OF SOME E2-ENHANCED HIPPOCAMPAL FUNCTIONS WITH LONG-TERM OVARIECTOMY

by

LINDSEY C. VEDDER, TERUKO M. BREDEMANN, DONGQI XING, ZHENGQIN YANG, SUSANNE OPARIL AND LORI L. MCMAHON

In preparation for submission

Format adapted for dissertation

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ABSTRACT

Cognitive decline associated with ovarian hormone loss in post-menopausal women is a common complaint in the course of normal aging. Some but not all women benefit from estrogen replacement therapy (ERT) and there may exist a defined critical during which time ERT can be initiated post-menopause and remain beneficial to cognition. We have previously shown that treatment of rats with the ovarian estrogen 17□-estradiol (E2) increases long-term potentiation (LTP) at CA3-CA1 synapses only if administered during the first 15 months after ovariectomy. The ability of E2 to increase LTP may directly relate to enhanced learning and memory. In the present study, we found that E2 preserves learning in the novel object recognition (NOR) task only when we have previously shown E2-increased magnitude of LTP. We also replaced 9-10 month post-OVX and 15 month post-OVX rats with E2 in silastic capsules that provided chronic diestrus levels of E2. These animals were all 19 months post-OVX at time of testing with either 8 or 3 months of E2 replacement. We found that LTP was only enhanced in 8 months of E2 replacement and neither group showed an increase in dendritic spine density. These data show that 8 months of E2 replacement is partially protective against the loss of E2-enhanced hippocampal function, while 3 months of

replacement is not at all protective against these losses. However, learning in NOR is preserved with both 8 and 3 months of replacement and supports that other areas of the brain involved in NOR are protected with this shorter 3 month replacement that compensates for the complete loss of E2-enhanced hippocampal function.

INTRODUCTION

Estrogen replacement therapy (ERT) administered post-menopause for the alleviation of hormone-related cognitive decline is effective in some, but not all women (Phillips and Sherwin, 1992; Sherwin, 1997; Shumaker et al., 2003; Shumaker et al., 2004)f. This lack of consistent benefit may be due to a critical period, or window of time, during which ERT may be initiated and still enhance cognition. Rodent studies support this critical window hypothesis, showing that replacement with the ovarian estrogen 17β-estradiol (E2) can enhance hippocampal dependent learning and memory only when given immediately or shortly after ovariectomy (OVX) (Gibbs, 2000; Daniel and Bohacek, 2010).

E2 is a potent modulator of hippocampal function. The magnitude of long-term potentiation (LTP) at CA3-CA1 synapses, a cellular correlate of learning and memory (Malenka and Bear, 2004), is increased both at proestrus, when E2 levels are highest in cycling rats (Warren et al., 1995; Bi et al., 2001) and when OVX rats are treated with E2 to simulate proestrus E2 levels (Smith and McMahon, 2005, 2006). E2-treatment in OVX rats also increases the density of dendritic spines on CA1 pyramidal cells (Gould et al., 1990), which likely causes an increase in the number of active synapses (Woolley et al., 1996). Consistent with this increase in number of synapses, proestrus levels of E2 also increases binding of and current mediated by the glutamategic NMDAR receptor (Woolley et al., 1997; Smith and McMahon, 2006). This increase in NMDAR function is specifically caused by NMDARs containing the NR2B subunit as pharmacologically

blocking this subunit prevents the E2-induced increase in LTP (Smith and McMahon, 2006). Transgenic overexpression of this NR2B subunit increases both LTP and learning (Tang, 1999) and we have shown that the E2-induced increases in LTP and current mediated by NR2B-containing NMDARs occur simultaneously with an increase in hippocampus-dependent learning and memory in young adult OVX rats (Vedder et al, submitted).

Previously, we have reported that the ability of E2 to enhance LTP is dependent upon the length of time of ovarian hormone deprivation, occurring 9, 15 but not 19 months post-OVX (Smith et al., 2010). This loss of E2-induced increases in LTP is not a direct effect of aging as the loss does not occur in rats that are aged with their ovaries intact and then tested at the same chronological age (21 months) as those 19 months post-OVX. E2 also only increases the NMDAR/AMPAR, current mediated by NR2B-containing NMDARs and dendritic spine density when it also increases LTP, further supporting that these mechanisms may underlie the ability of E2 to enhance plasticity. These findings support that a critical window of time exists during which E2 effectively enhances hippocampal function. Here, we further asked if, like in young adult rats (Vedder et al, submitted), the ability of E2 to enhance LTP in aging and through periods of long-term ovarian hormone loss predicts the ability of E2 to facilitate learning. We also determined whether chronic E2 replacement in a subcutaneous silastic capsule inserted between 9-11 months post-OVX or at 15 months post-OVX, time points at which we have previously shown E2-treatment still enhances hippocampal plasticity and morphology, will protect against the loss of E2 enhanced hippocampal function at 19 months post-OVX.

RESULTS

E2 preserves NOR learning 9 and 15, but not after 19 months post-OVX

We have previously shown that the E2-enhanced magnitude of LTP at CA3-CA1 synapses and enhanced NMDAR/AMPAR is lost after 19 months post-OVX (Smith et al., 2010). This loss in enhancement of LTP is not a direct consequence of aging as animals that were aged with their ovaries intact maintained enhanced LTP magnitude with E2 treatment, even though they were the same chronological age as animals 19 months post-OVX. Here we used NOR to determine whether learning with E2 treatment parallels the E2-enhanced LTP and NMDAR/AMPAR during long-term ovarian hormone deprivation and aging. We found that animals experiencing long-term ovariectomy are unable to remember the novel object with V treatment (Figure1A-B, Right; V, 9 months post-OVX, 0.55±0.084, $t(11)=0.68$ $p=0.26$, 15 months post-OVX, 0.53±7.1 $t(11)=0.49$, 19 months post-OVX, 62±0.064, $t(6)=1.97$,$p=0.1$) and that E2-treatment given at 9 months and 15 months-post-OVX preserves NOR learning (Figure 1A-B, Right; E2, 9 months post-OVX, 0.64±0.05, $t(12)=2.56$ $p<0.05$, 15 months post-OVX, 64± 5.33, $t(11)=2.81$,$p<0.005$). At the same duration of hormone loss we previously reported to show a lack of enhanced LTP magnitude with E2 treatment, we show here to no longer preserve NOR learning and memory (Figure 1C, Right; E2, 19 month post-OVX, 0.55±0.06, $t(10)=0.84$, $p=0.42$). No preference of object was seen in any group during the training session when both objects were novel and identical (Figure 1A-C, Left). Also, no differences in duration of time spent exploring objects during the training or testing sessions were observed in long-term ovariectomy animals (Figure 2) and therefore the preservation of learning with E2-treatment was not due to a difference in motivation

to explore or attention to objects for these groups. These results are in agreement with the hypothesis that the E2-enhanced hippocampal physiology contributes to E2's ability to enhance learning and memory.

E2 preserves NOR in animals aged with ovaries intact

We reported previously that the loss of E2-enhanced LTP magnitude is not dependent on chronological age of the animal, but is critically linked to the length of time post-OVX (Smith et al., 2010). Here we further show that E2 is able to preserve learning in animals aged with ovaries intact (Figure 1D; E2, 65.23±0.04%, $t(4)=4.29$, $p<0.01$) that are the same chronological age as those 19 months post-OVX, where E2 preservation of learning is lost. Again, V-treated animals did not learn this task (Figure 1D, Right, V,55.54±0.03%, $t(7)=1.61$, $p=0.08$). No preference of object or side was seen during the training session (Figure 1D, Left, V, 52.90±0.07%, $t(7)=0.43$, $p=0.68$, E2,49.45±0.04%, $t(4)=0.28$, $p=0.79$). Ovary intact animals have a small, yet significant increase in the total amount of time spent investigating objects during the training session (Figure 2D Left, V,15.58±5.03, E2, 21.27±2.61, $t(11)=2.31$ $p<0.05$). This increase in attention during the training session may contribute to the preservation of learning NOR in these animals.

Animal weights and serum E2 levels in chronic replacement studies

As we have previously reported that the length of time of hormone deprivation is critical for E2 to enhance the magnitude of LTP, we next asked if this window of time can be lengthened with chronic E2 replacement. After behavioral analysis, animals 9-11 or 15 months post-OVX were replaced with either 25% E2 diluted in chol or chol alone

(Figure 3A). The replacement of E2 significantly reduced the weight of animals compared to Chol-treated controls in both groups (Figure 3D 7-8 months of replacement; Chol, 801.56±47.33g E2, 642±27.48g, $t(22)=3.14$ $p<0.005$, Figure 3F 3 months of replacement; Chol, 804.10±50.91g E2, 676.14±18.61g, $t(23)=2.58$, $p<0.05$). Serum collection in replaced animals was collected prior to removal surgery to verify the circulating E2 levels supplied by the capsules (Table 1). We found pre-capsule removal serum E2 levels were undetectable in all chol-replaced animals. Animals replaced with Average serum E2 levels in E2-replaced groups was 50.09± 5.70 pg/mL for animals replaced for 7-8 months and 60.93 ± 32.89 pg/mL for animals replaced for 3 months. These averages did not differ significantly (Table 1, $t(10)=0.55$ $p=0.60$). These values may be an over estimate of average E2 serum levels during replacement as some animals included in the data sets had E2 levels below the EIA detectable limit (N=5, 7-8 months of replacement; N=9, 3 months of replacement). Due to this overestimation we considered the average serum E2 level during replacement to be closer to diestrus levels as shown previously with this same E2 replacement protocol (Gibbs, 2000). Blood was also collected through cardiac puncture just prior to brain slice preparation to further measure the E2 levels that were exogenously supplied with our acute two day injection protocol. Estrogen levels were within physiological range for all groups and were unexpectedly significantly higher in Chol treated animals (Table 1, 7-8 months of replacement; Chol, 109.05±17.38, E2, 47.39±9.48, $t(23)=3.40$ $p<0.005$; 3 months of replacement; Chol 80.09 ± 12.43, E2, 41.28±10.57, $t(29)=2.29$, $p<0.05$.)

Replacement Uterine weights

Complete removal of ovaries were verified in each animal after sacrifice for brain slice preparation. Animals used for 9 month post-OVX and 15 month post-OVX NOR experiments are the same animals used in 7-8 and 3 month replacement studies therefore do not have separate uterine weights (See Figure 3A for experimental timeline). In chronic replacement experiments, both Chol and E2 replaced groups were treated with the same two daily injections of E2 (40μg/kg) prior to the final experiment and therefore all groups had uteri consistent with E2 exposure. E2 replaced rats had significantly higher uterine weights than Chol treated rats in both replacement conditions (Figure 3E, 7-8 months of replacement; Chol, 0.31±0.03g E2, 0.43 ± 0.03g, $t(22)=3.12$, $p<0.005$, Figure 3G, 3 months of replacement; Chol, 0.27 ± 0.03g E2, 0.38 ± 0.02g , $t(23)=2.91$, $p<0.01$. These results suggest that 7-8 and 3 months of E2 replacement may also protect the uterine responsiveness to E2.

7-8, but not 3 months of E2 replacement protects the ability of E2 to enhance the magnitude of LTP at 19 months post-OVX.

Capsules were removed and at least four weeks later, E2 was replaced using the same two daily injection of E2 (40μg/Kg) previously shown to enhance magnitude of LTP in young adult animals (Smith and McMahon, 2005, 2006). We find that in animals replaced with E2 for 8 months, E2 treatment is able to enhance LTP magnitude at CA3-CA1 synapses (Figure 4A1-2; E2, 1.30±0.04%, V, 1.14±0.04%, $t(11)=2.60$, $p<0.05$). This increase in LTP is no longer seen in animals only replaced with E2 for 3 months (Figure 4B1-2; E2, 1.9±0.03%, V, 1.7±0.03%, $t(20)=0.32$, $p=0.37$). These results support that there is a mechanism initiated after 15 months post-OVX that leads to the loss of E2-

enhanced hippocampal plasticity that cannot be prevented or reversed with 3 months of E2 replacement.

Neither 7-8 nor 3 months of E2 replacement protects the ability of E2 to enhance the dendritic spine density at 19 months post-OVX.

Previously, we and others have reported an increase in dendritic spine density with E2 treatment in OVX rats (Gould et al., 1990; Woolley et al., 1990; Smith and McMahon, 2005; Smith et al., 2010). This increase, like the increase in LTP, NMDAR/AMPAR and learning is dependent on the duration of ovarian hormone deprivation, occurring at 15, but no longer at 19 months post-OVX (Smith et al., 2010). We sought to determine whether 7-8 or 3 months of chronic E2 replacement could protect against the loss of E2-enhanced dendritic spine density at 19 months post-OVX. We found that E2-replacement is not able to enhance dendritic spine density in either duration of E2 replacement (Figure 5A1-2, 7-8 months of E2 replacement; Chol, 22.31±1.84, E2, 21.06±1.03, $t(12)=0.59$, $p=0.56$, Figure B1-2, 3 months of E2 replacement; Chol, 21.43±1.86, E2, 21.28±1.48, $t(18)=0.06$, $p=0.95$). This surprising finding reveals that not all E2-induced mechanisms of enhanced hippocampal function are maintained with chronic E2 replacement.

7-8 and 3 months of E2 replacement protects against the loss of NOR learning at 19 months post-OVX

The ability of E2 to enhance learning and memory correlates precisely with the ability of E2 to enhance LTP and NMDAR/AMPAR at CA3-CA1 synapses in both

young adult OVX rats (Vedder et al, submitted) and during periods of long-term OVX as shown currently. As we further examine the window of time post-OVX during which E2 is able to enhance hippocampal function, it is critical to determine whether chronic E2 replacement can protect against the loss of E2-preserved learning and memory at 19 months post-OVX. Here, we show that E2 is able to enhance NOR in replaced with either 7-8 or 3 months of E2 (Figure 6A, Right, 7-8 months of E2 replacement; E2, 61.25±5.2%, $t(11)=2.15$, $p<0.05$, Figure 6B, Right, 3 months of E2 replacement; E2, 59.43±4.2%, $t(14)=2.27$, $p<0.05$). Just as seen in earlier NOR experiments, V-treated animals did not significantly learn in either group, (Figure 6A-B, Right, 8 months of E2 replacement; V, 54±7%, $t(6)=0.64$, $p=0.54$, 3 months of E2 replacement; V, 56±8.9%,$t(8)=0.72$, $p=0.25$). No preferences were seen during the training or testing sessions in either group (Figure 6A-B). The amount of time spent exploring during the training and testing sessions did not differ in either group suggesting that the ability of E2 to enhance NOR was not due to an increase in attention or motivation to explore in these animals (Figure 7).

7-8 months, but not 3 months, of E2 replacement enhances the ratio of Erα mRNA levels compared to ERβ levels.

Levels of ERs have been shown to change throughout aging and periods of long-term ovarian hormone deprivation, and it may be the ratio of ERα to ERβ that determines the effectiveness of E2 replacement (For review see(Foster, 2011)). Currently, we report that E2 treatment in rats replaced with 7-8 months of E2 increases, while nonsignificantly, the ratio of ERs to favor Erα (Figure 8D, 8 months of E2 replacement,

E2, 0.54±0.3, t(14)=1.40, p=0.09). This is not seen in animals replaced for only 3 months (Figure 8D, 0.44±0.3, t(3)=1.55, p=0.89) or in Chol replaced rats (Figure 8D, 0.47±0.5, t(8)=0.74, p=0.48) where ERβ mRNA levels remain elevated above Erα. ER mRNA levels from young adult OVX rats show a significant predominance for Erα over ERβFigure 8D, 0.63±0.04, t(5=3.23, p<0.05). No differences were seen between absolute values of ERα (Figure 8A), ERβ (Figure 8B) or the sum of ERα and ERβ (Figure 8C).

DISCUSSION

Since the Women's Health Initiative Memory Study in 2004 reported no benefit of ERT in post-menopausal women (Shumaker et al., 2003; Shumaker et al., 2004), mounting evidence has surfaced supporting the critical period hypothesis(Gibbs, 2000; Lord et al., 2006; Sherwin, 2006; B. B. Sherwin, 2007; Daniel and Bohacek, 2010; Smith et al., 2010). This hypothesis, that there exists a duration of time after ovarian hormone loss when E2 replacement remains beneficial to cognitive function, is now further supported with data reported here in a rodent model of long-term ovarian hormone loss. We find that E2 preservation of NOR learning and memory is dependent on the length of time which lapses after OVX (Figure 1A-C) and perfectly mirrors the ability of E2 treatment to enhance LTP and NMDAR/AMPAR we have shown previously (Smith et al., 2010). This facilitation of learning and memory with E2 thus only occurs if E2 is treated up to 15 months post-OVX and is absent after 19 months post-OVX. As we have previously reported, the mechanisms increasing LTP up to 15 months post-OVX are in parallel with mechanisms utilized in young adult OVX rats (Smith and McMahon, 2005, 2006; Smith et al., 2010). Thus, E2 increases the dendritic spine density, potentially

providing new synapses, and further increases the current mediated by NR2B-containing NMDARs to increase the ratio of NMDAR/AMPARs, which subsequently increases LTP up until 15 months of hormone deprivation. The loss of E2-effectiveness in hippocampus is directly caused by more than 15 but less than 19 months of ovarian hormone loss and is unrelated to chronological age. We now add that E2 is able to preserve NOR learning in animals aged with ovaries intact, data that strongly supports that the E2-induced increase in LTP is directly causing the E2 preservation of learning in our long-term ovariectomy model.

The ability of E2 treatment to prevent the loss of enhanced hippocampal function in animals aged with ovaries intact suggests that the cycling of ovarian hormones through the lifetime of the animal is protective. This protection may require cycling ovarian hormones, or it may only require the replacement of E2 within a critical period of time. Using a chronic low dose replacement of 25% E2 in subcutaneous silastic capsules we found that 7-8 months of E2 replacement initiated between 9-11 months post-OVX does not fully protect against the loss of E2-enhanced hippocampal function observed at 19 months-post OVX. This method of replacement is therefore not as effective as the protection of hippocampal function provided when rats are aged ovary intact (Smith et al., 2010). While LTP is enhanced with E2 treatment in these animals, the density of dendritic spines in CA1 pyramidal cells is not. Adams et al has also presented an absence of E2-enhanced dendritic spine density in animals 24 months old, however these animals did have an increase in the number of NR2B-containing NMDARs located within the synapse (Adams et al., 2004). It is therefore possible that E2 could enhance synaptic function without also enhancing dendritic spine density to increase the magnitude of LTP

at CA3-CA1 synapses. In animals replaced with E2 for three months, when E2 is replaced later (15 months post-OVX), we find that neither hippocampal synaptic plasticity nor morphology is enhanced. It has been suggested that shorter treatments with E2 administered immediately after OVX for a short duration of time may protect against later cognitive deficits related to hormone loss (Rodgers et al., 2010). Whether E2 is able to protect against the loss of hippocampal synaptic plasticity and morphology at 19 months post-OVX with this shorter 3 month replacement initiated sooner post-OVX is yet to be determined.

Even though E2 does not enhance hippocampal plasticity and morphology in animals replaced with E2 for 3 months, we still find that E2 preserves NOR learning with both replacement protocols. While we have shown that the ability of E2 to enhance NOR in dependent on NR2B-containing NMDARs within the hippocampus, we do not know whether the preservation of NOR through chronic E2 replacement also uses this mechanism. It is likely that E2 is protective to other areas of the brain involved in NOR allowing for its preservation. Novel object recognition has been shown to be critically dependent on the perirhinal cortex(Mumby and Pinel, 1994; Bussey et al., 1999; Barker et al., 2007) and entorhinal cortex(Bellgowan et al., 2009) and exploration of E2's effects in these areas of the brain is lacking.

Deciphering which receptor E2 binds to enhance hippocampal function is an extraordinary challenge. In rats, studies support a more critical role for ERα in the ability of E2 to enhance learning and memory (Rodgers et al., 2010), however a role for ERβ cannot be excluded (Phan et al., 2011). ERα expression levels decrease in rats during aging and periods of long-term ovarian hormone deprivation (Adams et al.,

2002; Mehra et al., 2005), and there is a trend for this effect in humans (Tohgi et al., 1995). Studies in mice, however, support that E2 uses ERβ to enhance learning and memory (Liu et al., 2008), however the contribution of receptor subtype may be task specific as agonists for Erα have been shown to enhance novel object recognition and placement tasks and also to enhance dendritic spines (Phan et al., 2011). These contradictions in results may be related to a species-specific difference in the concentration of these receptors and the ratio of Erα to ERβ may be the more crucial factor determining E2s effects on hippocampal function (Foster, 2011). Our findings in the current study support that a switch occurs after 19mPOVX in which ER is dominant. We show that ERα is the dominant receptor in young adult female rats (Figure 8D). However, after 19 months POVX, there is less ERα mRNA than ERβ as shown in cholesterol replaced rats and a reversal in the predominant ER to ERα occurs with 7-8, but not 3 months of E2 replacement. These results coincide with the ability of E2 to enhanced LTP and suggest that the level of ERα in comparison to ERβ is key to the preservation of E2-enhanced hippocampal function during LTO. 8 months of E2 replacement does not fully protect against change in ER dominance, as the ratio of ERα is not increased to that seen in young adult animals. Further investigations into the ER ratios in aged ovary intact animals and animals at various time points post OVX, i.e. 9 and 15 months post-OVX are required to fully understand the role of ERs and their ratios in the regulation of hippocampal function by E2.

Women and rats differ in ovarian senescence in critical ways that may contribute to the ability of E2 to remain effective in a rat model and not in women at late stages in life. Whereas women undergo menopause, after which there is a sustained loss of

ovarian function and thus hormone production (Klinga et al., 1982), rodents undergo a loss of hypothalamic regulation and enter into states of constant estrus or diestrus (Hung et al., 2003) during which there remains ovarian estrogen and progesterone production (Chakraborty and Gore, 2004). Our model of long-term ovariectomy more closely mimics a human menopause model than allowing animals to age intact as it results in loss of ovarian hormones. Chronic E2 replacement only partially protects against losses in hippocampal function after 19 months post-OVX and may need to be initiated closer to OVX to remain beneficial, or may need to be administered in combination with progesterone (P). In our ovary intact model, levels of E2 and P that remain circulating up until experimentation may add protection that does not occur in chronic E2 replacement. This protection seen in ovary intact rats is also not present during normal ovarian senescence in humans, when ovarian hormone production stops post-menopause.

A mechanism which could be responsible for the loss of normal learning reported here and hippocampal function reported previously (Smith et al., 2010) in 19 months post-OVX rats is a loss in cholinergic innervation to hippocampus (Gibbs, 2010). E2 has been shown to enhance activity of choline acetyl transferase (ChAT)(Luine, 1985), ChAT mRNA levels (McMillan et al., 1996) as well as hippocampal ACh release (Marriott and Korol, 2003). Removal of ovarian hormones through OVX also decreases ACh release in hippocampus (Marriott and Korol, 2003). The ability of E2 to enhance learning can be prevented when cholinergic projections to hippocampus are damaged through medial septal lesion (Gibbs, 2002). The amount of degeneration of the cholinergic system, which occurs during OVX, is greater than what occurs during the normal course of aging (Marriott and Korol, 2003; Mitsushima et al., 2009). Therefore, periods of long-term

ovarian hormone loss may cause cholinergic degeneration to a level that E2 is no longer able to overcome, preventing beneficial effects of estrogen. In support of this role of cholinergic cell loss is a hallmark of Alziemer's Disease (AD) and ERT in women has been shown protect against the onset of AD in post-menopausal women (Paganini-Hill and Henderson, 1994, 1996; Henderson, 2009).

This study further defines the critical period during which E2 replacement remains beneficial to hippocampal function and links the ability of E2 to enhance LTP with E2-preserved NOR during periods of long-term ovarian hormone loss. We also find that chronic replacement is only partially protective against the loss of E2-enhanced hippocampal function, enhancing LTP, but not dendritic spine density. This is in contrast to the full protection of hippocampal plasticity and morphology seen in previously in animals aged with ovaries intact (Smith et al., 2010). More physiological replacement protocols may be necessary for the maintenance of E2-enhanced hippocampal plasticity and future experiments are needed to determine whether E2 along with progesterone are able to protect against the loss of E2-enhanced plasticity and morphology after 19 months of OVX.

METHODS

All experiments were performed in accordance with the Institutional Care and Use Committee of the University of Alabama at Birmingham.

Animals

Sprague Dawley rats were purchased from Charles River Laboratories and allowed at least one week to acclimate to the animal facility before surgery. Rats were kept on a 12hr/12hr light/dark cycle and given unlimited access to food and water.

Surgery

All surgeries were performed under isoflurane anesthesia and using aseptic conditions. Animals were subcutaneously injected with 10mg of Carprofen prior to all surgeries for analgesia.

Ovariectomy. Removal of the ovaries was performed bilaterally as previously described (Smith and McMahon, 2005). For long-term ovariectomy and chronic E2 replacement, ovaries were removed when animals were 7 weeks of age. For animals aged with ovaries intact, ovaries were removed 30 days prior to testing when animals were 20 months old. All uteri were removed at the end of experiments for verification of successful removal of ovaries and hormone treatment.

E2 capsule Insertion. E2 was replaced 2-6 weeks after NOR testing at either 9 or 15 months post-OVX. 25% E2 diluted in cholesterol or cholesterol alone was packed into silastic tubing (0.058“ ID, 0.077” OD, Dow Corning Corp) and sealed with type-A medical adhesive (Factor II, Inc). This protocol was previously shown to produce diestrus physiological levels of E2 (Gibbs, 2000). Capsules were rinsed with saline before being placed subcutaneously in the nape of the neck to clean off any residual powder on the outside of the capsules.

Blood collections and serum analysis

Survival blood collection was performed through puncture of the saphenous vein using an 18 gage sterile needle. To avoid over-handling and potential occlusion of E2-enhanced hippocampal function, a separate group of 3 animals were used to assay the levels of E2 supplied through silastic capsules (Figure 4). Animals were weighed at each timepoint of blood collection. Final blood collections were also taken with cardiac puncture prior to acute hippocampal slice preparation. Blood was set at room temperature for at least 20 minutes to coagulate before being spun for 20 minute at 3000 rpms for serum collection. Serum samples were stored at -80°C until assayed for E2 levels. E2 serum levels were assayed by Cayman Chemical using an EIA kit (Cayman Chemical).

Novel object recognition

The day before testing, animals were handled by being held by the experimenter for 1 minute and then placed in the empty NOR box (black plexiglas, 40cm x 40cm x 80 cm, lxwxh) for 10 minutes. On testing day, animals were trained by replacing them in the NOR box with two identical objects (plastic, rubber or glass objects matched in size). Animals were allowed to investigate the objects for 3 minutes and then were placed back in their home cages for two hours before testing. For testing, animals were again placed in the NOR box for three minutes, but one of the training objects (familiar object) was replaced with a new object (novel object). Sessions were video–taped and scored blind to animal treatment and object novelty. Investigative behaviors were defined as the animal's nose coming in direct contact or within 1 cm of the object. Animals were excluded if they 1. Did not spend at least 10s investigating objects during the training

session. 2. Did not investigate both objects during the testing session or 3. Did not spend at least 5 seconds of time investigating during the testing session.

Electrophysiology

Rats were perfused transcardially using ice-cold high sucrose, low Calcium, low NaCl artificial cerebral spinal fluid (aCSF) (mM, 85 NaCl, 2.5 KCl, 4 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 glucose and 75 sucrose), saturated with 95% O_2:5% CO_2 (pH 7.4) for 3 minutes before decapitation and brain removal. As described previously(Smith et al., 2010), acute hippocampal slices were prepared coronally at 400μM using a Leica Vibratome and stored for 1 hour at room temperature submerged in 95% O_2:5% CO_2 saturated normal aCSF (mM, 119 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$, and 10 glucose). All recordings were done fully submerged in 28°C normal ACSF saturated with 95% O_2:5% CO_2. Custom-made Labview software (from Dr. Richard Mooney, Duke University) was used for acquisition and analysis of data.

Field long-term potentiation. Stimulation of the schaffer collateral pathway was done as previously described (Smith and McMahon, 2005; Smith et al., 2010). After 20 minutes of stable baseline transmission, high frequency stimulation (HFS, 100 Hz, 0.5 s duration X 4 times, 20 s intervals, at a current 1.5X that used during baseline) was applied to induce LTP. Baseline transmission did not vary by more than 8%. Recordings were performed using an Axoclamp 2A amplifier (Molecular Devices).

Real-time quantitative RT-PCR analysis

Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA), and treated with DNAase I to remove genomic DNA. The protein- and DNA-free RNA was reverse transcribed to cDNA and analyzed using the SYBR Green RT-PCR kit (Applied Biosystems, Foster City, CA) and specific primers for ERα and ERβ. cDNA was amplified by PCR in the iCycler for 40 cycles and relative RNA levels were calculated using the iCycler software and a standard equation (Applied Biosystems, Foster City, CA). Unknowns were normalized to Ribosome Protein S9 rRNA.

Histology

During whole-cell recordings, cells were filled with biocytin and slices were placed in 4% paraformaldehyde in PBS after recordings and fixed overnight. Slices were washed 3 times for 10 minutes with PBS before a 2 hour incubation with streptavidin conjugated Alexa 488 (10μM in PBS+1% Triton-X). Slices were washed three more times 30 minutes each with PBS and mounted for imaging. Cells were imaged on a Leica Confocal microscope using Leica Application Suite Software. Z-stacks (0.2μM in thickness) were acquired using a 63X objective and 3X digital zoom. Cells were imaged and analyzed with experimenter blind to animal treatment. Dendritic spines were counted on 10μM sections of dendrite by sequentially moving through individual Z-stacks. 5 sections of dendrite were counted per cell with 1-3 cells included per animal.

Statistics

Significant learning and memory in NOR was defined using a one-sample t-test of

percent time investigating the novel object out of total time investigating compared to chance (50%). LTP experiments were normalized to the average value of the 20-minute baseline and the percentage of LTP for each slice was acquired by averaging 40 sweeps at 30 minutes post-HFS. The percentage of LTP was then averaged for each animal and statistical comparisons of LTP were between chol and E2 replaced groups using student t-tests. Comparisons of dendritic spine density were made between chol and E2 replaced animals within separate replacement durations using student t-test. Comparisons between estrogen receptor mRNA was made between treatment groups and replacement durations with cholesterol for both treatment durations averaged together as these did not significantly differ, using one-way ANOVA with Bonferonni post-hoc tests. For all tests a p value of less than 0.05 was considered significant.

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TABLES

Table 1. E2 levels in serum

	Replacement	Pre-Capsule Removal	Final (Acute Injections)
19mPOVX + 8	Chol	Non-detectable	109.05±17.38
	E2	50.09± 5.70	47.39±9.48
19mPOVX + 3	Chol	Non-detectable	80.09 ± 12.43
	E2	60.93 ± 32.89	41.28±10.57

FIGURES AND LEGENDS

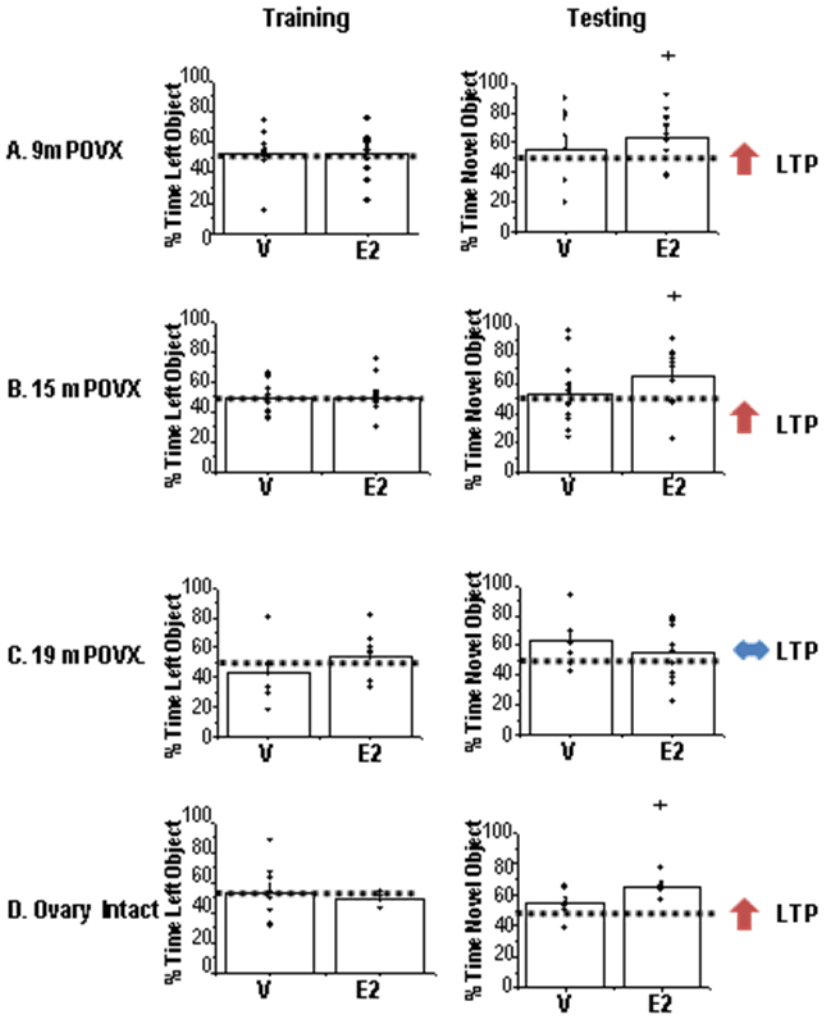


Figure 1. E2-preserved learning and memory is lost after 19mPOVX and this loss is not due to chronological age.

During training session for all groups, the percent of time spent on the left object was not different than chance (50%) supporting no preference of object or side during training session. (A-D, left). The percentage of time spent on the novel object was significantly enhanced from chance with E2 treatment in 9mPOVX (A, right) and 15mPOVX (B, Right) but not 19mPOVX (C, Right) rats. D. The loss of E2-preserved NOR is not due to chronological age as rats aged with ovaries intact, the same age as 19mPOVX animals, remain to exhibit preserved NOR learning and memory with E2-treatment. V-treated rats did not prefer an object during testing for any of the groups (A-D, Right). These results occur in sync with the ability of E2 to enhance LTP reported previously and represented to far right for each group (Smith et al., 2010). + indicates statistically significant NOR learning and memory.

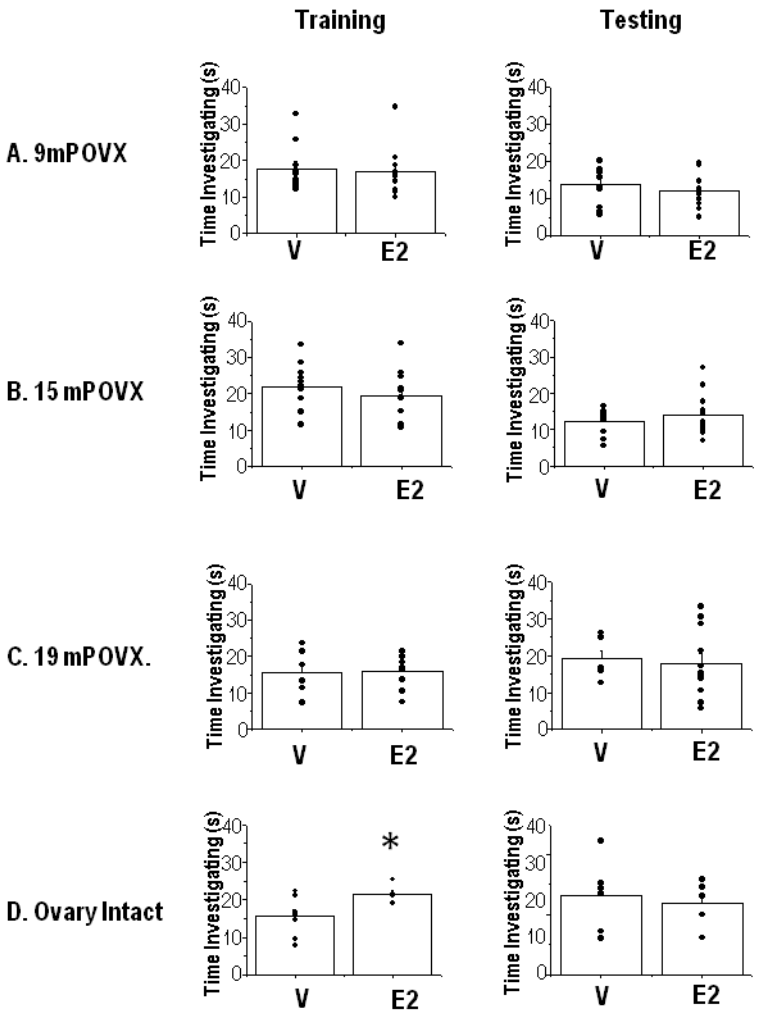


Figure 2. Total time spent investigating during training and testing for NOR in LTO and aging

Left, training and right, testing sessions for 9 months-post-OVX (A.) 15 months post-OVX (B.), 19mPOVX (C.) show total time spent investigating any object do not differ in these group. Animals aged with ovaries intact (D.) have significantly increased durations of time spent investigating objects during the training session.

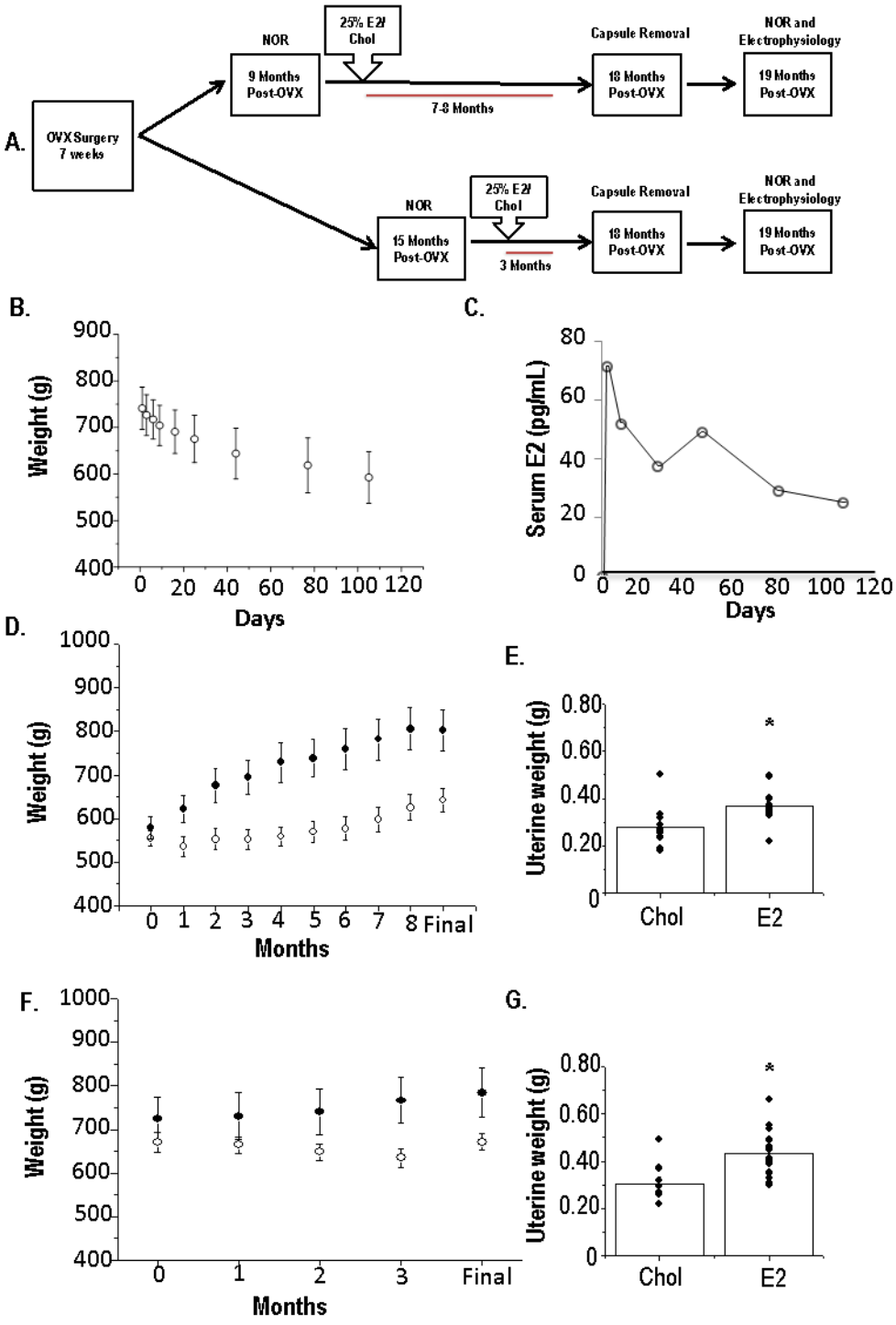
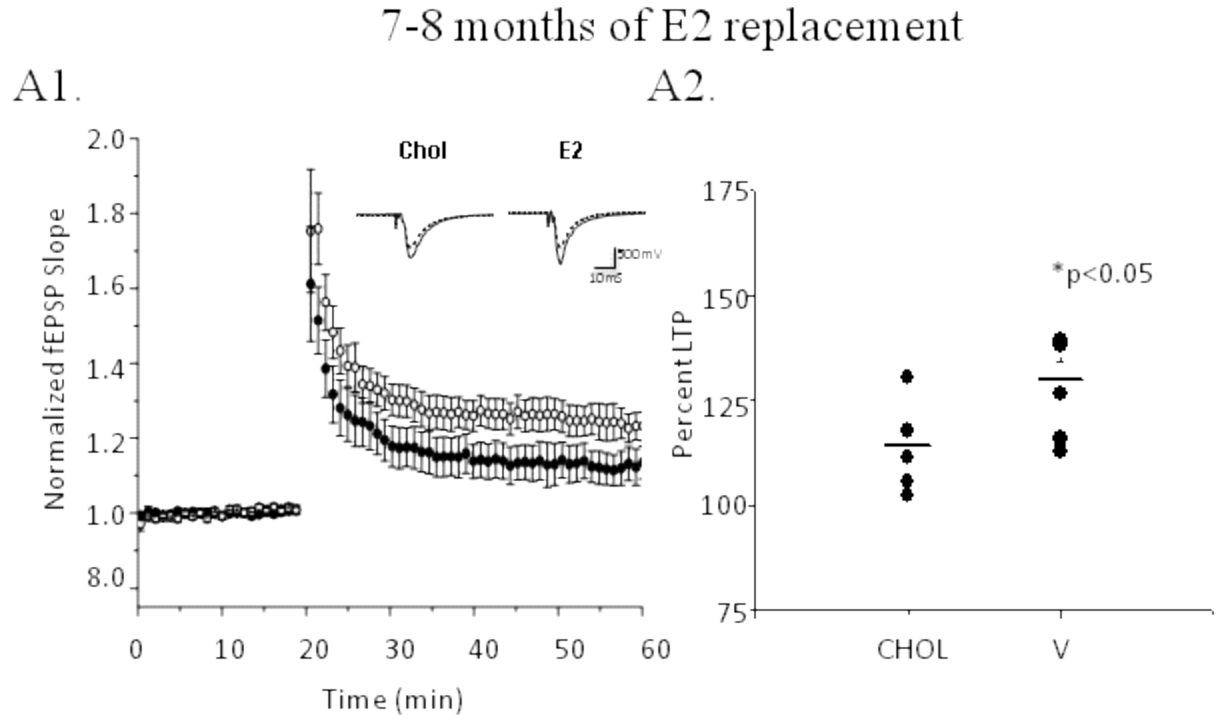

A.
OVX Surgery
7 weeks
NOR
9 Months
Post-OVX
25% E2/
Chol
7-8 Months
Capsule Removal
18 Months
Post-OVX
NOR and
Electrophysiology
19 Months
Post-OVX
NOR
15 Months
Post-OVX
25% E2/
Chol
3 Months
Capsule Removal
18 Months
Post-OVX
NOR and
Electrophysiology
19 Months
Post-OVX
B.
Weight (g)
Days
C.
Serum E2 (pg/mL)
Days
D.
Weight (g)
Months
E.
Uterine weight (g)
Chol
E2
F.
Weight (g)
Months
G.
Uterine weight (g)
Chol
E2

Figure 3. Representative weight loss and E2 levels in serum with E2 replacement through silastic capsules.

A. Timeline for E2 replacement. B. Effect of E2 replacement in silastic capsules on weight over the course of 3 months. C. Time course of E2 serum levels after silastic capsule implant containing 25% E2 in chol. Levels spike within 24 hours and rapidly decrease to level out within 5 days post-insertion, ranging between 25-60 pg/mL over the course of 3 months. D. Body weight for 7-8 months of E2 replacement in 19 month-post-OVX rats (open circles) compared to cholesterol (Closed circles). E. Uterine weights after acute E2 replacement in 7-8 month replaced 19 month post-OVX rats. F. Body weight for 3 months of E2 replacement in 19 month-post-OVX rats (open circles) compared to cholesterol (Closed circles). G. Uterine weights after acute E2 replacement in 7-8 month replaced 19 month post-OVX rats. *$p<0.05$


7-8 months of E2 replacement
A1.
A2.
Chol
E2
500mV
10ms
Normalized fEPSP Slope
Time (min)
Percent LTP
*p<0.05
CHOL
V

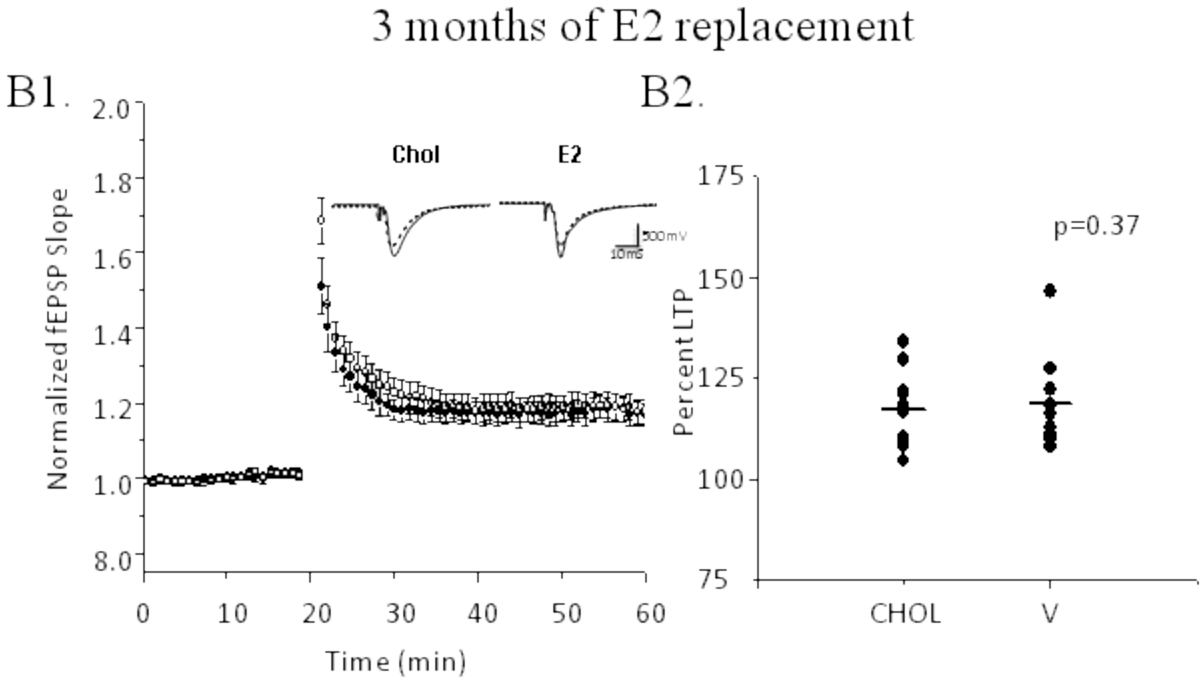

3 months of E2 replacement
B1.
B2.
Chol
E2
500mV
10ms
Normalized fEPSP Slope
Time (min)
Percent LTP
p=0.37
CHOL
V

Figure 4. 7-8 months but not 3 months of E2-replacement protects E2-induced increases in the magnitude of LTP at CA3-CA1 synapses

A1. Animals 19mPOVX with 7-8 months of E2 replacement exhibit enhanced magnitude of LTP with acute E2 injection. Inset shows representative waveforms for Chol and E2 replaced rats. Dotted lines represent baseline, and solid lines represent post-tetanus traces. A2. Average percent LTP with each animal presented as closed circles shows E2 replaced animals are significantly enhanced over Chol replaced animals, $p<0.05$. B1. Animals 19mpOVX with 3 months of E2 replacement do not show enhanced LTP magnitude with acute E2 injection. B2. Average percent LTP with each animal presented as closed circles shows animals with only 3 months of E2 replacement are not significantly enhanced over Chol replaced animals, $p=0.37$.

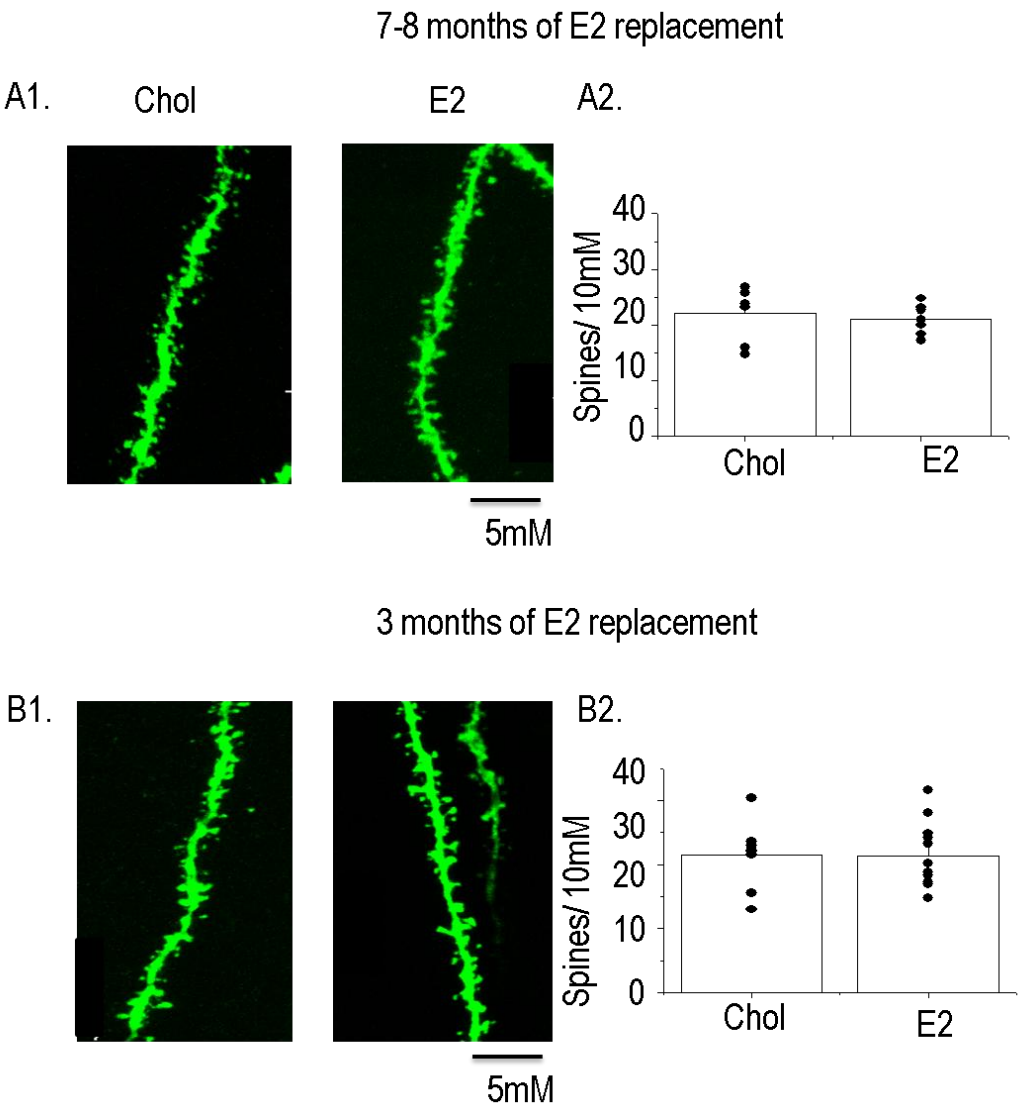

7-8 months of E2 replacement
A1.
Chol
E2
A2.
40
30
20
10
0
Spines/ 10mM
Chol
E2
5mM
3 months of E2 replacement
B1.
B2.
40
30
20
10
0
Spines/ 10mM
Chol
E2
5mM

Figure 5. Chronic E2 replacement does not prevent the loss of E2-enhanced dendritic spine density at 19mPOVX

A1. Representative confocal images of dendritic segments from animals replaced with 8 months of Chol or E2. A2. Average dendritic spine density for animals replaced with 8 months of Chol or E2 show no differenced between replacement conditions in the density of dendritic spines. B1. Representative confocal images of dendritic segments from animals replaced with 3 months of Chol or E2. B2. Average dendritic spine density for animals replaced with 3 months of Chol or E2 show no differenced between replacement conditions in the density of dendritic spines. Enclosed circles represent individual animal averages.

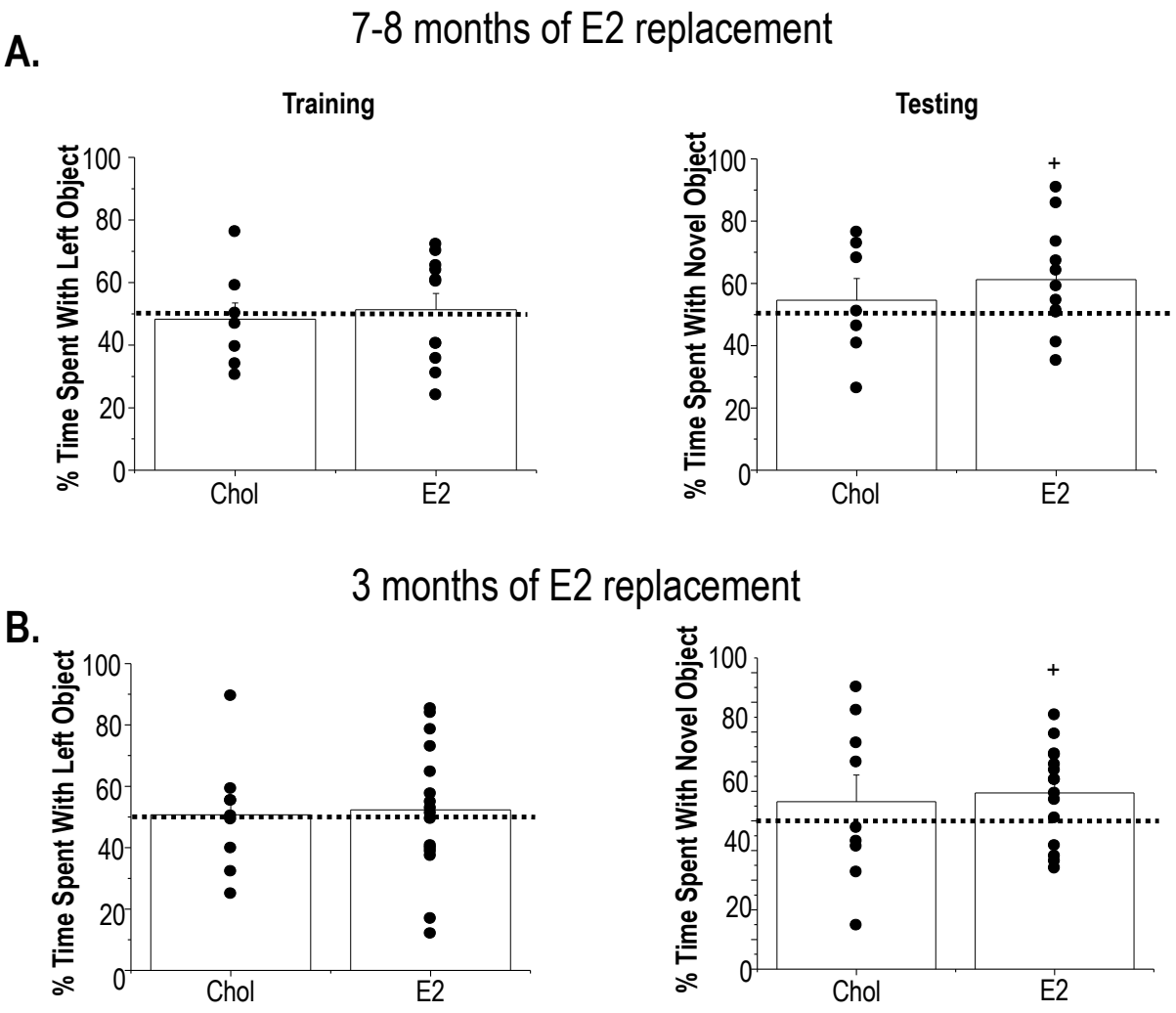


Figure 6. E2-replacement preserves the ability of E2 to induce NOR learning and memory.

During training session for both replacement conditions, the percentage of time spent on the left object was not different than chance (50%) supporting no preference of object or side during training session. (A1-B1). Both 8 (A2) and 3 (B2) months of E2-replacement preserves NOR learning and memory at 19mPOVX. + indicates statistically significant NOR learning and memory.

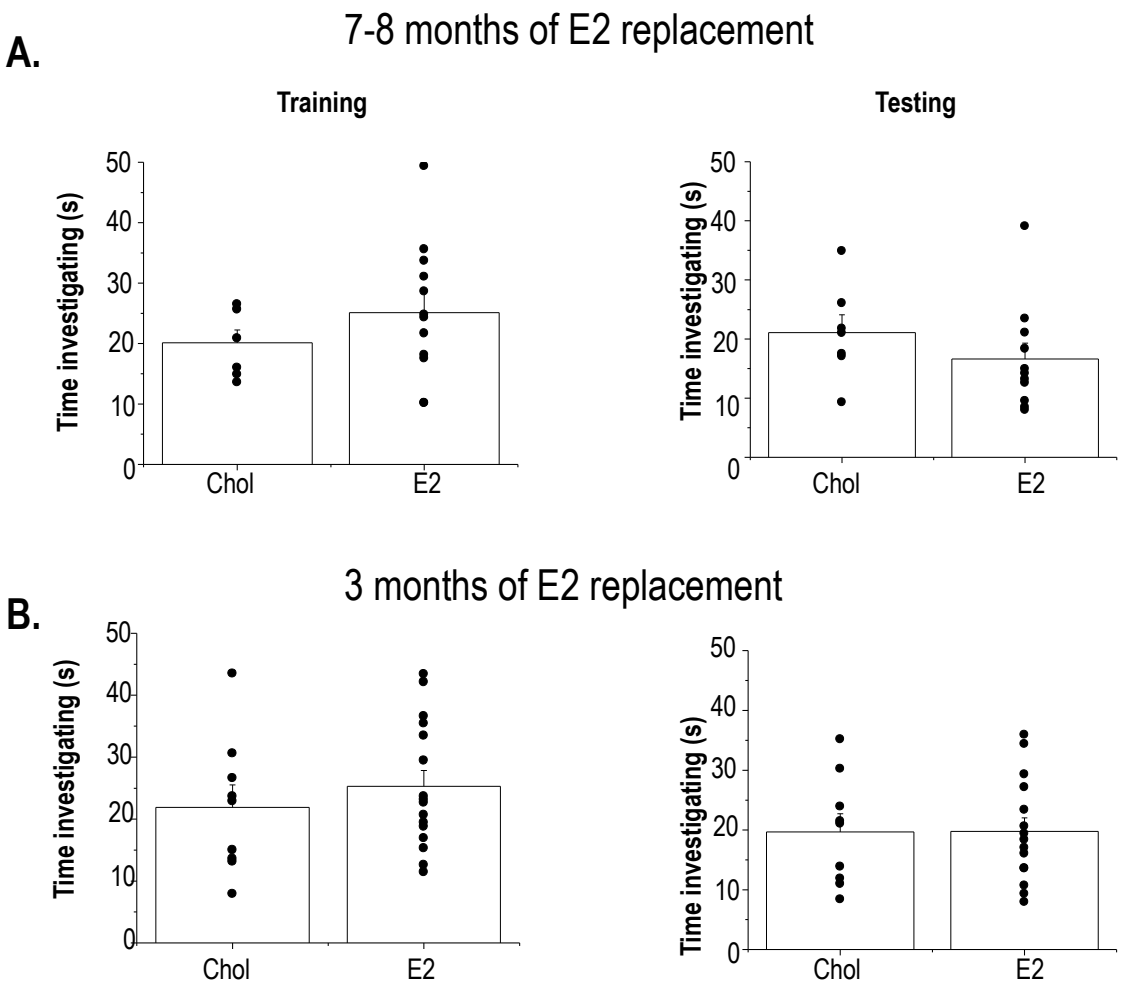


Figure 7. Total time spent investigating during training and testing for NOR in chronic E2-replacement

Left, training and right, testing sessions for 19mPOVX+8 (A.) 19mPOVX+3 (B.) show total time spent investigating objects do not differ in these groups.

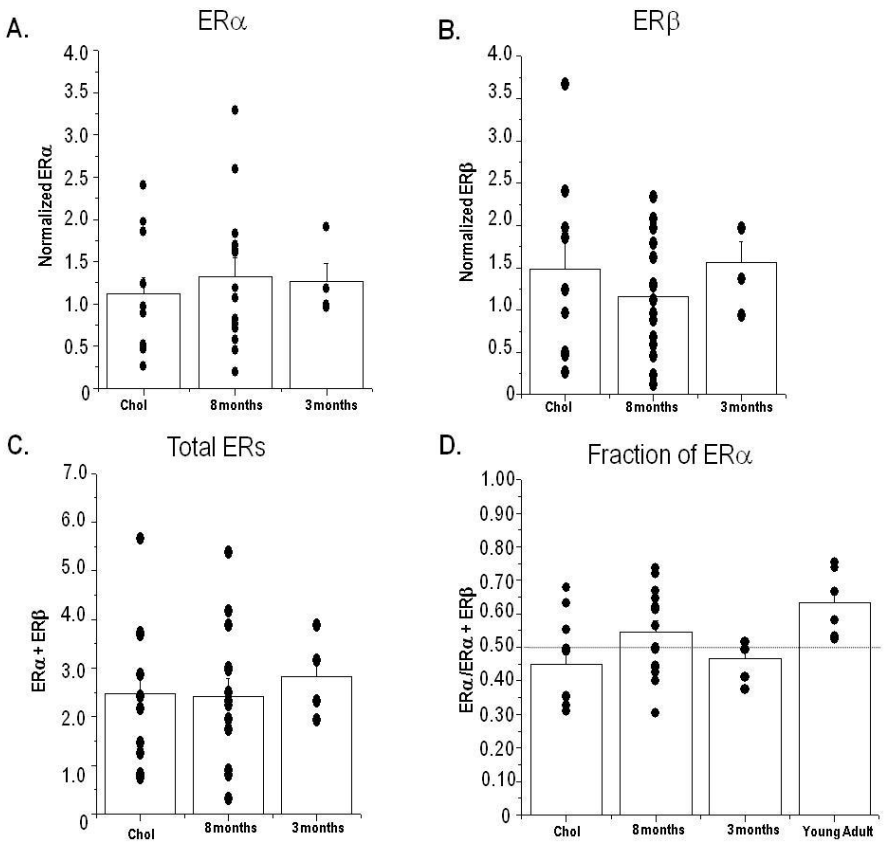


Figure 8. Fraction of ERα compared to ERβ is decreased in aging

Levels of ERα (A), ERβ (B.) and Total ERs (C.) do not differ significantly between chol treated and animals treated with E2 for 8 or 3 months. D. Fraction of Erα/ERβ is significantly decreased in aged compared to young adult OVX animals. Each enclosed circle represents individual animals. *$p<0.05$.

DISCUSSION

Estrogen and hippocampal function in young adult female rats

Previous research in our lab has shown that E2 enhances the magnitude of LTP in a time course that is tightly associated with enhanced dendritic spine density, NMDAR/AMPAR and current mediated by NR2B-containing NMDARs (Smith and McMahon, 2005) and that the E2-induced increase in NR2B-mediated current is causal to the E2-induced increase in LTP magnitude (Smith and McMahon, 2006). Results discussed in this section mechanistically link the E2-induced enhancements in hippocampal LTP and NMDAR/AMPAR with E2-enhanced learning, supporting a causal relationship between E2-enhanced LTP and learning and memory in young adult OVX rats.

E2-enhanced learning and memory occur simultaneously with enhanced LTP

It is currently a technical impossibility to directly measure LTP, dendritic spine density, and current mediated by NR2B-containing NMDA receptors in hippocampus during a learning and memory task in a behaving rodent. However, if these increases in synaptic physiology and morphology in hippocampus result in an increase in learning and memory, they should occur over the same time course. Here I show that significant E2-enhanced learning in NOR only occurs when LTP magnitude and NR2B-mediated NMDAR current was previously reported to be enhanced at 24 (E24), 48 (E48), but not 72 (E72) hours after E2 injection (Vedder et al submitted). E2-enhanced learning and

memory follows the same time course as enhanced LTP magnitude and not enhanced dendritic spine density, which remains elevated at E72 (Smith and McMahon, 2005). Therefore, just as is the case with E2-enhanced LTP magnitude (Smith and McMahon, 2005), an increase in dendritic spine density occurs along with enhanced learning and memory, but it does not precisely predict when learning and memory will be enhanced. These results support that the increase in LTP magnitude and NR2B-mediated NMDAR current are likely candidates for directly causing the increase in learning and memory as both occur simultaneously after treatment with E2.

E2-enhanced learning and memory is mechanistically linked to the E2-enhanced magnitude of LTP

If the E2-enhanced NOR is due to the increase in LTP magnitude, then a common mechanism must induce both learning and LTP. The E2-enhanced LTP is critically dependent on NR2B-containing NMDARs and if the E2-enhanced LTP causes the E2-enhanced learning, then blocking NR2B-containing NMDARs with the highly selective, activity dependent NR2B subunit antagonist Ro25-6981 (RO) (Fischer et al., 1997a) will also block E2-enhanced learning and memory. Systemic administration of 10mg/kg RO has been previously shown to prevent eye-blink conditioning in rats if administered systemically 30 minutes prior to training, showing that RO crosses the blood brain barrier (Valenzuela-Harrington et al., 2007). As the goal of this experiment was to investigate the role of NR2B-containing NMDARs in E2-enhanced NOR learning, to avoid affecting normal NOR learning in V-treated OVX rats, I administered RO systemically using a lower 5mg/kg concentration, 30 minutes prior to training. I found that this RO treatment does not alter NOR learning and memory in V-treated animals, but completely blocks

NOR learning and memory in E2-treated rats (Vedder et al submitted). This complete block of learning and memory in E2-treated animals with systemic administration of RO, along with no effect of RO with V-treatment supports an E2-induced increase in the role for NR2B subunits in NOR learning and memory.

Systemic administration of RO may be acting in multiple areas of the brain to block E2-enhanced learning and memory. To directly determine the role of hippocampal NR2B-containing NMDARs, I next targeted these receptors through infusion of 1 μL RO at 1μM (0.375μg) into CA1 20 minutes prior to NOR training. Intra-CA1 infusion of RO does not alter normal NOR in either V-treated or E2-treated rats, but there is a block of the E2-induced enhancement of learning and memory (Vedder et al submitted). This result directly mirrors previous electrophysiology studies from our lab showing that bath application of RO during LTP experiments only prevents the E2-induced increase in LTP magnitude, but does not block LTP in slices from either V-treated or E2-treated animals. This prevention of the E2-enhanced NOR learning and memory with intra-CA1 infusion of RO also supports results from Frick and colleagues, showing that E2-enhanced NOR is dependent on hippocampal NMDARs using intrahippocampal infusion of the NMDAR antagonist APV to prevent the E2-induced increase in NOR (Michael C. Lewis, 2008). In V-treated rats, using a 10X higher concentration of RO (1μL at 10μM, 3.75μg), NOR is completely blocked. This is the first presentation of data showing a hippocampal dependence of NR2B-containing NMDARs in this task in rats. This higher concentration of RO likely increases the number of NR2B-containing NMDARs that are antagonized, but it is possible that an increase in nonspecific binding of other NR2 subunits could result in the prevention of NOR learning and memory. However, an increase in

nonspecific targets of RO is unlikely due the 5000 times greater specificity of RO for NR2B (IC_{50} of RO is 0.009μM for NR2B versus 52μM for NR2A-containing NMDARs)(Fischer et al., 1997b).

E2-treatment enhances NR2B-containing NMDAR receptor density and phosphorylation

The ability of E2 to enhance current mediated by NR2B-containing NMDARs has been reported using electrophysiological techniques (Smith and McMahon, 2006; Smith et al., 2010; Snyder et al., 2010). Whether this increase in NR2B-mediated current is caused by an increase in the density of NR2B-containing NMDARs in the synapse, an increase in the phosphorylation of NR2B subunits, or both has been difficult to elucidate. Electron microscopy studies show E2-treatment increases the number of NR2B subunits within the synapse in aged animals, but E2 treatment has no effect on the number of these receptors in young adult OVX rats (Adams et al., 2004). These EM studies did not investigate the density of phosphorylated NR2B subunits so the lack of increased NR2B-containing NMDARs in young adult OVX animals does not account for whether or not these receptors may have increased function with E2-administration due to an increase in receptor phosphorylation. Biochemical studies have also attempted to determine the cause for this increase in NR2B function without success. Snyder et al investigated the protein density of NMDAR subunits in E2 or V treated rats within separate sub regions of hippocampus using crude synaptosomal fractions (Snyder et al., 2010). No differences were found between E2 and V treated animals in CA1 of dorsal hippocampus in NR1, NR2A or NR2B subunits. Snyder et al further used immunohistochemistry and analyzed subunit puncta, again finding no differences in NR1, NR2A or NR2B subunits between

E2 and V-treated rats. The lack of confirmation of increases in NMDAR subunit density and phosphorylation in biochemical and anatomical assays can be explained by the lower sensitivity of these assays in comparison to electrophysiological measurement. Assays measuring protein levels contain many potential caveats that could miss this enhancement in NR2B subunit density or phosphorylation. For example, the subcellular fractionation protocol to obtain a crude synaptosomal sample could dilute out small changes in synaptic densities of NMDARs as this fraction is not a pure sample of synaptosomes, as nonsynaptic membranes pollute this fraction (Hallett, 2008). Also, if there were lateral movement of receptors into the synapse from extrasynaptic locations rather than exocytosis of new receptors into the synaptic membrane, the total number of receptors would not change in a crude synaptosomal fraction. Electrophysiological approaches are innately more sensitive as they measure the addition of all functioning receptors in synapses. Using whole-cell electrophysiology, we included orthovanadate within the pipette solution to saturate phosphorylation. If E2 increases NMDAR current though phosphorylation alone, then saturation of phosphorylation in a slices from V-treated rats should mimic the NMDAR current in slices from E2-treated rats. If E2 only increases the density of NMDARs, then saturating phosphorylation will result in a parallel increase in NMDAR current in slices from both E2 and V-treated rats. Our results show that in slices from rats treated with E2, phosphorylation is already saturated, supportive of E2 enhancing phosphorylation of NMDARs (Vedder et al submitted). Also, there is a significant increase in NMDAR current with orthovanadate in slices from V-treated rats, but the increase is not to the level of NMDAR current in slices from E2 treated rats, supporting that the density of NMDARs is higher in E2 treated rats. Together, these

results show that E2 enhances both density of NMDARs and phosphorylation of NMDARs (Vedder et al submitted). It can thus be concluded that E2 enhances both NR2B phosphorylation and density to increase NMDAR activity.

Estrogen and hippocampal function in long-term ovarian hormone loss and aging

The effects of E2 in young adult rats cannot be assumed to persevere throughout aging and periods of long-term ovariectomy. In fact, studies in post-menopausal women and rodents support that changes do occur, either during normal aging, or during long-term ovarian hormone loss, that render E2 ineffective. This section will discuss the mechanisms of E2-effectiveness through periods of long-term ovarian hormone loss and aging in rats and further determine whether chronic replacement with E2 can protect against possible loss of these mechanisms.

Long-term potentiation

To our surprise, E2 is able to increase LTP at CA3-CA1 synapses until 15 months post-OVX, but is finally ineffective at 19 months post-OVX (Smith et al., 2010). Considering that it takes somewhere between 15 and 19 months of ovarian hormone loss for mechanisms involved in E2-enhanced LTP to deteriorate and that these mechanisms are protected when animals are aged with ovaries intact, it is of critical interest whether replacement of E2 is sufficient to protect against this loss of E2-enhanced LTP. We therefore determined whether the loss of E2-enhanced synaptic plasticity after 19 months post-OVX could be prevented through chronic E2 replacement in silastic capsules initiated at time points when E2 is still effective, between 10-11 months post-OVX or at

15 months post-OVX. When E2 is replaced for 7-8 months, and is initiated between 10-11 months post-OVX, the ability of E2 to enhance LTP is preserved, however in animals that are only replaced with E2 for 3 months, initiated at 15 months post-OVX, the E2-enhanced LTP magnitude is absent (Vedder et al in preparation). The effectiveness of 7-8 months of E2 replacement may be due to the duration of E2 replacement, or it may be due to the chronological age of the animals when replacement is commenced. Experiments from the laboratory of Jill Daniel show that rats OVX at 10-11 months of age treated immediately with E2 replacement for 40 days can produce lasting benefits as learning is enhanced up to 7 months later and is as effective as chronic E2-replacement (Rodgers et al., 2010). Therefore, a shorter replacement of E2 may be sufficient to protect against the loss of E2 enhanced LTP if it were administered when animals are younger, or immediately after ovariectomy. Alternatively, E2 may be required for longer durations of time to be protective against loss of LTP irrelevant of the age at which E2 is replaced and needs to be investigated.

Dendritic spines

It has long been a question of interest whether the increase in dendritic spine density with E2 treatment is required for E2 to enhance the LTP magnitude. As an increase in spines suggests an increase in synapses, it is could be postulated that the E2-induced increase in dendritic spines are required for the E2-induced increase in LTP magnitude to occur. Our data from young adult animals, as well as animals up to 15 months post-OVX, show that when E2-induces an increase in LTP magnitude, there is a coexistent increase in the density of dendritic spines (Smith and McMahon, 2005; Smith

et al., 2010), whether this increase in spines contributes to the enhanced magnitude of LTP is not known (Smith and McMahon, 2005). In conditions of long-term Ovariectomy, we find that at the time point when E2-enhanced LTP is lost (19 months post-OVX) there is also a loss of E2-enhanced dendritic spine density, and this loss is prevented when animals are aged with ovaries intact (Smith et al., 2010). This coexistence of enhanced dendritic spine density when LTP is enhanced by E2 diverges in our replacement model, where 7-8 months of E2 replacement enhances LTP, without an increase in dendritic spine density (Vedder et al, in preparation). It may be that a higher concentration of acute E2 treatment could increase dendritic spine density in these rats as higher E2 concentrations are required to enhance dendritic spine density after only 10 weeks of ovarian hormone loss (McLaughlin et al., 2008). Further, the route of administration of E2 may be critical for E2 to enhance dendritic spine density in aging. E2 in silastic capsules can increase dendritic spine density in young adult female rats (Garza-Meilandt et al 2006; McLaughlin et al 2010), but is not effective in aged female rats (Adams et al., 2001). It has been suggested that components of aging, such as decreased cholinergic innervation of hippocampus or decreased expression of ERs may render E2 less effective in aging leading to the need for higher concentrations of E2 (Gibbs 2009, Foster 2011). Consistent with our previous data, spines are not enhanced with 3 months of replacement, when LTP is also not enhanced (Vedder et al, in preparation). That spines are not enhanced while LTP is enhanced in the 19 months post-OVX animals replaced with 7-8 months of E2 provides evidence of a mechanism of E2-enhanced LTP magnitude that does not require a consequent enhancement of dendritic spine density. It is these data that supports a separate mechanism may be utilized to

enhance LTP from that which enhances dendritic spine density in rats. In support of this, Adams et al has shown an increase in synaptic NR2B-containing NMDARs in E2-treated aged female rats without an increase in synapse number compared to V-treated rats (Adams et al., 2001; Adams et al., 2004). In this study, animals were aged ovary intact and treated with silastic capsules containing 10% E2 in cholesterol at 24 months of age. The results of this experiment by Adams et al is in contrast to the increase in dendritic spine density that was observed in the animals aged ovary intact in the current study. An explanation for these differences in results is that our animals aged ovary intact were OVX 2-4 weeks prior to treatment, which has been shown to decrease dendritic spine density. The decreased dendritic spine density was likely reversed by E2 treatment, an effect that would not have been observed if animals were left intact. Therefore, the density of spines may simply not matter and the composition of NMDARs, i.e number of NR2B-containing NMDARS, that make up the synapses may attribute to the ability of E2 to enhance LTP with or without an overall change in dendritic spine density. The shape of a spine has been suggested to predict the synaptic composition of functional AMPARs (Matsuzaki et al., 2001) and NMDARs (Noguchi et al., 2005). Further analysis of the dendritic spine shape with E2 treatment in these studies of E2-replacement may shed light on whether E2 enhances the number of mature or stubby spines versus immature or thin spines, while not changing the overall dendritic spine density, accounting for the enhanced plasticity without an increase in dendritic spine density in rats replaced with 7-8 months of E2.

NMDAR/AMPAR

In young adult OVX rats, there is a dependence on an increase in the ratio of NMDARs to AMPARs for E2-enhanced LTP magnitude to occur (Smith and McMahon, 2005). This mechanism of the E2-induced increase in NMDAR/AMPAR is preserved in rats experiencing long-term ovariectomy up until 15 months post-OVX (Smith et al., 2010). This increase in NMDAR/AMPAR is also specifically due to NR2B-containing NMDARs just like that seen in a young adult OVX rats. At 19 months post-OVX, when E2-enhanced LTP is lost, there is also a loss of the enhanced NMDAR/AMPAR. This enhanced NMDAR/AMPAR is observed when animals are aged with their ovaries intact and therefore the significant increase in NMDAR/AMPAR with E2-treatment parallels E2-enhanced magnitude of LTP perfectly. These findings support that the mechanisms causing enhanced LTP with E2 treatment are preserved and the same in young adult OVX rats until 15months post-OVX. It is yet to be determined if rats 19 months post-OVX with 8 months of E2 replacement also preserves the E2-enhanced NMDAR/AMPAR to increase LTP. As E2 does not increase dendritic spine density under this protocol of E2 replacement, it is possible that a different mechanism of LTP enhancement is engaged, which does not depend upon enhanced NMDAR current. However, more likely E2 may cause an increase in NMDAR current without increasing the number of dendritic spines, such as increasing surface area of the synapse or implementing lateral movement of NMDARs into the synapse from extra synaptic locations.

Learning and Memory

In sync with the E2-induced increases in hippocampal plasticity and morphology, E2 preserves learning and memory in NOR up to 15 months post-OVX and is lost by 19 months post-OVX (Vedder et al, in preparation). This loss is also not a consequence of chronological age as E2-enhanced learning and memory in NOR is preserved in animals aged with ovaries intact. These results are fully expected given that the underlying mechanisms suspected to cause enhanced learning; ie. LTP, NMDAR/AMPAR, NR2B-mediated current, are all enhanced when learning is preserved with E2 treatment. Unexpectedly, E2-induced NOR learning and memory is preserved 19 month post-OVX replaced with either 7-8 or 3 months of E2. Given our previous studies, the ability of E2 to preserve NOR should not be possible in 19 month post-OVX rats with 3 months of replacement animals, when LTP is not enhanced with E2 treatment. This surprising finding could mean that the E2-enhanced LTP at CA3-CA1 synapses is simply not required for enhanced NOR. It is possible that even though E2-preserved NOR coincides with enhanced LTP in all non-replaced rats, that it does not directly cause preservation of learning in NOR and only occurs in parallel with enhanced learning. However, a more likely explanation is that E2 is acting in other brain regions such as perirhinal, entorhinal and prefrontal cortices (Mumby and Pinel, 1994; Bussey et al., 1999; Buffalo et al., 2006) to preserve NOR with only 3 months of E2 replacement. Also, being a task that involves visual and olfactory functions, all brain regions in those pathways are potential targets for E2. Effects of E2 on plasticity and morphology in these other brain regions involved in NOR are of great interest for future experimentations.

Does E2 enhance learning and memory or does it reverse deficits in learning and memory caused by ovarian hormone loss? From these studies, the answer to this question may depend the duration of ovarian hormone loss, as a major difference was observed between NOR learning and memory in young adult OVX rats and aged rats that experience long-term ovariectomy. After just 9 months of E2 deprivation, V-treated rats are no longer able to learn this task. Importantly the exact same protocols were used for young adult OVX rats and aged rats. While the end interpretation is the same, "E2 facilitates learning," in young adult OVX rats, E2 significantly enhances learning compared to V-treated rats that also learn to distinguish the novel from familiar object (Vedder et al submitted). In all long-term ovariectomy groups, as well as animals aged with ovaries intact, V-treated rats do not learn to distinguish the novel object from the familiar object (Vedder et al, in preparation). This distinction, along with the fact that E2 preserves NOR without enhancing CA3-CA1 LTP in 19 month post-OVX rats replaced with E2 for 3 months, further indicate that E2 benefits other brain areas that compensate for the loss of E2-enhanced hippocampal LTP. This absence of NOR learning in long-term ovariectomy may be due to chronological age as V-treated animals aged with their ovaries intact are not protected against this loss of significant learning (Vedder et al in preparation). In order to directly compare the results of rats aged with ovaries intact to our previous studies, rats were OVX 4 weeks prior to experimentation to remove any endogenous ovarian hormones. It may also be that the short 4 week deprivation of ovarian hormones prior to testing in our aged ovary intact animals causes deficits in the NOR task which requires E2-treatment to overcome. Studies of NOR in aging male rats show that learning in this task is preserved up to 24 months of age (Cavoy and Delacour,

1993) supporting that the loss of NOR learning and memory may be a direct consequence of duration of ovarian hormone loss. Future studies investigating the ability of intact female rats to learn NOR are required to fully determine whether the loss of NOR is due to aging processes.

It is critical to determine which brain area E2 effects to facilitate learning and memory. However, the finding of these experiments that E2 can facilitate learning and memory when replaced for a short 3-month period initiated 15 months post-OVX is of clinical significance. This result shows that E2 alone is at least partially protective of cognitive function if initiated within a critical period of time after ovariectomy (15 months) and supports that E2 replacement prevents some mechanisms of long-term ovarian hormone deficits in cognition. Future research should focus on the specific mechanisms degraded by ovarian hormone deprivation with the aim to more precisely target their prevention with replacement therapies.

Estrogen receptors

Expression of ERs have been shown to be species specific and change throughout normal aging, as well as periods of long-term ovarian hormone deprivation. Therefore, the ratio of ERα to ERβ may better predict the ability of E2 to benefit neuronal function (Foster, 2011). In this study, we report a significantly higher level of ERα compared to ERβ in CA1 of young adult OVX rats, that is lost after 19 months of ovarian hormone loss (Vedder et al, in preparation). Interestingly, there is a trend toward an increase in the level of ERα to ERβ when 19 month post-OVX rats are replaced with E2 for 7-8 months, conditions that also enhance the magnitude of LTP. This is not observed when rats are

replaced with E2 for only 3 months. These results are in disagreement with previous reports that ERβ is the predominant ER in CA1 of hippocampus in young adult rats (Mehra et al., 2005). However, the young adult rats in the current experiments were OVX for at least two weeks, which may alter estrogen receptor levels. These results are in agreement with previous works showing a reduction of ERα during aging (Mehra et al., 2005; Thakur and Sharma, 2006).

Potential mechanisms of E2 action in hippocampus

As previously mentioned, E2 enhances current mediated by NR2B-containing NMDARs to increase the magnitude of LTP and these receptors are also required for E2-enhanced learning in young adult rats. The many steps E2 takes to cause these effects are yet to be determined. This section will discuss the various hypotheses that have been proposed to fill in the gaps between increased E2 in hippocampus, either endogenously or exogenously, and increased hippocampal function.

Rapid versus genomic effects of E2

Estrogen could use a traditional ERα or ERβ and initiate gene transcription which is a slow process requiring hours to days, or E2 could engage a membrane bound ER resulting in the activation of signaling cascades to enhance hippocampal LTP, NR2B-mediated current, spines and learning and memory within 10s of minutes. We have previously shown that LTP at CA3-CA1 synapses is not enhanced 24 hours after a single 10μg E2 injection (Smith and McMahon, 2005). This suggests that a sustained increase in E2 for periods longer than 24 hours are required to enhance LTP in our model and

support a genomic mechanism. However, E2 can enhance NMDAR-mediated responses within minutes of bath application of 1nM E2 on acute hippocampal slices, increasing LTP (Foy et al., 1999). Also, direct infusion of E2 into the hippocampus after training in NOR results in an enhancement in the consolidation due to a membrane bound estrogen receptor and subsequent PKA and ERK activation (Fernandez et al., 2008; Michael C. Lewis, 2008). Recent data further supports that the ERα agonist (PPT) can increase dendritic spine density and learning within 40 minutes of a systemic PPT injection in vivo in OVX rats (Phan et al., 2011). It is most likely that the effects we observe here are caused by a combination of rapid and genomic effects of estradiol that use separate mechanisms to enhance hippocampal function. Future studies are required to determine the role of these separate mechanisms in to enhance LTP, NR2B-mediated current, dendritic spine density and learning and memory following an increase in circulating E2.

Local synthesis of E2

It has recently been shown that basal E2 levels within the hippocampus are significantly higher (6X) than those levels reported in serum (Hojo et al., 2004). This increased E2 concentration in hippocampus can be explained by the local synthesis of E2 by hippocampal neurons (Hojo et al., 2004; Kretz et al., 2004). Local E2 synthesis is required for basic cellular mechanisms in hippocampus such as proliferation and apoptosis of dentate gyrus granule cells (Fester et al., 2006). Inhibition of aromatase has been shown to prevent E2-induced enhanced dendritic spine density in hippocampal cultures (Kretz et al., 2004) and LTP in slices from male rats (Grassi et al., 2011). It is important to note that neither of these reports treated animals with E2 *in vivo* and the

effects of E2 on local E2 synthesis were rapid. It has not yet been determined whether local estrogen synthesis is required for long-term effects of E2, such as changes in hippocampal function at E24. Ovarian estrogen levels may regulate the amount of local estrogen synthesis occurring within hippocampus just as it does in the ovary by regulating GnRH (Prange-Kiel et al., 2008). Similarly to ovarian E2 production, intermediate levels of GnRH are most effective at increasing E2 levels in hippocampal cultures (Prange-Kiel et al., 2008). It is not known whether local estrogen synthesis, stimulated by our E2 injection protocol, is required for the E2-induced enhancement in learning and memory observed here, or for our previously reported increase in LTP, dendritic spine density and current through NR2B-containing NMDARs. Answers to these questions require future investigation. As aromatase inhibition may increase the concentration of the substrates in E2 synthesis, particularly testosterone, all effects of aromatase would need to be verified by preventing other steps of E2 synthesis that would not produce an excess of substrates. Knocking down the transporter for cholesterol into the mitochondria, steroidogenic acute regulatory protein (StAR), is an alternative way to prevent local estrogen synthesis that prevents an accumulation of testosterone with aromatase inhibition. Some initial studies show decreased dendritic spine density using StAR knockdown in hippocampal cultures (Rune et al., 2006), but these studies need to be further assayed *in vivo*. It is further important to investigate the how local estrogen synthesis may be effected by long-term ovarian hormone loss and aging.

E2-induced epigenetic modification

Post-translational modifications of histones or DNA allows for alterations in the

expression of genes in direct response to environmental exposures. While not yet shown to increase the length of an adult giraffe's neck, epigenetic modifications can persist through generations in a rather Lamarckian manner (Balter, 2000). These modifications have further been shown to critically affect cognitive mechanisms, including learning and memory (Levenson and Sweatt, 2005; Lubin et al., 2008; Day and Sweatt, 2011). While the underlying DNA sequence remains constant, an individual cell can alter its availability of DNA for transcription through post-translational modifications of histones or even the DNA directly. Recent reports support E2 enhances consolidation of memories using epigenetic mechanisms (Zhao et al., 2010).

Cells tightly regulate the availability of DNA to transcriptional processes post-mitotically. Histones are proteins that package DNA tightly into the nucleus, forming the nucleosome and preventing the availability of DNA to transcription. Acetylation of histone tails can loosen the attraction between DNA and histones, allowing for access of DNA to be transcribed leading to new protein synthesis. Increases in histone acetylation through inhibition of histone deacetylases (HDACs) in hippocampus has been shown to enhance learning and memory (Levenson et al., 2004). This inhibition of HDACs mimics the E2-induced enhancement of NOR in OVX female rats (Zhao et al., 2010). Intracerebroventricular infusion of E2 further increases acetylation of histone 3 (H3) in hippcampus within 30 minutes of infusion (Zhao et al., 2010). Methylation of DNA directly can cause suppression of gene transcription and has also been shown to be required for hippocampal dependent learning and memory (Levenson et al., 2006; Miller and Sweatt, 2007; Miller et al., 2008). The E2-induced increase in acetylation of H3 may be a result of E2-induced suppression in transcription of HDAC2 through increased DNA

methylation as E2 also increases the expression of the DNA methyltransferases 3A (DMNT3A) and 3B (DMNT3B) (Zhao et al., 2010). The role of epigenetic modification in the E2-induced increases in LTP and NR2B-mediated current has not yet been investigated. Considering that DMNT inhibition causes deficits in LTP, and that HDAC inhibition rescues this deficit (Miller et al., 2008), it is likely that E2-induced increases in H3 acetylation and decreased DMNT3A-B expression leads to increases in synaptic plasticity in CA3-CA1 synapses. The time course proposed for E2-induced epigenetic mechanisms is very rapid, within 30 minutes, and is dependent on a membrane bound ER(Fernandez et al., 2008). Whether these mechanism contribute to the sustained increases in synaptic plasticity and learning and memory days after E2 treatment is not known.

E2 modifications in cellular signaling

Multiple signaling molecules have been implicated in E2-induced enhancement of synaptic function. Of key interest to work in this dissertation are molecules modified by E2 that have been shown to be important for both learning and memory and synaptic plasticity.

Extracellular signal-regulator kinase (ERK) is critical for hippocampus dependent learning and memory (Atkins et al., 1998) as well as NMDAR dependent LTP (English and Sweatt, 1996). Inhibition of mitogen-activated protein kinase (MEK), which directly activates ERK, also prevents consolidation of object recognition memory (Kelly et al., 2003). The enhancement of object recognition memory consolidation with E2 treatment is further prevented by MEK inhibition (Fernandez et al., 2008). The role

of ERK in E2-enhanced hippocampal plasticity has not yet been investigated. Also, the role of ERK in E2-enhanced memory consolidation has only implicated the rapid effects of E2, occurring within two hours (Fernandez et al., 2008). Whether ERK activation is further required for the E2-induced enhancements in hippocampal function reported here up to 48 hours after E2 treatment has yet to be investigated.

Alpha calcium/calmodulin-dependent cam kinase II (αCaMKII) is considered a molecular switch; detecting calcium influx into the synapse, likely through NMDARs and is both necessary and sufficient for LTP induction (Silva et al., 1992; Bortolotto and Collingridge, 1998). Further, αCaMKII knock out mice show deficits in both LTP and spatial learning and memory(Silva et al., 1992). E2 has been shown to directly increase the activity of hippocampal αCAMKII (Sawai et al., 2002). As the NR2B subunit has been shown to bind with high affinity to αCAMKII (Strack and Colbran, 1998), the E2-induced increase in the function of NR2B-containing NMDARs may allow for this increase in activation of αCAMKII. Again, studies investigating the role of αCaMKII, like those investigating ERK, are on a rapid time scale within a few hours. Whether E2 in our model increases αCAMKII to increase LTP magnitude and thus learning up to 48 hours after treatment requires future investigation.

Brain derived neurotrophic factor (BDNF) has been shown to elicit many of the same effects on hippocampus as E2, including increasing the magnitude of LTP (Kang and Schuman, 1995; Figurov et al., 1996), NMDAR current (Levine and Kolb, 2000), dendritic spine density (Tyler and Pozzo-Miller, 2001) and learning (Tyler et al., 2002; Pang and Lu, 2004). The BDNF-induced increase in NMDAR current is also due specifically to NR2B-containing NMDARs (Levine and Kolb, 2000). These remarkably

similar effects between E2 and BDNF in CA1 of hippocampus support a direct interaction between E2 and BDNF. In support of this direct interaction, inhibiting TrkB receptors blocks the ability of E2 to increase dendritic spine density (Levine and Kolb, 2000). The BDNF gene contains an ERE (Sohrabji et al., 1995), providing a mechanism for estrogen to directly regulate the expression of BDNF and levels of BDNF in hippocampus fluctuate over the estrus cycle, increased during proestrus (Scharfman et al., 2003). It is yet unclear whether BDNF is involved in the E2-enhanced hippocampal function reported here.

Cholinergic innervation of hippocampus

The hippocampus is innervated by cholinergic projections from medial septum located in the basal forebrain (Mesulam, 1996). These projections are critical for attention as well as learning and memory (Everitt and Robbins, 1997). Significant loss of cholinergic neurons and cholinergic function is a hallmark of Alzheimer's Disease (Davies and Maloney, 1976), and occurs to some degree in normal aging (Morrison and Hof, 1997). This loss of cholinergic function has been correlated with age-related, as well as AD related cognitive decline in humans (Schliebs and Arendt, 2006). Antagonism of M1 muscarinic acetylcholine (ACh) receptors using scopolamine has been shown to induce cognitive deficits in both humans (Schmedtje et al., 1988) and rodents (Stone et al., 1988). In rat studies, MS lesion leads to deficits in learning and memory in a delayed matching to position version of the T-Maze (Johnson et al., 2002; Fitz et al., 2008). In NOR, learning in this task is associated with an enhancement in hippocampal ACh release (Ihalainen et al., 2010) and M1 receptor agonists have further been shown to

enhance NOR learning and memory (Cui et al., 2008).

Supported by the role of cholinergic innervation of hippocampus in learning and memory, cholinergic innervation is also critical for plasticity in hippocampus. In CA1, agonists of post-synaptic muscarinic acetylcholine receptors block the conductance of K+ channels (Cole and Nicoll, 1983; Madison et al., 1987) which causes an increase in NMDAR currents (Harvey et al., 1993; Marino et al., 1998). Consistent with a role for increasing activity of CA1 pyramidal cells, tetanic precondition of the MS decreases the threshold of CA3-CA1 LTP *in vivo* (Ovsepian et al., 2004).

Estrogen and cholinergic innervation to hippocampus have a reciprocal relationship, such that E2 enhances cholinergic function and cholinergic function is required for E2 to enhance hippocampal function. Specifically, E2 has been shown to increase the activity (Luine, 1985), mRNA levels (Gibbs, 1996) and protein levels (Gibbs and Pfaff, 1992; Bohacek et al., 2008) of the enzyme that synthesizes ACh, choline acetyl transferase (ChAT), in hippocampus. OVX decreases high affinity choline uptake in dorsal hippocampus, an effect that is reversed with E2 replacement (Luine, 1985). Estrogen may therefore act indirectly through cholinergic input into hippocampus to enhance potentiation at CA3-CA1 synapses and learning and memory. In support of this role for acetylcholine, E2 enhanced binding of NMDARs is prevented when muscarinic receptors are antagonized with scopolamine (Daniel and Dohanich, 2001). As the E2-induced increase in NMDAR current is caused by NR2B-containing NMDARs, it is likely that acetylcholine is also required for the E2-induced increase in NR2B-containing NMDARs and this has yet to be determined. Also, lesioning the medial septum prevents the increase in dendritic spine density with E2 treatment (Lam and Leranth, 2003) and

prevents the ability of E2 to enhance learning in the delayed matching to position task (Gibbs, 2002; Gibbs, 2007). Intrahippocampal antagonism of M2 muscarinic receptors decreases E2-enhanced working memory, suggesting that M2 receptors are specifically involved in E2-induced enhancement of hippocampal function (Daniel et al., 2005). Studies are yet required to determine a direct linkage between cholinergic innervation into hippocampus and E2-enhanced magnitude of LTP and to further determine whether these effects are causal to the ability of E2 to enhance learning. Future studies are also need to investigate whether decreased cholinergic function is causal to the loss of E2 effectiveness in hippocampal function after periods of long-term ovarian hormone loss.

Future directions

More needs to be known about E2-enhanced learning and memory

It is currently unknown whether this requirement of NR2B-containing NMDARs effects memory acquisition, consolidation or retention in our model of E2-induced increase in NOR. Research by others report that can E2-enhances the acquisition of memory and as our E2 replacement is administered prior to training, it is possible that E2 increases the function of NR2B-containing NMDARs to enhance acquisition in our model as well (Gibbs, 1999, 2000, 2002; Markham et al., 2002; Rhodes and Frye, 2004; Gibbs, 2007; Hammond et al., 2009). Also, as we prevent the E2-induced facilitation of NOR both with systemic and intra-CA1 administration of RO prior to NOR training supports a role for NR2B-containing NMDARs in the acquisition of the NOR task. However, E2 has been shown to enhance consolidation of NOR memory though both systemic and intrahippocampal administration of E2 immediately after training (Frye et

al., 2007; Harburger et al., 2007; Fernandez et al., 2008). As E2 serum levels in our treatment model have been shown to remain elevated above V-treated OVX serum levels for 5 days (Woolley and McEwen, 1993), and our experiments all occur within this time frame, it is not possible to differentiate between these mechanisms of enhanced learning or memory in our current study. Future experiments infusing RO immediately after training would allow for a more definitive distinction between acquisition and consoliation. Another experiment infusing RO 20 minutes prior to NOR testing would further determine whether NR2B-containing NMDARs are required for NOR retrieval.

Types of E2 replacement

Conjugated equine estrogens (Premarin) are commonly prescribed to post-menopausal women. Premarin in particular was used during the Women's Health Initiative Memory Studies (WHIMS), which resulted in negative effects on memory in postmenopausal women (Shumaker et al., 2003; Shumaker et al., 2004). It is conceivable that this cocktail of estrogens from pregnant mare urine, containing primarily estrogen and only 0.5% E2 (Gibbs, 2010), may not be as effective at benefiting learning and memory as pure E2, resulting in the detrimental effects on memory seen in the WHI and WHIMS. Recent evidence suggests that this is not the case as Premarin has been shown to increase neuroprotection in neuronal cultures (Zhao and Brinton, 2006), increase learning in novel object recognition (Prange-Kiel and Rune, 2006) and increase morris water maze retention (Acosta et al., 2009). Whether the mechanism of Premarin's effects on learning and memory is the same as E2's is unknown and Premarin's effects on synaptic plasticity have not yet been characterized.

Method of E2 administration

Much is yet to be understood about how different routes of administration may affect the ability of E2 replacement to enhance hippocampal function. While our acute injection protocol provides a systematic and reproducible effect on hippocampal plasticity, it is not the most clinically relevant method of delivery. Some hormone delivery methods, such as sub-dermal implants and skin patches, are similar to our silastic capsule method of E2 delivery. Our findings that chronic E2 replacement with silastic capsules does not protect against the loss of all E2-induced enhancements of hippocampal function could be due to the constant level of E2 supplied by the capsule. These levels remain elevated in our treatment, while in a cycling rat, these levels would spike and decline with each estrous cycle. The protection of E2-enhanced hippocampal function may thus require the fluctuation of estrogen, as well as progesterone. Previous studies investigating the ability of E2 to enhance learning in the DMTP T-maze in aged OVX rats show a trend toward more effective benefit on learning and memory when E2 or E2 + P are replaced in once weekly injections, versus through diffusion in a subcutaneous silastic capsule (Gibbs, 2000). The constantly elevated E2 levels with subcutaneous capsules may saturate cellular mechanisms that protect against loss of E2 function, and these potential complications would not occur if E2 levels were allowed to cycle. The most obvious method of replacement is to mimic the natural fluctuation of hormones throughout the estrous cycle, as production of ovarian hormones throughout the lifespan in ovary intact rats is fully protective against the loss of E2-enhanced hippocampal function at 19 months post-OVX (Smith et al., 2010). Further investigation

on the effects of various methods of hormone delivery on hippocampal plasticity is required to determine the most effective use of hormone replacement therapy.

It's not all about E2

While our rodent model shows that E2 is sufficient to induce beneficial effects on hippocampal function, progesterone (P) is also removed in our model. More research should be directed toward investigating the interplay between E2 and P effects on hippocampal function, as it would occur *in vivo.*

Studies investigating replacement of P in OVX rats have shown very similar enhancements in hippocampal function to those with E2 replacement. These include enhanced dendritic spine density and learning (Woolley et al, 1993(Harburger et al., 2007; Frye and Walf, 2008). Many women are placed on P only birth control post-partum (Kapp et al., 2010), when ovarian hormone levels have dropped significantly from what was experienced during pregnancy. Post-partum women often experience memory deficits as well as depression (Buckwalter et al., 2001), making studying the direct effects of P alone on hippocampal function of clinical importance. When P is replaced in a more physiological protocol, 4 hours after E2 treatment, P causes a synergistic increase in density of dendritic spines followed by a rapid decrease in density back to that of V-treated animals (Woolley and McEwen, 1993). The ability of E2 to enhance learning and memory may also be reversed by progesterone *in vivo*. This is supported by the negative correlation between progesterone levels and learning in visual recognition tasks in young adult cycling women (Phillips and Sherwin, 1992). It is therefore thought that P returns hippocampal activity to basal levels, a reset button of

sorts, readying the hippocampus for the next ovarian cycle. It is critical to fully understand the role of P as well as E2 in synaptic and cognitive function in order to formulate optimal hormone replacement therapies.

Summary and overall conclusions

From these studies, I have shown a tight linkage between the ability of E2 to enhance hippocampal plasticity and learning and memory in young adult OVX rats. Both E2-enhanced LTP and learning and memory occur over the same time course after E2 treatment and share the similar requirement for hippocampal NR2B-containing NMDARs. In rats aged with ovaries intact, mechanisms of E2-enhanced plasticity and learning and memory are preserved. In support of the critical period hypothesis of hormone replacement therapy, 19 months of long-term ovarian hormone loss results in the absence of E2-induced enhancement of hippocampal function, including enhanced LTP magnitude, NMDAR/AMPAR, current mediated by NR2B-containing NMDARs, dendritic spine density and NOR learning and memory. Replacement of chronic E2 at physiological levels is not sufficient to protect against the loss of all E2-enhanced hippocampal functions, supporting that E2 may use separate mechanisms to enhance synaptic physiology and morphology. Future investigation into the cause for the inferior effectiveness of E2 in rats with chronic E2 replacement compared to rats aged with ovaries intact is critical as it will better inform clinical studies toward effective use of hormone replacement therapy.

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APPENDIX A

IACUC APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: March 2, 2010

TO: McMahon, Lori
MCLM-701 0005
934-3523

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Estrogen and Hippocampal Plasticity
Sponsor: NIH
Animal Project Number: 091208635

On March 2, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Rats	C	203
Rats	B	203

Animal use is scheduled for review one year from December 2009. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 091208635 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
B10 Volker Hall
1670 University Boulevard
205.934.7692
FAX 205.934.1188

Mailing Address:
VH B10
1530 3RD AVE S
BIRMINGHAM AL 35294-0019