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A NEW APPROACH TO UNDERSTANDING THE MOLECULAR MECHANISMS THROUGH WHICH ESTROGENS AFFECT COGNITION

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Abstract

Traditional approaches to the study of hormones and cognition have been primarily observational or correlational in nature. Because this work does not permit causal relationships to be identified, very little is known about the specific molecules and cellular events through which hormones affect cognitive function. In this review, we propose a new approach to study hormones and memory, where the systematic blocking of cellular events can reveal which such events are necessary for hormones to influence memory consolidation. The discussion will focus on the modulation of the hippocampus and hippocampal memory by estrogens, given the extensive literature on this subject, and will illustrate how the application of this approach is beginning to reveal important new information about the molecular mechanisms through which estrogens modulate memory consolidation. The clinical relevance of this work will also be discussed.

Keywords

Estradiol; hippocampus; memory; object recognition; cell signaling; gene expression; ERK; estrogen receptors; selective estrogen receptor modulator; ER knockout; progesterone

1. Introduction

Considerable attention has been paid recently to understanding how ovarian hormones influence cognition and the function of brain regions critical for cognitive function. Such information is of great clinical relevance, given that significantly more women than men develop psychiatric disorders, such as depression and anxiety [1], and neurodegenerative conditions, such as dementia and Alzheimer's disease [2,3]. In particular, the relationship between the onset of age-related cognitive decline and the massive loss of estrogens and

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progestagens that accompanies menopause [4,5] has been of much recent interest, given the publication of data from the Women's Health Initiative (WHI) indicating that treatment with conjugated equine estrogens, either alone or in combination with a synthetic progestin, increased the risk of global cognitive decline and dementia in post-menopausal women [6–8]. Although not designed primarily to examine effects of hormone therapy on cognitive function, the WHI studies have raised many important questions regarding hormones and cognition, not the least of which is whether ovarian hormones should be used to prevent age-related cognitive decline.

The side effects of current estrogen treatments reported by the WHI (e.g., breast and uterine cancer, heart disease, and stroke [9–11]) preclude their widespread use to treat age-related cognitive decline or psychiatric disorders. Nevertheless, the development of drugs that mimic the effects of estrogens and natural progestagens in the brain may prove critical to maintaining cognitive health, given that ovarian hormones in adult females are trophic factors for cognitive regions of the brain such as the hippocampus [12], and that the loss of these hormones at menopause may render hippocampal neurons vulnerable to deterioration or exacerbate emerging age-related cognitive deficits. Therefore, the development of novel treatments that provide the beneficial effects of hormones on cognition, but do not produce their peripheral side effects, could improve the quality of life for millions of women. Identifying potential targets for drug development will require the discovery of the underlying molecular mechanisms in the brain through which estrogens and progestagens modulate cognition. Because many of these targets would lie downstream from estrogen and progesterone receptors, novel drugs could be developed to modulate the downstream effectors of hormone receptors rather than the receptors themselves, thus, leading to new drug treatments that produce the cognitive benefits of hormones without the adverse side effects of current therapies.

To illustrate how this molecular approach may be applied to hormones and cognition, this review will focus on the effects of the potent estrogen, 17 β -estradiol (E_2), on the hippocampus and hippocampal-dependent memory. The hippocampus, a bilateral medial temporal lobe structure that mediates the formation of memories involving spatial, relational, contextual, and object information [13–15], is exceedingly sensitive to levels of E_2 . This fact was first demonstrated in the early 1990's by seminal reports showing rapid effects of E_2 on CA1 dendritic spine density in the hippocampus of young female rats [16–19]. Further, the hippocampus is extremely vulnerable to the detrimental effects of aging and Alzheimer's disease [20,21], which has made it of particular interest to the study of ovarian hormone loss and age-related cognitive decline. In the nearly two decades since the publication of the initial spine density data, thousands of studies have examined the effects of E_2 on the hippocampus and on hippocampal-dependent memory. Nevertheless, because most previous research was correlational in nature, or examined either memory or hippocampal function independently, surprisingly little is known about the molecular and biochemical events in the hippocampus necessary for E_2 to influence memory formation. However, recent studies in rodents have begun to shed light on these events, and this research has provided interesting new avenues for the development of future hormone-based therapies. The goal of this review is to discuss this work and introduce a conceptual model that provides the framework for future research in this area.

2. Relating E_2 -induced changes in memory and the hippocampus

2.1. The Traditional Approach

The traditional approach used in the field of hormones and cognition has been largely observational and correlational. That is, subjects (e.g., humans, non-human primates, or rodents) are treated with hormones such as estradiol or progesterone (most often, systemically) and then some aspects of hippocampal function or hippocampal memory are tested, with the

resulting outcome that the treatments affect these variables or not. This approach has been invaluable to the field, as it has systemically documented the aspects of hippocampal function that are sensitive to hormones and the types of memory that can be modulated by hormones. Further, correlative studies that have measured both hippocampal function and memory have revealed important insights into the potential neural mechanisms underlying hormone effects on memory. However, after nearly 20 years of such work, we would argue that it is time to take this research to the next level, past observations and correlations, to more direct investigations that pinpoint the specific neurobiological alterations that are necessary for hormones to influence cognition.

Abundant evidence demonstrates that ovarian hormones such as E_2 rapidly and profoundly affect hippocampal morphology and physiology in both young and aging female rodents. For example, exogenous E_2 in young female rats and mice prevents the ovariectomy-induced decrease hippocampal CA1 dendritic spine density [18,22], enhances hippocampal synaptic excitability and LTP [23,24], increases release of growth factors and expression of synaptic proteins (e.g., synaptophysin and PSD-95) [25–27], and rapidly activates numerous cell signaling cascades including the extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways [28–31]. Similarly, numerous studies have shown that E_2 can enhance the acquisition and consolidation of various types of hippocampal memory (e.g., spatial, contextual, object recognition) in young females (e.g., [32–52], see [53] for review), and in middle-aged and aged females (e.g., [26,48,54–65], see [66] for review).

However, although some of these studies have reported that improvements in hippocampal memory are associated with increases in some aspect of hippocampal function, such data cannot definitively link these changes in a causative manner. As one example, we have previously shown in aged female mice that chronic estradiol benzoate treatment significantly improves spatial memory in the Morris water maze and increases hippocampal levels of the pre-synaptic protein synaptophysin [26]. Given that synaptophysin levels have been correlated with memory in humans and rodents [67–70], these data could suggest that an estradiol-induced increase in synaptophysin levels is responsible, at least in part, for the observed memory improvement. This conclusion is further supported by the fact that a dose of estradiol that did not improve memory also did not increase synaptophysin levels [26]. However, despite this clear association between increased synaptophysin levels and improved memory, no causal relationship can be drawn to show that increased hippocampal synaptophysin is necessary for estradiol to improve spatial memory. In fact, although somewhat unlikely, the estradiol-induced increases in both variables could be completely unrelated. As such, the failure to demonstrate that the specific hippocampal alteration(s) must occur in order for memory to be improved prevents definitive conclusions about the neurobiological mechanisms underlying hormone-induced behavioral change.

2.2. The Blocking Approach

To this end, we propose borrowing an approach from the extensive literature examining the neurobiology of learning and memory, where techniques such as post-training intracranial infusions of inhibitor drugs, gene knockouts, and gene silencing have allowed investigators to identify molecules at the receptor, cell signaling, epigenetic, genetic, and protein synthesis levels that are critical to the formation of long-term memories. This so-called “Blocking” approach [71] allows investigators to determine if a given event (e.g., receptor activation, protein phosphorylation, gene expression, protein synthesis) is necessary for the expression of a behavioral phenomena through the use of techniques such as those described above that prevent the occurrence of the event. If the behavior of interest is disrupted by the blocking

manipulation, then this result suggests that the targeted event was necessary for the behavior to occur.

This approach has the advantage of allowing investigators to test clearly defined hypotheses about the roles of specific molecules or cellular processes in hormonal modulation of cognition. As such, applying the blocking approach to the study of hormones and cognition can provide a powerful method of determining which molecules in the hippocampus and other brain regions are necessary for hormones to modulate cognitive processes like learning and memory. Further, this approach could be used more broadly, beyond the memory, towards the development of treatments for reducing mental illness (e.g., depression and anxiety disorders) in women, and potentially reducing the detrimental psychological effects of genetic disorders that predominantly affect females (e.g., Rett's syndrome).

2.3. The Downstream Molecule Model

To frame the following discussion of the application of the blocking approach to estrogenic modulation of memory, we suggest a conceptual model for future studies of hormones and cognition. This “Downstream Molecule” model is built upon the framework provided by data from the blocking approach as applied to the neurobiology of learning and memory. Essentially, the model stipulates that the effects of hormones on cognition are mediated by alterations in cell signaling, epigenetic mechanisms, gene expression, and new protein synthesis (Fig. 1). For any hormone, the blocking approach can be used to block each process to determine which molecules at each step of the model are critical for effects on cognition. For E₂ in the hippocampus, alterations in these molecules might lead to enhanced memory. Many effects of E₂ on memory and hippocampal function are rapid (e.g., [28–31]), and likely involve either novel membrane-bound hormone receptors or non-traditional effects of nuclear hormone receptors [29,31]. Studies using the blocking approach have already begun to determine which estrogen receptors and cell signaling pathways are critical to the effects of E₂ on memory consolidation. This work will be discussed in detail in the following sections.

3. Practical application of the blocking approach to hormones and cognition

As an example of the application of the blocking approach to cognition, studies from the learning and memory literature have demonstrated that phosphorylation (i.e., activation) of the cell signaling molecule ERK in the hippocampus is necessary for rodents to form long-term hippocampal-dependent memories. ERK is one of a family of mitogen-activated kinases (MAPKs) that is phosphorylated as part of a G-protein initiated signal transduction cascade. Upon phosphorylation, ERK translocates to the cell nucleus where its subsequent phosphorylation of molecules like RSK (ribosomal S6 kinase) can lead to phosphorylation of the transcription factor cAMP response element binding protein (CREB) and gene transcription [72]. MAPK kinase (or MEK) is the exclusive upstream activator of ERK, and its actions can be inhibited using a number of compounds [72]. Thus, the necessity of ERK activation for any process can be tested using MEK inhibitors that prevent ERK activation. Indeed, studies in which the MEK inhibitors U0126 or PD098059 have been infused into the hippocampus or cerebral ventricles prior to training report impaired spatial memory, contextual fear conditioning, and object recognition 24–48 hours later [73–76]. Administering the MEK inhibitor immediately after training (i.e., post-training) also blocks long-term memory formation [77]. This finding is important because post-training inhibitor treatments can better pinpoint effects of ERK activation to the memory consolidation phase of memory processing, given that pre-training treatments will also affect memory acquisition. Consistent with the pharmacological data, mice with a knockdown or conditional knockout of the p42 isoform of ERK have impaired spatial memory and contextual fear conditioning, reduced neocortical thickness, and altered neocortical neurogenesis [78,79], suggesting that p42 ERK is critical for memory and neural development. Collectively, this literature, which uses several blocking

techniques to prevent ERK activation, provides strong evidence that ERK activation is necessary for long-term hippocampal memory formation. As such, similar experimental approaches could be useful for identifying molecules, such as ERK, that are necessary for hormone-induced memory enhancement.

Our initial attempts to use the blocking approach to pinpoint molecules underlying E₂-induced memory modulation in female mice utilized three important elements: post-training treatments, rapidly metabolized E₂, and a one-trial learning task. Post-training hormone and inhibitor treatments were used because, as previously mentioned, pre-training treatments affect both memory acquisition and consolidation, thus, obscuring molecular events associated with each phase of memory formation. In addition, hormones such as E₂ affect performance factors (i.e., motivation, attention, and sensorimotor function) in addition to memory [80–82], thus, obscuring effects of E₂ on memory specifically. As such, administering E₂ immediately after training allows the effects of E₂ be pinpointed specifically to the memory consolidation phase of memory processing, and thus greatly aids in the identification of molecules necessary for memory formation.

Next, we used a form of E₂ encapsulated in 2-hydroxypropyl- β -cyclodextrin [29,62,83–86] to ensure that E₂ was not in the circulation during retention testing. Cyclodextrin-encapsulated hormones are metabolized within hours [87], and because E₂ is not in the circulation during training or testing, its specific effects on memory consolidation can be examined in the absence of non-mnemonic performance confounds. This form of E₂ has been shown to enhance hippocampal-dependent memory when injected intraperitoneally (i.p.) in doses of 0.1–0.4 mg/kg [83,86], which are high physiological/low pharmacological doses [88], and directly into the dorsal hippocampus and dorsal third ventricle in doses of 5–10 μ g per infusion [29,89].

Finally, we needed a hippocampal task that mice could learn in one trial and that involved minimal stress, given the well-documented interactions between gonadal and stress hormones (e.g., [90–92]). Because we had found that immediate post-training injection of 0.2 mg/kg E₂ (i.p.) could enhance novel object recognition in young ovariectomized mice [83], we thought this task might be useful for these studies. 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 arena [93]. Immediately after this training, mice are injected systemically or infused intracranially with E₂. Retention is then tested 48 hours later by presenting mice with one novel and one familiar (identical to training) object. Mice who remember the familiar object spend more time than chance (15 seconds) exploring the novel object. Although there has been some controversy about the extent to which novel object recognition requires the hippocampus versus other brain regions (e.g., perirhinal cortex, frontal cortex) [94–96], hippocampal involvement in our testing protocol has been demonstrated with complete hippocampal lesions and dorsal hippocampal inactivation [97,98]. Further, numerous studies from our lab have shown in young ovariectomized mice that immediate post-training intraperitoneal (i.p.) injection of 0.2 mg/kg E₂ enhances object recognition tested 48 hours later (e.g., [83,99]). Because the novel object recognition task involves rapid one-trial learning, we thought it might be an especially useful task in our efforts to identify the molecular mechanisms through which E₂ modulates memory consolidation.

4. Using the blocking approach to identify receptors and cell signaling molecules necessary for E₂ to enhance memory consolidation

4.1. Cell signaling

We turned to ERK as a potentially important molecule for the mnemonic effects of E₂, given its importance for long-term hippocampal memory formation. An increasing number of studies

have focused on rapid effects of E₂ in neurons, and *in vitro* data supported a link between E₂ and rapid effects on ERK activation and hippocampal function. For example, data from hippocampal cell culture studies had shown that E₂ increases ERK phosphorylation within 10–20 min of application [100,101], and that MEK inhibitors completely block not only this effect, but also E₂-mediated neuroprotection [100–103] and E₂-induced increases in synaptophysin protein levels [30]. In the intact rat, a single infusion of E₂ into the lateral ventricle increased ERK phosphorylation throughout the hippocampus within 5 minutes [104]. As such, evidence clearly demonstrated that E₂ could activate hippocampal ERK.

Based on these data, we hypothesized that the beneficial effects of 0.2 mg/kg E₂ on novel object recognition were dependent on dorsal hippocampal ERK activation. We first set out to measure whether 0.2 mg/kg E₂ increased ERK activation in the dorsal hippocampus of young ovariectomized mice. We found that 0.2 mg/kg E₂ (i.p.) increased phosphorylation of the p42 isoform of ERK (Fig. 2A), but not the p44 isoform of ERK (data not shown), 60 minutes after injection [29]. This increase was blocked by concurrent i.p. injection of the MEK inhibitor, SL327 (30 mg/kg; Fig. 2A) [29]. Next, mice were implanted with bilateral infusion cannulae directed at the dorsal hippocampus and were trained in the object recognition task. Immediately after training, mice were injected with 0.2 mg/kg E₂ and infused intrahippocampally (IH) with vehicle or the MEK inhibitor U0126 (0.5 µg/side of the hippocampus). U0126 blocked the beneficial effects of 0.2 mg/kg E₂ on novel object recognition tested 48 hours after training (Fig. 2B) [29], demonstrating that dorsal hippocampal ERK activation is necessary for E₂ to enhance object recognition. In addition, we found that infusion of E₂ into the dorsal hippocampus (5 µg/side) immediately, but not 3 hours, after training could also significantly enhance object recognition (Fig. 2C), further localizing the behavioral effects of E₂ to the dorsal hippocampus and demonstrating a relatively brief time window in which these effects occur [29]. We then wanted to see if IH infusion of U0126 would block the effects of intracranially infused E₂. In order to prevent tissue damage from repeated infusions into the hippocampus, we infused E₂ into the dorsal 3rd ventricle (ICV, 5 µg total), as a means of supplying E₂ to the hippocampus, concurrently with IH infusion of U0126. We found that ICV-infused E₂ increased phospho-p42 ERK levels within 5 minutes of infusion and enhanced 48-hour object recognition, and that these effects were blocked by U0126 (Z. Zhao, personal communication). Collectively, these data demonstrate that dorsal hippocampal ERK activation is necessary for systemically and intracranially administered E₂ to enhance object memory consolidation in young ovariectomized female mice. Further, these studies demonstrate the feasibility of the blocking approach to understanding the molecular events underlying E₂-induced memory modulation.

We have also used this approach to examine signaling upstream from ERK, specifically NMDA receptor activation and activation of protein kinase A (PKA). Immediately after object recognition training, young ovariectomized mice were injected with 0.2 mg/kg E₂ and infused IH with the NMDA antagonist APV (2.5 µg/side) or the PKA inhibitor Rp-cAMPS (18 µg/side). In addition to object recognition, ERK phosphorylation in the dorsal hippocampus was examined 1 hour after drug treatment. Both APV and Rp-cAMPS blocked the beneficial effects of 0.2 mg/kg E₂ on 48-hour object recognition and dorsal hippocampal phospho-p42 ERK levels [84], suggesting that dorsal hippocampal NMDA receptor and PKA activation upstream from ERK activation are also involved in the mnemonic effects of E₂. In addition, preliminary work from our lab shows that inhibition of PI3K or Akt also block the beneficial effects of E₂ on object recognition (L. Fan, personal communication), demonstrating the utility of this approach in understanding the roles that various signaling molecules play in mediating the effects of E₂ on memory consolidation.

4.2. Roles of nuclear estrogen receptors in mediating the effects of E₂ on memory consolidation

One of the most critical issues to the development of more selective estrogen treatments is to determine which estrogen receptors mediate the memory enhancing effects of E₂. Traditionally, the effects of E₂ were thought to be mediated by the nuclear estrogen receptors (ERs) ER α and ER β . ER α and ER β are found throughout the hippocampus [105,106], where they are located in the nucleus, dendritic spines, and axon terminals of pyramidal neurons [107]. In the classic (i.e., “genomic”) mechanism of estrogen action, estrogens pass through the outer cell membrane and bind to ER α and ER β in the cytoplasm, which then causes the receptors to dimerize and translocate the estrogen-ER complex to the cell nucleus, and the complex to bind estrogen response elements (EREs) on target genes [108]. Because the responses linked to gene transcription were thought to be too slow to account for the rapid activation of cell signaling in the hippocampus, it was unclear if these receptors played a role in mediating rapid effects of E₂ on hippocampal functioning. However, their presence in dendrites and axons [107] may suggest a potential mechanism for these effects. Such effects would be considered “non-genomic”, as they would not involve direct binding to an ERE.

Indeed, data from both *in vitro* and *in vivo* experiments demonstrate importance of rapid effects on cell signaling in mediating effects of E₂ on memory and the hippocampus. In hippocampal cell lines transfected with ER α or ER β , E₂ can increase p42 ERK phosphorylation within 15 minutes [100,101], suggesting that either receptor may mediate effects of E₂ on phospho-p42 ERK activation. These effects are blocked by MEK inhibition [100,101] and by the ER α /ER β antagonist ICI 182,780 [100,101], which blocks E₂-induced ER dimerization [109] and translocation into the cell nucleus [110]. In hippocampal cell culture or *in vivo*, E₂ can activate hippocampal ERK and PI3K 5–15 minutes after exposure, effects that are blocked by inhibitors of ERK and PI3K activation [29–31,104,111]. Further, E₂-induced ERK activation is also necessary for this hormone to increase hippocampal synaptic protein levels [30] and CA1 dendritic spine density [112], linking rapid effects of E₂ on hippocampal cell signaling to classic morphological alterations in this structure. The fact that E₂-induced increases in ERK activation and in both morphological alterations can be blocked by ICI 182,780 suggests that one or both nuclear ERs are critically involved in these effects. Consistent with these findings, we also found that the effects of 0.2 mg/kg E₂ on dorsal hippocampal phospho-p42 ERK levels and object recognition (Fig. 2B) were blocked by immediate post-training IH infusion of ICI 182,780 [29]. However, the rapidity of E₂'s effects on cell signaling and memory suggests that these effects result from “non-genomic” effects of nuclear ERs that do not directly involve EREs. Recent findings suggest that ER β can translocate to the plasma membrane upon exposure to E₂ [113], which would provide a means for this receptor to interact with proteins that activate cell signaling pathways.

If nuclear ERs do play a role in mediating rapid effects of E₂ on hippocampal function, then is one more important than the other? *In vivo*, two approaches may be especially useful in examining the relative contributions of nuclear ERs in mediating memory. The first approach employs ER knockout (ERKO) mice that lack functional copies of the gene for either nuclear ER α or ER β . Previous studies using ERKO females treated with E₂ suggest that E₂ may enhance memory primarily through activation of ER β . For example, ER α KO females receiving E₂ exhibit improved spatial memory and inhibitory avoidance relative to untreated ER α KO mice [114,115]. In contrast, ER β KO mice females receiving E₂ show no improvement in object recognition or object placement tasks [116]. ER α activation may impair hippocampal memory, as administration of E₂ in ER β KO females either impairs or fails to affect spatial and object memory relative to wild-type mice receiving E₂ [117–119].

The second approach to examine the role of nuclear ERs in memory employs pharmacological manipulation of ER α and ER β activation. Two selective estrogen receptor modulator (SERM)

drugs often used for this purpose are propylpyrazole-triol (PPT), which has a 410-fold greater affinity for ER α over ER β [120], and diarylpropionitrile (DPN), which has a 70-fold greater affinity for ER β over ER α [121]. PPT and DPN protect cultured hippocampal neurons from excitotoxicity, an effect that is blocked by MEK inhibition, but not by ICI 182,780 [31]. Both drugs have also been shown in culture to increase hippocampal phospho-p42 ERK levels within 10 minutes of application [31] and to increase CA1 dendritic spines within 2 hours of application [112]. Despite the fact that both PPT and DPN appear to promote hippocampal function *in vitro*, spatial memory in the Morris water maze is only improved by DPN among young ovariectomized rats tested after systemic post-training subcutaneous (s.c.) injections of 10 μ g PPT or DPN [122]. However, 0.9 mg/kg of both drugs enhanced novel object recognition [123] and object placement [124] in other studies of young ovariectomized rats, suggesting that effects of PPT and DPN on memory may be task- or dose-dependent.

Because both the knockout and SERM approaches have critical shortcomings (e.g., potential compensatory mechanisms for life-long gene knockouts and a lack of absolute receptor specificity of the SERMs), the combined use of both approaches using the same behavioral paradigm may provide important converging evidence about the roles of nuclear ERs in memory consolidation. Therefore, we recently used the same novel object recognition task to test the involvement of nuclear ERs in memory using both knockout mice and SERMs. First, vehicle or 0.2 mg/kg E₂ were given immediately post-training to young ovariectomized mice lacking either ER α (ER α KO, [125]) or ER β (ER β KO, [126]). E₂ enhanced 48-hour object recognition in ER α KO, but not ER β KO mice (Fig. 3A). Next, wild-type C57BL/6 mice were injected immediately post-training with 0.2 mg/kg E₂ (i.p.) or with 0.05 mg/kg (s.c.) of PPT or DPN. E₂ and DPN, but not PPT, enhanced object recognition 48 hours later (Fig. 3B). Together, these data suggest a role for ER β , but not ER α , in mediating effects of E₂ on object memory, which is consistent with previous studies on this subject.

However, many questions remain, and the blocking approach could be especially useful in revealing the answers. For example, direct intracranial infusions of PPT, DPN, and cell signaling inhibitors would shed light on whether the mnemonic effects of these drugs are dependent on hippocampal cell signaling. Further, conditional knockouts restricting ER α and ER β deletion to the dorsal hippocampus in adulthood would greatly facilitate our understanding the importance of hippocampal ER α and ER β to E₂-induced memory modulation.

4.3. Roles of non-nuclear estrogen receptors in mediating the effects of E₂ on memory consolidation

Although ICI 182,780 blocks many of the effects of E₂ and the SERMs on hippocampal neurons, it does not block all of their effects, including those on neuroprotection, synaptogenesis, or growth factor release [25,31]. As a result, it has been postulated that rapid non-genomic effects of E₂ on hippocampal neurons may also be mediated by membrane-bound ERs in the outer cell membrane, the precise identities of which are currently unknown [127, 128]. Although these receptors are not yet cloned, their function has been examined using a membrane-impermeable form of E₂ called BSA-E₂, in which E₂ is covalently linked to bovine serum albumin. Unlike E₂, BSA-E₂ does not activate ERE-mediated gene transcription [129]. Infusion of BSA-E₂ into the dorsal hippocampus or cerebral ventricles promotes ERK phosphorylation *in vivo* [29,104] and enhances hippocampal-dependent memory [29,50]. We recently showed for the first time that dorsal hippocampal ERK activation is necessary for BSA-E₂ to enhance novel object recognition in young ovariectomized mice. In this experiment, immediate post-training infusions of BSA-E₂ into the dorsal hippocampus (intrahippocampal, IH) or dorsal third ventricle (intracerebroventricular, ICV) significantly enhanced 48-hour object recognition (Fig. 4A) and robustly increased dorsal hippocampal phospho-p42 ERK activation 5 minutes after infusion (Fig. 4B) [29]. The importance of ERK activation in

mediating the effects of BSA-E₂ on novel object recognition was demonstrated by the fact that the effect of ICV BSA-E₂ infusion on object recognition was completely blocked by IH infusion of U0126 (Fig. 4A) [29]. Interestingly, unlike with E₂, the beneficial effects of ICV BSA-E₂ on object recognition (Fig. 4A) or phospho-p42 ERK activation (Fig. 4B) were not blocked by IH infusion of ICI 182,780 [29]. Further, the beneficial effects of ICV BSA-E₂ on object recognition were also not blocked by hippocampal inactivation with an IH infusion of the GABA_A agonist Muscimol (data not shown) [29]. Together, these data suggest that the effects of BSA-E₂ on object recognition depend on hippocampal activity, but not on ER α or ER β . Although these data demonstrate that the beneficial effects of E₂ on object memory consolidation can be mediated solely by putative membrane-bound ERs, the fact that ICI 182,780 blocks the beneficial effects of traditional E₂ on object recognition (Fig. 2B) [29] suggests an additional role for a nuclear ER. More work will be needed to understand how each ER contributes to E₂-induced memory enhancement.

4.4. Effects of progesterone on E₂-induced alterations in hippocampal cell signaling and memory

Very little is known about the effects of natural progestagens, such as progesterone, or synthetic progestins (such as those commonly used in hormone therapy) on memory and hippocampal function, despite the fact that levels of progesterone also plunge at menopause. The few studies that have examined effects of progesterone on hippocampal function report some similar effects of E₂ and progesterone on hippocampal function, although their effects are not identical. For example, progesterone has a biphasic effect on CA1 dendritic spine density, at first increasing spine density within the first 6 hours of treatment and then decreasing density below baseline over the next 18 hours [19]. These effects are likely mediated by the two nuclear progesterone receptors (PRA and PRB), which, like the ERs, are located in dendritic spines, nuclei, and axon terminals throughout the hippocampus [130]. The PR antagonist RU-486 blocks progesterone-induced increases in CA1 dendritic spine density [19], suggesting that one or both of the nuclear PRs mediate this effect. However, progesterone also rapidly activates both the ERK and PI3K/Akt signaling pathways in the hippocampus [111,131,132], and studies from mouse neocortex show that RU-486 does not prevent progesterone from phosphorylating ERK and Akt [133]. These data suggest non-genomic mechanisms similar to those of E₂, and indeed, multiple membrane-bound PRs have been identified in vertebrates and invertebrates [134,135]. Further, studies from my lab in which progesterone was administered systemically or into the dorsal hippocampus immediately after training show that progesterone can improve object recognition in young, middle-aged, and aged ovariectomized mice [65,136,137].

Although progesterone can facilitate memory and hippocampal functioning when given alone, numerous studies report that progesterone given in combination with E₂ reverses the beneficial effects of E₂. Indeed, in the WHI, the synthetic progestin medroxyprogesterone acetate appears to increase the risks of global cognitive decline and dementia in post-menopausal women more than conjugated equine estrogens alone [6–8]. In rodents, progesterone blocks E₂'s neuroprotective effects in the hippocampus [138] and neurotrophic effects in the entorhinal cortex [139]. Further, acute or chronic progesterone reverses the beneficial effects of E₂ on spatial memory tested in middle-aged ovariectomized rats [57] and aged ovariectomized mice [63]. However, the literature on E₂ plus progesterone treatment is quite inconsistent, and other studies in ovariectomized aging rats report beneficial effects of chronically administered E₂ plus progesterone treatment on spatial memory tested in aging rats [56,60].

Despite these discrepant results, we thought that the post-training approach employed for E₂ alone could be used to examine how progesterone interacts with E₂, given that both E₂ and progesterone increase ERK phosphorylation *in vitro*, and that our post-training treatments of each hormone separately improved memory in females throughout the adult lifespan. We

examined the effects of combined E₂ plus progesterone treatment on object recognition and dorsal hippocampal ERK activation using different dose combinations of behaviorally effective and sub-effective doses of both hormones. Previous studies had determined that 0.2 mg/kg E₂ and 10 or 20 mg/kg progesterone enhanced 48-hour object recognition, whereas 0.1 mg/kg E₂ and 5 mg/kg progesterone did not [83,136]. In the combination study [140], mice were injected with 0.1 or 0.2 mg/kg E₂ (i.p.) plus 5, 10, or 20 mg/kg progesterone (i.p.) immediately post-training, and then object recognition was tested after 48 hours and dorsal hippocampal phospho-ERK levels were measured after 1 hour. Ten and 20 mg/kg progesterone enhanced 48-hr object recognition regardless of the dose of E₂ with which it was paired (Fig. 5A) [140]. Conversely, 5 mg/kg progesterone did not enhance memory, and blocked the beneficial effects of 0.2 mg/kg E₂ on object recognition (Fig. 5A) [140]. Although 0.2 mg/kg E₂ significantly increased dorsal hippocampal phospho-p42 ERK levels (Fig. 5B) [140], 0.1 mg/kg E₂ had no such effect (data not shown). Interestingly, despite the fact that 10 and 20 mg/kg progesterone enhanced object recognition, all doses of progesterone blocked the 0.2 mg/kg E₂-induced increase in dorsal hippocampal phospho-p42 ERK levels (Fig. 5B) [140]. These data may suggest that the effects of progesterone or combined E₂ plus progesterone treatment influence ERK activation in a different time frame from E₂ alone (e.g., an increase in ERK activation may take place earlier than 1 hour after injection) or enhance memory through different mechanisms from E₂ alone (e.g., through signaling pathways such as PI3K/Akt).

The fact that so little is known about how E₂ and progesterone interact to influence memory and hippocampal function underscores the need for considerably more work on this subject. Understanding how estrogens and progestins affect cognitive function is of tremendous clinical importance, given that co-administration is recommended for treatment of menopausal women with a uterus because of the protection afforded by progesterone against uterine cancer [141]. Future work should aim to determine which hormone receptors and cell signaling pathways in the hippocampus mediate the mnemonic effects of E₂ plus progesterone treatment, and whether dose-dependent interactions result from differential receptor and cell signaling activation. Such information would greatly advance our understanding of how E₂ and progesterone together influence memory and hippocampal function, and would shed important light on how their interactions during the natural cycle may influence cognitive function.

5. Effects of E₂ on gene expression

Although the effects of E₂ on cell signaling do not appear to involve ERE-mediated gene transcription, the activation of cell signaling pathways can ultimately lead to gene transcription mediated by factors like CREB, c-Fos, and Elk-1. Certainly, ERK activation can lead to gene transcription [72], and the fact that ERK activation is necessary for E₂ to enhance object recognition suggests that ERK-induced gene alterations may play an important role in the mnemonic effects of E₂. The blocking approach has not yet been used to determine which genes are necessary for the effects of E₂ on hippocampal-dependent memory, but two recent DNA microarray studies have provided several interesting candidates.

In one study [85], we treated young ovariectomized mice with a single injection of vehicle or 0.2 mg/kg E₂ and then collected the dorsal hippocampus 60 minutes later. This dose of E₂ enhances both spatial memory and object recognition in young ovariectomized mice [83], so gene alterations could not be specifically associated with improvements in one type of memory. Genes were considered altered by E₂ if they showed a greater than 2-fold change in RNA expression levels compared to controls; 111 genes were up-regulated and 93 were down-regulated [85]. Of these, 17 up-regulated and 6 down-regulated genes have previously been implicated in learning and memory. mRNA expression changes in five genes were confirmed by quantitative real-time PCR (qRT-PCR) 1 hour after treatment, and resulting protein changes

were confirmed with Western blotting 3–4 hours after treatment. The five genes were as follows: Hsp70, a heat shock protein known to be estrogen responsive; Igfbp2, an IGF-I binding protein; Actn4, an actin binding protein involved in protein trafficking; Tubb2a, the major component of microtubules; and Snap25, a synaptosome-specific protein required for neurotransmitter release [85]. mRNA and protein for all genes were significantly up-regulated by E₂, with the exception of Igfbp2, which was down-regulated. Hsp70 is an integral part of the cytosolic estrogen receptor protein complex that keeps nuclear ERs in an inactive state until they bind to estrogens and translocate to the nucleus [142]. Hsp70 expression is also increased by E₂ in various cell types, including in the brain, where its expression is influenced by sex [143]. Hsp70-1 mRNA and protein levels have also been implicated in spatial learning and memory [144], so the Hsp70 gene may be an interesting candidate for future studies linking gene expression to E₂-induced memory modulation. Igfbp2 binds IGF-I in the serum and prevents it from activating the IGF-I receptor and initiating intracellular signaling cascades such as PI3K and ERK [145,146]. Thus, an E₂-induced down-regulation of Igfbp2 may lead to greater IGF-I availability, and subsequently to increased PI3K and ERK activation. The remaining genes, Actn4, Tubb2a, and Snap25, may be important in facilitating hormone effects on protein trafficking within cells [147], shuttling ERs to various locations within cells [148], or neurotransmitter release [149], respectively. Together, these data indicate that E₂ has numerous effects on the expression of various genes that potentially underlie E₂'s beneficial effects on cognition.

Another recent microarray study examined hippocampal gene expression after chronic estradiol treatment in young and middle-aged ovariectomized mice [150]. In this study, mice were given cyclic injections of oil vehicle or estradiol benzoate (5 µg) for 3 weeks prior to cued and spatial memory testing in the Morris water maze. Whole hippocampi were collected bilaterally after 5 weeks of treatment and analyzed with cDNA microarrays. Expression of a subset of genes was confirmed using oligonucleotide arrays and qRT-PCR. Behaviorally, middle-aged females treated with vehicle exhibited significant spatial learning and memory impairments relative to the other groups tested. Interestingly, more estradiol-induced alterations in gene expression were observed among middle-aged females (244 gene differentially expressed) compared to young females (58 genes differentially expressed) [150], which may be indicative of mechanisms in the middle-aged brain to counteract degenerative changes due to age and/or hormone loss. The oligonucleotide analysis of selected genes of interest found that estradiol reversed age-related increases in gene expression in 5 genes (Ldb2, Foxo3a, Hdac2, Lass2, and Kctd3) and age-related decreases in 2 genes (Fbxw8 and cbini) [150]. mRNA expression in two of these genes was confirmed with qRT-PCR (Hdac2 and Lass2). Hdac2, histone deacetylase 2, may be of particular interest for studies of hormones and cognition due to recent evidence that this gene negatively regulates hippocampal-dependent memory and hippocampal synaptic plasticity [151].

Although purely descriptive, these new studies provide the foundation for future work examining the roles that the aforementioned genes may play in mediating the beneficial effects of E₂ on object and spatial memory. Here again, the blocking approach can be used to test the importance of each gene in mediating the effects of E₂. Numerous techniques could be used to prevent transcription or translation of these genes, including RNA interference (RNAi), oligodeoxynucleotides directed against specific mRNAs, genetic manipulations (e.g., gene knockouts) and epigenetic manipulations (e.g., increasing DNA methylation). In the coming years, the application of these techniques to the study of hormones and cognition will undoubtedly reveal critical insights at the genomic and proteomic levels about how E₂-induced alterations in the hippocampus modulate memory processes.

6. Conclusions

In this review, we have proposed the application of a blocking approach to a Downstream Molecule model of hormones and cognition in order to identify molecules in the hippocampus through which E₂ modulates memory formation. Thus far, this approach has provided important insights into the cellular events in the dorsal hippocampus that are critical for acute E₂ treatments to enhance one type of hippocampal memory, object recognition, in young ovariectomized mice (Fig. 6). To date, our data regarding effects of E₂ alone demonstrate that phosphorylation of the p42 isoform of ERK is necessary for post-training E₂ treatment to enhance object recognition in young females [29]. This phosphorylation may be mediated by upstream activation of PKA, PI3K, and NMDA receptors [84]. Effects of E₂ on dorsal hippocampal ERK activation and object recognition can be mediated entirely by BSA-E₂[29], suggesting a critical role of membrane-bound ERs, although the nuclear ERs, specifically ER β , may also be involved through direct interactions with cell signaling cascades. Because alterations in gene transcription and protein translation will also likely be critical for long-term memory enhancements produced by E₂, future work should apply the blocking approach to understand which genes and proteins, perhaps starting with those already identified through microarray studies, are most critical to the mnemonic effects of E₂.

One important issue regarding the application of these data to the development of new drug treatments is whether the effects will generalize to other types of memory or to treatments given chronically. If common neurobiological principles govern the formation of all types of hippocampal memories regardless of type (e.g., spatial, contextual, object) and length of training (e.g. one-trial or multiple-trial), then the findings generated from acute post-training treatments should generalize more broadly. The fact that processes like ERK activation are critically necessary in the hippocampus for long-term memory formation in the spatial Morris water maze, contextual fear conditioning, and novel object recognition tasks [73–76] suggests that this might be the case. Such rapid processes likely serve as triggers that lead to the morphological and physiological changes (e.g. spine formation, enhanced LTP) responsible for long-term memory. Maintenance of these structural and physiological alterations may require chronic treatment with drugs that stimulate cell signaling, and so mechanisms identified through acute treatments may be pivotal to the success of chronic treatments.

Although this review has focused primarily on the modulation of hippocampal function and hippocampal memory by E₂, the Downstream Molecule model and blocking approach can be easily applied to any hormone, behavior, and brain region. E₂ and the hippocampus have been emphasized here simply because the blocking approach has not been widely applied to this area of study. The application of this approach to study the neurobiological mechanisms through which other hormones influence cognition will undoubtedly provide a much more complete understanding of how these hormones affect cognitive processes, and may ultimately lead to safer and more effective treatments for reducing the detrimental effects of aging, mental illness, and neurodegenerative disease on cognitive function.

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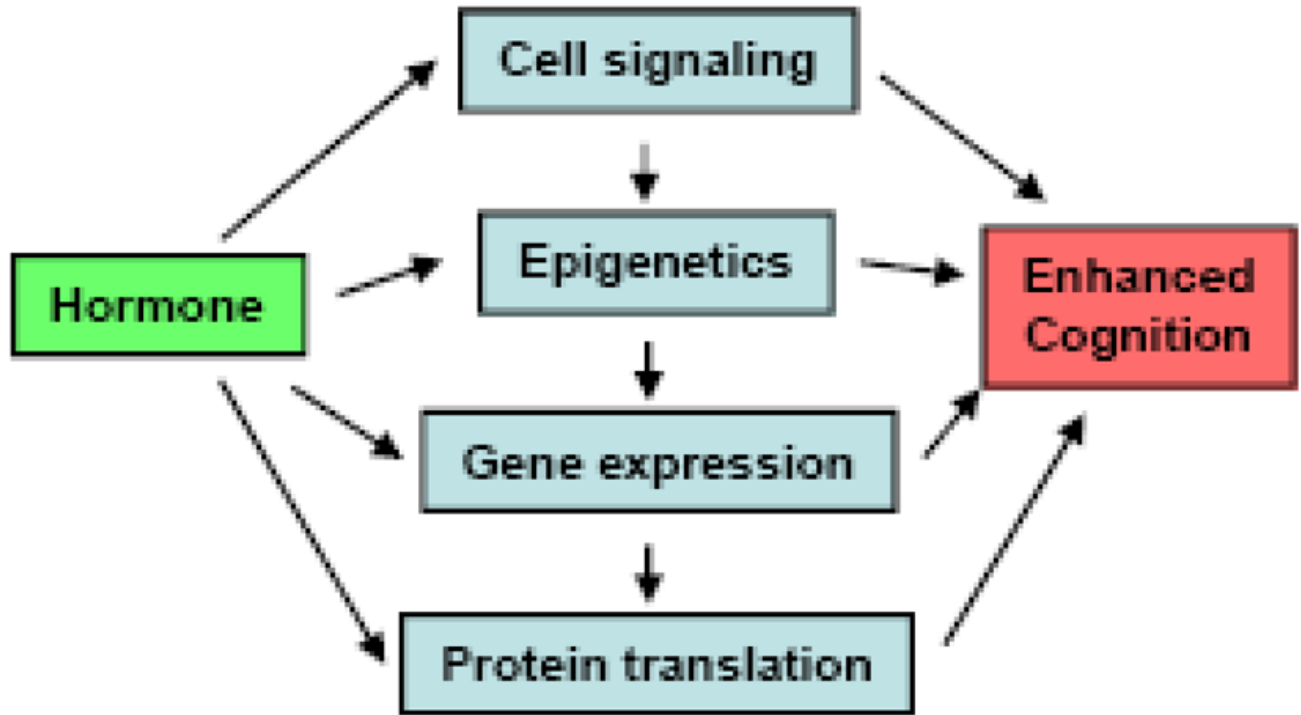
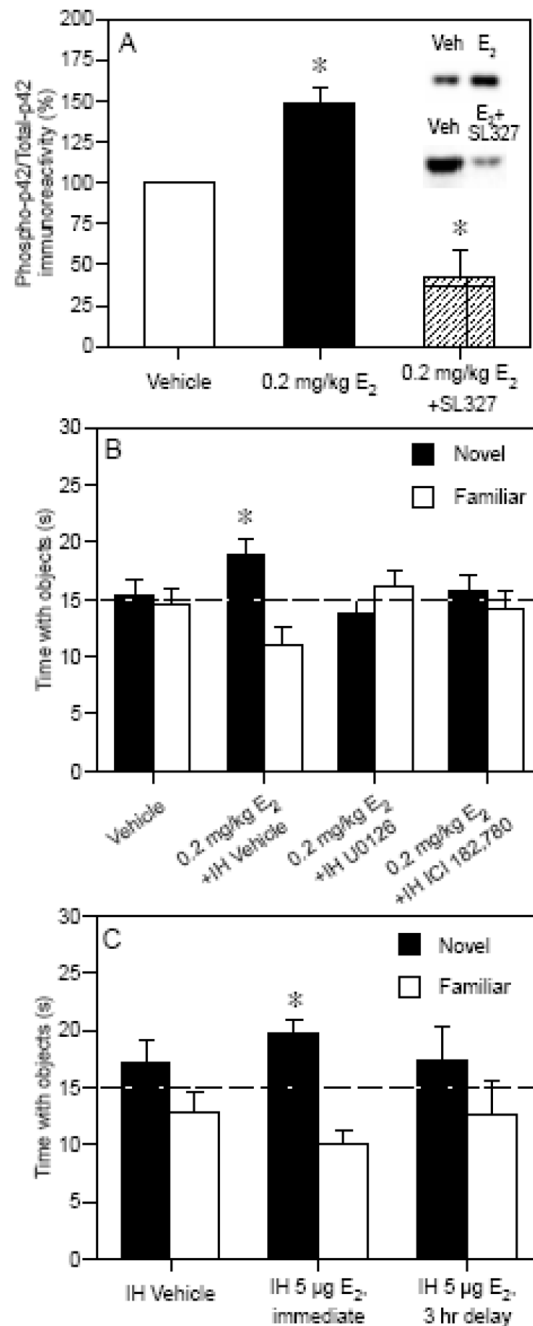


Fig. 1.
Schematic diagram of the "Downstream Molecule" model.

**Fig. 2.**

(A) Phospho-p42 ERK levels in the dorsal hippocampus 1 hr after 0.2 mg/kg E₂. E₂ significantly increased phospho-p42 ERK levels, and 30 mg/kg SL327 blocked this increase (**p* < 0.05 relative to vehicle). Bars represent mean (± SEM) % change from vehicle. Inset: Representative Western blots. (B) Mice receiving 0.2 mg/kg E₂ spent significantly more time with the novel object than chance (dashed line at 15 s, **p* < 0.05), indicating memory for the familiar object. This effect was blocked by IH infusion of U0126 (0.5 µg/side) or ICI 182,780 (5 µg/side). (C) Mice receiving immediate, but not 3-hr delayed, IH infusions of E₂ spent significantly more time than chance (dashed line at 15 s, **p* < 0.05) with the novel object, suggesting that the effects of E₂ on object recognition occur within 3 hours of infusion. Bars

in (B) and (C) represent the mean (\pm SEM) time spent with each object in seconds. Adapted from [29].

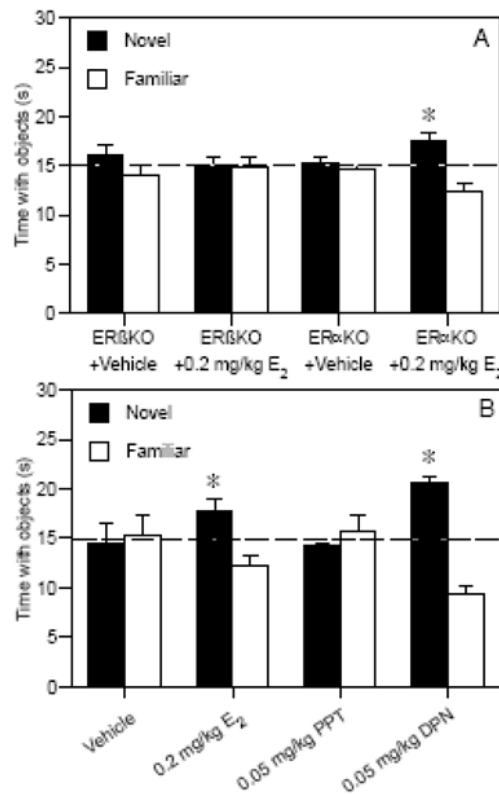
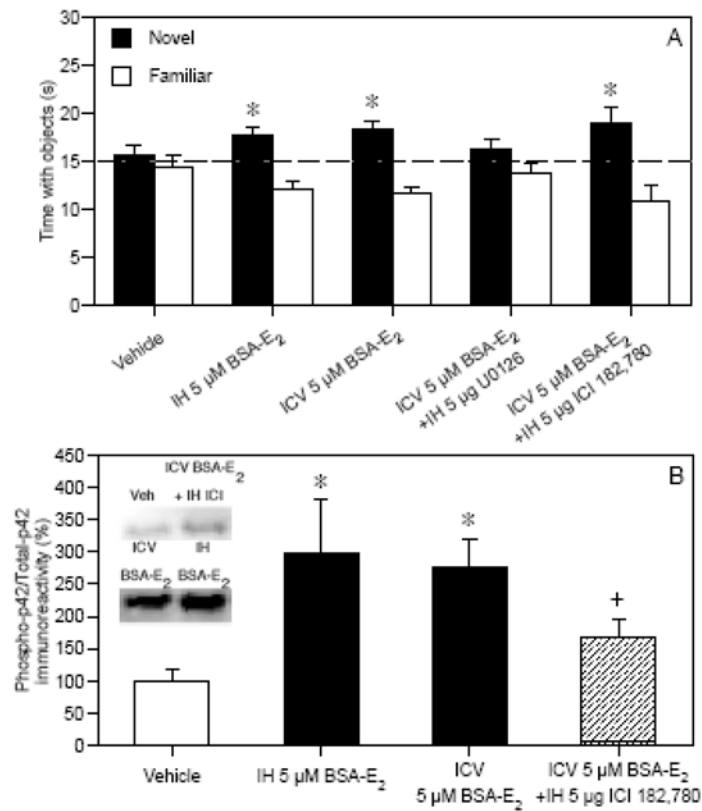


Fig. 3.

(A) 0.2 mg/kg E₂ (Sigma, St. Louis, MO) significantly increased the time spent with the novel object relative to chance (dashed line at 15 s, * $p < 0.05$) in ovariectomized ERαKO mice ($t(9) = 3.20$, $p < 0.05$), but not in ERβKO mice ($n=10$ /genotype), suggesting that only E₂-treated mice with ERβ could remember the familiar object. (B) In ovariectomized wild-type C57BL/6 mice, 0.2 mg/kg E₂ ($t(8) = 2.43$, $p < 0.05$) and 0.05 mg/kg DPN ($t(9) = 7.06$, $p < 0.05$), but not 0.05 mg/kg PPT, significantly increased the time spent with the novel object relative to chance (dashed line at 15 s, * $p < 0.05$). ER knockout and wild-type mice were obtained from Taconic Farms (Germantown, NY) and PPT and DPN were obtained from Tocris (Ellisville, MO). Bars in (A) and (B) represent the mean (\pm SEM) time spent with each object in seconds. Together, these data suggest a role for ERβ, but not ERα, in mediating beneficial effects of E₂ on object recognition.

**Fig. 4.**

(A) IH and ICV BSA-E₂ significantly increased the time spent with the novel object relative to chance (dashed line at 15 s, * $p < 0.05$), suggesting that BSA-E₂ enhanced memory for the familiar object. This effect was blocked by IH infusions of U0126, but not IH ICI 182,780. Bars represent the mean (\pm SEM) time spent with each object in seconds. (B) Phospho-p42 ERK levels in dorsal hippocampus 5 min after BSA-E₂ infusion; ICI 182,780 did not significantly reduce this effect. Bars are mean (\pm SEM) % change from vehicle (* $p < 0.05$ relative to vehicle; + $p > 0.05$ relative to all other groups). Inset: Representative Western blots. Adapted from [29].

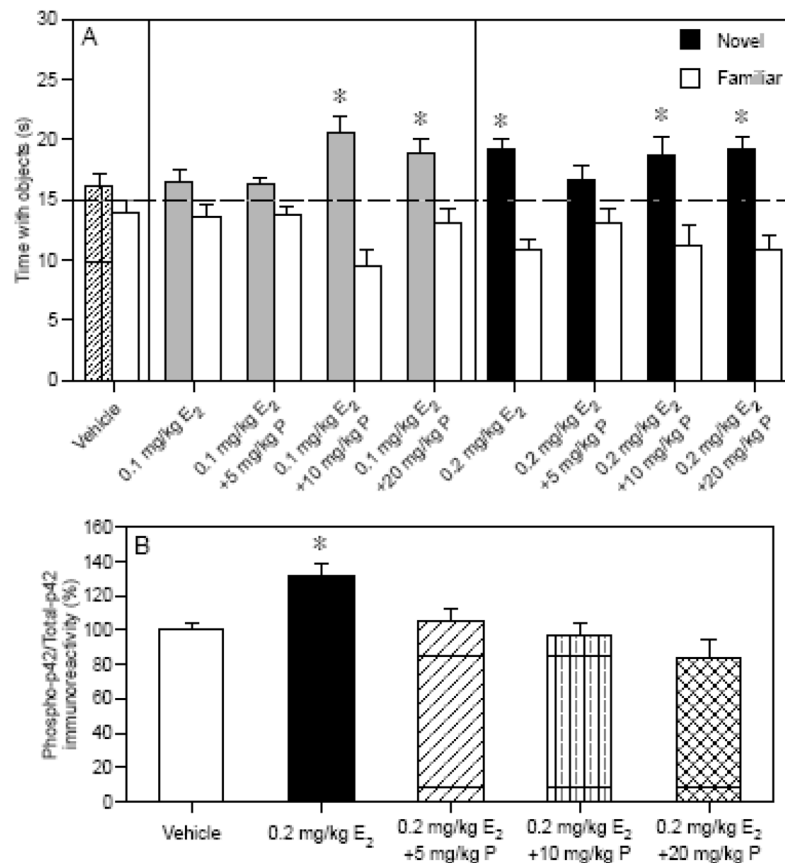


Fig. 5. (A) 0.2 mg/kg E₂, but not 0.1 mg/kg E₂, significantly increased the time spent with the novel object relative to chance (dashed line at 15 s). The 10 and 20 mg/kg doses of progesterone also significantly increased time spent with the novel object relative to chance regardless of the concomitant dose of E₂. Bars represent the mean (\pm SEM) time spent with each object in seconds ($*p < 0.05$ relative to chance). (B) All doses of progesterone blocked the increase in phospho-p42 levels induced by 0.2 mg/kg E₂ ($*p < 0.05$ relative to vehicle). Bars represent mean (\pm SEM) % change from vehicle. Adapted from [83,140].

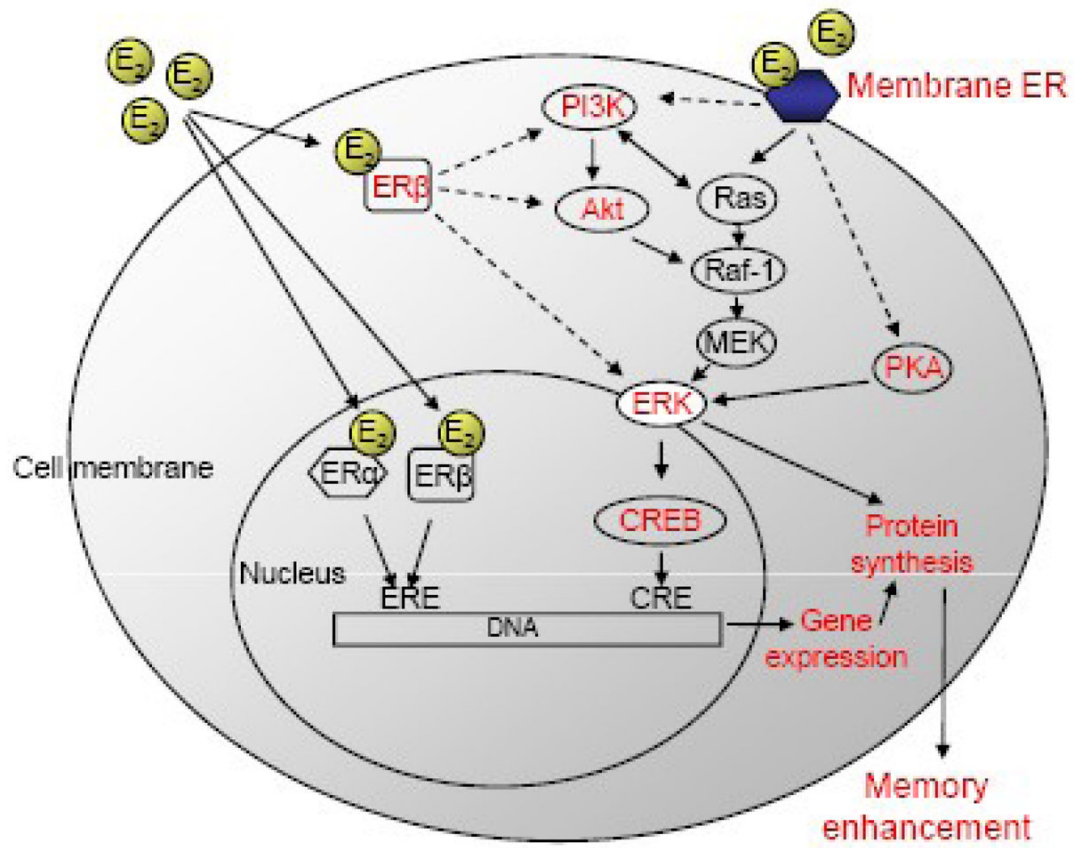


Fig. 6.

Hypothetical model of the molecular mechanisms in the dorsal hippocampus underlying E₂-induced enhancement of object memory consolidation. Solid lines represent events for which evidence exists, whereas dashed lines are hypothetical relationships. Terms in red indicate molecules or cellular processes that may be critical for the rapid effects of E₂ on object memory consolidation. E₂ binding to a membrane ER or ERβ may activate Ras, PI3K, Akt, and PKA, which can then activate ERK, which could stimulate epigenetic alterations, gene expression, and protein synthesis, and thereby improve memory.