

Building a Better Hormone Therapy?: How Understanding The Rapid Effects of Sex Steroid Hormones Could Lead to New Therapeutics for Age-Related Memory Decline

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A wealth of data collected in recent decades has demonstrated that ovarian sex-steroid hormones, particularly 17 β -estradiol (E₂), are important trophic factors that regulate the function of cognitive regions of the brain such as the hippocampus. The loss of hormone cycling at menopause is associated with cognitive decline and dementia in women, and the onset of memory decline in animal models. However, hormone therapy is not currently recommended to prevent or treat cognitive decline, in part because of its detrimental side effects. In this article, it is proposed that investigations of the rapid effects of E₂ on hippocampal function be used to further the design of new drugs that mimic the beneficial effects of E₂ on memory without the side effects of current therapies. A conceptual model is presented for elucidating the molecular and biochemical mechanisms through which sex-steroid hormones modulate memory, and a specific hypothesis is proposed to account for the rapid memory-enhancing effects of E₂. Empirical support for this hypothesis is discussed as a means of stimulating the consideration of new directions for the development of hormone-based therapies to preserve memory function in menopausal women.

Keywords: Estradiol, ERK, PI3K, epigenetic, aging

Perhaps no issue in women's health has been more controversial during the past decade than hormone replacement therapy for menopausal women. Several lines of clinical evidence suggest a rationale for the use of hormone therapy to prevent or reduce age-related cognitive decline in women. First, the loss of circulating ovarian sex steroid hormones (estrogens and progestogens) at menopause has been associated with impaired memory and executive functioning (Amore et al., 2007; Elsabagh, Hartley, & File, 2007; Fuh, Wang, Lee, Lu, & Juang, 2006; Gold et al., 2000; Halbreich et al., 1995; Thilers, Macdonald, Nilsson, & Herlitz, 2010), as well as an increased risk of Alzheimer's disease (Launer et al., 1999; Sherwin, 1999; Wolf & Kirschbaum, 2002; Yaffe et al., 2000; Yaffe, Sawaya, Lieberburg, & Grady, 1998; Zandi et al., 2002). Second, the use of estrogens in observational studies of menopausal women is associated with better verbal memory, working memory, and visuospatial function (Duff & Hampson, 2000; Duka, Tasker, & McGowan, 2000; Grodstein, Chen, Pollen, & Albert, 2000; Hogervorst, Boshuisen, Riedel, Willekes, & Jolles, 1999; Kampen & Sherwin, 1994; Maki, Zonderman, &

Resnick, 2001; Matthews, Cauley, Yaffe, & Zmuda, 1999; Steffens, Norton, Plassman, & Tschanz, 1999), and with a lower risk of dementia, particularly among women who initiated treatment during, or soon after, the menopause (LeBlanc, Janowsky, Chan, & Nelson, 2001; Sherwin & Henry, 2008; Yaffe et al., 1998). Third, randomized clinical trials of women treated with the potent estrogen 17 β -estradiol (E₂) near the onset of menopause also report beneficial effects of treatment on verbal and working memory (Joffe, Hall, Gruber, & Sarmiento, 2006; Phillips & Sherwin, 1992; Sherwin & Henry, 2008; Viscoli, Brass, Kernan, & Sarrel, 2005; Wolf et al., 1999). However, the Women's Health Initiative (WHI), the largest randomized clinical trial to date of the commonly prescribed conjugated equine estrogens (CEE), taken with or without a synthetic progestin, indicates that several years of treatment in postmenopausal women over 65 years of age significantly increases the risks of global cognitive decline, mild cognitive impairment, and dementia (Espeland et al., 2004; Rapp et al., 2003; Shumaker et al., 2004; Shumaker et al., 2003), and does not benefit verbal memory, working memory, attention, or spatial abilities (Resnick et al., 2009; Resnick et al., 2006). As such, CEE-based treatments are no longer recommended for maintaining cognitive function or preventing cognitive decline in postmenopausal women. Moreover, the WHI also reported small, but statistically significant, increases in breast cancer, heart disease, stroke, and blood clot after hormone treatment (Chlebowski et al., 2003; Rossouw et al., 2002; Wassertheil-Smoller et al., 2003), underscoring the inherent health risks to women of CEE therapy. As a result of these data, hormone therapy has become prescribed only for perimenopausal or recently menopausal women, who are recommended to use hormone therapy at the smallest effective dose for the shortest duration necessary to relieve menopausal

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symptoms (U.S. Department of Health and Human Services, National Institutes of Health, & National Heart, Lung, & Blood Institute, 2005).

Although this treatment strategy is prudent based on the available literature and currently available hormone formulations, a substantial literature accumulated over the past two decades has demonstrated that ovarian hormones, particularly E_2 , are critical trophic factors for regions of the brain that mediate cognitive function, including the hippocampus, basal forebrain, and prefrontal cortex (Brinton, 2001; Dumitriu, Rapp, McEwen, & Morrison, 2010; Garcia-Segura, Azcoitia, & Don Carlos, 2001; Wise, Dubal, Wilson, Rau, & Liu, 2001). Therefore, the loss of these hormones at menopause, if not mitigated by hormone therapy, may render neurons in these brain regions vulnerable to age-related deterioration and exacerbate emerging memory deficits. However, any form of systemic hormone therapy will carry risks of cancer and/or cardiovascular disease due to hormone binding at estrogen and progesterone receptors throughout the body. Therefore, it is reasonable to ask whether other treatment strategies could more effectively preserve cognitive function with fewer side effects. Selective estrogen receptor modulators (SERMs), such as tamoxifen, raloxifene, and soy phytoestrogens are obvious candidates, but are not generally effective at improving memory in postmenopausal women (Jenkins, Shilling, Fallowfield, Howell, & Hutton, 2004; Kreijkamp-Kaspers et al., 2004; Paganini-Hill & Clark, 2000; Rice, Graves, & Larson, 1995; Yaffe et al., 2001) or in animal models (Chen, Wu, Shi, & Xu, 2002a, 2002b; Gibbs, Gabor, Cox, & Johnson, 2004; Lacreuse, Wilson, & Herndon, 2002). Although research to develop more effective brain-specific estrogen receptor modulators is ongoing (e.g., Zhao et al., 2007), perhaps not all hormonal eggs should be placed in this single basket. Recent studies have revealed substantial new information about the neurobiological mechanisms involved in hormonal modulation of synaptic plasticity, neuroprotection, and memory function, and these data can be exploited to guide the development of new therapeutics for menopausal women that mimic the beneficial effects of sex steroid hormones on cognition without the peripheral side effects associated with current treatments. Moreover, identifying the cellular and molecular mechanisms through which sex steroid hormones modulate cognitive processes could have broad implications beyond hormone therapy by providing important insights into the etiology of sex differences in cognition and the increased risks to women of such diseases as Alzheimer's, depression, drug abuse, psychotic disorders, and anxiety disorders (Carroll & Anker, 2010; Grigoriadis & Robinson, 2007; Hedges, Staffend, & Meisel, 2010; Huber, Borsutzky, Schneider, & Emrich, 2004; Weinstock, 1999; Yaffe et al., 2007; Yaffe et al., 1998; Zandi et al., 2002). As such, the insights to be gained from identifying the neurobiological mechanisms underlying hormone-induced cognitive alterations in adult females of various ages could open new avenues for drug development for the treatment of multiple disorders for which women are at increased risk.

Of the various types of cognitive function affected by sex steroid hormones, memory has been the subject of the most intensive study because of the associations between menopause and memory dysfunction. As such, the remainder of this article will focus on memory. One potentially effective method of identifying new drug targets for reducing age-related memory decline is to pinpoint the neural mechanisms downstream from hormone recep-

tors through which sex steroid hormones influence memory. Some of the most easily targeted mechanisms may be the biochemical processes that are rapidly affected by hormones. Examples of such mechanisms are the rapid E_2 -induced activations of cell signaling and epigenetic mechanisms in the dorsal hippocampus that are necessary for E_2 to enhance consolidation of a specific type of memory (Frick, Fernandez, & Harburger, 2010; Zhao, Fan, & Frick, 2010). Because studies examining the rapid effects of E_2 on hippocampal biochemistry and hippocampal memory have begun to shed light on several critical molecules that could be potential targets for drug design, the present article will focus on rapid effects of E_2 in the hippocampus. However, other effects of E_2 in the hippocampus and related brain regions (e.g., prefrontal cortex, basal forebrain) with rapid or more long-term mechanisms of action (e.g., synaptic remodeling and neurophysiology, neurogenesis, neurotransmission, neuroprotection) will also be critical to the cognitive effects of sex steroid hormones throughout the adult life span. Thus, other parallel lines of investigation will undoubtedly reveal additional putative targets. Although these effects will not be the subject of this review, the reader is encouraged to explore this literature reviewed elsewhere (e.g., Barha & Galea, 2010; Dumitriu et al., 2010; Gibbs, 2010; Wise et al., 2001; Woolley, 2007). The goal of this article is not to provide a comprehensive review of all rapid effects of estrogens, but rather, to stimulate new ways of thinking about the development of future hormone therapies. To this end, I will propose a model to guide such investigations and illustrate the model's potential for drug target discovery using specific examples of the biochemical events that underlie the E_2 -induced facilitation of memory consolidation. As background, the following section will provide an overview of research from rodent models on the effects of E_2 on the hippocampus and memory in young and aging females. The remaining sections will then describe our conceptual model for understanding the rapid effects of E_2 on memory and review data collected that provide support for the model.

Estradiol, The Hippocampus, and Memory

In females, sex steroid hormones (estrogens, androgens, progesterone) are produced primarily in the ovaries, where E_2 and progesterone levels fluctuate from low levels early in the cycle to peak levels mid-to-late cycle that stimulate ovulation and thicken the endometrial lining. In rodents, the laboratory mammals most commonly used to study the effects of hormones on memory, E_2 and progesterone levels surge every 4–5 days and then return to baseline within 24 hours. Sex steroid hormones are also synthesized in the rodent hippocampus (Hojo et al., 2004; Kretz et al., 2004), although the mechanisms regulating their synthesis are unknown. A recent PET study has demonstrated the presence of aromatase, the synthetic enzyme for E_2 , in the hippocampus of both men and women, suggesting that local E_2 synthesis occurs within the hippocampus in humans as well (Biegon et al., 2010). With respect to hormone therapy, the effects of sex steroid hormones on hippocampal function are of particular interest given the importance of this structure to the consolidation of spatial, relational, and contextual memories (Eichenbaum, 1997, 2002; Squire, 1992) and its vulnerability to the detrimental effects of aging and Alzheimer's disease (DeToledo-Morrell, Stoub, & Wang, 2007; Driscoll & Sutherland, 2005). The hippocampus is exceptionally

sensitive to levels of E_2 in circulation, as illustrated by the evidence that elevated levels of E_2 during the natural reproductive (termed estrous) cycle or after exogenous administration significantly enhance hippocampal synaptogenesis (particularly in the CA1 subregion; Stone, Rozovsky, Morgan, Anderson, & Finch, 1998; Woolley & McEwen, 1992, 1993), neurogenesis (Ormerod, Lee, & Galea, 2003; Tanapat, Hastings, Reeves, & Gould, 1999), and synaptic physiology (Foy et al., 1999; Warren, Humphreys, Juraska, & Greenough, 1995; Woolley, 2007). Although some work shows that elevated E_2 and progesterone levels during the estrous cycle are associated with enhanced spatial reference memory (Frick & Berger-Sweeney, 2001), spatial working memory (Pompili, Tomaz, Arnone, Tavares, & Gasbarri, 2010), and spatial strategy use (Korol, Malin, Borden, Busby, & Couper-Leo, 2004), other studies report no such enhancements in tests of spatial or other hippocampal-dependent memories (Berry, McMahan, & Gallagher, 1997; Frye, 1995; Markham & Juraska, 2007; Sutcliffe, Marshall, & Neill, 2007; Walf, Rhodes, & Frye, 2006; Warren & Juraska, 1997), which highlights the difficulty of assessing the effects of rapidly fluctuating hormone levels on hippocampal memory. More reliably, exogenous E_2 administered to ovariectomized young adult rodents enhances several types of hippocampal-dependent memory, including spatial reference memory, spatial working memory, nonspatial working memory, memory for both the location and identity of objects (see Figure 1), inhibitory avoidance and trace eyeblink conditioning (see Conrad & Bimonte-Nelson, 2010; Daniel, 2006; Frick, 2009; Gibbs, 2010) for recent reviews of this literature). However, even in ovariectomized females, not all studies report a beneficial effect of E_2 on hippocampal memory (e.g., Galea, Lee, Kostaras, Sidhu, & Barr, 2002; Galea et al., 2001), and the observance of an E_2 -induced improvement can depend on numerous methodological variables including dose (Holmes, Wide, & Galea, 2002; Wide, Hanratty, Ting, & Galea, 2004), duration of treatment (Luine, Richards, Wu, & Beck, 1998), route of administration (Garza-Meilandt, Cantu, & Claiborne, 2006), extent of daily handling (Bohacek & Daniel, 2007), cognitive demand of the task (Bimonte & Denenberg, 1999), and whether E_2 was administered prior to training (Daniel, Fader, Spencer, & Dohanich, 1997; Gresack & Frick, 2004).

Age-related estrous cycle dysfunction begins at 9–12 months of age in rats (Finch, Felicio, Mobbs, & Nelson, 1984) and 13–14 months of age in mice (Nelson, Karelus, Bergman, & Felicio, 1995); reproductive senescence in rodents is characterized by a gradual lengthening of cycle duration and ultimate cessation of cycling, accompanied by a significant decrease in circulating levels of E_2 and progesterone (LeFevre & McClintock, 1988; Lu, Hopper, Vargo, & Yen, 1979; Nelson et al., 1995). In both rats and mice, the age-related decline of hippocampal memory, particularly of spatial reference memory tested in the Morris water maze, occurs earlier in females than in males, and the onset of this premature spatial memory loss in females coincides with the loss of regular hormone cycling during middle-age (Frick, Burlingame, Arters, & Berger-Sweeney, 2000; Markowska, 1999). Indeed, expression of the canonical intracellular estrogen receptors (ERs) alpha and beta ($ER\alpha$ and $ER\beta$) is decreased in the aged female hippocampus (Adams et al., 2002; Mehra, Sharma, Nyakas, & Vij, 2005; Waters et al., 2011; Yamaguchi-Shima & Yuri, 2007), and the responses of CA1 pyramidal neurons to E_2 (e.g., dendritic spine density, NMDA receptor number, and $ER\alpha$ immunoreactiv-

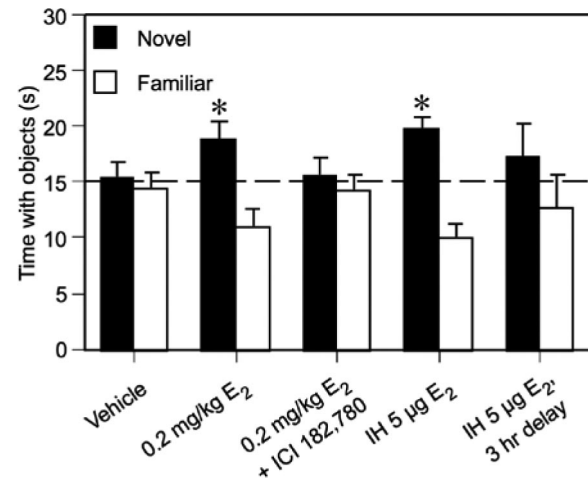


Figure 1. E_2 enhances novel object recognition when injected intraperitoneally or infused directly into the dorsal hippocampus (intrahippocampal, IH). Mice were exposed to two identical objects until they accumulated 30 seconds of exploration. They were then injected with 0.2 mg/kg E_2 immediately after training or infused bilaterally with 5 μ g E_2 /side of the dorsal hippocampus. Forty-eight hours later, mice accumulated 30 seconds exploring an object identical to that used in training (familiar) and a novel object. More time spent with the novel object than chance (dashed line at 15 seconds) indicated memory for the familiar object. Mice receiving 0.2 mg/kg E_2 spent significantly more time than chance with the novel object ($*p < 0.05$), indicating that E_2 enhanced memory for the familiar object. Dorsal hippocampal infusion of the nuclear estrogen receptor antagonist ICI 182,780 (5 μ g/side) blocked this effect, suggesting a role of estrogen receptors α or β in facilitating this memory enhancement. Dorsal hippocampal infusion of 5 μ g/side E_2 immediately, but not 3 hours, after training also significantly enhanced object recognition ($*p < 0.05$ relative to chance). Bars represent the mean \pm standard error of the mean (SEM) time spent with each object in seconds. Time spent with both objects must add up to 30 seconds. From 'Estradiol-induced enhancement of object memory consolidation involves hippocampal ERK activation and membrane-bound estrogen receptors,' by S. M. Fernandez, M. C. Lewis, A. S. Pechenino, L. L. Harburger, P. T. Orr, J. E. Gresack, G. E. Schafe, and K. M. Frick, 2008, *Journal of Neuroscience*, 28, pp. 8660–8667. Copyright (2008) by the Society for Neuroscience. Adapted with permission.

ity) differ in young and aged females (Adams, Fink, Janssen, Shah, & Morrison, 2004; Adams et al., 2002; Adams, Shah, Janssen, & Morrison, 2001; Miranda, Williams, & Einstein, 1999). Nevertheless, the aging female hippocampus remains responsive to some E_2 treatments, as illustrated by the observation that E_2 treatment in aged female rodents started within a week of ovariectomy can increase synaptogenesis and growth factor levels, increase $ER\beta$ immunoreactivity in CA1 spine synapses, activate protein kinases, normalize intracellular calcium homeostasis, phosphorylate NMDA receptors, and block the induction of long-term depression (Bi, Foy, Thompson, & Baudry, 2003; Fernandez & Frick, 2004; Foster, 2005; Frick, Fernandez, & Bulinski, 2002; Miranda et al., 1999; Waters et al., 2011). Further, chronic E_2 in ovariectomized middle-aged and aged rodents can significantly improve hippocampal-dependent spatial reference memory, spatial working memory, and novel object recognition (see Frick, 2009 for a review of this literature), although these effects depend critically

on numerous factors, including age at treatment, duration of hormone deprivation prior to treatment, duration and type of treatment, coadministration of a progestin, and environmental stimulation (Frick, 2009). Of these factors, duration of hormone deprivation has recently emerged as particularly critical, due to several studies in both animal models and humans supporting the existence of a critical period after the onset of menopause during which hormone treatment must commence to affect hippocampal function and cognition (Daniel, Hulst, & Berbling, 2006; Fan et al., 2010; Gibbs, 2000; Hamilton et al., 2011; Sherwin & Henry, 2008; Sherwin, 2007). As will be discussed below, this critical period may be linked to the inability of E_2 in aging females to rapidly activate biochemical processes essential for memory consolidation (Fan, Zhao, Orr, Chambers et al., 2010; Hamilton et al., 2011).

In both young and aging females, the effects of estrogens on hippocampal memory are likely mediated by estrogen receptors. In hippocampal neurons, $ER\alpha$ and $ER\beta$ are localized in multiple cellular compartments, including the nucleus, dendritic spine synapses, and axon terminals (Milner et al., 2005; Milner et al., 2001; Waters et al., 2011). Therefore, these intracellular receptors may mediate some effects of E_2 on hippocampal memory via traditional “genomic” mechanisms, whereby the intracellular E_2 -receptor complex acts as a nuclear transcription factor by binding to an estrogen response element on DNA and stimulating gene transcription (Figure 2A). Studies of $ER\alpha$ or $ER\beta$ knockouts ($ER\alpha$ KOs and $ER\beta$ KOs) show that deletion of either receptor can impair spatial reference memory in the Morris water maze (Fugger, Foster, Gustafsson, & Rissman, 2000; Rissman, Heck, Leonard, Shupnik, & Gustafsson, 2002) and that the deficit induced by $ER\alpha$ knockout can be rescued by lentiviral delivery of the $ER\alpha$ gene to the hippocampus (Foster, Rani, Kumar, Cui, & Semple-Rowland, 2008). However, E_2 can still improve spatial memory, inhibitory avoidance, and novel object recognition in female $ER\alpha$ KO mice (Frick et al., 2010; Fugger, Foster, Gustafsson, & Rissman, 2000; Liu et al., 2008), suggesting that $ER\alpha$ need not be present for E_2 to influence these types of memory. Some data suggest that $ER\alpha$ activation may actually impair hippocampal memory (Fugger et al., 2000; Liu et al., 2008; Rissman, Heck, Leonard, Shupnik, & Gustafsson, 2002; Walf, Koonce, & Frye, 2008). In contrast, neither exogenous E_2 nor high proestrus hormone levels facilitate memory in female $ER\beta$ KO mice tested in object recognition or object placement tasks (Walf, Koonce, Manley, & Frye, 2009; Walf et al., 2008), indicating that $ER\beta$ must be present for E_2 to enhance these types of memory. Pharmacological manipulation of the ERs also supports the importance of $ER\beta$ in hippocampal memory, although some evidence indicates an additional role for $ER\alpha$. The $ER\beta$ -selective agonist diarylpropionitrile (DPN, Figure 2B) most consistently mimics the beneficial mnemonic effects of E_2 ; various doses improve spatial memory in the water maze, object placement, and object recognition in ovariectomized rats and mice (Frick et al., 2010; Jacome et al., 2010; Rhodes & Frye, 2006; Walf et al., 2008; Walf et al., 2006). In contrast, several studies report that the $ER\alpha$ -selective agonist propylpyrazole-triol (PPT, Figure 2B) has no effect in the spatial water maze or object recognition tasks (Frick et al., 2010; Jacome et al., 2010; Rhodes & Frye, 2006), whereas others found that PPT enhances object recognition in female rats (Frye, Duffy, & Walf, 2007; Walf et al., 2006). Collectively, the balance of knockout and pharmacological studies support a more important role of $ER\beta$ than $ER\alpha$ in medi-

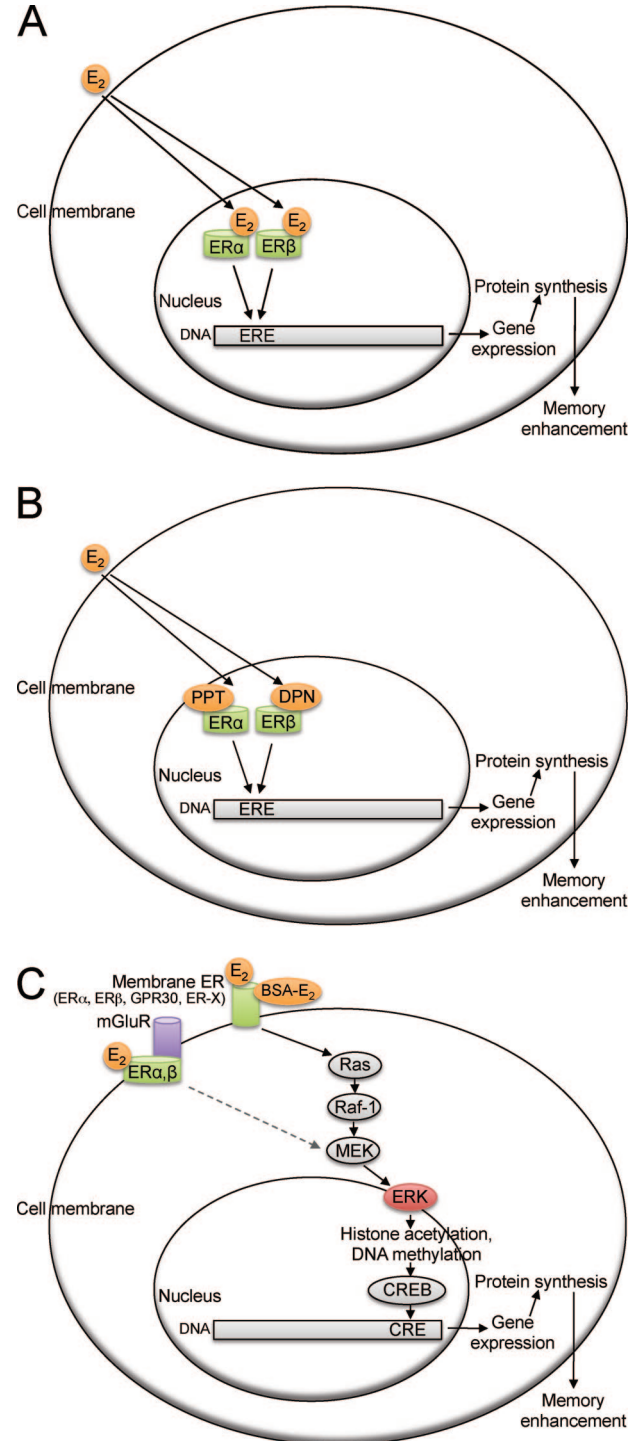


Figure 2. (A) Schematic diagram illustrating the traditional “genomic” mechanism of E_2 action. See text for description. ERE = estrogen response element. (B) Schematic diagram illustrating the putative genomic mechanisms of action of the $ER\alpha$ agonist PPT (propylpyrazole-triol) and $ER\beta$ agonist DPN (diarylpropionitrile). (C) Schematic diagram illustrating putative “non-genomic” mechanisms of E_2 action. See text for description. BSA- E_2 = bovine serum albumin-conjugated E_2 ; mGluR = metabotropic glutamate receptor; ERK = extracellular signal-regulated kinase; MEK = MAPK kinase; CREB = cAMP response element binding protein; CRE = cAMP response element.

ating the mnemonic effects of E_2 , although more research must be done to further support this conclusion.

In addition to genomic actions, the presence of ERs in spine synapses suggests that E_2 may also initiate more rapid “non-genomic” mechanisms at the membrane surface that bypass nuclear estrogen response elements (Figure 2C). Such membrane-initiated effects would include the rapid activation of intracellular signaling cascades and immediate early genes critical for memory consolidation (Fernandez et al., 2008; Guerra-Ariaza et al., 2009; Nilsen & Brinton, 2002, 2003; Webb, Lopez, Uht, & Kushner, 1995; Zhao & Brinton, 2007); however, the specific receptor mechanisms that mediate these membrane effects remain subject to debate (Toran-Allerand, 2000). Rapid effects may be facilitated by ER α and ER β , which can translocate to the plasma membrane upon exposure to E_2 (Razandi, Pedram, Greene, & Levin, 1999; Sheldahl, Shapiro, Bryant, Koerner, & Dorsa, 2008). At the membrane, these receptors interact with metabotropic glutamate receptors to stimulate the phosphorylation of the transcription factor cAMP response element-binding protein (CREB) in a manner dependent on activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling pathway (Boulware, Kordasiewicz, & Mermelstein, 2007; Boulware & Mermelstein, 2009; Boulware et al., 2005). Both CREB and ERK play critical roles in hippocampal long-term memory (Adams & Sweatt, 2002). Indeed, dorsal hippocampal infusion of the ER α /ER β antagonist ICI 182,780 blocks the rapid E_2 -induced increase in ERK phosphorylation (i.e., activation) and object recognition memory consolidation in young females (see Figure 1; Fernandez et al., 2008), suggesting a role for ER α and/or ER β in the effects of E_2 on the hippocampus and memory. Further, the ER agonists PPT and DPN rapidly increase ERK activation in primary hippocampal neurons (Zhao & Brinton, 2007). Although this evidence supports the involvement of intracellular ER α and ER β associated with membrane proteins, other studies suggest a role for integral membrane ERs that bind E_2 on the outer surface of the plasma membrane. Studies using a membrane-impermeable form of E_2 (E_2 covalently linked to bovine serum albumin, called BSA- E_2) report that membrane restricted E_2 enhances 48-hr object recognition memory (Figure 3A; Fernandez et al., 2008; Frye & Rhodes, 2002) and activates hippocampal ERK signaling within 5 minutes of administration (Figure 3B) (Fernandez et al., 2008; Kuroki, Fukushima, Kanda, Mizuno, & Watanabe, 2000). These effects are not significantly blocked by ICI 182,780 (Figure 3A & 3B) (Fernandez et al., 2008), suggesting that membrane ERs alone can mediate the effects of E_2 on hippocampal function. Another putative integral membrane ER is a G-protein coupled receptor called GPR30 (or GPER), which is expressed at high levels in the hippocampus (Brailoiu et al., 2007). GPR30 is structurally unrelated to ER α and ER β , but binds E_2 with high affinity, and can mediate rapid E_2 signaling in numerous cell lines (Moriarty, Kim, & Bender, 2006; Prossnitz, Arterburn, & Sklar, 2007). Preliminary data show that the GPR30 antagonist G-15 impairs spatial working memory in young ovariectomized rats (Hammond & Gibbs, 2011), suggesting that GPR30 may mediate the mnemonic effects of E_2 . However, data from in vitro studies and GPR30 knockout mice have called into question whether GPR30 is truly an ER or, rather, simply collaborates with an ER to promote cell signaling (Langer et al., 2010). Regardless of the specific identity of membrane-associated ERs, several lines of data indicate that these ERs play

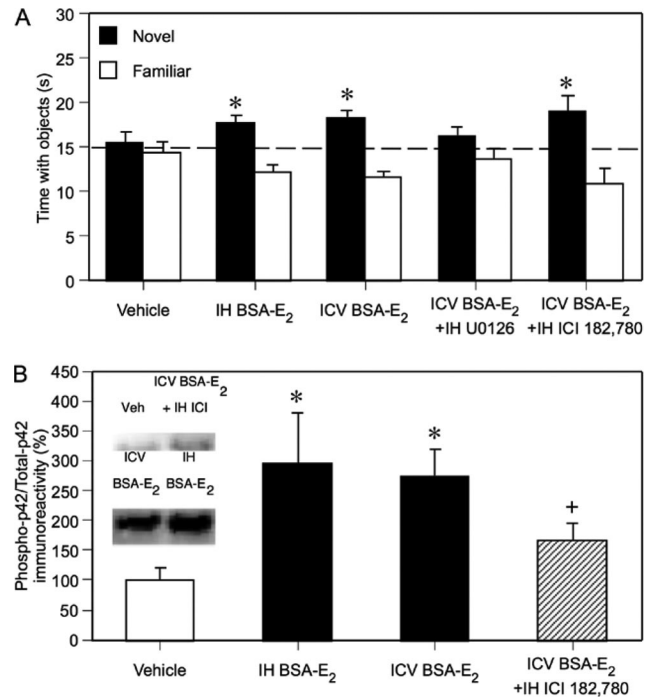


Figure 3. (A) BSA- E_2 (5 μ M) infused bilaterally into the dorsal hippocampus (intrahippocampal, IH) or unilaterally into the dorsal third ventricle (intracerebroventricular, ICV) significantly increased the time spent with the novel object relative to chance ($*p < 0.05$), suggesting that the membrane-impermeable BSA- E_2 enhanced memory for the familiar object. This effect was blocked by IH infusions of the ERK inhibitor U0126 (0.5 μ g/side), but not the ER antagonist ICI 182,780 (5 μ g/side). Bars represent the mean (\pm SEM) time spent with each object. (B) Levels of phosphorylated p42 ERK in dorsal hippocampus were significantly increased 5 minutes after BSA- E_2 infusion; ICI 182,780 did not significantly reduce this effect. Bars represent the mean (\pm SEM) % change from Vehicle ($*p < 0.05$ relative to Vehicle; $+p > 0.05$ relative to all other groups). Inset: Representative Western blots. From ‘Estradiol-induced enhancement of object memory consolidation involves hippocampal ERK activation and membrane-bound estrogen receptors,’ by S. M. Fernandez, M. C. Lewis, A. S. Pechenino, L. L. Harburger, P. T. Orr, J. E. Gresack, G. E. Schafe, and K. M. Frick, 2008, *Journal of Neuroscience*, 28, pp. 8660–8667. Copyright (2008) by the Society for Neuroscience. Adapted with permission. Also reprinted with permission from ‘A new approach to understanding the molecular mechanisms through which estrogens affect cognition,’ by K. M. Frick, S. M. Fernandez, and L. L. Harburger, 2010, *Biochimica et Biophysica Acta - General Subjects*, 1800, pp. 1045–1055. Copyright (2010) by Elsevier.

an important role in mediating the effects of E_2 on the hippocampus. Elucidating how these receptors initiate biochemical processes that promote memory formation will be vital to understanding the neurobiological mechanisms underlying estrogenic modulation of memory.

Building a Better Hormone-Based Therapy: The Downstream Molecule Model as a Guide

Our own research into putative new targets for drug development has been guided by a conceptual model that we have termed the “Downstream Molecule” model (see Figure 4). The goal of this

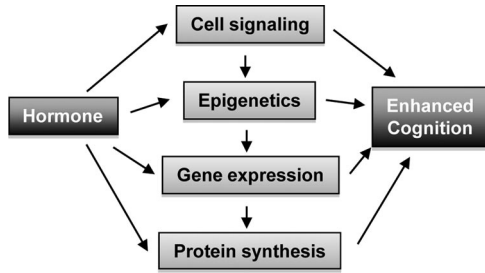


Figure 4. Schematic diagram of the Downstream Molecule model. The model proposes that hormones can enhance memory by influencing hippocampal cell signaling, epigenetic processes, gene expression, or protein synthesis either independently (angled arrows to the right of “Hormone”) or in sequence (downward arrows in the center). These four biochemical processes may enhance cognition via sequential activation of cell signaling down through protein synthesis or via independent effects of each process (the latter indicated by angled arrows pointing from each process towards “Enhanced Cognition”). From ‘A new approach to understanding the molecular mechanisms through which estrogens affect cognition,’ by K. M. Frick, S. M. Fernandez, and L. L. Harburger, 2010, *Biochimica et Biophysica Acta - General Subjects*, 1800, pp. 1045–1055. Copyright (2010) by Elsevier. Adapted with permission.

model is to pinpoint the biochemical mechanisms in hippocampal neurons downstream from hormone receptors that underlie the memory-enhancing effects of E_2 and progesterone (Frick et al., 2010). Identifying the molecules and cellular mechanisms downstream from hormone receptors that are necessary for these hormones to enhance memory consolidation could lead to the development of nonsteroidal drugs that target these mechanisms rather than the receptors themselves. By circumventing hormone receptors, such drugs should mimic the beneficial effects of hormones on memory without the side effects of current hormone therapies. Further, because these drugs would be nonsteroidal, they could potentially be used to safely and effectively reduce age-related memory decline in both women and men.

Although this article focuses on E_2 , the Downstream Molecule model was conceptualized to apply to any hormone (Frick et al., 2010). The guiding hypothesis behind the model is that rapid, membrane-initiated actions of hormone receptors induce cell signaling events that lead to alterations in epigenetic processes, gene expression, and protein synthesis, leading to enhanced memory (see Figure 4) (Frick et al., 2010). Although hormones may affect the aforementioned processes in sequence leading to memory enhancement, they may also affect each of these processes directly, and each process may contribute uniquely to memory enhancement. Thus, the model can be used flexibly to account for hormone-induced mechanisms that affect memory directly or in conjunction with other cellular processes. With respect to E_2 , our specific hypothesis is that the membrane-initiated actions of estrogen receptors in the dorsal hippocampus induce the initial activation of cell signaling cascades including phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and protein kinase A (PKA), which then activate ERK/MAPK signaling, leading to activation of epigenetic processes such as histone acetylation and DNA methylation that enhance the expression of genes and synthesis of proteins that promote memory consolidation (Figure 5A). The empirical bases underlying this hypothesis will be enumerated in the following

sections. Within the context of this model, the failure of E_2 to enhance memory consolidation in aging females would result from age-related dysfunction in one or more of parts of the model (e.g., failure of E_2 to activate a cell signaling pathway or acetylate a core histone protein). The remainder of this article will discuss recent findings testing various parts of this model for estrogenic modulation of memory as a means of illustrating how this model could be used to identify the critical molecules necessary for hormones to affect cognition.

Testing Memory in the Downstream Molecule Model

Our attempts to pinpoint the molecules through which E_2 enhances memory formation have utilized several key components: (a) *a one-trial learning task* to directly link rapid E_2 -induced alterations in hippocampal biochemistry with a specific learning event, (b) *acute posttraining E_2 administration* to pinpoint effects of E_2 specifically to the consolidation phase of memory formation and avoid the confounding influence of E_2 effects on acquisition and performance factors (motivation, attention, arousal) inherent to pretraining E_2 treatments (McGaughy & Sarter, 1999; Morgan & Pfaff, 2001; Pfaff, Frohlich, & Morgan, 2002), (c) *a rapidly metabolized form of E_2* to minimize effects of exogenously administered E_2 on performance variables during testing, (d) *direct infusions into the dorsal hippocampus* to localize effects of E_2 and inhibitor drugs to a specific brain region, and (e) *coadministration of E_2 and inhibitors* of specific biochemical processes (e.g., phosphorylation, acetylation) to determine which molecules must be activated or silenced for E_2 to enhance memory (see Frick et al., 2010 for a more in-depth discussion of these components). Because these design elements allow us to directly link rapid E_2 -induced alterations in hippocampal biochemistry with a specific learning event, their combination permits the specific molecules and cellular processes necessary for E_2 to enhance memory consolidation to be pinpointed more easily than the use of chronic E_2 treatments and multitrial learning tasks. However, once critical molecules and/or cellular events underlying the acute effects of E_2 are identified, their involvement in mediating the mnemonic effects of chronic E_2 treatments will need to be assessed to validate each as a potentially suitable target for drug development.

All of our data from this line of work were collected in ovariectomized C57BL/6 mice because we have accumulated a wealth of information about the effects of aging and hormones on hippocampal memory in this mouse strain (reviewed in Frick, 2009; Frick & Benoit, 2010; Frick et al., 2010; Frick, Zhao, & Fan, 2011). Posttraining treatments were administered immediately after training in most cases, or 2–3 hours after training to establish the time window after training in which treatments are effective. A rapidly metabolized water-soluble form of E_2 encapsulated in 2-hydroxypropyl- β -cyclodextrin was used (Fan et al., 2010; Fernandez et al., 2008; Gresack & Frick, 2006; Gresack, Kerr, & Frick, 2007a; Lewis, Kerr, Orr, & Frick, 2008; Packard & Teather, 1997a, 1997b; Pechenino & Frick, 2009; Zhao et al., 2010) to ensure that E_2 was not in circulation during retention testing. Cyclodextrin-encapsulated hormones are metabolized within hours (Pitha & Pitha, 1985), and because E_2 is not in circulation during training or testing, its specific effects on memory consolidation can be examined in the absence of nonmnemonic performance confounds. This form of E_2 enhances hippocampal spatial and object

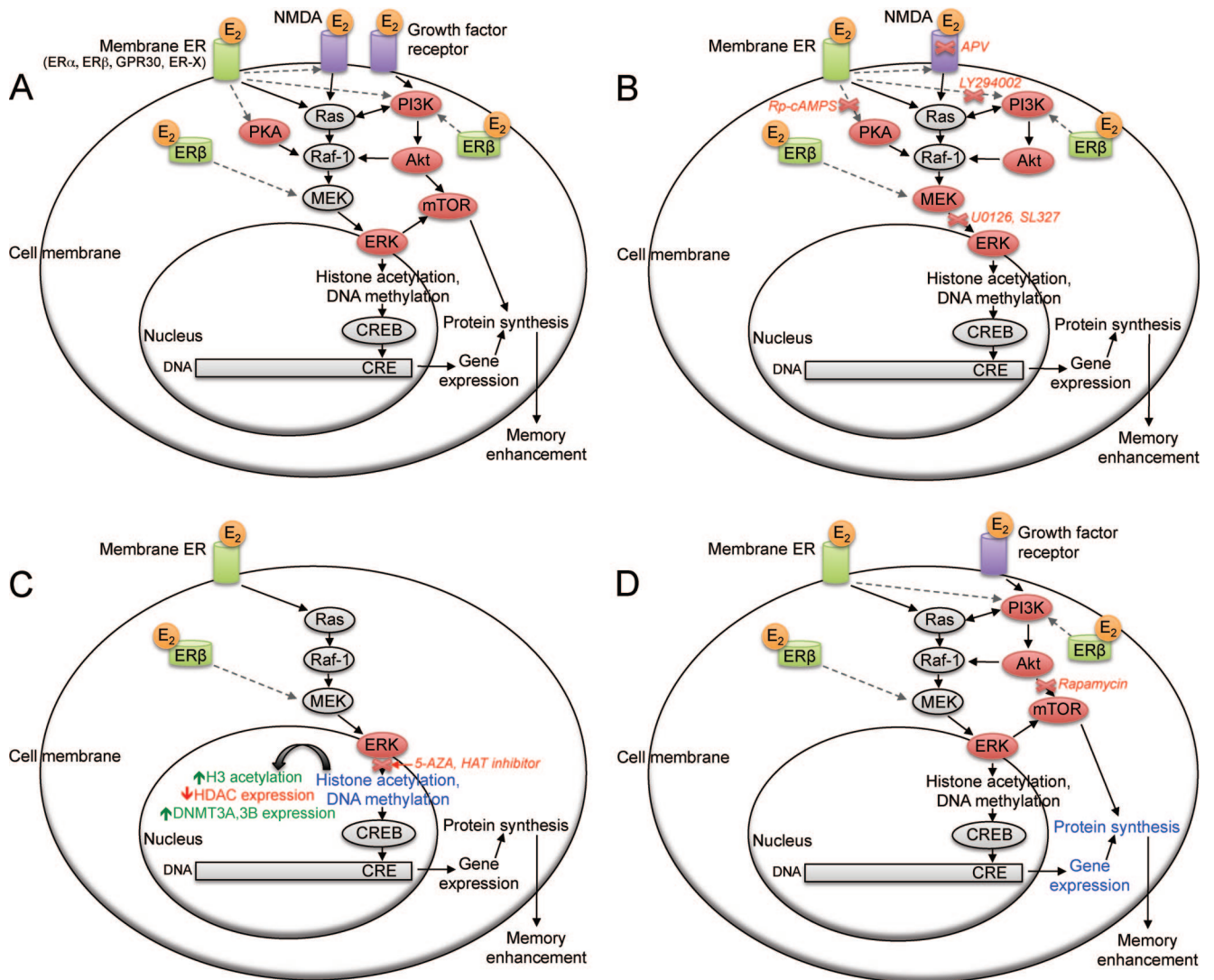


Figure 5. (A) Overall schematic model of the rapid molecular mechanisms uncovered thus far in dorsal hippocampal neurons underlying E_2 -induced enhancement of object memory consolidation in females mice. Solid lines represent events for which evidence exists, whereas dashed lines represent hypothetical relationships. We hypothesize that E_2 binding to a membrane ER or $ER\beta$ activates Ras, PI3K, Akt, and PKA, which can then activate ERK, and stimulate epigenetic alterations (e.g., histone H3 acetylation, increased DNMT3B expression, decreased HDAC2 expression), gene expression, and protein synthesis, thereby enhancing memory. See text for abbreviations and more complete description. Adapted from (Frick, Fernandez, et al., 2010). (B) Illustration highlighting the dorsal hippocampal cell signaling pathways discussed in the text, including the inhibitor drugs used to demonstrate their involvement in E_2 -induced enhancement of object recognition. (C) Illustration highlighting the epigenetic alterations produced in the dorsal hippocampus by E_2 and the inhibitor drugs used to demonstrate the involvement of histone acetylation and DNA methylation in E_2 -induced enhancement of object recognition. (D) Illustration highlighting the putative mechanisms involved in E_2 -induced gene expression and mTOR-mediated protein synthesis. In panels B-D, names of inhibitor drugs are in red italics and their inhibitory actions indicated by red Xs.

recognition memory when injected intraperitoneally (i.p.) in doses of 0.1–0.4 mg/kg (Gresack & Frick, 2006; Packard & Teather, 1997b) and directly into the dorsal hippocampus or dorsal third ventricle in doses of 5–10 μ g per infusion (Fernandez et al., 2008; Packard & Teather, 1997a). For dorsal hippocampal infusions, our infusion protocol results in approximately 1 mm³ of drug diffusion

(Lewis & Gould, 2007), and given the placement of our infusion cannulae, all effects of E_2 should be restricted to the dorsal hippocampus.

For our learning task, we selected novel object recognition because it is a hippocampal task that mice can learn in one trial and involves minimal stress, which is of importance due the well-

documented interactions between gonadal and stress hormones (e.g., Shors, Chua, & Falduto, 2001; Solomon & Herman, 2009; Wood & Shors, 1998). Although the extent to which the hippocampus is involved in novel object recognition has been a matter of recent debate (Dere, Huston, & De Souza Silva, 2007; Mumby, 2001; Winters, Saksida, & Bussey, 2008), the protocol used by our laboratory requires dorsal hippocampal activity as demonstrated by studies in which dorsal hippocampal infusions of the NMDA antagonist APV (Baker & Kim, 2002) or GABA_A antagonist muscimol (Fernandez et al., 2008) blocked object recognition memory consolidation. Therefore, all of our work has focused on the dorsal hippocampus as a locus of E₂'s effects on object recognition. Novel object recognition takes advantage of rodent's natural affinity for novelty. In our testing protocol, mice first accumulate 30 seconds exploring two identical objects in an open square arena (Frick & Gresack, 2003). Immediately after this training (i.e., posttraining), mice are injected systemically or infused directly into the dorsal hippocampus with E₂ and then returned to their home cages. Retention is tested 24 or 48 hours later by presenting mice with one novel and one familiar (identical to training) object. Mice that remember the familiar training object will spend more time than chance (15 seconds) exploring the novel object. In our experience, ovariectomized mice treated with vehicle do not remember the familiar object after 48 hours (Gresack et al., 2007a), so this delay allows us to observe memory-enhancing effects of E₂. As such, data from my laboratory reported below were collected 48 hours after training. We, and others, have repeatedly shown that E₂ administered immediately posttraining significantly enhances novel object recognition (see Figure 1) tested after various delays in young ovariectomized rats and mice when injected systemically (Fernandez et al., 2008; Gresack & Frick, 2006; Gresack et al., 2007a; Lewis et al., 2008; Luine, Jacome, & MacLusky, 2003; Walf et al., 2008; Walf et al., 2006) or infused directly into the dorsal hippocampus (Fan et al., 2010; Fernandez et al., 2008; Lewis et al., 2008; Zhao et al., 2010). Given the consistency of the E₂-induced enhancement across labs and testing protocols, we thought that novel object recognition would be a particularly suitable task for identifying the neurobiological mechanisms underlying the E₂-induced enhancement of memory.

It should be noted, however, that several important caveats apply to the discussion of our work that follows in subsequent sections. First, the novel object recognition task is the only task thus far used by my laboratory in support of the Downstream Molecule model. We have limited our investigation to this task because: (a) E₂ so consistently enhances memory consolidation in the task across various experiments and experimenters, (b) the task is neither appetitively nor aversively motivated, which significantly reduces stress due to an aversive stimulus (e.g., shock, water swimming) or lack of food or water, and (c) the task taps into a rodent's natural curiosity and, thus, mice will readily investigate the objects. However, other one-trial hippocampal tasks (e.g., object placement, contextual fear conditioning, active/passive avoidance) must also be used in future studies to determine the generalizability of the findings reported for object recognition to other forms of hippocampal memory. The second caveat, noted earlier in this review, is that memories modulated or molecules identified as a result of acute posttraining E₂ administration may not be the same as those involved in the effects of chronic E₂ administration on memory. Some evidence to suggest that the mechanisms underlying the mne-

monic effects of acute and chronic E₂ may be similar comes from behavioral work that directly compared the effects of acute and continuous E₂ treatment on spatial working memory in young ovariectomized rats; this work reported similar memory-enhancing effects of both treatments (Sandstrom & Williams, 2004). This similarity may stem from the involvement of hippocampally synthesized E₂ in learning and memory. Levels of E₂ in the rat are sixfold higher in the hippocampus than in plasma (Hojo et al., 2004). In vitro studies report that hippocampal E₂ synthesis is critical for the maintenance of hippocampal spines synapses and synaptic proteins in hippocampal cultures (Hojo et al., 2004; Kimoto, Ishii, Higo, Hojo, & Kawato, 2010; Kretz et al., 2004; Prange-Kiel et al., 2006). Moreover, hippocampal E₂ synthesis may depend on neural excitation, as demonstrated by the observation that stimulation of hippocampal slices with NMDA significantly increases hippocampal E₂ synthesis (Hojo et al., 2004). Together with a recent finding from songbirds that forebrain E₂ levels are rapidly regulated by voltage-gated calcium channels (Remage-Healey, Dong, Maidment, & Schlinger, 2011), these findings may indicate that brain synthesized E₂ can fluctuate quickly in response to depolarization-inducing stimuli such as NMDA-dependent learning. These data raise the possibility that E₂ is synthesized and released in response to specific learning events in order to facilitate learning and memory formation. If so, then this local source of E₂ may be as (or more) important than circulating E₂ in regulating learning and memory; this notion is supported by the rather limited effects in gonadally intact subjects of cyclic hormone fluctuations on learning and memory (as discussed in the previous section). If hippocampally synthesized E₂ is released in response to a learning event, then this response would be similar to our acute posttraining paradigm in which E₂ is infused immediately after a learning event. As such, our investigations using acute posttraining E₂ may provide more naturalistic insights about the biochemical basis of estrogenic modulation than are revealed through the study of chronic E₂ administration. Although chronic E₂ is commonly used in animal models, it is important to note that this chronic E₂ administration is typically not cyclic, and chronic administration would be clinically appropriate as a cognition enhancer only in menopausal women. It is quite likely that hippocampal synthesis of E₂ would be reduced in this population, similar to the age-related decrease in ER α and ER β reported in the hippocampus of aged female rats (Adams et al., 2002; Mehra et al., 2005; Waters et al., 2011; Yamaguchi-Shima & Yuri, 2007). Therefore, chronic E₂ in menopausal women could serve to replace the E₂ that would otherwise have been made by the brain in response to a learning event, and provide a source of consistent E₂ to facilitate memory acquisition and consolidation whenever learning events occur. Although these ideas are purely speculative at this point given the dearth of tools available to accurately measure brain E₂ levels, emerging data strongly suggest that brain E₂ is dynamically regulated by events such as social interactions and auditory stimuli (Remage-Healey, Coleman, Oyama, & Schlinger, 2010; Remage-Healey et al., 2011; Remage-Healey, Maidment, & Schlinger, 2008). Thus, it is plausible that the biochemical mechanisms identified through studies of acute E₂ administration will be functionally relevant to situations of chronic E₂ treatment. Although the

involvement of specific biochemical processes in the mnemonic effectiveness of chronic E_2 treatments would be difficult to assess using infusions of inhibitor drugs as employed with acute posttraining treatments, mice with genetic alterations of particular biochemical pathways (e.g., cell signaling, protein synthesis) or epigenetic processes (e.g., histone acetylation, DNA methylation) could help assess whether the molecules critical for the mnemonic effects of posttraining E_2 are also necessary for chronic E_2 to enhance memory.

Finally, it should be noted that our initial tests of the Downstream Molecule model were primarily conducted in young ovariectomized females to first understand how E_2 works in an optimally functioning system. However, we have begun to test parts of the model with middle-aged and aged females as described below. The goal of future work with aging females will be to determine whether age-related dysfunction in one or more parts of the model account for the critical period of response to estrogen treatment, and whether the age-related loss of sex steroid hormones render some biochemical processes more vulnerable to the effects of aging than others.

Cell Signaling Pathways Critical for E_2 -Induced Memory Enhancement

Our first tests of the Downstream Molecule model focused on dorsal hippocampal cell signaling (Figure 5B). Initial attention centered on the ERK/MAPK signaling pathway, which must be phosphorylated in the hippocampus in order for this structure to form long-term memories (Adams, Roberson, English, Selcher, & Sweatt, 2000; Adams & Sweatt, 2002; Kelly, Laroche, & Davis, 2003; Sweatt, 2004). ERK is activated by numerous upstream kinases, including PI3K/Akt, PKA, and PKC (Adams & Sweatt, 2002; Chen et al., 2005). Two ERK isoforms, p42 (42 kDa) and p44 (44 kDa), are phosphorylated by the exclusive upstream activator MAPK kinase (MEK; Adams et al., 2000). Although both isoforms can be activated by hippocampal learning (Atkins, Selcher, Petraitis, Trzaskos, & Sweatt, 1998; Kelly et al., 2003), reports that deletion of p44 in mice has no effect on hippocampal learning or long-term potentiation (Selcher, Nekrasova, Paylor, Landreth, & Sweatt, 2001; Selcher et al., 2003) suggest that p42 may be more important than p44 in hippocampal learning and memory processes. Phosphorylation of p42 ERK leads to gene transcription, primarily through the transcription factor CREB (Adams et al., 2000), which interacts with histone acetyltransferases and other coactivators to promote gene transcription (Selvi, Cassel, Kundu, & Boutillier, 2010). Although other signal transduction cascades (e.g., cAMP/PKA, PKC) are involved in memory consolidation, particular attention focused on ERK because its activation is necessary for other signaling pathways to activate CREB (Adams & Sweatt, 2002; Impey et al., 1998; Murphy & Segal, 1997). An accumulating body of evidence also implicated ERK in the rapid effects of E_2 . For example, *in vitro* studies reported that E_2 could activate hippocampal ERK within 10 minutes, an effect blocked by inhibitors of MEK (Wade & Dorsa, 2003). Further, MEK inhibitors rapidly blocked E_2 -induced increases in synaptophysin levels, glutamate release, CA1 spine synapses, and neuroprotection in cultured hippocampal neurons (Bi, Broutman, Foy, Thompson, & Baudry, 2000; Ogiue-Ikeda et al., 2008; Yokomaku et al., 2003). Consistent with these rapid

effects *in vitro*, a single infusion of E_2 into the left lateral ventricle increased ERK phosphorylation throughout the hippocampus within 5 minutes (Kuroki et al., 2000). Other work demonstrated that phosphorylation of p42 ERK in the hippocampus was reduced by ovariectomy and restored by E_2 (Bi, Foy, Vouimba, Thompson, & Baudry, 2001). Collectively, these studies demonstrated that E_2 could rapidly regulate hippocampal ERK levels and that ERK activation was necessary for E_2 to affect several aspects of hippocampal function. However, despite the clear relationships among E_2 , ERK signaling, and hippocampal function, no study prior to the work described below had directly linked E_2 -induced memory improvement to any specific alteration in the hippocampus, including ERK signaling. We hypothesized that dorsal hippocampal ERK activation would be necessary for E_2 to enhance novel object recognition.

We had previously demonstrated in young ovariectomized mice (2–3 months old) that a single intraperitoneal (i.p.) injection of 0.2 mg/kg E_2 or bilateral dorsal hippocampal infusion of 5 μ g/side immediately after object recognition training significantly enhanced object recognition memory consolidation. That is, E_2 -treated mice spent more time with the novel object than chance (15 seconds) 48 hours after infusion (see Figure 1) (Fernandez et al., 2008; Gresack & Frick, 2006; Gresack et al., 2007a; Gresack, Kerr, & Frick, 2007b), demonstrating intact memory for the familiar object. Further, infusion of E_2 into the dorsal hippocampus 3 hours after training did not enhance object recognition (see Figure 1) (Fernandez et al., 2008), demonstrating a relatively brief time window in which object recognition memory consolidation occurs. Therefore, we measured whether these treatments could increase ERK activation in the dorsal hippocampus of young ovariectomized mice. We found that both systemic and intrahippocampal E_2 treatments increased phosphorylation of p42 ERK (Figure 6A), but not p44 ERK (data not shown), although the timing naturally differed given their different routes of administration; ERK was increased 60 minutes after i.p. injection and 5 minutes after dorsal hippocampal infusion (Fernandez et al., 2008). The effects of 0.2 mg/kg E_2 on phospho-p42 ERK levels were blocked by i.p. injection of the MEK inhibitor SL327 (30 mg/kg) (Figure 6A) and its effects on 48-hr object recognition were blocked by dorsal hippocampal infusion of the MEK inhibitor U0126 (0.5 μ g/side) (Figure 6B; Fernandez et al., 2008). These findings indicated that dorsal hippocampal ERK activation is necessary for E_2 to enhance object recognition. We next sought to determine if dorsal hippocampal infusion of U0126 would block the behavioral effects of intracranially infused E_2 . In order to prevent tissue damage from repeated infusions into the hippocampus, we infused E_2 into the dorsal third ventricle (intracerebroventricular (ICV), 5 μ g total) as a means of supplying E_2 to the hippocampus concurrently with a dorsal hippocampal infusion of the inhibitor U0126. ICV-infused E_2 enhanced 48-hr object recognition, and this effect was blocked by intrahippocampal (IH) U0126 infusion (Figure 6C; Fernandez et al., 2008; Zhao et al., 2010). Together, these data provided the first demonstration that dorsal hippocampal ERK activation is necessary for systemically or intracranially administered E_2 to enhance object memory consolidation in young ovariectomized female mice.

Support for the importance of ERK in the mnemonic effects of E_2 subsequently came from studies of aging females. In rats, the activation of p42 and p44 ERK in the hippocampus and basal forebrain of aged males is reduced relative to young males in response to growth factor treatment (Gooney, Messaoudi, Maher,

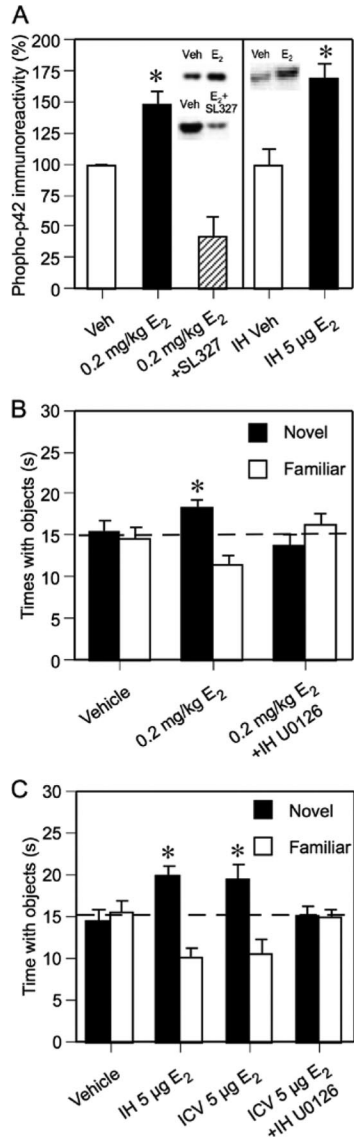


Figure 6. (A) Phospho-p42 ERK levels in the dorsal hippocampus were significantly increased 1 hour after 0.2 mg/kg E₂ injection or 5 minutes after dorsal hippocampal (intrahippocampal, IH) E₂ infusion. The MEK inhibitor SL327 (30 mg/kg) blocked the effect of 0.2 mg/kg E₂. Bars represent the mean (\pm SEM) % change from Vehicle ($^*p < 0.05$ relative to Vehicle). Inset: Representative Western blots. (B) Dorsal hippocampal infusion of the MEK inhibitor U0126 (0.5 μ g/side) blocked the memory enhancing effects of 0.2 mg/kg E₂ ($^*p < 0.05$ relative to chance, 15 seconds). (C) Object recognition was significantly enhanced by IH E₂ (5 μ g/side) or intracerebroventricular (ICV) E₂ (10 μ g) ($^*p < 0.05$ relative to chance). IH U0126 blocked the effect of ICV E₂. From 'Estradiol-induced enhancement of object memory consolidation involves hippocampal ERK activation and membrane-bound estrogen receptors,' by S. M. Fernandez, M. C. Lewis, A. S. Pechenino, L. L. Harburger, P. T. Orr, J. E. Gresack, G. E. Schafe, and K. M. Frick, 2008, *Journal of Neuroscience*, 28, pp. 8660–8667. Copyright (2008) by the Society for Neuroscience. Adapted with permission. Also from 'Epigenetic alterations regulate the estradiol-induced enhancement of memory consolidation,' by Z. Zhao, L. Fan, and K. M. Frick, 2010, *Proceedings of National Academy of Sciences, USA*, 107, pp. 5605–5610. Copyright (2010) by the National Academy of Sciences. Adapted with permission.

Bramham, & Lynch, 2004; Williams, Granholm, & Sambamurti, 2007; Williams, Bimonte-Nelson, & Granholm-Bentley, 2006). To determine if ERK phosphorylation was affected by aging in females, we measured levels of phospho-p42 and -p44 ERK in young (3 months old), middle-aged (17 months old), and aged (21 months old) female mice who were exposed to the object recognition testing chamber with or without objects. Although object training had no effect on ERK activation, age significantly reduced p42 ERK such that phospho-p42 levels in aged female mice were significantly lower than those of young and middle-aged females (Figure 7A; phospho-p44 ERK was not affected by age or training; Fan et al., 2010). These data naturally led to the prediction that E₂ should increase phospho-p42 ERK levels in young and middle-aged females, but not in aged females. Indeed, the same 5 μ g/side dose of E₂ that increased phospho-p42 ERK levels in young females (Fernandez et al., 2008) also significantly increased dorsal hippocampal phospho-p42 levels in middle-aged females (Figure 7C; Fan et al., 2010). As in young females, this increase was blocked by dorsal hippocampal infusion of 0.5 μ g/side U0126 (Figure 7C; Fan et al., 2010). However, in aged females, the same E₂ infusion had no effect on dorsal hippocampal phospho-ERK levels (A. Pechenino, personal communication), consistent with the age-related decrease in p42 ERK phosphorylation (Fan et al., 2010). These findings then led to another prediction: if dorsal hippocampal ERK activation is critical to the mnemonic effects of E₂, then E₂ should enhance object recognition in middle-aged, but not aged, females. Consistent with this hypothesis, dorsal hippocampal infusion of 0.5 μ g/side U0126 blocked the memory enhancing effects of 0.2 mg/kg E₂ (Figure 7B; Fan et al., 2010). However, in aged females, the same E₂ infusion had no effect on dorsal hippocampal phospho-ERK levels (A. Pechenino, personal communication), consistent with the age-related decrease in p42 ERK phosphorylation (Fan et al., 2010). These findings suggest several important conclusions with respect to E₂ and aging. First, that the hippocampus of middle-aged females can be as responsive to E₂ as that of young females. Second, that similar dorsal hippocampal signaling mechanisms appear to mediate E₂-induced object recognition memory enhancement in young and middle-aged females. Finally, that the inability of E₂ to activate ERK, and perhaps related signaling pathways (see below), in aged females may underlie its failure to enhance object recognition memory in advanced age. Our findings in aging females support the "critical period hypothesis," which maintains that E₂ benefits cognition only in recently menopausal women (Maki, 2006; Sherwin & Henry, 2008). Age-related dysfunction in one or more signaling pathways could help explain the etiology of this critical period. Better characterization of age-related alterations in these pathways could ultimately lead to treatments that delay cognitive decline or lengthen the critical period by ameliorating this dysfunction.

Not surprisingly, signaling mechanisms upstream from ERK are also required for E₂ to enhance object recognition in females. In one recent article, we examined the involvement of NMDA receptors and PKA signaling in the effects of E₂, given that activation of both is critical for ERK-mediated long-term memory formation (Adams & Sweatt, 2002). Young ovariectomized mice were injected immediately after object recognition training with 0.2 mg/kg E₂ and infused into the dorsal hippocampus with the PKA inhibitor Rp-cAMPS (18 μ g/side) or NMDA receptor antagonist APV (D-2-Amino-5-phosphonovaleric acid, 2.5 μ g/side). In another set of mice, ERK phosphorylation was examined 1 hr after treatment. Both drugs blocked the E₂-induced increase in 48-hr object rec-

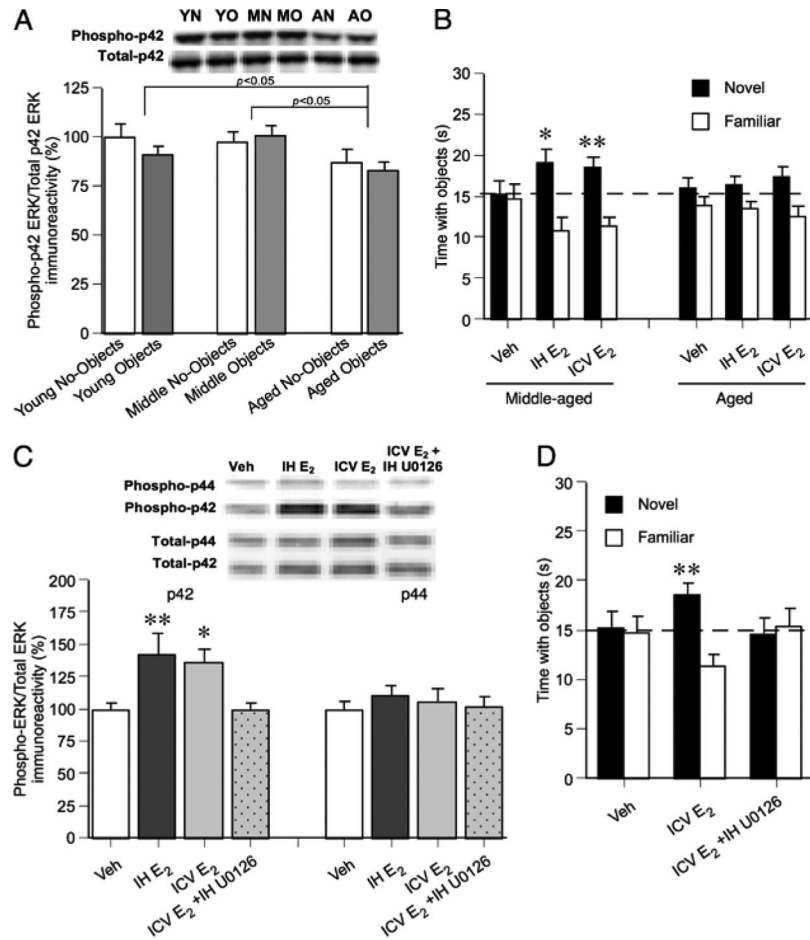


Figure 7. (A) Phospho-p42 ERK levels in the hippocampus of young, middle-aged, and aged females exposed to 2 identical objects or no objects in the testing arena. Levels were significantly decreased in aged females relative to young and middle-aged females. (B) In middle-aged females, intrahippocampal (IH, 5 μ g/side) and intracerebroventricular (ICV, 10 μ g) E_2 infusion significantly enhanced object recognition ($^{**}p < 0.01$, $^*p < 0.05$ relative to chance) in middle-aged, but not aged, females. (C) In middle-aged females, IH and ICV E_2 infusion significantly increased dorsal hippocampal activation of p42, but not p44, ERK 15 minutes after infusion ($^{***}p < 0.01$, $^*p < 0.05$ relative to Vehicle). The increase induced by ICV E_2 was blocked by the MEK inhibitor IH U0126. (D) In middle-aged females, the ICV E_2 -induced enhancement of 48-hour object recognition ($^{**}p < 0.01$ relative to chance) was blocked by IH U0126. Bars represent the mean (\pm SEM) % change from Vehicle (A&C) or chance (B&D). Insets: Representative Western blots. From 'Estradiol-induced object memory consolidation in middle-aged female mice requires dorsal hippocampal extracellular signal-regulated kinase and phosphatidylinositol 3-kinase activation,' by L. Fan, Z. Zhao, P. T. Orr, C. H. Chambers, M. C. Lewis, and K. M. Frick, 2010, *Journal of Neuroscience*, 30, pp. 4390–4400. Copyright (2010) by the Society for Neuroscience. Adapted with permission.

ognition and dorsal hippocampal phospho-p42 ERK levels (Lewis et al., 2008), suggesting that NMDA receptors and PKA signaling must be activated upstream from ERK to facilitate E_2 's rapid effects on object recognition memory.

The involvement of another signaling pathway, PI3K/Akt, was examined in the study of aging females described above. Activation of PI3K is necessary for hippocampal contextual memory retrieval, and activation of ERK during memory retrieval depends on PI3K activity (Chen et al., 2005). Further, evidence that dorsal hippocampal infusion of the PI3K inhibitor LY294002 prevents the depolarization-induced increase in ERK activation and ERK-mediated gene tran-

scription strongly suggests that PI3K is an upstream activator of ERK (Chen et al., 2005). PI3K is also crucial for hippocampal plasticity and object recognition (Horwood, Dufour, Laroche, & Davis, 2006; Kelly & Lynch, 2000; Lin et al., 2001), is rapidly activated by E_2 (Mannella & Brinton, 2006; Yokomaku et al., 2003), and is vital for E_2 -induced neuroprotection (Singh, 2001). Given the importance of neuroprotection for the aging brain, we thought that hippocampal PI3K/Akt signaling might play a pivotal role in E_2 -induced object recognition enhancement in aging females, perhaps by activating p42 ERK. In ovariectomized middle-aged females, infusion of E_2 into the dorsal hippocampus (5 μ g/side) or dorsal third ventricle (10 μ g total) sig-

nificantly increased dorsal hippocampal levels of phospho-PI3K (Figure 8A) and phospho-Akt (Figure 8B) 5 minutes later (Fan et al., 2010). In aged females, dorsal hippocampal infusion of 5 $\mu\text{g}/\text{side}$ E_2 had no effect on dorsal hippocampal levels of either phospho-PI3K or phospho-Akt (Fan et al., 2010), consistent with the inability of E_2 to enhance object recognition at this age (Fan et al., 2010). In middle-aged females, dorsal hippocampal infusion of the PI3K inhibitor LY294002 (0.0005 $\mu\text{g}/\text{side}$) prevented ICV-infused E_2 from increasing both object recognition (Figure 8C) and dorsal hippocampal p42 ERK activation (Figure 8D; Fan et al., 2010), suggesting that PI3K/Akt activation is necessary for E_2 to enhance object recognition and activate ERK. We recently replicated this finding in young ovariectomized females (Fan, Zhao, Orr, & Frick, 2010). Interestingly, the finding that LY294002 in middle-aged females prevented the E_2 -induced increase in dorsal hippocampal ERK suggests that PI3K activation occurs upstream from p42 ERK activation. In support of this notion, dorsal hippocampal infusion of U0126 (0.5 $\mu\text{g}/\text{side}$) had no effect on the ability of ICV-infused E_2 to increase dorsal hip-

pocampal phospho-PI3K levels (Figure 8E; Fan et al., 2010) as would be expected if E_2 -induced ERK activation occurs downstream from PI3K activation.

To summarize, our cell signaling data collected to date suggest several key points about the rapid biochemical mechanisms involved in E_2 -induced modulation of object recognition in females. First, systemic or intracranially administered E_2 can rapidly activate multiple cell signaling cascades (e.g., PI3K, Akt, ERK) in the dorsal hippocampus. Second, the E_2 -induced enhancement of object recognition memory consolidation is dependent on activation in the dorsal hippocampus of NMDA receptors and of the PI3K, Akt, PKA, and ERK signaling cascades, with activation of PI3K, Akt, and PKA occurring prior to ERK activation. Third, a single posttraining infusion of E_2 can enhance object recognition in both young and middle-aged females, and this effect depends on activation of PI3K and ERK in the dorsal hippocampus. Finally, the inability of E_2 to enhance object recognition in aged females may result from age-related dysfunction in PI3K, Akt, and/or ERK signaling.

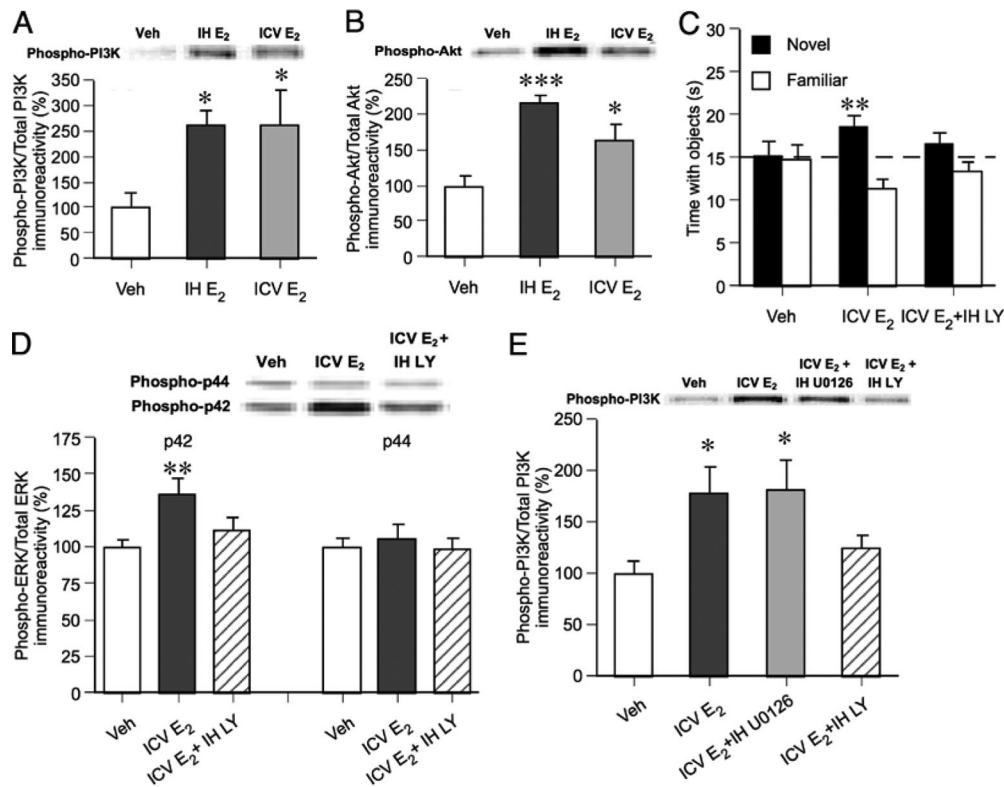


Figure 8. (A & B) In middle-aged females, intrahippocampal (IH, 5 $\mu\text{g}/\text{side}$) and intracerebroventricular (ICV, 10 μg) E_2 significantly increased dorsal hippocampal PI3K (A) and Akt (B) phosphorylation 5 minutes after infusion ($***p < 0.001$, $*p < 0.05$ relative to Vehicle). (C) The enhancement of 48-hour object recognition induced by post-training ICV E_2 was blocked by IH infusion of the PI3K inhibitor LY294002 (LY, 0.0005 $\mu\text{g}/\text{side}$) ($**p < 0.01$ relative to chance). (D) LY blocked the increase in dorsal hippocampal phospho-p42 ERK levels induced by ICV E_2 ($*p < 0.01$ relative to Vehicle); p44 ERK levels were not affected by either drug. (E) LY, but not the MEK inhibitor U0126 (0.5 $\mu\text{g}/\text{side}$), prevented ICV E_2 from increasing phospho-PI3K levels in the dorsal hippocampus. Bars represent the mean (\pm SEM) % change from Vehicle (A,B,D,E) or chance (C). Insets: Representative Western blots. From 'Estradiol-induced object memory consolidation in middle-aged female mice requires dorsal hippocampal extracellular signal-regulated kinase and phosphatidylinositol 3-kinase activation,' by L. Fan, Z. Zhao, P. T. Orr, C. H. Chambers, M. C. Lewis, and K. M. Frick, 2010, *Journal of Neuroscience*, 30, pp. 4390–4400. Copyright (2010) by the Society for Neuroscience. Adapted with permission.

The Role of Epigenetic Processes in Mediating the Effects of E₂

Because the aforementioned data suggested that ERK phosphorylation is a final common outcome for various signaling pathways activated by E₂ in the dorsal hippocampus, we next became interested in events downstream from ERK necessary for estrogenic modulation of memory. Because ERK can promote gene expression through mechanisms including CREB activation, E₂ might rapidly stimulate gene transcription via ERK activation. Gene transcription is regulated by epigenetic processes including histone modifications and DNA methylation. Both processes regulate the accessibility to DNA by transcription factors like CREB and *c-fos*. ERK activation in the dorsal hippocampus leads to epigenetic modifications, including histone acetylation, histone phosphorylation, and DNA methylation, that alter transcriptional regulation and produce lasting changes in gene expression and hippocampal memory (Chwang, O'Riordan, Levenson, & Sweatt, 2006; Levenson et al., 2004; Miller, Campbell, & Sweatt, 2008; Miller & Sweatt, 2007). Given the importance of ERK activation to the mnemonic effects of E₂, we reasoned that epigenetic processes might also be critical for E₂ to enhance memory consolidation (Figure 5C). Thus far, we have examined the roles of histone acetylation and DNA methylation in mediating the effects of E₂ on object recognition, and this work will be described in the sections below.

The basic element of chromatin is the nucleosome (Figure 9A), which consists of a core of four histone proteins (two each of histones H2A, H2B, H3 and H4) encircled by superhelical DNA (Luger, Mäder, Richmond, Sargent, & Richmond, 1997). Each core histone has a tail that can be modified by processes such as acetylation, phosphorylation, methylation, ubiquitination, and sumoylation (Kouzarides, 2007). Acetylation of lysine residues on histone tails relaxes the bond between the histones and DNA, which allows transcription factors to gain access to the DNA (Strahl & Allis, 2000). Therefore, histone acetylation is associated with an increase in gene expression. Histone acetylation is dynamically controlled by enzymes that acetylate lysine residues, termed histone acetyltransferases (HATs), and those that deacetylate these residues, termed histone deacetylases (HDACs, Figure 9A; Yang, 2007). The importance of histone acetylation in hippocampal memory formation has been demonstrated in studies of HDAC inhibitor drugs and mice with faulty HAT activity. Genetic disruption of HAT activity in mice impairs spatial memory, novel object recognition, and hippocampal synaptic plasticity, and these deficits are rescued by HDAC inhibitors (Alarcón et al., 2004; Barrett et al., 2011; Duclot, Jacquet, Gongora, & Maurice, 2010; Korzus, Rosenfeld, & Mayford, 2004; Maurice et al., 2008; Oliveira, Wood, McDonough, & Abel, 2007; Wood et al., 2005). In contrast, expression of certain HDACs, like HDAC2 and HDAC3, is detrimental to hippocampal memory, reduces synaptic plasticity, and suppresses the expression of plasticity-related genes in the hippocampus (Guan et al., 2009; McQuown et al., 2011). Indeed, HDAC inhibitors, such as trichostatin-A (TSA) and sodium butyrate, prevent deacetylation of histones by HDACs and enhance induction of hippocampal long-term potentiation (LTP) and memory in contextual fear conditioning, object recognition, and spatial memory tasks (Haettig et al., 2011; Stefanko, Barrett, Ly, Reolon, & Wood, 2009; Zhao et al., 2010). HDAC inhibitors also rescue

hippocampal memory deficits in mouse models of Alzheimer's disease (Kilgore et al., 2010; Ricobaraza et al., 2009) and traumatic brain injury (Dash, Orsi, & Moore, 2009), supporting the potential utility of these compounds in treating memory dysfunction. Hippocampal learning itself increases the acetylation of histone H3, but not H4, in the hippocampus, and this effect is dependent on activation of NMDA receptors and ERK (Levenson et al., 2004). Pharmacological activation of ERK also increases hippocampal histone H3, but not H4, acetylation, and ERK activation is necessary for other protein kinases (e.g., protein kinase C, PKC) to increase hippocampal H3 acetylation (Levenson et al., 2004). In total, the accumulated data suggest that histone H3 acetylation regulates long-term hippocampal memory formation and that ERK activation is essential to this process. Given that E₂ also regulates hippocampal memory consolidation in an ERK-dependent manner, we hypothesized that E₂ would increase hippocampal H3 acetylation and that the E₂-induced enhancement of object recognition would depend not only on hippocampal ERK activation, but also on hippocampal histone acetylation.

Before examining the role of histone acetylation in the mnemonic effects of E₂, we first demonstrated that histone acetylation regulates novel object recognition as tested by our protocol. We found that dorsal hippocampal infusion of the HDAC inhibitor TSA (16.5 mM/side) immediately (Figure 9B), but not 3 hours (data not shown), after training significantly enhanced 48-hr object recognition in young ovariectomized mice (Zhao et al., 2010). Consistent with its effects in other studies (Levenson et al., 2004), TSA also significantly increased acetylation of histones H3 (Figure 9C) and H4 (data not shown) in the dorsal hippocampus 30 minutes after infusion (Zhao et al., 2010). Although dorsal hippocampal infusion of E₂ (5 µg/side) also increased histone acetylation 30 minutes after infusion, its effects were specific to histone H3 (Figure 9C; Zhao et al., 2010), which is consistent with the effects of fear learning and pharmacological ERK activation on histone acetylation (Levenson et al., 2004). We next showed that dorsal hippocampal ERK activation is necessary for E₂ to influence histone acetylation, as illustrated by the finding that dorsal hippocampal infusion of U0126 completely blocked the E₂-induced increase in histone H3 acetylation (Figure 9D; Zhao et al., 2010). We then reasoned that the increase in histone H3 acetylation might result from a reduction in HDAC expression, and so measured protein levels of HDAC1 and HDAC2 at various times after dorsal hippocampal E₂ infusion. HDAC1 regulates cell proliferation and differentiation, and is neuroprotective in mouse forebrain (Kim et al., 2009). HDAC2 overexpression impairs several forms of hippocampal memory and reduces hippocampal synaptic plasticity in adult mice (Guan et al., 2009). Consistent with the detrimental effects of HDAC2 on hippocampal memory (Guan et al., 2009), dorsal hippocampal infusion of E₂ significantly decreased dorsal hippocampal levels of HDAC2 4 hours after infusion (Figure 9E), whereas HDAC1 levels were not affected (data not shown; Zhao et al., 2010). This finding suggests that E₂ represses the translation of proteins, such as HDAC2, that are detrimental to hippocampal memory. In support of this notion, a recent microarray study found that hippocampal HDAC2 expression was significantly increased, and spatial memory significantly impaired, in middle-aged ovariectomized rats relative to young rats (Aenlle, Kumar, Cui, Jackson, & Foster, 2009). Three weeks of E₂ treatment reduced HDAC2 expression and improved spatial mem-

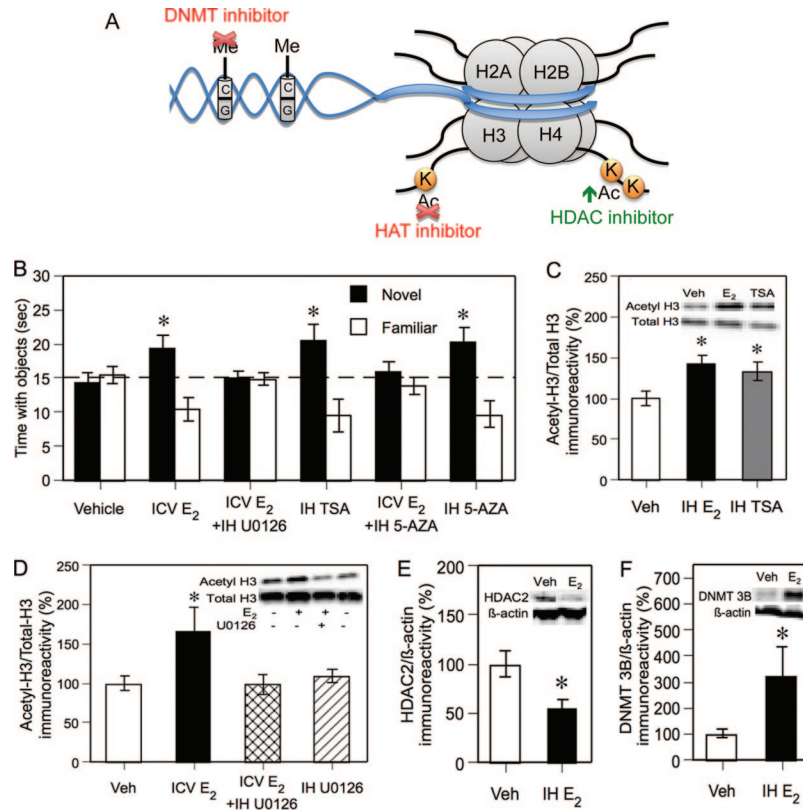


Figure 9. (A) Schematic diagram of the nucleosome illustrating the processes of histone acetylation and DNA methylation, and drugs that affect each. (B) Mice receiving intracerebroventricular (ICV) infusion of E₂ (10 μg), bilateral dorsal hippocampal (intrahippocampal, IH) infusion of the HDAC inhibitor TSA (16.5 mM/side) or DNMT inhibitor 5-AZA (100 μg/side) spent significantly more time than chance with the novel object ($p < 0.05$ relative to chance), demonstrating memory for the familiar object. IH infusion of the MEK inhibitor U0126 (0.5 μg/side) or 5-AZA completely blocked the ability of ICV E₂ to enhance object recognition. (C) IH infusion of E₂ or TSA significantly increased dorsal hippocampal histone H3 (lys 14) acetylation relative to Vehicle (Veh; $p < 0.05$) 30 minutes after infusion. (D) IH infusion of U0126 completely prevented ICV E₂ from increasing dorsal hippocampal histone H3 (lys 9,14) acetylation ($p < 0.05$ E₂ relative to Veh). (E) IH infusion of E₂ significantly decreased HDAC2 protein levels relative to Vehicle 4 hours after infusion ($p < 0.05$). (F) IH infusion of E₂ significantly increased DNMT3B protein levels relative to Vehicle 4 hours after infusion ($p < 0.05$). Bars represent the mean (\pm SEM) % change from Vehicle (B,C,D,E) or chance (A). Insets: Representative Western blots. Panels B-F from 'Epigenetic alterations regulate the estradiol-induced enhancement of memory consolidation,' by Z. Zhao, L. Fan, and K. M. Frick, 2010, *Proceedings of National Academy of Sciences, USA*, 107, pp. 5605–5610. Copyright (2010) by the National Academy of Sciences. Adapted with permission. Also from 'The epigenetics of estrogen: Epigenetic regulation of hormone-induced memory enhancement,' by K. M. Frick, Z. Zhao, and L. Fan, 2011, *Epigenetics*, 6, pp. 675–680. Copyright (2011) by Landes Bioscience. Reprinted with permission.

ory in middle-aged females (Aenlle et al., 2009), providing additional evidence linking E₂-induced repression of HDAC2 expression to improved memory. Finally, we recently demonstrated using a HAT inhibitor that histone acetylation is necessary for E₂ to enhance object recognition. Our preliminary data show that dorsal hippocampal infusion of this inhibitor prevents E₂ from enhancing 48-hr object recognition and increasing hippocampal histone H3 acetylation in young ovariectomized mice (Frick, Zhao, & Fan, 2010). As such, our data strongly suggest that ERK-driven histone acetylation is necessary for E₂ to enhance object recognition memory consolidation in young female mice.

We next examined the involvement of DNA methylation in the mnemonic effects of E₂. DNA methylation typically silences gene

expression, although the functional effects of this transcriptional repression depend on the specific genes altered. DNA (cytosine-5') methyltransferases (DNMTs, subtypes 1, 3A, and 3B) methylate cytosine residues in genomic regions with a high percentage of cytosine and guanine nucleotides (CpG islands; Figure 9A), which decreases transcriptional access to DNA. DNMT1 is a maintenance methyltransferase that transfers methylation to nascent DNA strands during replication (Bird, 2002). DNMTs 3A and 3B are de novo methyltransferases that catalyze the methylation of previously unmethylated cytosine residues (Bird, 2002). Evidence that the de novo methyltransferases are critical for hippocampal learning comes from studies of contextual fear conditioning, which show that fear learning significantly increases the expression of DNMT3A and DNMT3B,

but not DNMT1, mRNA (Levenson et al., 2006; Miller et al., 2008; Miller & Sweatt, 2007). PKC activation also increases expression of DNMT3A (Levenson et al., 2006), linking DNMT activity to cell signaling. Fear conditioning increases hippocampal methylation of genes that suppress memory (e.g., *protein phosphatase 1* or *PPI*) and decreases methylation of genes that facilitate memory (e.g., *reelin*; Miller & Sweatt, 2007), which more directly links methylation, gene expression, and memory formation. The necessity of DNA methylation for hippocampal learning was demonstrated by findings showing that DNMT inhibitors such as 5-Aza-deoxycytidine (5-AZA) and zebularine block hippocampal LTP and long-term fear memory consolidation (Levenson et al., 2006; Miller et al., 2008). These inhibitors also prevent the increase in histone H3 acetylation induced by fear conditioning or cell signaling activators (Levenson et al., 2006; Miller et al., 2008), suggesting that DNA methylation regulates histone acetylation. However, the interactions between DNA methylation and histone acetylation appear bidirectional, as the effects of 5-AZA on fear conditioning and LTP are blocked by HDAC inhibition (Miller et al., 2008).

To determine if DNA methylation is involved in the estrogenic modulation of object recognition, we first assessed in young ovariectomized mice whether dorsal hippocampal infusion of E₂ (5 µg/side) could affect the expression of DNMT1, DNMT3A, and DNMT3B. E₂ significantly increased DNMT3A and DNMT3B mRNA in the dorsal hippocampus 45 minutes after infusion (data not shown), and increased dorsal hippocampal DNMT3B protein 4 hours after infusion (Figure 9F; Zhao et al., 2010). DNMT1 mRNA and protein were not affected (Zhao et al., 2010). Our preliminary data show that dorsal hippocampal infusion of a HAT inhibitor prevents ICV-infused E₂ from increasing dorsal hippocampal DNMT3B levels (Frick et al., 2010), indicating that histone acetylation can regulate the expression of DNMT enzymes. To demonstrate that alterations in DNA methylation were necessary for E₂ to enhance object recognition, young ovariectomized mice were infused immediately or 3 hours after object training with 5-AZA into the dorsal hippocampus (100 µg/side) concurrently with ICV infusion of E₂. Infusion of 5-AZA prevented E₂ from enhancing 48-hr object recognition when given immediately (Figure 9B), but not 3 hours (data not shown), after training (Zhao et al., 2010), suggesting the importance of methylation soon after the learning event. Interestingly, 5-AZA enhanced object recognition when given alone (Figure 9B; Zhao et al., 2010), indicating that object recognition itself depends on DNA methylation. We hypothesize that in the absence of E₂, 5-AZA may prevent the methylation of memory promoter genes like *reelin*, which should then facilitate memory formation. The evidence that 5-AZA blocked the mnemonic effects of E₂ suggests that E₂ enhances memory, at least in part, by methylating memory repressor genes such as *HDAC2*, *HDAC3*, and *PPI*. Methylation patterns in these, and other, genes will need to be examined in future work.

In sum, our epigenetic data demonstrate that both histone H3 acetylation and DNA methylation play vital roles in mediating the beneficial effects of E₂ on object recognition memory consolidation in young females. However, considerably more work needs to be done to better understand how these, and other, epigenetic processes (e.g., histone phosphorylation) interact and are regulated by cell signaling pathways like ERK. Moreover, very little is known about how histone acetylation and DNA methylation are altered in the aging hippocampus. Thus, the role of epigenetic factors in age-related sex-steroid hormone loss, as occurs in meno-

pause, should also be explored, as epigenetic treatments (e.g., HDAC inhibitors) might prove beneficial for cognitive function in menopausal women.

Gene Expression and Protein Synthesis

The studies discussed above strongly suggest that epigenetic processes in the hippocampus are critical to the mnemonic effects of E₂. As such, one would expect hippocampal gene expression to also be affected by E₂. Although E₂-induced expression of individual genes of interest has been measured using quantitative real-time PCR (qRT-PCR) to measure levels of mRNA transcripts, several recent studies have used microarray techniques to assess E₂-induced changes in global hippocampal gene expression. These studies do not yet provide a comprehensive or cohesive picture of how E₂ affects gene expression in the hippocampus, in large part because the studies are few in number and differ in terms of type and duration of E₂ treatment, age of the subjects, and type of arrays used. However, they do provide interesting glimpses into the complexity of hippocampal gene expression changes in response to E₂ and suggest that age-related alterations in gene expression might be related to memory dysfunction.

One such study in young ovariectomized females expanded upon our findings showing that E₂ (0.2 mg/kg) enhances 48-hr novel object recognition and increases dorsal hippocampal p42 ERK phosphorylation 1 hr after injection (Fernandez et al., 2008; Gresack & Frick, 2006). To examine gene expression at the same point at which ERK was activated, mice were injected with vehicle or 0.2 mg/kg E₂ and the dorsal hippocampus was collected 1 hr later. We found that 111 genes were up-regulated and 93 were down-regulated by E₂ (Pechenino & Frick, 2009). Upregulated genes included those associated with cell structure and transport, transcription, metabolism, oxidative stress, inflammation, differentiation, growth factors, neuropeptides, and receptors. Downregulated proteins were associated with many of the same processes (e.g., transcription, metabolism, differentiation, and cell structure), but also with apoptosis, protein degradation, immune responses, and calcium regulation. Of these genes, 17 that were up-regulated and 6 that were down-regulated had been associated with learning and memory in previous studies. mRNA expression changes in five of these genes were confirmed by qRT-PCR 1 hour after treatment, and resulting protein changes were confirmed with Western blotting 3–4 hours after treatment. The genes were as follows: *Hsp70*, which codes for an estrogen-responsive heat shock protein (Olazabal, Pfaff, & Mobbs, 1992); *Igfbp2*, which codes for an IGF-I binding protein (Aberg, Brywe, & Isgaard, 2006; Chesik, De Keyser, & Wilczak, 2007); *Actn4*, which codes for an actin-binding protein that regulates hippocampal spine morphology (Nakagawa, Engler, & Sheng, 2004); *Tubb2a*, which codes for Tubulin-β, a major structural component of microtubules that binds directly to E₂ (Ramirez, Kipp, & Joe, 2001); and *Snap25*, which codes for a synaptosomal protein required for neurotransmitter release (Wang & Tang, 2006). mRNA and protein for all genes were significantly up-regulated by E₂, with the exception of *Igfbp2* mRNA and IGFBP2 protein, which were down-regulated (see Figure 10; Pechenino & Frick, 2009). Of these changes, those in *Hsp70* and *Igfbp2* may be particularly relevant to the mnemonic effects of E₂. *Hsp70* is an integral part of the cytosolic estrogen receptor protein complex that keeps nuclear ERs inactive until they bind estrogens and translocate to the nucleus (Whitesell & Lindquist, 2005). *Hsp70* expression is increased by E₂ in the brain

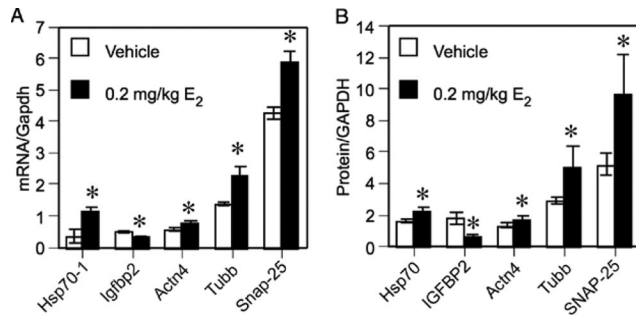


Figure 10. (A) mRNA expression measured 1 hour after E₂ injection. (B) Protein levels measured 3 or 4 hours E₂ injection. **p* < 0.05 relative to Vehicle. From ‘The effects of acute 17 β -estradiol treatment on gene expression in the young female mouse hippocampus,’ by A. S. Pechenino and K. M. Frick, 2009, *Neurobiology of Learning and Memory*, 91, pp. 315–322. Copyright (2009) by Elsevier. Adapted with permission.

(Olazabal et al., 1992) and Hsp70 isoforms, like Hsp70-1 and Hsp72, have been implicated in spatial learning and memory (Ambrosini, Mariucci, Tantucci, Van Hooijdonk, & Ammassari-Teule, 2005; Pizarro, Haro, & Barea-Rodriguez, 2003). In particular, knockout of Hsp72 in mice blocks spatial memory acquisition in the radial arm maze (Ambrosini et al., 2005). Although it is unclear how an E₂-induced increase in these heat shock proteins might benefit memory, Hsp72 and other Hsp70 isoforms should be further explored to determine their potential involvement in E₂-induced memory modulation. In addition to Hsp70, numerous interactions between IGF and E₂ have been previously documented (Mendez, Wandosell, & Garcia-Segura, 2006). IGFBP2 (insulin-like growth factor binding protein 2) sequesters IGF-I in serum and prevents it from activating elements of the IGF cascade such as PI3K and ERK (Aberg et al., 2006; Chesik et al., 2007). Thus, the E₂-induced down-regulation of Igfbp2 may increase IGF-I availability and subsequent PI3K and ERK activation. Because PI3K and ERK activation are necessary for E₂ to enhance object recognition, Igfbp2 could be a promising target for future drug development.

Two other recent studies examined whole hippocampal gene expression after acute or chronic estradiol treatment in young and middle-aged ovariectomized mice (Aenlle & Foster, 2010; Aenlle et al., 2009). One study examined gene expression in young, middle-aged, and aged females 6 and 12 hours after a single subcutaneous injection of vehicle or 5 μ g E₂ (Aenlle & Foster, 2010). At 6 hours, young and middle-aged females exhibited a significant increase in the number of genes whose expression was altered, including synaptic genes (whose expression was increased) and genes related to oxidative phosphorylation and mitochondrial dysfunction (whose expression was decreased). Several of the same genes were up- or down-regulated in both age groups. In aged females, considerably fewer genes were altered 6 hours after injection, and no changes were observed in synaptic genes. However, from 6–12 hours, the number of genes altered was substantially increased in aged females and decreased in young and middle-aged females. For aged females, many of the up-regulated genes were related to synaptic plasticity and various signaling pathways (cAMP, IGF-1) rapidly affected by E₂. No such increases were observed in young and middle-aged females. Although these findings might suggest that E₂-induced alterations in gene expression are delayed in aged females, the data do not support

this conclusion, as there was little correspondence between the genes altered in young females at 6 hours and aged females at 12 hours. Rather, the expression differences between young/middle-aged and aged females appears to reflect a fundamental disparity in how the female hippocampus responds to E₂ at these ages. As such, this discrepancy is consistent with the differential effects of acute E₂ on object recognition and hippocampal cell signaling in young/middle-aged and aged females discussed earlier (Fan et al., 2010; Gresack et al., 2007a), and suggests that differential gene expression may also contribute to the neurobiological mechanisms underlying the critical period hypothesis.

Another study from the same research group examined the effects of chronic E₂ treatment on hippocampal gene expression. Mice were given cyclic injections of vehicle or 17 β -estradiol benzoate (5 μ g) for 3 weeks prior to cued and spatial memory testing in the Morris water maze (Aenlle et al., 2009). After 5 weeks of treatment, gene expression was analyzed by cDNA microarray in the whole hippocampus, and expression of a subset of genes was confirmed using oligonucleotide arrays and qRT-PCR. Middle-aged females treated with vehicle exhibited significant spatial learning and memory impairments relative to young females or middle-aged females treated with E₂. Substantially more genes were altered by E₂ in middle-aged females than in young females, suggesting that gene expression mechanisms in the middle-aged hippocampus are more sensitive to chronic E₂ treatment than in the young hippocampus. An oligonucleotide analysis of seven selected genes indicated that E₂ reversed age-related increases in the expression of five genes (Ldb2, Foxo3a, Hdac2, Lass2, and Kctd3) and age-related decreases in the expression of two genes (Fbxw8 and cbini). mRNA expression in Hdac2 and Lass2 was confirmed with qRT-PCR. The observation that Hdac2 expression was increased with age and decreased by E₂ is consistent with our findings in young females that E₂ decreases HDAC2 protein levels (Zhao et al., 2010), and provides additional evidence that E₂-induced modulation of HDAC2 protein plays an important role in the estrogenic regulation of hippocampal memory.

Although few in number, these microarray studies suggest many genes whose regulation by E₂ might be particularly important for memory consolidation. The necessity of these genes for the mnemonic effects of E₂ must be tested in future studies using techniques that prevent their transcription or translation (e.g., RNA interference via oligonucleotides directed against specific mRNAs, gene knockout or overexpression). With respect to aging, however, this work clearly shows differential hippocampal gene expression throughout the female life span. In response to acute E₂ treatment, aged females showed a substantially different response than that of young and middle-aged females (Aenlle & Foster, 2010), a finding that corresponds well with the inability of acute E₂ to enhance object recognition and hippocampal cell signaling in aged females (Fan et al., 2010; Gresack et al., 2007a). Although the overall numbers of hippocampal genes altered in young and middle-aged females were similar, the specific genes affected at these two ages did differ somewhat (Aenlle & Foster, 2010). These differences may have contributed to the disparate response observed in these ages in response to chronic E₂ treatment, where the number of hippocampal genes altered was fourfold higher in middle-aged females relative to young females (Aenlle et al., 2009). Chronic E₂ also significantly improved spatial memory in middle-aged females (Aenlle et al., 2009), suggesting that alterations in genes

such as Hdac2 and Lass2 might contribute to the ability of E_2 to improve memory in middle-aged females. Although much more work is needed to link hippocampal gene expression changes to estrogenic effects on memory in young and aging females, these studies provide an important foundation for future research.

Given the widespread alterations in gene transcription observed after E_2 treatment, it stands to reason that protein synthesis would also be altered by E_2 . However, very few studies have examined the effects of E_2 on protein synthesis in the brain. In a rat model of cerebral ischemic injury, chronic treatment with E_2 prevented the injury-induced decrease in activation of the mammalian target of rapamycin (mTOR) signaling pathway (Koh et al., 2008). mTOR regulates protein synthesis at the level of translation initiation and can be activated by a number of upstream signaling pathways including PI3K, Akt, and ERK (Figure 5D; Kelleher, Govindarajan, Jung, Kang, & Tonegawa, 2004; Klann & Dever, 2004). mTOR is activated in the hippocampus by learning, and this activation is necessary for both hippocampal long-term potentiation and the formation of long-term fear, spatial, and object recognition memories (Bekinschtein et al., 2007; Dash, Orsi, & Moore, 2006; Gafford, Parsons, & Helmstetter, 2011; Myskiw et al., 2008; Richter & Klann, 2009; Tsokas, Ma, Iyengar, Landau, & Blitzer, 2007). The PI3K/Akt/mTOR pathway also regulates hippocampal dendritic spine morphology (Kumar, Zhang, Swank, Kunz, & Wu, 2005), and mTOR activation is critical for local protein synthesis within dendritic spines (Tsokas et al., 2005), thus, providing a potential mechanism by which E_2 could rapidly influence local protein translation within spines. Indeed, one recent study in cultured hippocampal neurons reported that E_2 rapidly induces mRNA translation in hippocampal dendrites (Sarkar, Smith, Logan, & Simpkins, 2010). E_2 increased activation of the mTOR pathway in hippocampal dendrites within 5 minutes, and this effect was dependent on ERK activation (Sarkar et al., 2010), demonstrating that cell signaling cascades like ERK must be activated for dendritic mTOR-mediated protein synthesis. Our own preliminary work shows that dorsal hippocampal infusion of E_2 significantly increases activation of the mTOR pathway within 15 minutes, and that the mTOR inhibitor rapamycin prevents E_2 from enhancing object recognition (Fan, Zhao, Orr, & Frick, 2010). Although considerably more research is warranted to understand the role of hippocampal protein synthesis in the rapid effects of E_2 on memory and hippocampal function, these early studies suggest that mTOR-mediated protein synthesis may be vital to the process (Figure 5D).

Summary and Conclusions

This article has discussed the application of the Downstream Molecule model to study the molecules and biochemical events in the hippocampus through which E_2 enhances memory. In vivo research conducted to date demonstrates that E_2 has rapid effects on hippocampal cell signaling, epigenetic processes, gene expression, and protein synthesis that appear to regulate memory in females, particularly object recognition memory. The data from our laboratory, thus far, suggest numerous interactions among cell signaling cascades and epigenetic processes that could ultimately influence protein synthesis and affect memory (see Figure 5). We have shown, for instance, that acute administration of E_2 rapidly activates hippocampal PI3K/Akt and PKA signaling, which then activates p42 ERK (Fan et al., 2010; Lewis et al., 2008). This E_2 -induced ERK activation leads to increased histone H3 acetylation, which may then increase DNMT3B

expression and, subsequently, reduce HDAC2 expression (Zhao et al., 2010). E_2 -induced epigenetic alterations surely influence gene expression, which may ultimately enhance memory through protein synthesis. Protein synthesis is likely pivotal to the beneficial effects of E_2 on memory, and emerging evidence suggests that this synthesis could either result from gene expression alterations in the nucleus or local synthesis within hippocampal dendrites mediated by ERK or other signaling cascades. Pharmacologically inhibiting activation of NMDA receptors and the PI3K, PKA, ERK, and mTOR pathways, histone acetylation, or DNA methylation prevents E_2 from enhancing object recognition in female mice (Fan et al., 2010; Fernandez et al., 2008; Lewis et al., 2008; Zhao et al., 2010), which suggests that each of these processes is necessary for E_2 to facilitate memory consolidation. Although the receptor mechanisms that mediate these processes remain unclear, the data thus far suggest that membrane ERs and ER β play an important role. However, more research on these receptors, as well as ER α and putative ERs such as GPR30, is necessary to more clearly elucidate the involvement of specific ERs. Future drug development could target any one of the biochemical processes discussed in this review to specifically modulate memory. However, it will be necessary to select potential targets with caution, given that these processes are common to many cells, including those that play no role in cognition. Thus, as we identify specific downstream targets, new drugs could result in unintended nonspecific consequences. Therefore, it is important that the targets of drug development be as specific to learning and memory as possible. It is also important to note that the aforementioned conclusions may be specific to the task and E_2 treatment that we have used to test the Downstream Molecule model. Therefore, future investigations must test whether these findings generalize to other forms of hippocampal memory and other methods of E_2 delivery (e.g., chronic treatment).

Although we have learned much in recent years about the molecular and biochemical bases through which E_2 enhances memory, this work merely scratches the surface in addressing the complexity of hormonal modulation of memory. Specific to the data discussed in this review, it will be critical to understand in future studies which other signaling pathways, epigenetic processes, genes, and protein synthesis mechanisms are involved in mediating E_2 's effects on memory, how these proteins and processes interact, and which ERs mediate these effects. In addition, pinpointing which molecules and biochemical processes are vital to the mnemonic effects of E_2 will help in determining why the responses of the aged female hippocampus to E_2 often differ from those of young females. It is also important to remember that progesterone levels fluctuate in parallel with E_2 , yet very little is known about the role that progesterone plays in modulating hippocampal function and memory formation. Thus, future research should aim to understand how these hormones work in concert to influence memory and other types of cognitive function.

Despite the caveats discussed in this article, the Downstream Molecule model provides a framework for testing the effects of any hormone, whether acutely or chronically administered, on memory and other forms of cognition. Shifting the drug discovery research focus from hormone receptors to rapid events downstream from these receptors that influence learning and memory formation may open the doors to new areas of investigation that could lead to the next generation of drugs for reducing age-related memory decline. The hope is that these new drugs would not be steroid hormones, thereby circumventing the side effects of hormones. Because their mecha-

nisms of action would mimic the effects of hormones on the hippocampus and memory, such treatments could be considered hormone-based. Although it will take some time for these hormone-based drugs to enter the clinic, the numerous recent advances in understanding how E_2 rapidly modulates memory provide new hope that such drugs might become available in the near future.

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