Review

Sex steroid hormones matter for learning and memory: estrogenic regulation of hippocampal function in male and female rodents

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Ample evidence has demonstrated that sex steroid hormones, such as the potent estrogen 17β-estradiol (E_2), affect hippocampal morphology, plasticity, and memory in male and female rodents. Yet relatively few investigators who work with male subjects consider the effects of these hormones on learning and memory. This review describes the effects of E_2 on hippocampal spinogenesis, neurogenesis, physiology, and memory, with particular attention paid to the effects of E_2 in male rodents. The estrogen receptors, cell-signaling pathways, and epigenetic processes necessary for E_2 to enhance memory in female rodents are also discussed in detail. Finally, practical considerations for working with female rodents are described for those investigators thinking of adding females to their experimental designs.

Hormones have long been known to play key roles in regulating learning and memory. Many hormones, including epinephrine, glucocorticoids, and insulin influence learning and memory via inverted U-shaped dose–response relationships in which memory is enhanced by moderate, but not low or high, hormone levels (Roozendaal 2000; Korol and Gold 2007; McNay and Recknagel 2011). Research from the past two decades has demonstrated that sex steroid hormones, particularly the potent estrogen 17β-estradiol (E_2), also regulate learning and memory in male and female rodents via an inverted U-shaped dose–response function that is influenced by estrogen receptor expression (Packard and Teather 1997a; Packard 1998; Foster 2012). Yet E_2 has not gained widespread acceptance as a hormonal modulator of memory in both females and males, perhaps because the majority of this research has been conducted in female rodents. Moreover, the common view of E_2 as a "female" hormone may contribute to the misperception that E_2 is not relevant for cognitive function in males. However, considerable evidence supports a vital role for E_2 in mediating neural function and behavior in male rodents. Therefore, it is important for investigators working with males to understand the ways in which E_2 and other sex steroid hormones (e.g., androgens, progestins, and other estrogens) may influence their brain regions and behaviors of interest.

Another reason for investigators to be cognizant of sex steroid hormone-induced regulation of learning and memory is that males and females may respond differently to various treatments and environmental factors. A classic example is that of acute stress, which enhances classical conditioning and increases apical CA1 dendritic spine density in male rats, but impairs classical conditioning and decreases CA1 spine density in female rats (Wood and Shors 1998; Shors et al. 2001). Therefore, investigators hoping to comply with National Institutes of Health policies that encourage the inclusion of females in biomedical research must be aware that adding females to a study is not as simple as adding another group. In some ways, females are fundamentally different from males, the most obvious of which is the presence of reproductive hormone cycling in females. In rodents, this 4–5 d cycle is termed the "estrous" cycle (Fig. 1) because ovulation leads to a state of behavioral estrus that signals sexual receptivity. No clear consensus has emerged regarding the influence of the estrous cycle on learning and memory, and it has been argued recently that the estrous cycle does not lead to more variability in females relative to males (Prendergast et al. 2014). Nevertheless, it is important that investigators consider the possible effects of sex steroid hormones on neural function and behavior in their experimental designs and data interpretation. However, when thinking about sex differences in brain function or behavior, it is important to note whether differences are due to the activational effects of circulating hormones in adulthood or from organizational effects of hormones in early development, as it has been argued that only the latter can be construed as a true sex difference (McCarthy and Konkle 2005).

Numerous recent reviews have discussed the effects of E_2 on learning and memory in females (Korol 2002; Foster 2005; Daniel 2006, 2013; Sherwin and Henry 2008; Barha and Galea 2010; Bimonte-Nelson et al. 2010; Gibbs 2010; Kim and Casadesus 2010; Choleris et al. 2012; Foster 2012; Frick 2012; Acosta et al. 2013; Chisolm and Juraska 2013; Ervin et al. 2013; Galea et al. 2013; Hogervorst 2013; Luine and Frankfurt 2013; Maki 2013; Bean et al. 2014; Fortress and Frick 2014; Luine 2014; Frankfurt and Luine 2015; Tuscher et al. 2015). As such, this review will not attempt to provide a comprehensive discussion of all effects of E_2 on learning and memory. Rather, the current review highlights effects of E_2 on hippocampal function and memory processes in both males and females to provide those working with either sex a sense of how E_2 might influence their behaviors of interest. E_2 will be the primary focus here because considerably more is known about the effects of E_2 on memory than any other sex steroid hormone. Moreover, the discussion below centers largely upon the hippocampus because of the extensive literature on the effects of E_2 in this structure. Where appropriate, information about the effects of E_2 in other brain regions will be mentioned. The sections below will discuss the localization of estrogen receptors, and the effects of E_2 on hippocampal spine density,

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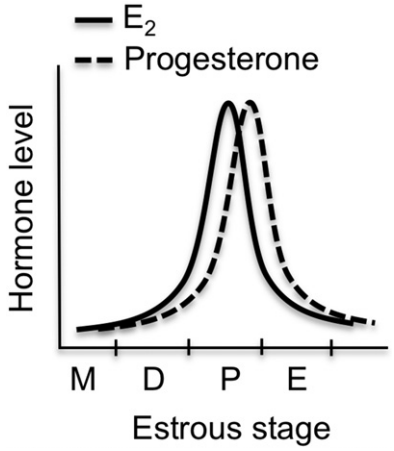

Figure 1. Illustration of serum E_2 and progesterone fluctuations during the 4–5 d rodent estrous cycle. Each stage lasts ~24 h. (M) metestrus (also called diestrus I), (D) diestrus (also called diestrus II), (P) proestrus, (E) estrus.

physiology, biochemistry, and learning and memory in male and female rodents. Finally, to aid those investigators who may seek to incorporate females in their experimental designs, the review will conclude by addressing practical considerations for working with female rodents.

Sex steroid hormones: a primer

To appreciate the complexity inherent to interpreting the biological effects of sex steroid hormones, it is necessary to understand their biosynthesis. Steroid hormones are one of four classes of hormones secreted by endocrine glands. All steroid hormones are synthesized from a cholesterol precursor (Fig. 2) and have a chemical structure that includes three six-carbon rings and one conjugated five-carbon ring (Compagnone and Mellon 2000; see Nelson 2011 for an introduction to steroid biosynthesis). Carbons are cleaved from the 27-carbon cholesterol precursor to generate two functionally distinct groups of steroid hormones, those that mediate the stress response (e.g., cortisol, corticosterone, and aldosterone) and the "sex steroid" hormones that regulate reproductive function (e.g., progestins, androgens, and estrogens). To generate both groups of hormones, six-carbons are cleaved from cholesterol to form the 21-carbon progestins (Nelson 2011). In mammals, these "progestational" hormones, which include pregnenolone and progesterone, are crucial for initiating and terminating mating behavior and for maintaining pregnancy (McCarthy and Becker 2002). These hormones are also essential precursors for all other steroid hormones. Pregnenolone is generated first from cholesterol, and is then converted into progesterone (Compagnone and Mellon 2000). Progesterone can be converted into several other hormones, including corticoids, pregnane neurosteroids, and other sex steroids (although a prohormone for corticoids, progesterone is typically considered a sex steroid hormone because of its importance to reproduction) (Compagnone and Mellon 2000). Because E_2 is the subject of this review, the remainder of this section will focus on sex steroid hormones.

Within specific tissues (e.g., the gonads, brain, adrenals, and adipose tissue), the enzymes necessary to convert progesterone to androgens (e.g., testosterone, androstenedione, and dihydrotestosterone) cleave two more carbons and an ethyl group from the 21-carbon progesterone structure to yield the 19-carbon androgens (Breedlove and Hampson 2002; Nelson 2011). Androgens derive their name from their ability to generate male (*Andros*) appearance, behaviors, and brain physiology during development and adulthood. In male mammals, these hormones are necessary for spermatogenesis, maintenance of the genitalia, and the development of secondary sex characteristics and muscle mass after puberty (Baum 2002; Nelson 2011). As such, androgens are involved in numerous behaviors that facilitate reproductive success, including courtship, copulation, and aggression (Baum 2002; Nelson 2011). Although the primary sources of androgens in male mammals are the testes, the brain also generates a substantial amount of sex steroid hormones independent of the testes (see "Hippocampally synthesized E_2 and memory").

Androgens are the precursors to all estrogens (e.g., E_2, estrone, and estriol). The enzyme aromatase cleaves a carbon from androgenic precursors to form the 18-carbon estrogens in a process called aromatization (McEwen and Krey 1984; Breedlove and Hampson 2002; Nelson 2011). In female mammals, estrogens are essential for many functions including copulatory and reproductive functions (e.g., stimulating ovulation), development of secondary sex characteristics, calcium metabolism, and water retention (McCarthy and Becker 2002; Nelson 2011). The primary sources of estrogens in females are the ovaries, which synthesize and release estrogens in response to signals from gonadotropin hormones released by the pituitary (Terasawa and Ojeda 2009). Estrogens are "estrous generating," in that they stimulate female sexual (estrous) behavior and regulate ovulation (McCarthy and Becker 2002; Nelson 2011). However, estrogens are also synthesized in the brain (see "Hippocampally synthesized E_2 and memory"), adipose tissue, and adrenals.

Finally, it is important to note that it is erroneous to think of androgens as "male" hormones, and estrogens and progestins as "female" hormones (Becker and Breedlove 2002; Nelson 2011). Both males and females synthesize considerable quantities of progestins, androgens, and estrogens (Terasawa and Ojeda 2009), but differ in the quantity of enzymes on hand to metabolize each hormone. For example, although both male and female gonads contain high levels of androgenic enzymes, ovaries also contain an abundance of aromatase for rapidly converting androgens to estrogens. Similarly, males produce copious quantities of progestins to make androgens, which can then be metabolized into estrogens and other metabolites. As such, any of these sex steroid hormones may affect learning and memory in either sex. However, interpretation of hormone effects is complicated by the fact that the

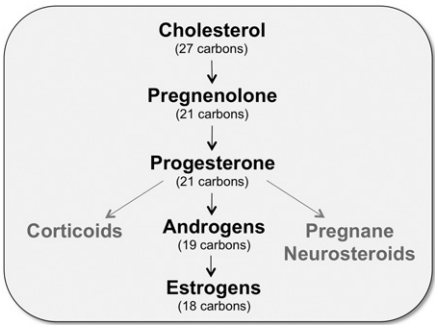

Figure 2. Simplified schematic of steroid hormone biosynthesis. A cholesterol precursor is cleaved to generate the progestins pregnenolone and progesterone. These hormones are essential precursors for all other steroid hormones. Progesterone can then be converted into several other hormones, including corticoids and pregnane neurosteroids (shown in gray). Progesterone can also be further metabolized to produce androgens. The enzyme aromatase cleaves an additional carbon from androgens to yield estrogens.

catabolism of one sex steroid leads to the synthesis of another. Therefore, it can be difficult to determine whether a given sex steroid influences biological processes as itself (e.g., by binding to its receptors) or via conversion to a metabolite. This issue can be addressed pharmacologically in several ways. To use progesterone as an example, the role of progesterone receptors in memory may be examined using progesterone receptor agonists (e.g., R5020) and antagonists (e.g., RU486). Effects mediated via metabolism may be assessed by comparing the effects of progesterone with those of a metabolite (e.g., allopregnanolone) or by co-infusing progesterone with an inhibitor that prevents its catabolism. Because the enzyme 5α-reductase converts progesterone into allopregnanolone, co-infusion of progesterone and a 5α-reductase inhibitor (e.g., finasteride) may be used to determine if allopregnanolone mediates the mnemonic effects of progesterone. Similarly, progesterone's catabolism into cortisol or corticosterone can be blocked an 11β-hydroxylase inhibitor (e.g., metyrapone) and its eventual catabolism into estradiol can be blocked by aromatase inhibitors (e.g., letrozole, fadrozole, or anastrozole). Although such experiments may seem excessive, they are important to determine the precise mechanisms through which a specific steroid hormone regulates memory.

Estrogen receptor localization and mechanism of action

ERα and ERβ as mediators of classical estrogen responses

Sex steroid hormones have traditionally been thought to affect cellular function via intracellular receptors that act as nuclear transcription factors. Because steroid hormones are lipids, they easily move through lipid bilayer plasma membranes to gain entry to the intracellular space. In the traditional model (typically called "classical" or "genomic"), the binding of sex steroid hormones to intracellular hormone receptors causes hormone-receptor complexes to translocate into the nucleus where they bind to hormone response elements on the DNA and initiate gene transcription (Fig. 3). Two intracellular estrogen receptors, ERα and ERβ, have been identified. Both ERs can be found throughout the brain, including the prefrontal cortex, hippocampus, entorhinal cortex, perirhinal cortex, basal forebrain, amygdala, thalamus, and cerebellum (Shughrue et al. 1997b, 2000; Osterlund et al. 2000; Shughrue and Merchenthaler 2000). In the medial prefrontal cortex of ovariectomized rats, ERα and ERβ have been observed in axons, terminals, dendrites, and dendritic spines of neurons, as well as in glia (Almey et al. 2014). Axons and dendrites exhibit a greater percentage of ERα-positive profiles, whereas terminals, spines, and glia exhibit a greater percentage of ERβ-positive profiles (Almey et al. 2014). Within the basal forebrain of ovariectomized rats, ERα colocalizes with choline acetyltransferase-positive cholinergic neurons (Shughrue et al. 2000). Because cholinergic neurons in the basal forebrain project to numerous brain regions, including the hippocampus and neocortex (Frotscher and Léránth 1985; Martinez-Murillo et al. 1990; Wainer et al. 1993), the localization of E_2 within basal forebrain cholinergic neurons may provide a mechanism for E_2 to regulate subcortical input to these structures. In many brain regions, including the infralimbic prefrontal cortex, entorhinal cortex, perirhinal cortex, amygdala, and basal forebrain, ERβ can also be found within parvalbumin-positive inhibitory interneurons (Blurton-Jones and Tuszynski 2002), suggesting that ERβ may regulate inhibitory tone in addition to pyramidal neuron excitability.

ERα and ERβ are distributed throughout the male and female rodent hippocampus (Shughrue et al. 1997a,b; Shughrue and Merchenthaler 2000; Mitra et al. 2003; Mitterling et al. 2010). In adult male and female rats and mice, ERα and ERβ are found in glia and within the nucleus, dendrites, dendritic spines, axons, and terminals of pyramidal neurons (Milner et al. 2001, 2005; Mitterling et al. 2010; Waters et al. 2011a). Findings that ERα-labeled dendritic spine profiles in the rat dentate gyrus were more numerous in proestrus females than in diestrus females and in males suggest the possibility of estrous cycle and sex differences in ER levels among hippocampal subregions (Romeo et al. 2005). In embryonic hippocampal neuron cultures, ERα is also found in vesicle clusters within GABAergic interneurons in CA1, where it mediates an E_2-induced decrease in hippocampal GABAergic neurotransmission that is thought to disinhibit pyramidal neurons (Murphy et al. 1998; Hart et al. 2007; Huang and Woolley 2012). Other work has localized ERα to glutamatergic and GABAergic synaptic vesicles in female rat hippocampal synaptosomes (Tabatadze et al. 2013). Thus, similar to ERβ in other brain regions, ERα in the hippocampus appears to regulate both inhibitory and excitatory tone.

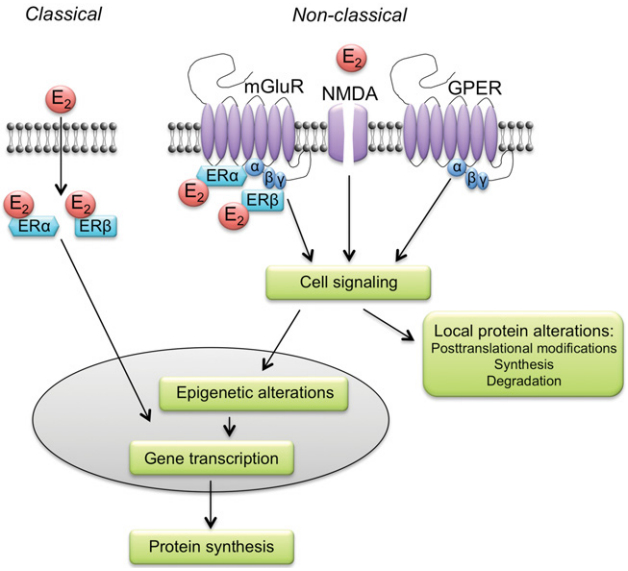

Figure 3. Classical and nonclassical mechanisms of E_2 action. In the classical mechanism (*left*), E_2 binds to ERα and ERβ in the cytoplasm, and then the E_2–ER complex translocates into the nucleus and binds to an estrogen response element on the DNA. Together with histone acetyltransferases and other co-regulators, the E_2–ER complex binds to the estrogen response element on DNA to facilitate gene transcription and protein synthesis. Nonclassical mechanisms (*right*) involve action at or near the plasma membrane that activates cell-signaling cascades. Within the dorsal hippocampus, cell-signaling enzymes associated with estrogenic regulation of hippocampal memory are phosphorylated by several membrane receptors including: (1) metabotropic glutamate receptor 1a (mGluR) and its interactions with ERα and ERβ, (2) NMDA receptors, and (3) G-protein-coupled estrogen receptor (GPER). The resulting activation of cell-signaling pathways (e.g., ERK) triggers epigenetic alterations (e.g., histone acetylation) that regulate gene transcription and protein synthesis. Cell-signaling alterations may also influence local protein levels in cellular compartments such as dendritic spines. (Adapted from Frick 2015 with permission from Elsevier © 2015.)

A role for nonclassical mechanisms in the effects of E_2

The positioning of hippocampal ERs in distal extranuclear cellular compartments like dendritic spines and terminals suggests possible nonclassical mechanisms of action for ERs at or within the plasma membrane (Fig. 3). Although nonclassical and classical mechanisms may ultimately lead to a similar cellular result (e.g., gene transcription), nonclassical mechanisms are typically thought to depend on activation of cell-signaling pathways

and/or epigenetic alterations, rather than nuclear binding to an estrogen response element on DNA. Systemic E_2 treatment increases the distribution of ERβ in dendritic spines and shafts in the adult female rat hippocampus (Waters et al. 2011b), which may position ERβ to interact with plasma membrane receptors in the dendrite to trigger cell signaling. In support of the notion that intracellular ERs act at the membrane are findings showing that E_2 causes ERβ to translocate to the plasma membrane in hippocampal-derived cell lines and rat primary cortical neurons (Sheldahl et al. 2008). At the membrane, ERα and ERβ in neonatal female rats interact with metabotropic glutamate receptor 1 (mGluR1) to rapidly activate hippocampal extracellular signal-regulated kinase (ERK) signaling and promote phosphorylation of cAMP response element binding protein (CREB) (Boulware et al. 2005, 2013). Interestingly, E_2 does not interact with mGluRs to increase ERK-dependent CREB phosphorylation in hippocampal cultures from neonatal male rats (Boulware et al. 2005), suggesting potentially important sex differences in estrogenic regulation of rapid cell signaling. In female rat hippocampal cultures, the ability of the ERs 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). Although the ability of ERs to associate with mGluRs and phosphorylate CREB through an *S*-palmitoylation process has not yet been tested in males, this finding may help to explain how ERα and ERβ can be shuttled to the plasma membrane to trigger rapid cell-signaling processes in females.

Another way in which E_2 may signal at the plasma membrane is by binding to ERs within the membrane itself. The identity of these ERs has been a source of much debate, as the physical structures of ERα and ERβ are not generally consistent with those of integral membrane proteins, and several other proposed membrane ERs (e.g., ER-X and Gq-ER) have proven difficult to clone (Levin 1999, 2011; Woolley 2007; Kelly and Rønnekleiv 2008). Evidence supporting the existence of membrane ERs comes from studies using bovine serum albumin-conjugated E_2 (BSA-E_2), which is too large to penetrate the plasma membrane. In vitro studies of primary rat hippocampal neurons or hippocampal cell lines demonstrate that BSA-E_2 localizes to the plasma membrane, where it rapidly induces calcium signaling, increases ERK phosphorylation and translocation into the nucleus, and increases CREB phosphorylation (Wade and Dorsa 2003; Boulware et al. 2005; Yang et al. 2010; Wu et al. 2011). In vivo, infusion of BSA-E_2 into the dorsal hippocampus or cerebral ventricles of ovariectomized rats or mice activates ERK, Akt, and CREB within 5–10 min (Fernandez et al. 2008; Yang et al. 2010). Moreover, dorsal hippocampal infusion of BSA-E_2 in adult ovariectomized mice induces a similar ERK-dependent enhancement of object recognition memory consolidation to that observed after dorsal hippocampal infusion of free E_2 (Fernandez et al. 2008), suggesting that membrane ERs mediate the memory-enhancing effect of E_2 by activating ERK signaling. Data such as these have led to a general acceptance that a membrane-associated ER facilitates the rapid nonclassical effects of E_2 (e.g., Micevych and Dominguez 2009) and have prompted some investigators to suggest that BSA-E_2 binds to ERα and ERβ localized within the membrane (Meitzen and Mermelstein 2011). Although one provocative report indicates that ERα in the hypothalamus has an extracellular domain and can be internalized by E_2 and mGluR1a ligands (Bondar et al. 2009), it is not yet widely accepted that ERα is an integral membrane protein.

However, BSA-E_2 may bind to other putative membrane ERs, such as G-protein-coupled estrogen receptor (GPER) (Filardo et al. 2000; Thomas et al. 2005; Funakoshi et al. 2006; Prossnitz et al. 2007). This former orphan G-protein-coupled receptor was previously called GPR30. Evidence that GPER strongly binds E_2 in peripheral tissues led recently to its official designation as an ER, despite an ongoing controversy about whether GPER is a true ER in the nervous system (Levin 2009; Langer et al. 2010; Maggiolini and Picard 2010; Barton 2012). GPER can be found throughout the brain in male and female rodents, including the hippocampus, prefrontal cortex, and striatum (Brailoiu et al. 2007). GPER is also found in the female rat basal forebrain, where it colocalizes with cholinergic neurons and, to a lesser extent, with GABAergic neurons (Hammond et al. 2011). Within the male and female mouse hippocampus, GPER has been observed in all lamina and exclusively at extranuclear sites within pyramidal neurons, interneurons, and glia (Waters et al. 2015). GPER has been localized to 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 may be regulated by estrogens and/or progestins. Sex differences have also been observed in relation to the cycle, such that estrus females exhibited fewer GPER-labeled axons in CA1 than males, whereas proestrus females exhibited more GPER-labeled glia in the dentate gyrus than males (Waters et al. 2015). Knockdown of GPER attenuates the neuroprotective effects of BSA-E_2 in the hippocampus of ovariectomized rats, indicating a role for GPER in E_2-induced neuroprotection (Tang et al. 2014). GPER activation also increases potassium-evoked acetylcholine release in the ovariectomized rat hippocampus in a manner similar to E_2 treatment (Gibbs et al. 2014). Importantly, systemic injections of GPER-specific compounds indicate that GPER activation facilitates spatial working memory in ovariectomized rats (Hammond et al. 2009, 2012), suggesting that GPER could mediate the mnemonic effects of E_2.

Estrogenic regulation of hippocampal morphology and physiology

Spine density

Interest in the effects of E_2 on nonreproductive regions of the brain intensified with the discovery in the early 1990s that both exogenous and endogenous estradiol increased the density of dendritic spines on CA1 pyramidal neurons in adult female rats (Gould et al. 1990; Woolley et al. 1990; Woolley and McEwen 1992, 1993). During the estrous cycle, spine density is highest during the proestrus phase (Woolley and McEwen 1992), which is characterized by high levels of estrogens and progesterone (Fig. 1). Spine density plunges ~30% within 24 h to reach its nadir during the estrus phase of the cycle (Woolley and McEwen 1992; Kato et al. 2013), which is characterized by low levels of estrogens and progesterone (Fig. 1). After bilateral ovariectomy, spine density decreases gradually and is significantly reduced 6 d after surgery (Gould et al. 1990; Woolley and McEwen 1993). Two systemic injections of estradiol benzoate given 24 h apart fully reversed this decrease within 48 h (although a significant increase was seen within 24 h), and an injection of progesterone 48 h later augmented the effects of estradiol on spines within the first 10 h of treatment (Woolley and McEwen 1993). Two systemic injections of the ERα agonist propyl pyrazole triol (PPT) or the ERβ agonist diarylpropionitrile (DPN) (Stauffer et al. 2000; Meyers et al. 2001) also significantly increase expression of the synaptic proteins PSD-95 and GluR1 in hippocampal CA1 (Waters et al. 2009), suggesting that E_2-induced spine increases in adult females could be mediated by either receptor. Additional support for a role

of ERα comes from work with hippocampal cultures from neonatal female rats, in which 7 d of treatment with PPT significantly increased CA1 spine density (Zhou et al. 2014). However, DPN significantly decreased CA1 spine density in these cultures (Zhou et al. 2014), suggesting that ERβ may suppress spinogenesis in early development. Some data support the conclusion that input from the basal forebrain is necessary for estradiol to enhance spines in adult female rats (Leranth et al. 2000). However, bath-applied E_2 can increase spines in male hippocampal slices (Mukai et al. 2007; Murakami et al. 2014; Hasegawa et al. 2015) and cultured embryonic hippocampal neurons (Murphy and Segal 1996), suggesting that subcortical input may not be essential, at least in an ex vivo system. Other work shows that two injections of estradiol benzoate spaced 24 h apart failed to increase CA1 spine synapse density in ovariectomized rats trained in the Morris water maze (Frick et al. 2004), indicating that stressful behavioral training may interfere with the effects of estradiol on CA1 spinogenesis.

It is important to note potential species differences in the effects of E_2 on CA1 dendritic spine density. One early report from ovariectomized mice found that 5 d of systemic estradiol benzoate injection did not increase the total number of spines, but rather increased the number of mushroom spines specifically (Li et al. 2004). However, other more recent studies report an increase in total CA1 spine density in mice after acute systemic treatment with E_2 or agonists of ERα and ERβ (Liu et al. 2008; Phan et al. 2011, 2012). Although the reasons for this discrepancy are unclear, these latter findings suggest parallels between the effects of E_2 on spine density in female rats and mice.

In male rats, bilateral gonadectomy significantly reduced CA1 spine synapse density, but this effect was not reversed by 2 d of systemic E_2 injection as observed in ovariectomized females (Leranth et al. 2003). Rather, the gonadectomy-induced decrease was reversed by 2 d of systemic testosterone or the nonaromatizable androgen dihydrotestosterone (DHT) (Leranth et al. 2003). The effects of androgens on spines are supported by data from male hippocampal slices showing that bath application of testosterone or the nonaromatizable DHT also increased CA1 spine density (Ooishi et al. 2012; Hatanaka et al. 2014). In contrast to the in vivo data, however, studies using hippocampal slices from adult male rats report that the density of dendritic spines in CA1 was increased within 2 h of bath application of E_2 (Murakami et al. 2006, 2014; Mukai et al. 2007; Ogiue-Ikeda et al. 2008; Ooishi et al. 2012). This increase was blocked by an inhibitor of ERK phosphorylation (Mukai et al. 2007; Murakami et al. 2014), linking ERK signaling to E_2-induced CA1 spinogenesis in males. The spinogenesis in male hippocampal slices appears to be mediated by ERα rather than ERβ, as illustrated by findings showing that CA1 spines were increased by bath application of PPT, but not DPN (Mukai et al. 2007). The role of ERα in CA1 spinogenesis among males is also supported by data from ER knockout mice demonstrating that bath-applied E_2 increased CA1 spine density in ERβ knockouts, but not in ERα knockouts (Murakami et al. 2014). Although the effects of E_2 on CA1 spines in male hippocampal slices have been observed in multiple studies, discrepancies between these effects and the aforementioned lack of effect of systemic E_2 on spines (Leranth et al. 2003) could be due to several factors, including different experimental systems (in vivo versus ex vivo) and timing. The increases induced by E_2, testosterone, and DHT in slice preparations were observed 2 h after treatment, whereas spines were assessed in the in vivo work 2 d after treatment. Thus, E_2-induced spine changes in males may be relatively transient and dissipate in vivo within 2 d.

One particularly intriguing aspect of the ex vivo spine data is the observation that significant changes could be observed within 2 h of treatment. This effect is not an artifact of the slice preparation, as several in vivo studies of ovariectomized rats have found that E_2 increased spine density in both CA1 and prefrontal cortex layer II/III within 30 min of a systemic injection (MacLusky et al. 2005; Inagaki et al. 2012). Moreover, agonists for ERα and ERβ increased CA1 dendritic spine density in ovariectomized mice within 40 min of a single systemic injection (Phan et al. 2011), indicating a role for both ERα or ERβ in rapid E_2-induced spinogenesis. At the present time, it is unclear how E_2 might facilitate CA1 spine formation. However, several mechanisms are possible, including inducing post-translational modifications of existing dendritic proteins, altering protein degradation, increasing constitutive protein synthesis, or triggering new protein synthesis, as all of these mechanisms have been implicated in hippocampal memory and/or synaptic plasticity (Holahan and Routtenberg 2007; Klann and Sweatt 2008; Routtenberg 2008; Abbas 2013; Jarome and Helmstetter 2014).

One potential mechanism through which new local protein synthesis may occur is via activation of mammalian target of rapamycin (mTOR) signaling, which phosphorylates several key components of the protein synthesis machinery (Hoeffer and Klann 2010). mTOR is necessary for the consolidation of several types of memories (Dash et al. 2006; Parsons et al. 2006; Bekinschtein et al. 2007; Myskiw et al. 2008) and, as will be discussed later, for E_2 to enhance consolidation of object recognition memory in ovariectomized mice (Fortress et al. 2013). mTOR signaling can be activated by a host of upstream kinases including phosphatidylinositol 3-kinase (PI3K), Akt, and ERK (Richter and Klann 2009; Hoeffer and Klann 2010; Laplante and Sabatini 2012), and phosphorylation of many of these kinases has been implicated in the ability of E_2 to increase CA1 spine density in hippocampal slices from male rats. For example, the aforementioned E_2-induced spine increase in male hippocampal slices was blocked by inhibitors of ERK, PI3K, protein kinase A (PKA), protein kinase C (PKC), and CaMKII (Hasegawa et al. 2015). Inhibitors of these signaling cascades also prevented E_2 from augmenting θ-bust-stimulated long-term potentiation (LTP) in male hippocampal slices (Hasegawa et al. 2015), suggesting a key role for activation of these cell-signaling cascades in E_2-induced synaptic potentiation. Similarly, an E_2-induced increase in cortical dendritic spine density observed 30 min after bath application in embryonic rat cultures was blocked by pharmacological inhibition of Rap/AF-6/ERK signaling (Srivastava et al. 2008). As with E_2, the spine increase induced by testosterone and DHT in males was blocked by inhibitors of ERK, PKA, PKC, LIM kinase (LIMK), and calcineurin (Ooishi et al. 2012; Hatanaka et al. 2014). As will be discussed below (see "Molecular mechanisms underlying E_2's effects on memory consolidation"), many of these same signaling cascades are involved in estrogenic regulation of hippocampal memory consolidation in ovariectomized mice, potentially linking E_2-induced alterations in CA1 spine density and LTP with memory formation.

Synaptic plasticity

Because dendritic spines are found on excitatory pyramidal neurons, increased spine density is thought to lead to enhanced synaptic plasticity. E_2 has numerous effects on neuronal excitability and plasticity in the brain. For example, acute E_2 regulates intrinsic plasticity in brain regions including the amygdala, striatum, cerebellum, and hippocampus (Nabekura et al. 1986; Smith et al. 1988; Mermelstein et al. 1996; Kumar and Foster 2002). In hippocampal slices from ovariectomized rats, bath-applied E_2 has been shown to increase spontaneous firing and suppress the afterhyperpolarization (Kumar and Foster 2002; Carrer et al. 2003). Bath-applied E_2 also increased kainate-induced currents in dissociated male and female CA1 neurons, an effect that was blocked

by inhibition of PKA activation and appeared to be independent of intracellular ERα and ERβ (Gu and Moss 1996; Gu et al. 1999).

Perhaps, the most notable effect of E_2 on hippocampal physiology is its ability to potentiate NMDA-dependent LTP in female CA3-CA1 synapses (for reviews, see Foy 2001; Woolley 2007; Smith et al. 2009). LTP results in a persistent increase in synaptic excitability thought to underlie memory formation. E_2 increases NMDA receptor binding in the female rat hippocampus, and the E_2-induced increase in CA1 dendritic spine density is correlated with synaptic input mediated by NMDA receptors (Weiland 1992; Woolley et al. 1997). Within the rat estrous cycle, the greatest degree of LTP in CA1 is observed on the afternoon of proestrus, at which point estrogen and progesterone levels peak (Warren et al. 1995). Similarly, exogenous E_2 increases baseline EPSP amplitude, reduces the threshold for LTP, and increases LTP amplitude in the hippocampus of male and female rodents (Teyler et al. 1980; Cordoba-Montoya and Carrer 1997; Foy et al. 1999, 2008; Bi et al. 2000; Fugger et al. 2001; Sharrow et al. 2002; Smith and McMahon 2005, 2006; Kramár et al. 2009; Smejkalova and Woolley 2010; Tanaka and Sokabe 2013; Kumar et al. 2015). Bath-applied E_2 and PPT facilitated NMDA-mediated transmission and LTP in the dentate gyrus of slices from juvenile males, whereas DPN suppressed both forms of plasticity (Tanaka and Sokabe 2012, 2013). These data suggest that ERα promotes, whereas ERβ represses, hippocampal synaptic plasticity in juvenile males. However, ERβ appears to facilitate, rather than suppress, plasticity in the adult hippocampus. In adult male hippocampal slices, the effects of E_2 on synaptic potentiation were mimicked by the ERβ agonist WAY200070, but not by PPT (Kramár et al. 2009), suggesting a selective role of ERβ in mediating the effects of E_2 on LTP in adult males. WAY200070 also enhanced LTP in hippocampal slices from adult wild-type females, but not slices from ERβ knockout mice (Liu et al. 2008), supporting an essential role for ERβ in regulating LTP in adult females as well.

Consistent with the localization of ERα to inhibitory interneurons in the hippocampus (Murphy et al. 1998; Hart et al. 2007; Tabatadze et al. 2013), bath-applied E_2 suppresses synaptic inhibition in hippocampal slices from ovariectomized rats (Huang and Woolley 2012). Interestingly, this effect was not observed in slices from male rats (Huang and Woolley 2012), which may stem from sex differences in levels of extranuclear ERα found in the hippocampus (Mitterling et al. 2010). The role of ionotropic glutamate receptors in estrogenic regulation of LTP may also be affected by sex, as illustrated by data showing that E_2 affected AMPA, but not NMDA, responses in adult male slices, whereas the effects of E_2 in adult female slices depended on NR2B-containing NMDA receptors (Smith and McMahon 2005, 2006; Kramár et al. 2009). However, other reports find that E_2 enhances NMDA responses in males (Foy et al. 1999; Tanaka and Sokabe 2013), suggesting similar effects of E_2 on NMDA receptors in both sexes. In females, the E_2-induced increase in NMDA-mediated excitatory postsynaptic currents was not due to an increase in NMDA receptor subunits or phosphorylation of NR2B (Snyder et al. 2011), so likely results from the recruitment of existing NMDA receptors to synaptic sites (Jelks et al. 2007; Snyder et al. 2011). Whether the E_2-induced alterations in hippocampal synaptic potentiation have direct functional consequences for the estrogenic regulation of learning and memory in either sex is unknown. However, an E_2-induced enhancement of object recognition in ovariectomized rats was found to require an increase in NR2B-containing NMDA receptors, linking E_2-induced alterations in LTP and memory in females (Vedder et al. 2013).

Actin polymerization is necessary in rats and mice for the formation of stable LTP (Krucker et al. 2000; Kramár et al. 2006). In both males and females, E_2 may facilitate hippocampal LTP through actin polymerization. In hippocampal slices from male rats, bath application of E_2 increased filamentous actin levels and actin polymerization in dendritic spines by activating the RhoA > RhoA kinase (ROCK) > LIMK > cofilin pathway, a key modulator of actin polymerization and stabilization (Kramár et al. 2009). The importance of actin dynamics in E_2-induced plasticity was demonstrated by studies showing that E_2's effects on LTP in male slices were completely blocked by latrunculin, a toxin that disrupts actin filament assembly (Kramár et al. 2009). In female rat slices, E_2 reversed ovariectomy-induced decreases in RhoA levels and actin polymerization (Kramár et al. 2009), suggesting that the RhoA > ROCK > LIMK > cofilin pathway may also be involved in E_2-induced plasticity in females.

Interestingly, recent work has shown that the ability of E_2 to increase LTP in ovariectomized rats is influenced by the duration of ovarian hormone deprivation prior to treatment. In these studies, two injections of E_2 given 24 h apart were unable to increase LTP in rats ovariectomized for 19 mo prior to treatment (Smith et al. 2010). Importantly, this loss was not due to aging, as rats of the same age ovariectomized just 1 mo prior to E_2 treatment exhibited increased LTP in response to E_2 (Smith et al. 2010). This research group also found that treatment with both acute and chronic E_2 was unable to enhance object recognition memory in rats ovariectomized for 19 mo prior to treatment (Vedder et al. 2014), suggesting important parallels between the synaptic and mnemonic responsiveness to E_2 in females. Other investigators have reported similarly detrimental effects of long-term ovariectomy on the ability of chronic E_2 to enhance spatial working memory (Daniel et al. 2006), supporting the idea that long-term hormone deprivation impairs E_2's capacity to facilitate hippocampal synaptic plasticity and memory. The reasons for this decreased responsiveness are not yet clear, but could be due to ubiquitination and degradation of estrogen receptors as observed in the CA1 of long-term ovariectomized rats (Zhang et al. 2011).

Although the effects of E_2 on synaptic plasticity are typically attributed to a postsynaptic mechanism of action, other data indicate that E_2 can potentiate glutamate transmission via a presynaptic mechanism. In hippocampal slices prepared from ovariectomized E_2-treated rats, bath-applied E_2 potentiated EPSCs by increasing the probability of glutamate release from specific inputs with an initially low probability of release (Smejkalova and Woolley 2010). This effect was mediated by ERβ, but not by ERα (Smejkalova and Woolley 2010). Although such presynaptic effects of E_2 would be expected based on the localization of ERs to axon terminals (Milner et al. 2001, 2005; Mitterling et al. 2010; Waters et al. 2011a), this novel study was the first to suggest presynaptic facilitation of excitatory transmission by E_2.

Finally, E_2 also affects hippocampal long-term depression (LTD), yet this has been far less studied than LTP. One series of studies showed that induction of LTD in CA1 by patterned low-frequency stimulation is impaired in ovariectomized rats relative to gonadally intact females and is restored by two injections of estradiol benzoate given 48 h before tissue collection (Desmond et al. 2000; Zamani et al. 2000; Day and Good 2005). The effects of E_2 on LTD in ovariectomized females required NMDA receptors and depended on conditioning frequency, as 2- or 4-Hz paired-pulse conditioning facilitated LTD induction, whereas 10-Hz conditioning blocked LTD induction (Zamani et al. 2000). These data have been interpreted to suggest that E_2 decreases the threshold for inducing LTD in adult females by activating group I mGlu receptors (Zamani et al. 2000; Shiroma et al. 2005). Similarly, 30 min of E_2 pretreatment enhanced NMDA-induced LTD in CA1, CA3, and the dentate gyrus of adult male rats (Mukai et al. 2007; Murakami et al. 2014). In contrast to the important role of ERβ in mediating LTP, this effect was mimicked by the ERα agonist PPT, but not by the ERβ agonist DPN, indicating a specific involvement of ERα in mediating LTD in the male hippocampus (Mukai

et al. 2007). However, not all studies find that E_2 facilitates LTD in male and female rodents. In hippocampal slices pretreated with E_2 for at least 30 min, the induction of LTD by patterned stimulation was unaltered in gonadally intact male rats (Vouimba et al. 2000) and blocked by E_2 in young ovariectomized rats (Sharrow et al. 2002). It has been suggested that the inconsistency between these studies and those showing that E_2 enhances LTP may result from differential influences of discrepant E_2 treatments on intracellular calcium or on activation of classical and nonclassical ER mechanisms (Sharrow et al. 2002; Shiroma et al. 2005; Foy et al. 2008).

In contrast to young adult rats, data from aged rats suggest that E_2 suppresses LTD in the aged hippocampus. For example, chronic E_2 treatment blocked the induction of LTD induced by patterned stimulation in hippocampal slices from aged ovariectomized rats that had undergone extensive behavioral testing (Foster et al. 2003). Similarly, 30 min of E_2 suppressed LTD induced by patterned stimulation in hippocampal slices from aged males (Vouimba et al. 2000; Foy et al. 2008). Enhanced LTD in aged rats is thought to play a role in age-related memory decline (Norris et al. 1996; Foster 1999), and E_2 is an important trophic factor that protects against such decline (Frick 2009). As such, suppression of LTD in aged subjects may be one mechanism through which E_2 reduces memory loss during aging.

Neurogenesis

Neurogenesis is another major morphological alteration in the hippocampus influenced by E_2 (for reviews, see Galea 2008; Pawluski et al. 2009; Galea et al. 2013). Among gonadally intact rats, proestrus females transiently exhibit more cell proliferation than males, such that higher numbers of bromodeoxyuridine (BrdU)-labeled cells are observed in females 2 d, but not 14 d, after BrdU injection (Tanapat et al. 1999). Cell proliferation is also substantially higher during proestrus than during estrus and diestrus, suggesting that elevated levels of estrogens and/or progestins enhance neurogenesis in females (Tanapat et al. 1999). This notion is supported by the observation that ovariectomy significantly reduces cell proliferation and increases numbers of degenerating pyknotic cells (Tanapat et al. 1999, 2005). Exogenous E_2 modulates neurogenesis in the dentate gyrus by regulating cell proliferation, although effects vary based on factors such as dose, sex, age, duration of ovariectomy, and timing of injection relative to BrdU labeling. In general, a single brief exposure to E_2 transiently increases cell proliferation in the dentate gyrus of ovariectomized rats (Tanapat et al. 1999, 2005; Banasr et al. 2001; Barha et al. 2009). Interestingly, an injection of progesterone given 48 h after E_2 treatment reverses the E_2-induced increase in cell proliferation (Tanapat et al. 2005), suggesting that the increased neurogenesis during proestrus results from elevated E_2 rather than progesterone. In addition to cell proliferation, a few reports indicate that acute or chronic E_2 increases cell survival and decreases pyknotic cells in ovariectomized rats (Tanapat et al. 1999; McClure et al. 2013). Both ERα and ERβ appear to mediate the effects of E_2 on hippocampal cell proliferation, as a single systemic injection of ERα or ERβ agonists increased dentate cell proliferation in ovariectomized rats (Mazzucco et al. 2006). Acute treatment with other forms of estrogens, including estradiol benzoate, 17α-estradiol, and estrone, also increases cell proliferation in ovariectomized rats in a dose- and time-dependent manner (Ormerod et al. 2003; Mazzucco et al. 2006; Nagy et al. 2006; Barker and Galea 2008; Barha et al. 2009). These data suggest that the ability to increase cell proliferation in female rats is a general feature of estrogens and not an effect specific to E_2.

In contrast to the effects of acute E_2 on hippocampal neurogenesis, chronic E_2 treatment (1–3 wk) administered via silastic capsules or daily injections has minimal effects on cell proliferation in ovariectomized rats (Perez-Martin et al. 2003; Tanapat et al. 2005; Barker and Galea 2008; McClure et al. 2013). The reasons for this lack of effect remain unclear, but the data indicate that chronically elevated levels of E_2 are not conducive to cell proliferation. During the rat estrous cycle, E_2 levels are elevated only during proestrus, so such chronically elevated E_2 levels are not characteristic of the natural hormonal milieu in females. As such, fundamental changes may occur in the brain during chronic treatment (e.g., downregulation of ERs) that do not occur after acute treatment. On the other end of the hormonal spectrum, long-term ovariectomy decreased hippocampal neurogenesis and prevented acute E_2 from increasing cell proliferation (Tanapat et al. 2005), which is consistent with the detrimental effects of long-term ovariectomy on LTP and memory discussed above (Daniel et al. 2006; Smith et al. 2010; Vedder et al. 2014). Collectively, these data suggest that either chronically elevated E_2 levels or long-term hormone deprivation may permanently alter the hippocampus to reduce its responsiveness to E_2.

Contrary to the beneficial effects of acute estrogens on neurogenesis in female rats, acute estrogen treatment does not increase cell proliferation in male rats. Castration of male rats significantly reduces 30-d cell survival without affecting cell proliferation (Spritzer and Galea 2007). Unlike in ovariectomized rats, neither E_2 nor estradiol benzoate increased cell proliferation or cell survival in gonadectomized male rats or mice (Spritzer and Galea 2007; Barker and Galea 2008; Zhang et al. 2010). However, acute or chronic testosterone or DHT increased cell survival in gonadectomized rats in an androgen receptor-dependent manner (Spritzer and Galea 2007; Hamson et al. 2013), indicating that androgens promote hippocampal neurogenesis in male rats by increasing cell survival. Interestingly, progesterone, but not testosterone, increased cell survival in a progesterone receptor-dependent manner in male mice (Zhang et al. 2010), suggesting that new cells in the male mouse hippocampus are more sensitive to progesterone than androgens. Moreover, the effects of progesterone were blocked by inhibitors of ERK and PI3K (Zhang et al. 2010), demonstrating a potentially important role for rapid cell signaling in progesterone-induced neurogenesis in male mice. Together, these findings demonstrate an important role for androgens and progesterone, but not estrogens, in hippocampal neurogenesis in male rodents.

Estrogenic modulation of learning and memory

The effects of E_2 on learning and memory in female rats and mice have been discussed at length in numerous comprehensive reviews (Korol 2002; Foster 2005; Daniel 2006, 2013; Sherwin and Henry 2008; Barha and Galea 2010; Bimonte-Nelson et al. 2010; Gibbs 2010; Kim and Casadesus 2010; Choleris et al. 2012; Foster 2012; Frick 2012; Acosta et al. 2013; Chisolm and Juraska 2013; Ervin et al. 2013; Galea et al. 2013; Hogervorst 2013; Luine and Frankfurt 2013; Maki 2013; Bean et al. 2014; Fortress and Frick 2014; Luine 2014; Frankfurt and Luine 2015; Tuscher et al. 2015). Therefore, we direct readers to these resources for a more detailed description of this literature than will be provided here. The sections below will provide a broad overview of the effects of the estrous cycle on memory, as well as the effects of gonadectomy and exogenous E_2 treatment on memory in female and male rodents.

Memory and the estrous cycle

If hippocampal dendritic spine density, LTP, and cell proliferation are increased during proestrus relative to other cycle stages, then one might expect forms of learning and memory in which the hippocampus is involved to also be enhanced during proestrus.

Some evidence does support this idea. For example, several studies report that spatial memory tested in the Morris water maze or object placement tasks was enhanced during proestrus relative to estrus and/or diestrus in mice and rats (Frick and Berger-Sweeney 2001; Frye et al. 2007; Paris and Frye 2008; Pompili et al. 2010). Consistent with these findings, rats in proestrus are more likely than those in estrus to use a spatial learning strategy (Korol et al. 2004). Rats tested during proestrus also exhibit facilitated eyeblink conditioning (Shors et al. 1998). However, the reported proestrus advantage in spatial tasks is inconsistent with other data in rats showing enhanced spatial reference memory in the Morris water maze during estrus relative to proestrus (Frye 1995; Warren and Juraska 1997; Sutcliffe et al. 2007) or no effect of the cycle on spatial reference memory in the water maze or radial arm maze, spatial working memory in the radial arm maze, or spatial novelty in a T-maze (Berry et al. 1997; Stackman et al. 1997; Conrad et al. 2004; Pompili et al. 2010). In tasks modeling selective attention, rats trained during proestrus failed to show latent inhibition (Arad and Weiner 2008; Quinlan et al. 2010). In object recognition tasks, rats and mice tested in proestrus have been reported to outperform those in diestrus and estrus, but other findings show intact object recognition memory in all phases of the cycle (Walf et al. 2006, 2009; Sutcliffe et al. 2007; Paris and Frye 2008). Conflicting effects of the cycle have also been reported in social recognition tasks, where one study reported intact social recognition in proestrus (Sánchez-Andrade and Kendrick 2011), but not estrus, whereas another found intact social recognition in both stages (Markham and Juraska 2007).

It is important to note that discrepancies among estrous findings may be amplified in this literature because so few studies have examined potential effects of the cycle on learning and memory. Thus, methodological differences between this handful of studies may create the false impression of discrepant findings. For example, water temperature in the Morris water maze has been shown to affect the observance of estrous cycle effects in rats, with proestrous rats outperforming estrus rats in warm (33°C) water and estrus rats outperforming proestrus rats in cold (19°C) water (Rubinow et al. 2004). Moreover, because ovarian hormone levels fluctuate rapidly, assessing learning and memory within a single phase of the cycle can be challenging. This is particularly so during the 24 h of proestrus, in which hormone levels rise throughout the day and peak in the evening. As such, studies in which subjects were tested early on the day of proestrus may find fewer effects of the cycle than those in which subjects were tested late in the day. Estrous cycle differences reported in various tests of learning and memory may not lead to substantial sex differences in learning and memory, but could contribute to increased variability with gonadally intact female groups. For mice, however, a recent meta-analysis indicated that the estrous cycle does not lead to greater variability in females relative to males among traits including learning and memory, attention, neuronal morphology, LTP, hormonal and immune function, and epigenetic processes (Prendergast et al. 2014). Given how few studies have examined the effects of the estrous cycle on various forms of memory, this area is particularly ripe for further investigation. See the section "Practical considerations for working with females" below for additional discussion about the use of cycling females in experimental designs.

Effects of exogenous E_2 on memory in females

Because bilateral ovariectomy provides better experimental control of circulating ovarian hormone levels than intact gonads, the vast majority of studies on estrogenic regulation of memory in rodents have been conducted using females that have had their ovaries removed. This approach eliminates the primary source of circulating estrogens and progestins, so it is important to realize that levels of several hormones will drop as a result. Ovariectomy itself impairs some forms of memory including spatial working memory in the radial arm maze, spatial reference memory in the Morris water maze, object recognition memory, and memory in a two-way active avoidance task (Singh et al. 1994; Daniel et al. 1999; Wallace et al. 2006; Gibbs and Johnson 2008; Monteiro et al. 2008). However, other studies report no effect or a memory-enhancing effect of ovariectomy in these tasks (Singh et al. 1994; Daniel et al. 1999; Bimonte-Nelson et al. 2003). In vivo, exogenous E_2 can be administered in numerous ways, the most common of which are systemic injection, silastic capsule implantation, slow-release pellet implantation, and intracranial infusion. Injections and infusions are most often used for acute treatments, whereas silastic capsules and pellets are typically used for chronic treatments.

In rats and mice, the effects of exogenous E_2 have been tested in a variety of tasks that tap into many forms of memory. In general, exogenous E_2 improves memory in adult ovariectomized rats and mice. For example, systemic E_2 given acutely or chronically improves spatial working memory in the radial arm maze, the Morris water maze, and alternation or delayed nonmatch to position tasks in the T-maze (O'Neal et al. 1996; Daniel et al. 1997; Fader et al. 1998, 1999; Luine et al. 1998; Bimonte and Denenberg 1999; Gibbs 1999; Daniel and Dohanich 2001; Sandstrom and Williams 2001, 2004; Bowman et al. 2002; Heikkinen et al. 2002; Holmes et al. 2002; Garza-Meilandt et al. 2006; Bohacek and Daniel 2007; Hammond et al. 2009). Nonspatial working memory in the T-maze is also improved by systemic E_2 (Wide et al. 2004), as is spatial reference memory in the radial arm and Morris water mazes (Packard and Teather 1997a; Heikkinen et al. 2002; Gresack and Frick 2006), recognition memory for the location and identity of objects (Vaucher et al. 2002; Luine et al. 2003; Walf et al. 2006; Frye et al. 2007; Fernandez et al. 2008; Inagaki et al. 2010; Zhao et al. 2010; Phan et al. 2012; Boulware et al. 2013), social recognition memory (Phan et al. 2012), inhibitory avoidance (Singh et al. 1994; Frye and Rhodes 2002, but see Foster et al. 2003), and trace eyeblink conditioning (Leuner et al. 2004). However, not all studies find that E_2 benefits memory in adult females under all conditions, as improvements often depend on methodological variables such as dose (Packard and Teather 1997a; Holmes et al. 2002; Leuner et al. 2004; Wide et al. 2004; Gresack and Frick 2006; Foster 2012; Phan et al. 2012), age at treatment (Savonenko and Markowska 2003; Gresack et al. 2007; Markham and Juraska 2007), duration of treatment (Luine et al. 1998; Markowska and Savonenko 2002), prior E_2 priming (Markowska and Savonenko 2002), route of administration (Garza-Meilandt et al. 2006), progesterone co-administration (Chesler and Juraska 2000; Bimonte-Nelson et al. 2006; Harburger et al. 2009), duration of daily handling (Bohacek and Daniel 2007), the cognitive demands of the task (Bimonte and Denenberg 1999), and duration of ovariectomy prior to treatment (Gibbs 2000; Daniel et al. 2006; Vedder et al. 2014). Understanding the extent to which these variables influence the response to E_2 has potential translational relevance. For example, findings that long-term ovariectomy substantially reduces the mnemonic response to E_2 in rodents support the critical period hypothesis developed to explain why menopausal hormone treatment benefits cognition more in younger menopausal women than in older post-menopausal women (Sherwin 2007). As such, duration of ovariectomy in rodents could be used as an experimental variable to develop treatments that lengthen this critical period in women. For more extensive discussion of these variables, see recent reviews that discuss the influence of estrogens on cognitive aging (Sherwin and Henry 2008; Frick 2009; Conrad and Bimonte-Nelson 2010;

Foster 2012; Chisolm and Juraska 2013; Maki 2013; Daniel et al. 2015).

To better understand the molecular mechanisms through which E_2 regulates memory formation, numerous investigators have administered acute E_2 immediately after training in a variety of tasks. When used with a water-soluble cyclodextrin-encapsulated form of E_2 that is metabolized within 24 h (Pitha and Pitha 1985; Pitha et al. 1986), post-training treatments allow both training and testing to occur in the absence of circulating E_2, which permits the memory-enhancing effects of E_2 to be isolated to the memory consolidation phase of memory processing (Frick et al. 2010). Cyclodextrin encapsulation also allows E_2 to be more easily infused into the brain, as E_2 must otherwise be dissolved in oil. The memory-enhancing effects of post-training cyclodextrin-encapsulated E_2 were first observed in gonadally intact male rats. In this initial work, bilateral intrahippocampal infusion of E_2 administered immediately, but not 2 h, after eight spatial Morris water maze training trials improved retention of the platform location 24 h later (Packard et al. 1996). Subsequent work in ovariectomized rats and mice showed that post-training systemic injection or intrahippocampal infusion of cyclodextrin-encapsulated E_2 enhanced spatial reference memory consolidation in the Morris water maze (Fig. 4A), spatial memory consolidation in an object location task, and object recognition memory consolidation (Fig. 4B; Packard and Teather 1997a,b; Fernandez et al. 2008; Lewis et al. 2008; Zhao et al. 2010, 2012; Boulware et al. 2013; Fortress et al. 2013; Pereira et al. 2014). Post-training systemic injection of noncyclodextrin-encapsulated E_2 or estradiol benzoate also enhanced spatial and object recognition memory in object-based tasks in both rats and mice (Luine et al. 2003; Walf et al. 2006, 2008; Frye et al. 2007; Inagaki et al. 2010). Similar to the original male study (Packard et al. 1996), delayed infusion of E_2 1–3 h after training had no effect on memory consolidation in female rodents (Walf et al. 2006; Frye et al. 2007; Fernandez et al. 2008), suggesting that the effects of E_2 on memory consolidation occur during a discrete time window after training. The cell-signaling, epigenetic, and receptor mechanisms that may underlie the beneficial effects of E_2 on object recognition memory consolidation in ovariectomized females will be discussed further in "Molecular mechanisms underlying E_2's effects on memory consolidation."

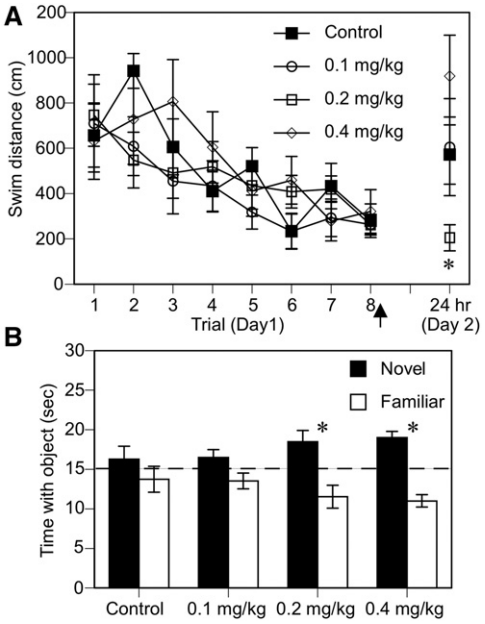

Figure 4. Post-training systemic E_2 injection enhances spatial memory consolidation in the Morris water maze and object recognition memory consolidation in ovariectomized mice. (*A*) Ovariectomized mice received eight hidden-platform training trials prior to E_2 administration. Immediately after the final training trial (arrow), mice were injected with cyclodextrin vehicle (Control) or one of three doses (0.1, 0.2, or 0.4 mg/kg) of cyclodextrin-encapsulated E_2. When memory for the platform location was tested 24 h later, only mice injected with 0.2 mg/kg E_2 remembered the platform location as indicated by the fact that mice in all other groups swam significantly longer distances on day 2 compared with mice in the 0.2 mg/kg group ($^*P < 0.05$). (*B*) Ovariectomized mice accumulated 30 sec exploring two identical objects and then were immediately injected with cyclodextrin vehicle (Control) or one of three doses (0.1, 0.2, or 0.4 mg/kg) of cyclodextrin-encapsulated E_2. During testing 48 h later, mice receiving 0.2 or 0.4 mg/kg E_2 spent significantly more time with the novel object than chance (dashed line at 15 sec), indicating that these doses enhanced object recognition memory consolidation ($^*P < 0.05$ relative to chance). In contrast, the control and 0.1 mg/kg groups did not spend more time than chance with the novel object. Error bars in both panels represent the mean ± SEM. (Adapted from Gresack and Frick 2006 with permission from Elsevier © 2015.)

Effects of exogenous E_2 on memory in males

Compared with females, far fewer studies have examined the effects of E_2 on memory in male rodents. Thus, broad conclusions are more difficult to draw. In general, the bulk of evidence suggests that gonadectomy impairs memory in males tested in the radial arm maze, T-maze, Barnes Maze, and object recognition tasks, as well as several operant tasks that test prefrontal cortex function (Ceccarelli et al. 2001; Kritzer et al. 2001, 2007; Daniel et al. 2003; Sandstrom et al. 2006; Aubele et al. 2008; Gibbs and Johnson 2008; Spritzer et al. 2008; Locklear and Kritzer 2014). However, the deleterious effect of gonadectomy does not extend to all types of memory, particularly spatial reference memory tested in the Morris water maze (Gibbs 2005; Sandstrom et al. 2006; Spritzer et al. 2008).

A handful of studies have administered E_2 or testosterone to gonadally intact males. One study found that chronic E_2 administered systemically improved spatial reference and working memory in the radial arm maze and T-maze in male mice (Heikkinen et al. 2002). Similarly, acute treatment with estradiol valerate (a synthetic form of E_2) improved one-trial passive avoidance in male rats (Vázquez-Pereyra et al. 1995). However, data from the Morris water maze are somewhat inconsistent. As mentioned above, a single post-training intrahippocampal infusion of E_2 enhanced 24-h retention of the learned platform position in male rats (Packard et al. 1996). However, another study that administered pretraining intrahippocampal infusions of estradiol valerate or testosterone found no beneficial effect of either hormone (Moradpour et al. 2006). Rather, the data suggested a dose-dependent effect of estradiol and testosterone, with higher doses of each hormone impairing spatial reference memory on certain days of testing, and all other doses having no effect (Moradpour et al. 2006). Numerous factors may have contributed to the discrepant outcomes, including differences in the type of estradiol used (natural versus synthetic), timing of infusion relative to testing (pre- versus post-training), and length of testing (single day versus multiple days).

Among studies of gonadectomized male rats, chronic treatment with systemic E_2, but not testosterone, enhanced spatial working memory in the radial arm maze and acquisition of a delayed-match-to-position task in the T-maze (Luine and Rodriguez 1994; Gibbs 2005; Gibbs and Johnson 2008). However, chronic systemic administration of either E_2 or testosterone reversed gonadectomy-induced impairments in a different spatial memory task, the Barnes maze (Locklear and Kritzer 2014). The discrepant effects of testosterone in these spatial tasks may result

from differential sensitivity of spatial working and reference memory to testosterone in males. E_2 can also enhance memory in nonspatial tasks in males. For example, chronic systemic E_2 treatment accelerated extinction of conditioned taste aversion in both male and female rats (Yuan and Chambers 1999). In a series of prefrontal-dependent tasks in an operant chamber, systemic E_2 administered chronically via pellets was found to enhance response withholding in male rats, but did not reverse gonadectomy-induced deficits in spatial alternation, light–dark discrimination, or progressive ratio responding (Kritzer et al. 2007). In contrast, testosterone tended to reverse these deficits (Kritzer et al. 2007). It should be noted that not all studies have reported a beneficial effect of E_2 on memory in castrated males. For example, chronic treatment with testosterone, but not E_2, reversed gonadectomy-induced deficits in object recognition (Aubele et al. 2008). Thus, as with spatial memory, some types of nonspatial memory in males may be more sensitive to the effects of E_2 or testosterone than others.

Although the effects of E_2 and testosterone on memory in male rodents are not always consistent with each other, the studies conducted thus far do show that gonadal hormones regulate learning and memory in males. On balance, most studies find a beneficial effect of E_2 and/or testosterone on memory in gonadally intact and gonadectomized male rodents. However, as noted above, some types of memory may be more amenable to modulation by one hormone or the other. Because estradiol is a metabolite of testosterone, it is important to determine if the effects of testosterone on learning and memory are due to testosterone acting on androgen receptors or rather due to its conversion to estradiol or another metabolite. Numerous methods can assist with this determination, including the use of nonaromatizable androgens like DHT, aromatase inhibitors (e.g., letrozole and fadrozole), and androgen or estrogen receptor knockout mice. Although somewhat laborious, isolating the role of androgens and estrogens in mediating memory is important to understanding the mechanisms through which these hormones influence learning and memory in both sexes.

Hippocampally synthesized E_2 and memory

Finally, it is important to remember that the gonads are but one source of estrogens in the body. Traditional views on the role of sex steroids in mediating memory have attributed estrogen effects to gonadally derived estrogens. However, the enzymes for synthesizing progestins, androgens, and estrogens are present within the adult male and female rodent hippocampus (Hojo et al. 2004), and aromatase activity in the hippocampus regulates hippocampal E_2 synthesis (Kretz et al. 2004). Surprisingly, E_2 levels are substantially higher in the hippocampus than in plasma in adult male and female rats (Hojo et al. 2009; Kato et al. 2013). In gonadally intact males, for instance, E_2 levels have been reported at 8.4 ± 1.5 nM in the hippocampus and only 0.014 ± 0.003 nM in plasma (Hojo et al. 2009). Castration decreased hippocampal E_2 levels by ~18%, but levels remained quite high at 6.9 ± 0.8 nM (Hojo et al. 2009). Thus, it would appear that gonadally derived E_2 makes a fairly minimal contribution to hippocampal E_2 levels in males. Interestingly, E_2 levels in the hippocampus of male rats were found to be substantially higher than those of gonadally intact female rats, even during proestrus (Kato et al. 2013). During proestrus, hippocampal E_2 levels were 4.3 ± 1.0 nM, compared with values of 1.0 nM and lower during other phases of the cycle (Kato et al. 2013). Relative to the nonproestrus phases of the cycle, ovariectomy did not significantly decrease hippocampal E_2 levels (Kato et al. 2013), suggesting that the hippocampus continues to synthesize E_2 even in the absence of ovaries. Moreover, as in males, hippocampal levels of E_2 were substantially higher than in plasma among intact and ovariectomized females (Kato et al. 2013), supporting the notion that the hippocampus is a significant source of E_2 in females as well. These findings suggest that the primary endogenous source of E_2 for hippocampal neurons in both males and females may be hippocampal neurons or glia (Garcia-Segura et al. 1999; Azcoitia et al. 2003), rather than the gonads.

Emerging data suggest that this hippocampally synthesized E_2 may be important for hippocampal synaptic plasticity and learning in males and females. In neonatal hippocampal slice cultures, aromatase inhibition decreased expression of synaptic proteins, dendritic spine density, and presynaptic boutons (Kretz et al. 2004; Prange-Kiel et al. 2006). A recent in vivo study found that systemic injections of the aromatase inhibitor letrozole were associated with impaired LTP and transient dephosphorylation of cofilin in gonadally intact male and female rats, as well as in ovariectomized rats (Vierk et al. 2012). However, the deficits were considerably more striking for females than for males (Vierk et al. 2012), perhaps indicating a greater reliance on hippocampally synthesized E_2 for LTP and spine alterations in females. The finding that mature spines, thin spines, and spine synapses were reduced by letrozole in females, whereas only thin spines were reduced by letrozole in males may support this conclusion (Vierk et al. 2012). Given that aromatase inhibition disrupts various aspects of hippocampal function in males and females, one might expect learning and memory to be disrupted as well. Such an effect has been observed in male zebra finches, where hippocampal infusion of the aromatase inhibitor fadrozole impaired spatial memory in a food-finding task (Bailey et al. 2013). Although not specific to the hippocampus, systemic injection of fadrozole either 30 min prior to or immediately after extinction training significantly impaired fear recall during testing in male rats (Graham and Milad 2014). In ovariectomized mice, we recently infused different doses of letrozole into the dorsal hippocampus and our preliminary data indicate that letrozole prevents memory consolidation in both the object recognition and object placement tasks (Tuscher et al. 2013). Collectively, these few studies suggest the intriguing possibility that hippocampal E_2 synthesis is necessary for hippocampal memory formation in both males and females.

Molecular mechanisms underlying E_2's effects on memory consolidation

The past few decades of research have revealed a great deal about the molecular mechanisms underlying memory consolidation. Numerous molecules are involved, including neurotransmitter receptors (e.g., NMDA and AMPA), cell-signaling kinases (e.g., ERK, PI3K, PKA, CaMKII, and mTOR), transcription factors, genes, and the enzymes and co-factors that regulate histone acetylation and DNA methylation (e.g., Silva et al. 1992; Guzowski and McGaugh 1997; Atkins et al. 1998; Impey et al. 1998a, b; Schafe et al. 1999; Selcher et al. 1999; Adams and Sweatt 2002; Wood et al. 2005; Horwood et al. 2006; Fischer et al. 2007; Ploski et al. 2008; Guan et al. 2009; Lee and Silva 2009; Sweatt 2009; Day and Sweatt 2010; Hoeffer and Klann 2010; Incontro et al. 2014; Jarome and Helmstetter 2014; Schoch and Abel 2014; Yiu et al. 2014). To examine the roles of these molecules in estrogenic memory modulation, investigators have borrowed a common approach used in neurobiology of learning and memory research in which intracranial infusions of E_2 and inhibitor drugs are combined with posttraining treatments in one-trial learning tasks. Although this work has barely scratched the surface, quite a bit has already been learned about the molecular underpinnings of estrogenic memory regulation (for recent reviews, see Frick et al. 2010;

Frick 2012; Fortress and Frick 2014; Frick 2015). The findings to date will be summarized in this section (see also Fig. 5).

Cell signaling

As mentioned earlier, E_2 can rapidly phosphorylate several cell-signaling cascades, and this phosphorylation is associated with the ability of E_2 to increase CA1 dendritic spine density and LTP (Hasegawa et al. 2015). Accordingly, many of these same cascades are necessary for E_2 to enhance memory consolidation. In our laboratory's studies on this topic, we infused E_2 into the dorsal hippocampus (5 μg/hemisphere) or dorsal third ventricle (10 μg) of ovariectomized mice immediately after training in an object recognition or object placement task. The object placement task is similar to object recognition except that one training object is moved to a new location in the arena during testing rather than being replaced with a novel object. A single post-training infusion of E_2 into the either brain region reliably enhances object recognition and object placement memory consolidation in young and middle-aged ovariectomized mice (Fernandez et al. 2008; Fan et al. 2009; Zhao et al. 2010, 2012; Boulware et al. 2013; Fortress et al. 2013, 2014; Pereira et al. 2014). If E_2 infusion is delayed until 3 h after training, then no memory enhancement is observed (Fernandez et al. 2008), which is consistent with other studies indicating a specific effect of E_2 on memory consolidation within 1–2 h of treatment (Packard et al. 1996; Walf et al. 2006; Frye et al. 2007).

In studies of potential cell-signaling mechanisms involved, the ERK, PI3K/Akt, PKA, and mTOR signaling pathways were of interest based on data showing that E_2 rapidly phosphorylates these signaling molecules in a variety of cell types, including hippocampal neurons (Watters et al. 1997; Wade et al. 2001; Wade and Dorsa 2003; Yokomaku et al. 2003; Manella and Brinton 2006). In ovariectomized mice, infusion of E_2 increased phosphorylation of p42 ERK, PI3K, Akt, and the mTOR effector proteins p70 ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) in the dorsal hippocampus within 5 min of infusion into the dorsal hippocampus or dorsal third ventricle (Fernandez et al. 2008; Fan et al. 2009; Boulware et al. 2013; Fortress et al. 2013). Intrahippocampal infusion of inhibitors of ERK (U0126), PI3K (LY298002), PKA (Rp-cAMP), or mTOR (rapamycin) prevented E_2 from phosphorylating these kinases and enhancing object recognition memory consolidation (Fig. 6; Fernandez et al. 2008; Lewis et al. 2008; Fan et al. 2009; Fortress et al. 2013), suggesting that activation of these signaling pathways is critical for E_2 to enhance object recognition memory consolidation. A pair of studies showed that E_2 first activated PI3K, followed by ERK and then mTOR signaling (Fan et al. 2010; Fortress et al. 2013), which is consistent previous studies showing that PI3K and ERK activate the mTOR pathway (Richter and Klann 2009; Laplante and Sabatini 2012). mTOR signaling is essential for local protein synthesis within neurons (Hoeffer and Klann 2010), and hippocampal infusions of rapamycin impair consolidation of object recognition, contextual fear, and spatial memories (Dash et al. 2006; Parsons et al. 2006; Bekinschtein et al. 2007; Myskiw et al. 2008). As such, the regulation of mTOR signaling suggests a way in which E_2 may rapidly regulate protein synthesis and LTP in the hippocampus (Hasegawa et al. 2015).

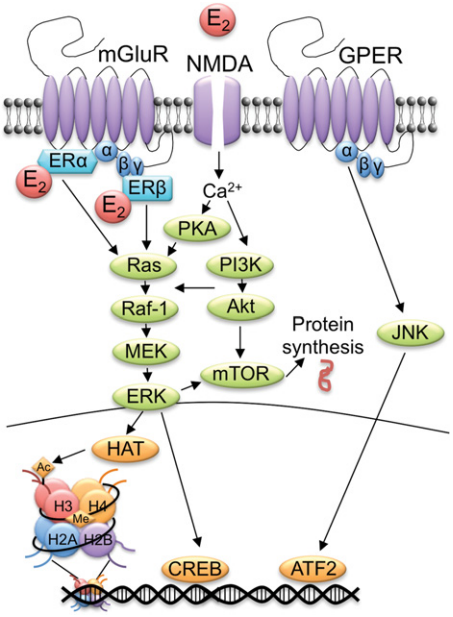

Figure 5. Schematic illustration of the molecular mechanisms required for E_2 and ERs to enhance hippocampal memory consolidation. Phosphorylation of the p42 isoform of ERK is necessary for E_2 to enhance object recognition memory consolidation. This phosphorylation is triggered by numerous upstream 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 and histone acetyltransferases (HAT) is also necessary for E_2 to increase histone H3 acetylation (Ac); E_2 increases H3 acetylation at the pII and pIV promoters of the *Bdnf* gene. DNA methylation is also essential for E_2 to enhance memory consolidation, although the specific cytosine residues methylated are unknown. Finally, GPER enhances memory consolidation by activating c-Jun N-terminal kinase (JNK), which facilitates gene expression via transcription factors such as ATF2. (Reprinted from Frick 2015 with permission from Elsevier © 2015.)

Receptors

Cell-signaling pathways are triggered by activation of plasma membrane receptors, and thus, it is of interest to determine which receptors may stimulate the cell-signaling changes involved in E_2-induced memory enhancement. One way in which to investigate the role of plasma receptors is with the use of the membrane-impermeable BSA-E_2. Infusion of BSA-E_2 into the dorsal hippocampus or dorsal third ventricle significantly increased the phosphorylation of p42 ERK in the dorsal hippocampus within 5 min and mimicked the memory-enhancing effects of E_2 (Fernandez et al. 2008). Interestingly, the effects of BSA-E_2 on p42 ERK and object recognition were not entirely blocked by the intracellular ER antagonist ICI 182,780 (Fernandez et al. 2008), suggesting that activation of plasma membrane receptors was sufficient to mediate the effects of E_2 on memory. But what are these plasma membrane receptors?

This issue remains unresolved, but two studies suggest an important role for glutamate receptors that may interact with ERα and ERβ at the membrane. In one study, dorsal hippocampal infusion of the NMDA receptor antagonist APV prevented systemically injected E_2 from enhancing object recognition, indicating a key role for NMDA receptors (Lewis et al. 2008). Nevertheless, it is not yet known how NMDA receptors may interact with E_2 or ERs. However, more recent work demonstrates that interactions among ERα, ERβ, and mGluR1a at the membrane are essential for E_2 to enhance both object recognition and object placement memory consolidation in ovariectomized mice (Boulware et al. 2013). C57BL/6 mice receiving dorsal hippocampal or dorsal third ventricle infusions of E_2, the ERα agonist PPT, or the ERβ agonist DPN (Stauffer et al. 2000; Meyers et al. 2001) immediately after object recognition and object placement training showed

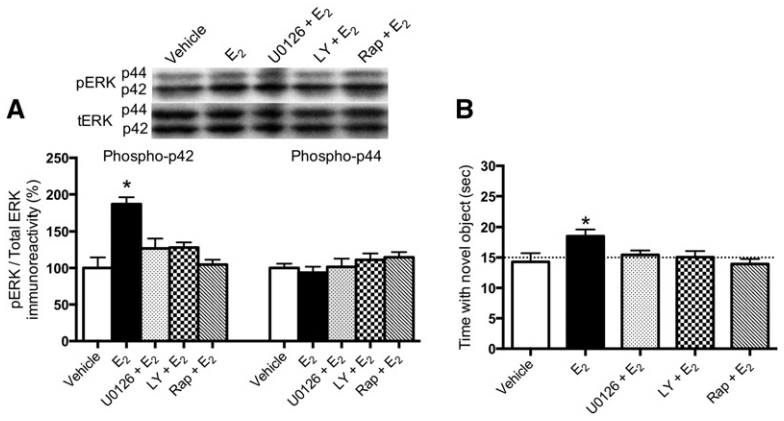

Figure 6. Dorsal hippocampal ERK, PI3K, and mTOR activation are involved in E_2-induced enhancement of object recognition memory consolidation in ovariectomized mice. (*A*) The phosphorylation of p42 ERK was significantly increased in young ovariectomized mice 5 min after bilateral dorsal hippocampal 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). (*B*) 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 (15 sec) with the novel object ($^*P < 0.05$). Error bars in both panels represent the mean ± SEM. Phosphorylated ERK levels were normalized to total ERK. Insets are representative Western blots of phosphorylated and total protein.

enhanced memory in both paradigms (Boulware et al. 2013). Interestingly, intrahippocampal infusion of DPN did not enhance object recognition memory in Swiss mice using the same infusion and testing parameters (Pereira et al. 2014), suggesting potentially important strain differences in the role of specific ERs in modulating memory in mice. In C57BL/6 mice, the ability of E_2 to increase p42 ERK and enhance memory was blocked by the mGluR1a antagonist LY367385 (Boulware et al. 2013), demonstrating an important contribution of mGluR1a to the memory-enhancing effects of E_2. Moreover, the effects of both agonists on memory was blocked by LY367385 or the ERK inhibitor U0126 (Fig. 7; Boulware et al. 2013), demonstrating that ERα and ERβ can mediate the effects of E_2 on ERK signaling and memory consolidation, and indicating an interaction between the ERs and mGluR1a. This interaction was further supported by co-immunoprecipitation experiments showing physical interactions among ERα, ERβ, mGluR1a, and the plasma membrane protein caveolin-1 (Boulware et al. 2013). Although it is not yet clear whether ERα and ERβ are physically located within membrane or just near the membrane, the data demonstrate that interactions between the classical ERs and mGluR1a at the membrane are necessary for E_2 to enhance object memory consolidation.

As discussed earlier, the putative membrane ER called GPER may also mediate the mnemonic effects of E_2. This possibility is suggested by studies in ovariectomized rats in which systemic injections of the GPER agonist G-1 agonist enhanced, whereas the GPER antagonist G-15 impaired, spatial working memory (Hammond et al. 2009, 2012). Consistent with these findings, our own preliminary data indicate that dorsal hippocampal infusion of G-1 enhanced, whereas G-15 impaired, object recognition and object placement memory in ovariectomized mice (Kim et al. 2013). Interestingly, however, G-1 did not activate ERK, PI3K, or Akt in the dorsal hippocampus, but instead phosphorylated the p46 and p54 isoforms of c-Jun N-terminal kinase (JNK) (Kim et al. 2013). The JNK inhibitor SP600125 prevented G-1, but not E_2, from enhancing object recognition and object placement memory (Kim et al. 2013), suggesting that GPER and E_2 may affect memory via different cell-signaling mechanisms. Interestingly, infusion of G-15 into the dorsal hippocampus did not prevent E_2 infused into the dorsal third ventricle from enhancing object recognition or object placement memory, indicating that GPER in the dorsal hippocampus may not regulate memory by acting as an ER. This interpretation is supported by a recent study in which a single systemic injection of E_2 or G-15 significantly increased, whereas G-1 significantly decreased, hippocampal cell proliferation in ovariectomized female rats (Duarte-Guterman et al. 2015). These findings indicate opposing effects of E_2 and GPER activation on hippocampal cell proliferation, and the authors interpret the data to suggest an

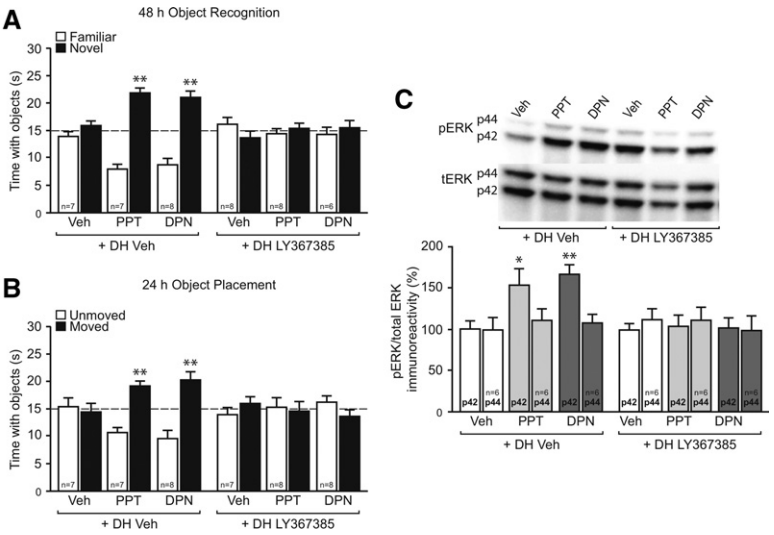

Figure 7. Dorsal hippocampal mGluR1a activation is essential in ovariectomized mice for ERα and ERβ to enhance object recognition and object placement memory consolidation, and to increase dorsal hippocampal ERK phosphorylation. (*A*,*B*) Immediate post-training infusion of PPT (0.2 pg) or DPN (20 pg) 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 sec, $^{**}P < 0.01$); 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 dorsal third ventricle infusion, PPT and DPN significantly increased phospho-p42 ERK levels ($^*P < 0.05$; $^{**}P < 0.01$ relative to vehicle); this effect was completely abolished by dorsal hippocampal infusion of LY367385. Bars represent mean ± SEM % change from vehicle. Phosphorylated ERK levels were normalized to total ERK. Insets are representative Western blots of phosphorylated and total protein. (Reprinted from Boulware et al. 2013).

estrogen-independent role for GPER in the adult hippocampus (Duarte-Guterman et al. 2015). However, this conclusion is complicated by two other recent reports that suggest a role for GPER in the modulatory effects of E_2 in the dorsal hippocampus. In one study, repeated systemic administration of G-1 had no effect on either ERK isoform in the dorsal hippocampus of gonadectomized male mice, but increased dorsal hippocampal ERK1/2 activation in gonadectomized female mice (Hart et al. 2014). Another recent study found that bath-applied G-15 prevented E_2 from increasing postsynaptic strength and phosphorylation of ERK1/2 in hippocampal slices from ovariectomized mice, suggesting a key role of GPER in mediating the effects of E_2 (Kumar et al. 2015). Although these findings contradict the aforementioned data suggesting independent roles of E_2 and GPER, it is difficult to directly compare the results because the experimental approaches used in the four studies were considerably different. As such, the nature of the potential interaction between GPER and E_2 in the dorsal hippocampus remains unclear, but is a topic of considerable importance for future study.

Finally, it should be noted that the role of ERs in mediating the effects of E_2 on memory might differ in males and females. As mentioned earlier, data from hippocampal cultures indicate that the E_2-induced regulation of CREB via mGluR1a signaling occurs in neonatal females, but not males (Boulware et al. 2005). Thus, E_2-induced memory enhancement in males may not depend on mGluR1-mediated activation of ERK as in females, although this potential sex difference has yet to be tested. Some evidence suggests sex differences in the role of specific ERs in memory. In one study, E_2 impaired spatial memory in the Morris water maze among female mice, but not males (Fugger et al. 1998). This E_2-induced impairment was not observed in female ERα knockout mice, suggesting a role for ERα in mediating the effects of E_2 on spatial memory in females but not males (Fugger et al. 1998). Interestingly, these investigators later reported no sex differences in the role of ERα in hippocampal synaptic responses (Fugger et al. 2001), indicating that other mechanisms likely account for the observed sex difference in spatial memory. More recently, deletion of ERα in mice impaired social recognition memory among females, but not males (Sánchez-Andrade and Kendrick 2011), suggesting sex differences in the role of ERα in multiple forms of memory. No studies to date have examined sex differences in the role of GPER in memory, but one recent study found that systemic administration of G-1 reduced anxiety in gonadectomized male, but not female, mice (Hart et al. 2014). As noted above, this study also found that G-1 increased dorsal hippocampal ERK1/2 activation in females, but not in males (Hart et al. 2014). In models of ischemic or hypoxic brain injury, GPER activation appears to be more beneficial for females than for males (Murata et al. 2013; Broughton et al. 2014). In a particularly dramatic example, G-1 increased neurological deficits and infarct volume after ischemic stroke in gonadally intact male rats, but reduced these parameters in ovariectomized rats (Broughton et al. 2014). Collectively, this work suggests the potential for sex differences in the roles of ERs in mediating memory formation. Thus far, the scant data reported thus far suggests a greater role for ERα and GPER in regulating spatial memory and anxiety, respectively, in female mice than in males.

Epigenetics

Cell-signaling cascades can regulate gene expression in multiple ways, for example, by phosphorylating transcription factors like CREB that promote gene transcription. Cell-signaling pathways can also trigger epigenetic alterations that alter the accessibility of DNA to transcription factors. For example, ERK phosphorylation appears to regulate histone acetylation (Levenson et al. 2004). Over the past decade, it has become clear that epigenetic processes such as histone acetylation and DNA methylation are important regulators of learning and memory mediated by numerous brain regions including the hippocampus (for recent reviews, see Sweatt 2009; Day and Sweatt 2010, 2011; Fischer et al. 2010; Gräff and Tsai 2013; Peixoto and Abel 2013; Jarome and Lubin 2014). Given the importance of ERK activation to estrogenic regulation of memory consolidation, our laboratory conducted a series of studies to examine whether histone acetylation and DNA methylation were involved in the effects of E_2 on memory consolidation in ovariectomized mice. Object recognition was used to address this issue because inhibitors of histone deacetylation enhanced memory in this task (Stefanko et al. 2009; Zhao et al. 2010).

DNA is wrapped tightly around a core of four histone proteins (H2A, H2B, H3, and H4) whose bond with DNA can be altered by post-translational modifications including acetylation, phosphorylation, methylation, ubiquitination, and SUMOylation. Histone acetyltransferase (HAT) enzymes add acetyl groups to histones, thereby relaxing the bond between histones and DNA, and allowing transcription factors access to the DNA for gene transcription. Histone deacetylase (HDAC) enzymes remove acetyl groups from histones, thereby tightening the bond between histones and DNA, and decreasing gene transcription. Infusion of the HAT inhibitor garcinol into the dorsal hippocampus of ovariectomized mice blocked object recognition memory consolidation (Zhao et al. 2012), indicating that histone acetylation is necessary for object recognition memory formation. Acetylation of histone H3 in the hippocampus is increased by contextual fear conditioning or ERK activation, the latter of which is necessary for other protein kinases to increase hippocampal H3 acetylation (Levenson et al. 2004). Thus, this work provides a possible link between E_2-induced regulation of ERK and H3 acetylation. Indeed, dorsal hippocampal infusion of E_2 in young and middle-aged ovariectomized mice selectively increased acetylation of H3 (Fig. 8A), but not H2A, H2B, or H4, in the dorsal hippocampus 30 min later (Zhao et al. 2010, 2012; Fortress et al. 2014). The E_2-induced increase in H3 acetylation was dependent on ERK activation, as infusion of the ERK inhibitor U0126 blocked the increase (Fig. 8A; Zhao et al. 2010). These data suggest that E_2-induced activation of ERK in the hippocampus mediates acetylation of H3, thereby likely regulating gene expression. Possible gene targets include *Bdnf*, as indicated by a recent study in which E_2 increased H3 acetylation at promoters II and IV of the *Bdnf* gene in both young and middle-aged ovariectomized mice (Fig. 8D). The regulation of *Bdnf* gene expression by E_2-induced H3 acetylation is supported by data showing that protein levels of BDNF and Pro-BDNF were increased in middle-aged females receiving dorsal hippocampus infusion of E_2 (Fortress et al. 2014).

E_2 also selectively decreased protein levels of HDAC2 (Fig. 8B) and HDAC3, without affecting levels of HDAC1 protein (Zhao et al. 2010, 2012; Fortress et al. 2014). HDAC2 and HDAC3 are potent negative regulators of hippocampal plasticity and memory (Guan et al. 2009; McQuown et al. 2011), so the E_2-induced reduction in these proteins supports the notion that E_2 regulates memory by altering histone acetylation. Importantly, the HAT inhibitor garcinol prevented E_2 from increasing H3 acetylation, decreasing HDAC2 protein, and enhancing object recognition (Fig. 8C) in ovariectomized mice (Zhao et al. 2012), demonstrating an essential role for histone acetylation in estrogenic regulation of object recognition memory consolidation.

Because histone acetylation and DNA methylation are interactive processes (Miller et al. 2008; Zhao et al. 2012), we also examined the role of DNA methylation in estrogenic regulation of memory. DNA methylation involves the addition of methyl groups to cytosine nucleotides located adjacent to guanine

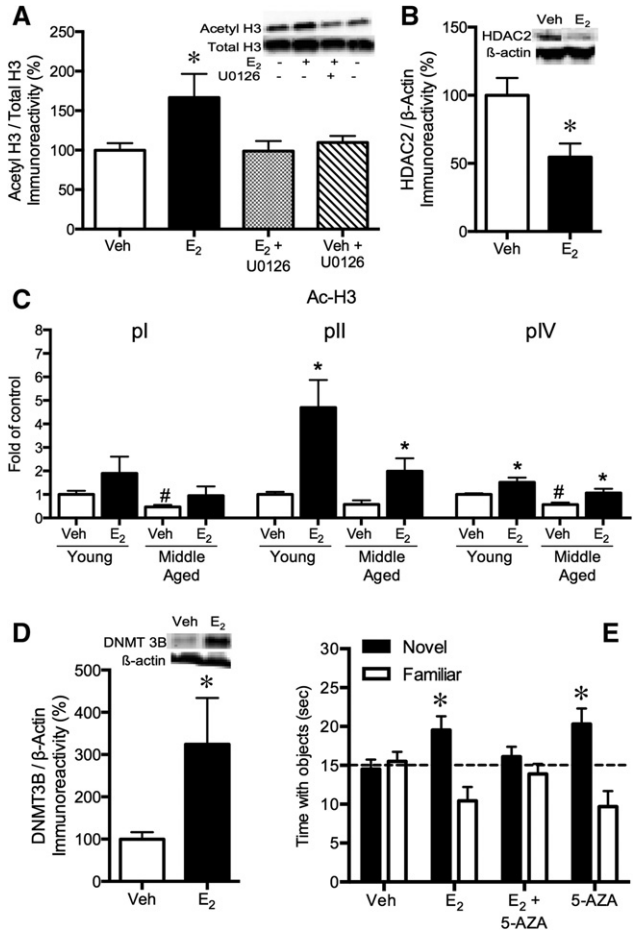

Figure 8. Histone acetylation and DNA methylation are critical for E_2 to enhance object recognition memory consolidation in ovariectomized mice. (*A*) Dorsal hippocampal infusion of 5 μg/hemisphere E_2 significantly increased acetyl H3 protein levels in the dorsal hippocampus within 30 min (*$P < 0.05$ relative to vehicle). This increase was blocked by the ERK activation inhibitor U0126, suggesting that the E_2-induced increase in H3 acetylation is dependent on ERK phosphorylation. (Adapted from Zhao et al. 2012.) (*B*) Dorsal hippocampal infusion of 5 μg/hemisphere E_2 significantly decreased HDAC2 protein levels in the dorsal hippocampus within 4 h relative to vehicle (*$P < 0.05$). (Adapted from Zhao et al. 2012.) (*C*) Chromatin immunoprecipitation analysis showed that dorsal 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. (*D*) Dorsal hippocampal infusion of 5 μg/hemisphere E_2 significantly increased DNMT3B protein levels in the dorsal hippocampus of young ovariectomized mice within 4 h relative to vehicle (*$P < 0.05$). (Adapted from Zhao et al. 2010.) (*E*) Dorsal hippocampal infusion of 5-AZA prevented E_2 from enhancing object recognition memory consolidation. E_2 or 5-AZA alone enhanced memory consolidation (*$P < 0.05$ relative to chance). (Adapted from Zhao et al. 2010.) For all panels, each bar represents the mean ± SEM. Acetyl H3 was normalized to total H3. HDAC2 and DNMT3B were normalized to β-actin. Insets are representative Western blots of phosphorylated and total protein.

nucleotides (CpG islands), which typically serves to silence gene transcription. Three DNA methyltransferase (DNMT) enzymes regulate DNA methylation. DNMT1 is a maintenance methyltransferase that transfers existing methyl marks during DNA replication. DNMT3A and DNMT3B are de novo methyltransferases that add methyl marks to previously unmethylated cytosines. Hippocampal learning selectively increases levels of DNMT3A and DNMT3B (Miller and Sweatt 2007), and dorsal hippocampal infusion of the DNMT inhibitor 5-azacytidine (5-AZA) blocks long-term contextual fear memory (Miller and Sweatt 2007; Miller et al. 2008). Together, these findings indicate an important role for de novo DNA methylation in hippocampal memory formation. Accordingly, dorsal hippocampal infusion of E_2 increased mRNA for DNMT3A and DNMT3B in the dorsal hippocampus of young ovariectomized mice within 45 min, whereas DNMT1 mRNA was unchanged up to 180 min after infusion (Zhao et al. 2010). DNMT3B protein levels were also increased 4 h after dorsal hippocampal E_2 infusion (Fig. 8D; Zhao et al. 2010), indicating that E_2 regulates the expression of this de novo methyltransferase. Moreover, infusion of 5-AZA immediately (Fig. 8E), but not 3 h, after training significantly enhanced memory consolidation (Zhao et al. 2010), suggesting that increasing methylation of certain genes (e.g., memory suppressor genes) enhances object recognition. However, infusion of 5-AZA blocked the memory-enhancing effects of E_2 (Fig. 8E; Zhao et al. 2010), indicating that DNA methylation is essential for E_2 to facilitate object recognition memory consolidation. At this point, it is not clear which genes are methylated by E_2, so additional studies using more sophisticated analyses (e.g., bisulfite sequencing) will be necessary to address this issue.

Practical considerations for working with females

The National Institutes of Health have issued new guidelines for inclusion of more females in biomedical research, which could lead to many more investigators using females in studies of learning and memory. For those investigators considering working with female rodents, there are many practical issues to consider. This final section will discuss briefly some of these issues.

An investigator's first decision is whether to ovariectomize female subjects. Gonadally intact females allow the effects of hormones to be observed in their natural state, which provides a more apt comparison with the vast learning and memory literature on gonadally intact males. However, the use of gonadally intact females is complicated by the estrous cycle, which could influence numerous aspects of learning, memory, and factors such as motivation or sensorimotor abilities that influence performance in learning and memory tasks. One way to address possible estrous-induced fluctuations in learning and memory is to limit testing to one particular phase of the cycle. The fact that each phase lasts ~24 h would limit the choice of tasks to rapidly learned one-trial learning tasks. This approach also requires regular monitoring of the cycle via a technique such as vaginal lavage (Long and Evans 1922). Alternatively, females could be tested in multi-day tasks (e.g., Morris water maze and radial arm maze) with daily cycle monitoring. Data could then be retrospectively analyzed by cycle phase, but interpretation would be complicated by the fact the same animals would be represented in multiple phase groups and not all subjects would begin and end testing in the same phase. A final option would be to ignore the cycle entirely. As mentioned earlier, effects of the cycle on memory are somewhat subtle and inconsistent, so they may wash out when averaging across multiple females. Thus, ignoring the cycle might be appropriate under certain circumstances. Indeed, it has been argued that the use of females in neuroscience research does not require monitoring of the cycle because females are not intrinsically more variable than males (Prendergast et al. 2014). However, it may be most prudent to first rule out possible effects of the cycle to ensure that effects are not limited to a specific phase.

Experiments using ovariectomized mice typically involve some form of E_2, progesterone, or E_2 plus progesterone replacement. As noted earlier, there are several options for chronic treatment depending on the scientific question, including silastic capsules, osmotic pumps, or hormone-secreting pellets. Such treatments have been widely used in female rodents and, as illustrated above, generally improve memory (Daniel et al. 1997, 2006; Gibbs 2000; Heikkinen et al. 2004). E_2 administered in the drinking water has also been shown to enhance spatial memory in middle-aged female mice (Fernandez and Frick 2004), but dosing is difficult to control. For acute treatments, systemic injections and intracranial infusions are excellent choices that allow for temporal control and observation of rapid effects. As such, these treatments are well suited for studies investigating the molecular and cellular mechanisms underlying hormonal regulation of memory (e.g., Fernandez et al. 2008; Boulware et al. 2013; Fortress et al. 2013; Pereira et al. 2014).

Finally, investigators studying age-related memory decline must keep in mind the impact of reproductive senescence on learning and memory. Female rodents undergo reproductive aging that is similar in many respects (although not identical) to menopause (LeFevre and McClintock 1988; Nelson et al. 1995; Wise 2000). This process is sometimes referred to as estropause. Reproductive function deteriorates gradually in both rats and mice, with the process starting at 9–12 mo of age in female rats and 13–14 mo of age in female mice (Nelson et al. 1981, 1982, 1995; Finch et al. 1984). With respect to spatial memory tested in the Morris water maze, impairments are evident at an earlier age in females than in males. In rats, spatial memory impairments are observed by 12 mo of age in females but not until 18 mo of age in males (Markowska 1999). In mice, the onset of spatial memory deficits is evident by 17 mo in females and by 25 mo in males (Frick et al. 2000). It is thought that the loss of estrogens due to age-related ovarian failure contributes to the premature spatial memory decline in female rodents, and studies showing that E_2 replacement can improve some types of memory in aging females support this idea (for reviews, see Sherwin and Henry 2008; Frick 2009; Conrad and Bimonte-Nelson 2010; Foster 2012; Chisolm and Juraska 2013; Maki 2013; Daniel et al. 2015). As such, investigators wishing to study learning and memory in middle-aged or aged rodents should be aware that females may exhibit a different pattern of age-related memory decline than males.

Conclusions

As we hope this review has illustrated, sex steroid hormones such as E_2 are crucial regulators of hippocampal morphology, plasticity, and memory in both male and female rodents. Given that receptors for these hormones are located throughout the brain, these hormones may modulate many types of memory mediated by several brain regions. Because sex steroid hormones affect learning and memory in both male and female rodents, it is important to understand how these hormones may regulate the forms of learning and memory in which your laboratory is interested. With E_2 as the example highlighted here, we hope this review has provided sufficient inspiration and guidance for other laboratories that do not traditionally work with hormones to begin examining the mechanisms through which E_2 and other hormones mediate memory in males and females.

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