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The progesterone-induced enhancement of object recognition memory consolidation involves activation of the extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin (mTOR) pathways in the dorsal hippocampus

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ABSTRACT

Although much recent work has elucidated the biochemical mechanisms underlying the modulation of memory by 17β-estradiol, little is known about the signaling events through which progesterone (P) regulates memory. We recently demonstrated that immediate post-training infusion of P into the dorsal hippocampus enhances object recognition memory consolidation in young ovariectomized female mice (Orr et al., 2009). The goal of the present study was to identify the biochemical alterations that might underlie this mnemonic enhancement. We hypothesized that the P-induced enhancement of object recognition would be dependent on activation of the ERK and mTOR pathways. In young ovariectomized mice, we found that bilateral dorsal hippocampal infusion of P significantly increased levels of phospho-p42 ERK and the mTOR substrate S6K in the dorsal hippocampus 5 min after infusion. Phospho-p42 ERK levels were downregulated 15 min after infusion and returned to baseline 30 min after infusion, suggesting a biphasic effect of P on ERK activation. Dorsal hippocampal ERK and mTOR activation were necessary for P to facilitate memory consolidation, as suggested by the fact that inhibitors of both pathways infused into the dorsal hippocampus immediately after training blocked the P-induced enhancement of object recognition. Collectively, these data provide the first demonstration that the ability of P to enhance memory consolidation depends on the rapid activation of cell signaling and protein synthesis pathways in the dorsal hippocampus.

Introduction

The roles of ovarian sex steroid hormones in mediating cognitive function have recently been the subject of intense study. The attention given to the mnemonic properties of sex steroids is due partly to the increased risk of dementia that accompanies menopause and the controversy surrounding the effects of ovarian hormone therapy on cognition in menopausal women (Craig et al., 2005; Sherwin, 2003; Sherwin and Henry, 2008). In particular, the effects of estrogens, such as 17β-estradiol (E_2), in cognitive regions of the brain like the hippocampus and prefrontal cortex have been studied extensively in recent years (see Daniel, 2006; Dumitriu et al., 2010; Frick, 2009; Sherwin and Henry, 2008; Woolley, 2007 for reviews). In contrast, the effects of progesterone (P) on memory and neural function, alone or in combination with estrogens, have been studied infrequently, despite the fact that these hormones can differentially affect brain regions such as the hippocampus. For example, rats treated with E_2 exhibited increased hippocampal dendritic spine density that peaked 2–3 days following treatment, whereas mice treated with P showed an initial peak in spine density 2–6 h following treatment and a subsequent decrease in spines 18 h after treatment (Woolley and McEwen, 1993). Studies that have examined the effects of exogenous P on memory, independent of E_2, suggest that the timing of P administration relative to training and testing is important in determining the effects of P on memory. For example, P administered chronically prior to training impairs footshock avoidance learning and spatial working memory in young ovariectomized mice and rats (Bimonte-Nelson et al., 2004; Farr et al., 1995), whereas acute pre-training P administration has no effect on spatial memory in the Morris water maze (Chesler and Juraska, 2000) or radial arm maze (Sato et al., 2004) in young ovariectomized rats. In contrast, a single intraperitoneal (i.p.) injection of P immediately after training (i.e., post-training) improves memory in Y-maze, inhibitory avoidance, and object recognition tasks in young ovariectomized rats (Frye and

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Lacey, 2000; Walf et al., 2006) and mice (Harburger et al., 2008). Acute post-training i.p. injection of a rapidly metabolized, water-soluble, form of P immediately after spatial water maze or object recognition training also enhances memory consolidation in aging female mice (Lewis et al., 2008b). The discrepancy between the effects of pre- and post-training P administration may result from methodological differences among the studies and/or the demonstrated anxiolytic and analgesic effects of P (Bitran et al., 1991a, b; Frye and Duncan, 1994). These latter effects are minimized by the use of water-soluble P and the post-training approach because this form of P is metabolized with hours (Pitha et al., 1986) and, therefore, is not in circulation during training or testing in this experimental design.

If P can enhance memory, as suggested by post-training studies, then what neural mechanisms may underlie this effect? One recent study of young ovariectomized female mice suggests that the dorsal hippocampus is crucial to the memory-enhancing effects of P (Orr et al., 2009). In this study, bilateral infusion of any of three doses of P (0.01, 0.1, or 1 μg) into the dorsal hippocampus immediately after object recognition training significantly enhanced object recognition tested 48 h later (Orr et al., 2009). Such enhancement was not observed when P was administered 2 h after training, suggesting a relatively narrow time window during which P exerts its modulatory effects. These effects may be linked to rapid regulation of synaptic plasticity, given the relatively narrow time frame in which the biphasic effects of exogenous P on hippocampal CA1 spine synapse density are observed (Woolley and McEwen, 1993). The extracellular signal-regulated kinase/mitogen activated kinase (ERK/MAPK) pathway is an excellent candidate to mediate at least some of the mnemonic and synaptogenic effects of P because of its central importance to cell signaling, gene transcription, and learning and memory processes (Adams and Sweatt, 2002). ERK/MAPK serves as a common final pathway for multiple signaling cascades such as phosphatidylinositol 3-kinase/Akt (PI3K/Akt) (Chen et al., 2005; Fan et al., 2010a) and protein kinase C (English and Sweatt, 1996), and acts as a mediator between these upstream activators and downstream effectors such as cAMP response element binding protein (CREB), which initiates gene transcription (Sweatt, 2001). Activation of the ERK/MAPK pathway is critical to late-phase long-term potentiation (LTP) in the hippocampus (Rosenblum et al., 2002), and is, consequently, necessary for long-term hippocampal memory as measured by several tasks, including object recognition (Fernandez et al., 2008) and contextual fear conditioning (Atkins et al., 1998b; Selcher et al., 1999). Moreover, ERK activation (i.e., phosphorylation) in the dorsal hippocampus is required for E_2 to enhance object recognition in young and middle-aged female mice (Fan et al., 2010a; Fernandez et al., 2008). This finding demonstrates a pivotal role of ERK in mediating the mnemonic effects of E_2 and raises the possibility that ERK activation is also involved in the mnemonic effects of P.

Indeed, some evidence indicates that P can increase ERK phosphorylation in the hippocampus. For example, in cultured neocortical and hippocampal neurons, P increases phosphorylation of the p42 isoform of ERK and induces the nuclear translocation of phosphorylated ERK (Nilsen and Brinton, 2002, 2003). This activation can be blocked with P receptor antagonists (Migliaccio et al., 1998). Further, the ability of P to promote the proliferation of neural progenitor cells and the survival of newborn neurons in the dentate gyrus is also dependent on ERK activation (Liu et al., 2009; Zhang et al., 2010). The only study to examine whether P affects ERK in vivo reported that a single intraperitoneal injection of P increased p42 and p44 ERK phosphorylation in the rat hippocampus after 24 h (Guerra-Araiza et al., 2009). Collectively, these findings support the conclusion that P activates hippocampal ERK. However, in vivo activation cannot yet be localized directly to the hippocampus because the effects of systemically administered P in other brain regions could have influenced hippocampal activity. Further, it is unknown whether P affects ERK biphasically, as it does CA1 dendritic spines, because previous in vivo work examined only a single time point. The present study addressed these issues of localization and timing by infusing P directly into the dorsal hippocampus and examining dorsal hippocampal ERK activation at multiple time points after infusion.

The rapid effects of P on CA1 dendritic spines suggest that P may alter protein synthesis very shortly after exposure. Long-term memory consolidation requires protein synthesis (Klann and Sweatt, 2008), and recent findings demonstrate that the ERK/MAPK pathway is critically involved in facilitating protein synthesis. ERK activates the mammalian target of rapamycin (mTOR) protein synthesis pathway (Kelleher et al., 2004; Mendoza et al., 2011; Tsokas et al., 2005), a pathway that regulates protein translation through downstream substrates such as ribosomal S6 kinase (S6K) (Hay and Sonenberg, 2004; Horwood et al., 2006). Protein synthesis mediated by the mTOR pathway is critical for the formation of late-phase LTP in the hippocampus, as observed both in hippocampal slices (Cammalleri et al., 2003) and in vivo (Sui et al., 2008). The importance of mTOR signaling in long-term hippocampal memory formation is highlighted by studies in male rats demonstrating that intrahippocampal infusion of the mTOR inhibitor rapamycin 15 min prior to training prevented expression of long-term, but not short-term, memory in a hippocampal-dependent inhibitory avoidance paradigm (Bekinschtein et al., 2007). Inhibitory avoidance alone increases phosphorylation of both mTOR and its downstream effector S6K (Bekinschtein et al., 2007). Further, post-training intrahippocampal infusion of rapamycin in rats impairs long-term spatial memory tested in a Morris water maze (Dash et al., 2006) and object recognition (Myskiw et al., 2008). Rapamycin also blocks the memory enhancing effects of various modulatory compounds, such as glucose (Dash et al., 2006), and therefore, the mnemonic effects of P may similarly depend on mTOR signaling. Interestingly, LTP in hippocampal slices requires activation of both ERK and mTOR (Gelinas et al., 2007), suggesting that ERK and mTOR may work in concert to mediate the P-induced enhancement in memory consolidation. However, the effects of P on mTOR signaling have never been examined. As such, another goal of the present work was to explore whether the P-induced enhancements in object recognition memory consolidation depend on P-induced modulation of mTOR signaling.

The present studies were designed to examine the roles of ERK and mTOR signaling in the P-induced enhancement of object recognition in young ovariectomized mice. The goals of Experiments 1a and 1b were to determine if dorsal hippocampal infusion of P increases ERK activation in the dorsal hippocampus, and whether the beneficial effects of P on object recognition memory are dependent on dorsal hippocampal ERK activation. Given the critical role of ERK in memory consolidation, we hypothesized that the ability of P to enhance object recognition memory depends on dorsal hippocampal ERK activation. We first examined whether P increased dorsal hippocampal p42 and p44 ERK phosphorylation 5, 15, or 30 min after bilateral dorsal hippocampal infusion of P. We next sought to determine if an inhibitor of the immediate upstream activator of ERK, MAPK kinase (MEK), blocks the memory-enhancing effects of P using the same post-training approach we have employed in our previous studies of P (Lewis et al., 2008b; Orr et al., 2009) and E_2 (Fan et al., 2010a; Fernandez et al., 2008; Zhao et al., 2010). Based on our previous work with E_2, we hypothesized that P would increase p42 ERK phosphorylation in the dorsal hippocampus within 5 min after infusion, and that the MEK inhibitor U0126 would block the effects of P on object recognition. Together, such findings would indicate that dorsal hippocampal ERK activation is necessary for P to enhance object recognition memory consolidation. The goals of Experiments 2a and 2b were to determine if dorsal hippocampal infusion of P increases activation of the mTOR substrate S6K in the dorsal hippocampus, and explore whether the P-induced enhancement in object recognition memory depends on dorsal hippocampal mTOR activation. We first measured whether P affected dorsal hippocampal S6K phosphorylation 5 or 15 min after bilateral dorsal hippocampal infusion of P. We next investigated whether rapamycin blocks the P-induced enhancement of object

recognition using the same post-training infusion approach employed in Experiment 1b (Lewis et al., 2008b; Orr et al., 2009). Because mTOR activation should occur downstream from ERK, we hypothesized that P would increase S6K phosphorylation in the dorsal hippocampus 15 min after infusion, and that rapamycin would block the effects of P on object recognition. Such data would indicate that the ability of P to enhance object recognition memory consolidation depends on dorsal hippocampal mTOR activation.

Materials and methods

Subjects

Female C57BL/6 mice (N = 134) were received from Taconic (Germantown, NY) at 9–10 weeks of age. Mice were housed five per shoebox cage prior to surgery, with ad libitum access to food and water in a room with a 12:12 light/dark cycle (lights on at 7:00). All behavioral testing was conducted during the light phase of the cycle. After surgery, mice were singly-housed. All procedures were approved by the Institutional Animal Care and Use Committee of Yale University, and conformed to the guidelines established by the National Institute of Health *Guide for the Care and Use of Laboratory Animals*.

Experiments 1a and 1b: Involvement of ERK signaling in the P-induced enhancement of object recognition memory

Experiment 1a assessed the effects of bilateral dorsal hippocampal infusion of P on ERK activation in the dorsal hippocampus by measuring levels of phosphorylated and total ERK (p42 and p44 isoforms) via Western blotting. Fifty-five mice were ovariectomized (to eliminate endogenous sources of ovarian hormones) and implanted bilaterally with cannulae into the dorsal hippocampus as described below (Fig. 1A). After surgical recovery, mice were infused bilaterally into the dorsal hippocampus with Vehicle or P and killed 5 min (n = 12/group), 15 min (n = 9 for Vehicle, n = 7 for P), or 30 min (n = 7 for Vehicle, n = 8 for P) later for measurement of phosphorylated and total ERK protein in the dorsal hippocampus.

Next, Experiment 1b used the MEK inhibitor U0126 to determine if ERK activation is necessary for P to enhance object recognition. Twenty-four mice were ovariectomized and implanted bilaterally with dorsal hippocampal cannulae (Fig. 1B). To prevent damage to the dorsal hippocampus from repeated infusions, P was infused into the dorsal third ventricle (abbreviated ICV for intracerebroventricular), which allowed U0126 to be bilaterally infused into the dorsal hippocampus (abbreviated IH for intrahippocampal). Thus, mice in this experiment were implanted with a triple cannula (one ICV and two IH), and each mouse received infusions into all three cannulae during each infusion session as in our previous work (Fan et al., 2010a; Fernandez et al., 2008). After surgical recovery, mice were habituated to the testing apparatus. Twenty-four hours later, they were trained in the object recognition task and then immediately infused intracranially with: 1) ICV and IH Vehicle (n = 9), 2) ICV P + IH vehicle (n = 8), or 3) ICV P + IH U0126 (a MEK inhibitor, n = 7). Object recognition testing was conducted 48 h later as described below.

Experiments 2a and 2b: Involvement of mTOR signaling in the P-induced enhancement of object recognition memory

Experiment 2a assessed the effects of bilateral dorsal hippocampal infusion of P on mTOR activation in the dorsal hippocampus by measuring levels of phosphorylated and total S6K via Western blotting.

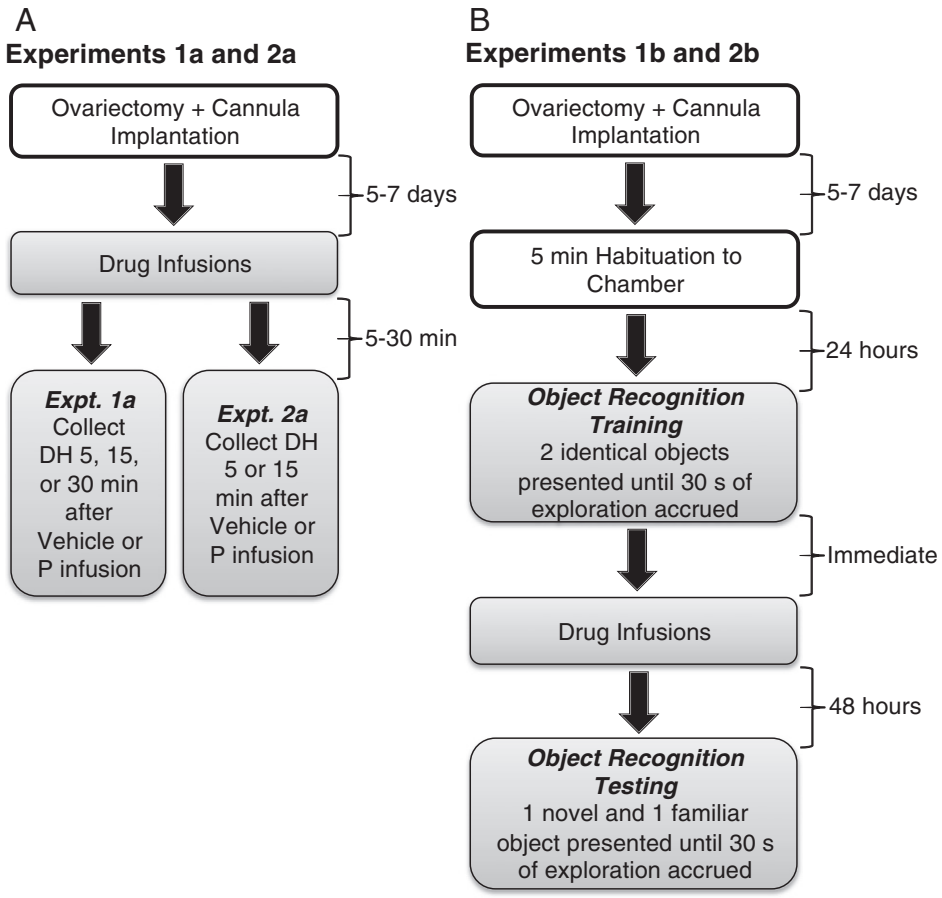

Fig. 1. (A) Experimental timeline for Experiments 1a and 2a. (B) Experimental timeline for Experiments 1b and 2b.

S6K is the kinase immediately downstream from mTOR (Raught et al., 2001) and is commonly used as an indicator of mTOR pathway activation (Dash et al., 2006; Gafford et al., 2011). Twenty-nine mice were ovariectomized and implanted bilaterally with dorsal hippocampal cannulae (Fig. 1A). After surgical recovery, mice were infused bilaterally into the dorsal hippocampus with Vehicle or P and killed 5 min (n = 7 for Vehicle, n = 6 for P) or 15 min (n = 7 for Vehicle, n = 9 for P) later for measurement of phosphorylated and total S6K protein levels in the dorsal hippocampus.

Experiment 2b used the mTOR inhibitor rapamycin to determine if mTOR signaling is necessary for P to enhance object recognition. Twenty-six female mice were ovariectomized and implanted triple cannulae as in Experiment 1b (Fig. 1B). After surgical recovery, mice were habituated to the testing apparatus. Twenty-four hours later, they were trained in the object recognition task and then immediately infused intracranially with: 1) ICV and IH Vehicle (n = 8), 2) ICV P + IH Vehicle (n = 9), or 3) ICV P + IH Rapamycin (n = 9). Object recognition testing was conducted 48 h later.

Surgery

Mice were ovariectomized and implanted with intracranial guide cannulae in the same surgical session as described previously (Lewis et al., 2008a; Orr et al., 2009). Mice were anesthetized with isoflurane gas (2% isoflurane in 100% oxygen) and placed into a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). Ovariectomy was conducted first. The ovaries and tips of the uterine horns were isolated and removed through bilateral dorsal incisions made at the tip of the pelvis. The muscle wall was sutured and the skin closed with wound clips. Immediately after ovariectomy, mice were implanted with stainless-steel guide cannulae (C232GC, 22 gauge, Plastics One, Roanoke, VA) with inserted dummy cannulae (C232DC; Plastics One) aimed at the dorsal hippocampus (1.7 mm posterior to Bregma, +/− 1.5 mm lateral to the midline, and 2.3 mm ventral to the surface of the skull; Paxinos and Franklin, 2003). In Experiments 1b and 2b, a third guide cannula was included, aimed at the dorsal third ventricle (0.5 mm posterior to Bregma), as described previously (Fernandez et al., 2008; Lewis et al., 2008a). Cannulae were affixed to the skull with dental cement that also served to close the wound. Mice in all experiments were allowed to recover from surgery for 5–7 days. During this time, they received 30 mg/kg ibuprofen in the drinking water as an analgesic. Previous studies using this cannulation procedure resulted in reliable placement within the dorsal hippocampus (Orr et al., 2009).

Drugs and infusions

During infusions, mice were gently restrained and dummy cannulae were replaced with injection cannulae (C232I; intrahippocampal: 28 gauge, extending 0.8 mm beyond the tip of the 1.5 mm guide cannula; intracerebroventricular: 28 gauge, extending 1.0 mm beyond the 2.0 mm guide cannula) that were attached to polyethylene tubing (PE50; Plastics One) connected to a 10 μl Hamilton syringe. Infusions were controlled by a microinfusion pump (KDS 100, KD Scientific; New Hope, PA) and conducted at a rate of 0.5 μl/min for 1 min at a volume of 0.5 μl/side of the dorsal hippocampus. Infusion cannulae were left in place for another minute to prevent drug diffusion up the cannula track. Methylene blue infused into the entorhinal cortex in this fashion diffuses approximately 1 mm^3 (Lewis and Gould, 2007), suggesting that our infusions were likely limited to the dorsal hippocampus.

Cyclodextrin-encapsulated progesterone (Sigma, St Louis, MO) was dissolved in physiological saline to a concentration of 0.2 μg/μl, resulting in doses of 0.1 μg/side of the dorsal hippocampus or 0.2 μg total into the dorsal third ventricle. The 0.1 μg/side dose of P infused into the dorsal hippocampus enhances 48-hour object recognition in young ovariectomized mice (Orr et al., 2009). Cyclodextrin-encapsulated P is more soluble than oil-based P formulations and cyclodextrin does not detrimentally affect the pharmacokinetic properties of steroid hormones (Brewster et al., 1995). Further, this form of P is metabolized within hours (Pitha et al., 1986) and is not in circulation during object recognition testing, thus, allowing memory consolidation to be examined in the absence of potential non-mnemonic confounds (e.g., motivation, anxiety; Gresack and Frick, 2006). The progesterone vehicle, 2-hydroxypropyl-β-cyclodextrin (HBC, Sigma), was dissolved in an equal volume of saline and contained the same amount of cyclodextrin as P for infusions. The MEK inhibitor 1,4-diamino-2,3-dicyano-1,4-bis (o-aminophenylmercapto) butadiene (U0126; Promega, Madison, WI) was dissolved in 50% dimethylsulfoxide (DMSO) in saline to a concentration of 0.5 μg/0.5 μl, and infused at a dose of 0.5 μg/side of the dorsal hippocampus. The mTOR inhibitor rapamycin (Sigma) was dissolved in 50% dimethylsulfoxide (DMSO) in saline to a concentration of 0.025 ng/0.5 μl, and infused at a dose of 0.025 ng/side of the dorsal hippocampus. We have previously demonstrated that these doses of U0126 and rapamycin do not, on their own, interfere with object recognition memory consolidation (Fan et al., 2010b; Fernandez et al., 2008). The vehicle for U0126 and rapamycin was 50% DMSO in saline. In Experiments 1b and 2b, where mice received two infusions into the dorsal hippocampus, DMSO vehicle or an inhibitor were always infused immediately before HBC vehicle or P.

Object recognition

Object recognition was conducted as described previously (Lewis et al., 2008b; Orr et al., 2009) approximately 1 week after surgery and was used to assess non-spatial hippocampal memory (Baker and Kim, 2002; Clark et al., 2000; Fernandez et al., 2008). Object recognition was conducted in three phases (habituation, training, and testing) in a white open field box (58 cm long by 58 cm wide by 46 cm high). Testing sessions were videotaped using a ceiling-mounted camera that was connected to a monitor and VCR outside of the room. Data were recorded on a computer running custom written software by an experimenter blind to the treatment each mouse received.

During habituation, mice were allowed to freely explore the empty testing box for 5 min. 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 the total amount of time spent exploring both objects equaled 30 s (exploration recorded when the front paws or nose contacted the object). Mice were then removed from the chamber, 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). Mice were then allowed to explore until the total time exploring the objects equaled 30 s. Unlike other object recognition protocols that maintain a constant trial time (e.g., 3 min), our procedure, which maintains a constant amount of exploration time, ensures that all mice have the same amount of exposure to the objects. During all phases of training, the box and objects were cleaned with 70% ethanol between mice.

The time spent with each object was recorded to assess object recognition. Because mice innately prefer novelty, a preference for the novel object (significantly more time than chance (15 s) spent with the novel object) indicates intact memory for the familiar object. Vehicle-treated ovariectomized mice do not demonstrate memory for the familiar object after a 48-hour delay (Lewis et al., 2008a; Orr et al., 2009), thus allowing for the observation of a P-induced memory enhancement at this delay. To assess whether non-mnemonic factors such as motor activity or motivation influenced task performance, elapsed time to accumulate 30 s of exploration was recorded, as was each instance in which a mouse ceased to explore an object and rather explored the testing box (termed “non-object exploration”).

Western blotting

As described above, mice in Experiments 1a and 2a were infused into the dorsal hippocampus with Vehicle or P and killed via cervical dislocation 5, 15, or 30 min after infusion. The dorsal hippocampus was immediately bilaterally dissected on ice and stored at −80 °C until homogenization. Western blotting was conducted as described previously (e.g. Fan et al., 2010a; Lewis et al., 2008a). Dorsal hippocampal samples were resuspended 1:50 w/v in hypotonic lysis buffer, homogenized with a probe sonicator (Branson Sonifier 250), suspended in sample buffer, and boiled for 5 min. Homogenates were electrophoresed on 10% Tris HCl gels (Bio-Rad, Hercules, CA) and blotted to an Immobilon PVDF membrane (Millipore Corporation, Billerica, MA). Western blots were blocked in 10% milk and incubated overnight with anti-phospho-p44/p42 ERK primary antibodies (1:2000, Cell Signaling, Danvers, MA) for Experiment 1a and with anti-phospho-S6K for Experiment 2a. Blots were then incubated with anti-rabbit-HRP (1:20,000; Cell Signaling) and developed using West Dura chemiluminescent substrate (SuperSignal West Dura, Thermo Scientific, Rockford, IL). Blots were imaged using a Kodak Image Station 440 CF (Kodak Scientific Imaging Systems), and densitometry conducted using Kodak 1D 3.6 software (Eastman Kodak). Following imaging, blots were stripped using 0.2 M NaOH and reprobed for anti-total-p44/p42 ERK (1:1000; Cell Signaling) in Experiment 1a and for anti-total-S6K in Experiment 2a. Phosphorylated ERK (pERK) and S6K (pS6K) levels were normalized to total-ERK (tERK) and total-S6K (tS6K) levels, respectively, and expressed as % immunoreactivity relative to Vehicle controls.

Data analysis

Data analyses were conducted using PASW (IBM Corporation, Somers, NY). For object recognition, separate one-sample *t*-tests were performed for each group to determine whether 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 object (Gresack and Frick, 2004). A one-way analysis of variance (ANOVA) with drug treatment as the independent variable was conducted on all other measures of performance from object recognition. For Western blotting data, independent samples *t*-tests were used at each time point to compare the % immunoreactivity between the Vehicle and P groups.

Results

Experiment 1a: P has a biphasic effect on dorsal hippocampal ERK activation

Five minutes after dorsal hippocampal infusion, dorsal hippocampal levels of phospho-p42 ERK were significantly higher in P-infused mice relative to Vehicle controls ($t(22) = -2.142$, $p = 0.044$; Fig. 2A). However, phospho-p42 ERK levels in P-infused mice decreased substantially 15 min after infusion, such that they were significantly lower than those in Vehicle-infused mice ($t(14) = 3.12$, $p = 0.006$). By 30 min after infusion, phospho-p42 ERK levels in P-infused mice returned to Vehicle levels and did not differ significantly between the two groups ($t(13) = 0.969$, $p > 0.05$). Phospho-p44 ERK levels in the dorsal hippocampus did not differ between the groups 5 and 30 min after infusion (5 min: $t(22) = -0.525$, $p > 0.05$; 15 min: $t(12) = -0.275$, $p > 0.05$; Fig. 2A). However, as with the p42 isoform, phospho-p44 ERK levels were significantly lower in P-infused mice relative to Vehicle 15 min after infusion ($t(14) = 2.305$, $p = 0.03$, Fig. 2B). Total p42 and p44 ERK expression did not differ between the groups at any time point ($ps > 0.05$).

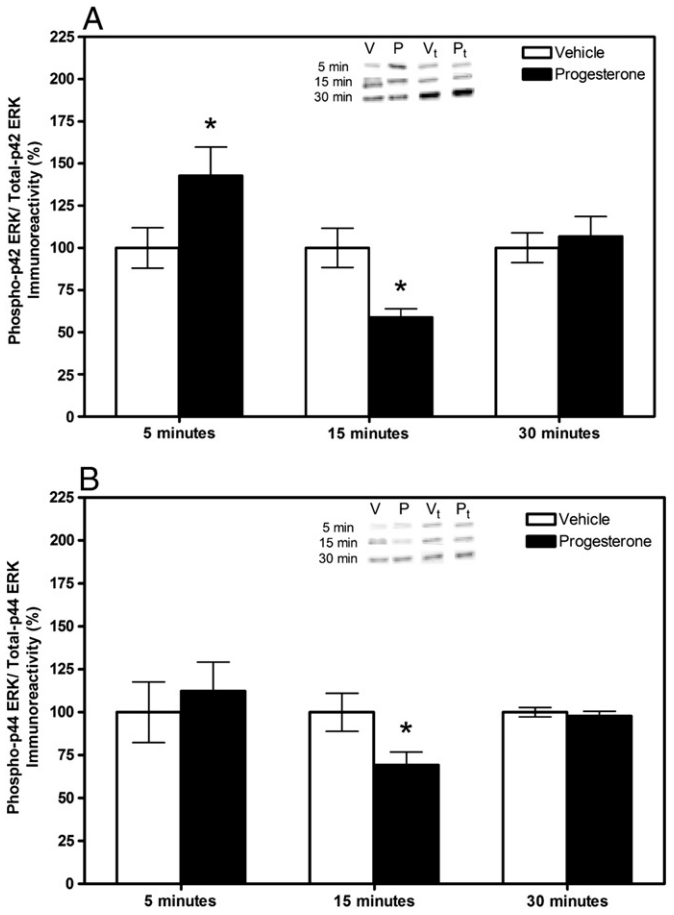

Fig. 2. (A) Relative to Vehicle, phospho-p42 ERK levels in the dorsal hippocampus were significantly increased by P 5 min after dorsal hippocampal infusion and decreased 15 min after infusion (*$p<0.05$ relative to Vehicle). Phospho-p42 ERK levels did not differ between the groups 30 min after infusion. (B) Phospho-p44 ERK levels in the dorsal hippocampus were significantly decreased by P 15 min after infusion (*$p<0.05$ relative to Vehicle), but did not differ between the groups 5 or 30 min after infusion. Bars represent mean ± standard error of the mean (SEM). Inset: Representative Western blots for each group.

Experiment 1b: The P-induced enhancement of memory depends on dorsal hippocampal ERK signaling

During object recognition testing, mice receiving P + Vehicle exhibited a significant preference for the novel object, whereas mice receiving Vehicle alone or P + U0126 did not (Fig. 3). Time spent with the novel object was not significantly different from chance (15 s) for groups receiving Vehicle alone ($t(8) = 0.35$, $p > 0.05$) or P + U0126 ($t(6) = 1.416$, $p > 0.05$). However, time spent with the novel object was significantly greater than chance for mice receiving P + Vehicle ($t(7) = 3.748$, $p = 0.007$). This pattern of data indicates that P enhanced recognition for the familiar object, and that MEK inhibition blocked this enhancement. These findings suggest that dorsal hippocampal ERK activation is necessary for P to enhance object recognition memory consolidation.

Neither the elapsed time to accumulate 30 s of exploration ($F(2,21) = 0.414$, $p > 0.05$) nor the instances of non-object exploration ($F(2,21) = 1.251$, $p > 0.05$) differed among the groups during testing (Table 1), indicating that non-mnemonic aspects of task performance, such as motor activity, anxiety, and motivation also did not differ significantly among the groups.

Experiment 2a: P rapidly increases dorsal hippocampal mTOR signaling

Five minutes after dorsal hippocampal infusion, mice receiving P + Vehicle had significantly higher levels of phoshpo-S6K relative to

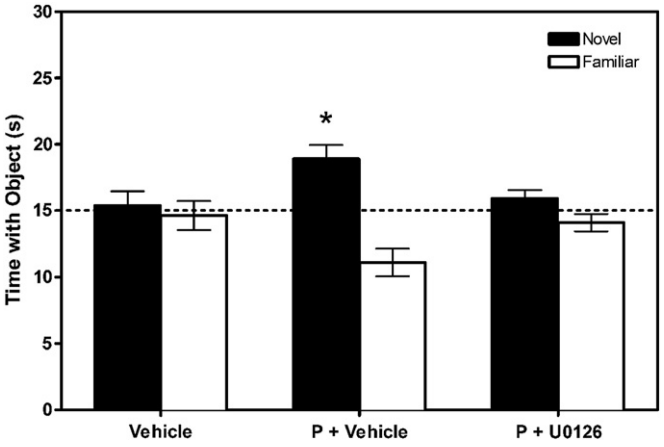

Fig. 3. Forty-eight hours after training, mice receiving P + Vehicle, but not Vehicle alone, spent significantly more time than chance (dashed line at 15 s, $^*p<0.05$) with the novel object, indicating that P enhanced memory for the familiar object. Mice receiving vehicle P + U0126 did not spend more time than chance with the novel object, indicating that MEK inhibition blocked the beneficial effects of P on object recognition. Bars represent mean ± SEM.

Vehicle ($t(11) = -3.496$, $p = 0.005$; Fig. 4A). This increase dissipated by 15 min after infusion, at which point levels of phospho-S6K did not differ between the groups ($t(14) = 1.188$, $p > 0.05$; Fig. 4A). Total S6K expression in the dorsal hippocampus did not differ between the groups at either time point ($p > 0.05$).

Experiment 2b: The P-induced enhancement of memory is dependent on dorsal hippocampal mTOR signaling

During testing, mice receiving P + Vehicle exhibited a significant preference for the novel object, whereas mice receiving Vehicle alone or P + Rapamycin did not (Fig. 4B). Time spent with the novel object did not significantly differ from chance (15 s) for the Vehicle ($t(7) = 0.623$, $p > 0.05$) or P + Rapamycin ($t(8) = 0.51$, $p > 0.05$) groups. In contrast, mice receiving P + Vehicle spent significantly more time than chance with the novel object ($t(8) = 4.365$, $p = 0.002$). The fact that rapamycin blocked the P-induced enhancement of object recognition suggests that activation of the mTOR pathway in the dorsal hippocampus is necessary for P to facilitate object recognition memory consolidation.

As in Experiment 1b, the groups did not differ in terms of the amount of time required to accumulate 30 s of exploration ($F(2,23) = 0.542$, $p > 0.05$) or the instances of non-object exploration ($F(2,23) = 0.315$, $p > 0.05$) (Table 1), indicating that P-induced alterations in the time spent with the novel object are not due to differences in motivation to explore the objects or testing box.

Table 1
Additional behavioral data collected during object recognition testing.

Group	Time to accumulate 30 s of exploration (s)	Instances of non-object exploration (#)
Experiment 1b		
Vehicle	446.26 ± 94.61	37.22 ± 2.70
P + Vehicle	312.16 ± 84.19	32.38 ± 1.90
P + U0126	376.90 ± 145.8	32.29 ± 3.13
Experiment 2b		
Vehicle	334.10 ± 94.73	35.88 ± 3.49
P + Vehicle	421.74 ± 77.48	39.00 ± 2.99
P + Rapamycin	329.47 ± 33.53	39.56 ± 3.85

Values represent the mean ± SEM.

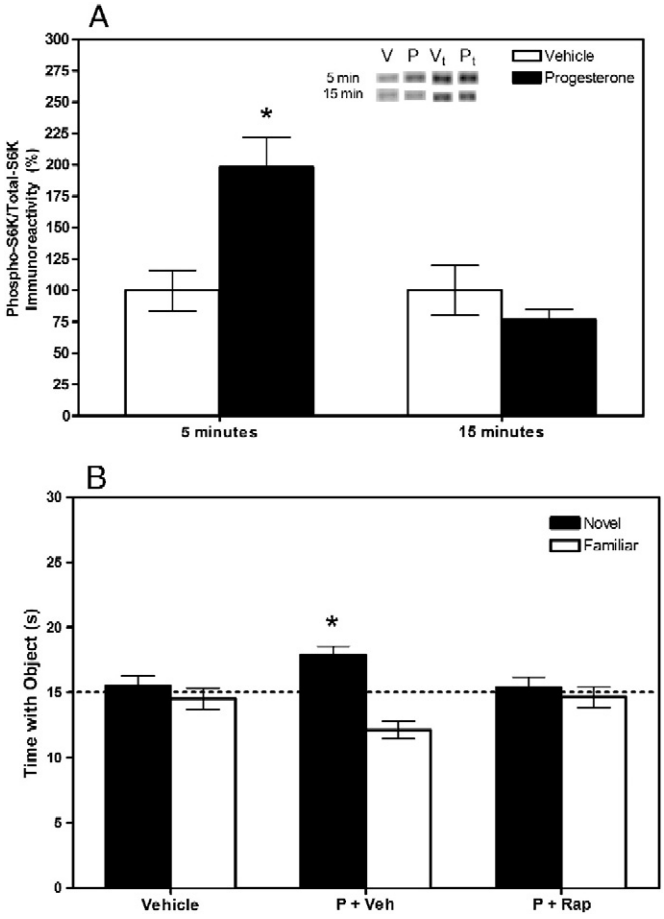

Fig. 4. (A) Phospho-S6K levels in the dorsal hippocampus were significantly increased by P 5, but not 15, minutes after dorsal hippocampal infusion ($^*p<0.05$ relative to Vehicle). Bars represent mean ± SEM. Inset: Representative Western blots for each group. (B) Forty-eight hours after training, mice treated with P + Vehicle spent more time than chance with the novel object ($^*p<0.05$), whereas mice receiving Vehicle alone or P + Rapamycin did not, indicating that mTOR inhibition blocked the beneficial effects of P on object recognition. Bars represent the mean ± SEM.

Discussion

The present data provide several important contributions to the scant literature on the modulation of memory by P. First, the findings illustrate that infusion of P into the dorsal hippocampus affects ERK in a biphasic manner, at first increasing p42 ERK phosphorylation 5 min after infusion and then decreasing both p42 and p44 ERK phosphorylation 15 min after infusion, before returning both isoforms to baseline 30 min after infusion. Second, the behavioral data demonstrate that dorsal hippocampal ERK activation is necessary for P to enhance object recognition memory consolidation in young females. Together, these findings represent the first evidence that P is capable of modulating hippocampal ERK phosphorylation and that this modulation is critical for P to facilitate object recognition memory consolidation. Finally, these data demonstrate for the first time that P can increase dorsal hippocampal mTOR signaling, and that this signaling is necessary for P to enhance object recognition memory consolidation. These findings represent the first known demonstration of interactions between a sex steroid hormone and mTOR signaling with respect to memory function.

The data from Experiments 1b and 2b demonstrating that ICV-infused P can enhance object recognition are consistent with several previous studies in which post-training i.p. injection of P enhanced object recognition and spatial memory in young and aging female rodents (Harburger et al., 2008; Lewis et al., 2008b). The data are

also consistent with our previous report that dorsal-hippocampally infused P can enhance object recognition memory consolidation in young females (Orr et al., 2009), indicating that infusion of P into the dorsal hippocampus is sufficient to produce a P-induced memory enhancement. In the present study, infusion of P into the dorsal third ventricle also significantly enhanced object recognition. Alone, this finding does not speak to the regional specificity of P's effects on memory, but coupled with the fact that dorsal hippocampal infusion of U0126 or rapamycin blocked the ability of ICV-infused P to enhance object recognition, demonstrates that the dorsal hippocampus is critical to the memory enhancing effects of P. Combined, these data indicate that the dorsal hippocampus is not only sufficient, but is also necessary, for P to enhance object recognition memory consolidation.

This study also provides the first demonstration that intrahippocampal infusion of P can alter ERK phosphorylation in the dorsal hippocampus. This finding is consistent with previous in vitro studies (Nilsen and Brinton, 2003) and with the ERK-dependence of P's beneficial effects on dentate neuron proliferation and survival (Liu et al., 2009; Zhang et al., 2010). Although previous in vivo work demonstrated that intraperitoneal injection of P increased ERK phosphorylation in the rat hippocampus (Guerra-Araiza et al., 2009), the present experiment extends these findings to show that direct dorsal hippocampal administration of P specifically activates p42 ERK, thereby providing support for the argument that ERK activation in the dorsal hippocampus is critically involved in P-induced enhancement of memory consolidation. Furthermore, compared to systemic injections, the dorsal hippocampal infusions used here allow more precise measurement of the time course of P effects on cell signaling events. However, it must be noted that cannula placements could not be recorded here because all brain tissue was collected for Western blotting. Therefore, we cannot exclude the possibility that infusions could have spread to adjacent areas (e.g., overlying neocortex or dorsal thalamus). Nevertheless, previous cannulations using this protocol resulted in consistent placements within the dorsal hippocampus (Orr et al., 2009), and limited infusion spread (Lewis and Gould, 2007), so we feel confident that infusions were restricted to the dorsal hippocampus.

P biphasically affected phosphorylation of p42 ERK, an isoform that is particularly critical for long-term memory formation (Atkins et al., 1998b; English and Sweatt, 1996; Selcher et al., 2001), and that is also modulated by E_2 (Fernandez et al., 2008). This biphasic effect stands in contrast to a previous study of cultured embryonic hippocampal neurons, which found that p42 ERK phosphorylation was increased by P 10, 30, and 60 min after exposure, but returned to baseline 120 min after exposure (Nilsen and Brinton, 2003). Nevertheless, a reduction in p42 ERK phosphorylation could have taken place between 60 and 120 min, so the possibility of a biphasic effect in this cell culture study cannot be excluded. The biphasic effect of P on phospho-p42 ERK levels is, however, consistent with other in vivo data from ovariectomized rats demonstrating that systemic P first increases, and then decreases, CA1 dendritic spine density before levels return back to baseline (Woolley and McEwen, 1993). Although the time scales for these biphasic effects differ considerably (i.e., hours for spines and minutes for ERK), the fact that P produced biphasic effects in two in vivo hippocampal studies suggests that the effects of P in the intact adult hippocampus may differ from those in embryonic hippocampal neurons. This speculation will need to be explored further in future studies.

It is notable that the biphasic effect of P on p42 ERK and the decrease in p44 ERK differs from the effects of E_2 on ERK. Using a different dorsal hippocampal infusion protocol, we recently found that E_2 increased p42 ERK phosphorylation 15 min after infusion, but not 5, 30, or 60 min after infusion (Fan et al., 2010b). Importantly, at no time point were phospho-p42 ERK levels decreased below baseline. Thus, it would seem that E_2 does not have a biphasic effect on ERK. Further, we have consistently found that neither IH nor ICV infusions of E_2 influence p44 ERK phosphorylation in the dorsal hippocampus (Fan et al., 2010a; Fernandez et al., 2008; Lewis et al., 2008a; Zhao et al., 2010), and thus, the effects of P on the p44 isoform at 15 min appear to be somewhat unique to P. Although p44 can be activated by hippocampal learning (Atkins et al., 1998; Kelly et al., 2003), it has not been considered critical for hippocampal learning and memory because its deletion mice has no effect on hippocampal learning or long-term potentiation (Selcher et al., 2001; Selcher et al., 2003). Although the function of the P-induced decrease in p44 ERK phosphorylation is unknown, it does parallel the reduction of the p42 isoform at the 15-minute time point. Whatever the specific cellular function of the decreases in these isoforms, the net effect of these fluctuating ERK levels appears to be an enhancement of object recognition.

The fluctuations of ERK activation produced by P may help to explain the differences in the effects of P given pre- and post-training. If P is administered prior to training, then much of the training may occur while ERK levels are either reduced or have returned to baseline. In this scenario, initial learning or memory consolidation may not take place contemporaneously with the P-induced increase in ERK that likely facilitates memory enhancement in the post-training design, thus leading to a null effect of P on memory (as in Chesler and Juraska, 2000; Sato et al., 2004). Indeed, the period during which memory consolidation takes place after pre-training administration may coincide with the later reduction in ERK activation and CA1 spine density, which could prove detrimental to memory. Such an effect could play a role in the detrimental effects of pre-training, chronically administered P on spatial working memory and footshock avoidance (Bimonte-Nelson et al., 2004; Farr et al., 1995). In contrast, post-training acute administration of P would likely provide at least an early temporal coincidence between memory consolidation and increases in p42 ERK phosphorylation and CA1 spine density, which may contribute to the observed memory enhancements produced by P in the Y-maze, inhibitory avoidance, and object recognition (Frye and Lacey, 2000; Harburger et al., 2008; Orr et al., 2009). Although thought provoking, this hypothesis will need to be tested more systematically in future work. Certainly, the analgesic effects of P via the binding of P metabolites, most notably 3α,5α-tetrahydroprogesterone, to hippocampal $GABA_A$ receptors are another factor that could contribute to the detrimental effects of pre-training P administration. Therefore, potential analgesic effects should be considered in future work comparing the effects of pre- and post-training P on memory consolidation.

The present data also demonstrate for the first time that dorsal hippocampal P infusion increases activation of the mTOR pathway and that the observed P-induced improvement in object recognition memory consolidation is dependent on this mTOR activation. The rapamycin-induced blockade of P-induced object recognition enhancement is consistent with previous work demonstrating that post-training intrahippocampal infusion of 0.018 ng/side and 0.9 ng/side rapamycin prevents the consolidation of object recognition (Myskiw et al., 2008) and spatial memory (Dash et al., 2006), respectively, in male rats. These data are also consistent with those of previous studies showing that rapamycin blocks the effects of other modulatory compounds such as glucose and tetrahydrocannabinol (THC), which depend on mTOR activation to affect memory (Dash et al., 2006; Puighermanal et al., 2009). As such, these data add to a growing literature demonstrating that hippocampal mTOR activation is necessary not only for memory consolidation but also for the modulation of memory consolidation by hormones and other factors.

Although ERK is most commonly associated with initiation of gene transcription through CREB, it can also rapidly promote protein translation through mTOR and S6K (Tsokas et al., 2005). ERK activates the mTOR pathway directly by phosphorylating S6K (Kelleher et al., 2004; Tsokas et al., 2005) and indirectly through interactions with the PI3K/Akt pathway (Tsokas et al., 2005), which phosphorylates mTOR. Thus, given the ERK-dependence of the P-induced enhancement of object recognition, it is perhaps not surprising that mTOR activation is also necessary for P to enhance object recognition. However, it is unclear from the

timing of S6K alterations whether this activation results from phosphorylation of ERK or another upstream kinase like PI3K or Akt. We hypothesized that S6K would not be altered by P until 15 min after infusion because S6K is activated downstream from ERK, which is activated 5 min after P infusion. Yet both ERK and S6K were increased 5 min after infusion, which calls into question whether ERK could have activated S6K within this brief time frame. However, S6K lies downstream from many other kinases (see Klann and Dever, 2004, for review), so the fact that it was activated so rapidly suggests that both S6K and the upstream kinases could be activated within the 5 min time point. Interestingly, P did not downregulate S6K 15 min after infusion as was observed with both isoforms of ERK, which might suggest that the effects of P on ERK phosphorylation are relatively independent of those regulating mTOR and S6K phosphorylation. This potential divergence could arise if P-induced activation of PI3K and Akt leads to ERK activation and mTOR activation independently (e.g., ERK via PI3K and mTOR via Akt). Alternatively, ERK may indeed activate S6K, but the dephosphorylation of both proteins may be differentially regulated by P and follow different time courses. Nevertheless, it is important to note that dorsal hippocampal phospho-S6K levels were increased by P, which is a novel finding and consistent with the effects of hippocampal learning on S6K activation (Bekinschtein et al., 2007; Gafford et al., 2011). Further, the increase in phospho-S6K levels 5 min after infusion indicates that P can rapidly affect protein translation and synthesis, and suggests interesting avenues for future study into how this synthesis leads to memory enhancement.

In conclusion, the present study provides several novel insights into the mechanisms of P-induced memory modulation. First, the data demonstrate that dorsal hippocampal activation of the ERK and mTOR pathways is necessary for P to enhance object recognition memory consolidation. These findings are consistent with our previous reports that post-training P administration is beneficial for object recognition and that the dorsal hippocampus is a critical locus of P-induced memory modulation. Second, the data demonstrate that P has a biphasic effect on dorsal hippocampal ERK signaling, similar to the biphasic effect of P on CA1 dendritic spine density (Woolley and McEwen, 1993). This biphasic effect affords an interesting contrast with E_2, which appears to only increase ERK activation. Finally, the current data provide the first evidence that P can rapidly activate the dorsal hippocampal mTOR pathway and, therefore, can likely rapidly regulate mTOR-related protein synthesis. Collectively, these data suggest that P may enhance memory consolidation by synergistically promoting ERK activation and mTOR-mediated protein translation, thereby ultimately leading to structural synaptic changes that facilitate memory consolidation.

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