Hormones and Behavior 74 (2015) 4–18

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Molecular mechanisms underlying the memory-enhancing effects of estradiol

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ARTICLE INFO

Available online 8 May 2015

Keywords:
Estrogen
Hippocampus
Estrogen receptor
Cell signaling
Epigenetic
ERK
mTOR
Histone acetylation
DNA methylation
GPER

ABSTRACT

This article is part of a Special Issue "Estradiol and cognition".

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 17β-estradiol (E_2) regulates hippocampal plasticity and memory. Recent research has demonstrated that rapid effects of E_2 on hippocampal cell signaling, epigenetic processes, and local protein synthesis are necessary for E_2 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 ERα and ERβ, 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_2 in regulating hippocampal memory formation. The present review discusses these findings in detail and suggests avenues for future study.

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 of 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 scientific bible of sorts for me. I marked it up, referred to it often, and carried it with me on faculty job interviews as an endocrinological 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 17β-estradiol (E_2) regulates hippocampal memory consolidation in female mice. These mechanisms underlie the so-called "rapid" effects of E_2 on hippocampal functioning, which encompass those that occur within minutes of E_2 exposure. *In vivo*, E_2 activates numerous cell-signaling cascades and alters epigenetic processes in the dorsal hippocampus within 5–30 min of treatment, and these actions are necessary for E_2 to enhance the consolidation of hippocampal memories (Bi et al., 2000, 2001; Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2013; Lewis et al., 2008; Zhao and Brinton, 2007; Zhao et al., 2010, 2012). *In vitro* studies report that rapid E_2-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_2 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_2 synthesis by hippocampal neurons may stimulate the rapid molecular alterations that are necessary for memory formation. Given the emerging importance of rapid E_2 effects

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http://dx.doi.org/10.1016/j.yhbeh.2015.05.001
0018-506X/

for hippocampal memory, this review will focus largely on findings detailing the rapid cell signaling, epigenetic, and receptor mechanisms necessary for E_2 to enhance hippocampal memory consolidation.

E_2 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_2 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_2 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_2 clearly promotes spinogenesis in multiple regions of the brain that regulate cognitive function. However, much less is known about the role of E_2 in mediating the function of brain regions other than the hippocampus. Within the hippocampus, dendritic spinogenesis is accompanied by the E_2-induced facilitation of synaptic plasticity. For example, E_2 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_2 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_2-induced enhancements in both object recognition and LTP occur within a similar time frame and require a functional increase in NR2B-containing NMDA receptors (Vedder et al., 2013), linking E_2-induced changes in LTP with hippocampal memory formation. Interestingly, the induction of LTP and spinogenesis by E_2 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_2 on hippocampal synaptic plasticity are regulated by many of the rapid molecular alterations that are also necessary for E_2 to enhance memory consolidation (see Cell-signaling mechanisms necessary for E_2-induced memory enhancement below).

A substantial literature shows that E_2 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; Glasper and Gould, 2013). Interestingly, the effects of E_2 on hippocampal neurogenesis appear to depend on timing of treatment, as E_2 given prior to the labeling of new neurons increases neuron proliferation and survival (McClure et al., 2013), whereas E_2 given after cell labeling decreases new neuron survival (Chan et al., 2014). These data suggest that E_2 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_2 regulate hippocampal morphology and synaptic plasticity? The canonical intracellular estrogen receptors, ERα and ERβ, are distributed throughout the dorsal–ventral extent of the hippocampus (Mitra et al., 2003; Mitterling et al., 2010; Shughrue et al., 1997a,b; Shughrue and Merchenthaler, 2000). Within hippocampal neurons, ERα and ERβ are located within the nucleus, dendritic spines, and axon terminals of pyramidal neurons and interneurons (Milner et al., 2001, 2005; Mitra et al., 2003; Mitterling et al., 2010; Waters et al., 2011). In the nucleus, ERα and ERβ 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 ERα and ERβ at these more distal sites; this mechanism is commonly referred to as "non-genomic" or "non-classical" (Fig. 1). Indeed, E_2 causes ERβ to translocate to the plasma membrane in hippocampal-derived cell lines and rat primary cortical neurons (Sheldahl et al., 2008). Moreover, both ERα and ERβ 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, 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 ERα and ERβ can be shuttled to the plasma membrane to trigger rapid cell-signaling processes. Other data implicate NMDA receptor activation in E_2-induced phosphorylation 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_2. As will be discussed below, E_2 activates numerous other cell-signaling cascades in hippocampal and cortical neurons within 5 min 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_2 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 ERα and ERβ, 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_2 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_2 (BSA-E_2) that cannot traverse the plasma membrane have demonstrated that BSA-E_2 rapidly activates signaling cascades including ERK, and induces similar ERK-dependent enhancement of object recognition memory consolidation to that observed with free E_2 (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_2, 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, 2012). GPER can be found in all lamina within the hippocampus, where it is located exclusively at extranuclear sites within pyramidal neurons, interneurons, and glia (Brailoiu et al., 2007; Waters et al.,

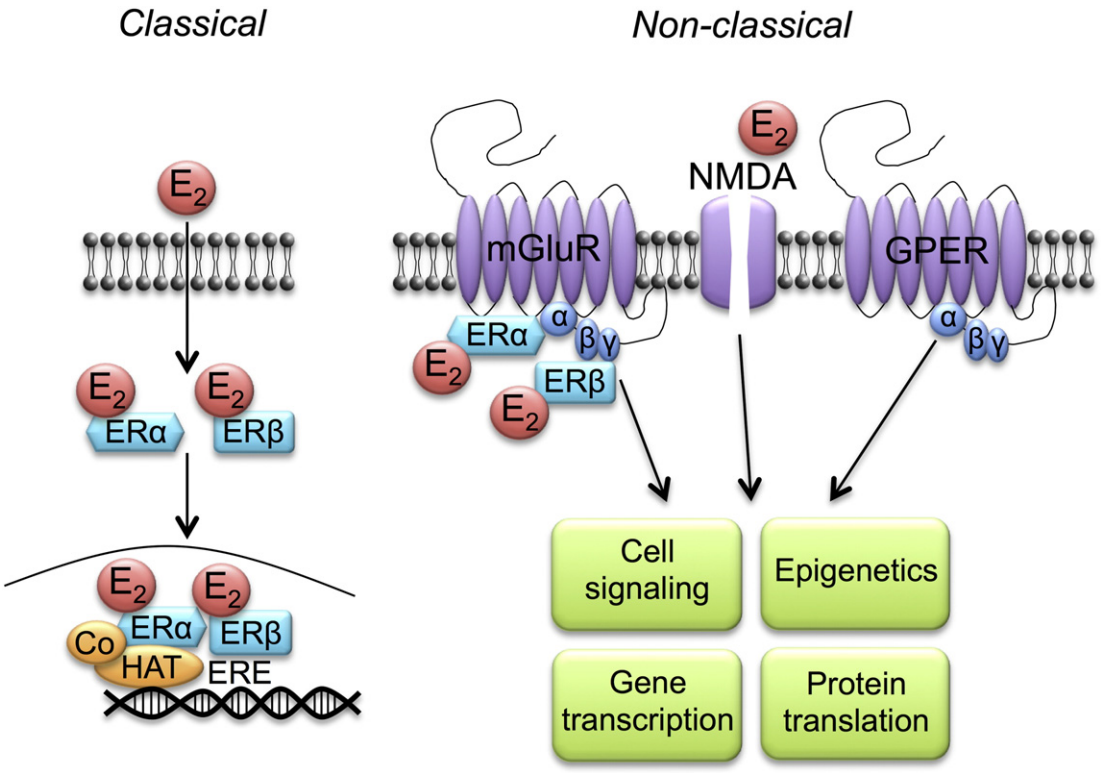

Fig. 1. Classical (genomic) and non-classical (non-genomic) mechanisms of E_2 action. In the classical mechanism (left), E_2 binds to ERα and ERβ in the cytoplasm, and then the E_2–ER complex translocates into the nucleus and binds to an estrogen response element (ERE) on the DNA. Together with histone 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_2 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_2 does not appear to mediate this effect. Effects of GPER activation on epigenetic processes, gene expression, and protein translation are not yet known.

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 postsynaptic 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 appear to differ from those of E_2.

Hippocampal E_2 synthesis

As indicated above, it is important to note that E_2 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_2 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_2 levels are significantly higher than plasma levels at every stage of the estrous cycle and after ovariectomy (Kato et al., 2013). Hippocampal E_2 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_2 levels during most of the estrous cycle. These findings suggest that the primary endogenous source of E_2 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_2 and its stereoisomer 17α-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_2 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 ERα agonist increased, whereas an ERβ 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_2 in regulating hippocampal spinogenesis.

Clues to the behavioral significance of *de novo* hippocampal E_2 synthesis come from studies of zebra finches, which report that E_2 is rapidly synthesized in the auditory caudo-medial nidopallium (NCM) of males during behavioral experiences such as exposure to female conspecifics or conspecific song (Remage-Healey et al., 2008). Our own preliminary data in female mice indicate that object learning stimulates dorsal hippocampal E_2 release within 30 min (Tuscher et al., 2013). Importantly, studies using aromatase inhibitors show that blocking hippocampal E_2 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_2 levels 30 min after training, an effect that is blocked by letrozole (Tuscher et al., 2013). A role for local E_2 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_2 in response to a learning event, and that this synthesis is necessary for hippocampal memory formation.

How might a role for hippocampally-synthesized E_2 be reconciled with the literature demonstrating that ovarian-synthesized E_2 influences hippocampal memory? At this point, the relative contributions of these two sources of E_2 to memory formation are unclear. Ovarian-derived steroids may prime the hippocampus and other brain regions to respond to locally-synthesized E_2. As such, E_2 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_2 levels are elevated. In this scenario, one might predict that aromatase inhibition would be most disruptive to memory consolidation during proestrus, when circulating E_2 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_2 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_2 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_2 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_2. Although ovariectomy reduces one potential interaction with hippocampal-derived E_2, it is unknown how exogenous E_2 treatments might affect the synthesis of E_2 within the hippocampus. Addressing these potential interactions will be an important issue for future study.

E_2 and hippocampal memory

Given the ample evidence demonstrating that E_2 regulates hippocampal morphology and physiology, it should be no surprise that E_2 also influences hippocampal memory. The effects of E_2 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_2 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_2 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_2 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_2 is beneficial for hippocampal memory in both female and male rodents.

Post-training E_2 treatment

In recent years, numerous laboratories have reported that a single post-training administration of E_2 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_2 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_2 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_2 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_2 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_2. In particular, we administer dorsal hippocampal or dorsal third ventricle infusions of a water-soluble form of E_2 immediately after training in one-trial object recognition and object placement tasks to pinpoint the molecular mechanisms within the dorsal hippocampus through which E_2 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_2 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_2 to enhance hippocampal memory consolidation. This approach requires the implantation of triple cannulae, which permits 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_2 infusion. Infusing E_2 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_2 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. Fig. 2 presents a schematic synthesis of our laboratory's findings to provide a guide for the discussion below.

Cell-signaling mechanisms necessary for E_2-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

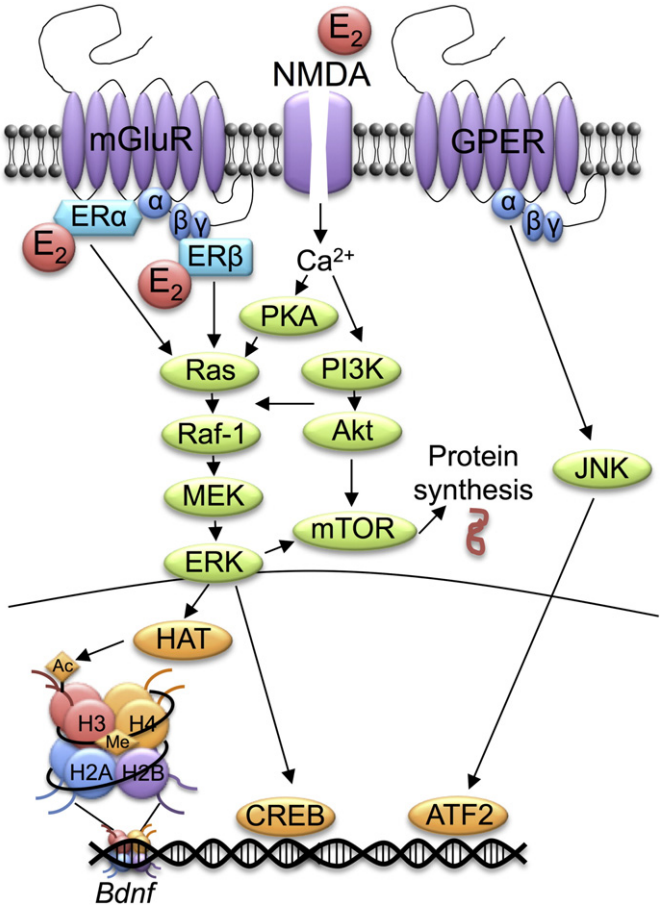

Fig. 2. Schematic illustration of the non-classical mechanisms required for E_2 and ERs to enhance hippocampal memory consolidation. Phosphorylation of the p42 isoform of ERK is necessary for E_2 to enhance object recognition memory consolidation. This phosphorylation is triggered by numerous upstream 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_2-induced phosphorylation of ERK, PI3K, and Akt elicits mTOR signaling, promoting local protein synthesis. E_2-activated ERK also transduces into the nucleus to phosphorylate the transcription factor CREB. Activation of ERK is also necessary for E_2 to increase histone H3 acetylation; E_2 increases H3 acetylation at the pII and pIV promoters of the *Bdnf* gene. DNA methylation is also necessary for E_2 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.

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_2 or BSA-E_2 increased ERK phosphorylation within 15 min 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_2 or BSA-E_2 increased ERK phosphorylation in the hippocampus within 5 min, 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_2 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_2 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_2 increased phosphorylation of the p42, but not p44, isoform of ERK in the dorsal hippocampus 60 min later (Fernandez et al., 2008; Lewis et al., 2008). Similarly, bilateral dorsal hippocampal infusion of 5 μg/hemisphere E_2 or ICV infusion of 10 μg E_2 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 min of infusion (Fernandez et al., 2008; Fortress et al., 2013; Zhao et al., 2012, 2010). Although these data were consistent with previous *in vitro* work, the key question was whether this E_2-induced ERK activation contributed to E_2-induced memory consolidation.

To address this issue, we allowed mice to accumulate 30 s 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_2. 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_2'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 h 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_2 and related compounds. In contrast

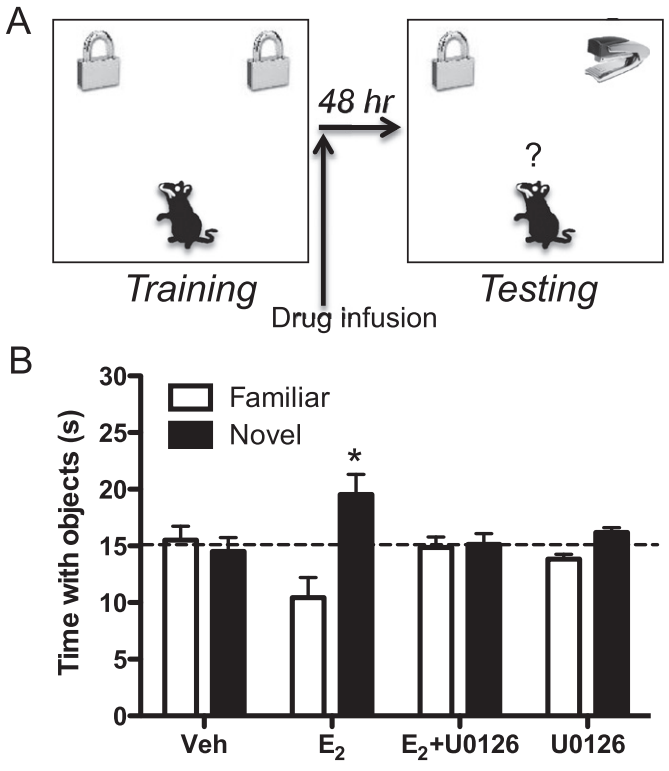

Fig. 3. Dorsal hippocampal ERK activation is necessary for E_2 to enhance object recognition memory consolidation. (A) Illustration of the object recognition protocol used in our laboratory. During training, mice accumulate 30 s of time exploring two identical objects. Immediately after training, mice receive an infusion of E_2 or other drugs into the dorsal hippocampus or dorsal third ventricle. Forty-eight hours later, mice accumulate 30 s 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 s) with the novel object. Adapted with permission from (Fortress and Frick, 2014; Frick, 2013). (B) ERK activation is necessary for E_2 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_2, but not vehicle, into the dorsal third ventricle spend significantly more time than chance (dashed line at 15 s) 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).

to vehicle-treated mice, mice receiving post-training dorsal hippocampal or ICV infusions of E_2 spent more time exploring the novel object than chance (Fernandez et al., 2008; Fortress et al., 2013; Zhao et al., 2012, 2010), indicating that E_2 enhances object recognition memory consolidation (Fig. 3B). Consistent with our hypothesis, U0126 prevented E_2 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_2 to enhance hippocampal memory consolidation. These data were the first to identify a specific molecular pathway through which E_2 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_2 (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_2'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_2 from enhancing object recognition memory in ovariectomized mice (Lewis et al., 2008). Next, we showed that E_2 significantly increased phosphorylation of PI3K and Akt in the dorsal hippocampus of young and middle-aged ovariectomized mice within 5 min of dorsal hippocampal or ICV infusion. In mice of both ages, dorsal hippocampal infusion of the PI3K inhibitor LY298002 prevented ICV-infused E_2 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_2 to facilitate object recognition memory consolidation (Fan et al., 2010; Fortress et al., 2013), but also that E_2 first activates PI3K before activating ERK.

mTOR

Interestingly, both PI3K and ERK activate the mammalian target of rapamycin (mTOR) cell-signaling pathway (Fig. 2) (Hoeffer 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) (Hoeffer 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_2-induced memory consolidation. Although dorsal hippocampal infusion of E_2 did not increase phosphorylation of mTOR itself, it did increase the phosphorylation of the downstream proteins S6K and 4E-BP1 (Figs. 4A,B) (Fortress et al., 2013), suggesting that E_2 activates mTOR signaling. These effects were blocked by dorsal hippocampal infusion of U0126, LY298002, and rapamycin (Figs. 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_2 from increasing dorsal hippocampal p42 ERK phosphorylation (Fig. 4C) (Fortress et al., 2013). Although we expected the PI3K and ERK inhibitors to block E_2-induced ERK phosphorylation given our previous data to this effect (Fan et al., 2010), it was surprising that rapamycin prevented E_2 from activating ERK. This interaction may be mediated by one of the two mTOR complexes that provide feedback regulating ERK and PI3K activity (Hoeffer and Klann, 2010; Klann and Dever, 2004). More importantly, rapamycin also prevented E_2 from enhancing object recognition memory consolidation (Fig. 4D) (Fortress et al., 2013), suggesting that dorsal hippocampal mTOR activation is necessary for E_2 to facilitate hippocampal memory formation. We have yet to determine exactly how mTOR regulates memory consolidation after E_2 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_2 facilitates this interaction in hippocampal neurons (Boulware et al., 2005) (Fig. 2). Thus, the E_2-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_2 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_2 on hippocampal memory consolidation, we thought that H3 acetylation might also be involved. In brief, we found that a dorsal hippocampal infusion of E_2 in ovariectomized mice significantly increases H3 acetylation in the dorsal hippocampus within 30 min, and this increase was blocked by U0126 (Zhao et al., 2010). These data suggest that the E_2-induced increase in H3 acetylation depends on dorsal hippocampal ERK activation. We also found that E_2 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_2 to enhance object recognition memory consolidation (Zhao et al., 2012). Collectively, these findings

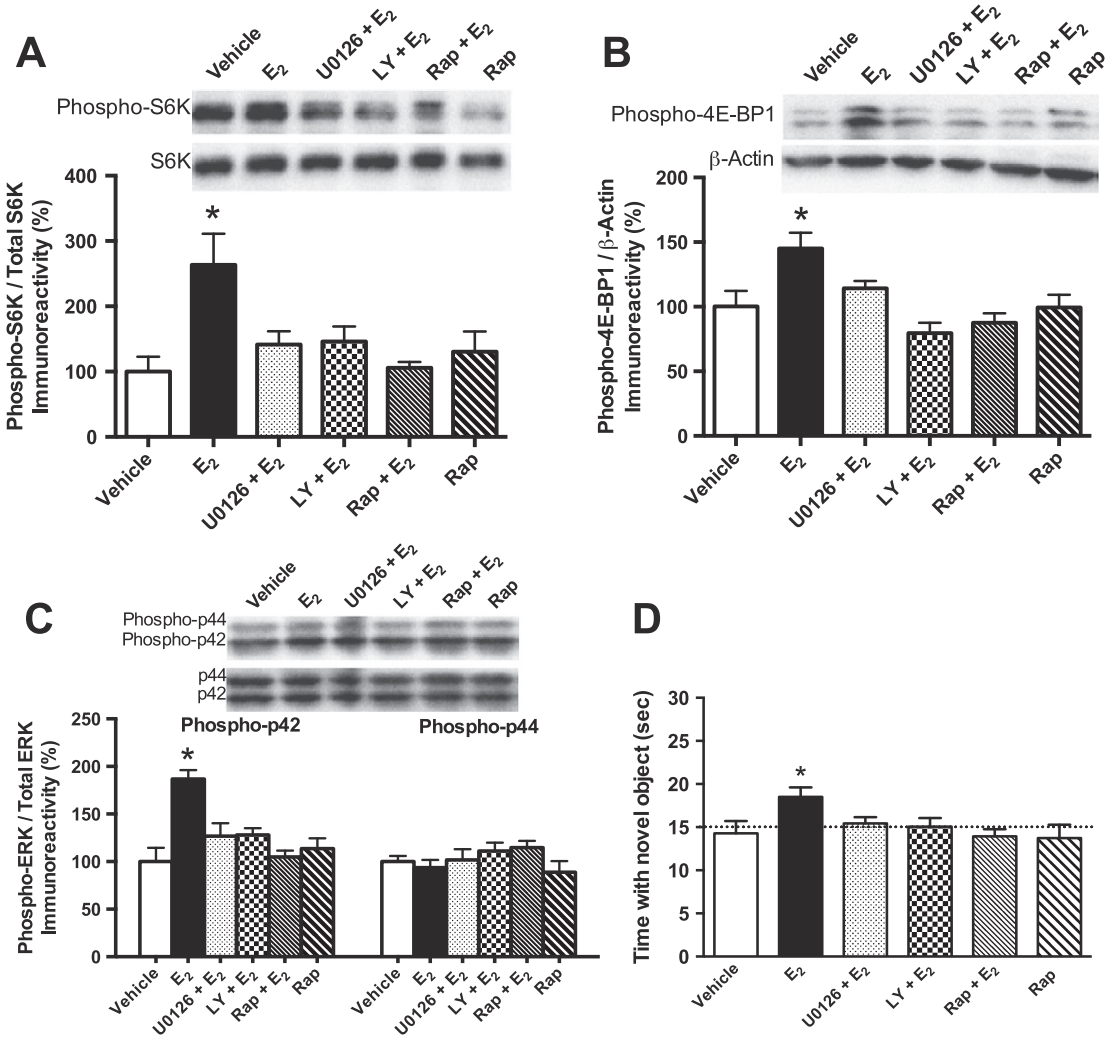

Fig. 4. Dorsal hippocampal mTOR activation is necessary for E_2 to enhance object recognition memory consolidation. The phosphorylation of S6K (A), 4E-BP1 (B), and p42 ERK (C) were significantly increased in young ovariectomized mice 5 min after bilateral dorsal hippocampal infusion of 5 μg/hemisphere E_2 (*$p < 0.05$ relative to vehicle). This effect was blocked by the ERK inhibitor U0126 (0.5 μg/hemisphere), the PI3K inhibitor LY298002 (0.005 μg/hemisphere), or the mTOR inhibitor rapamycin (0.25 μg/hemisphere) (A–C). (D) All three inhibitors also prevented E_2 from enhancing object recognition memory consolidation, as indicated by the fact that only mice infused with E_2 + vehicle spent more time than chance with the novel object (*$p < 0.05$). Error bars in all panels represent the mean ± SEM. Phosphorylated proteins were normalized to total protein or β-actin. Insets are representative Western blots of phosphorylated and total protein.
Adapted with permission from (Fortress et al., 2013).

demonstrate the central importance of histone acetylation to the memory-enhancing effects of E_2.

However, the specific genes regulated by E_2-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_2 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_2 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_2 infusion in middle-aged females (Fortress et al., 2014), indicating that E_2-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_2 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

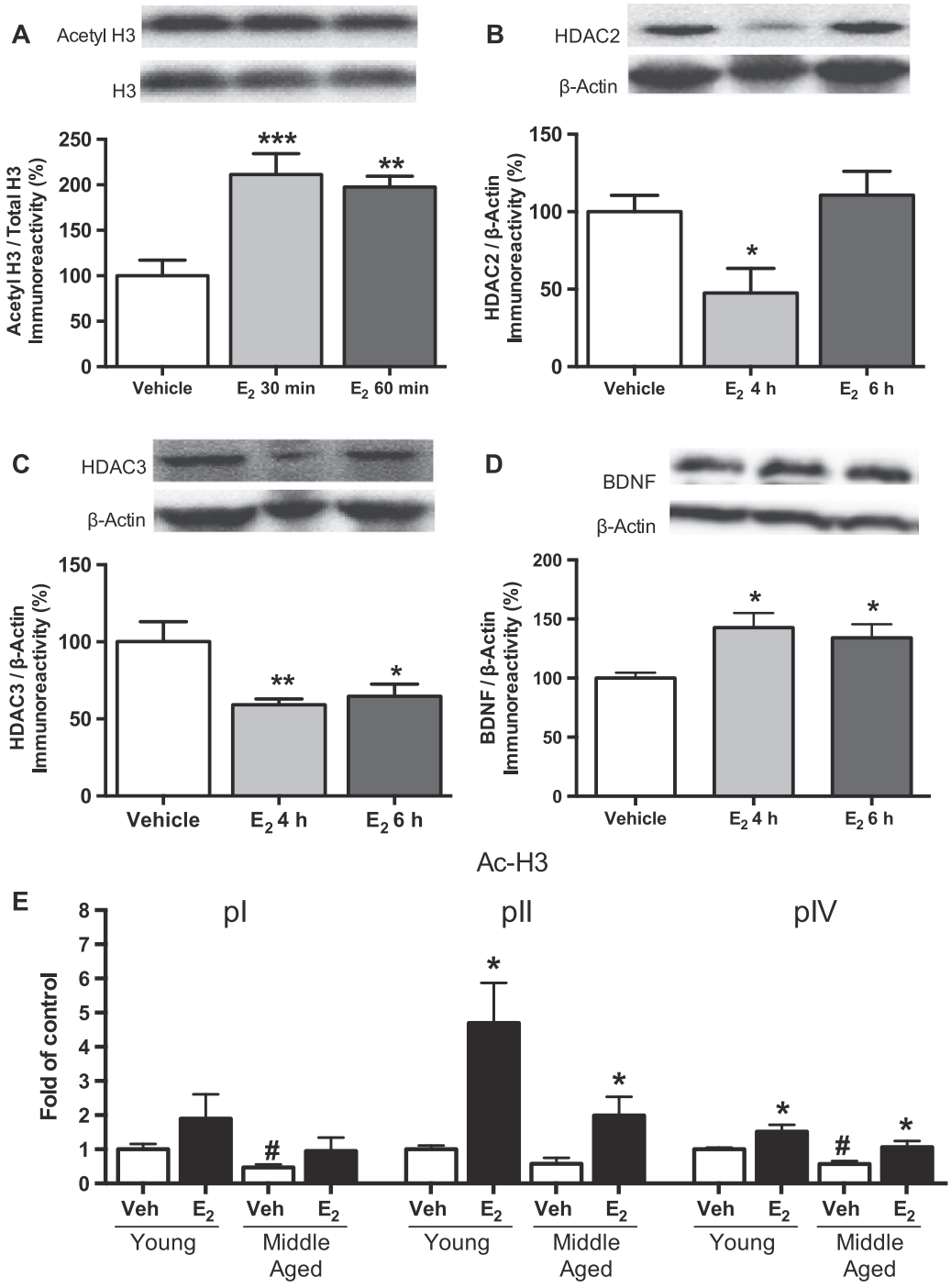

Fig. 5. E_2 facilitates dorsal hippocampal histone acetylation and BDNF protein expression in middle-aged ovariectomized mice. (A) Bilateral dorsal hippocampal infusion of 5 μg/hemisphere E_2 increased H3 acetylation 30 and 60 min later (***$p < 0.001$, **$p < 0.01$ relative to vehicle). Acetylated H3 protein was normalized to total H3 protein. Insets are representative Western blots of acetylated and total histone protein. (B,C) Hippocampal infusion of E_2 decreased protein levels of HDAC2 (B) 4 h after infusion, and of HDAC3 (C) 4 and 6 h after infusion (**$p < 0.01$, *$p < 0.05$ relative to vehicle). (D) Conversely, BDNF protein levels were increased in the dorsal hippocampus 4 and 6 h after bilateral dorsal hippocampal infusion of E_2 (*$p < 0.05$ 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_2 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).

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 *PP1* (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_2 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_2 regulated levels of the DNMT enzymes. We found in ovariectomized female mice that E_2 transiently increased mRNA for DNMT3A and DNMT3B 45 min after dorsal hippocampal infusion, whereas DNMT1 mRNA levels were unchanged up to 180 min after infusion (Zhao et al., 2012, 2010). Moreover, DNMT3B protein levels were significantly increased 4 h after dorsal hippocampal infusion of E_2 (Zhao et al., 2010), suggesting that E_2 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_2 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 3 h after training had no effect on memory consolidation. Together, these data demonstrate that DNA methylation is essential for E_2 to facilitate object recognition memory consolidation. At this point, however, we do not know which genes are methylated or demethylated by E_2, which is critical for better understanding how E_2 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_2'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_2'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. However, the picture has gotten more complex in recent years, as data have shown roles not only for ERα, ERβ, and GPER in mediating the mnemonic effects of E_2, 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 ERα and ERβ knockouts (ERαKO, ERβKO) suggest a role for both classical ERs in hippocampal memory. In the absence of exogenous E_2 treatment, ERαKO, but not ERβKO, mice exhibit impaired spatial memory, suggesting a key role for ERα in mediating baseline memory function (Bean et al., 2014). This role is supported by data showing that lentiviral delivery of ERα to the hippocampus improved spatial memory in the Morris water maze among female ERαKO mice (Foster et al., 2008). However, E_2 can enhance spatial memory, inhibitory avoidance, and object recognition in ERαKO mice, but cannot enhance object recognition or object placement in ERβKO mice (Frick et al., 2010; Fugger et al., 2000; Liu et al., 2008; Walf et al., 2008), suggesting an important role for ERβ in mediating at least some of the effects of exogenous E_2 treatment. Interestingly, lentiviral delivery of ERβ to the hippocampus of ERβKO mice has been shown to impair spatial memory, possibly through interactions with ERα (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_2 to induce neuritogenesis and PI3K/Akt activation in primary hippocampal cultures (Ruiz-Palmero et al., 2013), suggesting that GPER may regulate some effects of E_2 in the hippocampus. Another *in vitro* study found that siRNAs for ERβ 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_2 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 ERα and ERβ 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_2. Potential involvement of intracellular ERs in the effects of BSA-E_2 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, 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, 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 (Fig. 6A,B) (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 ERα antagonist MPP (1,3-Bis(4-hydroxyphenyl)-4methyl-5-[4-(2-piperidinylethoxy) phenol]-1H-pyrazole) or ERβ 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 ERα and ERβ 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_2's effects on memory. Because intracranial infusion of E_2 so reliably increases dorsal hippocampal ERK activation (Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2013; Zhao et al., 2012, 2010), we first measured the effects of intracranial PPT or DPN infusion on dorsal hippocampal ERK phosphorylation. As with E_2, 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 min of infusion (Fig. 6C) (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 ERα or ERβ 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_2 infusion (Fernandez et al., 2008).

The importance of ERK in mediating the effects of ERα and ERβ *in vivo* were also consistent with findings from cultured hippocampal primary neurons showing that membrane-localized ERα and ERβ 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_2 and the ER agonists. Indeed, we found that dorsal hippocampal infusion of the mGluR1a antagonist LY367385 prevented ICV-infused E_2, 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 ERα, ERβ, mGluR1, and ERK physically interact within hippocampal detergent-resistant membranes *in vivo* (Boulware et al., 2013). These data suggest that E_2-induced hippocampal memory consolidation is regulated by interactions among ERα, ERβ, 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 ERα and ERβ 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

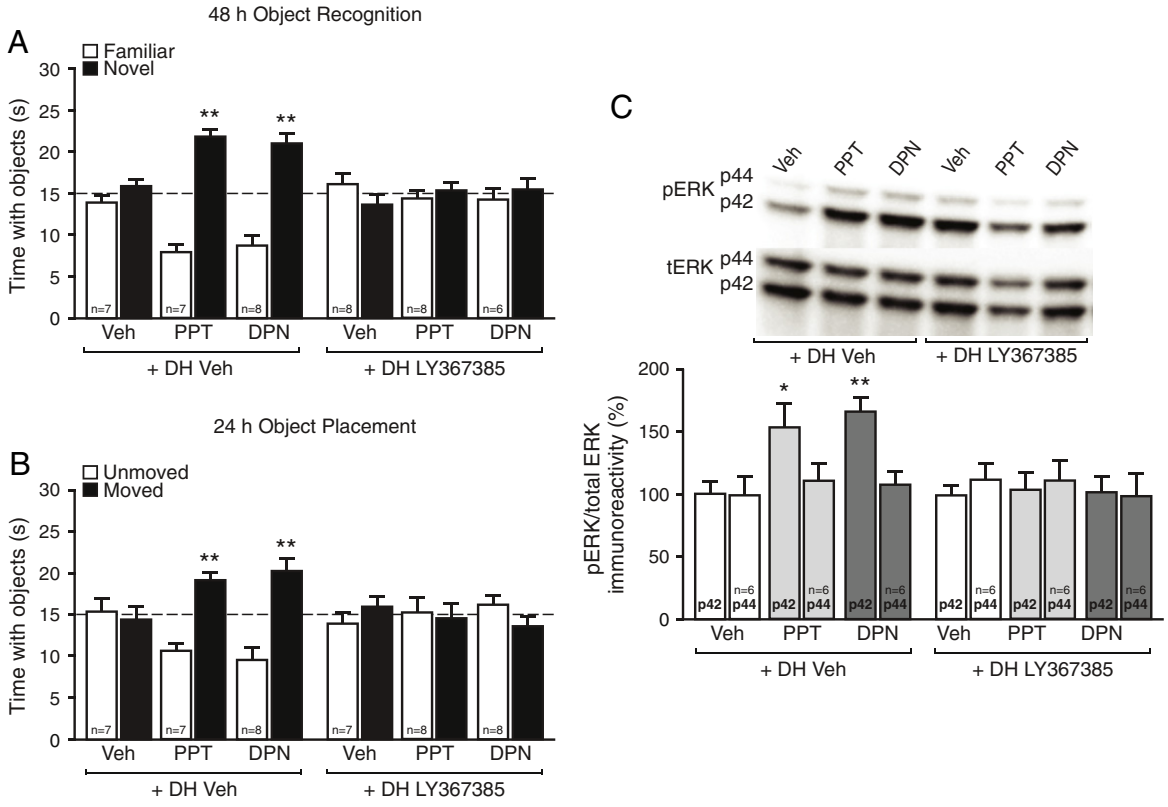

Fig. 6. Dorsal hippocampal mGluR1a activation is necessary for ERα and ERβ to enhance object recognition and object placement memory consolidation, and to increase dorsal hippocampal ERK phosphorylation. (A,B) Immediate post-training infusion of PPT (0.2 pg) or DPN (20 pg) into the dorsal third ventricle 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 pg/hemisphere). Bars represent the mean ± 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 abolished by dorsal hippocampal infusion of LY367385. Bars represent mean ± SEM % change from vehicle.
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targeted in a very general way by conjugating the large bovine serum albumin molecule to E_2, which restricts its activity to the plasma membrane. *In vitro*, BSA-E_2 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_2 mimicked exactly the effects of dorsal hippocampal E_2 infusion. That is, dorsal hippocampal or ICV infusion of BSA-E_2 increased dorsal hippocampal p42 ERK phosphorylation within 5 min and enhanced object recognition memory consolidation (Fernandez et al., 2008). Furthermore, as with E_2, ERK activation was necessary for BSA-E_2 to enhance object recognition memory consolidation (Fernandez et al., 2008). These data suggested that membrane-bound ERs might mediate the effects of E_2 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_2. GPER does appear to influence hippocampal memory formation and its effects on hippocampal memory are similar to those of E_2, 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, 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_2. Whereas E_2 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_2 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_2 (Kim et al., 2013). These data are consistent with a new report showing that the ability of E_2 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_2. This conclusion is supported by other work showing that G-1 does not rapidly increase Akt phosphorylation in the dorsal hippocampus, unlike E_2, which activates Akt between 5 and 30 min 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_2 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 tremendous 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_2 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_2 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_2 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_2 is likely a major player in memory formation, so we must better understand the role of *de novo* E_2 synthesis in regulating memory. In addition, future work should look beyond E_2 and the hippocampus to examine interactions with other brain regions and other hormones. In particular, examining the effects of E_2 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_2 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_2 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., 2014; 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_2 enhances memory, we may uncover new molecular targets for drug development that provide the mnemonic benefits of E_2 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 much more work needs to be done to fully understand how E_2 and other sex steroid hormones regulate cognition, recent advances pinpointing the neural mechanisms underlying the memory-enhancing effects of E_2 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, 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.

Acknowledgments

The University of Wisconsin-Milwaukee supported the writing of this manuscript. The empirical work from my laboratory described herein was supported by R01AG022525, R03MH065460, the American

Federation for Aging Research, a University of Wisconsin-Milwaukee Research Growth Initiative Award, the University of Wisconsin-Milwaukee, and Yale University. I would like to thank all of the students, postdoctoral fellows, and research staff from my laboratory whose work is reviewed in this article, and to Dr. Ashley Fortress, Jennifer Tuscher, and Jaekyoon Kim for critical comments on this manuscript.

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