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## Molecular mechanisms underlying the memory-enhancing effects of estradiol

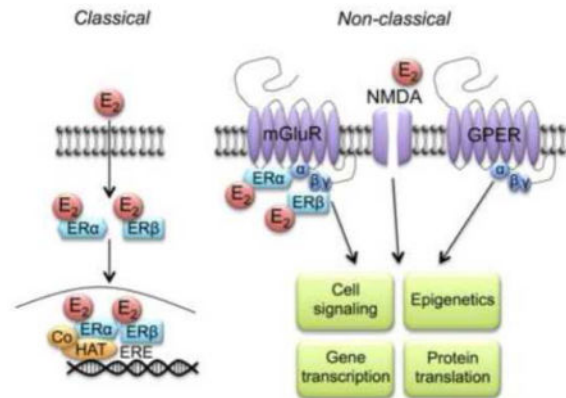
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### Abstract

Since the publication of the 1998 special issue of *Hormones and Behavior* on estrogens and cognition, substantial progress has been made towards understanding the molecular mechanisms through which 17 $\beta$ -estradiol (E<sub>2</sub>) regulates hippocampal plasticity and memory. Recent research has demonstrated that rapid effects of E<sub>2</sub> on hippocampal cell signaling, epigenetic processes, and local protein synthesis are necessary for E<sub>2</sub> to facilitate the consolidation of object recognition and spatial memories in ovariectomized female rodents. These effects appear to be mediated by non-classical actions of the intracellular estrogen receptors ER $\alpha$  and ER $\beta$ , and possibly by membrane-bound ERs such as the G-protein-coupled estrogen receptor (GPER). New findings also suggest a key role of hippocampally-synthesized E<sub>2</sub> in regulating hippocampal memory formation. The present review discusses these findings in detail and suggests avenues for future study.

### Graphical abstract



### Keywords

estrogen; hippocampus; estrogen receptor; cell signaling; epigenetic; ERK; PI3K; mTOR; histone acetylation; DNA methylation; GPER

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## Introduction

In 1998, *Hormones and Behavior* published a special issue entitled, “Estrogen Effects on Cognition across the Lifespan” (Volume 34(2), October). Guest edited by Christina Williams, the special issue featured papers from leaders in the fledgling field of “hormones and cognition”. The articles of the special issue deftly summarized the progress made in the relatively short time since estrogens were found to regulate dendritic spine density on pyramidal neurons in the hippocampus (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992, 1993). At the time, I was a postdoctoral fellow studying the relationship between age-related memory loss and biochemical alterations in the hippocampus and basal forebrain in mice. Our findings led me to learn about how sex steroid hormones influence the septo-hippocampal system and hippocampal memory. As such, the 1998 volume became a bible of sorts for me. I marked it up, referred to it often, and carried it with me on faculty job interviews as a sort of security blanket when I wanted to make sure I had my facts straight. Needless to say, my copy is well worn and I can still find it in my office at a moment’s notice. Although there remains much work to do, we have learned an enormous amount in the past 17 years about how estrogens regulate cognitive function. Given the tremendous advances made since 1998, it seems high time for another special issue that can serve to inspire young scientists in the way that the previous special issue inspired me.

In recent years, laboratories including my own have made progress towards elucidating the molecular mechanisms through which the potent estrogen 17 $\beta$ -estradiol (E<sub>2</sub>) regulates hippocampal memory consolidation in female mice. These mechanisms underlie the so-called “rapid” effects of E<sub>2</sub> on hippocampal functioning, which encompass those that occur within minutes of E<sub>2</sub> exposure. *In vivo*, E<sub>2</sub> activates numerous cell-signaling cascades and alters epigenetic processes in the dorsal hippocampus within 5–30 minutes of treatment, and these actions are necessary for E<sub>2</sub> to enhance the consolidation of hippocampal memories (Bi et al., 2000; Bi et al., 2001; Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2013; Lewis et al., 2008; Zhao and Brinton, 2007; Zhao et al., 2012; Zhao et al., 2010). *In vitro* studies report that rapid E<sub>2</sub>-induced activation of some of these same cell-signaling pathways promotes dendritic spine remodeling (Hasegawa et al., in press; Kramár et al., 2009; Srivastava et al., 2008), thus linking estrogenic regulation of spinogenesis to memory formation. Moreover, the discovery that E<sub>2</sub> is synthesized and released within the hippocampus (Hojo et al., 2004; Kretz et al., 2004; Prange-Kiel et al., 2006) raises the exciting possibility that learning-induced endogenous E<sub>2</sub> synthesis by hippocampal neurons may stimulate the rapid molecular alterations that are necessary for memory formation. Given the emerging importance of rapid E<sub>2</sub> effects for hippocampal memory, this review will focus largely on findings detailing the rapid cell signaling, epigenetic, and receptor mechanisms necessary for E<sub>2</sub> to enhance hippocampal memory consolidation.

## E<sub>2</sub> and the hippocampus

### Spinogenesis, neurogenesis, and long-term potentiation

Although they were controversial at the time of their publication, the groundbreaking findings showing that exogenous E<sub>2</sub> and progesterone increase dendritic spine density on

CA1 pyramidal neurons (Woolley and McEwen, 1993) provided incontrovertible evidence that so-called “ovarian” hormones influence hippocampal morphology. Numerous labs have since replicated these findings (e.g., (Frick et al., 2004; Inagaki et al., 2012; MacLusky et al., 2005; Murphy and Segal, 1996; Segal and Murphy, 2001)). Newer data show that E<sub>2</sub> also regulates dendritic spine density on neurons in the medial prefrontal cortex, somatosensory cortex, and amygdala (de Castilhos et al., 2008; Hao et al., 2006; Inagaki et al., 2012; Khan et al., 2013; Srivastava et al., 2008), as well as dendritic length in the basal forebrain (Saenz et al., 2006). As such, E<sub>2</sub> clearly promotes spinogenesis in multiple regions of the brain that regulate cognitive function. However, much less is known about the role of E<sub>2</sub> in mediating the function of brain regions other than the hippocampus. Within the hippocampus, dendritic spinogenesis is accompanied by the E<sub>2</sub>-induced facilitation of synaptic plasticity. For example, E<sub>2</sub> increases glutamate binding to hippocampal NMDA receptors and increases several measures of intrinsic excitability, leading to enhanced sensitivity of CA1 pyramidal neurons to NMDA-receptor mediated synaptic inputs (Carrer et al., 2003; Kumar and Foster, 2002; Wong and Moss, 1992; Woolley et al., 1997). E<sub>2</sub> also enhances long-term potentiation (LTP) at CA3-CA1 synapses (Bi et al., 2000; Foy et al., 1999; Kramár et al., 2009; Smith and McMahon, 2005; Vedder et al., 2013; Woolley et al., 1997), which is important because LTP is thought to underlie hippocampal memory formation. It was recently found that E<sub>2</sub>-induced enhancements in both object recognition and LTP occurred within a similar time frame and required a functional increase in NR2B-containing NMDA receptors (Vedder et al., 2013), linking E<sub>2</sub>-induced changes in LTP with hippocampal memory formation. Interestingly, the induction of LTP and spinogenesis by E<sub>2</sub> in hippocampal slices is blocked by inhibitors of several cell-signaling pathways, including extracellular signal-regulated kinase (ERK), protein kinase A (PKA), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), and calcium calmodulin kinase II (CaMKII) (Hasegawa et al., in press). These data suggest that the effects of E<sub>2</sub> on hippocampal synaptic plasticity are regulated by many of the rapid molecular alterations that are also necessary for E<sub>2</sub> to enhance memory consolidation (see *Cell-signaling mechanisms necessary for E<sub>2</sub>-induced memory enhancement* below).

A substantial literature shows that E<sub>2</sub> also regulates neurogenesis in the dentate gyrus of the hippocampus (e.g., (Galea et al., 2013; Ormerod et al., 2003; Tanapat et al., 1999)), generally facilitating the incorporation of new neurons into the established neural circuitry. In particular, numerous studies have investigated the role of hormones in mediating the effects of reproductive experience, stress, depression, and aging on hippocampal neurogenesis in both female and male rodents (Galea et al., 2013; Giasper and Gould, 2013). Interestingly, the effects of E<sub>2</sub> on hippocampal neurogenesis appear to depend on timing of treatment, as E<sub>2</sub> given prior to the labeling of new neurons increases neuron proliferation and survival (McClure et al., 2013), whereas E<sub>2</sub> given after cell labeling decreases new neuron survival (Chan et al., 2014). These data suggest that E<sub>2</sub> can enhance neurogenesis when levels are elevated at the time that new neurons are born, but perhaps not afterwards.

### Types and distribution of estrogen receptors

How might E<sub>2</sub> regulate hippocampal morphology and synaptic plasticity? The canonical intracellular estrogen receptors, ER $\alpha$  and ER $\beta$ , are distributed throughout the dorsal-ventral

extent of the hippocampus (Mitra et al., 2003; Mitterling et al., 2010; Shughrue et al., 1997a; Shughrue et al., 1997b; Shughrue and Merchenthaler, 2000). Within hippocampal neurons, ER $\alpha$  and ER $\beta$  are located within the nucleus, dendritic spines, and axon terminals of pyramidal neurons and interneurons (Milner et al., 2005; Milner et al., 2001; Mitra et al., 2003; Mitterling et al., 2010; Waters et al., 2011). In the nucleus, ER $\alpha$  and ER $\beta$  mediate the classical “genomic” effects of estrogens, in which an estrogen-ER complex binds to an estrogen response element on the DNA to promote gene transcription (Fig. 1). However, the localization of these receptors to dendritic spines and axon terminals suggests an additional mechanism of action for ER $\alpha$  and ER $\beta$  at these more distal sites; this mechanism is commonly referred to as “non-genomic” or “non-classical” (Fig. 1). Indeed, E<sub>2</sub> causes ER $\beta$  to translocate to the plasma membrane in hippocampal-derived cell lines and rat primary cortical neurons (Sheldahl et al., 2008). Moreover, both ER $\alpha$  and ER $\beta$  interact with metabotropic glutamate receptor 1 (mGluR1) to rapidly activate hippocampal ERK signaling and promote the phosphorylation of cAMP response element binding protein (CREB) (Boulware et al., 2013; Boulware et al., 2005). The ability of each ER to associate with mGluRs and phosphorylate CREB is dependent on S-palmitoylation (Meitzen et al., 2013), a post-translational modification associated with intracellular protein trafficking (Fukata and Fukata, 2010). This finding helps to explain how ER $\alpha$  and ER $\beta$  can be shuttled to the plasma membrane to trigger rapid cell-signaling processes. Other data implicate NMDA receptor activation in E<sub>2</sub>-induced activation of hippocampal ERK and facilitation of object recognition memory consolidation (Lewis et al., 2008), suggesting the involvement of multiple neurotransmitter receptors in the memory-enhancing effects of E<sub>2</sub>. As will be discussed below, E<sub>2</sub> activates numerous other cell-signaling cascades in hippocampal and cortical neurons within 5 minutes of intracranial infusion (Fan et al., 2010; Fortress et al., 2013; Manella and Brinton, 2006; Yokomaku et al., 2003). Although these rapid effects on cell signaling are necessary for E<sub>2</sub> to enhance memory consolidation (Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2013; Lewis et al., 2008), the potential role of classical genomic effects in memory formation should not be discounted, as classical and non-classical receptor mechanisms may interact to regulate memory.

In addition to ER $\alpha$  and ER $\beta$ , which are generally thought to be located intracellularly, the effects of estrogens may also be mediated by ERs located within the plasma membrane. Several putative receptors have been identified, including the recently renamed G-protein-coupled ER (GPER or GPER1, formerly GPR30), ER-X, and Gq-ER (Filardo et al., 2000; Qiu et al., 2003; Toran-Allerand et al., 2002). It is thought that these receptors bind E<sub>2</sub> and then transduce its signal by triggering second messenger cascades, which then lead to epigenetic alterations, gene transcription, and protein translation (Fig. 1). However, the existence of plasma membrane ERs has been the matter of extensive debate, largely because they have been difficult to clone (Levin, 1999). Nevertheless, studies using a bovine serum albumin-conjugated form of E<sub>2</sub> (BSA-E<sub>2</sub>) that cannot traverse the plasma membrane have demonstrated that BSA-E<sub>2</sub> rapidly activates signaling cascades including ERK, and induces similar ERK-dependent enhancement of object recognition memory consolidation to that observed with free E<sub>2</sub> (Fernandez et al., 2008; Wade et al., 2001; Watters et al., 1997). Although these findings suggest a role for membrane ERs in the mnemonic effects of E<sub>2</sub>, the specific receptors mediating these effects remain unclear. Currently, the best candidate is

GPER, which has been shown in ovariectomized rats to mediate spatial working memory acquisition (Hammond et al., 2009; Hammond et al., 2012). GPER has can be found in all lamina within the hippocampus, exclusively at extranuclear sites within pyramidal neurons, interneurons, and glia (Brailoiu et al., 2007; Waters et al., 2015). Within these cells, GPER has been observed within dendrites, dendritic spines, axons, terminals, and cell bodies, where it can generally be found at or near the plasma membrane in association with post-synaptic scaffolding proteins (Akama et al., 2013; Waters et al., 2015). Interestingly, GPER immunoreactivity in dendrites, spines, terminals, and axons differs in CA1, CA3, and the dentate gyrus during estrus relative to proestrus (Waters et al., 2015), suggesting that GPER expression is regulated by estrogens and/or progestins. However, as shall be discussed later, the molecular mechanisms through which dorsal hippocampal GPER regulates object memory consolidation differ from those of E<sub>2</sub>.

### Hippocampal E<sub>2</sub> synthesis

As indicated above, it is important to note that E<sub>2</sub> and other sex steroid hormones are synthesized within the hippocampus (Hojo et al., 2004; Ikeda et al., 2015; Kretz et al., 2004; Prange-Kiel et al., 2006). In fact, E<sub>2</sub> levels are substantially higher in the hippocampus than in the plasma among both male and female rats (Hojo et al., 2009; Kato et al., 2013). Among female rats, hippocampal E<sub>2</sub> levels are significantly higher than plasma levels at every stage of the estrous cycle and after ovariectomy (Kato et al., 2013). Hippocampal E<sub>2</sub> levels in ovariectomized female rats are similar to those of gonadally-intact females during diestrus, metestrus, and estrus (Kato et al., 2013), suggesting that ovariectomy may not radically alter hippocampal E<sub>2</sub> levels during most of the estrous cycle. These findings suggest that the primary endogenous source of E<sub>2</sub> for hippocampal neurons may be hippocampal neurons or glia (Azcoitia et al., 2003; Garcia-Segura et al., 1999), rather than the ovaries.

The aromatase inhibitor letrozole decreases levels of E<sub>2</sub> and its stereoisomer 17 $\alpha$ -estradiol in the hippocampus (Ikeda et al., 2015; Kretz et al., 2004), and has been used in numerous studies to examine the role of hippocampally-synthesized E<sub>2</sub> on hippocampal spine morphology and physiology. For example, in rat hippocampal slice cultures, letrozole reduced dendritic spine density, presynaptic bouton number, and synaptic protein levels (Kretz et al., 2004; Prange-Kiel et al., 2006). Letrozole treatment in hippocampal cultures also revealed divergent effects of ER agonists on dendritic spine density, such that an ER $\alpha$  agonist increased, whereas an ER $\beta$  agonist decreased, spine density in the presence of letrozole (Zhou et al., 2014). Further, systemic injections of letrozole have been associated with impaired LTP and transient dephosphorylation of the actin binding protein cofilin in gonadally-intact male rats and both intact and ovariectomized female rats (Vierk et al., 2012). Letrozole also reduced the numbers of mature spines, thin spines, and spine synapses in females, but only decreased thin spines in males (Vierk et al., 2012), suggesting a potential sex difference in the role of local E<sub>2</sub> in regulating hippocampal spinogenesis.

Clues to the behavioral significance of *de novo* hippocampal E<sub>2</sub> synthesis come from studies of zebra finches, which report that E<sub>2</sub> is rapidly synthesized in the auditory caudo-medial nidopallium (NCM) of males during behavioral experiences such as exposure to female conspecifics or conspecific song (Ramage-Healey et al., 2008). Our own preliminary data in

female mice indicate that object learning stimulates dorsal hippocampal E<sub>2</sub> release within 30 minutes (Tuscher et al., 2013). Importantly, studies using aromatase inhibitors show that blocking hippocampal E<sub>2</sub> synthesis impairs hippocampal-dependent memory. For example, infusion of the aromatase inhibitor fadrozole into the male zebra finch hippocampus impairs spatial memory in a food-finding task (Bailey et al., 2013). In ovariectomized female mice, our preliminary data indicate that letrozole infusion into the dorsal hippocampus prevents memory consolidation in both the object recognition and object placement tasks (Tuscher et al., 2013). Object training also appears to transiently increase dorsal hippocampal E<sub>2</sub> levels 30 minutes after training, an effect that is blocked by letrozole (Tuscher et al., 2013). A role for local E<sub>2</sub> in memory among rodents is supported by another recent report that systemic injection of fadrozole prior to or immediately after fear extinction training in male rats significantly impaired fear recall during testing (Graham and Milad, 2014). Combined, these data suggest the intriguing possibility that the hippocampus synthesizes E<sub>2</sub> in response to a learning event, and that this synthesis is necessary for hippocampal memory formation.

How might a role for hippocampally-synthesized E<sub>2</sub> be reconciled with the literature demonstrating that ovarian-synthesized E<sub>2</sub> influences hippocampal memory? At this point, the relative contributions of these two sources of E<sub>2</sub> to memory formation are unclear. Ovarian-derived steroids may prime the hippocampus and other brain regions to respond to locally-synthesized E<sub>2</sub>. As such, E<sub>2</sub> synthesized in the hippocampus in response to a learning event or other stimulus may produce greater synaptic potentiation and/or morphological alterations when circulating E<sub>2</sub> levels are elevated. In this scenario, one might predict that aromatase inhibition would be most disruptive to memory consolidation during proestrus, when circulating E<sub>2</sub> levels are the highest. Alternatively, circulating estrogens may simply be necessary for maintaining the general health of hippocampal neurons, but have little to do with memory formation *per se*. Some support for this idea comes from the well-established role of E<sub>2</sub> as a neuroprotective factor in the adult and aging hippocampus (Brinton, 2001; Garcia-Segura et al., 2001; Wise et al., 2001). The loss of this neuroprotection after ovariectomy or during aging may render hippocampal neurons less responsive to hippocampally-synthesized E<sub>2</sub> or more vulnerable to dysfunction due to aging, cellular damage, or ischemia. Future studies will need to test these possibilities and others to determine the unique roles of ovarian- and hippocampally-synthesized E<sub>2</sub> to memory formation. In reading the remainder of this review, it is important to note that the rodent subjects used were bilaterally ovariectomized, which eliminates ovarian-synthesized E<sub>2</sub>. Although ovariectomy reduces one potential interaction with hippocampal-derived E<sub>2</sub>, it is unknown how exogenous E<sub>2</sub> treatments might affect the synthesis of E<sub>2</sub> within the hippocampus. Addressing these potential interactions will be an important issue for future study.

## E<sub>2</sub> and hippocampal memory

Given the ample evidence demonstrating that E<sub>2</sub> regulates hippocampal morphology and physiology, it should be no surprise that E<sub>2</sub> also influences hippocampal memory. The effects of E<sub>2</sub> on hippocampal memory in rodents have been most commonly assessed in spatial tasks (e.g., Morris water maze, radial arm maze, delayed non-match to position, object location/placement) and in object recognition tasks. These effects have been

enumerated in hundreds of empirical papers and many outstanding reviews (e.g., see the following reviews: (Choleris et al., 2012; Conrad and Bimonte-Nelson, 2010; Daniel, 2006; Frick, 2009; Gibbs, 2010; Luine, 2014, 2015; Luine and Frankfurt, 2012; Tuscher et al., 2015)), so they will not be discussed extensively here. In brief, most studies of female rodents report that systemic or intracranial E<sub>2</sub> administration enhances memory acquisition and consolidation in spatial and object recognition tasks, although some studies found no such beneficial effects (Galea et al., 2001; Marriott et al., 2002). E<sub>2</sub> also enhances hippocampal memory in male rodents (Luine and Rodriguez, 1994; Packard et al., 1996), indicating that estrogenic regulation of hippocampal memory is not unique to females. In general, the effects of E<sub>2</sub> on hippocampal memory depend on many factors including dose, task type and difficulty, timing of injection relative to training, duration of treatment, duration of ovariectomy prior to treatment, presence of ovaries, number of prior pregnancies, and age at treatment (Frick, 2009; Tuscher et al., 2015; Workman et al., 2012). Nevertheless, the balance of studies supports the broad conclusion that E<sub>2</sub> is beneficial for hippocampal memory in both female and male rodents.

### Post-training E<sub>2</sub> treatment

In recent years, numerous laboratories have reported that a single post-training administration of E<sub>2</sub> systemically or intracranially enhances spatial and object recognition memory consolidation (see (Luine, 2015; Luine and Frankfurt, 2012; Tuscher et al., 2015) for reviews). For example, post-training administration of E<sub>2</sub> systemically or into the dorsal hippocampus enhances spatial reference memory consolidation in the Morris water maze in male rats, ovariectomized female rats, and ovariectomized female mice (Gresack and Frick, 2006; Packard et al., 1996; Packard and Teather, 1997a, b). In female rats and mice, post-training E<sub>2</sub> administered systemically or into the dorsal hippocampus or dorsal third ventricle also enhances spatial memory consolidation tested in an object placement (a.k.a., object location) task and object recognition memory consolidation tested in an object recognition task (Boulware et al., 2013; Gresack and Frick, 2006; Luine et al., 2003; Pereira et al., 2014; Walf et al., 2006). The beneficial effects of post-training E<sub>2</sub> on object recognition and/or object placement in young and middle-aged females have been reported in at least two-dozen papers from multiple laboratories in both rats and mice (see (Tuscher et al., 2015) for review), suggesting a broad effect that generalizes across species, dose, route of administration, testing protocol, and types of hippocampal memory.

Because post-training E<sub>2</sub> so consistently enhances hippocampal memory consolidation, my laboratory has used the post-training approach as a tool to understand the molecular mechanisms underlying the memory-enhancing effects of E<sub>2</sub>. In particular, we administer dorsal hippocampal or dorsal third ventricle infusions of a water-soluble form of E<sub>2</sub> immediately after training in one-trial object recognition and object placement tasks to pinpoint the molecular mechanisms within the dorsal hippocampus through which E<sub>2</sub> regulates memory consolidation. The benefits of this approach have been described in detail elsewhere (Frick, 2012; Frick et al., 2010), so will not be belabored here. However, it is important to note that the near simultaneous infusion of E<sub>2</sub> into the dorsal third ventricle (adjacent to the dorsal hippocampus) and of receptor antagonists or enzyme inhibitors into the dorsal hippocampus has allowed us to discern which receptors, cell-signaling pathways,

and epigenetic processes are necessary for E<sub>2</sub> to enhance hippocampal memory consolidation. This approach requires the implantation of triple cannulae, which allows us to determine if blocking receptor activation or cellular processes bilaterally in the dorsal hippocampus prevents the memory-enhancing effects of an intracerebroventricular (ICV) E<sub>2</sub> infusion. Infusing E<sub>2</sub> just outside of the dorsal hippocampus prevents tissue damage in the dorsal hippocampus that could result from multiple sequential infusions. Importantly, this method permits identification of specific proteins and enzymatic events in the dorsal hippocampus that are essential for E<sub>2</sub> to enhance hippocampal memory consolidation.

Our own efforts have focused on three realms of cellular functioning: cell signaling, epigenetics, and estrogen receptor mechanisms. Therefore, these processes will be discussed in detail in the remainder of this review. Figure 2 presents a schematic synthesis of our laboratory's findings to provide a guide for the discussion below.

## Cell-signaling mechanisms necessary for E<sub>2</sub>-induced memory enhancement

### ERK

Our initial studies focused on ERK signaling because ERK phosphorylation is necessary for rodents to form long-term hippocampal memories, including spatial memories, contextual fear memories, and object recognition memories (Atkins et al., 1998; Blum et al., 1999; Bozon et al., 2003; Kelly et al., 2003). Interestingly, early *in vitro* studies indicated that E<sub>2</sub> or BSA-E<sub>2</sub> increased ERK phosphorylation within 15 minutes of exposure in a variety of cell types including hippocampal neurons (Wade and Dorsa, 2003; Wade et al., 2001; Watters et al., 1997; Yokomaku et al., 2003). This activation was blocked by inhibitors of the enzyme mitogen activated protein kinase kinase (MAPKK or MEK), which is the exclusive upstream activator of ERK (Nilsen and Brinton, 2003; Yokomaku et al., 2003). Subsequent *in vivo* work demonstrated that an ICV infusion of E<sub>2</sub> or BSA-E<sub>2</sub> increased ERK phosphorylation in the hippocampus within 5 minutes, and that the classical nuclear estrogen receptor antagonist ICI 182-780 did not block this effect (Kuroki et al., 2000). These *in vivo* data suggested that E<sub>2</sub> activates ERK via a non-genomic mechanism, which together with evidence demonstrating that hippocampal ERK activation is necessary for hippocampal memory formation, led us to hypothesize that hippocampal ERK activation was also necessary for E<sub>2</sub> to enhance hippocampal memory consolidation.

Using ovariectomized 8–12 week-old C57BL/6 mice, we found that systemic injection of 0.2 mg/kg E<sub>2</sub> increased phosphorylation of the p42, but not p44, isoform of ERK in the dorsal hippocampus 60 minutes later (Fernandez et al., 2008; Lewis et al., 2008). Similarly, bilateral dorsal hippocampal infusion of 5 µg/hemisphere or ICV infusion of 10 µg E<sub>2</sub> increased phosphorylation of p42, but not p44, ERK, although on a much more rapid time scale; both intracranial infusions increased phospho-p42 levels within 5 minutes of infusion (Fernandez et al., 2008; Fortress et al., 2013; Zhao et al., 2012; Zhao et al., 2010). Although these data were consistent with previous *in vitro* work, the key question was whether this E<sub>2</sub>-induced ERK activation contributed to E<sub>2</sub>-induced memory consolidation.



To address this issue, we allowed mice to accumulate 30 seconds exploring two identical objects in a square open field and then infused the MEK inhibitor U0126 into the dorsal hippocampus immediately before an ICV infusion of E<sub>2</sub>. We used a dose of U0126 that has no effect on memory on its own (Fernandez et al., 2008) to ensure that any potential blockade of E<sub>2</sub>'s effect on memory was due to an interaction between the two drugs and not a nonspecific effect of U0126 on general memory formation. Memory was then tested 48 hours later by measuring the amount of time spent with a novel object and an object identical to that explored during training (familiar) (Fig. 3A). At this 48-hour delay, vehicle-infused ovariectomized female mice do not remember the familiar object (Gresack et al., 2007), which allows us to observe potential memory-enhancing effects of E<sub>2</sub> and related compounds. In contrast to vehicle-treated mice, mice receiving post-training dorsal hippocampal or ICV infusions of E<sub>2</sub> spent more time exploring the novel object than chance (Fernandez et al., 2008; Fortress et al., 2013; Zhao et al., 2012; Zhao et al., 2010), indicating that E<sub>2</sub> enhances object recognition memory consolidation (Fig. 3B). Consistent with our hypothesis, U0126 prevented E<sub>2</sub> administered ICV (Fig. 3B) or systemically from enhancing object recognition (Fernandez et al., 2008; Fortress et al., 2013; Zhao et al., 2010), demonstrating that dorsal hippocampal ERK activation is necessary for E<sub>2</sub> to enhance hippocampal memory consolidation. These data were the first to identify a specific molecular pathway through which E<sub>2</sub> facilitates memory formation.

### PKA and PI3K

We next sought to build on these findings by examining the contributions of signaling pathways upstream and downstream from ERK. Numerous upstream cell-signaling molecules, including PKA, PI3K, and Akt, activate ERK (Fig. 2) (Adams and Sweatt, 2002). These molecules also play key roles in hippocampal memory formation, and are activated in the hippocampus by E<sub>2</sub> (Adams and Sweatt, 2002; Horwood et al., 2006; Kelly and Lynch, 2000; Lin et al., 2001; Manella and Brinton, 2006; Nguyen and Woo, 2003; Shingo and Kito, 2005; Yokomaku et al., 2003). Therefore, it was of interest to determine whether these enzymes were involved in E<sub>2</sub>'s regulation of hippocampal ERK and memory consolidation.

We first found that infusion of the PKA inhibitor Rp-cAMPS into the dorsal hippocampus prevented a post-training systemic injection of E<sub>2</sub> from enhancing object recognition memory in ovariectomized mice (Lewis et al., 2008). Next, we showed that E<sub>2</sub> significantly increased phosphorylation of PI3K and Akt in the dorsal hippocampus of young and middle-aged ovariectomized mice within 5 minutes of dorsal hippocampal or ICV infusion. In mice of both ages, dorsal hippocampal infusion of the PI3K inhibitor LY298002 prevented ICV-infused E<sub>2</sub> from phosphorylating p42 ERK and enhancing object recognition memory consolidation (Fan et al., 2010; Fortress et al., 2013). These data demonstrate not only that dorsal hippocampal PI3K activation is necessary for E<sub>2</sub> to facilitate object recognition memory consolidation (Fan et al., 2010; Fortress et al., 2013), but also that E<sub>2</sub> first activates PI3K before activating ERK.

### mTOR

Interestingly, both PI3K and ERK activate the mammalian target of rapamycin (mTOR) cell-signaling pathway (Fig. 2) (Hoeffler and Klann, 2010; Laplante and Sabatini, 2012;

Richter and Klann, 2009). mTOR stimulates local protein synthesis by phosphorylating key components of the protein synthesis machinery, such as p70 ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E-binding proteins (4E-BPs) (Hoeffler and Klann, 2010). Stimuli that induce long-term potentiation activate mTOR signaling in hippocampal dendrites (Cammalleri et al., 2003; Tsokas et al., 2005), and hippocampal infusions of the mTOR inhibitor rapamycin impair consolidation of object recognition, contextual fear, and spatial memories (Bekinschtein et al., 2007; Dash et al., 2006; Myskiw et al., 2008; Parsons et al., 2006).

Given the important role of mTOR in hippocampal memory consolidation, we reasoned that mTOR activation might contribute to E<sub>2</sub>-induced memory consolidation. Although dorsal hippocampal infusion of E<sub>2</sub> did not increase phosphorylation of mTOR itself, it did increase the phosphorylation of the downstream proteins S6K and 4E-BP1 (Fig. 4A,B) (Fortress et al., 2013), suggesting that E<sub>2</sub> activates mTOR signaling. These effects were blocked by dorsal hippocampal infusion of U0126, LY298002, and rapamycin (Fig. 4A,B) (Fortress et al., 2013), as would be expected given that PI3K, ERK, and mTOR lie upstream of S6K and 4E-BP1. Likewise, all three inhibitors prevented E<sub>2</sub> from increasing dorsal hippocampal p42 ERK phosphorylation (Fig. 4C) (Fortress et al., 2013). Although we expected the PI3K and ERK inhibitors to block E<sub>2</sub>-induced ERK phosphorylation given our previous data to this effect (Fan et al., 2010), it was surprising that rapamycin prevented E<sub>2</sub> from activating ERK. This interaction may be mediated by one of the two mTOR complexes that provide feedback regulating ERK and PI3K activity (Hoeffler and Klann, 2010; Klann and Dever, 2004). More importantly, rapamycin also prevented E<sub>2</sub> from enhancing object recognition memory consolidation (Fig. 4D) (Fortress et al., 2013), suggesting that dorsal hippocampal mTOR activation is necessary for E<sub>2</sub> to facilitate hippocampal memory formation. We have yet to determine exactly how mTOR regulates memory consolidation after E<sub>2</sub> treatment, but suspect that local protein synthesis within dendrites is involved. This hypothesis remains to be tested in future studies.

### ERK-driven epigenetic alterations

Before leaving the subject of cell signaling, it is worth noting that ERK activation results in numerous cellular effects beyond triggering mTOR signaling. As mentioned previously, ERK translocates into the nucleus to phosphorylate the transcription factor CREB, and E<sub>2</sub> facilitates this interaction in hippocampal neurons (Boulware et al., 2005) (Fig. 2). Thus, the E<sub>2</sub>-induced activation of ERK in the hippocampus likely leads to gene transcription. Numerous studies have shown that gene expression is altered in the hippocampus after E<sub>2</sub> treatment (Aenlle and Foster, 2010; Aenlle et al., 2009; Noriega et al., 2010; Pechenino and Frick, 2009), although it is unclear if this is a direct result of CREB phosphorylation.

Alterations in gene transcription may arise not only from manipulating activity of transcription factors but also by altering the epigenetic mechanisms that regulate access to DNA. Epigenetic processes, such as histone acetylation and DNA methylation, are now well-accepted regulators of memory formation in numerous brain regions including the hippocampus (for recent reviews, see (Day and Sweatt, 2010, 2011; Fischer et al., 2010; Gräff and Tsai, 2013; Jarome and Lubin, 2014; Peixoto and Abel, 2013; Sweatt, 2009)). In

brief, DNA is tightly coiled around a nucleosome complex consisting of four histone proteins (H2A, H2B, H3, H4; Fig. 2). Histone proteins can be altered by numerous post-translational modifications that either relax the bond between the DNA and histone proteins (thereby permitting access to the DNA and increasing gene transcription) or tighten the bond (thereby reducing access to the DNA and decreasing gene transcription) (Fortress and Frick, 2014). Histone acetylation generally increases gene transcription. Acetyl groups are added to histone proteins by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs). Therefore, histone acetylation state is the result of a dynamic balance between the activities of these two classes of enzymes.

Early evidence suggested that either contextual fear conditioning or ERK activation increased acetylation of the H3 histone protein in the hippocampus (Levenson et al., 2004). ERK activation was also found to be necessary for other protein kinases to increase hippocampal H3 acetylation (Levenson et al., 2004), suggesting a key role for ERK in regulating hippocampal histone acetylation. Given the importance of ERK in mediating the effects of E<sub>2</sub> on hippocampal memory consolidation, we thought that H3 acetylation might also be involved. In brief, we found that a dorsal hippocampal infusion of E<sub>2</sub> in ovariectomized mice significantly increases H3 acetylation in the dorsal hippocampus within 30 minutes, and this increase was blocked by U0126 (Zhao et al., 2010). These data suggest that the E<sub>2</sub>-induced increase in H3 acetylation depends on dorsal hippocampal ERK activation. We also found that E<sub>2</sub> decreases protein levels of histone deacetylase 2 (HDAC2) in the dorsal hippocampus (Zhao et al., 2010), which is consistent with the role of this enzyme as a negative modulator of hippocampal plasticity and memory formation (Guan et al., 2009). In a follow-up study, we used a histone acetyltransferase inhibitor to show that dorsal hippocampal histone acetylation is necessary for E<sub>2</sub> to enhance object recognition memory consolidation (Zhao et al., 2012). Collectively, these findings demonstrate the central importance of histone acetylation to the memory-enhancing effects of E<sub>2</sub>.

However, the specific genes regulated by E<sub>2</sub>-induced histone acetylation remain a mystery. Our most recent work has begun to shed some light on this subject (Fig. 2). Consistent with our findings from young ovariectomized females, we found in middle-aged ovariectomized female mice that E<sub>2</sub> selectively increased H3 acetylation (Fig. 5A) and decreased protein levels of HDAC2 (Fig. 5B) and another negative regulator of hippocampal memory, HDAC3 (Fig. 5C) (Fortress et al., 2014; McQuown et al., 2011). We then examined H3 acetylation of specific promoters of the gene for brain derived neurotrophic factor (BDNF), a trophic factor essential for hippocampal memory formation (Bekinschtein et al., 2014; Heldt et al., 2007). Of the nine *Bdnf* promoters, we measured H3 acetylation in promoters I, II, and IV because hippocampal learning and/or aging regulate H3 acetylation of these promoters (Fuchikami et al., 2010; Lubin et al., 2008; Perovic et al., 2013). We found that dorsal hippocampal infusion of E<sub>2</sub> increased H3 acetylation of *Bdnf* pII and pIV in the dorsal hippocampus of both young and middle-aged females relative to vehicle-treated age-matched controls (Fig. 5E) (Fortress et al., 2014). For pII, the magnitude of the increase was greater in young females (~3.5 fold) than in middle-aged females (~1.5 fold), but the increase in pIV acetylation was similar in both ages (~0.5 fold). Accordingly, dorsal hippocampal protein levels of both BDNF (Fig. 5D) and Pro-BDNF were increased by E<sub>2</sub>

infusion in middle-aged females (Fortress et al., 2014), indicating that E<sub>2</sub>-induced H3 acetylation stimulated an increase in BDNF protein translation. Although these data suggest that epigenetic regulation of *Bdnf* could be an important mechanism underlying the beneficial effects of E<sub>2</sub> on hippocampal memory, much more work needs to be done to better understand the myriad other genes that are likely involved.

Although our data suggest that histone acetylation is important for estrogenic regulation of memory, it is far from the only epigenetic process involved. Early evidence from our laboratory indicates that DNA methylation also plays a role. DNA methylation involves the preferential addition of methyl groups to cytosine nucleotides located adjacent to guanine nucleotides (so-called CpG islands). DNA methylation typically silences gene transcription, but has been reported to increase transcription (Chahrour et al., 2008). DNA methylation is catalyzed by three enzymes called DNA methyltransferases (DNMTs). DNMT1 is a maintenance methyltransferase that transfers existing methyl marks during DNA replication. DNMT3A and DNMT3B are *de novo* methyltransferases that add methyl marks to previously unmethylated cytosines. Initial studies showed that contextual fear conditioning increases DNMT3A and DNMT3B, but not DNMT1, gene expression in the male rat hippocampus (Miller and Sweatt, 2007), suggesting that hippocampal learning stimulates *de novo* DNA methylation. Accordingly, dorsal hippocampal infusion of the DNMT inhibitor 5-AZA blocks contextual fear memory (Miller et al., 2008; Miller and Sweatt, 2007), indicating an important role for DNA methylation in hippocampal memory formation. But if methylation suppresses gene expression, then how can it facilitate memory? The key is the specific genes that are methylated. For example, 5-AZA prevents methylation of the memory suppressor gene *PPI* (Miller and Sweatt, 2007), indicating that methylation of genes that negatively regulate memory promotes memory formation. Conversely, contextual fear conditioning demethylates the memory promoter gene *reelin* (Miller and Sweatt, 2007), suggesting that demethylation of genes that positively regulate memory also promotes memory formation. Together, these data support the notion that hippocampal memory can be facilitated via a combination of methylation of memory suppressing genes and demethylation of memory promoting genes.

With respect to E<sub>2</sub> and memory, we were interested in DNA methylation because of its involvement in hippocampal memory and because histone acetylation and DNA methylation are interactive processes (Miller et al., 2008; Zhao et al., 2012). We first asked whether E<sub>2</sub> regulated levels of the DNMT enzymes. We found in ovariectomized female mice that E<sub>2</sub> transiently increased mRNA for DNMT3A and DNMT3B 45 minutes after dorsal hippocampal infusion, whereas DNMT1 mRNA levels were unchanged up to 180 minutes after infusion (Zhao et al., 2012; Zhao et al., 2010). Moreover, DNMT3B protein levels were significantly increased 4 hours after dorsal hippocampal infusion of E<sub>2</sub> (Zhao et al., 2010), suggesting that E<sub>2</sub> increases *de novo* DNA methylation. To determine if this methylation was involved in estrogenic regulation of memory, we infused 5-AZA into the dorsal hippocampus and found that it prevented E<sub>2</sub> infused ICV from enhancing object recognition memory consolidation. This effect was limited to the 1–3 hour memory consolidation window, as delaying infusion of 5-AZA for three hours after training had no effect on memory consolidation. Together, these data demonstrate that DNA methylation is

essential for E<sub>2</sub> to facilitate object recognition memory consolidation. At this point, however, we do not know which genes are methylated or demethylated by E<sub>2</sub>, which is critical for better understanding how E<sub>2</sub> affects gene expression. Additional studies using more sophisticated analyses (e.g., bisulfite sequencing) will be necessary to address this issue.

## Estrogen receptors

Absent from the aforementioned discussion of E<sub>2</sub>'s effects on memory, cell signaling, and epigenetics has been any mention of the receptors that might mediate these effects. This omission results largely because this information remains unclear. Therefore, pinpointing the receptor mechanisms underlying E<sub>2</sub>'s effects on memory and hippocampal function is an important subject for future study. Numerous investigators have examined the role of ERs in regulating hippocampal memory (for recent reviews, see (Bean et al., 2014; Ervin et al., 2013; Tuscher et al., 2015)), and some of these data will be discussed here. But the picture has gotten more complex in recent years, as data have shown roles not only for ER $\alpha$ , ER $\beta$ , and GPER in mediating the mnemonic effects of E<sub>2</sub>, but also for several neurotransmitter receptors (see below).

## Genetic manipulations

The contributions of specific ERs to cellular functioning have been examined using numerous methods, including ER-specific knockouts, siRNAs, and viral vector-mediated delivery of ERs. Studies using ER $\alpha$  and ER $\beta$  knockouts (ER $\alpha$ KO, ER $\beta$ KO) suggest a role for both classical ERs in hippocampal memory. In the absence of exogenous E<sub>2</sub> treatment, ER $\alpha$ KO, but not ER $\beta$ KO, mice exhibit impaired spatial memory, suggesting a key role for ER $\alpha$  in mediating baseline memory function (Bean et al., 2014). This role is supported by data showing that lentiviral delivery of ER $\alpha$  to the hippocampus improved spatial memory in the Morris water maze among female ER $\alpha$ KO mice (Foster et al., 2008). However, E<sub>2</sub> can enhance spatial memory, inhibitory avoidance, and object recognition in ER $\alpha$ KO mice, but cannot enhance object recognition or object placement in ER $\beta$ KO mice (Frick et al., 2010; Fugger et al., 2000; Liu et al., 2008; Walf et al., 2008), suggesting an important role for ER $\beta$  in mediating at least some of the effects of exogenous E<sub>2</sub> treatment. Interestingly, lentiviral delivery of ER $\beta$  to the hippocampus of ER $\beta$ KO mice has been shown to impair spatial memory, possibly through interactions with ER $\alpha$  (Han et al., 2013).

With respect to membrane ERs, GPER knockouts exist and siRNA has been used to knock down GPER receptor levels in the hippocampus (Kajta et al., 2013; Langer et al., 2010; Ruiz-Palmero et al., 2013). However, the effects of these manipulations on memory have not yet been tested. One recent *in vitro* study used GPER siRNA to show that GPER is necessary for E<sub>2</sub> to induce neuriteogenesis and PI3K/Akt activation in primary hippocampal cultures (Ruiz-Palmero et al., 2013), suggesting that GPER may regulate some effects of E<sub>2</sub> in the hippocampus. Another *in vitro* study found that siRNAs for ER $\beta$  and GPER blocked the neuroprotective effects of a specific phytoestrogen in primary hippocampal cultures (Kajta et al., 2013). Although these data provide support for the notion that GPER mediates the effects of E<sub>2</sub> in the hippocampus, it is not clear whether GPER fulfills such a role *in vivo*.

## Pharmacological manipulations

Because relatively few studies have been conducted to assess the effects of the genetic ER manipulations described above on memory, conclusions to be drawn from these studies are limited. Fortunately, a more substantial *in vivo* pharmacological literature exists from which to base hypotheses about the role of individual ERs in hippocampal memory. These studies describe the effects of selective ER agonists and antagonists on hippocampal memory (Bean et al., 2014; Ervin et al., 2013; Tuscher et al., 2015). Of these compounds, the most commonly used agonists for ER $\alpha$  and ER $\beta$  are propyl pyrazole triol (PPT) and diarylpropionitrile (DPN), respectively (Meyers et al., 2001; Stauffer et al., 2000). Membrane ERs can be targeted generally with BSA-E<sub>2</sub>. Potential involvement of intracellular ERs in the effects of BSA-E<sub>2</sub> is often reduced by co-administration the ER antagonist ICI 182-780 (Wakeling and Bowler, 1992). However, ICI 182-780 can also act a GPER agonist (Thomas et al., 2005), so its effects can be difficult to interpret. GPER is targeted more specifically with the agonist G-1 and antagonists G-15 or G-36 (Bologa et al., 2006; Dennis et al., 2009; Dennis et al., 2011). It is important to note, however, that all ER drugs are relatively, not absolutely, specific for their targeted receptor, so care must be taken to use doses that are low enough to prevent non-specific binding to other ERs.

## Classical ERs: Behavioral effects and molecular mechanisms

The vast majority of studies using PPT and DPN have administered these compounds systemically, making it difficult to pinpoint their effects to the hippocampus. Moreover, effects appear to depend on dose, type of memory tested, and task difficulty (Phan et al., 2011; Tuscher et al., 2015). In general, systemic DPN administered pre- or post-training enhances object recognition and object placement memory in ovariectomized rats and mice (Frick et al., 2010; Jacome et al., 2010; Phan et al., 2011; Walf et al., 2008; Walf et al., 2006), although one study found no effect of systemic DPN on object placement in ovariectomized rats (Frye et al., 2007). Although some studies report that systemic PPT does not affect object recognition and object placement memory (Frick et al., 2010; Jacome et al., 2010), others report beneficial effects in these tasks and in a social recognition task (Frye et al., 2007; Phan et al., 2011; Walf et al., 2006).

Only two studies have thus far infused PPT and DPN into the brain, and the results appear to be strain dependent. In ovariectomized Swiss mice, post-training dorsal hippocampal infusion of PPT, but not DPN, enhanced object recognition memory (Pereira et al., 2014). Yet in the same study, either PPT or DPN enhanced object recognition memory in ovariectomized C57BL/6 mice (Pereira et al., 2014). Our own work in ovariectomized C57BL/6 mice has shown that post-training infusion of PPT or DPN into the dorsal hippocampus or dorsal third ventricle at picrogram doses enhanced both object recognition and object placement memory consolidation (Boulware et al., 2013). Additional support for the role of both classical ERs in C57BL/6 mice comes from preliminary studies in our laboratory showing that post-training dorsal hippocampal infusion of the ER $\alpha$  antagonist MPP (1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyloxy) phenol]-1H-pyrazole) or ER $\beta$  antagonist PHTPP (4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol) dose-dependently impairs object recognition and object placement memory consolidation (Kim et al., 2014). As such, there is some agreement among studies for

involvement by dorsal hippocampal ER $\alpha$  and ER $\beta$  in mediating object and spatial memory consolidation, at least in C57BL/6 mice.

Given these findings, we sought to identify the molecular mechanisms through which PPT and DPN may enhance object recognition and object placement memory in C57BL/6 mice. Of chief interest was whether these mechanisms were similar to those underlying E<sub>2</sub>'s effects on memory. Because intracranial infusion of E<sub>2</sub> so reliably increases dorsal hippocampal ERK activation (Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2013; Zhao et al., 2012; Zhao et al., 2010), we first measured the effects of intracranial PPT or DPN infusion on dorsal hippocampal ERK phosphorylation. As with E<sub>2</sub>, infusion of PPT or DPN into the dorsal hippocampus (0.1 pg/hemisphere and 10 pg/hemisphere, respectively) or dorsal third ventricle (0.2 pg and 20 pg, respectively) increased levels of phospho-p42 ERK, but not phospho-p44 ERK, in the dorsal hippocampus within 5 minutes of infusion (Boulware et al., 2013). The ERK activation inhibitor U0126 blocked this increase in p42 ERK phosphorylation, as well as the enhanced object recognition and object placement memory consolidation induced by PPT and DPN (Boulware et al., 2013). These findings demonstrate that either ER $\alpha$  or ER $\beta$  can regulate object and spatial memory consolidation by rapidly activating dorsal hippocampal ERK signaling. As such, these data mirror the effects of dorsal hippocampal E<sub>2</sub> infusion (Fernandez et al., 2008).

The importance of ERK in mediating the effects of ER $\alpha$  and ER $\beta$  *in vivo* were also consistent with findings from cultured hippocampal primary neurons showing that membrane-localized ER $\alpha$  and ER $\beta$  regulate CREB by activating ERK and metabotropic glutamate receptors (mGluRs), including mGluR1a (Boulware et al., 2005). Therefore, we hypothesized that mGluR1a might play a role in the effects of both E<sub>2</sub> and the ER agonists. Indeed, we found that dorsal hippocampal infusion of the mGluR1a antagonist LY367385 prevented ICV-infused E<sub>2</sub>, PPT, or DPN from increasing p42 ERK phosphorylation in the dorsal hippocampus and from enhancing object recognition and object placement memory consolidation in ovariectomized C57BL/6 females (Fig. 6) (Boulware et al., 2013). Moreover, sucrose fractionation and coimmunoprecipitation experiments showed that ER $\alpha$ , ER $\beta$ , mGluR1, and ERK physically interact within hippocampal detergent-resistant membranes *in vivo* (Boulware et al., 2013). These data suggest that E<sub>2</sub>-induced hippocampal memory consolidation is regulated by interactions among ER $\alpha$ , ER $\beta$ , and mGluR1a at the membrane, which then rapidly trigger activation of ERK signaling. These data provide a putative mechanism for how intracellular ERs could rapidly activate cell-signaling pathways that are typically associated with G-protein-coupled receptors, and raise the intriguing possibility that ER $\alpha$  and ER $\beta$  may interact with many other receptors, including other neurotransmitter and G-protein-coupled receptors, to regulate memory.

### Membrane ERs: Behavioral effects and molecular mechanisms

The contributions of membrane-bound ERs to estrogenic regulation of memory have been difficult to test because these receptors have not been clearly identified. As mentioned earlier, these receptors can be targeted in a very general way by conjugating the large bovine serum albumin molecule to E<sub>2</sub>, which restricts its activity to the plasma membrane. *In vitro*, BSA-E<sub>2</sub> promotes synaptic plasticity, ERK translocation into the nucleus, and

neuroprotection in hippocampal CA1 pyramidal neurons (Carrer et al., 2003; Gu and Moss, 1998; Huang et al., 2004; Tang et al., 2014; Wu et al., 2011; Yang et al., 2010). In ovariectomized C57BL/6 mice, we found that dorsal hippocampal infusion of BSA-E<sub>2</sub> mimicked exactly the effects of dorsal hippocampal E<sub>2</sub> infusion. That is, dorsal hippocampal or ICV infusion of BSA-E<sub>2</sub> increased dorsal hippocampal p42 ERK phosphorylation within 5 minutes and enhanced object recognition memory consolidation (Fernandez et al., 2008). Furthermore, as with E<sub>2</sub>, ERK activation was necessary for BSA-E<sub>2</sub> to enhance object recognition memory consolidation (Fernandez et al., 2008). These data suggested that membrane-bound ERs might mediate the effects of E<sub>2</sub> on memory and cell signaling independent of intracellular ERs. However, the identity of the receptor(s) underlying these effects remains unclear.

Interestingly, emerging data suggest that the membrane ER GPER may *not* play a role in the memory-enhancing effects of E<sub>2</sub>. GPER does appear to influence hippocampal memory formation and its effects on hippocampal memory are similar to those of E<sub>2</sub>, PPT, and DPN (Hammond et al., 2009). Studies investigating the role of GPER in spatial working memory have shown that systemic administration of the GPER agonist G-1 enhances, whereas the GPER antagonist G-15 impairs, acquisition of a delayed match-to-position task (Hammond et al., 2009; Hammond et al., 2012). Similarly, our own preliminary data indicate that post-training dorsal hippocampal infusion of G-1 enhances, whereas G-15 impairs, object recognition and object placement memory consolidation in ovariectomized mice (Kim et al., 2013). However, the molecular mechanisms through which this regulation occurs appear to differ from those of E<sub>2</sub>. Whereas E<sub>2</sub> enhances memory by activating ERK, G-1 did not (Kim et al., 2013). Rather, the ability of G-1 to enhance memory consolidation in both object tasks depended instead on rapid activation of c-Jun N-terminal kinase (JNK) signaling (Kim et al., 2013). Moreover, the ability of E<sub>2</sub> to activate ERK and enhance object recognition and object placement memory consolidation was not dependent on JNK or G-1 activation, as dorsal hippocampal infusion of G-15 or the JNK inhibitor SP600125 did not block the effects of ICV-infused E<sub>2</sub> (Kim et al., 2013). These data are consistent with a new report showing that the ability of E<sub>2</sub> to increase dendritic spine density in hippocampal slices from male rats is blocked by inhibitors of many cell-signaling pathways (including ERK, PI3K, and PKA), but not by JNK inhibitors (Hasegawa et al., in press). Together, these data suggest that GPER facilitates memory consolidation in the dorsal hippocampus via a different cell-signaling mechanism than E<sub>2</sub>. This conclusion is supported by other work showing that G-1 does not rapidly increase Akt phosphorylation in the dorsal hippocampus, unlike E<sub>2</sub>, which activates Akt between 5 and 30 minutes after treatment (Akama and McEwen, 2003; Fortress et al., 2013; Waters et al., 2015). These findings beg the question of whether GPER really functions as an ER in the dorsal hippocampus. Whether GPER is a true ER has been the subject of intensive debate (Langer et al., 2010; Levin, 2009), and studies such as these are likely to fuel continued conversation. But if E<sub>2</sub> is not the endogenous ligand for GPER in the dorsal hippocampus, then what is? At this point, the answer is unknown, which highlights the need for much more research to better understand the molecular mechanisms through which GPER regulates hippocampal memory.



## Conclusions and future directions

The field has made considerable progress since the 1998 special issue of *Hormones and Behavior* on estrogens and cognition (Volume 34(2), October). We have learned a considerable amount in recent years about the molecular and cellular mechanisms through which E<sub>2</sub> regulates hippocampal morphology, synaptic plasticity, and memory. Many of these findings have challenged long held dogmas about how sex steroid hormones regulate neural and cellular function. For example, the discovery that E<sub>2</sub> and other sex steroid hormones are made within the hippocampus raises important questions about how *de novo* hormone synthesis regulates acute behavioral events such as a learning episode. In addition, the identification of non-classical effects of E<sub>2</sub> and other hormones has allowed memory researchers to tap into the vast neurobiology of memory literature for clues about the rapid molecular mechanisms through which these hormones might regulate memory formation. Of course, much more work is necessary to fully understand how a simple hormonal signal leads to a memory representation in the brain, but the work of the past two decades has given us a great start.

As we look to the future, many issues of importance emerge as areas for further study. For instance, hippocampally-synthesized E<sub>2</sub> is likely a major player in memory formation, so we must better understand the role of *de novo* E<sub>2</sub> synthesis in regulating memory. In addition, future work should look beyond E<sub>2</sub> and the hippocampus to examine interactions with other brain regions and other hormones. In particular, examining the effects of E<sub>2</sub> on memory mediated by other brain regions (e.g., prefrontal cortex, amygdala, perirhinal cortex, entorhinal cortex, and striatum) will provide a considerably more comprehensive view of how E<sub>2</sub> regulates learning and memory than has been provided to date by the field's primary focus on hippocampal-dependent memory tasks.

Finally, expanding our knowledge about the receptor, cell-signaling, and epigenetic mechanisms that mediate the gene expression and protein synthesis alterations that underlie the morphological, physiological, and behavioral effects of E<sub>2</sub> will also be essential to understanding why women are at greater risk than men of developing psychiatric and neurodegenerative diseases such as mood and anxiety disorders, and Alzheimer's disease (Kessler et al., 2005; Yaffe et al., 2007; Zandi et al., 2002). Although low estrogen levels have been implicated in elevated risks of these conditions among women (Graham and Milad, 2013; Meinhard et al., 2013; Milad et al., 2010; Yaffe et al., 2007), estrogen therapies are not attractive treatment options because of harmful side effects associated with such treatment, particularly breast cancer, heart disease, and stroke in women over age 65 (Rossouw et al., 2002). Because ERs are so widely distributed in tissues throughout female body, drugs that target ERs are almost certain to bind ERs in peripheral tissues and produce unintended consequences. Therefore, new avenues of research are needed to provide novel drugs that specifically target the cognition-enhancing mechanisms that lie downstream from ERs (Frick, 2012; Frick et al., 2010). For example, by identifying the cellular and molecular mechanisms downstream from ERs through which E<sub>2</sub> enhances memory, we may uncover new molecular targets for drug development that provide the mnemonic benefits of E<sub>2</sub> without detrimental side effects (Frick, 2012; Frick et al., 2010). Indeed, shifting the focus of drug discovery from finding better selective ER modulators to identifying novel

compounds that target rapid events downstream from ERs could lead to a new generation of drugs for reducing cognitive dysfunction in women.

Although we have much more work to do to fully understand how E<sub>2</sub> and other sex steroid hormones regulate cognition, recent advances pinpointing the neural mechanisms underlying the memory-enhancing effects of E<sub>2</sub> provide the hope that such drugs might become available in the near future. Fortunately, there are many investigators interested in studying these mechanisms at various levels of analysis, and so the coming years should provide a wealth of extraordinary new data. Undoubtedly, this work will make for another exceptional special issue of *Hormones and Behavior* in the future.

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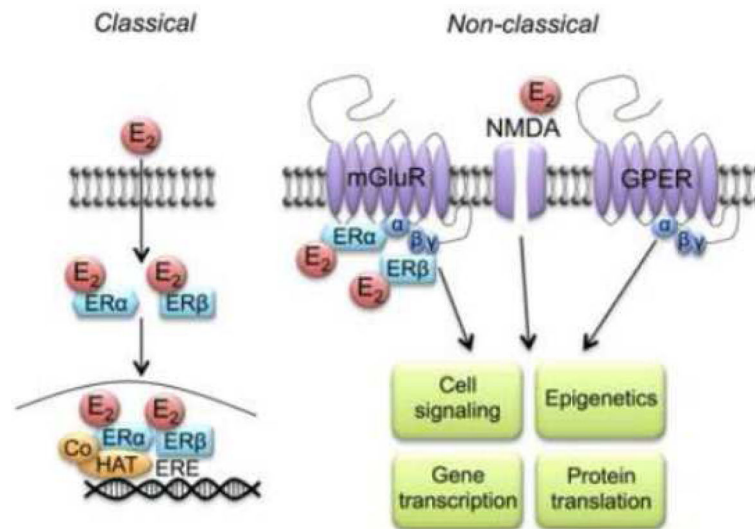
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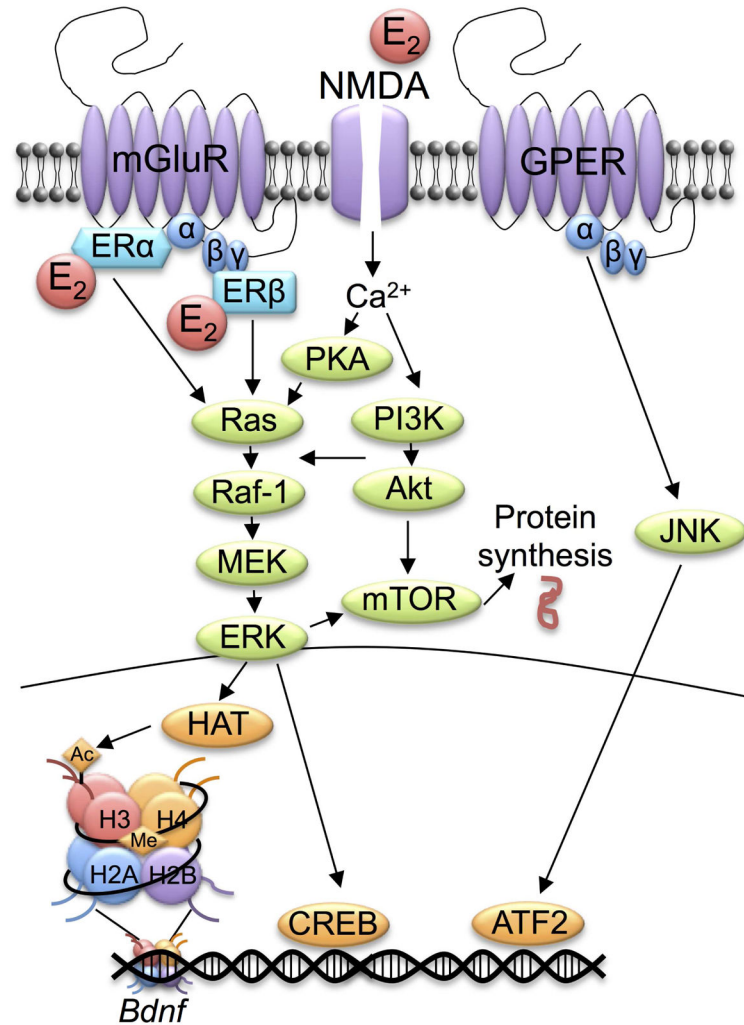
### Highlights

- 17 $\beta$ -Estradiol (E<sub>2</sub>) rapidly enhances hippocampal memory consolidation.
- Numerous molecular changes are necessary for E<sub>2</sub> to enhance hippocampal memory.
- The receptors mediating E<sub>2</sub>'s effects on memory are not well understood.
- This review discusses the molecular mechanisms through which E<sub>2</sub> enhances memory.

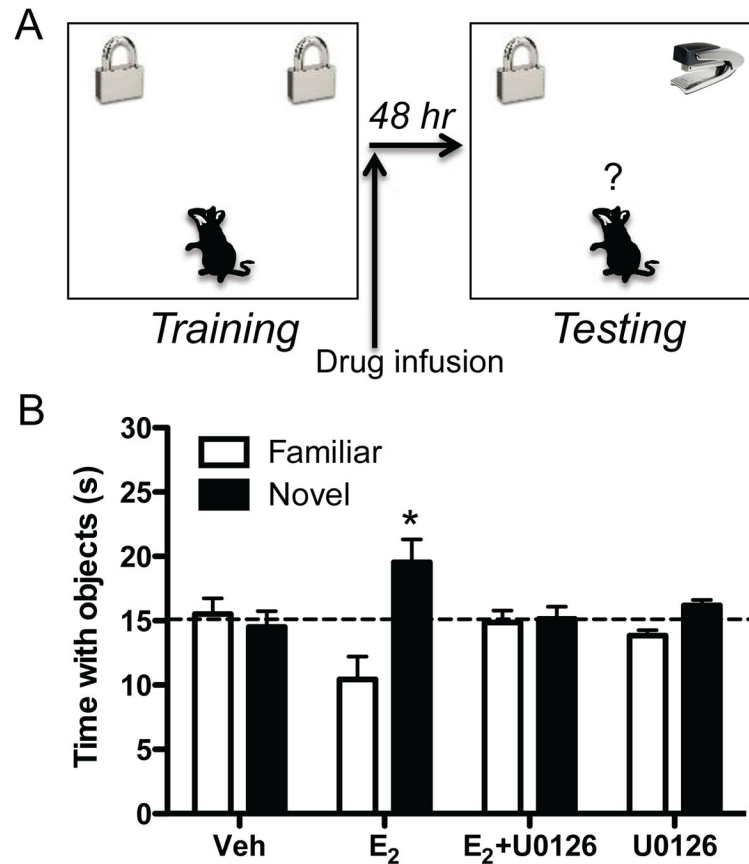


**Figure 1.**

Classical (genomic) and non-classical (non-genomic) mechanisms of E<sub>2</sub> action. In the classical mechanism (left), E<sub>2</sub> binds to ERα and ERβ in the cytoplasm, and then the E<sub>2</sub>-ER complex translocates into the nucleus and binds to an estrogen response element (ERE) on the DNA. Together with histone acetyltransferases (HAT) and other co-regulators (Co), the ERs facilitate gene transcription. Non-classical mechanisms (right) involve action at or near the plasma membrane. ERα and ERβ in the dorsal hippocampus interact with metabotropic glutamate receptor 1a (mGluR1a) to rapidly activate extracellular signal-regulated kinase (ERK) cell signaling, which triggers epigenetic alterations such as histone acetylation, local protein synthesis via the mammalian target of rapamycin (mTOR) cell-signaling pathway, and gene expression via the transcription factor cAMP response element binding protein (CREB). NMDA receptor activation in the dorsal hippocampus is also necessary for E<sub>2</sub> to activate ERK. G-protein-coupled estrogen receptor (GPER) rapidly activates c-Jun N-terminal kinase (JNK) cell signaling in the dorsal hippocampus, although E<sub>2</sub> does not appear to mediate this effect. Effects of GPER activation on epigenetic processes, gene expression, and protein translation are not yet known.

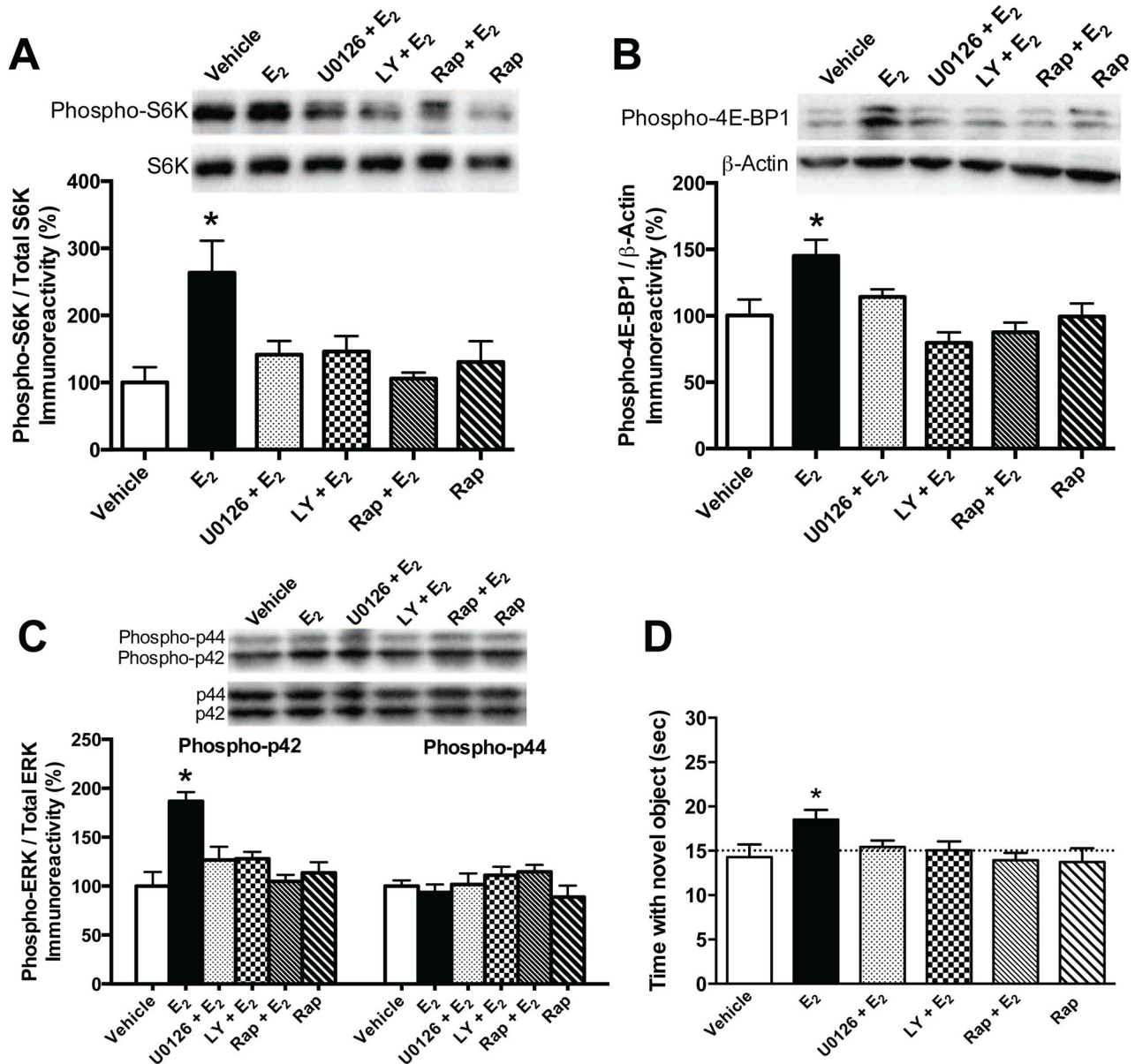


**Figure 2.** Schematic illustration of the non-classical mechanisms required for E<sub>2</sub> and ERs to enhance hippocampal memory consolidation. Phosphorylation of the p42 isoform of ERK is necessary for E<sub>2</sub> to enhance object recognition memory consolidation. This phosphorylation is triggered by numerous upstream events including interactions between mGluR1a and the canonical ERs (ERα and ERβ), and activation of NMDA receptors, protein kinase A (PKA), and phosphatidylinositol-3-kinase (PI3K). E<sub>2</sub>-induced phosphorylation of ERK, PI3K, and Akt elicits mTOR signaling, promoting local protein synthesis. E<sub>2</sub>-activated ERK also transduces into the nucleus to phosphorylate the transcription factor CREB. Activation of ERK is also necessary for E<sub>2</sub> to increase histone H3 acetylation; E<sub>2</sub> increases H3 acetylation at the pII and pIV promoters of the *Bdnf* gene. DNA methylation is also necessary for E<sub>2</sub> to enhance memory consolidation, although the specific genes methylated are unknown. Finally, GPER enhances memory consolidation by activating JNK, which facilitates gene expression via transcription factors such as ATF2.



**Figure 3.** Dorsal hippocampal ERK activation is necessary for E<sub>2</sub> to enhance object recognition memory consolidation. (A) Illustration of the object recognition protocol used in our laboratory. During training, mice accumulate 30 seconds of time exploring two identical objects. Immediately after training, mice receive an infusion of E<sub>2</sub> or other drugs into the dorsal hippocampus or dorsal third ventricle. Forty-eight hours later, mice accumulate 30 seconds with a novel object and an object identical to that explored during testing (familiar). Mice that remember the familiar object spend significantly more time than chance (15 sec) with the novel object. Adapted with permission from (Fortress and Frick, 2014; Frick, 2013). (B) ERK activation is necessary for E<sub>2</sub> to enhance object recognition memory consolidation in ovariectomized 8–12 week-old C57BL/6 mice. Forty-eight hours after training, mice infused with 10 μg E<sub>2</sub>, but not vehicle, into the dorsal third ventricle spend significantly more time than chance (dashed line at 15 sec) with the novel object (\**p* < 0.05), demonstrating enhanced object recognition. This effect is blocked by dorsal hippocampal infusion of the ERK activation inhibitor U0126 (0.5 μg/hemisphere), which has no detrimental effect on memory on its own at this dose (Fernandez et al., 2008). Error bars represent the mean ± standard error of the mean (SEM). Reprinted with permission from (Zhao et al., 2010).





**Figure 4.**

Dorsal hippocampal mTOR activation is necessary for E<sub>2</sub> to enhance object recognition memory consolidation. The phosphorylation of S6K (A), 4E-BP1 (B), and p42 ERK (C) were significantly increased in young ovariectomized mice 5 minutes after bilateral dorsal hippocampal infusion of 5  $\mu$ g/hemisphere E<sub>2</sub> ( $*p < 0.05$  relative to vehicle). This effect was blocked by the ERK inhibitor U0126 (0.5  $\mu$ g/hemisphere), the PI3K inhibitor LY298002 (0.005  $\mu$ g/hemisphere), or the mTOR inhibitor rapamycin (0.25  $\mu$ g/hemisphere) (A–C). (D) All three inhibitors also prevented E<sub>2</sub> from enhancing object recognition memory consolidation, as indicated by the fact that only mice infused with E<sub>2</sub> + vehicle spent more time than chance with the novel object ( $*p < 0.05$ ). Error bars in all panels represent the mean  $\pm$  standard error of the mean (SEM). Phosphorylated proteins were normalized to total

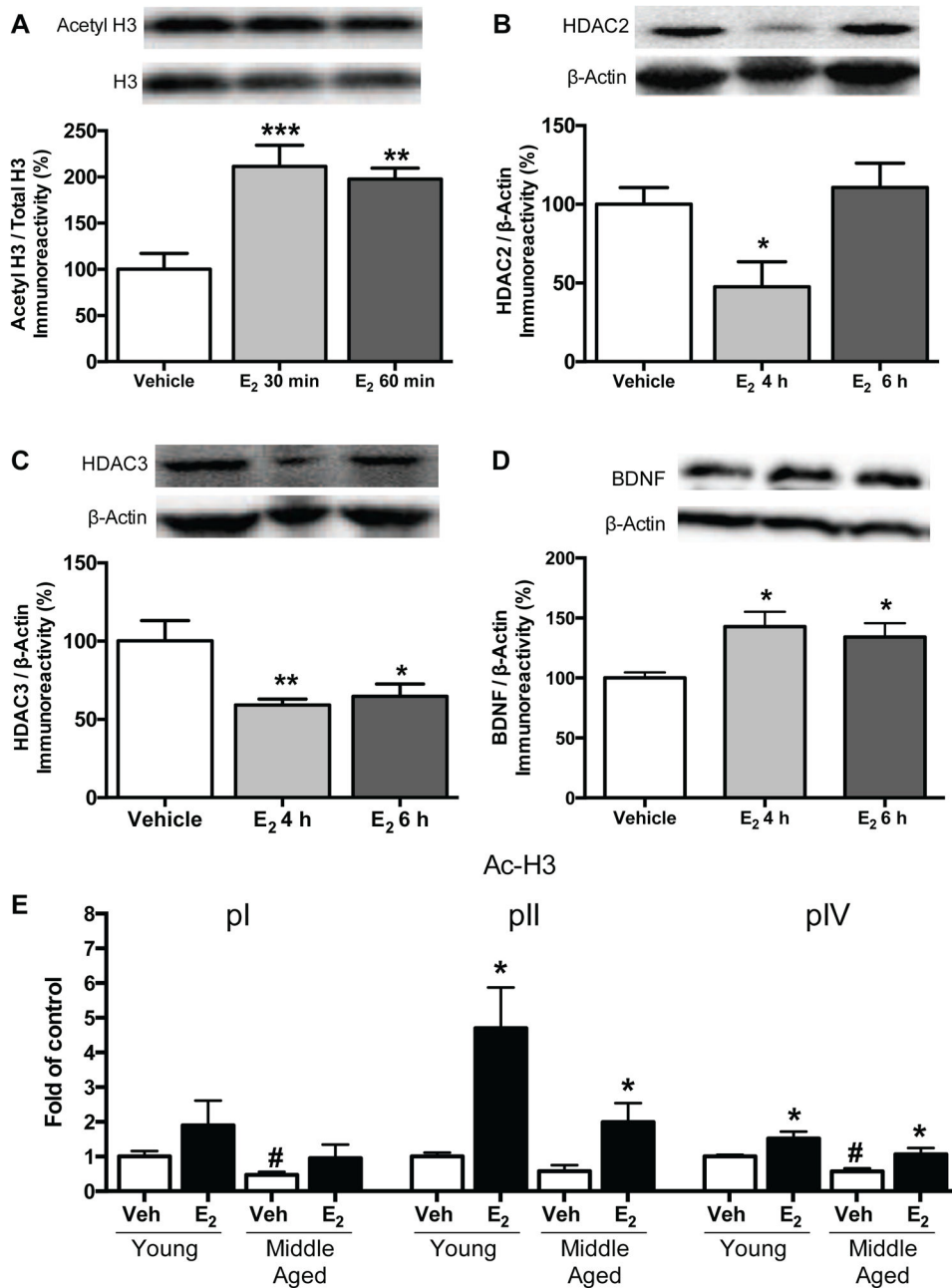
protein or  $\beta$ -actin. Insets are representative Western blots of phosphorylated and total protein. Adapted with permission from (Fortress et al., 2013).

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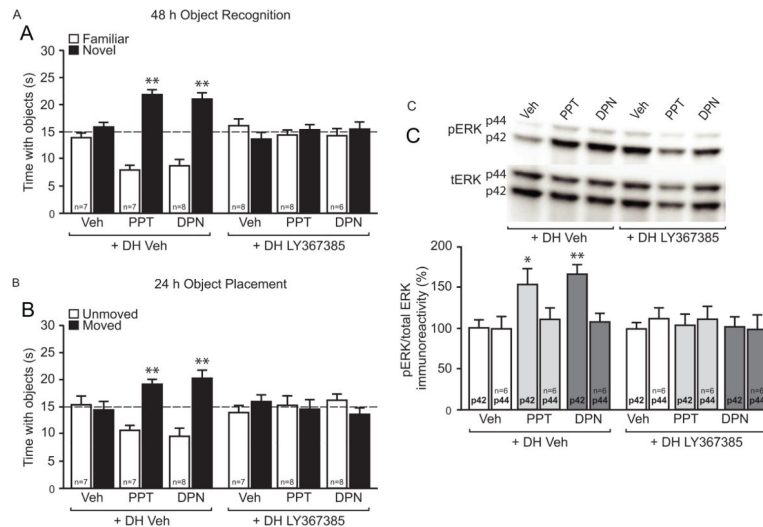
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**Figure 5.** E<sub>2</sub> facilitates dorsal hippocampal histone acetylation and BDNF protein expression in middle-aged ovariectomized mice. (A) Bilateral dorsal hippocampal infusion of 5 μg/ hemisphere E<sub>2</sub> increased H3 acetylation 30 and 60 min later ( $***p < 0.001$ ,  $**p < 0.01$  relative to vehicle). Acetylated H3 protein was normalized to total H3 protein. Insets are representative Western blots of acetylated and total histone protein. (B,C) Hippocampal infusion of E<sub>2</sub> decreased protein levels of HDAC2 (B) four hours after infusion, and of HDAC3 (C) four and six hours after infusion ( $**p < 0.01$ ,  $*p < 0.05$  relative to vehicle). (D) Conversely, BDNF protein levels were increased in the dorsal hippocampus four and six

hours after bilateral dorsal hippocampal infusion of E<sub>2</sub> (\**p* < 0.05 relative to vehicle). HDAC and BDNF proteins in panels B–D were normalized to β-actin protein. Insets are representative Western blots of protein and β-actin. (E) Chromatin immunoprecipitation analysis showed that hippocampal E<sub>2</sub> infusion increased acetylation of *Bdnf* promoters pII and pIV in both young and middle-aged ovariectomized mice (\**p* < 0.05 relative to age-matched controls). Among vehicle-infused controls, acetylation of pI and pIV was significantly lower in middle-aged females than in young females (#*p* < 0.05 relative to young vehicle-infused controls), indicating an age-related reduction in acetylation of these promoters. Data were normalized to LINE1 for each sample and then normalized to young vehicle-infused mice for each promoter region and represented as fold of control. For all panels, each bar represents the mean ± SEM. Adapted with permission from (Fortress et al., 2014).



**Figure 6.** Dorsal hippocampal mGluR1a activation is necessary for ER $\alpha$  and ER $\beta$  to enhance object recognition and object placement memory consolidation, and to increase dorsal hippocampal ERK phosphorylation. (A,B) Immediate post-training infusion of PPT (0.2  $\mu$ g) or DPN (20  $\mu$ g) into the dorsal third ventricle significantly increased the time spent with the novel object (A) and moved object (B) relative to chance (dashed line at 15 s, \*\* $p < 0.01$ ); these effects were blocked by dorsal hippocampal infusion of the mGluR1a antagonist LY367385 (10  $\mu$ g/hemisphere). Bars represent the mean  $\pm$  SEM time spent with each object. (C) Five min after infusion, PPT and DPN significantly increased phospho-p42 ERK levels (\* $p < 0.05$ ; \*\* $p < 0.01$  relative to vehicle); this effect was completely abolished by dorsal hippocampal infusion of LY367385. Bars represent mean  $\pm$  SEM % change from vehicle. Reprinted with permission from (Boulware et al., 2013).