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Divergent mechanisms for trophic actions of estrogens in the brain and peripheral tissues

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Abstract

17 β -estradiol (E₂) can enhance reproductive, cognitive, and affective functions; however, the mechanisms by which E₂ has these effects need to be better understood. Pleiotrophic effects of E₂ can occur via traditional and novel actions at various forms of estrogen receptors (ERs). In the central nervous system, trophic effects of E₂ may be related to beneficial effects of hormone replacement therapy (HRT). However, in peripheral reproductive tissues, E₂'s capacity to evoke growth can increase risk of cancers. This review focuses on investigations aimed at elucidating divergent mechanisms of steroids to promote trophic effects in the brain, independent of effects on peripheral reproductive tissues. First, actions of estrogens via ER α or ER β for peripheral growth (carcinogen-induced tumors, uterine growth) and hippocampus-dependent behaviors (affect, cognition) are described. Second, factors that influence these effects of estrogens are described (e.g. experience, timing/critical windows, non-ER mechanisms). Third, effects of estrogens at ER β related to actions of progestogens, such as 5 α -pregnan-3 α -ol-20-one (3 α ,5 α -THP) are described. In summary, effects of E₂ may occur via multiple mechanisms, which may underlie favorable effects in the brain with minimal peripheral trophic effects.

Keywords

Aging; Estropause; Menopause; Progesterone; Neurosteroid; Estrogen receptor

1. Introduction

Questions related to changes in responsiveness to estrogens during cancer progression and menopause have been driving forces in investigations of the mechanisms of estrogen action. A long-standing therapeutic approach for managing estrogen-related cancers, such as breast cancer, is to reduce estrogens' actions in peripheral target tissues. This can be accomplished

by depleting endogenous sources of estrogens through extirpation of peripheral endocrine glands (ovariectomy). Pharmacological interventions are used to inhibit estrogens (aromatase inhibitors) or antagonize estrogens' actions at traditional estrogen receptors (ERs). In contrast, management of menopausal symptoms has focused on restoring estrogen responsiveness in the central nervous system. This approach is based upon the premise that physical (e.g. hot flashes) and psychological (e.g. changes in cognitive function) symptoms become apparent when the ovaries fail to produce biologically-sufficient levels of estrogens (McKinlay and Jeffreys, 1974; Myers et al., 1990). Despite divergent approaches to manage treatment of cancer and menopause, the mechanisms underlying pro-oncogenic effects of estrogens in the periphery and brain are relevant for both areas. The focus of this review is on the mechanisms of estrogens for their growth-enhancing effects in the periphery and brain. The role of estrogen responsiveness for reproductive-related cancers and menopause is relevant when considering effects of hormone replacement therapies (HRT). Indeed, HRTs have therapeutic efficacy for managing many of the symptoms of menopause. However, a main concern of women considering HRT is the potential increased risk of breast and/or uterine cancer among some individuals. Thus, this review discusses the role and mechanisms of estrogens for trophic effects in the periphery and the brain in the context of HRT.

1.1. General overview of sources and functional effects of estrogens

Estrogens are a class of steroid hormones, named for their effects to generate the estrous cycle. However, estrogens have effects beyond their role in reproductive physiology. Estrogens are produced primarily in the theca interna cells of the ovaries from conversion of cholesterol to androstenedione or testosterone, which can then be aromatized to estrone and 17 β -estradiol (E₂), respectively, in the granulosa cells. Estrogens can also be produced in the corpus lutea and placenta. Non-traditional sources of E₂ include the adrenal glands, breasts, adipose tissue, and brain. These alternative sources of E₂ may be of particular functional importance when E₂ production from the ovaries declines with menopause, as these tissues continue to have the capacity to produce estrogens. For example, estrogen in breast tissue of postmenopausal woman derived from local metabolism of adrenal androstene-dione and DHEA, or from adipose tissue, is low but has sufficient biological activity to increase risk of breast cancer (Blair, 2010; Key et al., 2002; Liehr, 2000; Santen, 2008; Santen et al., 2008). Furthermore, effects of estrogens beyond those necessary for reproductive physiology are apparent during states, such as pregnancy, postpartum, and menopause, when there are robust alterations in endogenous levels of E₂. For example, during menopause, reductions in circulating E₂ from the ovaries occur coincident with physiological and/or psychological changes among many women. There is some support for E₂-based or E₂-mimetic HRTs to mitigate these effects. Indeed, clinical and basic sciences support the notion that E₂ has pervasive effects on physiological functions including, but not limited to, development, growth, energy homeostasis, immune system function, cardiovascular health, and bone maintenance. Moreover, there is accumulating evidence that E₂ contributes to central nervous system function, such as cognitive performance, neuroprotection, and affective responding. It is beyond the scope of this review to describe these varied effects of E₂ sufficiently. As such, this review will focus on the mechanisms of E₂ for growth in peripheral tissues that are particularly sensitive to proliferation (e.g. uterus, mammary glands) and limbic regions, such as the hippocampus, that may be particularly amenable to plastic changes that influence cognitive and affective function.

1.2. E₂'s growth-enhancing effects in the periphery: focus on the uterus and breast

The clinical value of investigating the effects and mechanisms of E₂'s trophic actions in peripheral tissues is unequivocal. The traditional view of E₂ involves its post-pubertal effects among females, where it is produced and secreted by the ovaries into circulation, to

act on distal tissues in the periphery, such as the mammary glands, uterus, bone, heart etc, as well as the brain (described below). For example, the uterus is a target for E₂'s trophic effects, where it increases cell proliferation in the endometrium cyclically. Moreover, E₂ potentiates proliferation of cells that line milk glands in breast to prepare for lactation. These are two examples of E₂'s trophic effects that are important for maintaining reproductive function. Notably, these tissues are also particularly susceptible to E₂-sensitive cancers.

Over 50 years ago, growth-enhancing effects of estrogens on the uterus of rats were attributed to actions at a binding site rather than solely through enzymatic actions (Jensen et al., 2010). This work was pivotal, launching many investigations of E₂ actions in breast cancer and treatment, in the decades since these effects and the binding site (what we now refer to as estrogen receptor α , ER α) was discovered. Early preclinical work using the "Huggin's Method," or exposure of female rats to a carcinogen, dimethylbenzanthracene (DMBA), that reliably produces mammary tumors, supported use of tamoxifen, a selective estrogen receptor modulator (SERM) that has antagonistic effects at estrogen receptors (ERs) in breast tissue (Huggins et al., 1961; Jensen et al., 2010; Jordan and Dowse, 1976; Jordan, 1976a,b).

Tamoxifen is still the most widely-used SERM for breast cancer treatment despite drawbacks to its use related to an incomplete understanding of the role of estrogen signaling in breast cancer and tamoxifen's mechanisms of actions. For example, many cases of ER α -positive cancer are unresponsive to tamoxifen treatment. Alternatively, many cases of ER-negative cancer are responsive to tamoxifen. Moreover, resistance to tamoxifen can also develop with prolonged use (Warner and Gustafsson, 2010). Tamoxifen has undesirable actions as an ER ligand in other E₂-sensitive tissues, such as bone and brain (Hunter et al., 2004; Warner and Gustafsson, 2010). Greater understanding of the role of E₂ signaling for trophic effects in peripheral tissues is important for normal reproductive physiology as well cancer processes.

1.3. E₂'s growth-enhancing effects in the brain: focus on the hippocampus

The role of E₂ to facilitate plasticity in the structure and function of the brain is of great interest. In particular, the hippocampus, well recognized for its plasticity in structure and functions related to neuroproliferation (e.g. cognition, affect, neuroprotection), is a target of E₂'s actions. Activity of the hippocampus is increased by E₂. Indeed, E₂ increases expression of the immediate early gene product, c-fos, detected by immunohistochemical staining (Rudick and Woolley, 2000). Moreover, there is an increase in dendritic spine density in the hippocampus with higher levels of endogenous E₂ or following E₂ administration to ovariectomized (OVX) rats (Gould et al., 1990; MacLusky et al., 2005; Woolley et al., 1990; Woolley and McEwen, 1993). A similar pattern is found for levels of the trophic factor, brain derived neurotrophic factor (BDNF), in the hippocampus across the estrous cycle and following exogenous E₂ administration (Gibbs, 1998, 1999; Frye and Rhodes, 2005). The proliferative effects of E₂ may also underlie its actions to increase neurogenesis in the hippocampus (reviewed in Galea, 2008).

Some functional effects that rely on the hippocampus are modulated by E₂. In support, there is improved performance of rats and mice in the object recognition, object placement, and inhibitory avoidance tasks during proestrus, when E₂ levels are higher relative to other phases of the cycle (Frye and Rhodes, 2005; Frye et al., 2007; Walf et al., 2006). There is accumulating evidence from clinical studies and animal models that E₂ loss contributes to cognitive decline (Acosta et al., 2009; Hogervorst and Bandelow, 2010; Wise et al., 2001). Moreover, E₂ administration enhances performance in several hippocampus-dependent cognitive tasks among young OVX or aged female rodents (Bowman et al., 2002; Frick et al., 2002; Frye et al., 2005; Frye and Rhodes, 2002; Gibbs et al., 2004; Li et al., 2004; Luine

et al., 2003; Packard, 1998; Wallace et al., 2006). Together, these data demonstrate that E₂ can have trophic effects in the hippocampus which underlie structural and behavioral plasticity. The potential mechanisms for these effects of E₂ in the hippocampus are discussed in the following sections of this review.

1.4. Interim summary—importance of elucidating E₂'s growth-enhancing effects in the periphery and brain

Do trophic effects of E₂ occur in the periphery independently of the brain and vice versa? Rates of clinical depression are increased among women that have been diagnosed with breast cancer or have entered menopause (Caplette-Gingras and Savard, 2008; Reich et al., 2008). These changes in depression with cancer are generally attributed to demographic factors, hormones, health status, age, etc, rather than the disease state or therapeutics (Caplette-Gingras and Savard, 2008; Reich et al., 2008). Further understanding of how these factors interact is important. We hypothesize that individual hormonal factors influence susceptibility to cancer or symptom severity in menopause, which also confers susceptibility to depression.

A relationship between diagnosis of a neuropsychiatric disorder related to E₂-responsiveness and another later in life has been reported. Higher sensitivities to changes in E₂ among some women contribute to postpartum depression; these women are then also more likely to have perimenopausal depression (Gregory et al., 2000). Of interest, is whether these women also have a differential susceptibility to E₂'s effects at its many targets. Another argument may be that individual factors, such as hormonal milieu, and mechanisms involved in these peripheral disease processes or symptomology may be unrelated to those involved in the concomitant psychological state/depression. In addition, whether the effects of anti-depressants on steroid metabolism enzymes may alter the effectiveness of tamoxifen to prevent recurrence, or worsening of, breast cancer is a clinically-relevant question (Kelly et al., 2010; Lash et al., 2010) related to discriminating the effects and mechanisms of E₂ in the brain and periphery. Given the described role of E₂ in mood processes, as supported by clinical and basic science findings (Walf and Frye, 2006), it is important to further investigate whether the effects and mechanisms of E₂ in the periphery and brain can be discriminated. Whether these changes in depression prevalence with aging and/or cancer are related to alterations in E₂ responsiveness and/or can be treated with HRT are of interest. The following sections present studies using animal models in our laboratory to investigate whether there are disparate mechanisms of E₂ in the periphery and brain for trophic processes.

2. What are the mechanisms of E₂ for trophic effects in the periphery and brain?

2.1. Brief overview of general approach taken to investigate steroids' effects and mechanisms in the brain and periphery

An approach that our laboratory has taken to begin to discriminate peripheral and central effects of steroids, like E₂, is to use a whole animal model. Behavioral endpoints involving hippocampal function (e.g. immobility in the forced swim test) as well as indices of cellular proliferation (i.e., uterine wet weight, incidence/size of DMBA-induced tumors) are analyzed concurrently in the same animal subjects. Effects are compared between animals in different endogenous hormonal milieu or following administration of steroids. This approach has enabled us to begin to address the role and mechanisms of E₂ as well as progestogens. Progesterone (P₄) and its metabolites co-vary with E₂ over endogenous cycles and have trophic effects, in aged and young rodents that have had their ovaries removed (OVX). Furthermore, comparisons are also made between compounds that have specific

effects at steroid receptors or between mice with these targets knocked out. Results from using this approach to investigate the mechanisms of E₂ and progestogens for their trophic actions are described in the next several sections of this review.

2.2. Brief overview of actions of E₂ at ERs

A traditional view of the actions of E₂ is that, like other steroid hormones (e.g. P₄, testosterone), the lipophilic nature permits ready diffusion across cell membranes to bind to cognate receptors (e.g. ERs, progesterin receptors; PRs, androgen receptors; ARs; Fannon et al., 2001; MacGregor and Jordan, 1998). The bound E₂ and ER form a complex that binds to acceptor sites on the chromatin within the nucleus. This attachment to specific acceptor sites enables particular portions of the DNA to be accessible, ultimately influencing protein synthesis and the cell's biological function (Etgen, 1984; Falkenstein et al., 2000; O'Malley and Means, 1974). Indeed, ERs act as transcription factors to regulate the expression of a multitude of genes. An example of one of the most well-known transcriptional actions is the regulation of PRs. Among adult OVX rats, low levels of PR expression in reproductively-relevant brain sites, such as the ventromedial hypothalamus, arcuate, central gray, and medial preoptic area, are reversed with E₂ administration (Lauber et al., 1991; MacLusky and McEwen, 1978, 1980). In addition to the many gene targets of estrogens, great complexity in the functional responses of E₂ is related to the fact that there are at least two characterized forms of the intracellular ER.

2.3. Localization of ERs influences E₂'s functional effects and whether the mechanisms for these effects can be considered traditional or novel

There are at least two forms of the ER that are regionally-defined, suggesting some functional specificity. The originally characterized binding site for E₂ (Jensen and Jacobson, 1962) was renamed ER α following the identification of a second type of ER, which was named ER β (Kuiper et al., 1996; Tremblay et al., 1997). Although these forms have different N-terminal regions, ER α and ER β share approximately 97% and 60% of their DNA- and ligand-binding domains, respectively, and have distinct actions for gene regulation (Green et al., 1986; Kuiper et al., 1996, 1997; Paech et al., 1997; Mitchner et al., 1998; Tena-Sempere et al., 2004; Shughrue et al., 1997a, 1997b). Furthermore, the expression of ER α and ER β differs between and within different tissues in the periphery (Gustafsson, 2003; Shughrue et al., 1997a, 1997b). Organs, such as the bladder, intestines, lungs, and glands, including pituitary and prostate, differ in their expression of ERs, with ER β localization greater than ER α in these cases (Kuiper et al., 1997; Matthews and Gustafsson, 2003; Nishihara et al., 2000; Välimaa et al., 2004).

There are also differences in expression between peripheral tissues and the brain. For example, in the periphery, ER β is expressed in the glandular epithelium of the uterus, but overall ER α expression in the uterus is significantly greater than ER β expression (Hiroi et al., 1999; Kuiper et al., 1996). In the brain, for example, ER α and ER β have been localized in the hippocampus (Mitra et al., 2003; Shughrue et al., 1997a, 1997b; 1998), but expression of ER β in the dorsal hippocampus is greater than ER α . Furthermore, intracellular location of ERs may underlie some of their different mechanisms of action. ER β , as compared to ER α , has been localized to a greater extent outside the nucleus and in the cytoplasm or trafficking to the membrane (Cammarata et al., 2004; Chen et al., 2005; Herrick et al., 2006; Sheldahl et al., 2008; Yang et al., 2004). Indeed, ER β is expressed in the mitochondria of neural, as well as peripheral, cells (Cammarata et al., 2004, 2005; Chen et al., 2005; Mehra et al., 2005; Solakidi et al., 2005; Yang et al., 2004), to which E₂ can be transported, or locally-synthesized (Felty and Roy, 2005; Moats and Ramirez, 2000, 1998). It has been proposed that unliganded ER β in the mitochondria increases measures of oxidative stress in primary hippocampal neurons (Yang et al., 2009). These differences in expression of ER α and ER β

suggest that E₂ acting at these sites may have distinct functions. As described in the sections that follow, these localization patterns likely underlie traditional steroid signaling at ER α in the nucleus and novel actions at ER β in the cytoplasm and/or membrane for their functional effects.

2.4. E₂ has dose-dependent effects on peripheral growth and affective behavior

The role of E₂ to increase the risk for breast cancer is widely recognized (Cauley et al., 1999; Clemons and Goss, 2001; Hankinson et al., 1998; Thomas et al., 1997; Toniolo et al., 1995). The effects of HRT to increase the risk of breast cancer to postmenopausal women are also well understood (Colditz et al., 1995; Magnusson et al., 1999). We have found similar effects in a rat model. Young OVX rats were either exposed to DMBA or acted as non-DMBA controls and then administered low (diestrus-like) or moderate (proestrus-like) dosing of E₂ or placebo oil vehicle, once weekly for nearly 3 mo, 44–48 h before behavioral testing. Tumor burden, uterine wet weight, and behavioral effects were assessed. Both dosages of E₂ increased tumor incidence, which was potentiated with DMBA exposure, compared to placebo vehicle (Walf and Frye, 2009). Both E₂ dosages also enhanced uterine weight, irrespective of DMBA exposure (Walf and Frye, 2009). Although DMBA did not significantly alter behavior of rats, rats administered moderate dosing of E₂ had reduced anxiety-like and depression-like behavior compared to rats administered vehicle (Walf and Frye, 2009). As such, E₂ can have trophic actions in ER α -associated tissues (i.e., mammary gland, uterus) coincident with behavioral changes in our model, which likely involve actions at ER β in the hippocampus (Walf and Frye, 2007, 2008). Using this model, we continued to investigate peripheral proliferative effects in the same animal subjects as behavioral effects were determined to elucidate the mechanisms involved.

2.5. Specific effects of ER α and ER β SERMs for peripheral growth and affective behavior

A follow-up study was conducted to determine whether effects at specific ER forms could discriminate trophic actions in the periphery versus the brain. Unlike what is observed with ER α and proliferation, the clinical literature suggests that the effects of ER β agonism may be protective. Although ER β is typically not highly expressed in mammary glands, prognosis in women with breast cancer that have greater expression of ER β is more favorable (Sugiura et al., 2007). We investigated in our animal model whether there would be differences between effects of ER α and ER β SERMs for tumor incidence, uterine weight, and affective behavior of OVX rats administered DMBA or not. Rats were administered 0.9 mg/kg dosages of E₂, an SERM with higher affinity for ER α than ER β (propyl pyrazole triol; PPT) or an SERM with higher affinity for ER β than ER α (diarylpropionitrile; DPN) or placebo (oil vehicle) once weekly for 8–12 weeks. Administration occurred 44–48 h before behavioral testing. The ER α -SERM, PPT, increased tumor burden (data not shown) and uterine proliferation (Fig. 1, left), similar to that observed with E₂. The ER β -SERM, DPN, did not have these proliferative effects. However, administration of DPN, but not PPT, reduced depression-like behavior similar to that seen with E₂ administration (Fig. 1, right). These data suggest that actions at ER β may underlie favorable psychological effects, whereas actions at ER α may underlie proliferative effects in the uterus and DMBA-induced tumors in our model.

The results of this study confirm what has been described about the majority of estrogens' beneficial effects on affect/mood occurring via actions of ER β and ER α being a target of proliferative effects in peripheral tissues. Moreover, these data extend this notion to suggest that these beneficial effects in the brain can occur independent of trophic actions in peripheral tissues with specific targeting of ER β versus ER α . In clinical studies, ER α is associated with deleterious trophic action in breast and uterine tissue; ER β is not (Lin et al., 2007; Park et al., 2006; Vinayagam et al., 2007). In animal models, we and others have

demonstrated with SERMs and ER α and ER β knock out mice that these receptors have distinct functions. In support, subcutaneous (SC) administration of ER β -selective SERMs can increase anti-anxiety and anti-depressive behavior compared to vehicle or ER α -selective SERMs (Lund et al., 2005; Walf et al., 2004; Walf and Frye, 2005b). E₂ or ER β SERMs do not reduce anxiety-like or depression-like behavior of ER β knockout mice (β ERKO; Imwalle et al., 2005; Krezel et al., 2001; Tomihara et al., 2009; Walf et al., 2008a; 2009a,b,c; Walf and Frye, 2005a). Some of these effects of SERMs may be through actions in the dorsal hippocampus. Infusions of E₂ and ER β -specific SERMs (1 μ g in β -cyclodextran) to the hippocampus increase anti-anxiety and anti-depressive behavior of OVX rats, similar to the effects observed with 10 μ g SC administration (Walf and Frye, 2005b, 2007; Walf et al., 2004). Moreover, ER β may play a more predominant role than ER α in E₂-enhanced hippocampally-mediated processes, such as cognitive processes (Chang et al., 2009; Day et al., 2005; Liu et al., 2008; Walf et al., 2008b; Walf and Frye, 2005b; Rhodes and Frye, 2006). However, the role of ER α for cognitive performance has also been described (Frye et al., 2007; Fugger et al., 2000; Luine et al., 2003; Mazzucco et al., 2006; Rhodes and Frye, 2006). Some of these discrepancies may be related to differences related to general arousal. ER α , rather than ER β , may be integral for E₂-facilitated sexual behavior and general behavioral arousal among rodents (Garey et al., 2003; Kudwa and Rissman, 2003; Mazzucco et al., 2008; Musatov et al., 2006; Ogawa et al., 1998, 1999, 2003). An important consideration is that ER α and ER β do not always act independently and may negate or enhance actions of the other receptor form. These actions are likely related to the tissue-specific transcriptional activities of ER α and ER β and their subsequent recruitment of specific steroid receptor co-activators. For a thorough review of this topic, the reader is referred to a recent publication (Tetel and Pfaff, 2010). Together, these data suggest that it may be possible to target a specific form of ER in to exploit favorable actions of E₂, while minimizing unwanted proliferative effects on breast, uterine, or other tissues that are susceptible to cancer.

3. Other experiential factors to take into consideration when investigating the role of ERs for E₂'s effects

3.1. Role of socially-relevant experiential factors—parity versus social isolation

Another important point for consideration is the role of individual differences and/or experience for observed effects of estrogens. For example, reproductive history contributes to breast cancer risk; pregnancies in young individuals can be protective (Trichopoulos et al., 2008). There are also examples of effects of parity for behavioral responses to E₂. Rats that have had more than one pregnancy (multiparous) outperform virgin or nulliparous rats in cognitive tasks as well as have reduced anxiety-like responding (Byrnes and Bridges, 2006; Gatewood et al., 2005; Kinsley et al., 1999; Macbeth and Luine, 2010; Paris and Frye, 2008; Pawluski et al., 2006). Multiparous rats have also been reported to have greater circulating E₂ compared to primiparous rats during pregnancy (Paris and Frye, 2008) but lower peak E₂ levels when not pregnant (Bridges and Byrnes, 2006). Responses to E₂ were altered in multiparous versus nulliparous rats for anxiety behavior (Walf and Frye, 2008). There are also effects of parity for ER α expression in the hippocampus (Pawluski et al., 2010) as well as reproduction-related regions (e.g. medial preoptic area; Byrnes et al., 2009), but the question remains whether reproductive experience alters expression of ER β in these regions. Indeed, polymorphisms in ERs have been identified as relevant for reproductive cancers as well as mental health (Figtree et al., 2009). Furthermore, other social experiences can alter these types of behavioral endpoints as well as proliferative effects. Low levels of social support (e.g. social isolation stress), both in people and in rodent breast cancer models, demonstrate that a lack of social supports can promote cancer progression (Fox et al., 1994; Grimm et al., 1996; Hermes et al., 2009; Kerr et al., 1997; Lillberg et al., 2003;

Reiche et al., 2004; Yee et al., 2008). Thus, these data demonstrate that socially-relevant experiential factors may influence trophic actions of steroids.

3.2. Role of timing of treatment

The timing of E₂ decline and its replacement is also an important consideration. Indeed, the notion that there is a time frame (i.e., critical window) when an individual may be most predisposed to beneficial effects of E₂ (or other steroid replacement) is referred to as the “window of opportunity.” A review of the evidence for this in clinical populations and the relevance for HRT can be found in several recent articles (Rocca et al., 2010; Sherwin, 2006, 2007, 2009). We and others have investigated this idea in animal models, and these data demonstrate that responsiveness to exogenous administration of E₂ most reliably benefits affective or cognitive responses when administered close to E₂ decline (Bohacek et al., 2008; Daniel et al., 2006; Gibbs, 2000; Walf et al., 2009b). As depicted in Fig. 2, young rats that were OVX for one week responded to physiological dosing of E₂ with improved affective behavior, as well as increased uterine wet weight, compared to controls (young OVX rats administered vehicle). We see that older rats (10–20 mo of age) that are left intact, or administered chronic E₂, had similar levels of affective performance and uterine weights as compared to controls. However, a delay in E₂ administration following OVX of aged rats significantly mitigated the beneficial behavioral effects of E₂ for depressive behavior in the forced swim test, while increasing uterine growth (Fig. 2). Although these data in animal models suggest that there are effects of E₂ timing following its decline in the nature of the response, this has been a particularly contentious topic because not all studies support this, especially in clinical populations (see Hogervorst and Bandelow, 2010). Furthermore, there are several other factors that can contribute to the response to HRT beyond age and timing effects.

3.3. Interactions between timing and type of treatment

One such factor is the nature of the HRT that is utilized. Recent studies in our laboratory and others have begun to investigate how formula of HRT, with a focus on the commonly prescribed HRT, conjugated equine estrogen (CEE; Premarin®), may contribute to the nature of responsiveness to such treatments using animal models. The beneficial effects of CEE have been inconsistent. In one study, there were no significant improvements in quality-of-life (affective and cognitive components) among 50–54 year old hysterectomized women that had been taking CEE for 1 or 3 years (Brunner et al., 2005). CEE is derived from pregnant mare urine and contains several components in addition to bioactive estrogens (one of which is E₂), such as androgens (Bhavnani, 1998; Stern et al., 1992), which suggest that CEE's complex pharmacological profile may modulate the nature of the response to such treatment as well as other individual variability among women. In animal models, there is evidence for beneficial effects of CEE. Acute administration of CEE reduces anxiety-like behavior in the elevated plus maze of intact middle-aged rats compared to age-matched rats administered vehicle (Walf and Frye, 2008) and reduces depression-like behavior in the forced swim test among young, OVX rats (Fig. 3, left). Moreover, acute CEE improved object memory of middle-aged rats (Walf and Frye, 2008). A similar pattern of effects was observed on indices of spatial reference and working memory of middle-aged OVX rats administered chronic CEE (Acosta et al., 2009). We have recently reported that decreased anxiety behavior of mice was only observed with CEE administration to wildtype, but not β ERKO mice (Walf, 2010), further suggesting the role of ER β for some of the actions of estrogens for affective responding. However, CEE has uterotrophic effects coincident with improving affect, as demonstrated in our middle-aged rat model (Fig. 3, right). Another clinically-relevant question is the role of over-the-counter treatments that often contain herbal supplements that have greater activity at ER β than ER α , such as soy isoflavones. We investigated the effects of chronic administration of a commercially-

available, herbal supplement, Women's Iso-Formula (AmeriSciences; Houston, TX), to female rats. Effects of this supplement, in particular, were examined because the manufacturer uses quality-control measures to ensure consistency and uniformity of this supplement, which contains β -sitosterol extract, black cohosh extract (root), soy isoflavone extract (bean, which contains daidzein, genistein, daidzin, genestin, and glycitein), kudzu (root), and red clover extract (flower). We found that this supplement did not have clear uterotrophic effects but did improve affective behavior over that observed in vehicle controls (Fig. 3 and see Friedman and Frye, 2011).

Determining if therapeutics can be developed that benefit brain function, likely through ER β , independent of proliferative effects in peripheral E₂-sensitive tissues is an important area of research that we are continuing to investigate. The highly selective ER β agonist, KB9520 (KaroBio AB, Huddinge, Sweden), was studied. We found that this compound had effects greater than physiological dosing of E₂ alone to decrease depressive behavior in the forced swim test, compared to vehicle. Notably, these effects occurred without any evidence of uterotrophic effects (Fig. 3). Together, these data suggest that additional studies investigating ER β -selective agents are necessary for the development of safe and effective HRT.

4. Interim summary—E₂'s trophic effects at ER α in the periphery and ER β in the brain

Together, these data provide proof-of-concept that actions at ER β underlie anti-anxiety and/or anti-depressive effects of E₂. Furthermore, the mechanisms for these effects in the brain (e.g. hippocampus) may be different from those in the periphery for trophic actions. How these protective effects can be differentiated from trophic actions in the periphery at E₂-sensitive tissues needs to be addressed further. Of interest are questions about other mechanisms of E₂ and its down-stream targets in the hippocampus and peripheral tissues. These questions will be addressed in the following sections.

5. Evidence in support of ER β and non-traditional estrogen signaling

5.1. Rapid, membrane actions of ER β in the hippocampus for affective responding

Data described thus far have focused on the notion of divergent effects of estrogens for growth-related processes, which may be ER-mediated. Other levels of complexity for estrogen signaling are beyond the scope of this review but deserve mention. Estrogen signaling is typically considered in the scope of two broad categories: 1) traditional or classical actions involving binding to intracellular ERs and having actions in the nucleus and 2) non-classical actions that are rapid and/or membrane-mediated and may induce signal transduction cascades. The rapid, membrane-mediated effects of E₂ for function (see Walf and Frye, 2008) have also been investigated. For example, E₂ that cannot penetrate the cell membrane (i.e., bound to BSA) improves cognitive performance in the inhibitory avoidance task of OVX rats (Frye and Rhodes, 2002; Walf and Frye, 2008). These effects of E₂ in the hippocampus were rapid (under 10 min; Frye and Rhodes, 2002; Walf and Frye, 2008), which suggests actions via a membrane target. Furthermore, blocking putative signaling pathways that are activated following these membrane actions, such as mitogen-activated protein kinase, in the hippocampus attenuates the effects of E₂ to enhance performance in this task (Walf and Frye, 2008).

Actions at ER β may be involved in some of these rapid and membrane-mediated effects. First, ER β has greater expression outside the nucleus and in the cytoplasm than ER α does (Cammarata et al., 2004; Chen et al., 2005; Herrick et al., 2006; Yang et al., 2004). Of particular interest in this regard is the expression of ER β in the mitochondria of cells in the

brain and peripheral E₂ targets and its role in metabolic function (Cammarata et al., 2004, 2005; Chen et al., 2005; Mehra et al., 2005; Solakidi et al., 2005; Yang et al., 2004). Furthermore, trafficking of ER β , but not ER α , to the membrane has been reported (Sheldahl et al., 2008), and ongoing studies are investigating the functional effects of such action. Second, rapid functional effects of SERMs with differential affinity for ER α and ER β further support the notion that actions at ER β are non-traditional. ER β -SERMs administered directly to the hippocampus or systemically can have rapid effects (Walf and Frye, 2007). Comparisons between placebo vehicle and SERMs with greater activity at ER α than ER β (PPT>17 α -E₂>17 β -E₂>coumestrol>DPN) on anxiety behaviors of OVX rats ten minutes following subcutaneous injections provides further support of rapid effects of ER β in the hippocampus for anxiety. As shown in Fig. 4, SERMs with greater activity at ER β improved affective responding in the open field (model of general anxiety), compared to vehicle. There were similar effects in the social interaction task, a model of social anxiety (data not shown). Interestingly, the ER α -SERM did not reduce anxiety-like responding in the open field, but there was an indication that this compound reduced anxiety-like behavior in the social interaction task. These preliminary data suggest that actions at ER β may be rapid and/or membrane-mediated and that there may be task specificity in whether SERMs have effects to reduce anxiety-like responding. It is also possible that there are interactions between the broad categories of E₂ action for behavior (Rhodes and Frye, 2006; Rissman, 2008; Vasudevan and Pfaff, 2007; 2008).

Other putative actions of estrogens for functional effects deserve mention. For example, there are other ERs to consider, such as ERX (Toran-Allerand et al., 2005). Some estrogens (referred to as “non-feminizing estrogens”) may also have functional effects at G-protein receptors, rather than ER α or ER β . STX is an estrogenic compound that does not bind ER α and ER β but has effects to activate potassium channels and phospholipase C signaling. STX reduces bodily weight gain following OVX of rodents, an effect which is similar to that of E₂ (Qiu et al., 2008; Roepke et al., 2008). In addition, the GPR30 estrogen target (Prossnitz and Maggiolini, 2009; Revankar et al., 2005; Thomas et al., 2005) is widely-expressed in the rodent central nervous system, including the hippocampus (Brailoiu et al., 2007). G1, a ligand of GPR30, increases survival of hippocampal neurons following experimental stroke of middle-aged female rats (Lebesgue et al., 2010). This pattern of protection with G1 is similar to that seen with E₂ and STX (Lebesgue et al., 2010), further suggesting that these G-protein-coupled E₂ targets are functionally-relevant. Notably, GPR30 does not seem to be a target for trophic effects in reproductive tissues (Otto et al., 2009). Together, these studies support the notion that ER β , as well as other substrates, in the hippocampus, may have beneficial functional effects.

5.2. Estrogens that do not bind ERs may have beneficial effects in the hippocampus

In addition to actions involving membrane targets and downstream signaling cascades, as briefly described above, there are other effects of “non-feminizing” estrogens that do not act at ERs, which should be considered in light of developing safer, more effective HRT. We have investigated compounds that are not active at ERs and would not have proliferative effects, on learning processes in an *in vivo* model. The estrogen analog, ZYC 5, is a synthetic compound, which has demonstrated low affinity for ER α or ER β (ED₅₀ of 100 nM; Xia et al., 2002). Previous work has demonstrated that such estrogen analogs are non-feminizing because uterine growth is not evoked coincident with beneficial effects on brain function (i.e., neuroprotection; Perez et al., 2005). An ER binding study in our lab demonstrated that neither ZYC 5 nor a similar synthetic, ZYC 13, bind appreciably in hippocampal tissue following infusion of these compounds, compared to E₂ which had binding parameters similar to previously published reports (Kuiper et al., 1997; Fig. 5, left). To assess putative functional effects of these synthetic compounds, OVX rats were

administered vehicle, E₂, ZYC 5, or ZYC 13 directly to the hippocampus (2 µg/2 µl) immediately following training in the inhibitory avoidance task. As shown in Fig. 5 (right panel), ZYC 5 and ZYC 13 increased crossover latencies in a manner similar to E₂, compared to vehicle. However, it should be noted that these crossover latencies were shorter than we have previously reported with hippocampal administration of E₂ using a different vehicle (β-cyclodextran; Frye and Rhodes, 2002; Rhodes and Frye, 2004). Although there may have been some intrinsic effect of the vehicle in this study, these data demonstrate that the non-ER active estrogens had similar effects in the hippocampus as E₂ to improve cognitive performance. Thus, not all beneficial effects of estrogen-like compounds in the brain require actions at either ERs.

5.3. E₂'s effects may also be through cellular targets involving progestogens

The focus of this review to this point has been action of E₂ through ERs and non-ER mechanisms. However, some non-traditional effects of E₂ may involve progestogens. Indeed, E₂ and P₄ do not act independently *in vivo*; rather, they fluctuate endogenously and co-vary. Moreover, many tissues have traditional, steroid receptor targets (ERs and PRs) for both of these classes of steroids. It is not surprising that important biological functions, such as reproduction and behaviors that may support successful reproduction (e.g. affect, cognition, stress responding), would have multiple targets and, even, interact (e.g. E₂ induction of PRs). For this reason, the question of a critical period for beneficial effects of HRT related to E₂'s actions should take into account progestogens. HRT to non-hysterectomized women typically involves combined estrogens and progestogens because estrogens, like CEE, promote uterine growth; whereas, progestogens thin out these tissues. However, the effects and mechanisms of progestogens in relation to E₂'s effects have been largely under-investigated. Evidence in support of the notion that some of E₂'s effects may involve progestogens follows.

In our laboratory, separate lines of research on the non-classical effects of E₂ and P₄ have converged to suggest that a common denominator for trophic effects may be the P₄ metabolite and neurosteroid, 3α,5α-THP. P₄, primarily from the ovaries, is metabolized by 5α-reductase to dihydroprogesterone, which is then converted to 3α,5α-THP by 3α-hydroxysteroid dehydrogenase. Ovarian E₂ and progestogens co-vary over reproductive cycles in women and rodents. Progestogens have necessary growth-enhancing effects that are critical for pregnancy/maternal behavior (tissue remodeling, changes in angiogenesis, ovulation, implantation, decidualization, parturition, breast development, etc) as well as a protective role in the uterus. However, progestogens can also have negative prothrombotic and other growth-enhancing effects in the periphery through actions at cognate PRs (Critchley et al., 2001; Conneely et al., 2007; Norman et al., 2007). For example, P₄ can have trophic effects in breast tissue through actions primarily involving PRs (Clarke and Sutherland, 1990). In contrast, P₄'s metabolite, 3α,5α-THP, can have trophic effects to facilitate cognition, affect, and neuroprotection, through non-PR actions (e.g. GABA, glutamate, cholinergic, and/or dopamine receptors; Frye, 2009; Gibbs and Aggarwal, 1998; Simpkins et al., 1997). Hence, progestogens may have disparate mechanisms for their positive and negative trophic effects.

3α,5α-THP can also be produced from central biosynthesis, particularly with physical (e.g. cold water swim) or social (e.g. mating) challenges (Frye, 2001a,b, 2009; Frye et al., 2007; Frye and Rhodes, 2006), suggesting a non-traditional functional effect of rapid production and action of this steroid through non-PR mechanisms. E₂ can enhance production of 3α,5α-THP, and its functional effects, by promoting activity of these requisite enzymes (Cheng and Karavolas, 1973; Malendowicz, 1976; Resko et al., 1986; Vongher and Frye, 1999). SC E₂ or an ERβ-SERM, DPN, to OVX rats improves object memory and has similar patterns to increase 5α-reductase expression in the hippocampus and plasma 3α,5α-THP levels (Jensen

et al., 2010). Administration of a low dosage of E₂ (2 µg/µl) via bilateral infusions to the hippocampus of OVX rats increases hippocampal 3α,5α-THP levels, compared to vehicle (Frye and Rhodes, 2005). Furthermore, administration of E₂ or DPN to wildtype, but not βERKO, mice improves object memory and hippocampal levels of 3α,5α-THP (Walf et al., 2008a, 2008b). Together, these data suggest an interaction between ERβ and progestogens.

5.4. ERβ versus PR versus P₄ metabolism for trophic effects

Knock out mouse models have provided an additional tool with which to investigate the role of ERβ, PRs, and steroid metabolism enzymes. We have used knock out mice with deletions of ERβ (βERKO), PR (PRKO), or 5α-reductase (5α-RKO), and wildtypes (WT), all bred onto a C57BL/6 background. A consistent background strain was used in this particular study because of reports that strain differences are observed in E₂-mediated growth of peripheral tissues (Bailey and Nephew, 2002; Harvell et al., 2000) and anxiety responses (Morgan and Pfaff, 2002). Mice were administered a physiological dosage of E₂ or P₄ or vehicle (to OVX WT as a control) before assessments of anti-depressive behavior and uterine growth. As shown in Fig. 6 (left), SC E₂ increased anti-depressive behavior among WT, but not βERKO or 5α-RKO, mice but increased uterine weight in all three of these groups (Fig. 6, right). Notably, the greatest increase in E₂-enhanced uterine growth was observed in βERKO mice. SC P₄ increased anti-depressive behavior of both WT and PRKO, but not 5α-RKO, mice and induced modest increases in uterine growth in all phenotypes, compared to controls (Fig. 6). These results suggest that both ERβ and 5α-reduction may be required for anti-depressive effects of E₂ and ERβ may be protective against uterine growth. Conversely, 5α-reduction, but not PRs, are required for P₄'s anti-depressive effects and uterine growth from P₄ may not depend on ERβ, PRs, or 5α-reduction. These findings from PRKOs support other studies in our laboratory that suggest PRs in the hippocampus or in the midbrain for learning and lordosis, respectively, are not required for progestogens' effects (Frye et al., 2006; Frye and Walf, 2010). Together, these data suggest that there are both autonomous and interacting mechanisms of action for E₂ and P₄'s trophic effects in the periphery and brain.

5.5. Reproductive status decline, HRT administration, and 3α,5α-THP levels

These data in mice suggest that mechanisms of E₂ and P₄ may not be independent and began to delineate some of the mechanisms for these effects. One mechanistic question that remains regarding differences in HRT effectiveness is the possible mechanisms downstream of E₂ involving 3α,5α-THP. Medroxyprogesterone acetate (MPA) is a commonly prescribed progestin therapy; however, questions about the efficacy of MPA for central nervous system function of people and in laboratory models have been noted. MPA is a synthetic progestin that has androgenic effects and little capacity to form 3α,5α-THP due to its effects to inhibit 3α-hydroxysteroid dehydrogenase activity (Penning et al., 1985). We have demonstrated that MPA does not increase 3α,5α-THP in plasma or brain and may mitigate P₄'s metabolism to 3α,5α-THP (Frye et al., 2010a,b). CEE is one of the estrogenic HRTs that is commonly paired with MPA. Unlike MPA, CEE increases 3α,5α-THP in plasma and the brain of female rats (Genazzani et al., 2004; Frye et al., 2010a,b). MPA does not protect against neurotoxicity *in vitro* or seizure activity or traumatic brain injury, cognitive performance, or anxiety behavior in rodents (Braden et al., 2010; Ciriza et al., 2006; Craig et al., 2005; Frye et al., 2010a,b; Frye et al., 2010a; Nilsen and Brinton, 2002; Toufexis et al., 2004; Wright et al., 2008; Zhao and Brinton, 2006;). Furthermore, MPA can attenuate effects of E₂ or P₄ administration to rodents for learning and memory across several tasks (Frye et al., 2010a; Lowry et al., 2010).

It is possible that the relative inability of MPA to form natural progestogens may be an underlying reason that the beneficial effects of MPA are minimal. To investigate this idea,

middle-aged rats were cycled daily and had reproductive function characterized by assessing normalcy of cyclicity, fertility, and fecundity (as per Walf et al., 2009a,b,c, 2011 and Paris et al., 2011). Middle-aged rats that had maintained reproductive status were compared to those that had declining reproductive status, on anxiety behavior in the open field and hippocampal $3\alpha,5\alpha$ -THP levels following administration of placebo vehicle, CEE, MPA, or CEE+MPA. Comparisons were made between these measures and the typical pattern observed in young, proestrous rats with physiological $3\alpha,5\alpha$ -THP levels in the hippocampus (Frye et al., 2000). Anti-anxiety behavior in the open field was increased only in middle-aged rats that had maintained reproductive status and were administered CEE or CEE+MPA (Fig. 7, bottom). In these subjects, $3\alpha,5\alpha$ -THP levels in the hippocampus were similar to those observed in proestrous rats (Fig. 7, top). Rats with decline in $3\alpha,5\alpha$ -THP levels did not have improvements in affective responding with treatment (Fig. 7). These studies in middle-aged rats characterized by reproductive function suggest that differences may be due to capacity for progestogen metabolism in specific brain regions (hippocampus, cortex>midbrain>striatum, cerebellum; Paris et al., 2011; Walf et al., 2011). Hence, HRT administration that increases $3\alpha,5\alpha$ -THP may be most effective, depending upon reproductive status of the individual.

6. The nature of trophic effects may underlie timing effects of therapeutics

6.1. Role of $3\alpha,5\alpha$ -THP formation

The data presented herein suggest that the dosing, formulation, and timing of steroid-based therapies may influence their effectiveness and safety with respect to trophic effects in the brain and peripheral tissues. The mechanisms that underlie the timing for functional effects of steroid-based therapies are not well-understood. Of interest is whether these effects are due to changes in expression/activity of multiple gene targets. For example, it is well recognized that the reduction in depression symptoms does not coincide with the biological effects on monoamines in the brain initiated by selective serotonin reuptake inhibitors (SSRIs). Changes in monoamines occur rapidly, but reports of depressive symptomology continue for several weeks following initiation of treatment. The mechanisms responsible for the delay of behavioral effects remain under investigation. It is possible that at least part of the efficacy of SSRIs may be related to their ability to increase $3\alpha,5\alpha$ -THP formation in the brain. Like E_2 , some SSRIs may directly increase activity of 3α -hydroxysteroid dehydrogenase enzyme, promoting conversion of dihydroprogesterone to $3\alpha,5\alpha$ -THP (Griffin and Mellon, 1999). Clinical data also support a role for brain levels of $3\alpha,5\alpha$ -THP to benefit mood (Guidotti and Costa, 1998; Uzunov et al., 1996). Cerebrospinal fluid levels of $3\alpha,5\alpha$ -THP are reduced in individuals with major depression, and this effect can be reversed with 8–10 weeks of SSRI treatment coincident with improvements in Hamilton depression ratings (Uzunova et al., 1998). Among women in the perimenopause or postmenopause that do not respond favorably to SSRIs to ameliorate depression symptoms, adjunctive treatment with E_2 -HRT can augment the therapeutic efficacy of SSRIs (Morgan et al., 2005; Rasgon et al., 2002; Schneider et al., 1997; Soares et al., 2003). A similar pattern has been observed among rodents (Estrada-Camarena et al., 2004; 2006a,b). The capacity to form $3\alpha,5\alpha$ -THP may underlie some of the timing effects for trophic actions of estrogens that are relevant for depression and other neuropsychiatric disorders that occur coincident with changes in hormonal milieu.

6.2. Role of hippocampus plasticity and histone changes

Recent investigations of the etiology and treatment of depression support further investigation of the role of E_2 's and progestogens' trophic actions for these effects. Depression may be related to a decline in hippocampal plasticity (Schmidt and Duman, 2007). Typical anti-depressant treatments increase neurogenesis in the hippocampus

(Duman et al., 2001), as do steroids (Galea, 2008), with similar results on hippocampal morphology/structure (Duman et al., 2001; Santarelli et al., 2003). Recent studies suggest that delay in clinical efficacy of anti-depressants, like SSRIs, may be due in part to changes in extensive gene sets that may underlie trophic actions in the brain (Charney and Manji, 2004; Drevets, 2001; Krishnan and Nestler, 2008). Moreover, histone modifications, such as acetylation and methylation, which evoke gene activity, are a likely target for some of the anti-depressant effects observed following chronic exposure. A postmortem analysis of the nucleus accumbens of individuals diagnosed with depression demonstrated increased levels of acetylated histone 3 and decreased levels of histone deacetylase 2, compared to individuals not diagnosed with depression (Covington et al., 2009). A similar pattern of effects was observed in social defeat, a mouse model of depression, and this was reversed with treatment with the SSRI, fluoxetine (Covington et al., 2009). A non-selective monoamine oxidase inhibitor reduced demethylation of histone H3 at lysine 4 in the nucleus of embryonic cancer cells (Lee et al., 2006). In the hippocampus, overexpression of histone deacetylase 2 inhibited plasticity (i.e., reduced number of synapses and dendritic spine density) and associative memory processes, and opposite effects were observed with deficiency in histone deacetylase 2 (Guan et al., 2009). In our rat model examining coincident changes in peripheral proliferation and hippocampal function following SERMs administration and DMBA exposure, we have observed divergent effects for acetylated and phosphoacetylated expression of histone H3 in tumors, uterus, and hippocampus (Walf, 2010). These data suggest that some of the specificity of the mechanisms of E₂ for functional, trophic effects may be at the level of chromatin. Epigenetic changes involving histones and other factors for growth and plasticity appear to be important for stress responding and relevant for mental health research (Hunter et al., 2009; Tsankova et al., 2007). Hence, further investigation of these effects, relevant for menopause HRT, is currently in progress using our model.

7. Summary

A maxim has been that steroids, such as estrogens and progestogens, have slow, long-term and distal effects but it is increasingly clear that steroids also have rapid, short-term, and proximate (from brain synthesis) effects. It is important to consider these rapid actions in the context of trophic effects that subserve plasticity throughout the lifespan. This review focused on the role of steroids to alter the likelihood of a biological response, such as affective responding or peripheral growth, which involves traditional actions at steroid receptors, actions that do not require cognate steroid receptors, as well as changes at the mitochondrial and chromatin level. Indeed, further investigation of these targets for divergent trophic actions of steroids in the brain and periphery is critical for the development of more effective and safer HRT.

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Fig. 1. Uterine proliferation, tumor weight, and depression-like behavior of ovariectomized (OVX) rats exposed to a chemical carcinogen, DMBA, and weekly administration of estradiol (E₂), an ER α -SERM (PPT), or an ER β -SERM (DPN) compared to OVX rats administered vehicle (control).

Fig. 2. Uterine proliferation (left) and depression-like behavior (right) of young, ovariectomized (OVX) rats administered estradiol (E₂), aged intact rats, or aged OVX rats administered chronic E₂ or E₂ following a delay, compared to OVX rats administered vehicle (control).

Fig. 3. Uterine proliferation (left) and depression-like behavior (right) of aged rats administered conjugated equine estrogens (CEE), an herbal supplement (Iso-Formula), or an ER β specific compound, KB9520, produced by Karo Bio AB, compared to ovariectomized rats administered vehicle (control).

Fig. 4.

Rapid changes in anxiety-like responding in an open field among ovariectomized (OVX) rats administered estrogens with greater affinity to ER α than ER β (PPT>17 α -estradiol (17 α -E $_2$)>17 β -E $_2$ >coumestrol (COUM)>DPN), subcutaneously ten minutes before testing, compared to OVX rats administered vehicle (control).

Fig. 5.

Effects of estradiol (E₂) or estrogen analogs (ZYC 5 and ZYC 13, which do not act at estrogen receptors) on estrogen receptor binding in the hippocampus (left) and inhibitory avoidance behavior (right) when administered to the hippocampus of young OVX rats compared to vehicle control. * indicates significantly different from all other groups.

Fig. 6. Effects of estradiol (E₂) or progesterone (P₄) to mice with targeted deletions of estrogen receptor β (β ERKO), progesterin receptor (PRKO), and 5 α -reductase (5 α -RKO), or not (wildtypes; WT), compared to vehicle controls.

Fig. 7. Effects of vehicle (VEH), conjugated equine estrogens (CEE), and/or medroxyprogesterone acetate (MPA) to middle-aged rats that had maintained reproductive status or had a decline in reproductive status for hippocampus 3α,5α-THP levels (top) and anti-anxiety-like behavior (bottom) compared to young, proestrous rats (control).