

Estradiol-Induced Object Memory Consolidation in Middle-Aged Female Mice Requires Dorsal Hippocampal Extracellular Signal-Regulated Kinase and Phosphatidylinositol 3-Kinase Activation

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We previously demonstrated that dorsal hippocampal extracellular signal-regulated kinase (ERK) activation is necessary for 17 β -estradiol (E₂) to enhance novel object recognition in young ovariectomized mice (Fernandez et al., 2008). Here, we asked whether E₂ has similar memory-enhancing effects in middle-aged and aged ovariectomized mice, and whether these effects depend on ERK and phosphatidylinositol 3-kinase (PI3K)/Akt activation. We first demonstrated that intracerebroventricular or intrahippocampal E₂ infusion immediately after object recognition training enhanced memory consolidation in middle-aged, but not aged, females. The E₂-induced enhancement in middle-aged females was blocked by intrahippocampal inhibition of ERK or PI3K activation. Intrahippocampal or intracerebroventricular E₂ infusion in middle-aged females increased phosphorylation of p42 ERK in the dorsal hippocampus 15 min, but not 5 min, after infusion, an effect that was blocked by intrahippocampal inhibition of ERK or PI3K activation. Dorsal hippocampal PI3K and Akt phosphorylation was increased 5 min after intrahippocampal or intracerebroventricular E₂ infusion in middle-aged, but not aged, females. Intracerebroventricular E₂ infusion also increased PI3K phosphorylation after 15 min, and this effect was blocked by intrahippocampal PI3K, but not ERK, inhibition. These data demonstrate for the first time that activation of dorsal hippocampal PI3K/Akt and ERK signaling pathways is necessary for E₂ to enhance object recognition memory in middle-aged females. They also reveal that similar dorsal hippocampal signaling pathways mediate E₂-induced object recognition memory enhancement in young and middle-aged females and that the inability of E₂ to activate these pathways may underlie its failure to enhance object recognition in aged females.

Introduction

Although recent clinical trials report that hormone therapy increases risks of global cognitive decline and dementia in postmenopausal women (Rapp et al., 2003; Shumaker et al., 2004), other studies demonstrate that estrogen therapy can reduce risks of cognitive decline and dementia in younger, recently menopausal, women (Henderson et al., 2005). These findings support the “critical period hypothesis” that posits that estrogen therapy preserves cognition only when initiated near menopause onset (Sherwin and Henry, 2008). Although compelling, the neurobiological mechanisms underlying this critical period have not been clearly elucidated in women or animal models. Because current estrogen therapies are associated with negative health outcomes (Rossouw et al., 2002), understanding the neural mechanisms through which estrogens protect memory in menopausal females could be instrumental to developing safer and more effective estrogen treatments for reducing age-related cognitive decline.

To this end, we recently demonstrated 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 17 β -estradiol (E₂) to enhance novel object recognition in young ovariectomized mice (Fernandez et al., 2008). In this study, a single intraperitoneal injection of E₂ given immediately after training significantly enhanced object recognition tested 48 h later and increased phosphorylation of the p42 isoform of ERK in the dorsal hippocampus 1 h after a subsequent injection (Fernandez et al., 2008). These effects were blocked by inhibiting MAPK kinase (MEK), the exclusive upstream activator of ERK (Fernandez et al., 2008). Post-training intraperitoneal E₂ injection also enhances 48 h object recognition in middle-aged, but not aged, ovariectomized mice (Gresack et al., 2007a,b), raising the possibility that age-related alterations in the ability of E₂ to activate cell signaling underlie its differential effects on object memory in aging females. However, it is unknown whether E₂ modulates object memory via similar signaling mechanisms in the aging female brain, given the myriad of age-related alterations that affect the hippocampus. Indeed, E₂ protects against the ovariectomy-induced decrease in phosphorylated p42 ERK levels in young, but not aged, female rats (Bi et al., 2003). Nevertheless, whether E₂ increases hippocampal ERK ac-

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tivation in middle-aged rodents has not been examined, so it is unclear whether ERK activation mediates the beneficial effects of E_2 on object recognition at this age.

Furthermore, ERK can be activated by other signaling cascades that are critical for hippocampal-dependent memory formation, including phosphatidylinositol 3-kinase (PI3K)/Akt (Chen et al., 2005). PI3K is crucial for hippocampal plasticity and object recognition (Kelly and Lynch, 2000; Lin et al., 2001; Horwood et al., 2006), is rapidly activated by E_2 (Yokomaku et al., 2003; Mannella and Brinton, 2006), and is vital for neuroprotection (Singh, 2001), which could be critically important for the aging brain. As such, hippocampal PI3K/Akt signaling may play a pivotal role in E_2 -induced object recognition enhancement in aging females, perhaps by activating p42 ERK.

The present study determined whether dorsal hippocampal ERK and PI3K activation are necessary for the beneficial effects of E_2 on object recognition in aging females. The data provide the first evidence that dorsal hippocampal PI3K and ERK activation is necessary for E_2 to enhance object memory consolidation in middle-aged female mice and that the inability of E_2 to activate these enzymes in aged females may underlie its failure to enhance object recognition in advanced aging.

Materials and Methods

Subjects. Young (3 months old), middle-aged (17 months old), and aged (21 months old) female C57BL/6 mice were obtained from the National Institutes on Aging colony at Charles River Laboratories. Mice were housed four to five per cage until surgery and were handled briefly before use. Mice were maintained on a 12 h light/dark cycle and had *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 Yale University Animal Care and Use Committee.

Surgery. Mice were ovariectomized and implanted with intracranial guide cannulae in the same surgical session as described previously (Fernandez et al., 2008; Lewis et al., 2008). All mice were implanted with stainless-steel guide cannulae (Plastics One) aimed at the dorsal hippocampus (bilaterally), dorsal third ventricle, or both brain regions. Mice were anesthetized with isoflurane gas (5% for induction, 2% for maintenance). Using a stereotaxic apparatus (David Kopf Instruments), guide cannulae (C232GC, 26 gauge; Plastics One) with inserted dummy cannulae (C232DC) were directed toward the dorsal hippocampus [-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 mm lateral to the midline, -3.0 (injection site) ventral to the skull surface], or both the hippocampus and dorsal third ventricle (triple guide; same coordinates as above for both regions) (Paxinos and Franklin, 2003). Each cannula was fixed to the skull with dental cement that also served to close the wound. Mice recovered 5–7 d before testing or drug treatment.

Drugs and infusions. Cyclodextrin-encapsulated E_2 (Sigma-Aldrich) was dissolved in physiological saline to a dose of $5.0 \mu\text{g}/0.5 \mu\text{l}$ and infused at $0.5 \mu\text{l}/\text{min}$ for 1 min/side of the dorsal hippocampus. This dose in young ovariectomized mice infused into the dorsal hippocampus facilitates object memory consolidation (Fernandez et al., 2008). Intracerebroventricular infusions were conducted at the same speed for 2 min. The vehicle, 2-hydroxypropyl- β -cyclodextrin (Sigma-Aldrich), was dissolved in saline to the same concentration of cyclodextrin as in the cyclodextrin- E_2 solution.

To demonstrate that E_2 -enhanced object recognition consolidation was dependent on dorsal hippocampal ERK activation, the MEK inhibitor U0126 [1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene] (Promega), at a concentration of $0.5 \mu\text{g}/0.5 \mu\text{l}$ in 50% dimethylsulfoxide (DMSO) in saline, was infused at a rate of $0.5 \mu\text{l}/\text{min}$ for 1 min/side of dorsal hippocampus, alone or concurrently with intracerebroventricular E_2 infusion. In young ovariectomized mice, intrahippocampal infusion of this dose of U0126 does not block ob-

ject recognition tested 24 h after training (Fernandez et al., 2008). The vehicle control for U0126 was 50% DMSO in saline.

To demonstrate that E_2 -enhanced object recognition consolidation was dependent on dorsal hippocampal PI3K activation, the PI3K inhibitor LY294002 [2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one] (Cell Signaling Technology), which inhibits phosphorylation of the p85 regulatory subunit of PI3K (Geltz and Augustine, 1998), was dissolved in 100% DMSO to $3 \mu\text{g}/\mu\text{l}$ as a stock solution and then serially diluted in physiological saline for infusion of various doses. We first wanted to determine a dose of LY294002 that did not impair object recognition tested 24 h after training. Previous studies in adult rats have shown that posttraining infusions of LY294002 at very low doses (equal to or <0.75 ng/side of hippocampus) do not cause retrograde amnesia in a step-down inhibitory avoidance task (Barros et al., 2001). Therefore, we infused very low doses in our 24 h pilot study; all doses (0.5, 0.005, and $0.0005 \mu\text{g}/\text{side}$ of the hippocampus) of LY294002 were infused at a rate of $0.5 \mu\text{l}/\text{min}$ and a volume of $0.5 \mu\text{l}/\text{side}$. Only a dose that did not impair 24 h object recognition was used in subsequent studies with E_2 . The vehicle control for LY294002 was 50% DMSO in saline.

During drug infusions, mice were gently restrained. 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 $10 \mu\text{l}$ Hamilton syringe. Infusions were controlled by a microinfusion pump (KDS 100; KD Scientific). Infusion cannulae remained in place for 1 min after infusion to prevent diffusion backup the cannula track. For behavioral testing, all mice were infused immediately after object recognition training.

Object recognition. The object recognition task was conducted as described previously (Frick and Gresack, 2003; Fernandez and Frick, 2004) and was used to assess nonspatial hippocampal-dependent memory (Clark et al., 2000; Baker and Kim, 2002). The task consisted of habituation, training, and testing phases conducted on separate days. During habituation, mice were allowed to freely explore an empty white box (60 cm width \times 60 cm length \times 47 cm height) for 5 min. No data were recorded. Twenty-four hours later, mice were rehabituated in the same box for 1 min and then placed in a holding cage while two identical objects were placed in the left and right corners (~ 5 cm from the walls) of the box. Mice were then immediately placed back into the box and allowed to freely investigate until they accumulated a total of 30 s exploring the objects. Exploration was recorded when the front paws or nose contacted either object. Mice were then immediately infused and returned to their home cage (or in some cases, killed 15 min later). After 24 or 48 h, object recognition was tested, using the same procedure as in training except that a novel object was substituted for one of the familiar training objects. Novel object location was counterbalanced across mice. Time spent with each object was recorded. Mice inherently prefer to explore novel objects; thus, a preference for the novel object [more time than chance (15 s) with the novel object] indicates intact memory for the familiar object. The use of 30 s total exploration time rather than a fixed trial duration minimizes confounding influences of group differences in activity. Young ovariectomized mice remember the familiar object after 24 h, but not 48 h (Gresack et al., 2007a), allowing impairing effects of drugs to be observed using the 24 h delay and enhancing effects of drugs to be observed using the 48 h delay. Elapsed time to accumulate 30 s of exploration was recorded to control for group differences in activity levels, but none were observed in any experiment (Table 1).

Western blotting. Mice were decapitated 5 or 15 min after intracranial infusions or 15 min after object recognition training, and the dorsal hippocampus was immediately dissected bilaterally on ice and frozen at -80°C until homogenized. Western blotting was conducted as described previously (Fernandez et al., 2008; Lewis et al., 2008). Briefly, all tissue samples were resuspended 1:50 w/v in lysis buffer and homogenized with a probe sonicator (Branson Sonifier 250). Homogenates were electrophoresed on 10% Tris-HCl gels and blotted to Immobilon-P polyvinylidene fluoride membranes (Millipore Corporation). Western blots were blocked and incubated with either anti-phospho-p44/42 MAPK (1:2000), anti-phospho-PI3K (1:1000), anti-phospho-Akt (1:1000), or total p44/42 MAPK (1:2000), total PI3K (1:1000), total Akt (1:1000) primary antibodies

Table 1. Mean \pm SEM elapsed time during object recognition testing

| Age | Delay | Treatment | Elapsed time (s) |
|-------------|-------|----------------------------------|---------------------|
| Middle-aged | 24 h | Vehicle | 387.95 \pm 71.07 |
| | | IH U0126 | 471.91 \pm 135.11 |
| Middle-aged | 24 h | IH LY294002, 0.0005 μ g | 270.22 \pm 31.47 |
| | | IH LY294002, 0.005 μ g | 291.88 \pm 52.51 |
| | | IH LY294002, 0.5 μ g | 468.25 \pm 64.55 |
| Middle-aged | 48 h | Vehicle | 342.43 \pm 58.42 |
| | | ICV E ₂ | 489.92 \pm 76.78 |
| | | IH E ₂ | 455.91 \pm 110.46 |
| | | ICV E ₂ + IH U0126 | 402.75 \pm 79.37 |
| | | ICV E ₂ + IH LY294002 | 441.78 \pm 67.32 |
| | | IH E ₂ | 297.73 \pm 39.48 |
| Aged | 48 h | Vehicle | 322.61 \pm 29.73 |
| | | ICV E ₂ | 297.73 \pm 39.48 |
| | | IH E ₂ | 294.25 \pm 58.36 |

ICV, Intracerebroventricular; IH, intrahippocampal.

(Cell Signaling Technology) overnight. Blots were then incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Cell Signaling Technology) and developed using West Dura chemiluminescent substrate (Pierce). Blots were then stripped and reprobed with antibodies for total p44/42 MAPK, total PI3K, or total Akt antibodies for phospho-protein normalization or with anti- β -actin antibody (1:5000; Sigma) for total protein normalization. Densitometry was conducted using Kodak 1D 3.6 software (Kodak Scientific Imaging Systems) on the Kodak Image Station 440 CF (Eastman Kodak).

Immunocytochemistry. In middle-aged mice, one side of the dorsal hippocampus was infused with vehicle, and the other side was infused with E₂. Fifteen minutes later, the mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused intracardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were postfixed and cryoprotected, frozen, and stored at -80°C . Before sectioning, a notch was placed along the left cortex to maintain laterality throughout the analyses. Forty micrometer frozen, serial, coronal sections were cut throughout the brain and stored in cryoprotectant for immunocytochemistry on free-floating sections. Every sixth section was collected and immunostained with phospho-p44/42 MAPK antibody as in a previous study (Fan et al., 2008; Tsuda et al., 2008) using Vectastain ABC kit (Vector Laboratories). Briefly, sections were blocked in 3% normal goat serum and incubated in rabbit anti-phospho-p44/42 MAPK antibody (1:1000; Cell Signaling Technology) overnight at room temperature. For control sections, the primary antibody was omitted. Sections were then incubated in biotinylated goat anti-rabbit IgG (1:200) for 1 h at room temperature, washed, and incubated in avidin-biotin solution (Vectastain ABC kit; 1:500; Vector Laboratories) in Tris-buffered saline for 1 h at room temperature. Sections were reacted with 3', 3'-diaminobenzidine solution (Sigma), washed, mounted on gelatin-coated slides, air dried, dehydrated, cleared, and coverslipped.

Statistical analyses. For object recognition data, separate one-sample *t* tests were performed for each group to determine whether the time spent with the novel object differed from 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 object (Gresack and Frick, 2004, 2006). For Western blotting experiments comprising multiple groups, differences between vehicle and treatment groups were evaluated using one-way ANOVA, followed by Fisher's least significant difference (LSD) *post hoc* tests. For Western blotting experiments with only two groups, separate two-tailed unpaired Student's *t* tests were performed between the vehicle and treatment group. For experiments examining the effects of object recognition training on phosphorylated and total protein levels, two-way ANOVA was first used to examine the effects of age and training, followed by Fisher's LSD *post hoc* tests. Next, two-tailed unpaired Student's *t* tests were performed between the training groups within each age to examine effects of training on protein levels within each age. Significance was determined at $p < 0.05$.

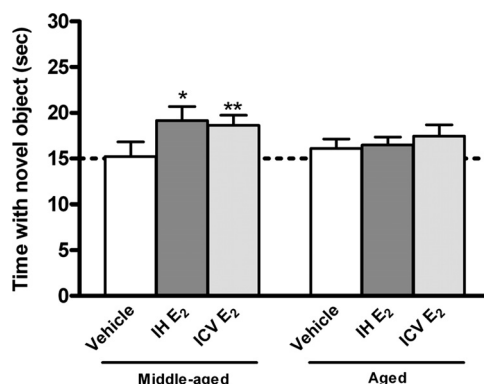


Figure 1. Object recognition in middle-aged and aged female mice. Middle-aged females receiving immediate posttraining intrahippocampal (IH) or intracerebroventricular (ICV) infusions of E₂ (intrahippocampal, 5 μ g/side; intracerebroventricular, 10 μ g total) but not vehicle spent significantly more time with the novel object than chance (dashed line at 15 s) 48 h after training, demonstrating memory for the familiar object. In contrast, aged females receiving vehicle or the same doses of intrahippocampal or intracerebroventricular E₂ spent no more time than chance with the novel object. Each bar represents the mean \pm SEM time spent with the novel object (* $p < 0.05$, ** $p < 0.01$ relative to chance).

Results

Intracranial infusions of estradiol enhance object recognition in middle-aged, but not aged, female mice

We previously demonstrated that a single posttraining intraperitoneal injection of E₂ enhances object recognition in middle-aged, but not aged, ovariectomized mice (Gresack et al., 2007a,b). One possible reason for the lack of effect in aged females is that too little of the systemically injected hormone reached the dorsal hippocampus to be behaviorally effective. Thus, we first sought to determine whether direct intrahippocampal or intracerebroventricular infusions of the same dose of posttraining E₂ that enhances object recognition in young ovariectomized mice (Fernandez et al., 2008) could also enhance object recognition in middle-aged ($n = 8$ –16 per group) and aged ($n = 9$ per group) ovariectomized mice. Immediately after training, mice were infused with vehicle or E₂ into the dorsal hippocampus (5 μ g/side) or dorsal third ventricle (10 μ g total), and retention was tested 48 h later. In middle-aged females (Fig. 1), both intrahippocampal and intracerebroventricular E₂ infusions significantly increased the time spent with the novel object relative to chance (15 s) ($t_{(7)} = 2.72$, $p < 0.05$ and $t_{(15)} = 3.26$, $p < 0.01$, respectively), suggesting that both intrahippocampal and intracerebroventricular E₂ enhanced novel object recognition in middle-aged female mice. Vehicle-treated mice did not exhibit a preference for the novel object ($t_{(9)} = 0.13$, $p > 0.05$). In contrast to the beneficial effects of E₂ in middle-aged females, aged females were unaffected by E₂. Neither the vehicle-treated nor the E₂-treated groups exhibited a preference for novel object 48 h after training ($t_{(8)} = 1.10$ –1.93, $p > 0.05$) (Fig. 1), suggesting that doses of intracranially infused E₂ that enhance object recognition in young and middle-aged females do not affect object memory consolidation in aged females.

Estradiol-induced enhancement of object recognition in middle-aged mice is dependent on dorsal hippocampal ERK activation

To determine whether the beneficial effects of E₂ on object recognition in middle-aged females were dependent on dorsal hippocampal ERK activation, the MEK inhibitor U0126 was bilaterally infused into the dorsal hippocampus concurrently with

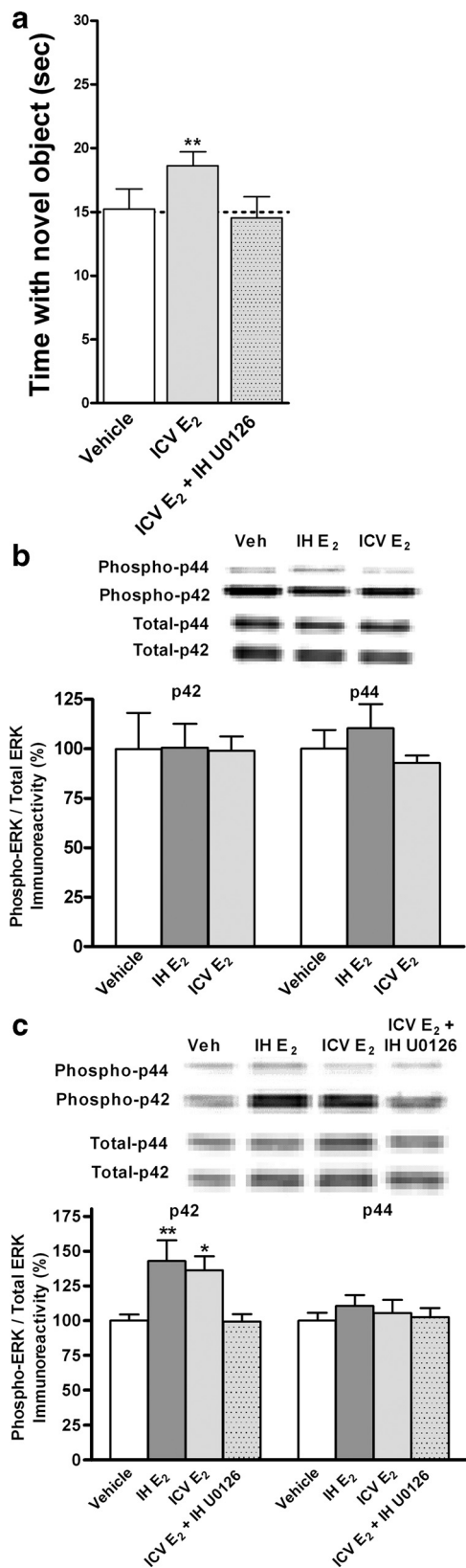


Figure 2. *a*, Intrahippocampal (IH) infusions of U0126 (0.5 μg/side) blocked the beneficial effects of intracerebroventricular (ICV) E₂ (10 μg) (vehicle and intracerebroventricular E₂ alone groups reprinted from Fig. 1). Mice receiving intracerebroventricular E₂ + intrahippocampal U0126 did not spend more time than chance with the novel object. Each bar represents the mean ± SEM time spent with the novel object (***p* < 0.01, relative to chance). *b*, Five minutes after infusion, levels of p42 and p44 ERK phosphorylation in the dorsal hippocampus were not affected by intrahippocampal or intracerebroventricular E₂ infusion.

intracerebroventricular E₂ infusion. To first demonstrate that effects of MEK inhibition on E₂-induced enhancement of 48 h object recognition did not result from general prevention of memory formation in this task, we identified a dose of U0126 that did not interfere with object recognition at a shorter delay. Previously, we showed in young ovariectomized mice that immediate posttraining infusion of U0126 into the dorsal hippocampus, at a dose of 0.5 μg/side, did not impair object recognition tested 24 h after training (Fernandez et al., 2008). Thus, we tested whether this dose of U0126 interfered with 24 h object recognition in middle-aged females. Mice were infused bilaterally into the dorsal hippocampus with vehicle (*n* = 10) or 0.5 μg of U0126 (*n* = 8) immediately after training. Twenty-four hours later, both the vehicle and U0126 groups spent significantly more time with the novel object than chance (19.45 ± 1.27 s for vehicle and 20.52 ± 0.6 s for U0126; *t*₍₉₎ = 3.51, *p* < 0.01; *t*₍₇₎ = 9.23, *p* < 0.001, respectively). These data suggest that 0.5 μg of U0126 does not prevent general memory formation in this task in middle-aged females. Therefore, this dose was used in all subsequent studies.

We then examined whether intrahippocampal infusion of 0.5 μg of U0126 affected the E₂-induced enhancement of object recognition by infusing U0126 bilaterally into the dorsal hippocampus concurrently with intracerebroventricular E₂ infusion. As in our previous work with a membrane-impermeable form of E₂ (Fernandez et al., 2008), E₂ was infused intracerebroventricularly in this study to provide E₂ to the brain while preventing tissue damage from repeated infusions into the dorsal hippocampus. As shown in Figure 2*a*, the intracerebroventricular E₂-induced enhancement in 48 h object recognition was completely blocked by intrahippocampal U0126 (*t*₍₉₎ = -0.27, *p* > 0.05 relative to vehicle), suggesting that E₂-induced facilitation of object recognition in middle-aged female mice is dependent on dorsal hippocampal ERK activation.

Estradiol increases p42 ERK activation in the dorsal hippocampus 15 min, but not 5 min, after infusion in middle-aged females

The fact that U0126 blocked the effects of E₂ on object recognition suggests that E₂ increases ERK activation in the dorsal hippocampus of middle-aged females. Therefore, we next sought to determine whether and when that activation takes place. Previous work showed that p42/p44 ERK activation in the dorsal hippocampus is increased 5 min after intracerebroventricular E₂ infusion in young male rats (Kuroki et al., 2000). Furthermore, we recently demonstrated that p42 ERK activation in the dorsal hippocampus is increased 5 min after dorsal hippocampal infusion of a membrane-impermeable form of E₂ in young ovariectomized mice (Fernandez et al., 2008). As such, we examined whether intrahippocampal or intracerebroventricular E₂ could increase ERK activation in middle-aged females 5 min after infusion. Middle-aged females received intrahippocampal or intracerebroventricular infusions of vehicle (*n* = 8) or E₂ (intrahippocampal, *n* = 7; intracerebroventricular, *n* = 6), and the dorsal hippocampus was collected bilaterally 5 min later for measurement of phos-

c, Fifteen minutes after infusion, levels of p42 ERK, but not p44 ERK, phosphorylation were significantly increased in the dorsal hippocampus by intrahippocampal (5 μg/side) or intracerebroventricular (10 μg) E₂ relative to vehicle controls (**p* < 0.05, ***p* < 0.01). The intracerebroventricular E₂-induced increase was blocked by intrahippocampal U0126 (0.5 μg/side). Phosphorylated p42/p44 protein levels were normalized to total p42/p44 protein levels. Each bar in *b* and *c* represents the mean ± SEM percentage change from vehicle controls. Insets in *b* and *c* are representative Western blots showing phosphorylated and total p42 and p44 ERK protein levels.

phorylated and total p42 and p44 ERK levels using Western blotting. Neither intrahippocampal nor intracerebroventricular E_2 infusion affected phosphorylation of p42 ERK ($F_{(2,18)} = 0.39, p > 0.05$) or p44 ERK ($F_{(2,18)} = 0.77, p > 0.05$) (Fig. 2*b*). This result led us to hypothesize that the temporal dynamics of ERK activation may differ in the young and aging female brain. Evidence for altered ERK activation as a result of aging comes from studies in which p42/p44 ERK activation in the hippocampus and basal forebrain of aged male rats is diminished relative to young males in response to growth factor treatment (Gooney et al., 2004; Williams et al., 2006, 2007). Therefore, we next examined ERK activation at a later time point, 15 min after infusion. To visualize where in the dorsal hippocampus this activation might occur, we infused mice ($n = 4$) on one side of the dorsal hippocampus with vehicle and the other side with $5 \mu\text{g}$ of E_2 , perfused them 15 min later, and stained coronal sections for phospho-p42/p44 ERK immunoreactivity. Immunoreactivity was increased primarily in the hilus of the dentate gyrus and in CA3. In the hilus (Fig. 3*c,d*), E_2 treatment increased phospho-ERK immunostaining in cells and processes relative to vehicle; estrogen receptors α and β (ER α and ER β) have been reported in interneurons, glia, dendrites, axons, and axon terminals of the hilus (Milner et al., 2001, 2005). In CA3 (Fig. 3*e,f*), increased immunolabeling was evident only in processes in the E_2 -treated side compared with vehicle; ER α and/or ER β have been localized to dendrites, dendritic spines, axons, and axon terminals of CA3 (Milner et al., 2001, 2005). Given the observed increase in ERK phosphorylation evident at 15 min immunocytochemically, we then quantified this increase using Western blotting. In addition to mice infused with vehicle and E_2 alone, a fourth group was added that received intracerebroventricular E_2 plus intrahippocampal U0126. The main effect of treatment was significant for p42 ERK phosphorylation ($F_{(3,22)} = 6.33, p < 0.01$) but not p44 ERK phosphorylation ($F_{(3,22)} = 0.39, p > 0.05$) (Fig. 2*c*). Both intrahippocampal E_2 ($n = 6$) and intracerebroventricular E_2 ($n = 6$) infusions significantly increased dorsal hippocampal phosphorylated p42 ERK levels relative to vehicle ($n = 7$; $p < 0.01$ and $p < 0.05$, respectively) (Fig. 2*c*). The intracerebroventricular E_2 -induced increase in p42 ERK phosphorylation was completely blocked by intrahippocampal infusion of U0126 ($n = 7$; $p > 0.05$ relative to vehicle) (Fig. 2*c*).

Estradiol increases PI3K and Akt activation in the dorsal hippocampus 5 min after infusion in middle-aged females

The PI3K/Akt pathway, which can activate ERK (Chen et al., 2005), is also critical for hippocampal synaptic plasticity and memory formation (Kelly and Lynch, 2000; Lin et al., 2001; Horwood et al., 2006) and can be activated *in vitro* within minutes of E_2 treatment (Yokomaku et al., 2003; Mannella and Brinton, 2006). As such, we thought that PI3K and Akt might also play a role in the E_2 -induced enhancement of object recognition. To examine this issue, we first measured the effects of intrahippocampal and

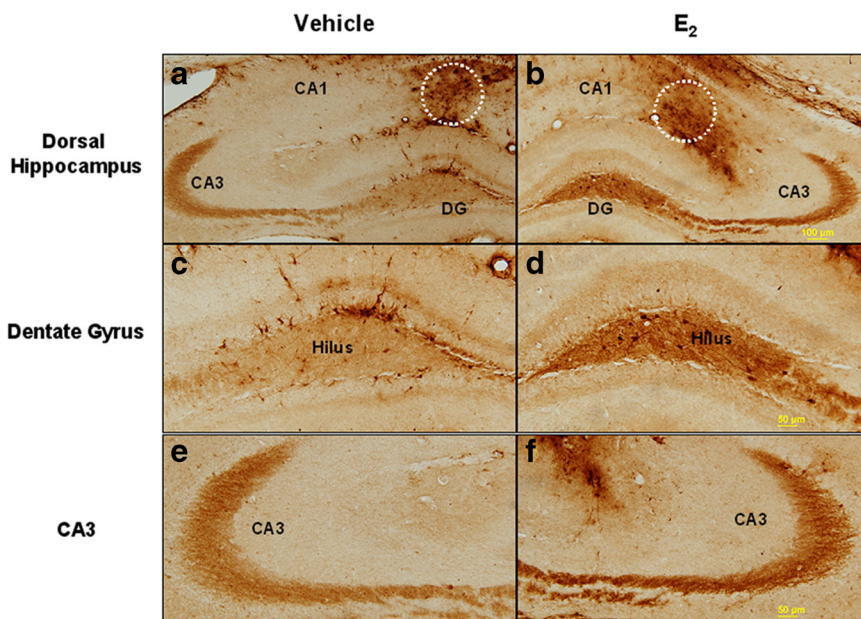


Figure 3. Low- and high-magnification light micrographs of phospho-p42/p44 ERK immunolabeling in the dorsal hippocampus of middle-aged mice. The photos are taken from the same mouse, in which the left side of the hippocampus is treated with vehicle and the right side is treated with $5 \mu\text{g}$ of E_2 . *a* and *b* shows low-magnification light micrographs of phospho-p42/p44 ERK immunoreactivity in the dorsal hippocampus. White-dashed circles are the sites anterior to infusion sites. Phospho-ERK immunoreactivity was increased in the hilus of the dentate gyrus (DG) and in the CA3 region of the E_2 -treated side. The bottom panels show the hilus of the dentate gyrus (*c*, *d*) and CA3 (*e*, *f*) at higher magnification. In the hilus, an increase in immunolabeled phospho-ERK cells and processes is evident in the E_2 -treated side compared with the vehicle-treated side. In the CA3 region, increased immunolabeling in processes, but not cell bodies, is visible in the E_2 -treated side compared with the vehicle-treated side.

intracerebroventricular infusions of E_2 on dorsal hippocampal PI3K and Akt phosphorylation using Western blotting. Middle-aged females received infusion of vehicle (intrahippocampal or intracerebroventricular; $n = 7$), intrahippocampal E_2 ($5 \mu\text{g}/\text{side}$; $n = 7$), or intracerebroventricular E_2 ($10 \mu\text{g}$; $n = 6$), and the dorsal hippocampus was dissected bilaterally 5 min later. Levels of phosphorylated PI3K protein differed significantly among the groups ($F_{(2,17)} = 5.29, p < 0.05$), such that both intrahippocampal and intracerebroventricular E_2 significantly increased PI3K phosphorylation relative to vehicle ($p < 0.05$) (Fig. 4*a*). Phosphorylated Akt protein levels also differed significantly among the groups ($F_{(2,16)} = 15.68, p < 0.001$), and, again, both intrahippocampal and intracerebroventricular E_2 infusions significantly increased Akt phosphorylation relative to vehicle ($p < 0.001$ and $p < 0.05$, respectively) (Fig. 4*b*). To determine whether the failure of E_2 to enhance object recognition in aged females might be attributable to an inability of E_2 to activate cell signaling pathways, aged females were infused with vehicle ($n = 6$) or E_2 ($n = 5$) into the dorsal hippocampus, and phosphorylation of PI3K and Akt in the dorsal hippocampus was measured 5 min later. Unlike in middle-aged females, E_2 in aged females did not increase phosphorylated PI3K (Fig. 4*e*) or Akt (Fig. 4*f*) levels relative to vehicle ($t_{(9)} = 0.35, p > 0.05$ and $t_{(9)} = 0.27, p > 0.05$, respectively).

Estradiol-induced enhancement of object recognition in middle-aged females is dependent on dorsal hippocampal PI3K activation

We next wanted to determine whether dorsal hippocampal PI3K activation was necessary for the beneficial effects of E_2 on object recognition in middle-aged females, and, therefore, we con-

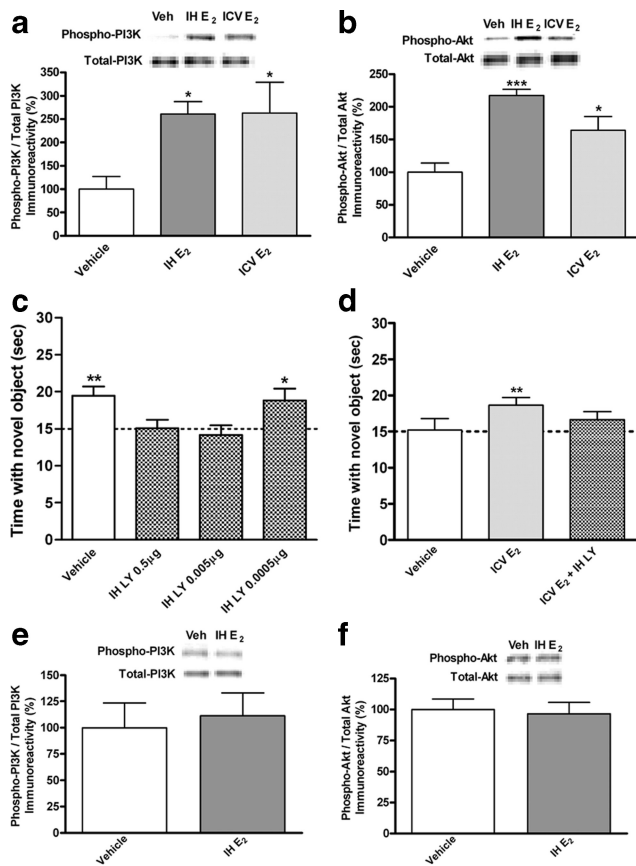


Figure 4. *a*, PI3K protein phosphorylation in the dorsal hippocampus of middle-aged mice significantly differed among the groups 5 min after infusion. Infusion of E₂ into either the dorsal hippocampus [intrahippocampal (IH), 5 μg/side] or dorsal third ventricle [intracerebroventricular (ICV), 10 μg] significantly increased phosphorylated PI3K protein levels relative to vehicle (Veh) (**p* < 0.05). Inset, Representative Western blots showing phosphorylated and total PI3K protein levels. *b*, Akt phosphorylation in the dorsal hippocampus of middle-aged mice significantly differed among the groups 5 min after infusion. Infusion of E₂ into either the dorsal hippocampus (5 μg/side) or dorsal third ventricle (10 μg) significantly increased phosphorylated Akt protein levels relative to vehicle (**p* < 0.05, ****p* < 0.001). Inset, Representative Western blots showing phosphorylated and total Akt protein levels. For *a* and *b*, each bar represents mean ± SEM percentage change from vehicle controls. *c*, Object recognition tested 24 h after training. Middle-aged mice receiving intrahippocampal vehicle or 0.0005 μg of LY294002 (LY) per side but not those receiving 0.005 or 0.5 μg of LY294002 per side spent significantly more time than chance with novel object, demonstrating that the 0.0005 μg/side dose of LY294002 did not impair object memory at this delay. *d*, Object recognition tested 48 h after training. Intrahippocampal infusions of LY294002 (0.0005 μg/side) blocked the beneficial effects of intracerebroventricular E₂ (10 μg) (vehicle and intracerebroventricular E₂ alone groups reprinted from Fig. 1). Mice receiving intracerebroventricular E₂ plus intrahippocampal LY294002 did not spend more time than chance with the novel object. For *c* and *d*, each bar represents the mean ± SEM time spent with the novel object (**p* < 0.05, ***p* < 0.01 relative to chance). *e*, *f*, Western blotting data for PI3K and Akt in aged female mice. Bilateral dorsal hippocampi were collected 5 min after dorsal hippocampal infusion of vehicle (*n* = 6) or E₂ (5 μg/side; *n* = 5). E₂ did not affect phosphorylated PI3K (*e*) or Akt (*f*) protein levels. For *e* and *f*, each bar represents the mean ± SEM percentage change from vehicle controls. Insets are representative Western blots for phosphorylated and total protein levels.

ducted several studies with LY294002, an inhibitor of PI3K activation. We first demonstrated that the effects of PI3K inhibition on E₂-induced enhancement object recognition did not result from general prevention of object memory formation, by identifying doses of LY294002 that did not interfere with object recognition after a 24 h delay. Immediately after training, middle-aged females received intrahippocampal infusions of vehicle (*n* = 10) or one of three doses of LY294002: 0.5 μg/side (*n* = 9), 0.005

μg/side (*n* = 6), or 0.0005 μg/side (*n* = 8). Twenty-four hours later, mice receiving vehicle or 0.0005 μg of LY294002 exhibited a significant preference for the novel object relative to chance ($t_{(9)} = 3.51$, *p* < 0.01 and $t_{(7)} = 2.38$, *p* < 0.05, respectively), whereas mice receiving 0.5 or 0.005 μg of LY294002 did not ($t_{(8)} = 0.07$, *p* > 0.05 and $t_{(5)} = -0.65$, *p* > 0.05, respectively) (Fig. 4*c*). Because these data suggest that 0.0005 μg of LY294002 did not prevent general object memory formation, the 0.0005 μg dose was used for all subsequent studies.

We then infused mice intrahippocampally with LY294002 (0.0005 μg/side) and intracerebroventricularly with E₂ (10 μg; *n* = 10) immediately after training and tested memory retention 48 h later. As shown in Figure 4*d*, the intracerebroventricular E₂-induced memory enhancement was completely blocked by intrahippocampal LY294002 ($t_{(9)} = 1.49$, *p* > 0.05 relative to chance), suggesting that the beneficial effects of E₂ on object recognition are dependent on dorsal hippocampal PI3K activation in middle-aged females.

Estradiol-induced dorsal hippocampal ERK activation is dependent on PI3K activation in middle-aged females

Our data indicate that both ERK and PI3K activities are required for E₂ to enhance object recognition in middle-aged females, and previous studies have shown that PI3K can activate ERK either directly or indirectly (Lin et al., 2001; Perkinson et al., 2002; Chang et al., 2003; Chen et al., 2005). Therefore, we hypothesized that E₂ might first activate the PI3K/Akt pathway, which could then activate ERK and lead to memory facilitation. To this end, we examined the effect of PI3K inhibition on ERK activation in the dorsal hippocampus 15 min after intracerebroventricular E₂ infusion. Middle-aged mice received intrahippocampal or intracerebroventricular vehicle (*n* = 7), intracerebroventricular E₂ at 10 μg (*n* = 6), or intracerebroventricular E₂ at 10 μg plus intrahippocampal LY294002 at 0.0005 μg (*n* = 8), and the dorsal hippocampus was dissected bilaterally 15 min later. Phosphorylation of p42 ERK differed among the groups ($F_{(2,18)} = 5.07$, *p* < 0.05), but p44 ERK phosphorylation ($F_{(2,18)} = 0.21$, *p* > 0.05) did not (Fig. 5*a*). The intracerebroventricular E₂-induced increase in p42 ERK phosphorylation (*p* < 0.01 relative to vehicle) was completely blocked by intrahippocampal infusions of LY294002, suggesting that PI3K activation is upstream from ERK.

To further demonstrate that E₂-induced increases in PI3K and Akt are upstream from ERK activation, we next examined PI3K and Akt phosphorylation 15 min after infusion with vehicle (intrahippocampal or intracerebroventricular, *n* = 6), intracerebroventricular E₂ at 10 μg (*n* = 6), intracerebroventricular E₂ at 10 μg plus intrahippocampal U0126 at 0.5 μg/side (*n* = 7), or intracerebroventricular E₂ at 10 μg plus intrahippocampal LY294002 at 0.0005 μg/side (*n* = 8). Phosphorylated PI3K levels differed significantly among the groups ($F_{(3,26)} = 3.00$, *p* < 0.05) (Fig. 5*b*); intracerebroventricular E₂ treatment significantly increased PI3K phosphorylation relative to vehicle (*p* < 0.05), and this effect was completely blocked by intrahippocampal infusion of LY294002. However, the increase produced by intracerebroventricular E₂ was not blocked by U0126 (*p* > 0.05 relative to vehicle) (Fig. 5*b*), suggesting that E₂-induced PI3K activation is not dependent on ERK activation. Phosphorylated Akt levels did not significantly differ among the groups 15 min after infusion (data not shown), suggesting that the E₂-induced increase in Akt phosphorylation seen 5 min after infusion is only transient.

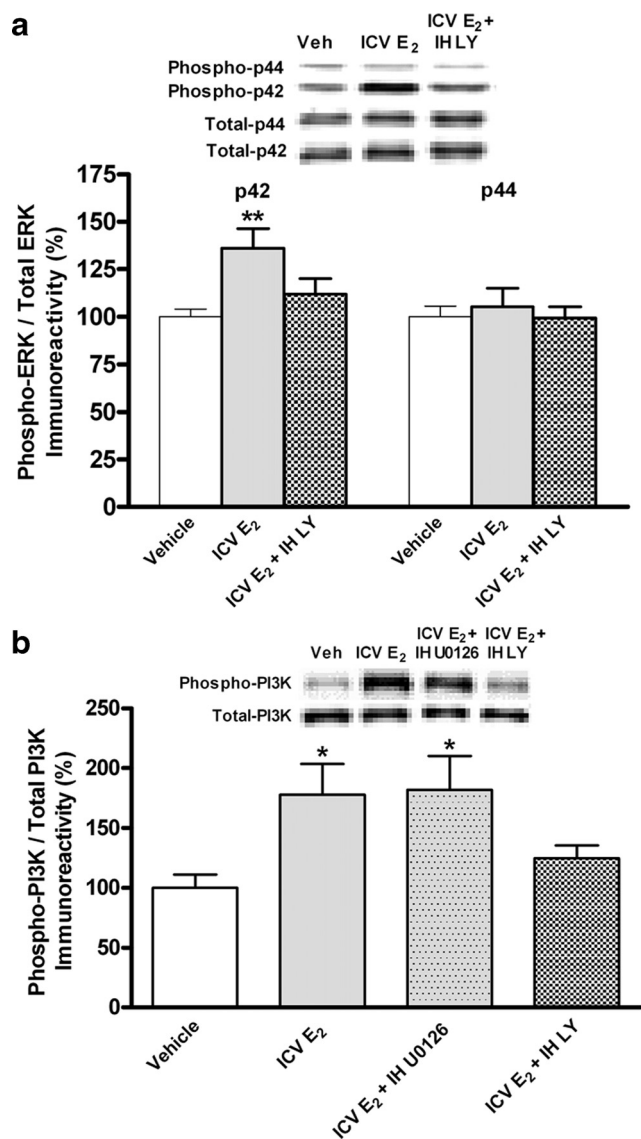


Figure 5. *a*, Dorsal hippocampal levels of phosphorylated p42 ERK, but not p44 ERK, were significantly increased by intracerebroventricular (ICV) E₂ (10 μg) in middle-aged mice 15 min after infusion (** $p < 0.01$ relative to vehicle). This effect was blocked by intrahippocampal (IH) infusion of 0.0005 μg of LY294002 (LY). Inset, Representative Western blots showing phosphorylated and total p42 and p44 ERK protein levels. *b*, Dorsal hippocampal levels of phosphorylated PI3K were significantly increased by intracerebroventricular E₂ (10 μg) in middle-aged mice 15 min after infusion (* $p < 0.05$ relative to vehicle). This increase was blocked by intrahippocampal infusion of 0.0005 μg of LY294002 ($p < 0.05$ relative to intracerebroventricular E₂) but not intrahippocampal infusion of 0.5 μg of U0126 (* $p < 0.05$ relative to vehicle). Inset, Representative Western blots showing phosphorylated and total PI3K protein levels. Each bar represents mean \pm SEM percentage change from vehicle controls.

Object recognition training does not differentially affect dorsal hippocampal ERK, PI3K, or Akt in ovariectomized young and aging female mice

Age differences in the ability of E₂ to enhance object recognition and activate the ERK/MAPK and PI3K/Akt signaling pathways may be attributable to age-related decline in the function of these signaling pathways or of the ability of object recognition training to stimulate these pathways, regardless of E₂ treatment. To demonstrate whether object recognition training differentially affects ERK, PI3K, and Akt levels across ages, we examined dorsal hippocampal total and phospho-ERK, -PI3K, and -Akt levels 15 min after object recognition training in young, middle-aged, and aged

ovariectomized mice ($n = 7$ or 8 per group). Mice were habituated to the object recognition apparatus and then, 24 h later, were placed in the apparatus with no objects or with two identical objects. Mice with objects were allowed to accumulate 30 s with the objects as in our standard training protocol. Control mice without objects were yoked to spend the same amount of time in the apparatus as mice that explored objects. Dorsal hippocampi were collected bilaterally 15 min after training based on data from Figures 2*c* and 5*b* showing that E₂ significantly increased ERK and PI3K phosphorylation in middle-aged females at this time point. The main effect of age was significant for phosphorylated p42 ERK levels ($F_{(2,40)} = 3.978, p < 0.05$) (Fig. 6*a*), and *post hoc* tests indicated that levels were significantly reduced in aged females relative to young and middle-aged females ($p < 0.05$). However, the main effect of training and age \times training interaction were not significant, suggesting no effect at any age of object training on p42 ERK phosphorylation measured after 15 min. Neither the main effects of age and training nor the age \times training interactions were significant for phosphorylated p44 ERK, PI3K, or Akt (Fig. 6*b–d*), suggesting no effect of age or training on activation of these proteins 15 min after training. To determine whether total protein levels were affected by age or training, we also examined total ERK, PI3K, and Akt protein levels normalized to β -actin in the same mice. Two-way ANOVA showed no significant main effects of age and training, or interactions, for total p42, p44, PI3K, or Akt levels (Fig. 7*a–d*). Additional two-tailed unpaired Student's *t* tests performed between the training groups within each age (data normalized to the age-matched control group mean) also showed no significant differences in levels of all phosphorylated or total proteins, suggesting no effects within each age of training on protein levels measured after 15 min.

Discussion

This report provides the first demonstration that intracranial E₂ infusion enhances object memory consolidation in middle-aged female mice and that this effect is dependent on both PI3K and ERK activation, with an early activation of PI3K/Akt observed 5 min after infusion and a later activation of ERK 15 min after infusion. Furthermore, studies with PI3K and ERK inhibitors suggest that E₂ first activates PI3K, which then activates ERK, illustrating a potential directionality of cell signaling that leads to enhanced object recognition. The involvement of PI3K signaling in estrogenic modulation of object recognition memory has not been demonstrated previously at any age, so these data also provide novel insights into the molecular mechanisms underlying these effects beyond those demonstrated previously in young females. Of importance to aging in particular, the data suggest that E₂ modulates object recognition via similar signaling mechanisms in young and middle-aged females and that these mechanisms remain functional in middle-aged, but not aged, females. Although we would expect similar cell signaling mechanisms to be involved in E₂-induced enhancement of other types of hippocampal memory, e.g., spatial memory, in which posttraining E₂ also enhances memory consolidation in females (Packard and Teather, 1997*a,b*; Gresack and Frick, 2006), this generalization has yet to be tested systematically at any age.

The fact that intrahippocampal and intracerebroventricular E₂ infusion enhanced object recognition in middle-aged females is consistent with effects of systemic posttraining E₂ injections on object recognition at this age (Gresack et al., 2007*a*). These data are also consistent with findings from young female mice (Fernandez et al., 2008) that dorsal hippocampal ERK activation is necessary for E₂ to enhance object recognition, as demon-

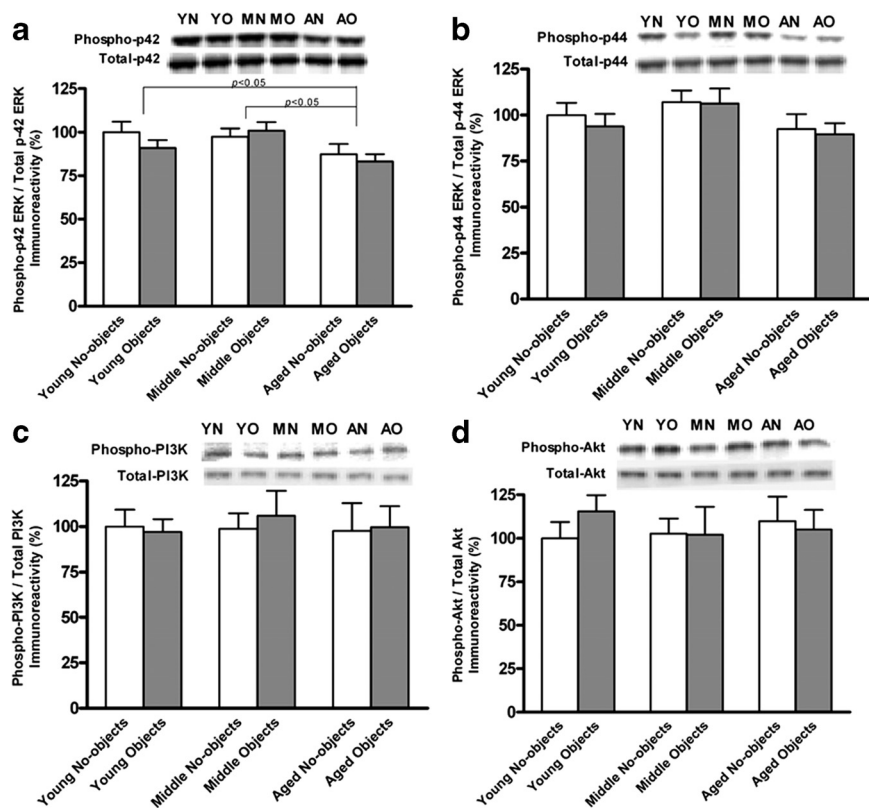


Figure 6. Western blotting data for phosphorylated ERK, PI3K, and Akt levels in young, middle-aged, and aged female mice after object recognition training. Dorsal hippocampi were collected bilaterally 15 min after training with objects or without objects (exploration times yoked to time for mice with objects). Each bar represents the mean \pm SEM percentage change from young no-objects controls. Insets are representative Western blots for phosphorylated and total protein levels. **a**, For p42 ERK, the main effect of age was significant, such that phosphorylation levels were lower in aged females relative to young and middle-aged females ($p < 0.05$ for both comparisons). However, training had no effect on phosphorylated p42 ERK levels measured after 15 min. **b–d**, Neither age nor training significantly affected p44, PI3K, or Akt phosphorylation. Two-tailed unpaired Student's *t* tests performed between the “no-objects” and “objects” groups within each age also showed no significant differences in levels of all phosphorylated proteins, suggesting no effects within each age of training on protein phosphorylation after 15 min.

strated by the fact that intrahippocampal U0126 blocked the beneficial effects of intracerebroventricular E_2 on object recognition. Interestingly, however, the time at which E_2 increased phosphorylated p42 ERK levels differed in the two ages, such that levels were increased 5 min after infusion in young females (Kuroki et al., 2000; Fernandez et al., 2008) and 15 min after infusion in middle-aged females. This delay in the temporal dynamics of E_2 -induced ERK activation in the dorsal hippocampus of middle-aged females may result from reduced estrogen receptor expression in the hippocampus. Hippocampal p42 ERK is activated *in vitro* by $ER\alpha$ and $ER\beta$ agonists (Zhao and Brinton, 2007) and *in vivo* by a form of E_2 that binds to membrane-bound ERs (Fernandez et al., 2008). Hippocampal $ER\beta$ expression is decreased by 12 months of age in female rats (Yamaguchi-Shima and Yuri, 2007), and age-related alterations in the expression or function of any ER could lengthen the time necessary for E_2 to increase p42 ERK phosphorylation. The delayed ERK activation in middle-aged females also could be attributable to age-related alterations in NMDA receptors, which, in young female mice, activate ERK and are involved in the E_2 -induced enhancement of object recognition (Lewis et al., 2008). In aged female rats, E_2 affects the synaptic distribution of NR2B subunits (Adams et al., 2004), which could influence downstream signaling activation. Interestingly, the magnitude of ERK activation induced by intracranial E_2 in middle-aged females ($\sim 50\%$ over vehi-

cle) is similar to that seen in young females (our unpublished observations), suggesting that, although the ERK response is delayed in middle-aged mice, E_2 can produce a similar degree of p42 ERK phosphorylation in young and middle-aged females.

This study provides the first evidence that PI3K activation is necessary for E_2 to enhance memory at any age. This conclusion is supported by the fact that the beneficial effects of E_2 on object recognition were blocked by intrahippocampal infusion of the PI3K inhibitor LY294002 and is consistent with literature demonstrating that hippocampal PI3K is required for several types of memory (Barros et al., 2001; Chen et al., 2005). The PI3K/Akt pathway is rapidly activated by E_2 in cultured hippocampal and neocortical neurons (Singh, 2001; Yokomaku et al., 2003; Mannella and Brinton, 2006), but this report is the first to our knowledge showing that E_2 can increase phosphorylation of PI3K itself. Phosphorylation of Akt is typically PI3K dependent and plays an important role in E_2 -induced neuroprotection (Wilson et al., 2002). E_2 increases hippocampal Akt phosphorylation *in vitro* (Akama and McEwen, 2003; Yokomaku et al., 2003), and phosphorylated Akt is increased during the high-estradiol phase of the rodent estrous cycle (Znamensky et al., 2003; Spencer et al., 2008). Interestingly, Akt in the present study was only activated 5 min after E_2 infusion, whereas PI3K was activated 5 and 15 min after infusion, suggesting that cell

survival may not be the primary outcome of E_2 treatment in our mouse model compared with ischemic models in which Akt phosphorylation can last 2 h after injury (Wilson et al., 2002). It should be noted that systemically injected progesterone can rapidly increase PI3K expression and phosphorylation of ERK and Akt in the hippocampus (Nilsen and Brinton, 2002, 2003; Guerra-Araiza et al., 2009), and so examining the effects of intrahippocampal infusions of E_2 plus progesterone on ERK, PI3K, and Akt activation should be addressed in future studies.

The fact that E_2 activated PI3K and Akt earlier (5 min) than ERK (15 min) suggests that E_2 activates PI3K and Akt upstream from ERK in middle-aged females. This conclusion is supported by the fact that intrahippocampal infusion of LY294002 blocked the E_2 -induced activation of both PI3K and ERK, whereas intrahippocampal infusion of U0126 blocked only ERK activation. Additional support comes from studies in embryonic neocortical cell culture, in which an interaction between $ER\alpha$ and the p85 regulatory subunit of PI3K leads to activation of Akt and ERK within the same neurons (Mannella and Brinton, 2006). Other studies in young adult rodents report that PI3K activates ERK directly or indirectly in the hippocampus, amygdala, and striatum (Lin et al., 2001; Perkinson et al., 2002; Chen et al., 2005), so a similar effect to that seen here would be expected in the hippocampus of young female mice.

Collectively, the present data suggest a model of the cell signaling mechanisms in the dorsal hippocampus underlying the beneficial effects of E_2 on object recognition memory. E_2 , binding to membrane or nuclear ERs activates PI3K, which then activates ERK directly or indirectly through Akt. ERK activation then leads to altered gene transcription and protein translation, consistent with previous work showing that ERK activation promotes both processes (Adams and Sweatt, 2002; Kelleher et al., 2004). Altered gene transcription and protein translation then lead to enhanced memory, possibly through multiple routes such as spino-genesis, neurogenesis, and increased growth factor levels (Woolley and McEwen, 1992; Miranda et al., 1999; Tanapat et al., 1999; Bimonte-Nelson et al., 2004; Fernandez and Frick, 2004). In support of this notion, both PI3K/Akt and ERK signaling have been shown to regulate hippocampal dendritic morphology through the mammalian target of rapamycin translational pathway (Kumar et al., 2005). Although other signaling pathways may also be involved, this model provides a framework for future investigations of E_2 -regulated memory modulation.

Another important finding from this study is that intracranial E_2 infusion enhanced object recognition memory in middle-aged, but not aged, female mice, which is consistent with findings from other studies that have compared effects of E_2 on hippocampal memory in middle-aged and aged female rodents (Savonenko and Markowska, 2003; Gresack et al., 2007a; Talboom et al., 2008). The present and previous studies support the critical period hypothesis, which maintains that E_2 benefits cognition only in recently menopausal women (Maki, 2006; Sherwin and Henry, 2008). The decreased responsiveness of the aged female hippocampus to E_2 may result from age-related reductions in hippocampal ER immunoreactivity and mRNA levels (Adams et al., 2002; Mehra et al., 2005; Yamaguchi-Shima and Yuri, 2007) and/or, as demonstrated in this report, by impairments in cell signaling. We show here that dorsal hippocampal ERK and PI3K activation are necessary for E_2 to enhance object recognition in middle-aged females, and, thus, the failure of a single posttraining E_2 injection to enhance object recognition in aged females may result from the inability of E_2 to activate PI3K (Fig. 4e) or of age-related reductions in phospho-p42 ERK protein (Fig. 6a). However, it should be noted that aged female mice are not completely unresponsive to E_2 , because E_2 can enhance various forms of hippocampal memory in aged rodents (Miller et al., 1999; Frick et al., 2002; Heikkinen et al., 2002; Vaucher et al., 2002; Foster et al., 2003), although the response of aged females to E_2 is influenced by numerous factors, including timing of treatment relative to testing (e.g., before and after training), priming (Savonenko and Markowska, 2003), duration of treatment, and dose (for review, see Frick, 2009). In particular, timing and treatment duration may be important factors for object recognition, because chronic pre-training E_2 treatment improves object recognition in aged female

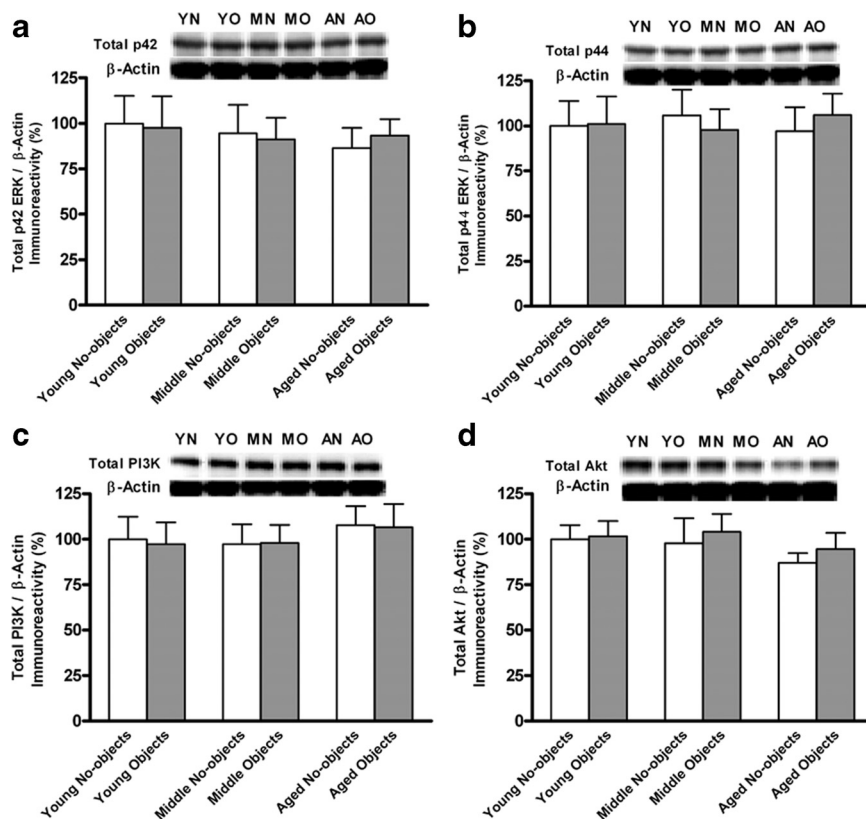


Figure 7. Western blotting data for total ERK, PI3K, and Akt in the young, middle-aged, and aged female mice shown in Figure 6. Each bar represents the mean \pm SEM percentage change from young no-objects controls. Insets are representative Western blots for total protein levels and β -actin levels. **a–d**, Neither age nor treatment affected total p42, p44, PI3K, or Akt levels. Two-tailed unpaired Student's *t* tests performed between the “no-objects” and “objects” groups within each age also showed no significant differences of training in levels of all four proteins after 15 min.

mice (Vaucher et al., 2002). Dose may also be an issue here, since the doses used in this study may have been too low to enhance memory in aged females. Although this explanation is less likely given that both systemic (Gresack et al., 2007a,b) and intracranial (present study) E_2 treatments fail to enhance object recognition in aged female mice, testing higher intracranial doses should help resolve this issue.

Finally, the data also indicate that, in the absence of E_2 , object recognition training does not activate the ERK/MAPK and PI3K/Akt pathways at any age after 15 min. This finding might suggest that object training does not activate these pathways and that the E_2 -induced enhancement of object recognition is mediated by a different set of cellular mechanisms than those that normally take place during object memory consolidation. Although this possibility cannot be conclusively discounted at present, it is not supported by previous studies in young mice showing that ERK phosphorylation in whole hippocampus is increased 10 min (Wang et al., 2004) or 30 min (Goeldner et al., 2008) after object recognition training. Moreover, our own data from young (Fernandez et al., 2008) and middle-aged (present study) vehicle-treated females shows that preventing ERK or PI3K activation in the absence of E_2 blocks 24 h object recognition. Together, these studies indicate that object recognition training activates ERK and that preventing this activation blocks learning. However, ERK and PI3K may be activated more slowly in middle-aged females, and, thus, the 15 min time point may have been too soon to observe E_2 -induced increases in these enzymes. Alternatively, ERK and PI3K may be activated more slowly in the

absence of E₂, regardless of age, with the more rapid E₂-induced activation leading to longer-lasting memory (i.e., 48 h rather than 24 h). Measuring protein levels at times longer than 15 min would help demonstrate the time course of training-induced activation of these signaling pathways.

In conclusion, the present study demonstrates that the E₂-induced enhancement of object recognition in middle-aged female mice is dependent on PI3K and ERK activation in the dorsal hippocampus. These data provide the first evidence that hippocampal cell signaling molecules are critically necessary for estrogenic enhancement of object memory consolidation in middle-aged females. Interestingly, middle-aged females are responsive to the same doses of E₂ as young females, and the signaling pathways that mediate effects of E₂ on object recognition are similar in young and middle-aged females, suggesting that the mechanisms through which E₂ modulates object recognition in females remain essentially intact during middle age. The failure of E₂ to enhance object recognition in aged females may be attributable to its inability to activate dorsal hippocampal PI3K and ERK. Together, the present data provide important new insights into the molecular mechanisms underlying estrogenic modulation of memory in aging females that could be pivotal to the design of future hormone therapies.

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