# 8

# Molecular Mechanisms Underlying Rapid Effects of Estradiol on Memory Consolidation

*KARYN M. FRICK, JAEKYOON KIM, WENDY A. KOSS, AND JENNIFER J. TUSCHER*

## INTRODUCTION

As illustrated by the chapters in this book, estrogens have myriad effects on the functioning of the hippocampus and neocortex, from inducing rapid biochemical changes in neurons and glia to remodeling dendritic processes and promoting the proliferation and maturation of new neurons. These effects can be mediated in the hippocampus and prefrontal cortex by both classical and nonclassical effects of estrogen receptors (ERs), the cellular distribution of which is detailed in Chapter 1 of this volume. Given the seminal role of the hippocampus and prefrontal cortex in mediating numerous forms of learning and memory, it is not surprising that the modulatory effects of estrogens on hippocampal and cortical cell signaling, gene expression, synaptic physiology, glial function, and neurogenesis have measurable and meaningful effects on memory formation.

Taking a cue from the focus in Part I of this book on the rapid effects of 17β-estradiol (E2) on hippocampal and prefrontal function, this first chapter of Part II will highlight how these effects facilitate the consolidation of memories. This work represents a point of convergence between two literatures, one demonstrating that E2 and other sex steroid hormones mediate rapid effects on neurons and neuronal cell lines (Watters et al., 1997; Wade et al., 2001; Fitzpatrick et al., 2002; Wade and Dorsa, 2003; Zhao and Brinton, 2007) and another using one-trial learning approaches to show that memory consolidation in numerous brain regions occurs within 1 to 3 hours of training and requires rapid activation of numerous cell signaling cascades linked to membrane neurotransmitter and growth factor receptors (McGaugh, 1966; McGaugh, 1989; Guzowski and McGaugh, 1997; Atkins et al., 1998; Selcher et al., 1999; Schafe et al., 2000; Adams and Sweatt, 2002). Identifying the receptor, cell-signaling, epigenetic, and spinogenic mechanisms through which E2 facilitates memory consolidation in both female and male mice has been a major focus of our laboratory's research efforts in the past decade, so this chapter will detail our work, along with those of other investigators who have used similar approaches to examine rapid effects of E2 on memory consolidation. Many other chapters in Part II will take more protracted view of estrogenic memory modulation in which effects on multiple aspects of learning and memory processing (e.g., acquisition, consolidation, and retention) are influenced in both the short and long terms. These chapters will address not only optimally functioning young adult animals, but will also discuss the contributions of estrogen loss to age-related memory decline in rodent and monkey models of menopause. As such, Part II will provide readers with a broad view of the effects of estrogens on learning and memory throughout the lifespan.

## E2-INDUCED ENHANCEMENT OF MEMORY CONSOLIDATION

The effects of E2 on memory consolidation were demonstrated more than 20 years ago by seminal studies from Mark Packard's laboratory. Packard was trained by Drs. Norman White at McGill University and James McGaugh at the University of California–Irvine, in whose laboratories he became steeped in a multiple memory systems approach that used posttraining drug treatments to probe the neural mechanisms in various brain regions necessary for memory consolidation (McGaugh, 1989; McGaugh and Roozendaal, 2009). In contrast to studies that administer drugs prior to training, and thus, influence not only multiple phases of memory formation (e.g., acquisition and consolidation) but also aspects of task performance unrelated to memory (e.g.,

motivation, anxiety, sensorimotor abilities), drugs in posttraining studies are administered immediately or at some delay after training, and are typically metabolized by testing the next day. As such, effects of posttraining drug administration can be pinpointed specifically to memory consolidation in a way that avoids the nonmnemonic performance confounds of pretraining treatments (McGaugh and Roozendaal, 2009). Packard and his colleagues were the first to use this approach to study the effects of estrogens on memory consolidation. Their first study was conducted in gonadally intact male rats, which is not surprising given that males were, and still remain, the predominant subject of basic learning and memory research. Rats were trained in eight consecutive trials in the Morris water maze, a large round water tank in which subjects use extramaze cues to locate and swim to an escape platform hidden just underneath the surface of the water (Packard et al., 1996). Given the navigation requirements of the task, it is thought to test hippocampal-dependent spatial memory (Morris et al., 1982). Immediately after the last training trial, rats received bilateral dorsal hippocampal infusions of saline or a water-soluble form of E2 encapsulated in cyclodextrin that is capable of being infused into the brain. Another group received E2 infusion 2 hours after training to determine how quickly E2-induced memories were consolidated. After infusion, rats were returned to their home cages and then given two test trials 24 hours after the final training trial to assess memory consolidation. Rats receiving saline or 2-hour delayed E2 infusions forgot the escape platform location overnight; the time these groups took to find the escape platform on the first trial of day 2 was significantly greater than that on the last trial of day 1 (Packard et al., 1996). However, rats receiving infusions of E2 immediately after training showed remarkably preserved memory; their performance on the first trial of day 2 was as good or better than that during the last trial of day 1 (Packard et al., 1996). These data suggested two important conclusions. First, E2 administered immediately posttraining significantly and specifically enhances spatial memory consolidation. Second, this consolidation occurs within 2 hours of training, suggesting mediation by rapid nonclassical effects of E2.

In follow-up studies, Packard and colleagues demonstrated similar memory-enhancing effects of either systemic or intrahippocampal E2 treatment in ovariectomized female rats (Packard and Teather, 1997b, 1997a). As in males, the consolidation-promoting effects of E2 were limited to within the first 2 hours after training, as the performance of rats receiving the 2-hour delayed E2 treatment on day 2 was as poor as saline-treated rats (Packard and Teather, 1997b, 1997a). E2-induced enhancements in both males and females depended on activation of muscarinic cholinergic receptors, as muscarinic receptor antagonists blocked these effects (Packard et al., 1996; Packard and Teather, 1997b). Importantly, the doses of the antagonists used in combination with E2 had no detrimental effect on memory consolidation themselves; that is, they were behaviorally subeffective doses (Packard and Teather, 1997b). Thus, the poor performance of rats given a memory-enhancing dose of E2 in combination with a behaviorally subeffective dose of antagonist could be attributed to an interaction between E2 and the mechanism targeted by the antagonist, rather than a memory-impairing effect of the antagonist itself. As such, this approach represents a powerful way to interrogate the molecular mechanisms necessary for modulators like E2 to regulate memory (Frick et al., 2010) and is a method we will return to later in this chapter.

The E2-induced enhancement of memory consolidation observed by Packard and colleagues over 20 years ago has stood the test of time. It has been replicated by several labs and expanded to other types of memory. For example, Victoria Luine and colleagues adapted the posttraining approach to one-trial object recognition and spatial recognition tasks (Luine et al., 2003). Unlike the water maze, which requires several training trials, these tasks involved a single training trial in which rats were allowed to explore two identical objects placed near the upper corners of a square open field. The ability to remember the identity of the training objects (i.e., object recognition) was later tested by substituting a novel object for a familiar training object. Because rodents have a natural predilection for novelty, more time spent exploring the new object indicates intact recognition memory for the identity of the old training object. The ability of subjects to remember the location of the training objects in the object placement (or object location) version of the task is tested by moving one of the training objects to a new location (e.g., a lower corner of the open field). Increased time spent with the moved object indicates intact spatial memory for the original training locations. Using this approach, Luine and colleagues found that immediate, but not 2-hour delayed, systemic injection of E2 or the synthetic estrogen diethylstilbestrol significantly enhanced

both object recognition and spatial memory consolidation in ovariectomized rats (Luine et al., 2003). Similar findings have been published from Cheryl Frye's laboratory (Walf et al., 2006; Frye et al., 2007). As discussed in more detail in Chapter 10, the Luine group has replicated this finding many times and has correlated E2-induced enhancements in memory consolidation with rapid (i.e., within 30 minutes) increases in dendritic spine density in both the hippocampus and prefrontal cortex in ovariectomized female and gonadally intact male rats (MacLusky et al., 2005; Inagaki et al., 2010; Jacome et al., 2010, 2016; Inagaki et al., 2012).

## RAPID EFFECTS OF E2 ON MEMORY CONSOLIDATION IN FEMALE MICE

The beneficial effects of E2 on memory consolidation observed in rats ended up translating remarkably well to mice. In the late 2000s, our laboratory published a series of studies using Packard's Morris water maze protocol and a modified version of Luine's object recognition protocol to show that systemic injection of Packard's effective dose of cyclodextrin-encapsulated E2 (0.2 mg/kg) enhanced memory consolidation in both tasks among ovariectomized C57BL/6 mice (Gresack and Frick, 2004, 2006; Gresack et al., 2007a, 2007b; Fernandez et al., 2008; Lewis et al., 2008; Harburger et al., 2009; Frick et al., 2010). Since 2008, we have used Packard's memory-enhancing 5 μg/hemisphere dose to show that bilateral dorsal hippocampal infusion of E2 significantly enhances memory consolidation in the object recognition and object placement tasks among young and middle-aged ovariectomized mice (Fernandez et al., 2008; Fan et al., 2010; Zhao et al., 2010, 2012; Boulware et al., 2013; Fortress et al., 2013, 2014; Kim et al., 2016; Figure 8.1). Similar object recognition memory enhancements induced by posttraining systemic (0.2 mg/kg) or intrahippocampal (5 μg/hemisphere) E2 have been reported by investigators using ovariectomized Swiss mice (Pereira et al., 2014). Most recently, our laboratory showed that

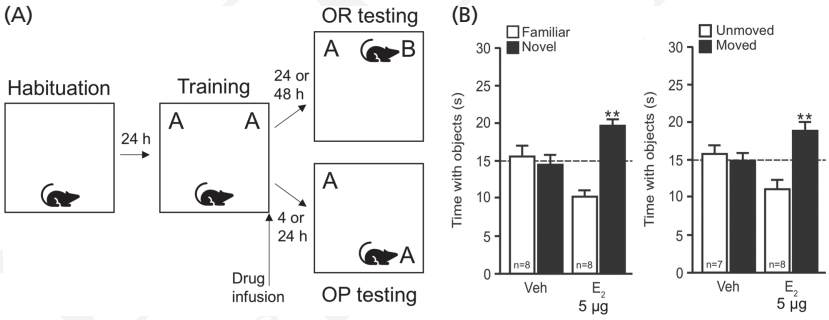


**FIGURE 8.1.** Effects of dorsal hippocampal E2 infusion on memory consolidation in the object recognition and object placement tasks. (A) Schematic diagram of the object recognition and object placement protocols used to study effects of E2 on memory consolidation. Mice are first habituated to an empty testing arena for 5 minutes/day for 2 days. Twenty-four hours after the last habituation trial, mice are trained with two identical objects placed near the upper corners of the arena. Mice remain in the arena until they have explored the objects for a total of 30 seconds or until 20 minutes have elapsed. Immediately after training, mice are infused with E2 or other drugs. Object recognition is tested 24 or 48 hours later by substituting a novel object for one of the training objects. Because mice prefer novelty, more time spent exploring the novel object than chance (15 seconds) indicates memory for the familiar training objects. Because vehicle-infused mice remember the identity of the training objects 24, but not 48, hours after training, a 24-hour delay is used to test memory-impairing effects of drugs, whereas a 48-hour delay is used to test the memory-enhancing effects of drugs. During object placement testing, one of the training objects is moved to a lower corner of the arena. Mice who remember the locations of the training objects will spend more time than chance exploring the moved object. Object placement testing occurs 4 or 24 hours after training because vehicle-infused mice remember the object locations 4, but not 24, hours later. (B) Representative data showing that E2 enhances 48-hour object recognition and 24-hour object placement memory consolidation. Vehicle-infused mice spend similar amounts of time with the familiar and novel/moved objects, whereas mice receiving 5 μg/hemisphere $E_2$ spend significantly more time than chance (dashed line at 15 seconds; $^{**}p < 0.01$ relative to chance) with the novel (left) and moved (right) objects, indicating enhanced memory consolidation. Panel B modified from Boulware et al. (2013).

5 μg/hemisphere E2 enhances consolidation in the object recognition and object placement tasks among gonadally intact and gonadectomized male mice (Koss et al., 2018). We also recently found that bilateral infusion of 5 μg/hemisphere E2 into the prefrontal cortex of ovariectomized mice enhanced consolidation in the object recognition and object placement tasks, demonstrating that estrogenic regulation of other brain regions connected to the dorsal hippocampus can mediate memory formation (Tuscher et al., 2019).

Collectively, the consistent data from mice and rats published over two dozen studies from multiple laboratories demonstrates that the memory-enhancing effects of E2 generalize across rodent species and behavioral tasks, to both sexes, and between brain regions. Given the robust and reliable effects of E2 in mediating hippocampal memory consolidation, our laboratory has spent the past decade using the object recognition/placement tasks to identify the underlying neural mechanisms necessary for this phenomenon. The remaining sections of this chapter detail our findings thus far.

However, before we begin, it is worth noting three methodological specifics about our laboratory's approach. First, mice trained in our object recognition and object placement protocols are required to remain in the testing arena until they have accumulated 30 seconds of total object exploration, rather than until a set time (e.g., 5 minutes) has elapsed. We feel this criterion is important to ensure that all mice have equal exposure to the objects prior to drug infusion, and thus effects during testing are not biased by differing amounts of time taken to encode object information. Second, when E2 is infused into the brain on its own, we infuse it bilaterally into the dorsal hippocampus. However, when we co-infuse E2 and a kinase inhibitor or receptor antagonist, we infuse the inhibitor/antagonist bilaterally into the dorsal hippocampus and E2 into the adjacent dorsal third ventricle. We do not infuse both compounds into the dorsal hippocampus to protect the tissue from possible damage due to back-to-back infusions. In our hands, the effects of E2 infused into the dorsal hippocampus and dorsal third ventricle have been identical, both in terms of behavioral and biochemical effects. Finally, the effects reported herein of drug infusion on kinase phosphorylation occur 5 minutes after infusion unless noted otherwise.

## Involvement of ERK and Related Kinases

Our initial studies focused on cell-signaling mechanisms, given ample evidence that activation of various cell-signaling cascades is necessary for memory consolidation. We began by targeting extracellular signal-regulated kinase (ERK) because of two unrelated literatures. One showed that phosphorylation (i.e., activation) of the p42 isoform of ERK in the hippocampus was necessary for contextual fear memory in gonadally intact male rats (Atkins et al., 1998; Selcher et al., 1999). Similarly, other studies demonstrated that ERK phosphorylation in the male rat hippocampus was also necessary for the consolidation of spatial and object recognition memories (Blum et al., 1999; Selcher et al., 1999; Bozon et al., 2003; Kelly et al., 2003), supporting a consensus view that ERK phosphorylation is essential for various types of hippocampal memory consolidation. Interestingly, the timing of ERK involvement in memory consolidation mirrored that of E2; blocking ERK activation immediately, but not 1 hour, after training had no detrimental effect on memory consolidation, suggesting that ERK activation within an hour after training is necessary for consolidation (Blum et al., 1999).

At around the same time, an unrelated in vitro literature showed that E2 or a membrane-impermeable form of E2 (bovine serum album-conjugated E2 [BSA-E2]) could increase ERK phosphorylation within 15 minutes in numerous cell types including hippocampal neurons (Watters et al., 1997; Wade et al., 2001; Wade and Dorsa, 2003; Yokomaku et al., 2003). Moreover, intracerebroventricular infusion of either type of E2 increased hippocampal ERK phosphorylation within 5 minutes in vivo, an effect that was not blocked by the ER antagonist ICI 182-780 (Kuroki et al., 2000). Together, these data suggested that E2 could rapidly activate hippocampal ERK in a manner not contingent on ER-dependent gene transcription. The time frame in which E2 increased ERK phosphorylation fit within the 1-hour window in which ERK was necessary for memory, leading us to connect these two literatures. We hypothesized that if E2 could rapidly activate hippocampal ERK activation and hippocampal ERK activation was necessary for memory consolidation, then ERK activation should be necessary for E2 to enhance memory consolidation.

We tested this hypothesis in a 2008 study (Fernandez et al., 2008) that has been replicated many times since. To briefly summarize, we showed that E2 specifically increases the phosphorylation of the p42 isoform of ERK within 60 minutes of a systemic injection (0.2 mg/kg E2) or within 5 minutes of an infusion into either the dorsal hippocampus or dorsal third ventricle

of young adult ovariectomized C57BL/6 mice (Fernandez et al., 2008; Lewis et al., 2008; Zhao et al., 2010, 2012; Boulware et al., 2013; Fortress et al., 2013; Kim et al., 2016). Similar phosphorylation occurs in middle-aged ovariectomized mice, although after 15 minutes rather than 5 minutes, but does not occur in aged ovariectomized mice (Fan et al., 2010). In both young and middle-aged females, the ability of E2 to facilitate memory consolidation in the object recognition and object placement tasks was blocked by co-infusion of an ERK phosphorylation inhibitor (Fernandez et al., 2008; Fan et al., 2010; Zhao et al., 2010), demonstrating that ERK phosphorylation is necessary for the memory-enhancing effects of E2 in female mice. This finding was particularly novel because it was the first to show that rapid activation of cell signaling was essential for E2 to influence memory formation. In support of the involvement of rapid effects at the cell membrane rather than nuclear-mediated mechanisms, the effects of E2 on object recognition memory consolidation and dorsal hippocampal p42 ERK phosphorylation in young females were mimicked by infusion of the membrane-impermeable BSA-E2 and were only partially blocked by the ERα/β antagonist ICI 182,780 (Fernandez et al., 2008). The ER mechanisms mediating these rapid effects on ERK will be discussed later in the chapter; the following sections will first discuss our subsequent findings with ERK and other signaling pathways (see Figure 8.2 for a schematic model of the molecular mechanisms thus far shown to regulate E2's effects on memory and hippocampal dendritic spinogenesis and gene expression).

ERK phosphorylation represents a point of convergence for many cell-signaling pathways in that it can be activated by numerous

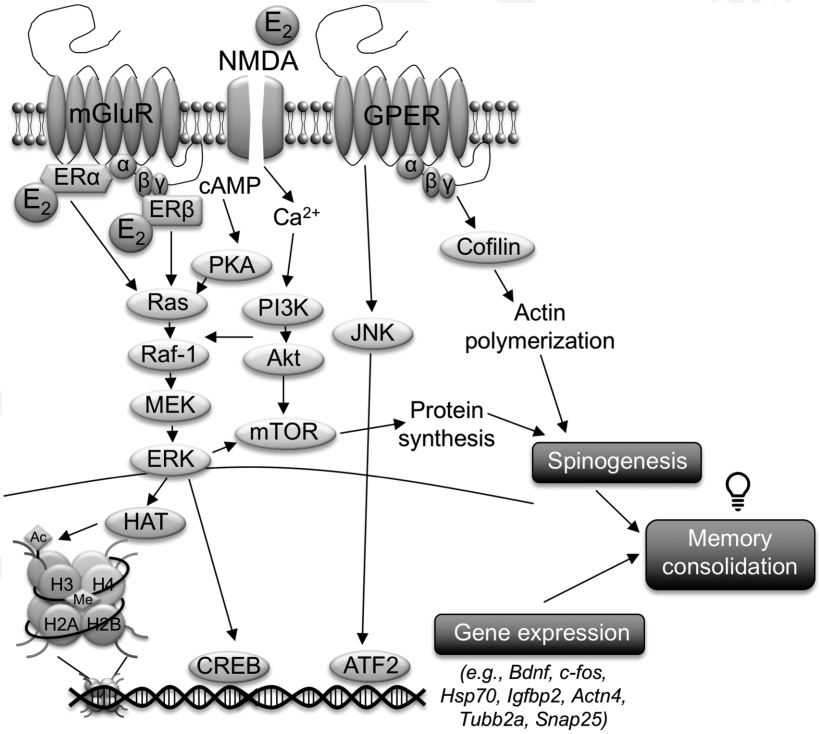


**FIGURE 8.2.** Schematic diagram illustrating the molecular events known to mediate the rapid effects of E2 on memory consolidation in female mice. Activation of NMDA and mGluR1 receptors by E2 via interaction with estrogen receptors (ERs) ERα and ERβ at the membrane promotes signaling by PKA, PI3K/Akt, ERK, and mTOR, which then facilitate epigenetic changes including histone acetylation of *Bdnf* promoters, DNA methylation, local protein synthesis, and dendritic spinogenesis. Activation of GPER by the agonist G-1 phosphorylates JNK and cofilin, leading to increased gene expression, actin polymerization, and CA1 pyramidal neuron spinogenesis. Inhibition of kinase phosphorylation (PKA, PI3K, ERK, mTOR), histone acetylation, or DNA methylation prevents E2 from enhancing memory consolidation. Blocking JNK phosphorylation or actin polymerization prevents G-1 from enhancing memory consolidation and increasing CA1 dendritic spine density. Adapted from Frick et al. (2015) (See color plate).

upstream signaling kinases including protein kinase A (PKA), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), and Akt. Many of these pathways are activated by E2 and are central to its effects in the hippocampus, as illustrated by the fact that the induction of long-term potentiation and dendritic spinogenesis by E2 in hippocampal slices is blocked by inhibitors of ERK, PKA, PKC, PI3K, and calcium calmodulin kinase II (CaMKII; Yokomaku et al., 2003; Shingo and Kito, 2005; Manella and Brinton, 2006; Ogiue-Ikeda et al., 2008; Hasegawa et al., 2015; Hojo et al., 2015). Accordingly, infusion of PKA or PI3K inhibitors into the dorsal hippocampus blocks the beneficial effects of E2 on object recognition memory consolidation in young ovariectomized mice (Lewis et al., 2008; Fortress et al., 2013), indicating that signaling kinases upstream from ERK are also necessary for E2 to enhance memory consolidation.

### ERK-Mediated Genetic and Epigenetic Processes

ERK phosphorylation triggers numerous events inside neurons, including gene transcription. For example, ERK can phosphorylate the transcription factor cAMP response element binding protein (CREB) to influence gene transcription in the nucleus. In cultured hippocampal pyramidal neurons from neonatal female rats, E2 increases CREB phosphorylation within 5 minutes in a manner dependent on activation of both ERK and metabotropic glutamate receptor 1a (mGluR1a; Boulware et al., 2005). E2 also increases CREB phosphorylation in the dorsal hippocampus of young adult ovariectomized mice within 5 minutes of infusion (Koss et al., 2018).

However, CREB is not the only mechanism through which E2 may regulate gene transcription. Gene microarray studies have shown that E2 rapidly alters the expression of many genes in the hippocampus, including histone deacetylase 2 (HDAC2; Aenlle et al., 2009; Aenlle and Foster, 2010), an enzyme involved in epigenetic regulation of gene transcription. DNA is tightly coiled around octamers of histone proteins whose tails can be posttranslationally modified in many ways to regulate the binding of DNA to the histone octamer (Luger et al., 1997). Acetylation of histone tails by histone acetyltransferase enzymes (HATs) relaxes the bonds between DNA and the histone complex, thereby allowing transcription factors access to the DNA and increase gene transcription (Strahl and Allis, 2000; Yang and Seto, 2007). Conversely, HDAC enzymes remove acetyl groups from histone tails, which maintains the close bond between DNA and histones and restricts transcription factor access, thus preventing gene transcription (Strahl and Allis, 2000; Yang and Seto, 2007). Overexpression of HDACs, particularly HDAC2 and HDAC3, impairs many forms of hippocampal memory, blocks hippocampal synaptic plasticity, and reduces hippocampal spinogenesis (Guan et al., 2009; McQuown et al., 2011). Thus, HDAC2 and HDAC3 are considered memory repressors.

Given E2's role as a memory enhancer, it is perhaps not surprising that E2 reduces HDAC expression. Dorsal hippocampal E2 infusion decreases levels of HDAC2 and HDAC3 protein in young and middle-aged ovariectomized mice (Zhao et al., 2010; Fortress et al., 2014). In both cases, these reductions were associated with enhanced object memory consolidation, as well as with a specific increase in acetylation of the core histone called H3 (Zhao et al., 2010; Fortress et al., 2014). HAT inhibition blocks hippocampal memory consolidation (Zhao et al., 2012), demonstrating that H3 acetylation is necessary for this process. Moreover, H3 acetylation is increased by hippocampal learning in an ERK-dependent manner (Levenson et al., 2004; Chwang et al., 2006). Accordingly, dorsal hippocampal infusion of E2 increases dorsal hippocampal H3 acetylation within 30 minutes, an effect that is blocked by ERK inhibition (Zhao et al., 2010).

The genes that are regulated E2-induced histone acetylation remain unclear, although one recent study implicates the growth factor brain-derived neurotrophic factor (BDNF). BDNF is essential for hippocampal synaptic plasticity and memory formation, and levels of BDNF mRNA and protein are increased in the brain by E2 (Singh et al., 1995; Sohrabji et al., 1995; Gibbs, 1998; Scharfman et al., 2003). The *Bdnf* gene consists of nine exons, and the transcription of each is driven by its own unique promoter (Aid et al., 2007). Of these, promoters I (pI), pII, and pIV drive expression of the most abundant transcript variants in the brain (Baj et al., 2013). We recently found that dorsal hippocampal infusion of E2 significantly increased H3 acetylation of pII and pIV in young and middle-aged ovariectomized mice and increased BDNF and pro-BDNF protein levels in middle-aged females (Fortress et al., 2014). These alterations were associated with E2-induced enhancements of object recognition and object placement memory consolidation (Fortress et al., 2014), suggesting

that epigenetic regulation of *Bdnf* promotes the expression of BDNF, which then helps to facilitate memory formation. Unpublished data from our laboratory support the importance of BDNF signaling to E2-induced memory enhancement, as dorsal hippocampal antagonism of the high-affinity BDNF receptor TrkB blocks the beneficial effects of E2 on object recognition and object placement memory consolidation in young ovariectomized mice (Gross and Frick, unpublished observations). Collectively, these data suggest that E2 can influence gene expression via ERK-dependent H3 acetylation, which regulates levels of other modulatory factors such as BDNF that may mediate memory consolidation.

### Downstream Effects on Protein Synthesis and Dendritic Spine Density

Aside from influencing gene transcription, E2 may also mediate memory consolidation by facilitating local protein translation within dendrites to increase CA1 pyramidal neuron dendritic spine density. Such increases are associated with learning in spatial and recognition tasks (Moser et al., 1994; O'Malley et al., 2000; Wallace et al., 2006; Kim et al., 2017), suggesting that new spines may underlie hippocampal memory formation. As detailed elsewhere in this volume, elevated levels of E2 are associated with increased CA1 dendritic spine density in female and male rodents. During the natural estrous cycle, CA1 spine density is highest when E2 levels are most elevated (i.e., during the proestrus phase of the cycle) and exogenous systemic or intrahippocampal E2 treatment increases CA1 spine density significantly in female and male rodents both in vitro and in vivo (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992, 1993; Leranth et al., 2003; Frick et al., 2004; MacLusky et al., 2005; Murakami et al., 2006, 2014; Mukai et al., 2007; Ogiue-Ikeda et al., 2008; Phan et al., 2011, 2012, 2015; Inagaki et al., 2012; Ooishi et al., 2012; Kato et al., 2013; Hasegawa et al., 2015; Hojo et al., 2015; Tuscher, Luine et al., 2016). As such, increased CA1 dendritic spine density is a hallmark of estrogenic effects on the hippocampus. However, relatively little is known about the mechanisms through which E2 regulates dendritic spinogenesis.

New spines require new proteins, which may be generated in the cell body or locally at the synapse. Exogenous E2 can increase CA1 spine density within 30 minutes (MacLusky et al., 2005; Inagaki et al., 2012; Tuscher, Luine et al., 2016), suggesting rapid effects that could depend on local protein synthesis. An important mechanism regulating local protein synthesis in hippocampal neurons is activation of the mammalian target of rapamycin (mTOR) signaling pathway. mTOR phosphorylates core components of the translational initiation machinery, including p70 ribosomal S6 kinase (S6K) and eIF4E-binding proteins (4E-BPs; Kelleher et al., 2004; Tsokas et al., 2005; Hoeffer and Klann, 2010). In gonadally intact male and ovariectomized female rodents, hippocampal-dependent learning activates mTOR and, conversely, the mTOR inhibitor rapamycin prevents the consolidation of contextual fear, spatial, and object recognition memories (Dash et al., 2006; Parsons et al., 2006; Bekinschtein et al., 2007; Myskiw et al., 2008; Fortress et al., 2013). mTOR is activated by numerous upstream kinases, including ERK and PI3K (Klann and Sweatt, 2008; Richter and Klann, 2009; Hoeffer and Klann, 2010), both of which are necessary for E2 to enhance object memory consolidation (Fernandez et al., 2008; Fortress et al., 2013). Activation of mTOR signaling increases new protein synthesis, synaptic plasticity, and memory formation, and inhibitors that prevent phosphorylation of ERK (e.g., U0126) or mTOR (e.g., rapamycin) block these effects (Kelleher et al., 2004; Tsokas et al., 2005; Myskiw et al., 2008; Richter and Klann, 2009; Hoeffer and Klann, 2010), suggesting critical roles for ERK and mTOR phosphorylation in dendritic spinogenesis and memory consolidation.

Given the rapid effects of E2 on CA1 dendritic spines, we hypothesized that mTOR signaling would be involved in the beneficial effects of E2 on memory consolidation in females. As with inhibitors of ERK and PI3K, dorsal hippocampal infusion of rapamycin prevented E2 from enhancing object recognition and object placement memory consolidation in young ovariectomized mice, suggesting that mTOR-mediated local protein synthesis is essential for the memory-enhancing effects of E2 in females (Fortress et al., 2013). E2-indcued activation of ERK, PI3K, and mTOR was later observed in hippocampal slices from adult male rats within 15 minutes of bath application (Briz and Baudry, 2014). In a subsequent study, we found that dorsal hippocampal activation of either ERK or mTOR was necessary for E2 to increase CA1 dendritic spine density in young ovariectomized females (Tuscher, Luine et al., 2016; Figure 8.3), supporting our initial hypothesis that mTOR regulates the spinogenic effects of E2 on CA1 pyramidal neurons.

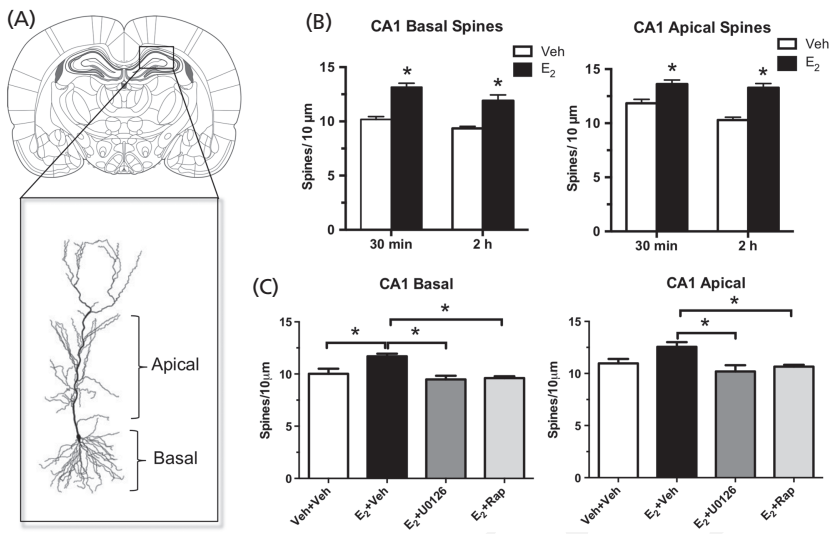


**FIGURE 8.3.** E2-induced CA1 dendritic spinogenesis is dependent on ERK and mTOR activation in the dorsal hippocampus of ovariectomized mice. (A) Illustration of the apical and basal CA1 dendrites analyzed. (B) Bilateral dorsal hippocampal infusion of 5 µg/hemisphere E2 increased CA1 pyramidal apical and basal spine density 30 minutes and 2 hours later (*$p < 0.05$ relative to vehicle-treated controls). (C) Mice received bilateral dorsal hippocampal infusion of vehicle, the ERK phosphorylation inhibitor (U0126), or mTOR activation inhibitor (rapamycin) immediately before intracerebroventricular infusion of vehicle or E2, and spines were counted 2 hours later. U0126 or rapamycin prevented E2 from increasing apical and basal spine density (*$p < 0.05$ relative to mice receiving E2 + vehicle). Panels B and C adapted from Tuscher et al. (2016).

## ER Involvement in Memory Consolidation

The data reviewed thus far indicate that E2 enhances memory consolidation by rapidly activating cell-signaling pathways including PI3K, PKA, ERK, and mTOR, which then influences the gene transcription, local protein translation, and increased dendritic spine density necessary for the formation and retention of a memory. However, the specific mechanisms through which E2 initiates cell signaling remain an area of active investigation. Some studies implicate involvement of intracellular ERα and ERβ acting at the cell membrane in concert with neurotransmitter receptors. Other work indicates involvement of the membrane ER G-protein-coupled ER (GPER). The following section discusses current knowledge about the involvement of these receptors to date.

### Roles of Membrane-Associated ERα and ERβ in Memory Consolidation

ERs are generally divided into two groups based on their cellular localization, intracellular (ERα and ERβ) or membrane (e.g., GPER, ER-X). ERα and ERβ are localized widely throughout the brain (Shughrue, Scrimo et al., 1997; Shughrue, Lane et al., 1997; Shughrue and Merchenthaler, 2000; Shughrue et al., 2000). Although traditionally thought to be localized to the cell nucleus, ERα and ERβ can be found in the nuclei, dendrites, dendritic spines, axons, and axon terminals of hippocampal pyramidal neurons, as well as in interneurons, granule cells, and astrocytes (Milner et al., 2001). In the "classical" mechanism of ER action, estrogens bind ERα or ERβ in the cytoplasm, causing the receptors to dimerize and translocate into the nucleus, where they bind to estrogen response elements (ERE) on DNA and act as transcription factors to regulate gene expression (Cheskis et al., 2007). ER nuclear translocation peaks within about an hour (Walters, 1985), suggesting that this mechanism is too slow to account for E2-induced alterations that occur within a few minutes. Thus, more rapid "nonclassical" mechanisms mediated by ERs near or within the membrane have been posited to account for these effects. As previously discussed, ERα and ERβ are localized to many nonnuclear cellular compartments in pyramidal neurons, like dendritic spines, where they tend to cluster near the postsynaptic density (Milner et al., 2001). This proximity to the postsynaptic membrane allows ERα and ERβ to be close to the site of

activity-dependent changes in the spine head. Indeed, within 5 minutes of estradiol exposure, monomeric ERβ translocates to the plasma membrane prior to the onset of cell-signaling (Sheldahl et al., 2008), suggesting a potential involvement in the cell signaling alterations that regulate memory formation.

Numerous studies have implicated ERα and ERβ in hippocampal-dependent spatial and object recognition memory using ER-specific knockouts, siRNAs, and viral vector-mediated delivery of ERs (Fugger et al., 2000; Foster et al., 2008; Liu et al., 2008; Walf et al., 2008; Frick et al., 2010; Han et al., 2013; Bean et al., 2014). To specifically target memory consolidation, we and other investigators have administered agonists and antagonists of ERα and ERβ immediately posttraining. Of these, the most commonly used are the ERα agonist propyl pyrazole triol (PPT) and ERβ agonist diarylpropionitrile (DPN), which have ~400-fold and 70-fold selectivity for their respective ER (Stauffer et al., 2000; Meyers et al., 2001). In general, systemic injection of PPT or DPN enhances memory consolidation in the object recognition and object placement tasks among young ovariectomized rats and mice, although the effects of DPN on object placement differ by species and of PPT on object recognition differ by dose (Walf et al., 2006; Frye et al., 2007; Walf et al., 2008; Frick et al., 2010; Hanson et al., 2018). Dorsal hippocampal infusion of PPT or DPN enhances both object recognition and object placement in C57BL/6 mice, but only PPT enhances object recognition in Swiss mice (Boulware et al., 2013; Pereira et al., 2014; Hanson et al., 2018). More recently, we worked with collaborators to test effects of a novel ERβ agonist, ISP358-2, which exhibits 750-fold selectivity for ERβ over ERα (Hanson et al., 2018). Posttraining administration of ISP358-2 or DPN via dorsal hippocampal infusion, intraperitoneal injection, or oral gavage significantly enhanced memory consolidation in the object recognition and object placement tasks among young ovariectomized mice (Hanson et al., 2018), supporting a beneficial effect of selective ERβ activation on memory consolidation. Collectively, the ER agonist data suggest that activation of ERα or ERβ in the hippocampus is sufficient to promote memory consolidation in females.

To determine whether ERα and ERβ are also necessary for memory consolidation in ovariectomized females, we recently infused the ERα antagonist MPP (1,3-Bis(4-hydroxyphenyl)-4methyl-5-[4-(2-piperidinylethoxy) phenol]-1H-pyrazole dihydrochloride) or ERβ antagonist PHTPP (4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo [1,5-a]pyrimidin-3-yl]phenol) into the dorsal hippocampus immediately after object recognition and object placement training (Kim and Frick, 2017). PHTPP impaired memory consolidation in both tasks, whereas MPP impaired memory only in object placement (Kim and Frick, 2017). These data indicate that both ERs are necessary for spatial memory consolidation in young ovariectomized mice, but that only ERβ is necessary for object recognition consolidation.

Together, the ER agonist and antagonist data suggest that ERβ is both necessary and sufficient for object recognition and spatial memory consolidation, whereas ERα is necessary and sufficient for spatial memory consolidation but not necessary for object recognition consolidation. Collectively, however, pharmacological data suggest a key involvement of both classical ERs in memory consolidation among young ovariectomized females.

Because activation of both ERs facilitates memory consolidation, we were curious to determine if these receptors were conduits through which E2 promotes ERK-dependent memory consolidation. This work was inspired by the previously discussed seminal study showing that ERα and ERβ interacted at the cell membrane with mGluR1a to rapidly increase the phosphorylation of ERK and CREB (Boulware et al., 2005). In a follow-up study, we used PPT and DPN to determine whether ERα and ERβ interacted with mGluR1a in vivo to activate ERK and enhance memory consolidation. As with E2, we found that infusion of PPT or DPN into the dorsal hippocampus of young ovariectomized mice increased ERK phosphorylation within 5 minutes and enhanced memory consolidation in the object recognition and object placement tasks, effects that were blocked by the ERK inhibitor U0126 (Boulware et al., 2013; Figure 8.4A). The ability of E2, PPT, and DPN to increase ERK phosphorylation and enhance memory was blocked by dorsal hippocampal infusion of an mGluR1a antagonist (Boulware et al., 2013; Figure 8.4B), suggesting key interactions between the ERs and mGluR1a at the membrane. In support of this conclusion, we used sucrose fractionation to show that ERα, ERβ, and mGluR1a were present within hippocampal detergent-resistant membranes, and co-immunoprecipitation to demonstrate physical interactions among ERα, ERβ, and mGluR1a at the membrane (Boulware

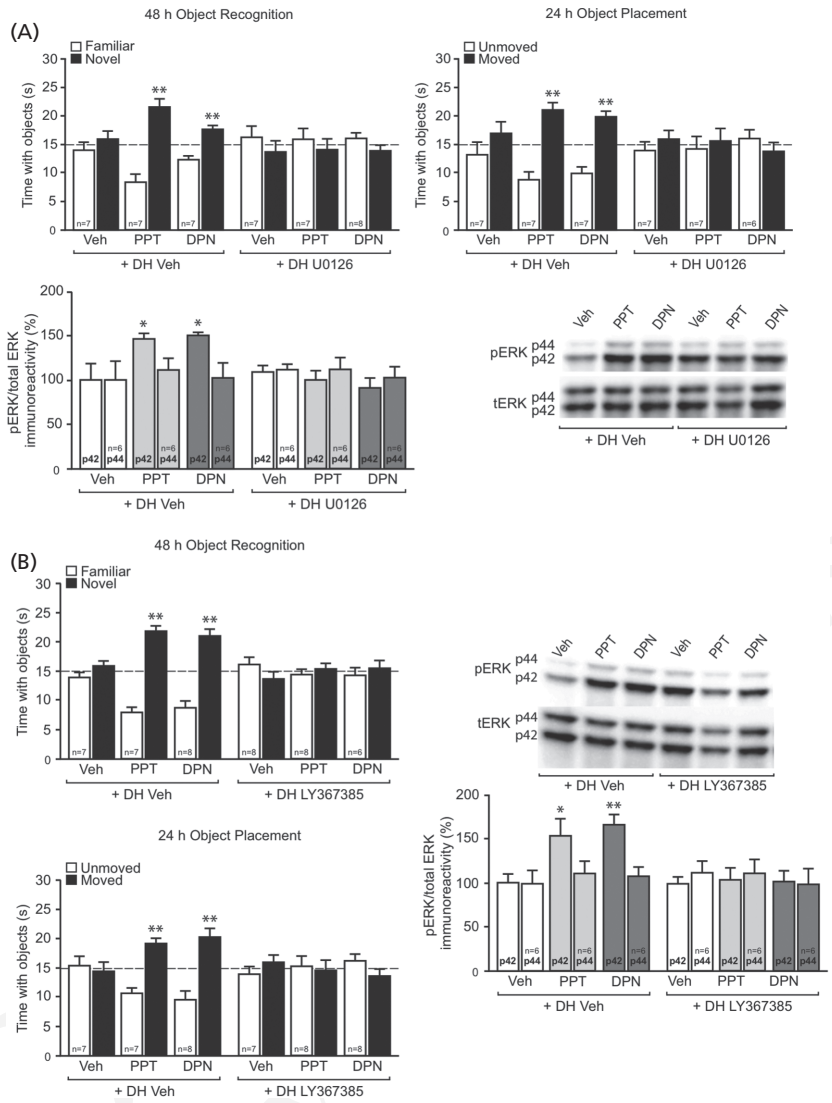


**FIGURE 8.4.** The ability of ERα and ERβ to enhance memory consolidation depends on mGluR1a activation and ERK phosphorylation. (A) Ovariectomized mice received dorsal hippocampal infusion of vehicle or U0126 immediately prior to intracerebroventricular infusion of vehicle, the ERα agonist PPT, or the ERβ agonist DPN. Mice infused with vehicle plus PPT or DPN exhibited enhanced memory consolidation in the object recognition and object placement tasks, as well as increased p42 ERK phosphorylation. U0126 blocked these effects. (B) Ovariectomized mice received dorsal hippocampal infusion of vehicle or the mGluR1a antagonist LY367385 immediately prior to intracerebroventricular infusion of vehicle, PPT, or DPN. As in Panel A, mice infused with vehicle plus PPT or DPN exhibited enhanced memory consolidation and increased p42 ERK phosphorylation, whereas those receiving LY367385 plus PPT or DPN did not. $*p < 0.05$, $**p < 0.01$ relative to chance (dashed line at 15 seconds) or relative to vehicle (pERK). Reprinted from Boulware et al. (2013).

et al., 2013). These data are consistent with the notion that ERα and ERβ act in females at the plasma membrane to trigger ERK signaling via interactions with mGluR1. Given our previous work in ovariectomized mice showing that an NMDA receptor blocker prevents E2 from increasing ERK or enhancing object recognition memory consolidation (Lewis et al., 2008), similar ER interactions may also occur with NMDA receptors. In support of this notion, inhibition of the NR2b subunit of NMDAR receptors has also been shown to block E2-mediated

enhancement of object recognition memory (Vedder et al., 2013).

## The Curiously E2-Independent Effects of GPER

In addition to ERα and ERβ, E2 may regulate memory by binding to membrane ERs, including GPER, ER-X, and Gq-mER. GPER is the most well characterized and studied membrane ER to date, despite being only recently classified as such (it was formerly known as the orphan GPCR called GPR30; Funakoshi et al., 2006). GPER is localized to several brain regions, including the hippocampus (Brailoiu et al., 2007). Like ERα and ERβ, GPER is expressed within astrocytes and pyramidal neurons, in which it can be found in dendrites, dendritic spines, axons, axon terminals, and cell bodies (Akama et al., 2013; Waters et al., 2015). One recent study using digital droplet PCR to measure transcripts of all three ERs actually reports that GPER is by far the predominant ER transcript in the hippocampus and prefrontal cortex of male and female rats (Hutson et al., 2019), suggesting that GPER may mediate many of E2's effects in the hippocampus. GPER is a seven-transmembrane domain receptor including the heterotrimeric G protein subunits Gαβγ (Filardo and Thomas, 2005), which can regulate signaling pathways in vitro, including a SRC-like tyrosine kinase (Quinn et al., 2009), PKA (Thomas et al., 2005), PI3K/Akt (Maggiolini and Picard, 2010), and the Notch signaling pathway (Ruiz-Palmero et al., 2011). As such, GPER is a prime candidate to mediate the rapid effects of E2 on cell signaling and memory consolidation.

Pharmacological studies have examined the role of GPER in memory processes using the selective GPER agonist, G-1, and selective antagonist, G-15 (Bologa et al., 2006; Dennis et al., 2009). In young ovariectomized rats and mice, systemic pretraining treatment with G-1 facilitates spatial working memory, object recognition, object placement, social recognition, and social learning, whereas systemic treatment with G-15 impairs these forms of learning and memory (Hammond et al., 2009, 2012; Ervin et al., 2015; Gabor et al., 2015; Lymer et al., 2017). Similarly, our laboratory recently showed that posttraining infusion of G-1 and G-15 into the dorsal hippocampus of young ovariectomized mice enhances and impairs, respectively, memory consolidation in the object recognition and object placement tasks (Kim et al., 2016). Because GPER activation appeared to mimic the beneficial effects of E2 on hippocampal memory, we next wondered whether it did so via ERK activation, as we observed with ERα and ERβ. Interestingly, G-1 did not increase phosphorylation of ERK, PI3K, or Akt in the dorsal hippocampus of young ovariectomized mice (Kim et al., 2016). Rather, G-1 increased phosphorylation of the p46 and p54 isoforms of c-Jun N-terminal kinase (JNK), as well as the downstream transcription factor ATF2 (Kim et al., 2016). These alterations, as well as the memory-enhancing effects of G-1, were blocked by dorsal hippocampal infusion of G-15 or an inhibitor of JNK (SP600125), but not by the ERK inhibitor U0126 (Kim et al., 2016; Figure 8.5A). Together, these data suggest that the effects of GPER on memory are independent of ERK activation. However, these findings are inconsistent with reports that systemic G-1 increases dorsal hippocampal ERK in ovariectomized mice (Hart et al., 2014) and that bath-applied G-15 blocks E2-induced ERK phosphorylation in hippocampal slices (Kumar et al., 2015). Although it is somewhat difficult to reconcile these conflicting findings, data from our laboratory and others do strongly suggest differing effects of GPER and E2 on the hippocampus and memory consolidation. For example, systemic E2 increases, whereas G-1 decreases, new cell proliferation in the hippocampal dentate gyrus of ovariectomized rats (Duarte-Guterman, Leiblich et al., 2015). In our work, E2 and G-1 have similar effects on memory, but differ in terms of the hippocampal cell-signaling pathways used to facilitate memory. In support of this dissociation, we found that E2 did not increase phosphorylation of either JNK isoform in the dorsal hippocampus of ovariectomized mice, nor were the memory-enhancing effects of E2 blocked by JNK inhibition (Kim et al., 2016; Figure 8.5B). Furthermore, the memory-enhancing effects of E2 were also not blocked by G-15 (Kim et al., 2016), suggesting that dorsal hippocampal GPER does not mediate the effects of E2 memory consolidation in female mice.

To further investigate the mechanisms through which E2 and GPER influence the hippocampus, we next examined effects of E2 and G-1 on CA1 dendritic spine density. As previously described, either systemic or dorsal hippocampal E2 treatment increases CA1 dendritic spine density in ovariectomized rats and mice within 30 minutes (MacLusky et al., 2005; Inagaki et al., 2012; Tuscher, Luine et al., 2016). Similarly, systemic G-1 treatment dose-dependently increases CA1 dendritic spine density in ovariectomized CD1 mice within

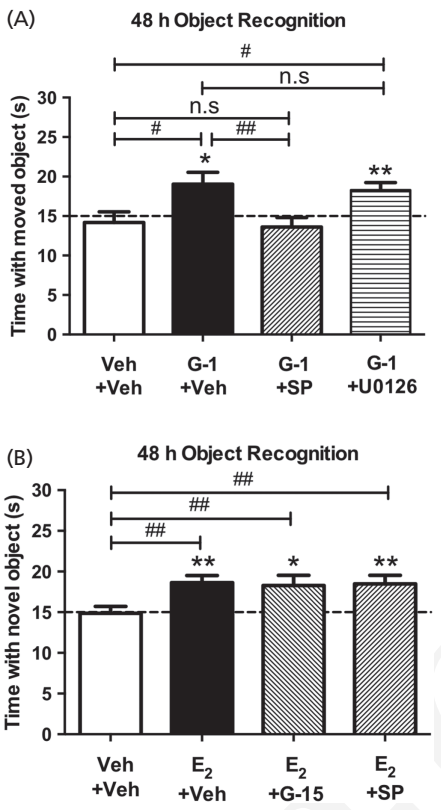


**FIGURE 8.5.** The memory-enhancing effects of GPER activation and E2 depend on different cellular mechanisms. (A) Ovariectomized mice received dorsal hippocampal infusion of vehicle, the JNK phosphorylation in inhibitor SP600125 (SP), or U0126 immediately prior to intracerebroventricular infusion of vehicle or the GPER agonist G-1. G-1 enhanced object recognition memory 48 hours later, and this effect was blocked by SP, but not U0126, suggesting that the memory-enhancing effects of GPER activation depend on JNK, but not ERK, signaling. (B) Ovariectomized mice received dorsal hippocampal infusion of vehicle, SP, or the GPER antagonist G-15 immediately prior to intracerebroventricular infusion of vehicle or $E_2$. The $E_2$-induced enhancement of object recognition memory consolidation was not blocked by SP or G-15, suggesting that it does not depend on JNK or GPER activation. (*$p < 0.05$, **$p < 0.01$ relative to chance; #$p < 0.05$, ##$p < 0.01$ relative to vehicle). n.s. = nonsignificant. Adapted from Kim et al. (2016).

40 minutes (Gabor et al., 2015), so we expected dorsal hippocampal G-1 infusion to do the same. As predicted, dorsal hippocampal infusion of E2 or G-1 increased CA1 spine density in ovariectomized C57BL/6 mice 40 minutes later (Kim et al., 2017). The effect of G-1 was blocked by G-15 and, consistent with its effects on JNK signaling, by JNK inhibition (Kim et al., 2017), indicating that JNK mediates GPER's effects on both spines and memory consolidation, and suggesting that dendritic spinogenesis is necessary for GPER to enhance memory.

To test this hypothesis, we examined G-1's effects on the phosphorylation of cofilin, an actin-binding protein that regulates spine growth and stability. Actin is one of the major components of the cellular scaffold that maintains cell shape, and is highly enriched within dendritic spines (Cingolani and Goda, 2008). Actin exists in two forms: filamentous F-actin, which is composed of monomeric G-actin (Penzes and Cahill, 2012). The formation of F-actin from G-actin, a process called polymerization, is regulated by the actin-binding proteins profilin and cofilin. Whereas profilin promotes organization and stabilization of the actin cytoskeleton, cofilin severs the actin cytoskeleton and promotes spine destabilization (Spence and Soderling, 2015; Wioland et al., 2017). Cofilin is inactivated via phosphorylation by signaling kinases, which prevents actin depolymerization, and promotes F-actin elongation, the growth and maturation of spines, and synaptic plasticity (Chen et al., 2007). Relevant to this discussion, multiple sources support the estrogenic regulation of actin polymerization. For example, systemic E2 injection in young ovariectomized mice increases dorsal hippocampal expression of the F-actin binding protein alpha actinin-4 (Pechenino and Frick, 2009). As detailed in Chapter 5 in this volume, E2 administered to hippocampal slices increases F-actin in spines and promotes induction of long-term potentiation, both of which are blocked by latrunculin A, an inhibitor of de novo F-actin formation (Kramár et al., 2009). Other studies report that inactivation of cofilin is an important step for E2-induced spine formation (Yuen et al., 2011; Briz and Baudry, 2014). Despite the fact that E2 and G-1 differentially affect hippocampal cell signaling, both increase CA1 spine density, so it was plausible that actin polymerization was involved in the spinogenic effects of G-1. Indeed, our data showed that dorsal hippocampal E2 or G-1 infusion significantly increased hippocampal cofilin phosphorylation within 5 minutes (Kim et al., 2017). However, the effects of E2 were not blocked by G-15 (Kim et al., 2017), again suggesting independent effects of E2 and GPER. As in our previous work, the effects of G-1 were mediated by JNK signaling, as a JNK inhibitor blocked the effects of G-1 on cofilin

(Kim et al., 2017). Moreover, we found that dorsal hippocampal infusion of latrunculin A blocked the G-1-induced increase in CA1 spine density and memory consolidation, demonstrating that actin polymerization is essential to the effects of G-1 (Kim et al., 2017). The data suggest a mechanism in which G-1 induction of JNK phosphorylation triggers cofilin phosphorylation, which then promotes increased CA1 spine density, thereby leading to enhanced memory consolidation (Figure 8.2).

## ROLE OF THE PREFRONTAL CORTEX IN ESTROGENIC REGULATION OF MEMORY CONSOLIDATION

Thus far, our discussion has focused exclusively on the dorsal hippocampus, in large part because most is known about how E2 influences this brain region. However, E2 can influence learning and memory mediated by other brain regions, including the prefrontal cortex, striatum, amygdala, and perirhinal cortex (Zurkovsky et al., 2007; Gervais et al., 2013; Maeng et al., 2017). Our laboratory has begun to investigate the role of the prefrontal cortex in estrogenic regulation of memory because it mediates similar types of learning and memory as the dorsal hippocampus, and evidence supports a functional connection between the two brain regions (Jay et al., 1992; Hoover and Vertes, 2007; Churchwell and Kesner, 2011; Warburton and Brown, 2015; Kitamura et al., 2017). Relevant to studies of estrogenic modulation of memory consolidation, we recently reported that posttraining chemogenetic inactivation of the prefrontal cortex (Figure 8.6A), dorsal hippocampus, or both structures blocks memory consolidation in the object recognition and object placement tasks among young ovariectomized mice (Tuscher et al., 2018), suggesting that both regions are essential for the object recognition and spatial memory tasks regulated by E2.

Within the medial prefrontal cortex (mPFC) of female rats, ERα, ERβ, and GPER are expressed almost exclusively at extranuclear sites and are most abundant in axons and axon terminals where they are poised to affect synaptic transmission (Almey et al., 2014). In support, chronic systemic E2 treatment influences mPFC levels of monoamine neurotransmitters in ovariectomized rats (Luine et al., 1998), and the proestrus (high estrogen) phase of the estrous cycle in mice is associated with increased mPFC NMDA receptor transmission and synaptic plasticity (Galvin and Ninan, 2014). Consistent with its effects on neurotransmission and plasticity, numerous studies have reported that systemic E2 increases dendritic spine density and synaptic protein levels in the mPFC among gonadectomized female and male rats (Hajszan et al., 2007; Chisholm and Juraska, 2012; Inagaki et al., 2012; Velázquez-Zamora et al., 2012), which led us to further examine estrogenic regulation of spines in this brain region. In one recent study, we were surprised to find that dorsal hippocampal infusion of E2 significantly increased basal spine density in the mPFC two hours later (Tuscher, Luine et al., 2016). Interestingly, this increase was dependent on ERK and mTOR activity in the dorsal hippocampus (Tuscher, Luine et al., 2016). This finding indicates that hippocampal activity regulates mPFC spinogenesis and suggests that these two brain regions may work in concert to mediate the memory-enhancing effects of E2.

However, no role for E2 in the mPFC in memory consolidation had yet been established, so to further investigate this issue, we first infused E2 bilaterally into the mPFC of ovariectomized mice. We found that mPFC E2 infusion produced similar enhancements in object recognition (Figure 8.6B) and object placement memory consolidation as dorsal hippocampal E2 infusion (Tuscher et al., 2019). E2 also significantly increased apical spine density in the mPFC two hours later (Tuscher et al., 2019; Figure 8.6B). This work provides the first demonstration of a significant role for mPFC E2 in memory consolidation, and suggests that E2 in either the mPFC or dorsal hippocampus can facilitate memory consolidation in ovariectomized mice. We next investigated the extent to which the mPFC and dorsal hippocampus interact to mediate the memory-enhancing effects of E2. To determine whether mPFC activity is necessary for E2 in the dorsal hippocampus to facilitate consolidation, we chemogenetically inactivated the mPFC in ovariectomized mice receiving a posttraining dorsal hippocampal infusion of E2. Chemogenetic suppression of the mPFC prevented E2 in the dorsal hippocampus from enhancing object recognition and object placement memory consolidation (Tuscher et al., 2019; Figure 8.6C), suggesting that concurrent activity in the dorsal hippocampus and mPFC is necessary for E2 to enhance memory formation. This work opens new areas for future research into the mPFC's role in memory formation and in understanding the circuitry required for E2 to regulate memory consolidation.

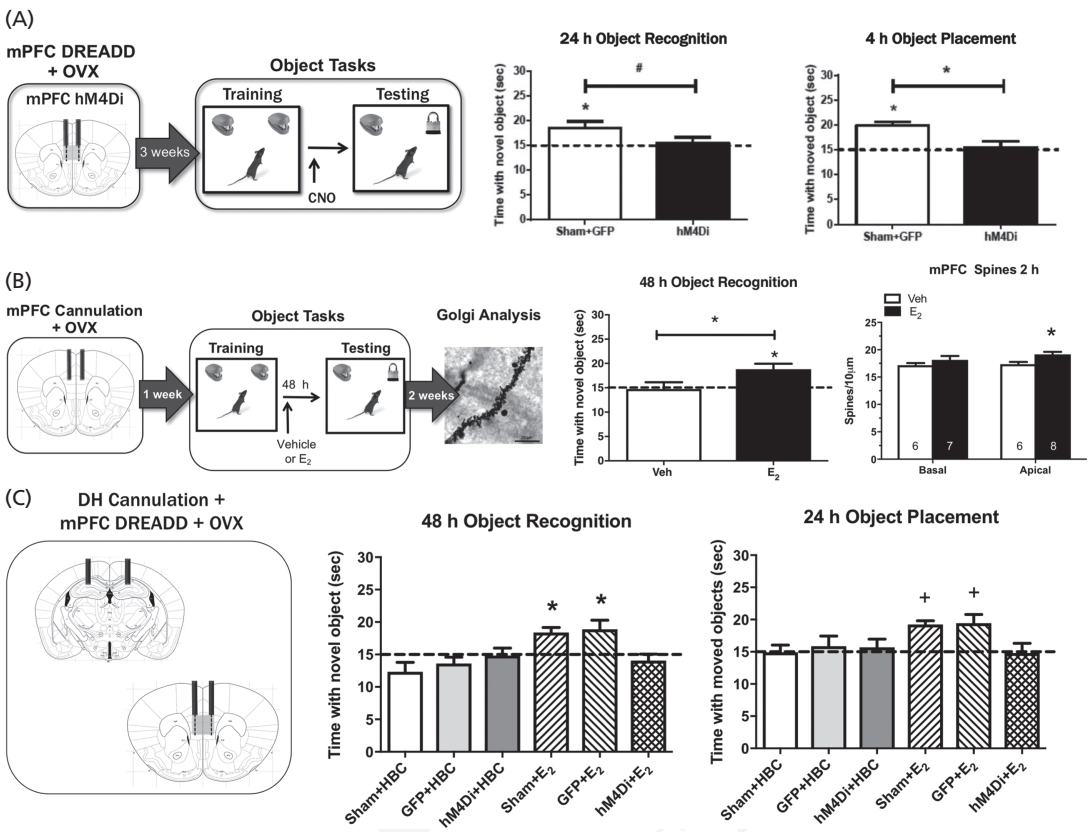


**FIGURE 8.6.** The mPFC is essential for memory consolidation and regulates the memory-enhancing effects of dorsal hippocampal E2. (A) Ovariectomized mice received mPFC infusion of saline (sham) or were infused with an inhibitory DREADD (Designer Receptors Exclusively Activated by Designer Drug) virus to deliver a mutated human $G_i$-coupled muscarinic receptor (hM4Di; AAV-CaMKIIα-HA-hM4Di-IRES-mCitrine, $2.1 \times 10^{12}$ particles/ml, serotype 8) or a control eGFP control virus (AAV-CaMKIIα-eGFP, $2.1 \times 10^{12}$ particles/ml, serotype 8) into the mPFC. Three weeks later, mice received object training and then immediately received an intraperitoneal (i.p.) injection of the synthetic DREADD ligand clozapine-N-oxide (CNO) to activate the virus. The hM4Di virus hyperpolarizes excitatory neurons, thereby temporarily inhibiting their firing. Inactivation of mPFC activity impaired object recognition and object placement memory consolidation tested 24- and 4-hours later, respectively. $^*p < 0.05$ relative to chance, $\#p < 0.05$ relative to Sham-GFP controls. Adapted from Tuscher et al. (2018) with permission. (B) Ovariectomized mice received bilateral mPFC infusions of vehicle or 5 µg/hemisphere $E_2$ immediately after object training. E2 significantly enhanced the consolidation of object recognition and object placement (not shown), and increased apical mPFC spine density 2 hours later. $^*p < 0.05$ relative to chance and vehicle-treated mice. (C) Ovariectomized mice were implanted with bilateral cannulae into the dorsal hippocampus and were infused into the mPFC with saline (sham), eGFP virus, or hM4Di virus. Three weeks later, they received dorsal hippocampal infusion of 2-hydropropyl-β-cyclodextrin (HBC) vehicle or E2 immediately after object training. hM4Di-mediated inhibition of mPFC activity blocked the memory-enhancing effects of hippocampally infused $E_2$, indicating that the mPFC and hippocampus work in concert to mediate the memory-enhancing effects of $E_2$. $^*p < 0.05$ relative to chance, all HBC-infused groups, and the hM4Di-$E_2$ group. $+p < 0.05$ relative to chance and the Sham + HBC-infused and hM4Di-E2 groups. Panels B and C adapted from Tuscher et al. (2019).

## RAPID EFFECTS OF EXOGENOUS E2 ON MEMORY IN MALE MICE

Our discussion of the neural mechanisms through which E2 regulates memory consolidation has focused primarily on ovariectomized females because the vast majority of studies in the field, and in our laboratory, have used these subjects. But what about males? Sex differences in memory and hippocampal function have been reported

by numerous investigators (see recent reviews for more information, e.g., Romeo et al., 2004; Heise et al., 2014; Vierk et al., 2014; Duarte-Guterman, Yagi et al., 2015; Frick et al., 2015; Hamson et al., 2016; Shors, 2016; Koss and Frick, 2017; Pike, 2017; Choleris et al., 2018; Korol and Wang, 2018), so it makes sense to ask if E2 utilizes the same molecular mechanisms to regulate memory consolidation in males. Recall from earlier in the chapter that systemic posttraining E2 enhances spatial memory consolidation similarly in male and female rodents (Packard et al., 1996; Packard and Teather, 1997a, 1997b; Gresack and Frick, 2006). Also remember that activation of cell signaling cascades such as ERK, PI3K, and PKA are required for E2 to increase long-term potentiation and CA1 spine density in male hippocampal slices (Mukai et al., 2007; Ogiue-Ikeda et al., 2008; Murakami et al., 2014; Hasegawa et al., 2015; Hojo et al., 2015). Thus, we hypothesized that the molecular mechanisms through which E2 mediates memory consolidation in males would be similar to those in females.

To address this issue, we examined ERK phosphorylation, given the ample evidence that ERK activation is necessary for E2 to enhance object recognition and spatial memory consolidation in ovariectomized mice (Fernandez et al., 2008; Fan et al., 2010; Zhao et al., 2010; Boulware et al., 2013; Fortress et al., 2013). We first compared the effects of bilateral dorsal hippocampal E2 infusion on consolidation in the object recognition and object placement tasks in ovariectomized female mice and gonadally intact male mice, and found that E2 enhanced consolidation in both groups (Figure 8.7A), suggesting no sex differences in the ability of E2 to regulate memory consolidation (Koss et al., 2018). We also found similar E2-induced enhancements in gonadectomized and gonadally intact males (Figure 8.7B), indicating that exogenous E2 can facilitate memory consolidation in the absence of circulating gonadal hormones in males (Koss et al., 2018). Surprisingly, E2 did not increase p42 ERK or Akt phosphorylation in the dorsal hippocampus of gonadectomized or gonadally intact males (Figure 8.7D) as it does in females, nor did the ERK inhibitor U0126 prevent E2 from enhancing memory consolidation in either group of males (Koss et al., 2018; Figure 8.7C). These data indicate an interesting sex difference in the molecular mechanisms used by E2 to mediate memory consolidation that is consistent with other recent reports of sex differences in the types of ERs that regulate glutamatergic transmission in the hippocampus and in the dependence of hippocampal long-term potentiation on PKA activation (Oberlander and Woolley, 2016; Jain et al., 2018). The picture that has begun to emerge from these studies is that males and females may achieve the same phenotypic end (i.e., memory enhancement, long-term potentiation) via different molecular mechanisms (i.e., receptor activation, kinase activation). Such differences at the subcellular level could be of primary importance for the future development of drugs to reduce memory dysfunction in conditions which differ in risk or severity between the sexes, such as Alzheimer's disease, depression, addiction, or neurodevelopmental disorders. As such, more research is clearly needed to address this potentially critical issue.

One obvious area for future research is to understand the molecular mechanisms that *are* mediating the effects of E2 on memory in males. An initial clue comes from our study of ovariectomized females, intact males, and gonadectomized males. In all three groups, phosphorylation of the transcription factor CREB was increased in the dorsal hippocampus by E2, suggesting the involvement of a kinase upstream from CREB in males (Koss et al., 2018). However, this kinase is not ERK, as U0126 did not prevent E2 from increasing CREB phosphorylation in gonadectomized or gonadally intact males (Koss et al., 2018). Thus, future work should seek to identify the signaling kinases activated by E2 in males and whether these kinases are necessary for E2 to enhance memory consolidation in males. The resulting data could stimulate additional work to determine other cellular mechanisms through which E2 regulates memory consolidation in males.

## RAPID EFFECTS OF HIPPOCAMPALLY SYNTHESIZED E2 ON MEMORY

Finally, no discussion of E2's rapid effects on the hippocampus would be complete without consideration of the possible influence that hippocampally synthesized E2 has on memory consolidation. Removal of the gonads is standard procedure in most studies of estrogens and memory because the ovaries and testes are considered the primary sources of endogenous sex steroid hormones. However, brain regions including the hippocampus can make their own sex steroid hormones, as indicated by the presence of synthetic enzymes including aromatase, the enzyme that converts androgens to

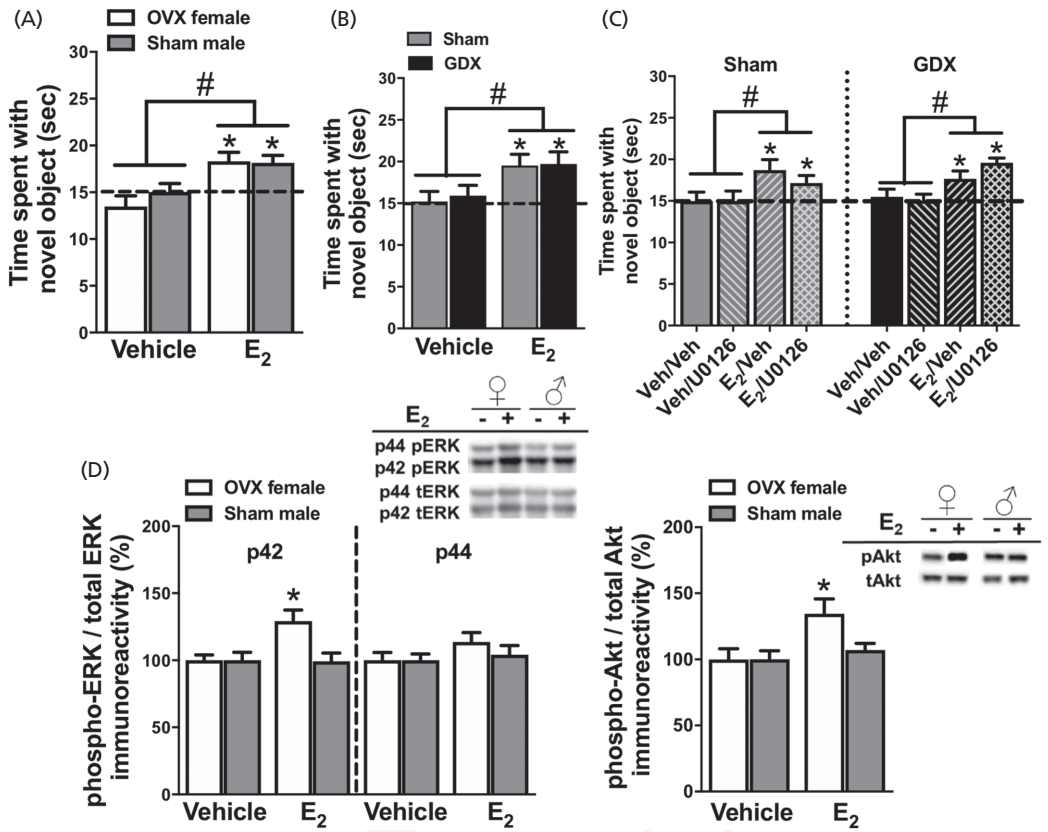


**FIGURE 8.7.** The E2-induced enhancement of memory consolidation in male mice is not mediated by ERK activation. (A, B) Bilateral dorsal hippocampal infusion of E2 enhances object recognition and object placement (not shown) memory consolidation relative to chance (*$p < 0.05$) and to vehicle infusion (#$p < 0.05$) in ovariectomized (OVX) female, sham-gonadectomized male, and gonadectomized (GDX) male mice. (C) Sham and GDX mice received dorsal hippocampal infusions of vehicle or U0126 and dorsal third ventricle infusions of vehicle or E2 immediately after object training. U0126 did not block the memory-enhancing effects of E2 in object recognition or object placement (not shown). (D) E2 increased p42 ERK and Akt phosphorylation in OVX females, but not sham males or GDX males (not shown). *$p < 0.05$ relative to vehicle. Adapted from Koss et al. (2018).

estrogens (Stoffel-Wagner et al., 1999; Prange-Kiel et al., 2003; Hojo et al., 2004; Azcoitia et al., 2011). Moreover, hippocampal levels of E2 in ovariectomized female rats are comparable to those of intact females in diestrus, metestrus, and estrus (Kato et al., 2013), suggesting that the hippocampus is a local source of E2 independent of the ovaries. Decades of work in avian species have demonstrated that brain-synthesized estrogens are essential for numerous behaviors, including mating, social interactions, and vocal communication (Remage-Healey et al., 2010; Chao et al., 2015; Cornil et al., 2018; de Bournonville et al., 2019) and are increased in response to sensory (e.g., auditory) stimulation (Remage-Healey et al., 2008, 2010; Vahaba et al., 2017). This literature suggests a functional role for hippocampally synthesized E2 in memory consolidation, yet little is known about the extent to which this locally synthesized E2 regulates memory in either sex. Evidence in support of a role for hippocampal E2 in memory comes from studies of cultured hippocampal rat neurons in which aromatase inhibitors like letrozole decreased synaptic protein levels, dendritic spine density, and neurogenesis (Kretz et al., 2004; Fester et al., 2006; Prange-Kiel et al., 2006, 2013). Furthermore, pretraining hippocampal aromatase inhibition in gonadally intact male zebra finches impairs spatial memory formation (Bailey et al., 2013; Bailey et al., 2017), suggesting a role for the rodent hippocampus in memory consolidation. Our work using dorsal hippocampal infusions of letrozole to test this putative role in female and

male mice is detailed in the sections below. For additional information on the effects of aromatase inhibition on memory and cognition, see Chapters 9 and 22.

### Aromatase Inhibition in Female Mice

We first assessed the effects of letrozole infusion on memory consolidation in ovariectomized mice. Mice received bilateral dorsal hippocampal infusion of letrozole immediately or three hours after training in the object recognition and object placement tasks. Infusion of letrozole immediately, but not 2 or 3 hours, after training dose-dependently blocked memory consolidation in both tasks (Tuscher, Szinte et al., 2016). Letrozole also blocked an increase in hippocampal E2 levels observed 30 minutes after object training (Tuscher, Szinte et al., 2016), suggesting that object learning stimulates de novo E2 synthesis in the hippocampus. However, letrozole did not prevent exogenous E2 from enhancing memory in both tasks, indicating that de novo E2 is not necessary for the memory-enhancing effects of exogenous E2. Collectively, these data demonstrate an essential role for hippocampal E2 synthesis in object recognition and spatial memory consolidation among ovariectomized mice. The findings suggest that object learning triggers local E2 synthesis, which presumably then facilitates memory consolidation via ER binding and downstream cell signaling.

### Aromatase Inhibition and Androgen Receptor Antagonism in Male Mice

We next investigated whether hippocampal E2 synthesis was also important for memory consolidation in male mice. A similar role for hippocampal E2 in males could not be assumed because systemic treatment letrozole reduces CA1 spine density and long-term potentiation more in ovariectomized females than in gonadally intact males (Zhou et al., 2010; Fester et al., 2012; Vierk et al., 2012), suggesting that the male rodent hippocampus may be less sensitive to aromatase inhibition than that of females. Indeed, data regarding the role of hippocampal E2 in memory among males are somewhat inconsistent. Whereas two studies report modest improvements in spatial memory and working memory after intrahippocampal or systemic aromatase inhibition (Moradpour et al., 2006; Alejandre-Gomez et al., 2007), other studies found that aromatase inhibition impairs fear extinction recall in gonadally intact male rats (Graham and Milad, 2014) and passive avoidance in gonadectomized male rats (Nayebi et al., 2014). Most recently, a study comparing effects of aromatase inhibition on spatial memory in gonadally intact and gonadectomized male mice reported that treatment in both groups impaired memory and reduced hippocampal dendritic spine density, synaptic protein levels, local protein synthesis, androgen receptor levels, and GPER levels (Zhao et al., 2018). This association between aromatase inhibitor-induced memory deficits and hippocampal alterations provided the strongest evidence to date for a critical role of hippocampally synthesized E2 in memory among male rodents. However, the specific role of de novo E2 synthesis in memory consolidation remained unknown.

To address this issue, gonadally intact and gonadectomized male mice received immediate posttraining dorsal hippocampal infusions of the same dose of letrozole that impaired consolidation in ovariectomized females. Interestingly, letrozole impaired object recognition and object placement consolidation in gonadectomized males, but not intact males (Koss and Frick, 2019). The letrozole-induced impairment in gonadectomized males is consistent with our findings in ovariectomized females (Tuscher, Szinte et al., 2016) and suggests that hippocampal E2 synthesis is essential to memory consolidation in the absence of gonadal steroids. However, the fact that intact males were not impaired indicated that circulating androgens, or a rise in hippocampal androgen synthesis due to aromatase inhibition, may mitigate against the detrimental effects of aromatase inhibition among intact males. As such, we examined the effects of androgen receptor antagonism on memory in intact males and found a dose-dependent impairment in both tasks (Koss and Frick, 2019). This finding indicated a key role for androgen receptors in memory consolidation among intact males and led us to posit that androgen receptor activation may protect intact males from the memory-impairing effects of letrozole. To test this hypothesis, intact males received dorsal hippocampal infusions of a nonmemory-impairing dose of the androgen receptor antagonist flutamide in conjunction with a dorsal third ventricle infusion of letrozole. This co-infusion blocked memory consolidation in both tasks (Koss and Frick, 2019), indicating that hippocampally synthesized E2 is essential in males only when androgen receptors are blocked. As such, these data suggest that hippocampal E2 synthesis and androgen receptors may

operate together in males to mediate memory consolidation.

## CONCLUSIONS AND FUTURE DIRECTIONS

We have learned much in the past couple of decades about the mechanisms through which E2 influences hippocampal-dependent memory (see summary in Table 8.1). Our laboratory has attempted to contribute to this body of knowledge by pinpointing the neural mechanisms through which E2 enhances memory consolidation. Our studies thus far indicate that exogenous E2 facilitates consolidation in ovariectomized females via actions in the mPFC and dorsal hippocampus, the latter of which involves binding to ERα and ERβ at the membrane, which interact with mGluR1 to trigger ERK signaling and stimulate histone acetylation and gene expression, mTOR signaling and local protein synthesis, and dendritic spinogenesis in both CA1 and the mPFC (Figure 8.2). Activation of GPER in ovariectomized females also leads to enhanced memory consolidation and increased CA1 dendritic spine density, but via JNK signaling, rather than ERK (Figure 8.2). Hippocampally synthesized E2 also plays a critical role in memory consolidation in ovariectomized females. Among males, E2-induced enhancements in memory consolidation do not involve ERK signaling, but may involve ERK-independent CREB phosphorylation. Although both hippocampal E2 synthesis and androgen receptor activation regulate memory consolidation in males, the presence of circulating androgens protects against the loss of de novo E2 synthesis in the dorsal hippocampus. However, the nature of a potential interaction between de novo E2 synthesis and androgen receptors is unknown.

This research can progress further forward by extending the scope of inquiry in several different directions. First, we must greatly expand our knowledge of the specific molecular mechanisms through which E2 regulates memory consolidation. These include additional cell-signaling and receptor mechanisms involved in estrogenic regulation of memory, as dozens of cell-signaling pathways and hormones, neurotransmitters, and growth factor receptors are likely involved. This work could also benefit from detailed genomic, epigenomic, proteomic, and metabolomic studies. Second, we must reach beyond the hippocampus to first identify the underlying neural circuitry regulated by E2 and then investigate the molecular mechanisms within these brain regions necessary for E2 to modulate memory consolidation. Third, most existing data were collected in female subjects only, and recent reports of sex differences in hippocampal cell signaling and ER utilization suggest that considerably more research in males is warranted. Direct comparisons between males and females, both gonadally intact and gonadectomized, would provide a much more complete understanding of how E2 influences memory consolidation. Finally, determining the extent to which the mechanisms that mediate acute effects of E2 on memory consolidation also regulate the chronic effects of E2 on memory acquisition and retention will be a major advance toward translating what we have learned in rodents to humans. Broadening our inquiries in these four directions will provide fundamental advances that will greatly enrich our understanding of how E2 modulates memory consolidation.

**TABLE 8.1.** SUMMARY OF THE MOLECULAR MECHANISMS KNOWN TO REGULATE MEMORY CONSOLIDATION IN FEMALE AND MALE MICE

| Treatment | Variable | OVX Female | GDX Male | Intact Male |
|---|---|---|---|---|
| E2 | Memory | ↑ | ↑ | ↑ |
| | ERK activation | ↑ | – | – |
| | PI3K activation | ↑ | ? | ? |
| | Akt activation | ↑ | – | – |
| | CREB activation | ↑ | ↑ | ↑ |
| Letrozole | Memory | ↓ | ↓ | – |
| Flutamide | Memory | ? | ? | ↓ |
| Letrozole + Flutamide | Memory | ? | ? | ↓ |

*Notes*: OVX = ovariectomized. GDX = gonadectomized. ERK = extracellular signal-regulated kinase. CREB = cAMP response element binding protein

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