17β-Estradiol regulates histone alterations associated with memory consolidation and increases *Bdnf* promoter acetylation in middle-aged female mice

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Histone acetylation is essential for hippocampal memory formation in young adult rodents. Although dysfunctional histone acetylation has been associated with age-related memory decline in male rodents, little is known about whether histone acetylation is altered by aging in female rodents. In young female mice, the ability of 17β-estradiol (E2) to enhance object recognition memory consolidation requires histone H3 acetylation in the dorsal hippocampus. However, the extent to which histone acetylation is regulated by E2 in middle-aged females is unknown. The mnemonic benefits of E2 in aging females appear to be greatest in middle age, and so pinpointing the molecular mechanisms through which E2 enhances memory at this age could lead to the development of safer and more effective treatments for maintaining memory function without the side effects of current therapies. Here, we show that dorsal hippocampal infusion of E2 rapidly enhanced object recognition and spatial memory, and increased histone H3 acetylation in the dorsal hippocampus, while also significantly reducing levels of histone deacetylase (HDAC2 and HDAC3) proteins. E2 specifically increased histone H3 acetylation at *Bdnf* promoters pII and pIV in the dorsal hippocampus of both young and middle-aged mice, despite age-related decreases in pI and pIV acetylation. Furthermore, levels of mature BDNF and pro-BDNF proteins in the dorsal hippocampus were increased by E2 in middle-aged females. Together, these data suggest that the middle-aged female dorsal hippocampus remains epigenetically responsive to E2, and that E2 may enhance memory in middle-aged females via epigenetic regulation of *Bdnf*.

Epigenetic processes such as histone acetylation are essential for memory formation in the hippocampus and other cognitive regions of the brain (Vecsey et al. 2007; Fischer et al. 2010; Sharma 2010; Graff and Tsai 2013a,b; Peixoto and Abel 2013). DNA is supercoiled around four histone proteins (H2A, H2B, H3, and H4), each of which has an amino acid tail that can be acetylated to relax chromatin structure and increase transcription. Acetyl groups are added by lysines by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs) (Yang 2007). Considerable evidence suggests that histone acetylation promotes memory formation. For example, hippocampal contextual learning increases H3 acetylation (Levenson et al. 2004), whereas expression of certain HDACs, such as HDAC2 and HDAC3, impairs spatial, contextual, and object recognition memories mediated by the hippocampus (Guan et al. 2009; Haettig et al. 2011; Hawk et al. 2011; McQuown et al. 2011). Furthermore, HDAC inhibitors enhance hippocampal memory, synaptic plasticity, and gene expression in young rodents (Stefanko et al. 2009; Zhao et al. 2010), supporting the notion that histone acetylation facilitates the expression of genes necessary for memory formation.

However, much less is known about the contributions of histone acetylation to memory formation in the aging brain. To date, few studies have examined this issue. In middle-aged mice, impaired spatial and contextual memory has been associated with deficits in learning-induced H4 acetylation in the hippocampus (Peleg et al. 2010; Dagnas and Mons 2013). Treatment with HDAC inhibitors reverses these deficits in middle-aged mice (Peleg et al. 2010), which is consistent with other data from middle-aged male rats showing that aging increases HDAC activity in the hippocampus (Dos Santos Sant’Anna et al. 2013). HDAC inhibition can also reverse memory deficits in mouse models of Alzheimer’s disease (Kilgore et al. 2010). Although these studies suggest that dysregulated hippocampal histone acetylation in middle age leads to memory impairment, it is notable that none specifically examined histone acetylation in females. The onset of memory decline occurs earlier in female rats and mice than in males, and is associated with the loss of estrous cycling (Markowska 1999; Frick et al. 2000). In the hippocampus of middle-aged females, levels of the primary estrogen receptors (ERα and ERβ) are reduced (Yamaguchi-Shima and Yui 2007; Bohacek and Daniel 2009), and prolonged ovarian hormone deprivation reduces the ability of the most biologically active estrogen, 17β-estradiol (E2), to regulate estrogen receptor levels (Daniel et al. 2006; Bohacek et al. 2008; Bohacek and Daniel 2009). Nevertheless, exogenous E2 can still reverse hippocampal memory deficits in middle-aged female rodents (Markham et al. 2002; Fernandez and Frick 2004; Bimonte-Nelson et al. 2006; Daniel et al. 2006), demonstrating that the middle-aged hippocampus remains somewhat responsive to E2. Indeed, our laboratory has shown that a bilateral infusion of E2 into the dorsal hippocampus enhances object recognition...
memory in middle-aged female mice in a manner dependent on rapid activation of phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) (Fan et al. 2010). Given our previous reports in young female mice that bilateral infusion of E2 into the dorsal hippocampus increases histone H3 acetylation within 30 min in an ERK-dependent manner (Zhao et al. 2010, 2012), this finding might suggest an important role for E2-induced histone acetylation in memory formation among middle-aged females. The E2-induced increase in H3 acetylation in young females is blocked by HAT inhibition, as is an E2- induced decrease in HDAC2 protein 4 h after infusion (Zhao et al. 2010, 2012). These data suggest that ERK-driven histone acetylation is essential for E2 to regulate memory in young females. However, the extent to which histone acetylation is necessary for E2 to enhance memory in middle age remains unknown, as are the downstream gene targets necessary for E2 to enhance memory at any age.

One potential gene target involved in estrogenic regulation of hippocampal memory consolidation is brain-derived neurotrophic factor (BDNF). BDNF is crucial for synaptic plasticity and hippocampal memory formation (Heldt et al. 2007; Bekinschtein et al. 2014). The Bdnf gene consists of eight untranslated exons and one protein coding exon (Aid et al. 2007). In the hippocampus, BDNF transcripts can be uniquely regulated by L-type calcium channels (Tao et al. 1998) and aging (Chapman et al. 2012; Perovic et al. 2013). Epigenetic regulation of Bdnf in the hippocampus by HDAC inhibition (Koppel and Timmusk 2013) or contextual fear conditioning (Lubin et al. 2008) has also been shown to increase the expression of specific Bdnf exons. E2-induced activation of L-type calcium channels increases CREB phosphorylation to promote gene transcription and exert neurotrophic effects in hippocampal neurons (Boulware et al. 2005; Wu et al. 2005; Zhao et al. 2005), and high levels of E2 during the estrous cycle are associated with increased hippocampal CA1 excitability in a BDNF-dependent manner (Scharfman et al. 2003). Further, aging and ovariectomy significantly decrease the expression of BDNF mRNA in the hippocampus in rodents (Singh et al. 1995; Sohrabji et al. 1995; Chapman et al. 2012; Perovic et al. 2013). Exogenous E2 administered to ovariectomized rodents (Singh et al. 1995; Sohrabji et al. 1995) or elevated E2 in the estrous cycle are associated with increased BDNF mRNA or protein levels (Gibbs 1998; Scharfman et al. 2003). Because E2 modulates many of the same neural mechanisms responsible for epigenetic regulation of Bdnf, and E2 can rapidly activate cell signaling independent of transcriptional genomic mechanisms to enhance hippocampal memory (Packard and Teather 1997b; Fernandez et al. 2008; Zhao et al. 2010), we reasoned that Bdnf may be a key gene that is epigenetically regulated by E2.

Here, we demonstrate that the female middle-aged dorsal hippocampus remains epigenetically responsive to E2. Our findings suggest that E2 may facilitate memory consolidation in the novel object recognition and object placement tasks by increasing histone H3 acetylation at Bdnf promoters in the dorsal hippocampus.

Results

Estradiol enhances novel object recognition and object placement memory consolidation in middle-aged female mice

We first sought to replicate our previous findings that a post-training infusion of 5 µg of E2 into the dorsal hippocampus of ovariectomized middle-aged female mice enhances novel object recognition (NOR) memory consolidation (Fan et al. 2010). Mice first explored two identical objects until they had accumulated 30 sec of object exploration (Fig. 1A). Immediately after this training, mice received bilateral dorsal hippocampal infusion of vehicle or 5 µg of E2. Memory for the training objects was tested 48 h later by allowing the mice to explore an object identical to that used in training (i.e., familiar) and a novel object. As in our previous work (Fan et al. 2010), middle-aged mice infused with E2 into the dorsal hippocampus immediately post-training spent significantly more time than chance with the novel object 48 h later (t(16) = 3.41, P = 0.004) (Fig. 1B). In contrast, vehicle-infused mice did not exhibit a significant preference for the novel object (t(16) = −1.56, P = 0.14) (Fig. 1B), suggesting that E2 enhanced NOR memory consolidation in middle-aged females. We next determined whether the beneficial effects of dorsal hippocampal E2 infusion in middle-aged females extend to other forms of memory mediated by the hippocampus. The object placement (OP) task tests hippocampal-dependent spatial memory, and E2 administered to the dorsal hippocampus immediately after training enhances OP memory consolidation in young female mice (Boulware et al. 2013). In the present study, middle-aged females were trained in the OP task 2 wk after NOR testing (Fig. 1A). The training procedure for OP was identical to that for NOR. Immediately after training, the mice were bilaterally infused with vehicle or 5 µg of E2 into the dorsal hippocampus. Twenty-four hours after training, one of the identical objects was moved to a lower corner of the testing arena and mice were again allowed to accumulate 30 sec of exploring the objects. Neither vehicle- nor E2-infused mice showed a significant preference for the moved object in the novel location (data not shown). Because spatial memory decays more rapidly with aging than object memory (Wimmer et al. 2012), we next tested the mice using a shorter 4-h delay. Indeed, 4 h after training, mice infused with E2 spent significantly more time than chance with the moved object (t(17) = 7.14, P < 0.001) (Fig. 1C), demonstrating an intact memory for the unmoved object. In comparison, vehicle-infused mice did not spend more time than chance with the moved object (t(17) = 0.18, P = 0.86) (Fig. 1C). These data show for the first time that acute post-training infusion of E2 into the dorsal hippocampus enhances spatial memory consolidation in middle-aged females, and demonstrate E2 can enhance the consolidation of multiple forms of hippocampal memory in middle age.

Estrogenic effects on histone acetylation are restricted to H3

We next determined the extent to which this memory-enhancing dose of E2 triggers epigenetic alterations in the middle-aged dorsal hippocampus. We began with histone acetylation because this process regulates hippocampal memory and the genes necessary for synaptic plasticity (Guan et al. 2009; Graff et al. 2012). Of the four core histones (H2A, H2B, H3, and H4), acetylation of H3 is particularly important in regulating hippocampal learning and memory (Leveson et al. 2004), as well as estrogenic modulation of learning and memory. We previously found in young female mice that dorsal hippocampal infusion of 5-µg E2 significantly increases acetylation of H3 (but not H2B or H4) 30 min later in an ERK-dependent manner, and that histone acetylation is required for E2 to enhance NOR memory consolidation (Zhao et al. 2010, 2012). Because E2 enhances memory consolidation among middle-aged females in a manner similar to young females (Fig. 1; Fan et al. 2010), we hypothesized that E2 would also increase histone acetylation among middle-aged females in an H3-specific manner. Middle-aged mice (n = 27) were bilaterally infused into the dorsal hippocampus with vehicle or 5-µg E2, and the dorsal hippocampus was collected bilaterally 30 or 60 min later for Western blot analysis. The 60-min time point was included in case alterations in histone acetylation were delayed beyond the
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30-min time point by aging. E2 significantly increased acetylated H3 levels relative to vehicle (F(2,23) = 10.91, P = 0.0005) (Fig. 2A) both 30 min (P < 0.0001) and 60 min (P < 0.01) after infusion, suggesting that the E2-induced increase in H3 acetylation lasts at least 1 h. In contrast to H3, E2 did not affect acetylation of H2A (F(2,17) = 0.2, P = 0.83) (Fig. 2B), H2B (F(2,24) = 0.8, P = 0.46) (Fig. 2C), or H4 (F(2,21) = 0.29, P = 0.75) (Fig. 2D) at either 30 or 60 min, suggesting that effects of E2 on histone acetylation are specific to H3.

Estradiol decreases levels of histone deacetylases 2 and 3

HDACs remove acetyl groups from histone tails, thereby condensing the chromatin and decreasing gene transcription. HDAC2 is a potent negative regulator of both hippocampal memory and synaptic plasticity (Guan et al. 2009). We previously showed that dorsal hippocampal infusion of E2 in young females significantly decreases HDAC2, but not HDAC1, protein in the dorsal hippocampus 4 h after infusion (Zhao et al. 2010, 2012). HDAC3 also negatively regulates object memory consolidation (McQuown et al. 2011), but the effects of E2 on HDAC3 levels in the hippocampus were unknown at any age. Therefore, we next used Western blotting to measure levels of HDAC1, HDAC2, and HDAC3 protein in the middle-aged dorsal hippocampus 4 and 6 h after bilateral dorsal hippocampal infusion of vehicle or 5-μg E2. We hypothesized that E2 would decrease both HDAC2 and HDAC3 levels in middle-aged females because E2-induced memory enhancement is associated with decreased HDAC2 protein expression in young females (Zhao et al. 2010, 2012) and because E2 enhances memory in middle-aged females (Fig. 1). As expected, E2 had no effect on HDAC1 protein levels at either time point (F(2,22) = 0.61, P = 0.6) (Fig. 3A), but significantly decreased HDAC2 (F(2,21) = 5.39, P = 0.01) (Fig. 3B) and HDAC3 (F(2,21) = 6.05, P = 0.008) (Fig. 3C) protein levels in the dorsal hippocampus. For HDAC2, this decrease was evident 4 h after infusion of E2 (P < 0.05), whereas HDAC3 levels were reduced both 4 h (P < 0.01) and 6 h (P < 0.05) after infusion. These data suggest that E2 decreases levels of two key HDACs whose expression is associated with impaired memory.

Estradiol increases BDNF protein and regulates acetylation of Bdnf promoters

Traditionally, the estrogenic regulation of Bdnf has been attributed to classical genomic signaling mechanisms due to regulation of the Bdnf gene via an estrogen response element (ERE) (Sohrabji et al. 1995). However, as our lab and others have recently shown, E2 can act in rapid, nonclassical signaling pathways that may ultimately regulate gene transcription independent of the ERE (Bjornstrom and Sjoberg 2005; Spencer et al. 2008b; Fan et al. 2010). The Bdnf gene consists of eight 5′ untranslated exons and one 3′ exon (exon IX) that encodes the BDNF protein (Aid et al. 2007), with the ERE located on Exon V (Sohrabji et al. 1995). Transcription of each exon is driven by its own unique promoter (Aid et al. 2007). Of these promoters, pI, pII, pIV, and pVI are the most common in the brain (Baj et al. 2011). In the hippocampus, memory consolidation is associated with increased histone H3 acetylation at the pIV promoter (Lubin et al. 2008), and fear conditioning increases H3 acetylation at pI and pIV (Fuchikami et al. 2010). In contrast, aging is associated with a decrease in pI and pIII BDNF mRNA in the hippocampus of male rats (Perovic et al. 2013), but changes in acetylation have not been examined. Because E2 increases hippocampal BDNF mRNA (Singh et al. 1995; Sohrabji et al. 1995) and requires ERK-dependent H3 acetylation to enhance memory consolidation (Zhao et al. 2010), we hypothesized that E2 may increase acetylation of H3 at Bdnf promoters pI, pII, and pIV. Further, a role for E2 in regulating H3 acetylation of dorsal hippocampal Bdnf promoters has not been demonstrated at any age, so we used chromatin immunoprecipitation (ChIP) to examine effects in both young and middle-aged female mice.

Our control studies demonstrated specificity of the ChIP assay. We first confirmed, using Western blotting, that the pan-acetyl histone H3 was present in the nuclear fraction, but not the cytoplasmic fraction following our shearing protocol (Fig. 4B). Following immunoprecipitation, we observed a reduction in the signal for acetyl H3 in the supernatant samples immunoprecipitated with acetyl H3, suggesting antibody binding to beads coincubated with acetyl H3 rather than control IgG serum (Fig. 4B). Before testing our primers of interest, we used quantitative real-time PCR (qPCR) to ensure that our amplification was specific to the promoters of interest. We found no amplification in an IgG-only sample using primers for our negative control, LINE1 (LINE1). Following immunoprecipitation with acetyl H3, we observed a modest increase in the amplification of LINE1 (LINE1) and a threefold increase in the Nr4a2 positive control (Nr4a2)
Figure 2. Dorsal hippocampal histone H3 acetylation is increased by E2. (A) Bilateral dorsal hippocampal infusion of 5-µg E2 significantly increased bulk acetyl H3 levels relative to vehicle 30 min ([**P < 0.001]) and 60 min ([***P < 0.001]) later. In contrast, E2 had no effect on the acetylation of histones H2A (B), H2B (C), or H4 (D). All proteins were normalized to total histone (each bar represents the mean ± SEM percent change from vehicle). (Insets) Representative Western blots of acetylated and total histone protein.

Discussion

Because E2 is a potent regulator of hippocampal function, the age-related loss of E2 likely contributes to the increased risk of cognitive decline and dementia in postmenopausal women (Zandi et al. 2002; Yaffe et al. 2007). However, our previous and present work suggests that the dorsal hippocampus of the young and middle-aged female hippocampus is similarly responsive to E2. For example, the present data replicate our previous report that post-training dorsal hippocampal infusion of E2 enhances NOR memory consolidation (Fan et al. 2010), and extends this finding to show that dorsal hippocampal E2 infusion also enhances spatial memory consolidation in middle-aged females. Our previous work also demonstrated that rapid ERK phosphorylation in the dorsal hippocampus is necessary for E2 to enhance NOR in both young and middle-aged mice (Fernandez et al. 2008; Fan et al. 2010), which suggests that the middle-aged female hippocampus remains at least partially responsive to E2. Because ERK-induced histone acetylation in the dorsal hippocampus is necessary for E2 to enhance NOR in young females (Zhao et al. 2010, 2012), we hypothesized that E2 would increase histone acetylation in the middle-aged dorsal hippocampus. Indeed, the pattern of E2-induced histone modifications was nearly identical in young and middle-aged females. As in young females (Zhao et al. 2012), E2 selectively increased dorsal hippocampal histone H3 acetylation 30 min after dorsal hippocampal infusion, and decreased dorsal hippocampal levels of HDAC2 (but not HDAC1) 4 h after infusion. This study also extends our previous work to show that E2 decreases levels of HDAC3 in the dorsal hippocampus both 4 and 6 h after infusion. Moreover, we show for the first time that E2 can regulate H3 acetylation of Bdnf promoters and levels of pro-BDNF and BDNF proteins in the dorsal hippocampus of both young and middle-aged females. Together, this work provides the first evidence that the female middle-aged dorsal hippocampus remains epigenetically responsive to E2, and that E2 may enhance memory consolidation in middle-age females by increasing histone H3 acetylation of Bdnf promoters in the dorsal hippocampus.

Our data from middle-aged females showing that the consolidation of NOR and OP was enhanced 48 h and 4 h after dorsal hippocampal E2 infusion are consistent with our previous findings in young female mice (Boulware et al. 2013). These data are also consistent with reports that systemic E2 enhances NOR and OP memory 4 h after treatment in young female rats (Luine et al. 2003; Walf et al. 2013). These findings suggest that the hippocampus is necessary for the mnemonic effects of E2.
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Figure 3. E2 decreases levels of histone deacetylases. (A) Bilateral dorsal hippocampal infusion of 5 µg of E2 did not alter HDAC1 protein levels in the dorsal hippocampus at either time point. (B) E2 significantly decreased HDAC2 protein levels 4 h after infusion (\(P < 0.05\)) later, relative to vehicle. All proteins were normalized to β-Actin (each bar represents the mean \([\pm SEM]\) percent change from vehicle). (Insets) Representative Western blots of total and phosphorylated protein.

et al. 2006; Frye et al. 2007). However, differences in the timing of spatial memory enhancement between young and middle-aged females should be noted. In young female mice, E2 and estrogen receptor agonists enhance OP 24 h after dorsal hippocampal infusion (Boulware et al. 2013), whereas the present study found that E2 does not enhance OP at this delay in middle-aged females. Rather, effects of E2 on OP were seen here at the shorter 4 h post-infusion delay, suggesting that aging compromises the ability of E2 to enhance spatial memory. However, these data may reflect a detrimental effect of aging on spatial memory rather than a decreased responsiveness to E2, given reports that OP is more susceptible to aging than NOR (Wang et al. 2009; Wimmer et al. 2012). Despite potential age-related decline in spatial memory, memory consolidation in both NOR and OP was facilitated by dorsal hippocampal infusion of E2, suggesting that the molecular mechanisms within the dorsal hippocampus that support object recognition and spatial memory consolidation remain responsive to E2 in middle-aged females.

These mechanisms may include chromatin modifications, as suggested by the present data. As in young females (Zhao et al. 2010), E2 increased histone H3 acetylation in the dorsal hippocampus 30 min after infusion and decreased HDAC2 levels in the dorsal hippocampus 4 h after infusion. Not only were these effects present in middle-aged females, but they were also persistent, lasting at least 60 min for H3 acetylation and 6 h for HDAC3 levels. Such persistent changes may be evident in young females as well, but have not yet been examined. With respect to histone acetylation, the specificity of E2-induced changes to H3 is consistent with our previous work in young females (Zhao et al. 2010, 2012) and with contextual fear-induced changes in the hippocampus of male rats (Levenson et al. 2004; Chwang et al. 2006). With respect to HDAC levels, the failure of E2 to regulate HDAC1 levels is consistent with our previous data from young female mice (Zhao et al. 2010, 2012). These data also support evidence that hippocampal HDAC1 is necessary for the extinction of fear memory, but not for NOR memory (Bahari-Javan et al. 2012). In contrast, the E2-induced reduction of dorsal hippocampal HDAC2 and HDAC3 levels corresponds with other studies showing that these HDACs negatively regulate hippocampal memory (Guan et al. 2009; McQuown et al. 2011). Notably, Hdac2 knockout mice display enhanced acetylation of genes that regulate synaptic plasticity and memory (Guan et al. 2009) and shRNA knockdown of Hdac2 restores memory deficits in a mouse model of Alzheimer’s disease (Graff et al. 2012). Similarly, specific deletion of Hdac3 in the dorsal hippocampus enhances long-term NOR memory (McQuown et al. 2011). Here, we provide the first evidence that E2 reduces HDAC3 protein in the dorsal hippocampus, thus supporting HDAC2 and HDAC3 as negative regulators of memory.

Findings showing that E2 administered 2 or 3 h after training does not enhance spatial memory or object recognition (Packard and Teather 1997a;b; Fernandez et al. 2008) support the temporal specificity of E2’s effects on the memory consolidation phase of memory formation. Therefore, E2-induced changes in cell signaling and epigenetic modifications likely follow a specific time course in order to facilitate memory consolidation. Acute systemic injections of E2 in young female rats can increase spine density in the hippocampus within 30 min (Inagaki et al. 2012), an effect that is ERK-dependent in vitro (Srivastava et al. 2008). Our laboratory has shown in young female mice that E2 increases the phosphorylation of p42 ERK and activates ERK-driven mammalian target of rapamycin (mTOR) protein synthesis pathway 5 min after dorsal hippocampal infusion (Fernandez et al. 2008; Boulware et al. 2013; Fortress et al. 2013b), which suggests a possible increase in synaptogenesis as early as 5 min after infusion. We also found in young females that E2 increases dorsal hippocampal HAT activity 30 min after infusion, and that ERK activation is necessary for the E2-induced acetylation of H3 30 min after infusion (Zhao et al. 2010). In young ovariectomized female mice, histone acetylation must be increased within 3 h of NOR training to facilitate memory consolidation (Zhao et al. 2010, 2011), and is essential for the reduction in E2-induced HDAC2 observed 4 h after infusion (Zhao et al. 2010, 2012). Therefore, the data suggest that E2-induced alterations in histone acetylation occur within the time frame of memory consolidation, and lead to subsequent alterations in levels of HDAC2 protein. This evidence leads us to hypothesize that E2-induced changes in H3 acetylation are responsible for the initial consolidation of the memory, and that a subsequent decrease in HDAC2 expression may be involved in the maintenance of the memory. Support for this notion comes from evidence that HDAC inhibitors increase the persistence of object recognition memories for up to a
regions. Age-related decreases in acetylation were evident among vehicle-infused females at the pI and Chromatin immunoprecipitation analysis of histone H3 acetylation at the pII and pIV promoters 30-min post-infusion in both young and middle-aged mice (Zheng et al. 2011). E2 increases histone H3 acetylation of promoters is closely associated with hippocampal learning and aging (Lubin et al. 2008; Fuchikami et al. 2010; Perovic et al. 2013). We found that aging significantly decreased H3 acetylation of pI, pII, and pIV, and tended to decrease acetylation of pl. These findings are generally consistent with data from male rats showing significant age-related reductions in Bdnf pl and plv (Perovic et al. 2013). Despite these reductions, we found that E2 significantly increased H3 acetylation of pl and plv, but had no effect on pl. The specific roles of each Bdnf mRNA transcript in hippocampal function are still unclear. Bdnf pl is located largely in the soma of CA1 pyramidal neurons, whereas pl and plv are located primarily in proximal and distal dendrites, respectively (Baj et al. 2011). Interestingly, plv is regulated by calcium through L-type calcium channels (Zheng et al. 2011). E2 activates the L-type calcium channels necessary for ERK signaling (Wu et al. 2005; Sarkar et al. 2006), and we have shown that ERK signaling is required for H3 acetylation (Zhao et al. 2010). Activation of L-type calcium channels by ERK promotes CREB phosphorylation (Wu et al. 2005), which can facilitate epigenetic changes by increasing CBP occupancy at gene promoters important for synaptic plasticity (Bousiges et al. 2010). In fact, a synthetic CBP analog has recently been shown to increase acetylation of Bdnf pl in addition to increasing BDNF mRNA and protein levels (Chatterjee et al. 2013). As such, E2 may increase H3 acetylation at plv by activating L-type calcium channels, thereby phosphorylating ERK, and increasing the expression of Bdnf mRNA at distal dendrites. These changes could increase the pool of resident mRNA for local BDNF synthesis to facilitate synaptic plasticity, but this speculation has yet to be tested. It will also be of interest in future studies to determine how E2 regulates expression of other Bdnf transcripts and the extent to which E2 increases H3 acetylation of promoters for other synaptic plasticity genes like Egr1 and c-fos, the latter of which is transcribed within 15 min after dorsal hippocampal E2 infusion in young female mice (Zhao et al. 2010).

E2 also increased pro-BDNF and BDNF protein levels in middle-aged females 4 and 6 h after dorsal hippocampal infusion. Combined with our ChIP data, these results suggest that E2 induces a transcriptionally permissive state at the Bdnf promoter within 30 min, which may increase levels of Bdnf transcripts and BDNF protein within 4 h. Evidence that BDNF is transcribed within 30–60 min and translated within 4–6 h is supported by other studies demonstrating that visual experience affects BDNF synthesis in visual cortex (Schwartz et al. 2011) and that vibrissae stimulation affects BDNF synthesis in somatosensory cortex and hippocampus (Nanda and Mack 2000). Although protein changes in the order of hours could suggest regulation of the BDNF gene by classic genomic signaling through nuclear estrogen receptors at the BDNF ERE (Sohrabi et al. 1995), the present data instead implicate rapid nonclassical epigenetic regulation of BDNF. Other rapid regulation of BDNF by E2 has been observed; for example, E2 facilitates hippocampal synaptogenesis by increasing BDNF release through a rapid PKA-dependent mechanism (Sato et al. 2007). Interestingly, exogenous BDNF increases expression of Bdnf plv mRNA within 1 h of application (Zheng et al. 2012). These data suggest a feedback mechanism in which BDNF levels regulate BDNF signaling. Such a mechanism is supported by in vitro evidence that BDNF increases BDNF protein by nitrosylating cysteine residues on HDAC2 (Nott

| Figure 4. | E2 increases histone H3 acetylation of Bdnf promoters in the dorsal hippocampus. (A) Chromatin immunoprecipitation analysis of histone H3 acetylation at the Bdnf pl, pII, and pIV promoter regions. Age-related decreases in acetylation were evident among vehicle-infused females at the pl and plv promoters (\( P < 0.05 \) compared to young vehicle-infused mice). E2 increased H3 acetylation at the pl and plv promoters 30-min post-infusion in both young and middle-aged mice (\( P < 0.05 \) vs. age-matched control). 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. Each bar represents the mean ± SEM. (B) Western blot demonstrating specificity of sonication parameters. Pan-acetyl histone H3 was not evident in the cytoplasmic fraction (lane 1), but was present in the nuclear fraction (lane 2). The presence of H3 acetylation (Ac-H3) was reduced in the IP supernatant removed from the incubation containing the beads and the acetyl-H3 antibody (lane 3), but remained fully present in the IP supernatant with beads not containing acetyl-H3 antibody (lane 4). (C) qPCR data showing the absence of nonspecific binding in the IgG only control when amplified using primers for LINE1 (LINE1), no difference in the expression of the LINE1 negative control (LINE1), and a threefold increase in expression of the Nr4a2 positive control (Nr4a2) in vehicle- and E2-infused mice.

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et al. 2008; Graff and Tsai 2013a). Thus, one way in which E2 may facilitate memory consolidation in middle-aged females is by priming this BDNF positive feedback loop. Indeed, E2 levels are positively correlated with the expression of activated TrkB receptors (Spencer et al. 2008a). However, the potential interactions between E2 and the BDNF feedback loop will need to be examined in future studies. It should be noted, however, that our studies cannot definitively exclude other mechanisms regulating pro-BDNF and BDNF protein levels. It is unclear whether ERα and ERβ are involved in estrogenic regulation of histone acetylation, but both receptors are present in neurons within the hippocampus. Both ERα and ERβ have been localized to nuclei, axons, and dendritic spine synapses of CA1 pyramidal neurons in the hippocampus (Milner et al. 2001, 2005), and ERα is present in GABAergic interneurons (Murphy et al. 1998b) and cholinergic axons and terminals (Towart et al. 2003) within the hippocampus. In 2- to 3-wk-old hippocampal cell cultures, E2 down-regulates BDNF immunoreactivity in GABAergic interneurons, which reduces inhibition and increases excitatory tone and dendritic spine density in pyramidal neurons (Murphy et al. 1998a). Similarly, other data suggest that estrogenic stimulation of the basal forebrain cholinergic system disinhibits hippocampal pyramidal neuron by reducing GABAergic neurotransmission (Rudick et al. 2003). These data suggest complex interactions between E2 and BDNF involving multiple neuron types within the hippocampus. As such, future studies should specifically examine epigenetic regulation of BDNF in glutamatnergic, GABAergic, and cholinergic cell types to gain a better understanding of how E2 regulates BDNF and pro-BDNF proteins.

In conclusion, our findings provide the first evidence that the middle-aged female dorsal hippocampus remains epigenetically responsive to E2, and suggest that E2 may enhance memory in middle-aged females via epigenetic regulation of Bdnf. Because women are at greater risk than men for developing Alzheimer’s disease (Yaffe et al. 2007) and mental illnesses such as depression, anxiety, and mood disorders (Kessler et al. 2005), these findings may provide key insight into the hormonal regulation of cognition in women. Understanding how E2 epigenetically regulates Bdnf in females throughout the lifespan will provide essential new information for the development of more effective treatments for women with neurodegenerative and neuropsychiatric illnesses.

Materials and Methods

Subjects

Middle-aged (16-mo-old) female C57BL/6 mice were obtained from the National Institutes on Aging Aged Rodent Colony at Charles River Laboratories. For chromatim immunoprecipitation studies, young (3-mo-old) female C57BL/6 mice were obtained from Taconic. All mice were singly housed and maintained on a 12-h light–dark cycle (lights on at 07:00) with ad libitum access to food and water. All procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee.

Surgery

All mice were bilaterally ovarietomized and implanted with guide cannulae within the same surgical session as described previously (Fortress et al. 2013b). Using a stereotaxic apparatus, bilateral stainless-steel guide cannulae (22-gauge, C232G, Plastics One) were implanted into the dorsal hippocampus (−1.7 mm AP, ±1.5 mm ML, −2.3 mm DV [injection site]) and were fitted with dummy cannulae (C232DC) to preserve the integrity of the guide cannulae. Dental cement (Darby Dental) was used to affix the cannulae to the skull surface and also served to close the wound. Mice were allowed to recover for 7 d before the start of behavioral training.

Drugs and infusions

All mice were infused with vehicle or E2. Cyclodextrin-encapsulated E2 (Sigma-Aldrich) was dissolved to a concentration of 5 μg/0.5 μL in physiological saline as described previously (Fortress et al. 2013b). The vehicle, 2-hydroxypropyl-β-cyclodextrin (HBC, Sigma-Aldrich), was dissolved in saline to the same concentration of cyclodextrin used in the cyclodextrin–E2 solution. During infusions, mice were gently restrained and dummy cannulae were replaced with infusion cannulae (C232I, 26 gauge extending 0.8 mm beyond the 1.5-mm guide cannulae), which were attached to PE50 tubing connected to a 5-μL Hamilton syringe. Using a microinfusion pump (KDS 100, KD Scientific), mice were bilaterally infused into the dorsal hippocampus at a rate of 0.5 μL/min for 1 min, resulting in an E2 dose of 5 μg/hemisphere. Infusion cannulae remained in place for 1 min to prevent diffusion back up the cannula track. Infusions occurred immediately after behavioral training (i.e., post-training). Infusions were spaced out by 2 wk to allow the acute effects of E2 to dissipate, and all mice received the same number of infusions.

Behavioral testing

To examine the effects of E2 on object recognition and spatial memory, mice (n = 79) were tested in both novel object recognition (NOR) and object placement (OP) (Fig. 1A) tasks. NOR was first used to assess nonspatial hippocampal-dependent memory as described previously (Fortress et al. 2013b). Mice were handled for 30 sec/d for 3 d prior to habituation. A small Lego was placed in the home cage from the start of handling until training to habituate the mice to the objects. Mice were habituated to the testing arena for 2 d, during which time they were allowed to freely explore the empty, white testing arena (60-cm W × 60-cm L × 47-cm H) for 5 min. Twenty-four hours after the second habituation session, mice
were rehabilitated to the testing arena for 2 min and, then placed in a holding cage while two identical objects were placed in the Northeast and Northwest corners of the box (~5 cm from the walls). Mice were then immediately returned to the testing arena and allowed to freely explore both objects until they accumulated a total of 30 sec of exploration time, with a maximum of 20 min allowed for completion of training. Exploration was recorded when the front paws or nose contacted either object. Immediately after training, mice were removed from the box, gently restrained, and were bilaterally infused with vehicle or E2 into the dorsal hippocampus. Forty-eight hours after infusion, mice were returned to the testing arena and allowed to accumulate 30 sec exploring an object identical to that explored during training (familiar) and a new (novel) object. Novel object location was counterbalanced across mice. Time spent exploring each object was recorded using ANYmaze software (Stoelting). More time than chance (15 sec) spent exploring the novel object indicated memory for the familiar object.

Two weeks after the completion of NOR, spatial memory was tested using an OP protocol as described previously (Boulware et al. 2013). Mice were rehabilitated to the testing arena for 2 min and were then allowed to explore two identical objects placed in the Northeast and Northwest corners as in NOR. Immediately after training, mice were infused with vehicle or E2 as above. Either 4 or 24 h after training, mice were returned to the testing arena, and exploration of the identical objects had been blocked by placing them in the Southeast or Southwest corner. The location of the moved object was counterbalanced across mice. Mice were again allowed to accumulate 30 sec with the objects. More time than chance (15 sec) spent exploring the moved object indicated memory for the unmoved object.

Western blotting

Two weeks after the completion of OP, mice were again infused with vehicle (n = 16) or E2 (n = 36) into the dorsal hippocampus, and dorsal hippocampal tissues were collected bilaterally on ice 30 min, 60 min, 4 h, or 6 h later for measurement of histone acetylation (H2A, H2B, H3, and H4), and levels of HDAC1, HDAC2, and HDAC3 protein. Our previous studies in young females showed that dorsal hippocampal infusion of E2 increased H3 acetylation 30 min later and decreased HDAC2 protein 4 h later (Zhao et al. 2010, 2012). However, because we also found that activation of ERK is delayed in middle-aged females relative to young females (Fan et al. 2010), and that ERK activation is necessary for E2 to increase H3 acetylation (Zhao et al. 2010), we included the 60-min and 6-h time points here to account for potential age-related delays in histone modifications. Dorsal hippocampal tissues were maintained at ~80°C until homogenization.

Tissues were prepared as described previously (Fortress et al. 2013a), with the exception of those used for histone extraction. For tissue collected at 30 and 60 min to measure histone acetylation, tissues were dounce homogenized with a Teflon homogenizer after resuspension in 1.5 w/v of 1× Laemmli buffer diluted from 5× Laemmli buffer (50% glycerol in water containing 587 mM Tris HCl, 38 mM Tris Base, 173 mM SDS) containing 100 mM PMSF, and 1× Protease Inhibitor Cocktail. Homogenates were then incubated in a 37°C water bath for 10 min and centrifuged at 12,000 rpm for 5 min at 4°C. For all other proteins to be assayed at the 4- and 6-h time points, tissues were homogenized in 1:25 w/v dilution in lysis buffer and homogenized with a probe sonicator (Branson Sonifier 250). After total protein content was measured for all samples (Fernandez et al. 2008), 20 μg of sample was electrophoresed on various gradients of Tris-HCl gels and transferred to PVDF membranes using the TransBlot Turbo system (Bio-Rad). Membranes were blocked and incubated with the following anti-rabbit primary antibodies overnight at 4°C: acetyl-H2A (Lys 5), acetyl-H2B (Lys 12) (1:1000, Cell Signaling Technology), acetyl-H3 (pan), acetyl-H4 (Lys 12), HDAC1, HDAC2, or HDAC3 (1:1000, Cell Signaling Technology). The next day, membranes were washed and incubated in secondary antibody (anti-rabbit HRP, 1:5000, Cell Signaling Technology), and then developed in West Dura Chemiluminescent substrate (Pierce). For normalization of histone proteins, blots were stripped and reprobed with anti-rabbit antibodies for corresponding total histone proteins H2A, H2B (1:1000, Cell Signaling Technology), H3, or H4 (1:2000, Millipore). Anti-β-Actin (1:5000, Cell Signaling Technology) was used to normalize all other proteins. Images were captured and densitometry was performed using the Carestream Gel Logic 6000 Pro. All normalized proteins were expressed as a percentage relative to vehicle controls.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using a previously published protocol (Kenney et al. 2012). Thirty minutes after infusion with vehicle or E2, young (n = 15) and middle-aged mice (n = 13) were cervically dislocated, and the dorsal hippocampus was rapidly dissected, flash frozen, and stored at ~80°C until use. Tissues were minced and proteins cross-linked by placing them for 10 min in 1% formaldehyde on a rotator at room temperature. Glycine (200 mM) was then added for 5 min to stop the cross-linking reaction, and the tissue was then centrifuged at 2500 rpm for 2 min at 4°C. The supernatant was removed and the samples were washed three times with ice-cold phosphate buffered saline (PBS) containing protease and phosphatase inhibitors (PPI) (Pierce), with centrifugation at 2500 rpm for 2 min at 4°C between each wash. To lyse the cells, tissue was homogenized on ice in 500 μL of lysis buffer (1% SDS, 10 mM Tris-HCl, 1 mM EDTA, 1.5 mM MgCl2, 0.5% Igepal-CA630) using a sterile pestle homogenizer and centrifuged at 5500 rpm for 5 min at 4°C. The supernatant was then removed and 150 μL of nuclear lysis buffer (1× PPI, 50 mM Tris-HCl [pH 8.1], 5 mM EDTA, 1% SDS) was added. For chromatin shearing, samples were incubated on ice for 10 min and then sonicated on ice using a probe sonicator (10 cycles of 15-sec on, 120-sec off at 25% power) (Branson Sonifier 250). These conditions yielded chromatin fragments in the 100–800 base pair range. Following sonication, 5 μL of sample was eluted in ChIP elution buffer (1% SDS, 0.1 M NaHCO3) and 0.1 μg/μL proteinase K (Invitrogen), and then incubated for 2 h at 62°C on a rotator. Cross-links were then reversed by incubation at 95°C for 10 min. Resulting DNA was purified using QiaQuick spin columns (Qiagen). The following day, purified DNA fragments were used to check DNA concentration and efficiency of chromatin shearing. To detect the size of base pair fragments, samples were electrophoresed in 1.5% agarose in TAE buffer containing Sybr Safe and visualized using UV luminescence on the ChemiDoc MP Gel Imaging System (Bio-Rad). All fragments of DNA were determined to be in the 100–800 base pair range. For the immunoprecipitation (IP) process, 2 μg of DNA was mixed with IP buffer (16.7 mM Tris-HCl [pH 8.1], 1.1% Triton X-100, 0.01% SDS, 167 mM NaCl) with 1× PPI to a volume of 500 μL. Five microliters of the IP mix was removed as 1% input prior to the addition of beads and antibody. This process resulted in three tubes for each sample: input, acetyl-H3, or IgG control. For tubes receiving acetyl-H3, 5 μL of pan-acetyl H3 antibody (Millipore #06-599) was added. For IgG control tubes, 1 μL of rabbit IgG (Cell Signaling Technology) was added. Twenty microliters of protein A magnetic beads (Millipore) were then added to the acetyl-H3 and IgG tubes, and incubated overnight on a rotator at 4°C. Following incubation, the acetyl-H3 and IgG tubes were washed once each with low salt (20 mM Tris HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton-X, 0.1% SDS), high salt (20 mM Tris HCl, 500 mM NaCl, 2 mM EDTA, 1% Triton-X, 0.1% SDS), LiCl (10 mM Tris HCl, 250 mM LiCl, 1 mM EDTA, 1% deoxocholate, 1% Igepal-CA630), and TE (10 mM Tris HCl, 1 mM EDTA) wash buffers. All tubes were then incubated in elution buffer (0.1 M NaHCO3, 1% SDS) and 0.1 μg/μL proteinase K (Invitrogen) for 2 h at 62°C. Cross-links were reversed via incubation at 95°C for 10 min. DNA was isolated and purified using QiaQuick spin columns (Qiagen) and eluted twice in nuclelease-free water to a total volume of 200 μL.

Quantitative real-time PCR (qPCR)

For each qPCR reaction, 9 μL of DNA was combined with 250 nM primer solution and 10 μL of EVAGreen SYBR Green Master Mix

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Statistical analyses

For NOR and OP, independent sample t-tests were used to determine if the time each group spent with the novel or moved objects differed significantly from chance (15 sec) (Boulware et al. 2013; Daniel JM, Hulst JL, Berbling JL. 2006. Estradiol replacement enhances working memory in middle-aged rats when initiated immediately after ovariectomy but not after a long-term period of ovarian hormone deprivation. *Endocrinology* 147:607–614.


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