Epigenetic alterations regulate estradiol-induced enhancement of memory consolidation

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The involvement of epigenetic alterations in mediating effects of estrogens on memory is unknown. The present study determined whether histone acetylation and DNA methylation are critical for the potent estrogen 17β-estradiol (E_2) to enhance object recognition memory. We show that dorsal hippocampal E_2 infusion increases acetylation of dorsal hippocampal histone H3, but not H4—an effect blocked by dorsal hippocampal inhibition of ERK activation. Further, intrahippocampal inhibition of ERK activation or DNA methyltransferase (DNMT) activity blocked the memory-enhancing effects of E_2. Consistent with these effects, E_2 decreased levels of HDAC2 protein and increased DNMT expression in the dorsal hippocampus. These findings provide evidence that the beneficial effects of E_2 on memory consolidation are associated with epigenetic alterations, and suggest these can be triggered by dorsal hippocampal ERK signaling.

object recognition | dorsal hippocampus | ERK | histone acetylation | DNA methylation

The specific molecular mechanisms underlying the mnemonic effects of estrogens remain largely unknown. We recently showed that activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling cascade in the dorsal hippocampus is necessary for the potent estrogen 17β-estradiol (E_2) to enhance novel object recognition in young female mice (1). In this study, i.p. injection of E_2 immediately after object recognition training significantly enhanced long-term memory and increased p42 ERK phosphorylation in the dorsal hippocampus; both effects were blocked by inhibiting MAPK kinase (MEK), the exclusive upstream activator of ERK (1).

Activated ERK can promote the expression of genes associated with learning and memory, in part, by activating transcription factors such as CREB (2, 3). ERK may also increase gene expression by regulating epigenetic mechanisms necessary for memory formation, such as histone acetylation and DNA methylation; recent findings suggest that ERK activation influences both processes (4–7). Thus, these mechanisms may also play a role in estrogenic modulation of memory. DNA is wound around a core of eight histone proteins, two each of histones H2A, H2B, H3, and H4. Acetylation of lysine residues on histone tails relaxes the bond between histones and DNA, allowing transcriptional access. Histone acetylation is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (8). Genetic disruption of HAT activity impairs hippocampal memory, and these deficits are rescued by HDAC inhibitors (9). HDAC inhibitors such as trichostatin A (TSA) prevent deacetylation of histones H3 and H4, and enhance induction of hippocampal long-term potentiation (LTP), long-term contextual fear conditioning (CFC), and novel object recognition (4, 10–12). Acetylation of hippocampal histone H3, but not H4, is increased following ERK activation or CFC (4, 5), and ERK activation is necessary for other protein kinases (e.g., protein kinase C, PKC) to increase hippocampal H3 acetylation (4). Collectively, these data suggest that histone acetylation regulates long-term memory formation, and that ERK activation is essential to this process. Given that ERK activation is necessary for E_2 to enhance object recognition, histone acetylation may also regulate effects of E_2 on memory consolidation.

DNA methylation typically silences gene expression, and its functional effects depend on the genes altered. DNA (cytosine-5′) methyltransferases (DNMTs) methylate cytosine residues in CpG islands, thereby decreasing transcriptional access. Expression of DNMT3A and DNMT3B, but not DNMT1, mRNA is increased after CFC, and DNMT inhibitors such as 5-Aza-deoxycytidine (5-AZA) block hippocampal LTP, impair CFC, and prevent PKC activation and CFC from increasing histone H3 activation (6, 13, 14). Accordingly, CFC increases methylation of memory suppressor genes (e.g., *PP1*) and decreases methylation of memory promoter genes (e.g., *reelin*) in the hippocampus (13, 14). Expression of DNMT3A is increased by PKC activation (13), linking DNMT activity to cell signaling, and effects of 5-AZA on CFC and LTP are blocked by HDAC inhibition (6), suggesting that histone acetylation regulates DNA methylation. Interestingly, the gene for HDAC2 negatively regulates hippocampal memory and synaptic plasticity (15); thus, E_2 may regulate hippocampal function by affecting HDAC2 methylation.

The present study examined the involvement of dorsal hippocampal histone acetylation and DNMT expression in the beneficial effects of E_2 on object memory. The data suggest that dorsal hippocampal histone acetylation and DNA methylation are critically involved in estradiol-induced memory enhancement, and link these processes to hippocampal ERK activation. This report provides evidence that epigenetic alterations regulate the effects of E_2 on memory, and reveals an association between E_2-induced modulation of hippocampal cell signaling and epigenetic mechanisms in regulating memory.

Results

Histone Deacetylase Inhibition Enhances Object Recognition. We first determined whether novel object recognition was sensitive to epigenetic alteration. Mice receiving bilateral dorsal hippocampal infusions of vehicle immediately after training spent more time than chance (15 s) with the novel object after 24 h ($t_{(5)} = 10.93, P < 0.001$; Fig. 1), indicating intact object recognition at this delay. We have previously shown that vehicle-infused females do not remember the familiar object after 48 h (1), and therefore this delay was used to observe the memory-enhancing effects of drugs in this study. Immediately after training, mice received bilateral dorsal hippocampal infusion of vehicle or TSA (16.5 mM/side). After 48 h, mice receiving TSA, but not vehicle, spent significantly more time than chance with the novel object ($t_{(9)} = 7.5, P < 0.01$; Fig. 1). The TSA-induced preference for the novel object indicates that blocking dorsal hippocampal histone deacetylation enhances object recognition. To demonstrate that this memory consolidation occurs within hours of infusion, other mice received dorsal hippocampal infusions of the same dose of TSA 3 h after training. These mice did

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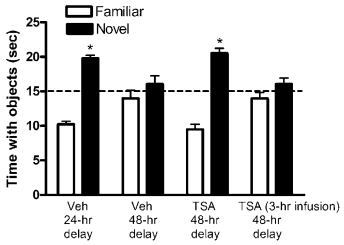

Fig. 1. Histone acetylation enhances object recognition. Mice infused with TSA (16.5 mM/side, $n = 10$), but not vehicle (Veh; 50% ethanol, $n = 5$), into the dorsal hippocampus immediately after training spent significantly more time with the novel object than chance (dashed line at 15 s; $*P < 0.05$) 48 h later, indicating enhanced memory for the familiar object. Vehicle mice ($n = 6$) spent more time than chance with the novel object 24 h after infusion ($*P < 0.05$), suggesting that these mice can remember the familiar object at a shorter delay. Mice infused with TSA ($n = 7$) 3 h after training did not spend more time than chance with the novel object. Bars represent the mean ± SEM for each object.

not show a preference for the novel object after 48 h (Fig. 1), indicating that the beneficial effects of TSA occur within 3 h of infusion.

E_2-Induced Memory Enhancement Is Associated with Dorsal Hippocampal ERK Activation and Histone H3 Acetylation. We next determined whether E_2 increased dorsal hippocampal histone acetylation, and if so, whether this effect depended on ERK activation. We first showed that dorsal hippocampal E_2 infusion significantly enhances object recognition and dorsal hippocampal ERK activation. Immediately after training, mice received intrahippocampal (IH) infusion of vehicle or E_2 (5 μg/side). After 48 h, mice receiving E_2, but not vehicle, spent significantly more time with the novel object than chance ($t_{(8)} = 4.3$, $P < 0.01$), demonstrating memory for the familiar object (Fig. 2*A*). Next, mice received IH infusions of vehicle or E_2 and were killed 5 min later for bilateral dissection of the dorsal hippocampus. E_2 significantly increased phosphorylation of p42 ($t_{(8)} = 2.9$, $P < 0.02$), but not p44, ERK (Fig. 2*B*). We next examined if IH E_2 infusion affects histone H3 and H4 acetylation. TSA, which increases H3 and H4 acetylation in the hippocampus (4), was a positive control. Mice received IH infusions of vehicle, E_2, or TSA, and dorsal hippocampi were collected 30 min later. Treatment significantly affected histone H3 ($F_{(2,16)} = 5.7$, $P < 0.02$) and H4 ($F_{(2,18)} = 4.0$, $P < 0.05$) acetylation. Consistent with previous work (4), TSA infusion increased acetylation of both histones relative to vehicle ($P < 0.03$; Fig. 2 *C* and *D*). E_2 increased acetylation of histone H3 ($P < 0.01$), but not H4, relative to vehicle (Fig. 2 *C* and *D*). These data suggest that dorsal hippocampal histone H3 acetylation may play a role in the beneficial effects of E_2 on object recognition.

Dorsal Hippocampal ERK Activation Is Necessary for E_2 to Enhance Histone H3 Acetylation. Because dorsal hippocampal ERK activation is necessary for systemically injected E_2 to enhance object recognition (1), we thought that ERK activation may also be necessary for intracranial E_2 to increase dorsal hippocampal histone H3 acetylation. Therefore, we first determined if intrahippocampal MEK inhibition blocked the effects of intracranially infused E_2 on object recognition. E_2 was infused intracerebroventricularly (ICV) into the dorsal third ventricle to provide E_2 to the brain while preventing tissue damage from repeated infusions into the dorsal hippocampus. Mice were infused ICV with vehicle or E_2 (10 μg), and into the dorsal hippocampus with vehicle or the MEK inhibitor U0126 (0.5 μg/side). As seen with IH E_2 (Fig. 2*A*), ICV E_2, but not vehicle, infusion significantly increased time spent with the novel object relative to chance 48 h after training ($t_{(5)} = 2.7$, $P < 0.05$; Fig. 3*A*). This effect was completely blocked by concurrent IH U0126 ($t_{(5)} = 0.2$, $P > 0.05$; Fig. 3*A*). IH U0126 alone did not affect object recognition (Fig. 3*A*). Next, effects of IH U0126 on histone acetylation were examined. Mice were infused ICV with vehicle or E_2 and

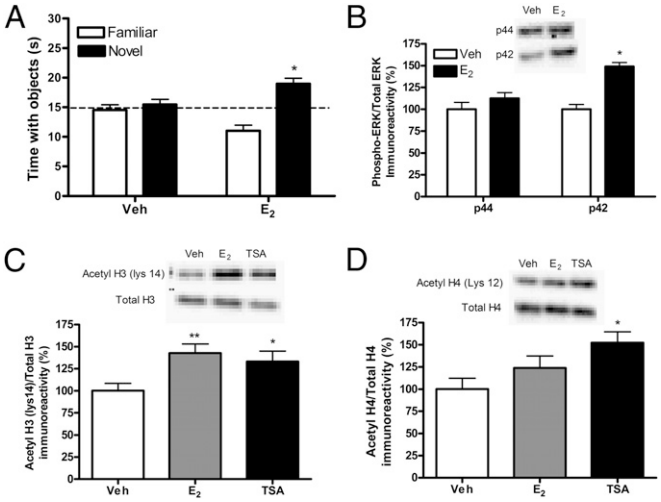

Fig. 2. IH E_2 infusion enhances object recognition and increases dorsal hippocampal p42 ERK activation and histone H3 acetylation. (*A*) Immediately after training, mice received IH Veh [2-hydroxypropyl-β-cyclodextrin (HBC), $n = 9$] or E_2 (5 μg/side, $n = 5$) infusion. After 48 h, mice infused with E_2, but not Veh, spent more time than chance ($*P < 0.05$) with the novel object. Bars represent mean ± SEM for each object. Relative to Veh, IH E_2 significantly increased dorsal hippocampal: (*B*) p42, but not p44, ERK phosphorylation 5 min after infusion ($n = 5$ or 6), and (*C* and *D*) histone H3 (Lys-14), but not H4 (Lys-12), acetylation 30 min after infusion ($n = 6$–8). IH TSA (16.5 mM/side) significantly increased dorsal hippocampal H3 and H4 acetylation 30 min after infusion. Bars represent mean ± SEM percent change from Veh ($*P < 0.05$, $**P < 0.01$ relative to Veh, set at 100%).

IH with vehicle or U0126. Two polyclonal antibodies were used to recognize acetylation on histone H3; one on lysine 14 and the other on lysine 9 and 14. A significant treatment effect was observed for H3 (Lys-9,14) ($F_{(3,18)} = 3.3$, $P < 0.05$), and a trend for an effect was observed for H3 (Lys-14) ($F_{(3,18)} = 2.5$, $P = 0.09$), but no effect was observed for H4 (Fig. 3 *B*–*D*). Histone H3 (Lys-9,14) acetylation was increased 30 min after ICV E_2 infusion ($P < 0.02$) relative to vehicle, and this increase was blocked by concurrent IH U0126 infusion ($P < 0.02$ relative to E_2 alone; Fig. 3*C*). Histone H4 acetylation was not altered by E_2, U0126, or their combination (Fig. 3*D*). Together, these findings suggest that inhibition of dorsal hippocampal ERK activation by U0126 reverses E_2-induced enhancement of dorsal hippocampal histone H3 acetylation and memory consolidation.

E_2-Induced Histone H3 Acetylation Is Associated with Down-Regulation of HDAC1 and HDAC2. To better understand the molecular mechanisms through which E_2 might increase histone H3 acetylation, we measured protein and mRNA expression of the HDAC enzymes 1 and 2 (HDAC1 and HDAC2) in dorsal hippocampus after IH E_2 infusion. Both HDACs are expressed in adult mouse hippocampus, where HDAC1 is expressed in glia and neural progenitor cells, and HDAC2 is expressed in neurons and neural progenitors (16). HDAC1 regulates cell proliferation and differentiation and is neuroprotective in mouse forebrain (17). HDAC2 negatively modulates hippocampal memory and synaptic plasticity in adult mice (15). Therefore, E_2 may increase histone H3 acetylation by increasing HDAC1 expression and reducing HDAC2 expression. We first examined dorsal hippocampal HDAC mRNA expression 5, 15, 45, 90, and 180 min after IH vehicle or E_2 infusion. HDAC1 mRNA was significantly altered by E_2 ($F_{(5,18)} = 6.8$, $P < 0.001$; Fig. 4*A*); expression was increased relative to vehicle 15 min after infusion ($P < 0.01$), and then gradually decreased to below vehicle 180 min after infusion ($P < 0.02$). HDAC2 mRNA expression was slightly, but not significantly ($F_{(5,22)} = 1.2$, $P = 0.36$), decreased 15, 45, 90, and 180 min after E_2 infusion (Fig. 4*C*). We next measured

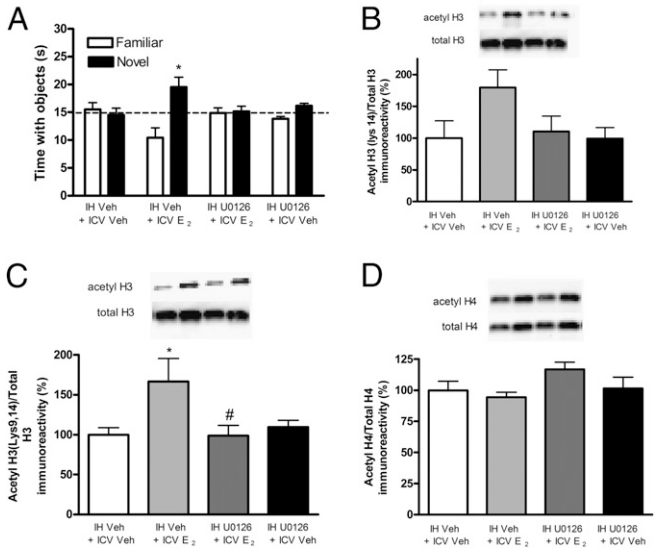

Fig. 3. Inhibition of ERK by U0126 reverses E_2-induced histone H3 acetylation and blocks beneficial effects of E_2 on object recognition. (*A*) Mice received ICV infusion of Veh (HBC) or E_2 (10 μg) and IH infusion of Veh (100% DMSO) or U0126 (0.5 μg/side) immediately after training. After 48 h, ICV E_2 alone, but not Veh alone, significantly increased time with the novel object relative to chance (*$P < 0.05$); this effect was blocked by IH U0126. U0126 alone did not affect memory consolidation. Bars represent mean ± SEM for each object, $n = 4$–6. (*B*) ICV E_2 increased histone H3 (Lys-14) acetylation 1.8-fold relative to Veh 30 min after infusion; IH U0126 infusion blocked this effect. (*C*) ICV E_2 significantly increased histone H3 (Lys-9,14) acetylation relative to Veh (*$P < 0.05$); IH U0126 infusion blocked this effect (#$P < 0.05$ relative to ICV E_2). (*D*) Neither E_2 nor U0126 altered H4 acetylation. Bars represent mean ± SEM percent change from Veh (100%). $n = 5$ or 6 in *B*–*D*.

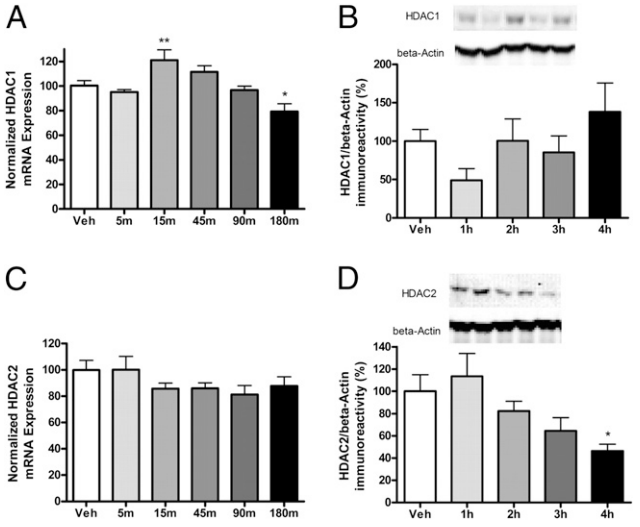

Fig. 4. Dorsal hippocampal HDAC1 and HDAC2 mRNA (*A* and *C*) and protein (*B* and *D*) expression after IH E_2 ($n = 4$–9). (*A*) Relative to Veh, HDAC1 mRNA expression was significantly increased 15 min after IH E_2 infusion (5 μg/side), and decreased 180 min after infusion (**$P < 0.01$). (*B*) HDAC1 protein levels were not significantly affected by E_2, but tended to decrease 1 h after infusion. (*C*) Although not significant, HDAC2 mRNA expression was decreased from 15 to 180 min after IH E_2 infusion. (*D*) HDAC2 protein expression was significantly decreased relative to Veh 4 h after IH E_2 (*$P < 0.05$). Bars represent mean ± SEM percent change from Veh (100%).

HDAC protein levels 1, 2, 3, and 4 h after IH vehicle or E_2 infusion. Although HDAC1 protein levels tended to decrease 1 h after E_2 infusion, no significant effect of E_2 was seen ($F_{(4,15)} = 1.8, P = 0.19$; Fig. 4*B*). However, HDAC2 protein levels were affected by E_2 ($F_{(4,14)} = 3.1, P < 0.04$), exhibiting a gradual decrease from 1 to 4 h (Fig. 4*D*), and becoming significantly reduced after 4 h ($P < 0.03$). These findings suggest that E_2-induced histone H3 acetylation may be associated with reduced HDAC2 expression.

DNA Methylation Is Necessary for E_2 to Enhance Memory Consolidation. Having determined that E_2 alters histone acetylation in a manner dependent on ERK activation, we next turned to DNA methylation. Expression of DNMT enzymes is dynamically regulated by hippocampal learning. DNMT1 is a maintenance methyltransferase that copies methylation to nascent DNA strands during replication, whereas DNMT3A and DNMT3B are de novo methyltransferases that add new methyl residues to DNA (18). mRNA for DNMT3A and 3B, but not DNMT1, in the hippocampus is increased 30 min after CFC (14), suggesting that learning preferentially affects de novo methylation in the hippocampus. mRNA for the immediate-early gene *c-fos* in the hippocampus is also significantly increased by CFC (14). Therefore, we hypothesized that E_2 might also increase dorsal hippocampal expression of DNMT3A, DNMT3B, and *c-fos*. *c-fos* served as a positive control, given that E_2 increases *c-fos* mRNA levels by directly stimulating *c-fos* gene transcription (19). We first measured dorsal hippocampal DNMT mRNA 5, 15, 45, 90, and 180 min after IH E_2 infusion (5 μg/side). Vehicle-treated mice were killed at various points after infusion. DNMT1 mRNA levels were not altered by E_2 at any time (Fig. 5*A*). Both DNMT3A and DNMT3B mRNA were significantly increased by E_2 ($F_{(5,17)} = 3.4, P < 0.03$ and $F_{(5,18)} = 2.9, P < 0.05$, respectively), peaking 45 min after infusion ($P < 0.01$ for both subtypes; Fig. 5 *B* and *C*). *c-fos* mRNA was also significantly increased by E_2 ($F_{(5,18)} = 7.1, P < 0.001$), peaking 15 min after infusion ($P < 0.01$; Fig. 5*D*). Next, dorsal hippocampal DNMT protein levels were measured 1, 2, 3, and 4 h after IH E_2 infusion. E_2 significantly affected DNMT3B (Fig. 5*G*; $F_{(4,15)} = 3.2, P < 0.04$), but not DNMT1 (Fig. 5*E*) or DNMT3A (Fig. 5*F*), protein levels. DNMT3B protein was significantly increased relative to vehicle 4 h after infusion ($P < 0.01$; Fig. 5*F*), consistent with elevated DNMT3B mRNA expression 45 min after infusion (Fig. 5*C*).

To determine if increased DNMT expression was necessary for E_2 to enhance memory, we examined if the nonspecific DNMT inhibitor 5-AZA could block the beneficial effects of E_2 on object recognition. 5-AZA is a cytidine analog that causes demethylation and blocks actions of DNMTs (13). Mice received ICV infusion of vehicle or E_2 (10 μg) concurrently with IH infusion of vehicle or 5-AZA (100 μg/side). As before (Fig. 3*A*), mice infused with E_2 alone ($t_{(7)} = 2.5, P < 0.05$), but not vehicle alone, exhibited a significant preference for the novel object 48 h after training (Fig. 5*H*). However, mice infused with E_2 plus 5-AZA showed no preference for the novel object (Fig. 5*H*), suggesting that 5-AZA blocked the memory-enhancing effects of E_2. These data suggest that DNA methylation is necessary for E_2 to enhance memory consolidation. Interestingly, 5-AZA alone significantly enhanced 48-h object recognition ($t_{(7)} = 2.7, P < 0.04$; Fig. 5*H*), suggesting that in the absence of E_2, inhibition of DNA methylation is beneficial for memory consolidation. To demonstrate that the effects of 5-AZA on memory occur within hours after infusion, other mice received IH 5-AZA plus ICV vehicle 3 h after training; these mice did not prefer the novel object 48 h after training (Fig. 5*H*), suggesting that the epigenetic modifications that lead to memory enhancement occur within 3 h of 5-AZA infusion.

Discussion

This report presents evidence that alterations in histone acetylation and DNA methylation are associated with E_2-induced enhancememt of memory consolidation. Further, the data demonstrate that dorsal hippocampal ERK activity regulates both mnemonic and epigenetic effects. CFC in male rats increases acetylation of hippocampal histone H3, but not H4, and systemic

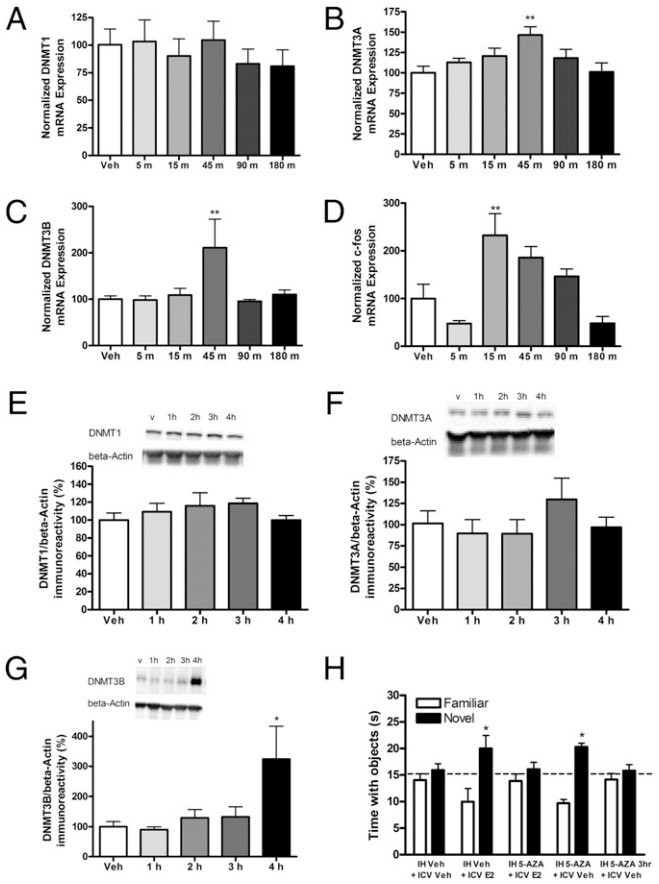

Fig. 5. (*A*) DNMT1 mRNA levels were not affected by IH E_2 infusion (5 μg/side). (*B* and *C*) DNMT3A and DNMT3B mRNA was significantly increased relative to Veh 45 min after IH E_2 (**$P < 0.01$). (*D*) c-fos mRNA was significantly increased relative to Veh 15 min after IH E_2 (**$P < 0.01$). (*E*) DNMT1 protein was not changed by IH E_2 infusion. (*F*) DNMT3A protein was slightly, but not significantly, increased 3 h after IH E_2. (*G*) DNMT3B protein was significantly increased relative to Veh 4 h after IH E_2 (*$P < 0.05$). (*H*) Mice receiving ICV E_2 (10 μg), but not Veh (ICV HBC + IH 0.8% acetic acid), immediately after training spent significantly more time with the novel object than chance (*$P < 0.05$) after 48 h; this effect was blocked by IH 5-AZA (100 μg/side). Mice receiving IH 5-AZA plus ICV Veh ($n = 8$) immediately after training spent significantly more time with the novel object than chance (*$P < 0.05$) after 48 h; this effect was time limited, as infusion of IH 5-AZA plus ICV Veh 3 h after training ($n = 6$) did not increase time with the novel object after 48 h.

MEK inhibition blocks this effect (4, 5). This work also suggests that the pathways mediating acetylation of histones H3 and H4 differ (4). Consistent with these findings, IH infusion of E_2 in the present study significantly increased acetylation of histone H3, but not H4, and this increase was blocked by IH MEK inhibition, demonstrating that ERK activation is necessary for E_2 to acetylate histone H3 in the dorsal hippocampus. The fact that ERK activation is necessary for other protein kinases to increase hippocampal H3 acetylation in male rats (4) suggests that ERK signaling may critically link cell-signaling mechanisms with histone acetylation. ERK activation also regulates histone phosphorylation induced by CFC (5), so ERK may mediate numerous changes in histone biochemistry related to estrogenic modulation of memory. Further, E_2 in females rapidly activates other hippocampal signaling cascades important for memory and neuroprotection [e.g., phosphatidylinositol 3-kinase (20) and Akt (21)], so involvement of these signaling cascades in E_2-induced histone alterations should be investigated in future work.

Given the importance of dorsal hippocampal ERK activation to E_2-induced memory enhancement, the present data suggest that histone H3 acetylation is also involved in E_2-induced enhancement of object recognition. This conclusion is supported by the present data showing that TSA, which increases histone H3 and H4 acetylation, enhanced memory consolidation. However, more definitive conclusions about the necessity of histone acetylation, and H3 acetylation in particular, in mediating the effects of E_2 on memory will require blocking histone acetyltransferase (HAT) activity. Genetic disruption of HAT activity in mice lacking the gene for CBP (CREB binding protein) impairs object recognition and spatial memory, and these deficits are rescued by HDAC inhibitors (9). Several HAT inhibitor drugs (e.g., garcinol and H4K16CoA) have recently been developed (22, 23), although none have yet been tested in neuronal cell culture or in vivo. Thus, future studies should explore the effects of these drugs on memory, and determine if their infusion into the hippocampus blocks the memory-enhancing effects of E_2.

The fact that E_2 decreased dorsal hippocampal HDAC2 protein levels may suggest a mechanism for the observed increase in acetylated H3. HDAC overexpression in mice decreases histone acetylation, and overexpression of HDAC2, but not HDAC1, impairs hippocampal-dependent memory formation, decreases CA1 spine density, and impairs hippocampal LTP (15). Conversely, histone acetylation, hippocampal memory, spine density, and LTP are enhanced in HDAC2 knockout mice (15). Accordingly, deficits observed in HDAC2 overexpressing mice can be reversed by an HDAC inhibitor (15), which suggests that the primary target of this, and perhaps other, HDAC inhibitors that improve memory is HDAC2. Given that HDAC2 so extensively represses hippocampal plasticity and memory, this enzyme may play a role in deacetylating histones such as H3 and, further, in mediating effects of E_2 on H3 acetylation. However, the timing of E_2-induced alterations in H3 and HDAC2 may rule out this possibility, as H3 acetylation was increased 30 min after infusion, whereas HDAC2 protein levels were not decreased until 4 h after infusion. Nevertheless, H3 acetylation is increased as late as 2–4 h after TSA injection (10) and 3–24 h after environmental enrichment (11), so the time frame for H3 acetylation may be longer than the time point tested here. The decrease in HDAC2 levels observed at 4 h may also be outside of the time frame in which memory consolidation takes place, as infusion of E_2 (1) or epigenetic inhibitors (present study) 3 h after training does not enhance object recognition. However, the decreasing levels of HDAC2 protein 2–3 h after infusion may be biologically significant, although levels are not statistically different from vehicle until 4 h after infusion. Alternatively, E_2 may induce rapid protein modifications that repress existing HDAC2 activity without immediately altering transcription or translation; for example, cigarette smoke decreases HDAC2 activity in lung tissue within 30 min by phosphorylation and ubiquitination of HDAC2 protein (24). This issue will need to be addressed in future studies. Although recent work shows no role for HDAC1 in hippocampal memory (15), HDAC1 is neuroprotective in forebrain neurons (17), and E_2-induced alterations in HDAC1 may be involved in the neuroprotective effects of E_2 on hippocampal neurons (25). Understanding whether HDAC1 or HDAC2 modulate E_2-induced increases in H3 acetylation and object recognition could be of clinical significance, given that nearly a dozen HDAC inhibitors are already in clinical trials for the treatment of various cancers (26). The ability of HDAC inhibitors to enhance memory in rodents suggests that these drugs may be promising treatments for neurodegenerative disease.

The present study also suggests that DNA methylation is necessary for E_2 to enhance object recognition. IH infusion of E_2 significantly increased dorsal hippocampal DNMT3A and DNMT3B, but not DNMT1, mRNA levels 45 min after infusion, indicating that E_2 increases de novo DNA methylation in the dorsal hippocampus. In addition, dorsal hippocampal DNMT3B protein levels were

significantly increased 4 h after infusion. Although a bit late for immediate memory consolidation, this increase may support long-term memory consolidation or influence memory retrieval processes activated during testing. Further, as for HDAC2, rapid protein modifications of DNMT3B induced by E_2 could also affect this protein's activity without immediately affecting protein levels. Nevertheless, given that immediate IH E_2 infusion enhances memory consolidation, these data suggest that increased expression of DNMT3A or DNMT3B may be involved in this enhancement. In support, IH 5-AZA completely blocked the beneficial effects of E_2 on object recognition. It is worth noting that 5-AZA alone enhanced object recognition, suggesting that in the absence of E_2, inhibition of DNMTs can benefit memory consolidation. Nevertheless, the data from combined E_2 + 5-AZA treatment suggest that E_2 infusion leads to methylation of a gene that negatively regulates memory, for example HDAC2. However, numerous other genes could be suitable targets, such as *PP1* (protein phosphatase 1), a memory suppressor gene methylated in male rats after CFC (14). Interestingly, IH 5-AZA in male rats also blocks increases in hippocampal histone H3 acetylation induced by CFC (6, 13), which suggests direct interactions between DNA methylation and histone H3 acetylation. In support, the HDAC inhibitor sodium butyrate prevents 5-AZA from impairing CFC in male rats (6), indicating a dynamic balance between effects of histone acetylation and DNA methylation on hippocampal memory.

The present data suggest that the beneficial effects of E_2 on memory are associated with genetic and epigenetic events in the dorsal hippocampus, many of which may be regulated by ERK (Fig. S1 shows a putative model). E_2 may stimulate ERK activation by binding to plasma membrane-bound estrogen receptors (ERs), which can rapidly increase hippocampal p42 ERK phosphorylation in vivo (1), or to nuclear ERs in the cytosol (ERα or ERβ), which can activate hippocampal ERK in vitro (25). ERK activation and translocation into the nucleus would then activate transcription factors (e.g., CREB), induce histone modifications (e.g., histone H3 acetylation), and alter DNA methylation (e.g., DNMT expression), thereby increasing transcription of genes that promote hippocampal synaptic plasticity and enhance memory consolidation. Although the present study did not demonstrate that ERK activation directly affects DNA methylation, IH 5-AZA blocks increases in histone H3 acetylation induced by PKC activation (13), linking cell signaling to DNA methylation. The fact that PKC can activate ERK (27) may then provide a link between hippocampal ERK activation and DNA methylation, although this must be tested in future work. An important caveat to this model is that an E_2 surge immediately after learning may be uncommon outside of the laboratory, and therefore the epigenetic alterations observed after posttraining E_2 may not be involved when E_2 is elevated before and during learning. Thus, future studies examining the role of epigenetics in regulating memory during the estrous cycle and after pretraining E_2 treatment will be critical to determining whether our findings generalize to learning in other hormonal milieus.

In conclusion, the present study provides evidence that histone acetylation and DNA methylation play a pivotal role in regulating the beneficial effects of E_2 on memory consolidation. As such, this work provides important insights into the molecular mechanisms underlying estrogenic modulation of memory. The data suggest that increased acetylation of memory promoter genes, plus increased methylation of memory suppressor genes, may be crucial for E_2 to enhance memory consolidation. Understanding how epigenetic alterations contribute to estrogenic modulation of memory consolidation may ultimately reveal novel targets for therapeutic intervention of age-related memory loss and dementia in menopausal women.

Materials and Methods

Subjects. Female C57BL/6 mice were obtained from Taconic at 12 weeks of age and housed individually in shoebox cages in a room (22-23 °C) with a 12/12-h light-dark cycle. Food and water were provided ad libitum. Mice were handled briefly before use. Procedures were conducted from 10:00 to 17:00 h in a quiet room, and experimenters conducting behavioral testing were blind to the treatment each mouse received. All procedures were approved by the Yale University Animal Care and Use Committee and are consistent with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Surgery. Mice were bilaterally ovariectomized and implanted with guide cannulae during the same surgical session as described previously (1). Mice were anesthetized using isoflurane gas (5% for induction, 2% for maintenance) in 100% oxygen and placed in a stereotaxic apparatus (Kopf Instruments). Immediately after ovariectomy, mice were implanted with stainless-steel intracranial guide cannulae (C232GC; 26 gauge; Plastics One) with inserted dummy cannulae (C232DC) aimed at the dorsal hippocampus [bilaterally; −1.7 mm posterior to bregma, ±1.5 mm lateral to midline, −2.3 mm (injection site) ventral to skull surface], dorsal third ventricle [−0.5 mm posterior to bregma, ±0.0 lateral to the midline, −3.0 (injection site) ventral to the skull surface], or both loci (triple guide; same coordinates as above for both regions) (28). Guide cannulae were anchored to the skull with dental cement that also closed the wound. Mice recovered for at least 5 days before testing.

Drugs and Infusions. During infusions, mice were gently restrained, and dummy cannulae were replaced with injection cannulae (C232I; intrahippocampal: 26 gauge, extending 0.8 mm beyond the 1.5-mm guide; intracerebroventricular: 28 gauge, extending 1.0 mm beyond the 2.0-mm guide) attached to polyethylene tubing (PE50) connected to a 10-μL Hamilton syringe. Infusions were controlled by microinfusion pump (KD Scientific) and were conducted at a rate of 0.5 μL/min for 1 min. Infusion cannulae were left in place for another minute to prevent drug diffusion up the cannula track.

Cyclodextrin-encapsulated 17β-estradiol (Sigma) was dissolved in physiological saline to a concentration of 10 μg/μL, resulting in doses of 5 μg/side of the dorsal hippocampus and 10 μg/ICV infusion (*SI Text*). Vehicle, 2-hydroxypropyl-β-cyclodextrin (HBC; Sigma) was dissolved in an equal volume of saline, and contained the same amount of cyclodextrin as E_2 for both IH and ICV infusions. All other drugs were infused IH only. The MEK inhibitor 1,4-diamino-2,3-dicyano-1,4-bis (*o*-aminophenylmercapto) butadiene (U0126) (Promega) was dissolved in 100% DMSO to concentration 1 μg/μL, for a dose of 0.5 μg/side. Trichostatin A (TSA; Sigma) was dissolved in 50% ethanol in saline to concentration of 16.5 mM/side. 5-Aza-2′-deoxycytidine (5-AZA; Sigma) was dissolved in 0.8% acetic acid and diluted with saline to 200 ng/μL, for a dose of 100 μg/side. Vehicles for each drug were based on their respective diluents.

Object Recognition. Novel object recognition, conducted as previously reported (29), evaluated nonspatial hippocampal memory (30). Briefly, mice were habituated to an empty white chamber by allowing them to freely explore for 5 min (no data were collected). After 24 h, mice were rehabituated to the empty chamber for 1 min and then placed in a holding cage while two identical objects were placed near the left and right corners of the chamber. Mice were returned to the chamber for training and allowed to freely explore until they accumulated a total of 30 s exploring the objects (exploration recorded when the front paws or nose contacted the object). Mice were then removed, immediately infused, and returned to their home cage. After 48 h, object recognition was tested by substituting a novel object for a familiar training object (novel object location counterbalanced across mice). Time spent with each object was recorded; because mice inherently prefer to explore novel objects, a preference for the novel object [more time than chance (15 s) spent with the novel object] indicates intact memory for the familiar object. Vehicle-treated mice remember the familiar object after 24 h, but not 48 h (29), and thus a 48-h delay is used to observe memory-enhancing effects of E_2.

Western Blotting. Both dorsal hippocampi were dissected rapidly on dry ice at various times after infusion as follows: 5 min for ERK, 30 min for histones, and 1, 2, 3, and 4 h for HDAC1, HDAC2, and the DNMTs (*SI Text*). Samples were resuspended and homogenized. Total protein content of the lysates was measured, sample buffer was added, and samples were boiled. Samples were loaded onto 18%, 10%, or 7.5% Tris-HCl polyacrylamide gel (Bio-Rad) for electrophoresis and, after separation, transferred to PVDF membranes (Millipore). Membranes were incubated overnight at 4 °C with rabbit polyclonal or monoclonal antibodies recognizing phospho-p44/42 ERK (Thr202/Tyr204) (1:1,000; Cell Signaling), acetylated histone H3 (Lys-14) (1:2,000; Upstate), acetylated histone H3 (Lys-9,14) (1:5000, Upstate), acetylated histone H4

(1:2,000; Upstate), DNMT1 (1:500; Abcam), DNMT3A (1:1,000, Cell Signaling), DNMT3B (1:1,000, Cell Signaling), HDAC1 (1:1,000; Upstate), and HDAC2 (1:1,000; Upstate). After TTBS wash, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling) or with HRP-conjugated anti-mouse IgG (Sigma) and developed using enhanced chemiluminescence (Pierce). Blots were then stripped for 1 h and reprobed with antibodies for total p44/42 ERK (1:2,000; Cell Signaling), total histone H3 (1:5,000; Upstate), total histone H4 (1:5,000; Upstate), or monoclonal β-Actin (1:5,000; Sigma) for normalization. Signal was detected using a Kodak Image Station 440CF and quantified by densitometry using Kodak 1D 3.6 software. Data were expressed as percent immunoreactivity relative to vehicle controls. Treatment effects were measured within single gels.

Quantitative Real-Time PCR (qRT-PCR). Dorsal hippocampi were dissected 5, 15, 30, 45, 90, and 180 min after IH E_2 infusion and stored at −20 °C in RNAlater buffer (Ambion). Total RNA was extracted using TRIzol reagent (Invitrogen) as previously described (31, 32). Total RNA concentration was determined by reading absorbance at 260 and 280 nm on a SmartSpec 3000 Spectrophotometry (Bio-Rad) and adjusted to 50 ng/μL. RNA was reverse transcribed to cDNA in the presence of random hexamers using the SuperScript First-Strand Synthesis Kit (Invitrogen). cDNA from each sample was subjected to qRT-PCR using the QuantiTect SYBR Green PCR Kit and ABI 7900HT real-time PCR system (Applied Biosystems). Specific predesigned and preoptimized primers were purchased from QuantiTect Primer Assay PCR (Qiagen). Quantitation of PCR products was performed using the ΔΔCt method. Quantities of mRNA were normalized to the housekeeping gene GAPDH.

Statistical Analyses. Statistical analyses were conducted using SPSS 14.0 (SPSS Inc.). For each behavioral experiment, separate one-sample *t* tests were performed for each group to determine if the time spent with the novel object differed from chance (15 s). This analysis was used because time spent with the objects is not independent; time spent with one object reduces time spent with the other (29). For Western blotting and qRT-PCR with two groups, separate two-tailed unpaired Student's *t* tests were performed between treatment and vehicle groups for each normalized protein or mRNA. For Western blotting and qRT-PCR with multiple groups, one-way analyses of variance (ANOVAs) were conducted, followed by Fisher's LSD post hoc tests. Significance was determined at $P < 0.05$. Data are expressed as mean ± SEM.

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