Estradiol-Mediated Spine Changes in the Dorsal Hippocampus and Medial Prefrontal Cortex of Ovariectomized Female Mice Depend on ERK and mTOR Activation in the Dorsal Hippocampus

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Dendritic spine plasticity underlies the formation and maintenance of memories. Both natural fluctuations and systemic administration of 17β -estradiol (E_2) alter spine density in the dorsal hippocampus (DH) of rodents. DH E_2 infusion enhances hippocampal-dependent memory by rapidly activating extracellular signal-regulated kinase (ERK)-dependent signaling of mammalian target of rapamycin (mTOR), a key protein synthesis pathway involved in spine remodeling. Here, we investigated whether infusion of E_2 directly into the DH drives spine changes in the DH and other brain regions, and identified cell-signaling pathways that mediate these effects. E_2 significantly increased basal and apical spine density on CA1 pyramidal neurons 30 min and 2 h after infusion. DH E_2 infusion also significantly increased basal spine density on pyramidal neurons in the medial prefrontal cortex (mPFC) 2 h later, suggesting that E_2 -mediated activity in the DH drives mPFC spinogenesis. The increase in CA1 and mPFC spine density observed 2 h after intracerebroventricular infusion of E_2 was blocked by DH infusion of an ERK or mTOR inhibitor. DH E_2 infusion did not affect spine density in the dentate gyrus or ventromedial hypothalamus, suggesting specific effects of E_2 on the DH and mPFC. Collectively, these data demonstrate that DH E_2 treatment elicits ERK- and mTOR-dependent spinogenesis on CA1 and mPFC pyramidal neurons, effects that may support the memory-enhancing effects of E_2 .

Key words: CA1; dendritic spine density; estrogen; mPFC; pyramidal neuron; spinogenesis

Significance Statement

Although systemically injected 17β -estradiol (E_2) increases CA1 dendritic spine density, the molecular mechanisms regulating E_2 -induced spinogenesis *in vivo* are largely unknown. We found that E_2 infused directly into the dorsal hippocampus (DH) increased CA1 spine density 30 min and 2 h later. Surprisingly, DH E_2 infusion also increased spine density in the medial prefrontal cortex (mPFC), suggesting that estrogenic regulation of the DH influences mPFC spinogenesis. Moreover, inhibition of ERK and mTOR activation in the DH prevented E_2 from increasing DH and mPFC spines, demonstrating that DH ERK and mTOR activation is necessary for E_2 -induced spinogenesis in the DH and mPFC. These findings provide novel insights into the molecular mechanisms through which E_2 mediates dendritic spine density in CA1 and mPFC.

Introduction

The ability of 17β -estradiol (E_2) to increase dendritic spine density on CA1 pyramidal neurons in ovariectomized female rats is

one of the most seminal and replicated findings in behavioral neuroendocrinology (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1993; MacLusky et al., 2005; Phan et al.,

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2012), yet the molecular mechanisms regulating E₂-induced spinogenesis in the hippocampus and elsewhere in the brain remain largely unclear. In the dorsal hippocampus (DH), E₂ significantly increases CA1 dendritic spine density within 30 min of systemic injection (MacLusky et al., 2005; Inagaki et al., 2012). The E₂-induced facilitation of spinogenesis peaks 2–3 d after systemic injection (Woolley and McEwen, 1993) and is associated with enhanced hippocampal synaptic plasticity (Woolley et al., 1997; Foy et al., 1999; Mukai et al., 2007) and memory formation (Inagaki et al., 2012; Phan et al., 2012). E₂ also affects spinogenesis in other brain regions that mediate learning and memory, such as the medial prefrontal cortex (mPFC; Kesner et al., 1996; Inagaki et al., 2012; Yang et al., 2014), where E₂ increases pyramidal spine density from 30 min to 24 h after systemic injection (Inagaki et al., 2012; Phan et al., 2012). Given the putative role of spinogenesis in synaptic plasticity, estrogenic regulation of spines in regions, such as the DH and mPFC, is likely instrumental for E₂ to regulate memory formation (Luine and Frankfurt, 2013).

However, the molecular mechanisms through which E₂ regulates *in vivo* spine density are unclear. Spinogenesis requires protein synthesis, which could occur via nuclear transcriptional or by local protein synthesis within dendrites. The rapidity with which E₂ mediates spinogenesis suggests the involvement of local protein synthesis mechanisms such as mammalian target of rapamycin (mTOR) signaling (Hoeffer and Klann, 2010). mTOR is activated by multiple kinases, including extracellular signal-regulated kinase (ERK; Ma et al., 2007; Winter et al., 2011). Within the DH, activation of ERK and mTOR is necessary for E₂ to enhance object recognition and spatial memory consolidation in ovariectomized female mice (Fortress et al., 2013). Specifically, DH infusion of the ERK inhibitor U0126 or mTOR inhibitor rapamycin prevents E₂ from enhancing memory and increasing p42 ERK phosphorylation (Fernandez et al., 2008; Fortress et al., 2013). Given the importance of DH ERK and mTOR activation for E₂-induced memory enhancement, we hypothesized that these signaling pathways would also be involved in E₂-induced spinogenesis. Although ERK activation is necessary for E₂ to increase dendritic spines in cultured cortical and hippocampal neurons and slices (Mukai et al., 2007; Srivastava et al., 2008; Hasegawa et al., 2015; Murakami et al., 2015), the involvement of ERK and mTOR activation in E₂-mediated spinogenesis *in vivo* is unknown.

We tested whether DH infusion of E₂ regulates dendritic spine density in the DH and other brain regions, and examined the contribution of DH ERK and mTOR activation to E₂-induced spinogenesis. DH-infused E₂ increased dendritic spine density within 30 min in the DH and within 2 h in the mPFC, suggesting that E₂-induced DH spinogenesis may drive mPFC spinogenesis. Moreover, E₂-induced spine changes in both brain regions required ERK and mTOR activation in the DH, providing the first evidence that specific cell-signaling pathways regulate E₂-induced spinogenesis *in vivo*.

Materials and Methods

Subjects. Experiments used 8- to 12-week-old female C57BL/6 mice (Taconic Biosciences) as subjects ($n = 5$ –7/group). Mice were group-housed until surgery, after which they were singly housed. Mice were maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. All procedures were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Surgery. Immediately before surgery, mice received 5 mg/kg of Rimadyl subcutaneously for pain management. They were anesthetized with

isoflurane in 100% oxygen and placed in a stereotaxic apparatus for ovariectomy and cannula implantation (Boulware et al., 2013; Fortress et al., 2013). Mice were implanted with stainless steel bilateral guide cannulae (Plastics One) aimed at the DH only [C232GC, 22 gauge; -1.7 mm AP, ± 1.5 mm ML, and -2.3 mm DV (injection site)] or at the DH and dorsal third ventricle [intracerebroventricular (ICV); C232GC, 22 gauge; -0.9 mm AP, ± 0.0 mm ML, and -2.8 mm DV (injection site)]. Cannulae were fixed to the skull with dental cement (Darby Dental) that also closed the wound. Dummy cannulae (C232DC; Plastics One) were used to prevent clogging. Postsurgical analgesia was provided by MediGel CPF (ClearH₂O). Mice recovered 1 week before infusion.

Drugs and Infusions. In Experiment 1, mice received bilateral DH infusions of vehicle or E₂ and were killed 30 min or 2 h later. Hydroxypropyl- β -cyclodextrin (HBC)-encapsulated E₂ (Sigma-Aldrich) was dissolved in 0.9% sterile saline to $10 \mu\text{g}/\mu\text{l}$ and infused at $0.5 \mu\text{l}/\text{min}$ for 1 min/hemisphere (Fernandez et al., 2008; Fortress et al., 2013). The vehicle was HBC (Sigma-Aldrich) dissolved in saline to the same concentration of cyclodextrin present in the E₂ solution. In Experiment 2, mice received DH infusion of DMSO vehicle or a cell-signaling inhibitor, followed immediately by ICV infusion of HBC vehicle or E₂ (Fortress et al., 2013). The MEK inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenyl-mercapto)butadiene (U0126; Promega) was dissolved in 50% DMSO to $1.0 \mu\text{g}/\mu\text{l}$, and the mTOR inhibitor rapamycin was dissolved in 50% DMSO to $0.025 \text{ ng}/\mu\text{l}$. Bilateral DH infusions of $0.5 \mu\text{l}$ DMSO vehicle, U0126, or rapamycin were immediately followed by ICV infusion ($0.5 \mu\text{l}/\text{min}$ for 2 min) of $10 \mu\text{g}/\mu\text{l}$ E₂ or HBC vehicle. Bilateral DH infusion of $5 \mu\text{g}$ E₂ or ICV infusion of $10 \mu\text{g}$ E₂ enhances object recognition and object placement memory in ovariectomized mice (Boulware et al., 2013; Fortress et al., 2013).

Golgi impregnation and spine counting. Mice were cervically dislocated and decapitated 30 min or 2 h after infusion. Golgi impregnation was performed as described previously (Frankfurt et al., 2011) using the Rapid GolgiStain Kit (FD NeuroTechnologies). Secondary basal dendrites and tertiary apical dendrites were counted blindly from pyramidal neurons in dorsal hippocampal CA1 and layer II/III of the prelimbic mPFC. Spines were also counted on granule cells and neurons from the ventromedial hypothalamic nucleus (VMN). Dendrites from six cells/region/brain were included in the analysis, and five to seven brains were quantified/group. Neurons were chosen for analyses as described previously (Luine et al., 2006). Spines were counted on a Nikon Eclipse E400 microscope under oil ($100\times$) using a hand counter, and dendritic length was measured using Spot Advanced v5.0 Windows (Diagnostic Instruments). Spine density was calculated by dividing spine number by dendrite length. Data were expressed as number of spines/ $10 \mu\text{m}$ dendrite.

Data Analysis. For Experiment 1 (E₂ only), two-way ANOVAs (treatment $\times$ time) were conducted separately for apical and basal spine density in each brain region. For Experiment 2 (E₂ plus inhibitors), one-way ANOVAs were conducted to measure treatment effects on spines 2 h postinfusion. *Post hoc* tests (Tukey, Fisher's least significance difference) assessed between-group differences.

Results

DH E₂ infusion increased CA1 spine density 30 min and 2 h later

Main effects of treatment and time were significant for basal (Fig. 1A; $F_{(1,22)} = 63.37$, $p < 0.0001$ and $F_{(1,22)} = 8.93$, $p = 0.007$, respectively) and apical (Fig. 1B; $F_{(1,22)} = 46.46$, $p < 0.0001$ and $F_{(1,22)} = 7.25$, $p = 0.013$, respectively) CA1 dendrites. Relative to vehicle, E₂ increased CA1 basal ($p < 0.0001$) and apical ($p < 0.01$) spine density within 30 min. This effect remained significant for both basal and apical dendrites 2 h after infusion (p values < 0.0001). These data demonstrate that DH E₂ infusion induces a rapid increase in apical and basal CA1 spine density that lasts at least 2 h.

DH E₂ infusion did not affect spine density in the dentate gyrus

To demonstrate specificity of E₂ effects within the DH, we examined dendritic spine density in the dentate gyrus, which exhibits

Figure 1. E₂ increased CA1 spine density 30 min and 2 h after DH infusion. Relative to vehicle, basal (A) and apical (B) CA1 spine density were increased 30 min and 2 h after DH infusion of 5 μ g/hemisphere E₂. C, E₂ did not significantly alter dentate gyrus spine density at either time point. Bars represent the mean $\pm$ SEM. * p < 0.05. D, Photomicrograph of Golgi-impregnated secondary basal dendrites of CA1 pyramidal cells (A, E₂; B, vehicle). Arrows denote spines. Under oil 100 $\times$.

increased neurogenesis in response to E₂ (Tanapat et al., 2005; Galea et al., 2006; Barha et al., 2009). The main effect of time was significant ($F_{(1,20)} = 18.13$, $p = 0.0004$), such that fewer spines were observed 2 h after infusion than 30 min after infusion (Fig. 1C). However, E₂ had no effect on spines at either time point ($p > 0.05$).

DH infusion of E₂ increased basal spine density in mPFC 2 h after infusion

Main effects of treatment ($F_{(1,20)} = 18.47$, $p = 0.0004$) and time ($F_{(1,20)} = 17.3$, $p = 0.0005$) were significant for basal mPFC dendrites (Fig. 2A), as was the main effect of treatment ($F_{(1,20)} = 5.0$, $p = 0.037$) for apical mPFC dendrites (Fig. 2B). DH E₂ infusion did not significantly alter apical or basal spine density in the mPFC 30 min later, although a trend for an increase was evident for both types of spines. Indeed, E₂ significantly increased basal ($p < 0.01$), but not apical, spine density 2 h later (Fig. 2A,B). Thus, DH E₂ infusion increased basal spine density in the mPFC within 2 h, suggesting that estrogenic regulation of DH spinogenesis may alter mPFC spine morphology.

E₂ did not influence spine density in the VMN

The effects of DH E₂ infusion on mPFC spines might result from exogenous E₂ diffusing outside of the DH. Because systemic E₂ treatment increases spinogenesis in the VMN, we examined spine density in this region (Frankfurt et al., 1990). The main effect of time was significant ($F_{(1,22)} = 24.1$, $p < 0.0001$), such that fewer spines were observed 2 h after infusion than 30 min after infusion (Fig. 2C). However, no effect of E₂ was observed at either time point (p values > 0.05). These data support a more specific effect of DH E₂ infusion on mPFC spine density.

E₂-induced spine changes in the DH and mPFC depend on DH ERK and mTOR signaling

To determine whether E₂-induced spine changes in CA1 and mPFC depend upon rapid activation of ERK and mTOR signaling, mice received bilateral DH infusions of DMSO vehicle, the ERK inhibitor U0126, or the mTOR inhibitor rapamycin, followed immediately by ICV infusion of HBC vehicle or E₂. Brains were collected 2 h later. Importantly, the inhibitor doses used do not impair object recognition memory or p42-ERK phosphorylation on their own, yet block the memory-enhancing effects of E₂ (Fortress et al., 2013). In CA1, the main effect of group was significant for basal ($F_{(3,22)} = 10.59$, $p = 0.0002$; Fig. 3A) and apical ($F_{(3,22)} = 6.11$, $p = 0.004$; Fig. 3B) spines. Mice receiving E₂+vehicle exhibited a significant increase in basal ($p < 0.01$) and apical ($p < 0.05$) CA1 spine density 2 h after infusion relative to vehicle controls. This increase was blocked for basal and apical spines (Fig. 3A,B) in mice receiving DH infusions of U0126 ($p < 0.001$ relative to E₂+vehicle) or rapamycin ($p < 0.01$ relative to E₂+vehicle), suggesting that ERK and mTOR activation are necessary for E₂ to increase CA1 spine density.

Interestingly, DH ERK and mTOR activation also regulated mPFC spine formation. mPFC spine density was significantly altered 2 h after infusion for basal ($F_{(3,19)} = 11.49$, $p = 0.0002$) and apical ($F_{(3,19)} = 7.04$, $p = 0.002$) spines. As in CA1, mice infused with E₂+vehicle exhibited significantly greater basal ($p < 0.01$) and apical ($p < 0.01$) spine density than vehicle controls. Again, these increases were blocked by U0126 ($p < 0.01$ relative to E₂+vehicle) or rapamycin ($p < 0.05$ relative to E₂+vehicle), indicating that activation of ERK or mTOR in the DH is necessary for DH-infused E₂ to increase mPFC spine density.

Figure 2. DH E_2 infusion increased mPFC basal spine density 2 h later. mPFC basal spine density was significantly increased relative to vehicle 2 h after DH E_2 infusion (**A**). E_2 did not significantly alter spine density on mPFC apical dendrites (**B**) or in the VMN (**C**) at either time point. Bars represent the mean $\pm$ SEM. * $p < 0.05$. **D**, Photomicrograph of Golgi-impregnated secondary basal dendrites of pyramidal cells in the mPFC (layer II/III; **A**, E_2 ; **B**, vehicle). Arrows denote spines. Under oil 100 $\times$.

Discussion

Our findings provide several novel insights into the mechanisms through which E_2 induces dendritic spine density in CA1 and mPFC. DH infusion of E_2 significantly increased basal and apical spine density on CA1 pyramidal dendrites within 30 min, an effect that persisted for 2 h. DH or ICV infusion of E_2 also significantly increased basal spine density on mPFC pyramidal neurons within 2 h. Although systemic E_2 injection increases spinogenesis in these brain regions (Inagaki et al., 2012; Luine and Frankfurt, 2012), this is the first demonstration that DH or ICV E_2 infusion increases CA1 and mPFC spine density, and that DH E_2 infusion drives mPFC spine changes. Importantly, DH E_2 infusion did not alter spines in the dentate or VMN, suggesting an effect specific to CA1 and mPFC. DH infusion of U0126 or rapamycin blocked the spine increase observed in CA1 and mPFC 2 h after ICV E_2 infusion, indicating that E_2 -mediated spinogenesis requires ERK and mTOR activation. Together, these data suggest the involvement of local protein translation in E_2 -mediated spine alterations, and indicate that E_2 -induced DH cell signaling regulates mPFC spine density.

These data provide novel evidence that E_2 -induced hippocampal alterations impact spine density in the mPFC. The CA1 findings were consistent with classic studies demonstrating that naturally elevated or systemically injected E_2 increases CA1 dendritic spine density (Gould et al., 1990; Woolley et al., 1990). The mPFC findings were more surprising. Although a dose of systemic E_2 that enhances hippocampal memory increases CA1 and mPFC spine density in ovariectomized rats (Inagaki et al., 2012) and mice (Phan et al., 2012), the dependence of spine changes in one region on E_2 -induced alterations in the other has never been investigated. The present results highlight a previously unexplored interaction between the DH and mPFC that may have important implications for understanding how E_2 regulates

memory. Presently, the mechanisms through which DH E_2 infusion affects mPFC spine density are unclear. Sparse projections from dorsal CA1 and subiculum to the mPFC exist, as do indirect projections through the nucleus reuniens of the thalamus and ventral hippocampus (Jay et al., 1992; Hoover and Vertes, 2007). The functional relevance of these projections is supported by evidence that collaboration between these brain regions is important for episodic-like memory tasks (Warburton and Brown, 2015) and delayed spatial working memory (Churchwell and Kesner, 2011). Notably, DH E_2 infusion affected basal, but not apical, spines in mPFC, which may reflect differences between presynaptic excitatory input to apical and basal domains. For example, input to basal spines in mPFC layer II/III reportedly originates from local circuitry (Spruston, 2008). Therefore, our data suggest that DH E_2 infusion may influence local prefrontal synaptic activity, but additional experiments are necessary to substantiate this interaction. Interestingly, ICV E_2 infusion increased both basal and apical spines, which could suggest that the more anterior ICV infusion affects a subpopulation of DH pyramidal neurons that project to both apical and basal dendrites. Nevertheless, blocking ERK and mTOR signaling in the DH prevented ICV-infused E_2 from increasing spines in the mPFC, pinpointing the DH as a key regulator of ICV E_2 -mediated spinogenesis.

Our data suggest that ERK and mTOR activation is necessary for E_2 to increase CA1 and mPFC spine density. ERK activation is essential for synaptic plasticity and hippocampal-dependent memory (English and Sweatt, 1997; Atkins et al., 1998; Selcher et al., 1999). Moreover, E_2 rapidly increases DH ERK phosphorylation *in vitro* (Boulware et al., 2005; Zhao and Brinton, 2007) and *in vivo* (Fernandez et al., 2008), and ERK activation is necessary for E_2 to enhance hippocampal-dependent object recognition and spatial memory in female mice (Fernandez et al., 2008; Boul-

Figure 3. The increased CA1 and mPFC apical and basal spine density induced by ICV E₂ was blocked by ERK or mTOR inhibition. Two hours after ICV E₂ infusion, basal and apical spine density was significantly increased in the mPFC (**A**, **B**) and CA1 (**C**, **D**) relative to vehicle. These effects were blocked by DH infusion of U0126 or rapamycin. Bars represent the mean $\pm$ SEM. * p < 0.05 relative to all other groups. **E**, Photomicrograph of Golgi-impregnated secondary basal dendrites of CA1 pyramidal cells (A, vehicle; B, E₂ + vehicle; C, E₂ + U0126). Arrows denote spines. Under oil 63 $\times$.

ware et al., 2013; Fortress et al., 2013). The current work extends these findings by demonstrating that E₂-induced ERK activation is also necessary for CA1 and mPFC spinogenesis *in vivo*. These results are consistent with *in vitro* data showing that ERK inhibition blocks E₂-induced spinogenesis in the CA1 of male hippocampal slices (Mukai et al., 2007; Hasegawa et al., 2015) and in cortical neuron cultures (Srivastava et al., 2008).

ERK phosphorylation triggers downstream mTOR-mediated protein synthesis (Hoeffer and Klann, 2010), which is also essential for hippocampal synaptic plasticity (Tang et al., 2002) and hippocampal-dependent memory (Bekinschtein et al., 2007; Gafford et al., 2011). Furthermore, ERK-induced activation of mTOR signaling is required for E₂ to enhance object recognition memory in female mice (Fortress et al., 2013). Here, rapamycin blocked the

increased CA1 and mPFC spine density 2 h after E₂ infusion, suggesting that E₂-mediated spinogenesis depended on DH mTOR activation. Although E₂ increases mTOR phosphorylation in hippocampal slices (Briz and Baudry, 2014), these findings provide the first *in vivo* demonstration that mTOR activation is necessary for E₂ to increase spine density in either brain region. Interestingly, E₂-induced spinogenesis in the mPFC required mTOR activation in the DH, further supporting that DH cell signaling regulates mPFC spine density. Together with the aforementioned behavioral and *in vitro* data, these results suggest that estrogenic activation of mTOR-mediated protein synthesis may be essential for the local translation of transcripts that support the formation or maintenance of new spines. Because other signaling mechanisms regulate E₂-mediated spinogenesis in cultured hippocampal or cortical neurons, including PI3K, PKA, PKC, CaMKII, and the RhoA > ROCK > LIMK > cofilin > actin pathway (Srivastava et al., 2008; Hasegawa et al., 2015), the role of these mechanisms in mediating E₂-induced spinogenesis *in vivo* should be tested in future studies.

In conclusion, the present findings demonstrate that DH E₂ infusion rapidly increases CA1 and mPFC dendritic spine density in an ERK- and mTOR-dependent fashion. These data shed new light on the molecular mechanisms underlying E₂-induced spinogenesis *in vivo*, and suggest that the DH and mPFC may interact to mediate the memory-enhancing effects of E₂. Because systemic E₂ treatments that enhance memory also increase spine density in CA1 and mPFC (Velázquez-Zamora et al., 2012; Luine and Frankfurt, 2013), the current findings have important implications for understanding how estrogenic regulation of neural circuitry influences memory formation.

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