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The Role of Brain-synthesized E2 in Hippocampal Learning and Memory Consolidation in Female Mice

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THE ROLE OF BRAIN-SYNTHESIZED E_2 IN HIPPOCAMPAL LEARNING AND MEMORY CONSOLIDATION IN FEMALE MICE

by

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ABSTRACT

THE ROLE OF BRAIN-SYNTHESIZED E_2 IN HIPPOCAMPAL LEARNING AND MEMORY CONSOLIDATION IN FEMALE MICE

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The potent estrogen 17β-Estradiol (E_2) plays a critical role in neuroprotection, serving as an important trophic factor for neurons in the hippocampus, basal forebrain, and prefrontal cortex (Brinton, 2001). In the hippocampus, E_2 promotes neurogenesis (Tanapat et al., 1999, Prange-Kiel et al., 2006), protects against cell death after ischemic injury (Garcia-Segura et al., 2001, Zhao and Brinton, 2007), and helps maintain spine morphology crucial for synaptic connectivity and memory (Woolley et al., 1990; Gould et al., 1990, Woolley and McEwen, 1992, Li et al., 2004). However, the mechanisms through which E_2 promotes synaptic plasticity and enhances memory function are largely unknown. It has been principally assumed that E_2's effects on memory are due to E_2 synthesized from the ovaries, which are the primary endogenous source of E_2 in pre-menopausal females. However, E_2 is also synthesized locally in the adult brain of a variety of species, where it regulates synaptic plasticity and can be synthesized in response to behavioral experiences (Prange-Kiel et al., 2003, Kretz et al., 2004, Remage-Healey et al., 2008, Azcoitia et al., 2011). Although *de novo* E_2 may be a critical regulator of memory, very little is known about the functional role of local E_2 synthesis in brain

regions important for cognition, or the potential mental health implications of a reduction in local E_2 synthesis (e.g., during menopause or in neurodegenerative disease). Therefore, the present study examined the role of locally synthesized estrogens in hippocampal memory consolidation. Three studies were used to determine if 1) hippocampal E_2 synthesis is necessary for the consolidation of hippocampus-dependent object recognition and spatial memories, 2) experience-induced changes occur in hippocampal E_2 levels after behavioral training, and 3) local E_2 synthesis contributes to the memory-enhancing effects of exogenous E_2. To block hippocampal E_2 synthesis, we bilaterally infused into the DH an inhibitor of aromatase, the enzyme that synthesizes E_2 from testosterone. We first found that blocking E_2 synthesis in the DH during consolidation impaired both object recognition and spatial memory consolidation. We next found that local E_2 levels are acutely increased in the DH after object training. This increase in E_2 is blocked by DH infusion of an aromatase inhibitor at a dose that impairs memory consolidation *in vivo*. Finally, aromatase inhibition did not prevent exogenous E_2 from enhancing hippocampal memory, suggesting that hippocampal E_2 synthesis is not necessary for exogenous E_2 to enhance hippocampal memory consolidation. Combined, these data demonstrate for the first time in mammals that hippocampally-synthesized E_2 is necessary for hippocampus-dependent memory consolidation.

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INTRODUCTION

The hippocampus and memory

Perhaps one of the most well known historical examples for the role of temporal lobe structures, such as the hippocampus, in memory comes from observations of memory loss in Henry Molaison, more commonly known as patient H.M. In 1953, H.M. underwent a surgical procedure to reduce the frequency and severity of the debilitating seizures from which he often suffered (Squire, 2009). His neurosurgeon, William Scoville, bilaterally removed a sizable portion of tissue from his medial temporal lobes, including his hippocampus. Although the surgery did reduce his seizure activity, H.M. experienced profound anterograde memory impairment, as well as some retrograde memory loss (Schmolck et al., 2002). Several observations of H.M.'s abilities post-surgery provided critical insight into the existence of different types of memory, which are distributed across several brain regions. For example, despite his severe anterograde memory impairment, his skill retention for certain motor tasks (i.e., mirror-drawing) improved across days, even though he had no memory of previously attempting the task. His intact ability to remember facts and recall remote memories from early in life and childhood, suggested that the medial temporal structures removed were likely critical for consolidation of short-term memories to long-term memory, but were not the final storage site for long-term memories (Squire and Wixted, 2011). Overall, the observations suggested the existence of two larger classes of memory: declarative and non-declarative. Declarative memory includes conscious knowledge of events (episodic memory) and facts (semantic memory). What we now call declarative

memory was largely impaired in H.M., and as such, the involvement of medial temporal structures removed during his surgery has been the focus of most research on declarative memory. However, his non-declarative memory—which includes skill and habit learning, simple conditioning, priming, and perceptual learning—was largely spared. The contrast between H.M.'s loss of certain types of memory, but not other cognitive abilities, formed the basis of our current understanding of memory systems, and supported the notion that different types of memory might be stored in different neural networks. Thus, early observations of patient H.M. created a foundation for future investigations into the structures that subserve different memory systems, and ushered a new era of systems-level experimental research in clinical and preclinical models.

Since the initial insights provided by H.M. a half century ago, a great deal of information has been gleaned from studying the neural circuitry that supports our memory systems, including the prefrontal cortex, amygdala, and hippocampal region. For the purpose of this thesis, the "hippocampal region" is defined to include CA fields, subiculum, and dentate gyrus, and is often extended to include the neighboring parahippocampal region (i.e., entorhinal, perirhinal, and postrhinal cortices) (see Fig. 1; (Squire, 2008, Squire and Wixted, 2011). There are three primary pathways utilized to transfer information into and throughout the hippocampus. Information first enters from the entorhinal cortex and is directed to granule cells in the dentate gyrus via the perforant pathway. Granule cells in the dentate gyrus then relay information through the mossy fiber pathway to the pyramidal cells of CA3. Next, information travels from

the CA3 subfield to the CA1 subfield via the Schaffer collateral pathway (Squire, 2008). Finally, information flows out of the hippocampus through the adjacent subiculum. The hippocampal formation and adjacent parahippocampal regions are largely conserved in terms of anatomical and circuit connectivity across most mammalian species (Manns and Eichenbaum, 2006). Together, these structures collaborate and operate in parallel to support a number of cognitive functions, including the formation and storage of memories. A great deal of research has been dedicated to understanding how external (i.e., behavioral experiences, lesions, pharmacological or genetic manipulations) or internal factors (i.e., changes in growth factors or hormonal milieu) influence the cognitive processes regulated by these structures.

Figure 1

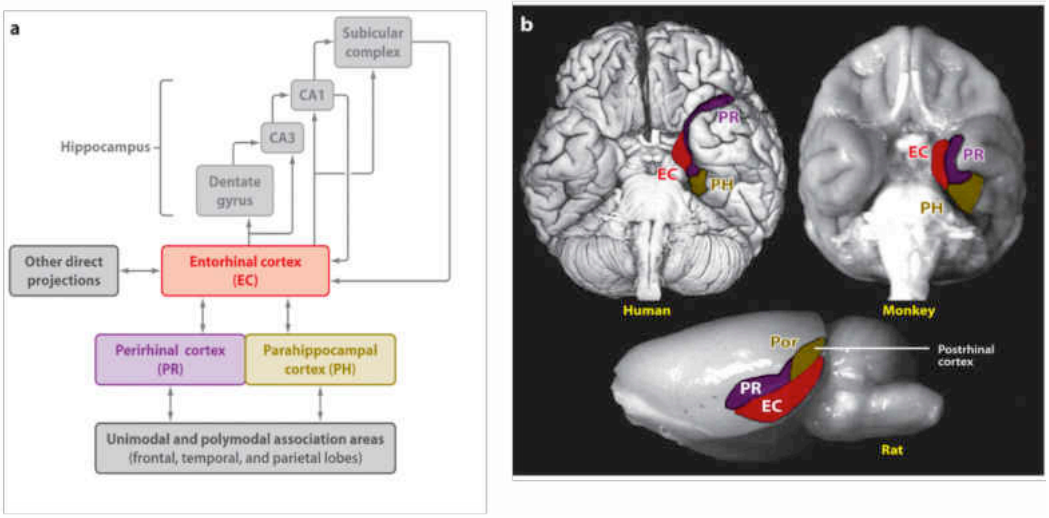

Figure 1. Overview of the medial temporal lobe memory system for declarative memory, which includes the hippocampus and the perirhinal, entorhinal, and parahippocampal cortices. (a) Schematic of the medial temporal lobe memory system. (b) Ventral view of a human brain (upper left), monkey brain (upper right), and a sagittal view of a rat brain (lower center). In humans, the hippocampus lies beneath the cortex of the medial temporal lobe. Its anterior region lies below the posterior entorhinal (shown in red) and perirhinal (shown in purple) cortices, and the main body of the hippocampus lies beneath the parahippocampal cortex. The parahippocampal cortex is also known as the postrhinal cortex in the rat brain. Abbreviations: EC, entorhinal cortex; PH, parahippocampal cortex (shown in brown); Por, postrhinal cortex; PR, perirhinal cortex. Figure from Squire and Wixted, 2011.

In recent years, the sex steroid hormone 17β-estradiol (E_2) has been shown to play an important role in mediating hippocampal function. Although our laboratory and others have discovered much about how E_2 regulates hippocampal memory, many important questions remain. Chief among these is the role of hippocampally-synthesized E_2 in memory formation and storage. The experiments of this thesis were designed to address this issue. To provide context to frame the importance of this question, the following sections will discuss estrogen biosynthesis and evidence supporting the role of E_2 in hippocampal learning and memory.

Biosynthesis of estrogens in the peripheral and central nervous systems

Biosynthesis of estrogen compounds in the peripheral and central nervous systems depend upon the cytochrome P450 protein aromatase, a potent enzyme that catalyzes androgen and cholesterol precursors to estrogens in a process known as aromatization (Azcoitia et al., 2011). The aromatization reaction involves the cleavage of androgen precursors at carbon 19 to produce one of three biologically significant aromatic estrogen compounds: estrone, estriol, and E_2 (Nelson, 2000). Of the three, E_2 is the most abundant and potent circulating estrogen in non-pregnant females prior to reproductive senescence (Gillies and McArthur, 2010). The aromatase enzyme is expressed in a variety of tissues throughout the body, including the gonads, adrenal glands, liver, bone, skin, blood vessels, adipose tissue and the brain (Simpson et al., 2002, Santen et al., 2009, Gillies and McArthur, 2010). For nearly half a century, the majority of neuroendocrinology research has focused on how peripherally-synthesized

estrogens released into circulation travel through the blood to act on the brain as a distant target. However, new research is beginning to expand upon this traditional view to include alternative mechanisms of action, such as local *de novo* E_2 synthesis by neurons and glia within the brain. The present study begins to address some of the important questions for this novel area of research. To provide more context for this study, the sections below will provide additional background about the role of E_2 in regulating hippocampal memory.

E_2 levels across the lifespan

Sex steroid hormones are potent regulators of brain function across the lifespan. They begin exerting their effects on the brain prenatally, where hormone concentrations differ between males and females from about the first trimester of gestation (Nelson, 2000). This *in utero* exposure to estrogens guides the organization of sexually dimorphic brain regions involved in regulating reproductive behavior, gonadotropin secretion, and cognitive function in both males and females (Schwarz and McCarthy, 2008). Neural networks 'organized' during this early developmental window can then be 'activated' later in life by another surge of hormones, for example during puberty or adulthood. In females, natural fluctuations in E_2 levels occur across the month-long menstrual cycle in humans, and the 4-5 day estrous cycle in rodents (Long and Evans, 1922). Performance in mnemonic tasks and changes in neural plasticity have been reported to fluctuate along with these normal changes in endogenous hormone levels (Phillips and Sherwin, 1992, Frick and Berger-Sweeney, 2001, Tuscher et al., 2014),

and are also regulated by exogenous E_2 treatment (Daniel et al., 2006, Galea et al., 2008, Frick, 2009, Gibbs, 2010, Choleris et al., 2012, Frick, 2012, Luine and Frankfurt, 2012, Maki, 2012), the later of which will be discussed in greater detail below. Finally, a precipitous loss of sex steroid hormones occurs in females during middle age, and a considerable amount of research has investigated how this hormone loss may contribute to age-related cognitive decline (Foster et al., 2003, Frick, 2009, Daniel and Bohacek, 2010, Rodgers et al., 2010, Smith et al., 2010) and susceptibility to neurodegenerative diseases, such as Alzheimer's disease (Zandi et al., 2002, Li et al., 2012, Long et al., 2012, Li et al., 2014).

Organizational and activational effects of E_2 on hippocampal memory

Numerous studies have demonstrated that E_2 can enhance learning and memory in tasks that require the hippocampus (Packard and Teather, 1997, Fader et al., 1998, Daniel et al., 1999, Luine et al., 2003, Daniel, 2006, Daniel et al., 2006, Fernandez et al., 2008, Lewis et al., 2008, Walf et al., 2008, Fan et al., 2010, Zhao et al., 2010); see Daniel et al., 2006 and Tuscher et al., 2014 for review). Some of the earliest work to demonstrate that aromatization during perinatal development plays a critical role in organizing the brain into a distinct male or female pattern, came from studies investigating sex differences in spatial working memory (Williams et al., 1990). Williams and colleagues showed that spatial working memory performance in the radial arm maze was critically influenced by exposure to sex steroid hormones during the first 10 days of life. For example, adult male rats castrated before postnatal day 10 exhibited

"feminized" memory performance. That is, castrated males did not experience the typical neonatal surge of E_2 that occurs after aromatization (which leads to masculinization and defeminization of the male brain), and as a result, made more errors than control males (Williams et al., 1990). Conversely, exposing newborn females to estradiol benzoate during this same critical period after birth prevented feminization, which occurs in the absence of E_2 during this period. Instead, females treated with estradiol benzoate exhibited "masculinized" performance in adulthood, such that estrogen-treated females made fewer errors than control females (Williams et al., 1990). In other words, these experiments demonstrated early exposure to sex steroid hormones appear to cause long-lasting organizational effects on spatial reference and working memory in male and female rodents (Williams, 1990). Although it had been widely accepted that sex steroid hormones influence behavior for nearly a century, this study by Williams and colleagues was one of the first to demonstrate a role for the organizational effects of sex steroid hormones in hippocampal learning and memory.

In addition to their organizational effects on the brain, sex steroid hormones also exert activational effects across the adult lifespan. In the two decades since the publication of the aforementioned data by Williams and colleagues, hundreds of studies have examined the activational effects of exogenous E_2 on learning and memory on adults of a variety of species, including songbirds, rodents, non-human primates, and humans (for review see: (Frick, 2009, Bimonte-Nelson et al., 2010, Hammond and Gibbs, 2011, Frick, 2012, Maki, 2012, Schlinger and Remage-Healey, 2012). In general, E_2 treatment has largely been reported to enhance memory in tasks involving the

hippocampus. For example, young female rodents treated with exogenous E_2 exhibit enhanced spatial memory in the object placement, Morris water maze, radial arm maze, and T-maze tasks (Daniel et al., 1997, Fader et al., 1998, Luine et al., 1998, Fader et al., 1999, Bimonte et al., 2002, Bowman et al., 2002, Sandstrom and Williams, 2004). E_2 can also facilitate memory in a number of non-spatial tasks, as demonstrated in the object recognition (Fernandez et al., 2008, Boulware et al., 2013, Fortress et al., 2013), social recognition (Phan et al., 2012), inhibitory avoidance (Singh et al., 1994, Rhodes and Frye, 2004), fear conditioning (Chang et al., 2009, Barha et al., 2010, Milad et al., 2010, Zeidan et al., 2011, Lebron-Milad and Milad, 2012), and trace eyeblink conditioning (Leuner et al., 2004) tasks. Collectively, these studies provide evidence that E_2 treatment can benefit hippocampal memory in a number of behavioral tasks. However, it should also be noted that several other factors impact the effects of E_2 on memory in the aforementioned tasks, including dose, duration of treatment, age, length of ovarian hormone deprivation prior to treatment, type of memory being tested, timing of administration relative to testing, task difficulty, and reproductive history (Daniel, 2006, Acosta et al., 2009, Frick, 2009, Acosta et al., 2010, Luine, 2014). Nonetheless, the balance of studies supports the conclusion that E_2 facilitates hippocampal memory. The molecular mechanisms through which E_2 exerts these beneficial effects are discussed in the following section.

Molecular mechanisms through which E_2 regulates memory

Classical estrogen receptors and genomic mechanism of action

There are two isoforms of the classical intracellular estrogen receptors (ERs), ERα and ERβ, which are found within the cytoplasm and nucleus of the cell. Localization of ERα, which was the first isoform characterized, dates back to the 1960s, when radioactively labeled E_2 was used to detect it's presence within cell nuclei of a variety of rat tissues (Jensen, 1962b). The original mapping of ERα focused on it's distribution in the uterus, mammary glands, pituitary glands, and brain, but later investigations examined expression in specific brain regions (McEwen, 2001), which will be described in greater detail below. It was not until several decades later that ERβ, an ER with a distinct but similar binding affinity to ERα, was discovered in rat uterus (Kuiper et al., 1997). ERα and ERβ have similar ligand-binding domains and affinity for E_2, but regulate different gene targets in a tissue and cell-specific manner (Tee et al., 2004). Outside of the central nervous system, both ERα and ERβ are highly expressed in the ovaries, testes, and uterus, although each receptor has it's own unique pattern of distribution. For example, ERα is expressed at moderate to high levels in the pituitary, kidney, epididymis, and adrenals, whereas ERβ is more highly expressed in prostate, lung, and bladder (McEwen, 2001).

Within the central nervous system, many of the brain regions that support memory formation and storage also express these classical intracellular ERs. Both ERα and ERβ have their own distinct patterns of expression in the cerebral cortex, basal forebrain, amygdala, and hippocampus in a variety of species, including mouse, rat, non-human primates, and humans (Shughrue et al., 1997, Osterlund et al., 2000, Shughrue

and Merchenthaler, 2000, Shughrue et al., 2000, Milner et al., 2001, Milner et al., 2005). In the basal forebrain, which sends cholinergic projections to the hippocampus and neocortex, both ERα and ERβ are expressed, although ERα is more abundant (Shughrue et al., 2000). ERα and ERβ are also both expressed in the dorsal and ventral hippocampus, predominantly in pyramidal neurons of the CA1 and CA3 subfields, although ERβ is more prevalent than ERα in these subfields (Shughrue and Merchenthaler, 2000). Studies examining the ultrastructural localization of ERs within neurons report ERα in the nuclei and cytoplasm of GABAergic interneurons, and in the cytoplasm of pyramidal and granule cells (Milner et al., 2001). Both receptors are found in dendritic spines and axon terminals of pyramidal neurons, however ERβ is more prevalent at these types of extranuclear sites (Milner et al., 2001, Milner et al., 2005). In addition, both ERs are also expressed in extranuclear sites in the rat medial prefrontal cortex (Almey et al., 2014), which receives indirect projections from the dorsal hippocampus (DH) via the ventral hippocampus (Hoover and Vertes, 2007).

Classical intracellular hormone receptors, such as ERα and ERβ, are located within the cytosol and consist of 3 essential domains: the C- terminal (the site for hormone binding), the central domain (which binds DNA), and the N-terminal (which interacts with DNA binding proteins to affect transcription activation) (Nelson, 2000). The classical "genomic" action of E_2 is initiated once the hormone dissociates from a carrier protein at the site of it's target tissue, diffuses through the target cell's outer membrane, and binds ERα or ERβ within the cytoplasm (Nelson, 2000). Once the estrogen-receptor complex is formed, it translocates to the nucleus, where it binds to

estrogen response elements on the DNA. Here, the complex acts as a transcription factor, and can initiate the transcription of E_2-sensitive genes important for maintaining neural circuits that ultimately influence behavior and cognition (Jensen, 1962a, Heldring et al., 2007). Changes in gene expression elicited by such nuclear receptor-hormone interactions, occur slowly (on the scale of tens of minutes to hours), and typically yield long lasting changes.

Membrane ERs and rapid cell-signaling mechanisms

E_2 can also influence cell function in a non-classical manner, by binding to membrane-bound estrogen receptors (mERs; e.g., GPER/GPR30, Gq-mER;(Srivastava and Evans, 2013), or by interacting with neurotransmitter receptors (e.g., mGluRs, NMDARs; (Boulware et al., 2005, Lewis et al., 2008, Boulware et al., 2013), to rapidly activate intracellular signaling pathways on the order of seconds to minutes (Gillies and McArthur, 2010). Although these mechanisms are often referred to as "non-genomic", this designation should be thought of as way to distinguish a separate mode of action of mERs from classical nuclear receptor activation, and should not be taken literally, as activation of mERs can ultimately influence gene transcription. For example, E_2 activation of membrane receptors rapidly initiates cell-signaling cascades like ERK and PI3K (Fernandez et al., 2008, Fan et al., 2010), whose downstream effects result in activation of transcription factors like CREB (Wade and Dorsa, 2003, Boulware et al., 2005). Activation of mERs can also induce post-translational epigenetic modifications such as histone acetylation and DNA methylation (Zhao et al., 2010), and initiate mTOR-

mediated protein synthesis (Fortress et al., 2013). Therefore binding of these non-classical receptors can still ultimately result in modifying the expression of genes important for neural plasticity and cognition.

Studies investigating the cell-signaling mechanisms initiated by E_2 have primarily focused on circulating endogenous or exogenously administered estrogens. Relatively few studies have investigated the effects of brain-synthesized estrogens on neuronal morphology and physiology, and even fewer have examined the functional relevance of local E_2 synthesis at the behavioral level. This relatively novel area of investigation in neuroendocrinology will be discussed in the following section.

E_2 synthesis in the adult brain

The beneficial effects of E_2 on memory and hippocampal function have largely been attributed to gonadally-derived estrogens acting on the brain via a traditional endocrine mechanism. However, accumulating evidence shows that E_2 can also be synthesized locally in the brain from cholesterol or androgen precursors, challenging the long-held dogma that the brain is merely a target for peripheral endocrine glands. Instead, this capacity for local steroidogenesis suggests that neurosteroids like E_2 are poised for acute and precise control of neural circuitry, and may act on a rapid timescale similar to traditional neurotransmitters (Balthazart and Ball, 2006, Saldanha et al., 2011). Despite these intriguing findings, little is currently known about the functional significance of local E_2 synthesis, particularly in brain regions critical for regulating learning and memory, like the hippocampus.

In vitro work conducted over the past few decades has been instrumental in expanding our understanding of neurosteriodogenesis in the adult vertebrate brain. This is in part due to the discovery that aromatase, and all other necessary precursors for E_2 synthesis from the point of cholesterol (Sanghera et al., 1991, Abdelgadir et al., 1994, Wehrenberg et al., 2001), are expressed in several regions of the adult brain in a multitude of species, including songbirds, rodents, non-human primates, and humans (Roselli et al., 1985, Roselli and Resko, 1989, Vockel et al., 1990, Ivanova and Beyer, 2000, Azcoitia et al., 2011). Although originally studied in the context of neurodevelopment and sexual differentiation of brain regions involved in gonadotropin secretion and sexual behavior (Naftolin et al., 1971, Naftolin et al., 1975, Dohler et al., 1984, Baum and Tobet, 1986, MacLusky et al., 1987); for reviews see MacLusky and Naftolin, 1981, (Naftolin et al., 1975, Goy, 1980, Bakker and Baum, 2008), new roles for aromatase and brain-synthesized hormones have recently emerged in the adult brain, including the regulation of neuronal survival, neurogenesis, and the modulation of synaptic function (Azcoitia et al., 2001, Garcia-Segura et al., 2003, Kretz et al., 2004, Fester et al., 2006, Garcia-Segura, 2008, Fester et al., 2012).

De novo hippocampal E_2 synthesis

De novo E_2 synthesis was first demonstrated in adult rodent hippocampal neurons *in vitro* (Prange-Kiel et al., 2003). It was first observed that hippocampal neurons could generate E_2 in steroid-free media from a cholesterol precursor with the aid of StAR (steroidogenic acute regulatory protein), cytochrome P450scc, and 3β-

hydroxysteroid, key enzymes for steroidogenesis that were previously described in hippocampal neurons (Furukawa et al., 1998, Wehrenberg et al., 2001). It was further demonstrated that this *de novo* E_2 synthesis could be markedly reduced in a dose-dependent manner when the aromatase inhibitor letrozole was added to the medium (Prange-Kiel et al., 2003). This *in vitro* phenomenon has since been replicated and extended by several other labs (Hojo et al., 2004, Kretz et al., 2004, Fester et al., 2012). For example, subsequent *in vitro* studies showed that the letrozole-mediated reduction in rat hippocampal E_2 levels was accompanied by decreased spine synapse density and number of presynaptic boutons, suggesting that the loss of local E_2 may lessen synaptic connectivity (Kretz et al., 2004, Zhou et al., 2010, Fester et al., 2012). This notion is supported by other *in vitro* studies demonstrating that aromatase inhibition also decreases the expression of the presynaptic membrane protein synaptophysin and the postsynaptic protein spinophilin, key components of spine formation in rat hippocampal neurons (Kretz et al., 2004, Fester et al., 2012).

In support of these *in vitro* findings, systemic injections of letrozole were also shown to decrease hippocampal synaptic protein levels in both intact and ovariectomized female mice (Zhou et al., 2010), suggesting a critical role of *de novo* E_2 synthesis in hippocampal synaptic function. The physiological significance of compromised synaptic protein levels following letrozole is supported by data showing that systemic letrozole also impairs long-term potentiation (LTP), a putative molecular mechanism underlying memory formation, in gonadally intact male and female mice, as well as ovariectomized female mice (Vierk et al., 2012). Interestingly, aromatase

inhibition has similar effects on hippocampal synapse density and synaptic proteins in male and female rodent cell cultures, whereas systemic treatment produces synapse loss only in females, despite the down-regulation of synaptic proteins in both sexes (Fester et al., 2012). Sex differences are also observed in the extent of LTP impairment; whereas 7 days of systemic letrozole suppresses hippocampal LTP by roughly 20% in males, this same treatment completely abolishes LTP in the female hippocampus (Vierk et al., 2012). These discrepancies suggest the existence of compensatory mechanisms preventing synapse loss in males, although the specific mechanisms through which this may occur remain unknown. Taken together, these studies not only provide evidence that adult male and female rodent hippocampal neurons are capable of *de novo* E_2 synthesis, but further demonstrate that local E_2 is critical for the maintenance of hippocampal synapses and regulation of synaptic proteins. Despite these compelling data from electrophysiological recordings and hippocampal cell cultures, relatively little is known regarding the role of brain-derived E_2 in mediating E_2's effects on memory. This disparity highlights a gap in our current knowledge of the relative contributions of ovarian-generated and brain-generated E_2 to hippocampal function *in vivo*.

Role of brain-synthesized E_2 in hippocampus-dependent behavior

Despite mounting evidence for a role of local E_2 synthesis in *ex vivo* and *in vitro* experiments, little is known about the behavioral relevance of brain-synthesized E_2 *in vivo*. However, some investigators have recently demonstrated that blocking *de novo* E_2 synthesis can impact performance in certain cognitive tasks involving the hippocampus.

In a study published earlier this year, Milad and colleagues showed that systemic treatment with the aromatase inhibitor fadrozole prior to fear extinction training impaired the consolidation of extinction memories in male rats (Graham and Milad, 2014). Interestingly, male rats receiving fadrozole four hours after extinction training had no deficits in fear extinction recall the following test day, suggesting *de novo* synthesis is particularly important for the consolidation period after training. Further, co-administration of exogenous E_2 rescued the observed deficits in extinction recall (Graham and Milad, 2014). Together, these findings suggest acute effects of *de novo* E_2 synthesis may be necessary for the extinction of fear memories. Although this study provides intriguing evidence regarding the role of *de novo* E_2 synthesis in the consolidation of fear memories, blockade of E_2 synthesis was not limited to any particular brain region, nor were E_2 levels measured in any of the brain regions that might contribute to the cognitive processes involved in the extinction of fear memories. Therefore the question of which brain regions require local E_2 synthesis for the consolidation of memories in rodents remains unanswered.

Two recent publications in avian species also support the notion that local *de novo* E_2 synthesis is important for aspects of hippocampal function, such as spatial navigation. One study conducted with male zebra finches demonstrated that systemic administration of fadrozole prior to testing impaired performance in a spatial task that required subjects to remember the location of a food source (Rensel et al., 2013). This finding suggests that local E_2 synthesis may be important for the retrieval of spatial memories. Another zebra finch study utilizing a modified T-maze showed that blocking

E_2 synthesis directly in the hippocampus impaired spatial memory (Bailey et al., 2013). However, the chronic nature of aromatase inhibitor administration (a hippocampally implanted silicone pellet) did not permit a distinction among the acquisition, consolidation, or retrieval phases of spatial memory in this study. Collectively, however, these findings suggest that *de novo* E_2 synthesis is critical for memory formation in hippocampus-dependent tasks in male rodents and zebra finches. However, no study to date has examined the role of brain-synthesized E_2 in female rodents *in vivo*, nor have any studies addressed the role of hippocampally-synthesized E_2 during memory consolidation in rodents.

Experience-induced changes in locally-synthesized E_2

Other studies conducted in songbirds have shown that local E_2 levels in the caudomedial nidopallium (NCM), a brain region critical for song learning and recognition, fluctuate in an experience-dependent fashion (Remage-Healey et al., 2008). Specifically, *in vivo* microdialysis studies have demonstrated that conspecific song playback and social interactions increase levels of *de novo* E_2 in the male zebra finch NCM within 30 minutes (Remage-Healey et al., 2008). These findings are supported by evidence that aromatase is expressed in the soma and synaptic terminals of neurons in the zebra finch brain, which would allow for local and acute control of E_2 synthesis during behavior (Remage-Healey et al., 2009). In fact, studies have shown that aromatase activity is elevated in the synaptic terminals in the forebrain of zebra finches engaged in singing, relative to non-singing males (Remage-Healey et al., 2009). *In vivo*

retrodialysis of fadrozole suppresses local E_2 in the zebra finch forebrain, and subsequently disrupts song preference within 30 min of delivery (Remage-Healey et al., 2008, Remage-Healey et al., 2010). These behavioral changes are accompanied by decreased burst firing and single unit bursts in auditory neurons that typically occur during song presentation (Remage-Healey et al., 2010). Collectively, these findings demonstrate acute experience-dependent changes in E_2 occur in the zebra finch forebrain, and that suppression of this increase disrupts neuronal activity important for song encoding and subsequent processing that influences song preference behavior in zebra finches (Remage-Healey et al., 2008, Remage-Healey et al., 2009, Remage-Healey et al., 2010, Remage-Healey et al., 2012, Remage-Healey and Joshi, 2012). Although *de novo* E_2 synthesis has also been observed in the rodent hippocampus, these data have been collected primarily from acute slices or dispersion cultures *in vitro* (Prange-Kiel et al., 2003, Hojo et al., 2004, Kretz et al., 2004, Fester et al., 2012, Vierk et al., 2012). Nevertheless, the *in vitro* evidence suggests that rodents, much like songbirds, have the ability to produce *de novo* E_2 that may fluctuate in an experience-dependent manner. However, the extent to which a learning event can regulate *de novo* E_2 synthesis *in vivo* remains to be determined in rodents. Determining if local E_2 production is increased in response to a behavioral event will be critical to elucidating the role of local E_2 synthesis in memory consolidation.

Role of local E_2 in the effects of exogenous E_2

In addition to the possibility that local E_2 may increase in an experience-dependent manner to facilitate learning and memory, local E_2 synthesis may also interact with exogenous E_2 to potentiate the beneficial mnemonic effects of each source of estrogen. To date, no studies examining the beneficial effects of exogenous E_2 on memory have considered the potential contributing role that local *de novo* E_2 may play in enhancing memory. One possibility is that locally-synthesized E_2 may prime hippocampal neurons to facilitate the beneficial mnemonic effects of exogenous or ovarian E_2. In support of this notion, *in vitro* data has shown that pre-treatment of hippocampal cells with the aromatase inhibitor anastrozole prevents exogenous E_2 from increasing expression of PSD95 and Arc mRNA and protein (Chamniansawat and Chongthammakun, 2012). Further, exogenous E_2 failed to reverse a letrozole-induced down-regulation of the presynaptic protein synaptophysin in hippocampal slices from male and female rats (Kretz et al., 2004). Together, these studies suggest that local E_2 synthesis may be necessary for exogenous E_2 to regulate mRNA transcription and expression of proteins related to synaptic plasticity. Although these findings suggest genomic regulation, local E_2 may also acutely affect intracellular localization of ERs (i.e., recruitment to the membrane). For example, membrane ERs activated by locally-synthesized E_2 may interact with neurotransmitter receptors (e.g., metabotropic glutamate or NMDA receptors) to rapidly activate cell-signaling cascades like ERK and PI3K (Fernandez et al., 2008, Fan et al., 2010) in order to facilitate the effects of exogenous E_2. Evidence for this possibility comes from studies in songbirds, where an important link between brain-synthesized E_2 and ERK activation has been reported. In

zebra finches, local E_2 levels are increased in the NCM by song playback and social interactions, and these fluctuations trigger a rapid increase in ERK phosphorylation (Remage-Healey et al., 2008, Remage-Healey et al., 2010, Remage-Healey et al., 2011, Pinaud and Tremere, 2012, Tremere et al., 2012). Blocking local E_2 synthesis in the NCM with an aromatase inhibitor reduces local E_2 levels and suppresses ERK phosphorylation within 30 min of infusion (Tremere et al., 2012). This finding supports the notion that local E_2 may facilitate memory consolidation after a learning event by activating the MAPK/ERK pathway. Collectively, these studies not only provide evidence that synaptic proteins are regulated by local E_2, but also, suggest that some effects of exogenous E_2 may require the ability to locally synthesize E_2.

Goals of this thesis

Given the well-established effects of E_2 on the hippocampus, and the critical role of this structure in several types of learning and memory, the goal of this thesis was to determine the contribution of hippocampally-synthesized E_2 to hippocampal memory consolidation. To accomplish this goal, three experiments were designed to determine whether: 1) local E_2 synthesis is necessary for the consolidation of hippocampus-dependent recognition and spatial memories, 2) experience-induced changes in hippocampal E_2 synthesis occur in mice, and 3) local E_2 synthesis is necessary for exogenous E_2 to enhance hippocampal memory consolidation. Together, these experiments take a critical first step toward defining the role of local E_2 synthesis in regulating memory consolidation in female mammals. Further, this work will enable

subsequent studies investigating the molecular mechanisms through which *de novo* E_2 mediates cognition across the lifespan.

METHODS

Subjects. All experiments used young (8-12 week old) female C57BL/6 mice (Taconic, Cambridge City, IN). Mice were housed in groups of up to five until surgery, after which they were singly housed. Mice were maintained on a 12 h light/dark cycle with ad libitum access to food and water. All experimental protocols and procedures were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee and are in accordance with National Institutes of Health guidelines or Guide for the Care and use of Laboratory Animals.

Surgery. All surgeries were conducted at least one week prior to behavioral testing as described previously (Fernandez et al., 2008). Mice were anesthetized with isoflurane (5% for induction, 2% for maintenance) in 100% oxygen and placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). Female mice underwent ovariectomy and cannulae implantation in the same surgical session. Ovaries, oviducts, and tips of the uterine horn were clamped and bilaterally removed via two dorsal incisions in the muscle wall near the tips of the pelvis. After completion of ovariectomy, mice were implanted with stainless steel bilateral guide cannulae (Plastics One, Roanoke, VA) aimed at the DH or at the DH and dorsal third ventricle (intracerebroventricular; ICV). First, an incision in the scalp was made to expose the skull, followed by alignment of

Bregma and Lambda along the horizontal plane. Small perforations were made with a 26 ½ GA needle for placement of DH guide cannulae (C232GC, 22 GA; −1.7 mm posterior to bregma, ±1.5 mm lateral to midline, and −2.3 mm (injection site) ventral to the skull surface) and ICV guide cannula (C232GC, 22 GA; -0.9 mm posterior to bregma ± 0.0 mm, -2.8 mm (injection site) ventral to the skull surface). Cannulae were fixed to the skull with dental cement (Darby Dental Supply, New York, NY) that served to close the wound. Dummy cannulae (C232DC; Plastics One) were used to prevent clogging of the cannula tracts. Mice received 10% ibuprofen in their drinking water 5 days post surgery and were allowed a minimum of one week to recover before behavioral testing.

Drugs and Infusions. The aromatase inhibitor letrozole (Selleck Chemicals, Houston, TX) was dissolved in sterile 0.9% saline and 2% dimethyl sulfoxide (DMSO) to concentrations of 0.01, 0.05, and 0.1 μg/μl and infused bilaterally into the DH immediately after training. Vehicle-infused controls received infusions of sterile 0.9% saline and 2% DMSO at the same rate and total volume. Hippocampal infusions were conducted at a rate of 0.5 μl/min for 1 min per hemisphere as described previously (Fernandez et al., 2008, Zhao et al., 2010, Zhao et al., 2012, Fortress et al., 2013), resulting in letrozole doses of 0.005, 0.025, and 0.05 μg/hemisphere. For experiments also involving ICV infusion of E_2, cyclodextrin-encapsulated E_2 (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile 0.9% saline to a concentration of 10 μg/μl. Vehicle ICV infusions consisted of 2-hydroxypropyl-β-cyclodextrin (HBC; Sigma-Aldrich, St. Louis, MO) dissolved in saline to the same concentration of cyclodextrin present in the cyclodextrin-encapsulated E_2

solution. ICV infusions were conducted at the same rate as DH infusions (0.5 µl/min) for 2 min total, to allow for infusion of the same total volume at the same rate as DH infusions.

Behavioral Testing. Object recognition (OR) and object placement (OP) were used to measure object recognition and spatial memory as we have previously described. Previous work from our laboratory (Gresack and Frick, 2006, Fernandez et al., 2008) and others (Luine et al., 2003, Li et al., 2004, Walf et al., 2008) has established that each of these tasks is sensitive to E_2 and involves the DH (see also Tuscher et al., 2014 for review). Mice were first handled for one min/day for three days prior to habituation. After the first day of handling, a Lego was placed in each home cage to habituate the mice to objects during the remaining handling days and habituation period. After three days of handling, mice were habituated to the apparatus for two consecutive days by allowing them to explore the empty white arena (60 cm x 60 cm x 47 cm) for 5 min/day. For the OR task, mice first accumulated 30 seconds exploring two identical objects placed 5 cm from the upper left and right corners of the arena during the training phase (Fig. 2A). Immediately after training, mice were infused into the DH or into the DH and dorsal third ventricle with their respective drug treatments. These post-training infusions allow us to pinpoint effects of aromatase inhibition specifically to the memory consolidation period, while also minimizing the confounding effects of hormones on performance factors (e.g., motivation, anxiety) during training or retention testing (McGaugh, 1989, Frick and Gresack, 2003). Further, we have previously shown

that E_2's ability to facilitate memory consolidation is restricted to within a three hour window after training, as mice infused three hours post-training do not remember the familiar object during OR testing (Fernandez et al., 2008). Therefore, the inability to recall the familiar object following letrozole treatment should be due to inhibition of *de novo* E_2 synthesis during only the consolidation phase of memory formation. Object recognition memory was then tested 24 hours later by measuring the amount of time spent with the novel and familiar object. OR memory consolidation was demonstrated if the mice spent more time than chance (15 seconds) with the novel object during testing. At this time point, vehicle-infused ovariectomized females show intact object recognition (Fortress et al., 2013, Boulware et al., 2013), thereby allowing us to observe potential memory-impairing effects of letrozole. Training and testing for OP was identical to OR, except that testing was conducted four hours after training, and involved moving one of the identical training objects to a new location in the arena (lower right or lower left corner; Fig. 2B). Spatial memory consolidation was demonstrated if the mice spent more time than chance with the moved object. At this four-hour delay, vehicle-infused ovariectomized females show intact OP memory (Boulware et al., 2013), thereby permitting observation of letrozole-induced memory impairments.

Figure 2

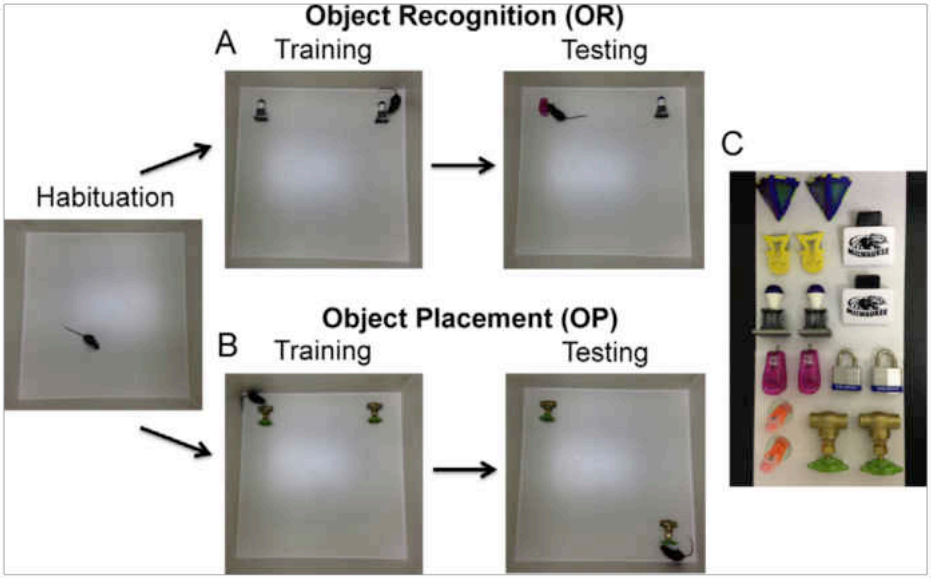

Figure 2. Overview of behavioral testing protocols. Mice are first habituated to an empty arena prior to beginning behavioral training (habituation). (A) In object recognition (OR), mice accumulate 30 seconds exploring two identical novel objects placed in the arena (training). Retention is tested 24 or 48 hours later by presenting mice with one novel and one familiar object (testing). Mice who remember the familiar object spend more time than chance (15 seconds) exploring the novel object. (B) Object placement (OP) uses the same apparatus and general procedure, but during testing, one training object moves to a new location in the arena, rather than being replaced with a new object. (C) Object pairs used in our laboratory's OR and OP protocols.

E_2 Measurement. E_2 levels were measured in the DH using an EIA assay following dual liquid-solid phase extraction (Chao et al., 2011). Briefly, frozen DH tissue was first homogenized in 0.1 M phosphate buffer, followed by three rounds of ether extraction. The final organic phase was dried under air in a 50˚C water bath, followed by re-suspension in 250 µl of 0.1 M phosphate buffer prior to solid phase extraction. Re-suspended samples were then eluted through C18 columns under vacuum pressure and washed with ddH_2O to remove hydrophilic polar compounds. Hydrophobic compounds, including steroids, were then removed with a series of washes with 100% methanol,

followed by an evaporation step under air in a 50˚C water bath. After drying, samples were suspended in EIA buffer. E_2 concentrations were then measured from EIA plates per the manufacturer's instructions using a Thermo Multiskan EX plate reader (Thermo Corp) with a 450 nm filter and Ascent Software 2.6 (Remage-Healey et al., 2008). The EIA assay used to measure brain estrogen levels in this experiment is highly specific for E_2 (cross-reactivity: 14% for E_2-3-glucuronide; 12% for estrone; 1% for E_2-17-glucuronide; < 0.10% for other major steroids including testosterone). Data are expressed in pg E_2 per mg wet weight of DH tissue, and were analyzed using one-way ANOVAs to measure effects of learning or drug treatments.

Western blotting. After infusion, a subset of mice was cervically dislocated and decapitated, and the DH was immediately dissected on wet ice. Tissue samples were then resuspended in a 1:50 weight/volume dilution in lysis buffer containing phosphatase and protease inhibitors, and homogenized via sonication (Branson Sonifier 250, Danbury, CT). Protein concentrations of the homogenates were measured using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA; Bradford, 1976). All samples were normalized to two µg/µl using 5X SDS/PAGE loading buffer and 1X lysis buffer, followed by a five minute boil to denature proteins. Ten microliter aliquots were then loaded onto a 10% precast gel (Bio-Rad) and underwent electrophoresis, followed by transfer to a Midi membrane using the TransBlot Turbo transfer system (Bio-Rad). Membranes were blocked in 5-10% milk to prevent non-specific binding, and then incubated with anti-phospho-p44/42 ERK (Thr202/Tyr204) (1:1000; Cell Signaling

Technology, Danvers, MA). Membranes were stripped and reprobed with anti-total p44/42 ERK antibody (1:2000; Cell Signaling Technology) for protein normalization. Membranes were then incubated with anti-rabbit IgG HRP-linked secondary antibody (1:20,000; Cell Signaling Technology) for one hour, and developed using Clarity Western enhanced chemiluminescence (Bio-Rad). A ChemiDocMP gel imager (Bio-Rad) was used for signal detection of the protein expression, and accompanying analysis/quantification software was used to perform densitometry.

Data analysis. All statistical analyses were conducted using GraphPad Prism 6 (La Jolla, CA). OR and OP data were analyzed using one sample *t*-tests to determine if the time spent with the novel or moved object differed significantly from chance (15 seconds; Fortress et al., 2013; Boulware et al., 2013). This analysis is used because time spent with the objects is not independent; time spent with one object reduces time spent with the other object (Frick and Gresack, 2003). EIA data were analyzed using a two-way ANOVA, followed by Tukey's post hocs when appropriate, to determine effects of post-training drug treatments over time. For Western blotting, phospho-p42 ERK levels were normalized to total p42/p44 ERK and β-Actin levels, and were expressed as % immunoreactivity of vehicle controls. One-way ANOVA was used to analyze each antibody, followed by Tukey's post hocs for between-group comparisons. Statistical significance for all analyses was determined as $p \leq 0.05$.

RESULTS

Experiment 1: Blocking hippocampal E_2 synthesis during consolidation impairs object recognition and object placement memory.

To determine if hippocampal E_2 synthesis is necessary for the consolidation of object memories, young female mice were first ovariectomized and then bilaterally implanted with cannulae aimed at the DH one week prior to the start of behavioral training. Immediately after OR training, mice received bilateral DH infusion of vehicle or one of three doses of the aromatase inhibitor letrozole (0.005, 0.025, or 0.05 μg/hemisphere; n=10/group). Object recognition memory was tested 24 hours later, a time point at which young ovariectomized vehicle-treated mice remember the familiar object (Fernandez et al., 2008, Zhao et al., 2010, Boulware et al., 2013, Fortress et al., 2013). Mice treated with vehicle spent significantly more time than chance with the novel object ($t_{(8)} = 2.433$, $p = 0.0410$; Fig 3A), demonstrating intact memory for the familiar training object. However, mice treated with 0.005, 0.025, or 0.05 μg/hemisphere letrozole spent similar amounts of time with the familiar and novel objects (0.005 μg: $t_{(5)} = 1.45$, $p = 0.21$; 0.025 μg: $t_{(5)} = 0.49$, $p = 0.64$; 0.05 μg: $t_{(6)} = 0.13$, $p = 0.90$; Fig. 3A), demonstrating impaired OR memory consolidation.

Figure 3

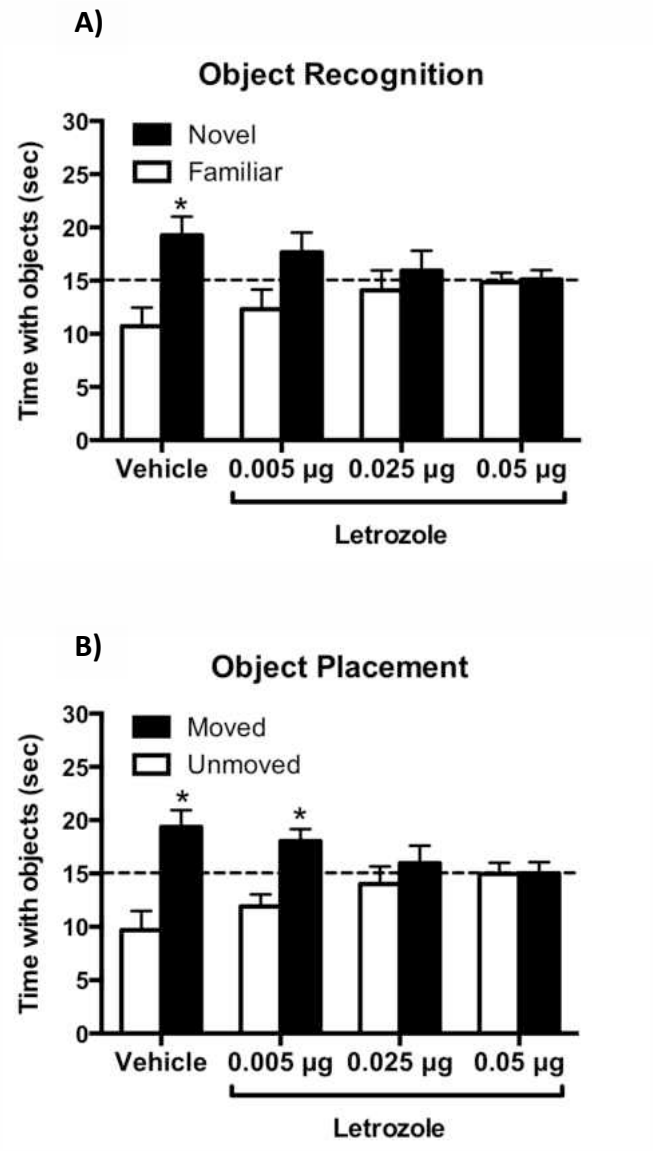

Figure 3. Letrozole impairs object recognition and object placement memory consolidation. (A) Mice receiving bilateral DH infusion of vehicle, but not 0.005 µg, 0.025, or 0.05 µg letrozole, immediately after training spent more time than chance (dashed line at 15 sec, $*p < 0.05$) with the novel object 24 h after training, suggesting that all doses of letrozole blocked object recognition memory consolidation. (**B)** Mice receiving bilateral DH infusions of vehicle or 0.005 µg letrozole, but not 0.025 or 0.05 µg letrozole, immediately after training spent more time than chance ($*p < 0.05$) with the moved object 4 h after training. These data suggest that the 0.025 or 0.05 µg doses of letrozole blocked spatial memory consolidation. n=6-9/group.

Next, to determine if local E_2 is also essential for spatial memory consolidation, mice received bilateral DH infusion of vehicle or one of the same three doses of letrozole (n=6-9/group) immediately after OP training. Spatial memory was then tested four hours later, a time point at which young ovariectomized vehicle-treated females remember the unmoved object (Boulware et al., 2013). Mice receiving either vehicle ($t_{(7)}$ = 2.75, p = 0.03) or 0.005 µg/hemisphere letrozole ($t_{(6)}$ = 2.7, p = 0.04) spent significantly more time than chance with the novel object (Fig. 3B), demonstrating intact memory, whereas those infused with 0.025 or 0.05 µg/hemisphere letrozole exhibited impaired OP memory (0.025 µg: $t_{(6)}$ = 0.58, p = 0.58; 0.05 µg: $t_{(8)}$ = 0.02, p = 0.98; Fig. 3B). Together, these data provide the first evidence that local E_2 synthesis in the DH is necessary for the consolidation of hippocampus-dependent object recognition and spatial memories in young female mice.

Experiment 2: Letrozole blocks an experience-induced increase in dorsal hippocampal E_2 levels.

We next measured levels of E_2 in the DH at various points after object training to determine: 1) the extent to which letrozole reduced E_2 levels within the DH, and 2) if letrozole could block an experience-induced increase in DH E_2 levels. Two weeks after the completion of behavioral testing, mice were trained with two new identical objects and then immediately received a DH infusion of vehicle or the lowest dose of letrozole shown to impair memory in both OR and OP (0.025 µg/hemisphere). The DH was then dissected 30, 60, and 120 min later on wet ice and stored at -80 ^{o}C prior to dual liquid

and solid phase extraction for quantification of DH E_2 content by EIA. A two-way ANOVA revealed a significant interaction between treatment and time ($F_{(2, 21)} = 8.55$, $p = 0.002$; Fig. 4) and main effect of treatment ($F_{(2, 21)} = 8.58$, $p = 0.002$). Post hoc analyses revealed that DH E_2 levels were significantly higher 30 min after training in mice infused with vehicle relative to those infused with letrozole ($t_{(7)} = 4.06$, $p = 0.005$; Fig. 4). DH E_2 levels in vehicle-treated mice returned to baseline by 60 min after training, and were similar to those of letrozole-treated mice at the 60 min and 120 min time points (60 min: $t_{(8)} = 1.11$, $p = 0.31$; 120 min: $t_{(6)} = 0.82$, $p = 0.44$; Fig. 4). These data suggest that letrozole suppresses an experience-induced increase in DH E_2 levels 30 min after infusion, at a dose shown to impair OR and OP memory consolidation *in vivo*.

Figure 4

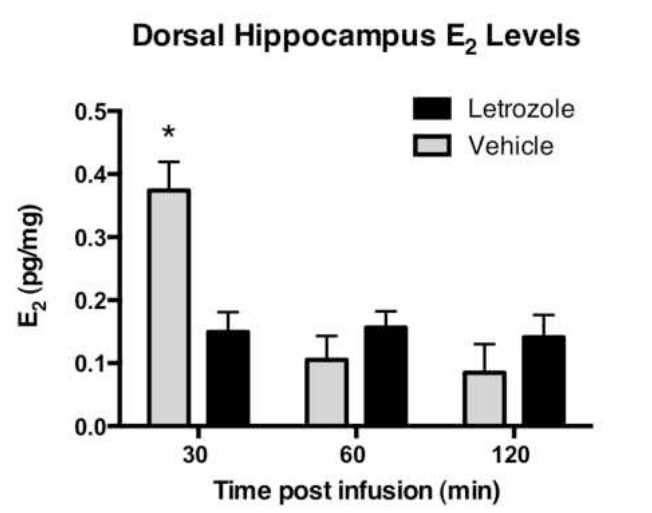

Figure 4. Letrozole reduces hippocampal E_2 levels at a dose that impairs spatial and object recognition memory consolidation. Mice receiving bilateral DH infusion of 0.025 µg letrozole had significantly lower DH E_2 levels than vehicle-treated mice 30 min after infusion (*$p < 0.05$). DH E_2 levels in vehicle-treated mice returned to baseline by 60 min after training, and were similar to that of letrozole-treated mice at the 60 min and 120 min time points. n=3-7/group.

Experiment 3: Local E_2 synthesis is not necessary for exogenous E_2 to enhance hippocampal memory consolidation.

Previous work from our laboratory has demonstrated that 5 μg E_2 infused bilaterally into the DH or 10 μg infused into the dorsal third ventricle (ICV) enhances the consolidation of object recognition and object placement memories in young ovariectomized female mice in an ERK-dependent manner (Fernandez et al., 2008, Zhao et al., 2010, Boulware et al., 2013, Fortress et al., 2013). However, these studies did not take into account the potential contribution of local E_2 synthesis to the observed memory enhancements. To determine if local and exogenous E_2 may synergistically facilitate memory consolidation, we next investigated the role of local E_2 synthesis in the memory-enhancing effects produced by exogenous E_2. Mice were ovariectomized and then implanted with bilateral DH cannulae and a unilateral ICV cannula as in our previous work (e.g., Boulware et al., 2013, Fortress et al., 2013). Immediately after object training, mice received a DH infusion of 2% DMSO in sterile saline vehicle or letrozole (0.025 μg), followed immediately by an ICV infusion of HBC vehicle or 10 μg E_2. OR and OP memory were then tested 24 or 48 hours later, respectively, because exogenous E_2 enhances memory in these tasks at these time points (e.g., Boulware et al., 2013, Fortress et al., 2013). Mice receiving vehicle infusion into both the DH and dorsal third ventricle did not spend more time than chance with the novel object in the OR task ($t_{(8)} = 1.21$, $p = 0.26$; Fig. 5A), or the moved object in the OP task ($t_{(8)} = 1.46$, $p = 0.18$; Fig. 5B). In contrast, mice who were bilaterally infused with vehicle or 0.025 μg letrozole into the DH, followed by ICV infusion of E_2, spent more time than chance with

the novel object 48 h after OR training (veh/E_2: $t_{(7)} = 3.26$, $p = 0.01$; let/E_2: $t_{(5)} = 3.05$, $p = 0.03$; Fig. 5A).

Figure 5

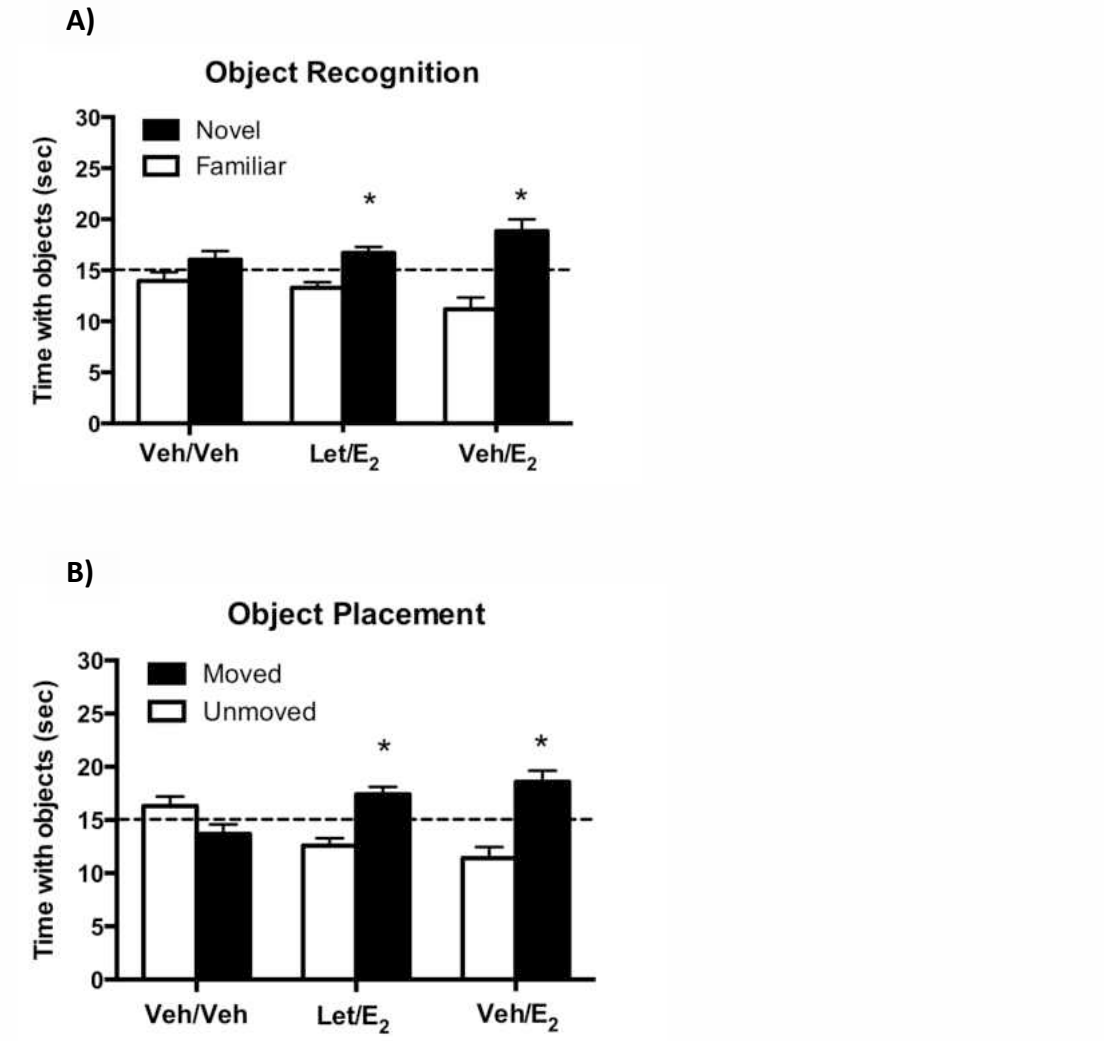

Figure 5. Local E_2 synthesis is not necessary for exogenous E_2 to enhance object recognition or spatial memory consolidation. Mice receiving infusions of vehicle into the DH and dorsal third ventricle did not spend more time than chance with the novel object (A) or moved object (B). In contrast, mice receiving bilateral infusion of vehicle or 0.025 μg letrozole into the DH, followed by ICV infusion of E_2, spent more time than chance (dashed line at 15 sec, $^*p < 0.05$) with the novel object 48 h after training (A) and the moved object 24 h after training (B). These data suggest that inhibition of local E_2 synthesis in the DH is not sufficient to prevent exogenous E_2 from enhancing object recognition or spatial memory consolidation. n=6-9/group.

Similarly, mice receiving the same treatments (bilateral DH infusion of vehicle or 0.025 μg letrozole, followed by ICV infusion of E_2) also spent more time than chance with the moved object 24 h after OP training (veh/E_2: $t_{(7)} = 3.38$, $p = 0.01$; let/E_2: $t_{(6)} = 3.5$, $p = 0.01$; Fig. 5B). Together, these data suggest that inhibition of local E_2 synthesis in the DH does not prevent exogenous E_2 from enhancing object recognition or spatial memory consolidation, and suggest that local *de novo* E_2 synthesis is not necessary for exogenous E_2 to enhance memory, at least at the 10 μg dose.

Finally, we examined whether letrozole could prevent exogenous E_2 from activating p42 ERK. Two weeks after completion of behavioral testing, mice were infused with their respective treatments, and DH tissues were dissected 5 minutes later (n=8/time point) based on previous work from our laboratory demonstrating that exogenous E_2 influences DH cell signaling 5 minutes after infusion (Fernandez et al., 2008, Fan et al., 2010, Zhao et al., 2010) and work in zebra finches showing that aromatase inhibition blocks an experience-induced increase in ERK phosphorylation (Pinaud and Tremere, 2012). When data were normalized to the housekeeping protein β-actin, we observed an overall significant effect of treatment on phospho-p42 ERK levels ($F_{(2,21)} = 4.63$, $p = 0.022$; Fig. 6A). Consistent with previous studies published by our lab (Fernandez et al., 2008, Lewis et al. 2008, Fan et al. 2010, Zhao et al. 2010) Fisher's LSD post-hoc comparisons revealed that E_2 significantly increased p42-ERK phosphorylation 5 minutes after infusion ($p < 0.05$; Fig. 6A) relative to vehicle-treated mice. Mice that were infused with both letrozole and E_2 did not exhibit p42-ERK levels significantly higher than vehicle mice, although the post-hoc test was nearly significant

(p = 0.053; Fig. 6A). When normalized to total ERK, the main effect of treatment was not significant, although a priori post-hoc tests indicated that mice treated with vehicle + E_2 exhibited significantly higher p42-ERK phosphorylation relative to vehicle-treated mice ($p < 0.05$; Fig. 6B). Again, the letrozole + E_2 group had lower phospho-p42 ERK levels than mice treated with vehicle + E_2, but the letrozole group did not significantly differ from any other group (Fig. 6B). Although both normalizations indicate that our letrozole + E_2 group had lower p42-ERK levels than the E_2 alone group, these data suggest that letrozole does not fully block the effects of exogenous E_2 on ERK phosphorylation.

Figure 6

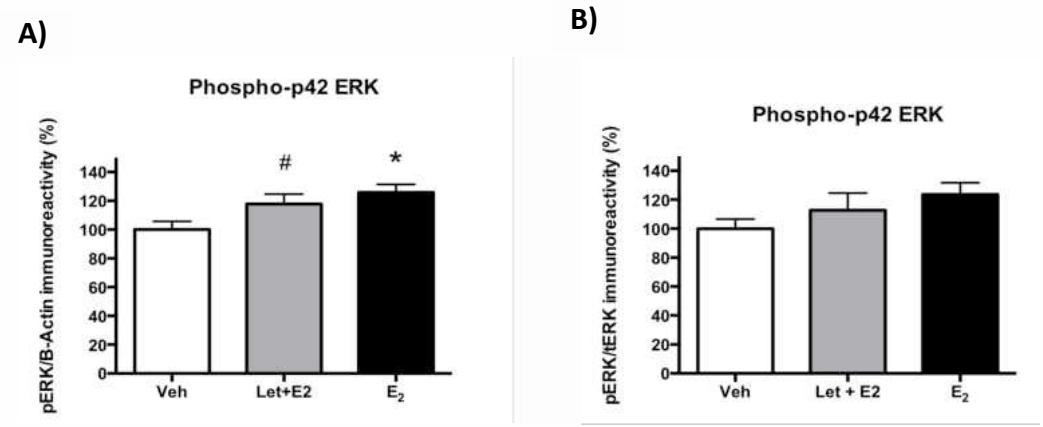

Figure 6: Inhibition of local E_2 synthesis attenuates, but does not completely block, the effects of exogenous E_2 on p42-ERK activation. Mice receiving bilateral infusion of vehicle followed by ICV infusion of E_2, had significantly higher levels of phospho p42 ERK (*$p < 0.05$) relative to mice infused with vehicle into both the DH and dorsal third ventricle ($p > 0.05$). Bilateral infusion of 0.025 µg letrozole into the DH, followed by ICV infusion of E_2, did not result in significant elevation of phosphor-p42 ERK levels, however, we did observe a trend toward significance (p = 0.0675). These data suggest that inhibition of local E_2 synthesis in the DH attenuates p42 ERK levels, but does not entirely block exogenous E_2 from increasing p42-ERK activation. A) phospho-p42 ERK levels normalized to B-Actin. B) phosphor-p42 ERK levels normalized to tERK.

DISCUSSION

The current findings provide novel insight into the functional role of brain-derived estrogens on learning and memory in rodents. First, we demonstrated that hippocampal E_2 synthesis is necessary for object recognition and spatial memory consolidation in young ovariectomized mice. Next, we found that DH E_2 levels increase 30 min after novel object training, and that this increase is blocked by DH infusion of an aromatase inhibitor at a dose that impairs recognition and spatial memory consolidation *in vivo*. Together, these data suggest E_2 synthesis can increase acutely in an experience-dependent manner, and that this increase is necessary for the consolidation of recognition and spatial memories. We also found in a subsequent experiment that local E_2 synthesis is not necessary for exogenous E_2 to enhance object recognition and spatial memory consolidation, at least at the 10 μg E_2 dose used. Collectively, these findings are the first to demonstrate that *de novo* E_2 synthesis is necessary for the consolidation of hippocampus-dependent memories in female rodents. To our knowledge, these are the first rodent data to use intrahippocampal infusions to directly assess the specific involvement of hippocampal E_2 synthesis in memory formation. Further, these experiments are the first to address whether or not *de novo* E_2 synthesis in the dorsal hippocampus is necessary for the memory-enhancing effects of exogenous E_2 in rodents.

Our data showing that letrozole blocks OR and OP memory consolidation are consistent with previous *in vitro* studies suggesting *de novo* E_2 synthesis regulates the expression of synaptic proteins, synaptic spine density, and LTP (Kretz et al., 2004,

Fester et al., 2012, Vierk et al., 2012, Vierk et al., 2014). Our present findings are also consistent with recently published *in vivo* studies showing that *de novo* E_2 synthesis is important for spatial memory in zebra finches (Bailey et al., 2013, Rensel et al., 2013), and a study conducted in male rats demonstrating the necessity of *de novo* E_2 synthesis in the extinction of fear memories (Graham and Milad, 2014). Taken together with the present data, these results suggest regulation of memory by *de novo* E_2 synthesis may be a general property of both male and female adult vertebrate brains, and that this process is particularly important during the consolidation phase of memory in male and female rodents. Although zebra finch studies have demonstrated that increases in local E_2 synthesis drive p42 ERK activation, the mechanisms through which *de novo* E_2 facilitates memory consolidation in rodents is currently unknown. Therefore, future studies should address whether local E_2 synthesis is necessary and/or sufficient to activate the MAPK/ERK pathway and it's downstream targets, which would ultimately influence the expression of genes that support learning and memory.

The current study also provides the first evidence that a learning experience can increase local E_2 synthesis in the rodent hippocampus. The fact that the increase in E_2 synthesis in vehicle-treated mice observed 30 min after training was not observed at 60 or 120 min later, suggests a transient increase in hippocampal E_2 synthesis driven by object training. This transient post-training increase in hippocampal E_2 synthesis was suppressed by a DH infusion of the aromatase inhibitor letrozole. These data confirm that DH infusion of letrozole suppresses local E_2 synthesis in the DH at a dose that impairs recognition and spatial memory consolidation *in vivo*. The observed experience-

induced increase in hippocampal E_2 levels in our mice is consistent with avian studies reporting experience-induced changes in the male zebra finch forebrain after social interactions with female zebra finches or exposure to different auditory stimuli (Remage-Healey et al., 2008, Remage-Healey et al., 2010, Remage-Healey et al., 2011). Also, our finding that DH delivery of the aromatase inhibitor letrozole suppresses local E_2 synthesis is consistent with zebra finch studies showing suppression of acute changes in E_2 in the NCM of male zebra finches after retrodialysis of the aromatase inhibitor fadrozole (Remage-Healey et al., 2008, Remage-Healey et al., 2010). In the zebra finch forebrain, it has also been established that rapid changes in E_2 synthesis are dependent on Ca^{2+} influx, much like classical neurotransmitters (Remage-Healey et al., 2011). Evidence from zebra finches also shows that blocking *de novo* E_2 synthesis in the NCM disrupts neuronal response properties during the processing of auditory stimuli, such as spike rate and burst firing activity (Remage-Healey et al., 2010). Further, suppressing *de novo* E_2 synthesis in the NCM changes firing rate and stimulus selectivity in the HVC, a structure that receives indirect afferent input from the NCM. Thus, suppressing the ability of one region to synthesize E_2 impacts auditory processing in other downstream target regions that receive input from the NCM (Remage-Healey and Joshi, 2012). Although the present study did not measure the electrophysiological consequences of blocking local E_2 in female mice *in vivo*, the avian data and *ex vivo* rodent data suggest such investigations may provide valuable additional insight into the mechanism through which local E_2 synthesis influences cognitive function, and should be addressed in future studies.

Finally, the present findings also suggest that local E_2 synthesis is not essential for exogenous E_2 to exert it's beneficial effects on object recognition and spatial memory consolidation. Further, the fact that p42 ERK levels in our letrozole + E_2 group trend toward significance suggest that inhibition of local E_2 synthesis does not block the activating effects of exogenous E_2 on p42 ERK activation. These findings do not align with previously reported *in vitro* data showing that treatment of hippocampal cells with an aromatase inhibitor prevents exogenous E_2 from increasing mRNA and protein expression of synaptic plasticity markers PSD-95 and Arc (Chamniansawat and Chongthammakun, 2012), and presynaptic marker synaptophysin in hippocampal slice cultures (Kretz et al., 2004). There are several possible reasons for this discrepancy. First, the previous studies were conducted *in vitro* and the duration of exposure to the aromatase inhibitor was chronic (4 – 12 days), instead of the single acute infusion used in the current study. It is possible that chronic delivery in our study would have prevented exogenous E_2 from enhancing hippocampal memory consolidation. Second, the synaptic proteins measured in the *in vitro* studies discussed above may not be necessary for E_2 to enhance object recognition or spatial memory consolidation, as the necessity of these specific proteins has not been directly evaluated in our behavioral paradigms. Third, the dose of E_2 used in the present study may have been too high for local inhibition to matter, as much lower doses of E_2 were use in the aforementioned *in vitro* studies (10^{-7} - 10^{-12} M E_2 compared to 10 µg E_2 in this study). Finally, the timing of aromatase inhibitor and E_2 administration may have played an important role in our findings. That is, local E_2 synthesis may not have been sufficiently suppressed by the

time exogenous E_2 was administered. Thus, exogenous E_2 may have activated the cell-signaling cascades (i.e., ERK, PI3K, mTOR) necessary for E_2 to enhance memory before letrozole had had time to suppress local E_2 levels. Delaying infusion of E_2, perhaps by 30-60 min, may have better allowed for an observation of interactions between local and exogenous E_2. Despite the fact that our Western data do not show a significant increase in p42 ERK levels after infusion of both letrozole and E_2, these data do trend toward a significant elevation similar to our E_2-treated group. It is possible that these intermediate levels of phosphorylation are reflective of early interference with ERK phosphorylation, and that aromatase inhibition may suppress ERK phosphorylation more substantially over time. To address this question, future studies should examine p42 ERK phosphorylation at different time points after aromatase inhibition.

In conclusion, the present study provides novel insights into the functional role of hippocampally-synthesized E_2 on hippocampal memory in female mice. This is the first study to demonstrate the necessity hippocampal E_2 synthesis for the consolidation of object recognition and spatial memories in female mice. Our finding that DH E_2 levels are elevated within 30 min of behavioral training demonstrates that rodents synthesize E_2 in the hippocampus in an experience-dependent manner. However, the specific mechanisms regulating E_2 synthesis remain to be tested in future studies. Finally, our data also suggested that local E_2 synthesis is not necessary for exogenous E_2 to enhance object recognition and spatial memory consolidation, or increase p42 ERK phosphorylation, at least at the 10 μg E_2 dose used. Collectively, these findings provide important new insight into the contribution of hippocampal E_2 synthesis to learning and

memory, and build a foundation for future studies to investigate the molecular mechanisms through which *de novo* E_2 mediates cognition across the lifespan.

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