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Estradiol or diarylpropionitrile administration to wildtype, but not estrogen receptor beta knockout, mice enhances performance in the object recognition and object placement tasks

Alicia A. Walf[a], **Carolyn Koonce**[a], and **Cheryl A. Frye**[a,b,c,d]

a*Dept. of Psychology, Research- The University at Albany – State University of New York, Albany, NY 12222*

b*Dept. of Biological Sciences, Research- The University at Albany – State University of New York, Albany, NY 12222*

c*The Centers for Neuroscience, Research- The University at Albany – State University of New York, Albany, NY 12222*

d*Life Sciences, Research- The University at Albany – State University of New York, Albany, NY 12222*

Abstract

Cognitive processes mediated by the hippocampus and cortex are influenced by estradiol (E_2); however, the mechanisms by which E_2 has these effects are not entirely clear. As such, studies were conducted to begin to address the role of actions at the β form of the intracellular estrogen receptor (ERβ) for E_2's cognitive effects in adult female mice. We investigated whether E_2 improved performance of wildtype (WT) and ERβ knockout (βERKO) mice in tasks considered to be mediated by the cortex and hippocampus, the object recognition and object placement tasks. WT and βERKO mice were ovariectomized (ovx) and E_2 (0.1 mg/kg), an ERβ selective ER modulator (SERM), diarylpropionitrile (DPN; 0.1 mg/kg), or oil vehicle was administered to mice following training in these tasks. We hypothesized that if E_2 has mnemonic effects, in part, due to its actions at ERβ, then WT mice administered E_2 or DPN would have improved performance compared to vehicle WT controls, which would not be different from βERKO mice administered vehicle, E_2 or DPN. Alternatively, activation of ERα (with E_2, which is a ligand for both ERα and ERβ) may produce opposing effects on cognition and/or the activation of ERα and ERβ vs. either receptor isoform alone may produce a different pattern of effects. Results obtained supported the hypothesis that ERβ activation is important for mnemonic effects. Ovx WT, but not βERKO, mice administered E_2 or DPN had a greater percentage of time exploring a novel object in the object recognition task and a displaced object in the object placement task. Thus, actions at ERβ may be important for E_2 or SERMs to enhance cognitive performance of female mice in the object recognition and placement tasks.

Keywords

estrogen; SERMs; allopregnanolone; hippocampus; cortex; learning; memory

address for correspondence: Cheryl A. Frye, Ph.D., Department of Psychology, The University at Albany-SUNY, Life Sciences Research Building 01058, 1400 Washington Avenue, Albany, NY 12222, (518) 591-8839 voice, (518) 591-8848 facsimile, cafrye@albany.edu.

1. Introduction

Steroid hormones, such as estradiol (E_2), can have trophic effects in the central nervous system. However, results of recent clinical trials suggest that E_2-based replacement therapies are not beneficial to all women (Almeida, Lautenschlager, Vasikaran, Leedman, Gelavis and Flicker, 2006; Espeland, Rapp, Shumaker, Brunner, Manson, Sherwin, Hsia, Margolis, Hogan, Wallace, Dailey, Freeman and Hays, 2004; LeBlanc, Janowsky, Chan and Nelson, 2001; Shumaker, Legault, Kuller, Rapp, Thal, Lane, Fillit, Stefanick, Hendrix, Lewis, Masaki and Coker, 2004; Yaffe, Sawaya, Lieberburg, and Grady, 1998). These initial reports included findings from women seventy years old and older, who had been post-menopausal for an average of 20 years. More recent re-analyses of these data suggest that E_2-based therapies can confer some benefit to women when treatment begins closer to the onset of menopause (reviewed in Sherwin, 2007). Because of the clear clinical relevance, and the need for a greater understanding of E_2's effects for cognitive behavior, more research is needed in this area.

Because age, duration of E_2 deprivation, and other factors may influence the effects of E_2 on cognitive processes, it can be advantageous to investigate effects of E_2 in animal models, where these factors can be controlled. The validity of using an animal model to investigate effects of E_2 is supported by studies with older rodents. For instance, in studies using middle-age female rats and mice, spatial memory decline coincides with the transition to reproductive senescence, which is characterized by decline in ovarian function and E_2 levels (Markowska, 1999; Frick, Burlingame, Arters and Berger-Sweeney, 2000). As well, the effects of long-term E_2-replacement to enhance working memory are only observed when replacement begins at time of ovariectomy (ovx), rather than when initiated months post-ovx (Daniel, Hulst and Berbling, 2006). Comparisons of endogenous fluctuations in, and/or exogenous administration, of E_2 to young rodent are another approach to utilize to investigate the effects of E_2 for cognitive performance. Studies using this approach suggest that the capacity for E_2 to alter cognitive processes appear to be E_2 concentration- and/or task-dependent. During proestrus, or when proestrous-like levels of E_2 are mimicked in ovx rodents, performance in tasks that involve hippocampus-mediated 'place' strategy are enhanced, perhaps due the favorable effects of high E_2 on hippocampal function during consolidation (Frye and Rhodes, 2002; Korol and Kolo, 2002; Korol, Malin, Borden, Busby, and Couper-Leo, 2004; Rhodes and Frye, 2004) or increases in hippocampal dendritic spine density, synaptic proteins, or long-term potentiation (Brake, Alves, Dunlop, Lee, Bulloch, Allen, Greengard, and McEwen, 2001; Choi, Romeo, Brake, Bethea, Rosenwaks, and McEwen, 2003; Cordoba Montoya and Carrer, 1997; Day and Good, 2005; McEwen, Akama, Alves, Brake, Bulloch, Lee, Li, Yuen, and Milner, 2001; Warren, Humphreys, Juraska, and Greenough, 1995; Woolley and McEwen, 1992). Notably, these high, physiological E_2 levels can impair performance in other hippocampally-mediated tasks with aversive components, which implies that some of E_2's effects may occur when E_2 is present during training and testing and/or may be due to altering stress responses (e.g. Chesler and Juraska, 2000; Frye, 1995; Koch, 1998; Wood and Shors, 1998). As well, performance in tasks that are mediated by the striatum and/or dependent upon response learning is enhanced with lower levels of E_2 (Daniel and Lee, 2004; Davis, Jacobson, Aliakbari, and Mizumori, 2005; Holmes, Wide, and Galea, 2002; Korol and Kolo, 2002; Quinlan, Graffe, Duncan, and Brake, 2006; Nofrey, Ben-Shahar, and Brake, 2007; Wide, Hanratty, Ting, and Galea, 2004. Given these differences in E_2's effects, the present study investigates the effects of post-training E_2 regimen that produces proestrous-like E_2 levels in young adult, ovx mice for performance in tasks mediated by the hippocampus and cortex (i.e. object placement and object recognition).

In addition to elucidating the nature and neural substrates of E_2's effects on cognitive performance, it is also important to ascertain the mechanisms by which E_2 enhances cognitive performance. E_2 receptors (ERs) have been localized to the hippocampus of both rats (Shughrue, Lane and Merchenthaler, 1997) and mice (Shughrue, Scrimo, Lane, Askew and

Merchenthaler, 1997). Further, ER expression is reduced among middle-age female rats (Wilson, Rosewell, Kashon, Shughrue, Merchenthaler and Wise, 2002). E_2 binds with a high affinity to ER isoforms, ERα and ERβ. Both ERα and ERβ are expressed in the hippocampus and cortex (Shughrue and Merchenthaler, 2001, Shughrue et al., 1997 and Shughrue et al., 1998), albeit ERβ expression may predominate in these regions. Although, E_2 can have actions through both ERα and ERβ, whether actions at ERβ may underlie some cognitive effects of E_2 are of interest. ERβ knockout mice (βERKOs) administered E_2 had delayed acquisition of learning in the water maze compared to their wildtype (WT) counterparts (Rissman et al., 2002). Moreover, ERβ selective ER modulators (SERMS) or dietary phytoestrogens, which have selective actions at ERβ, enhance performance in hippocampal tasks, such as the water maze, radial arm maze, and inhibitory avoidance tasks (Lund, West, Tian, Bu, Simmons, Setchell, Adlercreutz, Lephart, 2001; Rhodes and Frye, 2006) and performance in tasks mediated by both the hippocampus and cortex (i.e. object recognition and object placement) in some, but not all, studies (Frye, Duffy, and Walf, 2007; Luine, Jacome and MacLusky, 2003; Walf, Rhodes, and Frye, 2006). Given these different patterns of effects with SERMs, it is important to further investigate the role of ERβ for E_2's effects on performance in hippocampally- and/or cortically-mediated tasks, such as the object placement and object recognition tasks. We hypothesized that if E_2 has mnemonic effects, in part, due to actions at ERβ, then WT mice administered E_2 or an ERβ SERM, diarylpropionitrile (DPN), would have improved performance in the object placement and object recognition tasks, compared to vehicle WT controls, which would not be different from βERKO mice administered vehicle, E_2 or DPN. Alternative hypotheses about residual effects of ERα and/or ERβ, and the effects of the ERβ knockout and/or non-ER-mediated effects could be determined with the following comparisons. Residual activation of ERα for observed effects would be supported if there are differences between WT and βERKO mice administered E_2 (which is a ligand for both ERα and ERβ). Residual activation of ERβ for observed effects would be supported if there are differences between WT mice administered E_2 and DPN. Differences due to the knockout of ERβ alone could be accounted for by differences in WT and βERKO mice administered vehicle. Differences in effects of vehicle or DPN to βERKO mice would suggest non-ERβ-mediated effects of DPN.

2. Methods

All procedures were approved by the Institutional Animal Care and Use Committee of The University at Albany, and conformed to the guidelines established by The National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.1 Mouse Husbandry

Subjects were 8–10 weeks old, female mice (N=104). Mice were group-housed (4/5 per cage) in polycarbonate cages (45 × 24 × 21 cm) in a temperature-controlled room (21 ± 1 °C) in the Laboratory Animal Care Facility in The Life Sciences Research Building at The University at Albany. Mice were maintained on a 12/12-hour reversed light cycle (lights off at 8:00 am) with continuous access to Purina Rodent Chow and tap water in their home cages at all times.

2.2 Strain and Genotyping

WT (n=56) and homozygous βERKO (n=48) mice were raised on a C57BL/6 background and derived from breeder pairs obtained from Jackson Laboratories (Bar Harbor, ME). To determine the genotype of mice, DNA was isolated from tails and analyzed by PCR. PCR was conducted in the laboratory of Dr. Anne Messer at The Wadsworth Center by K. Manley, in the Molecular Core Facility at SUNY Albany, or in our laboratory. Briefly, DNA was denatured at 94°C for 3 min, followed by 35 cycles of amplification: 94°C for 30 secs, 60°C for 30 secs, 72°C for 30 secs and a final primer extension step at 72°C for 2 min. Specific primers used:

ESR2-1, which lies upstream of insertion site in exon 2 (5'-GTTGTGCCAGCCCTGTTACT-3'), ESR2-1, which lies downstream of the insertion site in exon 2 (5'-TCACAGGACCAGACACCGTA-3'), and ESR2-3, a neo gene-specific primer (5'-GCAGCCTCTGTTCCACATACAC-3'). Primers were obtained from Integrated DNA Technologies (Coralville, IL). Bands of approximately 106 and 160 base pairs were amplified for WT and βERKO mice, respectively. For this study, only WT and homozygous βERKO mice were included.

2.3 Screening procedure

Before inclusion in the study, the general health of mice and their normative responses to external stimuli were evaluated (Crawley, Chen, Puri, Washburn, Sullivan, Hill, Young, Nadler, Moy, Young, Caldwell, and Young, 2007). Observers, who were blind to the genotypes of mice, performed these evaluations so that potential differences in these measures could be ruled out as contributors to genotypic differences in behavioral responses to E_2 or DPN in the present study. Measures included: the general appearance of mice (clean fur, whiskers, posture, gait, muscle tone) and normative behavior (fur grooming, nest-building with Nestlet cotton squares provided in home cage, huddling with cage mates, ability to cage climb, paw withdrawal when gently pulled) and reflexes (blinking to cotton swab placed close to eyes, ear twitch when cotton swab is gently placed on ear). No differences were noted in WT or βERKO mice for these measures and all mice were included in the study.

2.4. Ovariectomy

Approximately 7 to 10 days prior to behavioral testing, mice were ovariectomized to remove the primary endogenous source of steroid hormones. Briefly, mice were anesthetized using sodium pentobarbital (80 mg/kg, IP, or to effect). An incision in the skin and then abdominal wall was made away from the midline at the level of the pelvis. The ovary, oviduct, and top of the fallopian tubes were ligated and removed. The abdominal wall was sutured. The skin was closed with surgical adhesive and wound clips. After recovery from anesthesia, mice were returned to group-housing in their home cages.

2.5. Hormone treatment

After ovariectomy, mice were randomly-assigned to receive subcutaneous (SC) injections of vehicle (vegetable oil), 17β-estradiol (E_2; 0.1 mg/kg; Sigma Chemical Co., St. Louis, MO), or an ERβ-selective SERM, DPN (0.1 mg/kg; Meyers et al., 2001; Tocris Chemical Co., Ellisville, MO) immediately after the completion of training in each task. E_2 and DPN were dissolved in vegetable oil. Dosing for both was based upon pilot studies and published reports (Frye et al., 2007; Walf et al., 2006; 2007), which indicated that this regimen of E_2 produced proestrous-like levels of E_2 in plasma (Edwards, 1970; Frye & Vongher, 1999) and in brain (see Table 1). Mice were randomly assigned to groups to be administered E_2 (WT, n = 18; βERKO, n=19), DPN (WT, n = 18; βERKO, n=26) or vehicle (WT, n = 22; βERKO, n=27).

2.6. Habituation/Behavioral testing

To habituate mice to handling, mice were picked up for 5 min/day for 5 consecutive days and exposed to a novel stimuli/situation on days 1–4 (new clean cage, weighing scale, novel open field testing chamber (which was utilized in subsequent behavioral tasks that are described below), SC injection of oil vehicle; as per Frye, Sumida, Dudek, Harney, Lydon, O'Malley, Pfaff, Rhodes, 2006). This procedure occurred one week prior to the start of behavioral testing. The mice were housed in a room with a 12:12 reversed light/dark cycle (lights off at 07:00). All training and testing was performed in the core behavioral testing facility, which is adjacent to the animal housing area. Mice were transported in their home cages on a cart to the behavioral testing facility. Each mouse was individually housed prior to training (which occurred between

0900–1100 hours) and was returned to their home cage after testing (between 1300 and 1500). Approximately 7–10 days after ovariectomy, mice were tested in the object recognition or object placement task using a 4-hr delay. Seven to 10 days later, mice were tested in whichever task (object recognition or object placement) that they had not been tested in previously. Hormone injections were given at the completion of training, as described below. The majority of experimental mice were tested once in each task. A few mice were tested in either task twice. Analyses of data from the few mice that were tested in the same task on more than one occasion did not reveal test decay effects, as has been reported (Frye and Walf, 2008; Luine et al., 2003). Whether mice were tested initially in the object recognition or object placement task was randomized and counterbalanced across groups.

2.6.1. Object recognition—The object recognition task, a test of non-spatial reference memory, was conducted in an open field (46 × 57 × 30 cm), constructed of white laminate and located in a quiet room under dim lighting. This same open field was utilized in the habituation procedure described above. Small, plastic, washable toys that have distinct circular (oranges, lemons, apples) or cone-like shapes (buoys, bottles) were utilized as target stimuli. A digital camera was mounted on the ceiling above the testing chamber and connected to a computer with a video-tracking system that objectively monitored and quantified animals' movements (Any-maze- Stoelting, Inc., Wood Dale, IL). Testing, which takes advantage of the natural affinity of mice for novelty, was conducted as described previously (Walf et al., 2006). Briefly, during training, each mouse was placed in the open field box for 180 secs and allowed to explore two identical objects (placed approximately 15 cm from the northeast and northwest walls). After 180 secs, each mouse was removed from the box, immediately injected with vehicle or E_2 or DPN and returned to its individual holding cage. Mice were tested in the choice phase 4 hrs later. One familiar object (identical to that which was used in training) and one novel object were placed in the same location of the box as was used during training. Whether the novel object was placed in the northeast or northwest location was counterbalanced within and between groups. The time spent exploring each object was recorded during both the training and testing phases using Anymaze video tracking system. Object exploration was scored when the mouse was sniffing, climbing on, or touching the object and was a body's length or less away from the object (i.e. less than 3 cm) while facing the object and/or oriented towards it. Four hours later, the time mice spent exploring the objects were recorded during testing. An increased percentage of time spent exploring the displaced object compared to the total amount of time spent exploring both objects during testing (duration spent with novel object/(duration spent with novel object + duration spent with familiar object) × 100) was considered an index of enhanced performance in this task. Chance levels of performance are when mice spend 50% of the total exploration time investigating each object.

2.6.2. Object placement—The object placement task, a test of spatial reference memory, was conducted as per Frye et al., 2007, using methods similar to that described above. Mice were trained in the open field box for 180 secs and allowed to explore two identical objects (placed approximately 15 cm from the northeast and northwest walls). After 180 secs, each mouse was removed from the box, immediately injected with vehicle, E_2, or DPN and returned to its individual holding cage. Mice were tested 4 hrs later. The same objects used in training were employed but one was placed in the same location of the box as was used during training and the other was displaced to a novel location (southeast or southwest). Whether the northeast or northwest object was displaced was counterbalanced within and between groups. An increased percentage of time spent exploring the displaced object compared to the total amount of time spent exploring both objects during testing (duration spent with displaced object/(duration spent with displaced object + duration spent with non-displaced object) × 100) was considered an index of enhanced performance in this task. Chance levels of responding are considered when mice spend 50% of the total exploration time investigating each object.

2.7. Tissue Collection/Dissection

One week after the completion of object recognition and placement testing, some mice that were initially assigned to receive vehicle, E_2, or DPN were re-injected their assigned condition. One hr later, mice were cervically-dislocated, decapitated, and brains were rapidly dissected from the skull and placed on dry ice. This time frame was used because E_2 has to be present within 1 hour of training for post-training estradiol to enhance memory (Frye et al., 2007; Packard and Teather, 1997; Walf et al., 2006). Whole brains were then stored at −80°C until immediately prior to radioimmunoassay. The cortex and hippocampus were dissected out from whole brain on ice immediately before radioimmunoassay (as per Frye and Walf, 2008).

2.8. Radioimmunoassay of Steroid Hormones

To address the effects of hormone treatments and possible genotypic differences in E_2 or progestin biosynthesis, E_2, progesterone (P_4), and its metabolite, 5α-pregnan-3α-ol-20-one (3α,5α-THP), were measured in cortex and hippocampus, as described below, using previously reported methods (Frye and Bayon, 1999). Radioactive probes utilized, E_2 (NET-317, 51.3 Ci/mmol), P_4 (NET-208: specific activity = 47.5 Ci/mmol), and 3α,5α-THP (NET-1047: specific activity = 65.0 Ci/mmol), were purchased from Perkin Elmer (Boston, MA).

E_2, P4, and 3α,5α-THP were extracted from brain tissues that had been processed with a glass/glass homogenizer in 50% MeOH;1% acetic acid. Brain tissues were centrifuged at 3,000 × *g*, followed by chomatographic separation of the supernatant using Sepak-cartridges equilibrated with 50% MeOH:1% acetic acid. Steroids were eluted with increasing concentrations of MeOH (i.e. 50% MeOH followed by 100% MeOH). Solvents were evaporated to dryness in a Savant. Immediately before radioimmunoassay set-up, samples were reconstituted in 150 µl phosphate assay buffer.

The E_2 antibody (E#244, Dr. G.D. Niswender, Colorado State University, Fort Collins, CO), which typically binds between 40% and 60% of [^{3}H] E_2, was used in a 1:40,000 dilution and bound 54% in the present study. Because of concerns about cross-reactivity between E_2 and DPN, only effects of E_2 and vehicle administration are reported here. The P_4 antibody (P#337), obtained from Dr. G.D. Niswender (Colorado State University), when used in a 1:30,000 dilution typically binds between 30% and 50% of [^{3}H]P_4, and bound 48% in the present study. The 3α,5α-THP antibody (#921412-5), obtained from Dr. Robert Purdy (Veterans Medical Affairs, La Jolla, CA), when used in a 1:5000 dilution binds between 40–60% of [^{3}H] 3α,5α-THP and bound 47% in the present study.

The range of the standard curves, prepared in duplicate, was 0–1000 pg for E_2, and 0–8000 pg for P4 and 3α,5α-THP. Standards were added to assay buffer followed by addition of the appropriate antibody (described above) and ^{3}H steroid. Total assay volumes were 750 µl for E_2 and P_4, and 950 µl for 3α,5α-THP. All assays were incubated overnight at 4°C.

Separation of bound and free steroid was accomplished by rapidly adding dextran-coated charcoal to assay tubes. Following 20 minute incubation with charcoal, samples were centrifuged at 3000 *g* for 20 minutes and the supernatant was decanted into a glass scintillation vial with 5 ml scintillation cocktail (Scintiverse BD). Sample tube concentrations were calculated using the logit-log method of Rodbard and Hutt (1974), interpolation of the standards, and correction for recovery with Assay Zap. The inter- and intra-assay reliability co-efficients were: 0.05 and 0.06 for E_2, 0.08 and 0.10 for P_4, and 0.09 and 0.10 for 3α,5α-THP.

2.9. Data analyses

Two-way analysis of variance (ANOVA) was utilized with hormone (E_2, DPN, and/or vehicle) and genotype (WT, βERKO) as independent variables using Statview and/or SuperANOVA statistical software. *Post-hoc* analyses were run, as appropriate, to determine group differences. The α level for statistical significance was 0.05.

3. Results

3.1 Hormone Measures

E_2 administration similarly increased concentrations of E_2 in cortex and hippocampus of WT and βERKO mice. Compared to vehicle, E_2 administration significantly increased E_2, but neither P4, nor 3α,5α-THP, levels in the prefrontal cortex ($F_{2,93} = 16.60$, $P < 0.01$; Table 1). E_2 and DPN administration similarly increased concentrations of 3α,5α-THP in the hippocampus of WT and βERKO mice. E_2 administration significantly increased E_2 ($F_{2,93} = 24.16$, $P < 0.01$; Table 2) and 3α,5α-THP ($F_{2,93} = 2.94$, $P < 0.05$; Table 2), and DPN increased 3α,5α-THP levels in the hippocampus compared to vehicle administration

3.1 Behavioral Measures

3.1.1 Object Recognition—There were no statistical differences between groups in the time spent exploring the objects on the training trials. All mice investigated both objects during training (mean ± sem in secs): WT + vehicle (left: 9.0 ± 3.0, right: 6.7 ± 2.5), βERKO + vehicle (left: 16.4 ± 7.6, right: 6.3 ± 2.4), WT + E_2 (left: 8.5 ± 4.8, right: 14.4 ± 7.4), βERKO + E_2 (left: 7.9 ± 3.4, right: 9.0 ± 3.2), WT + DPN (left: 5.8 ± 3.1, right: 4.4 ± 1.6), βERKO + DPN (left: 13.7 ± 6.7, right: 6.3 ± 2.7).

Administration of E_2 or DPN to WT, but not βERKO, mice significantly increased the percentage of time spent investigating the novel object, over that of vehicle-administered control mice, during the test phase (Figure 1). Hormone and genotype condition interacted ($F_{2,124} = 3.79$, $P< 0.03$) to influence the percentage of time spent investigating the novel object (vs. the familiar object as a function of the total time exploring both objects during testing). There were also main effects of genotype ($F_{1,124} = 24.19$, $P< 0.01$) and hormone condition ($F_{2,124} = 5.44$, $P< 0.01$) during testing. The time mice spent with each object (mean ± sem in seconds) is as follows: WT + vehicle (novel: 10.1 ± 4.0, familiar: 8.0 ± 3.0), βERKO + vehicle (novel: 6.6 ± 2.4, familiar: 9.5 ± 4.0), WT + E_2 (novel: 12.8 ± 5.6, familiar: 7.9 ± 3.9), βERKO + E_2 (novel: 11.0 ± 4.1, familiar: 10.3 ± 4.2), WT + DPN (novel: 4.8 ± 1.0, familiar: 2.3 ± 0.3), βERKO + DPN (novel: 5.3 ± 1.7, familiar: 8.6 ± 3.3).

3.1.2 Object Placement—There were no statistical differences between groups in the time spent exploring the locations during the training trial. All mice investigated both objects during training (mean ± sem in seconds): WT + vehicle (left: 3.3 ± 0.4, right: 4.3 ± 0.8), βERKO + vehicle (left: 3.6 ± 0.5, right: 2.9 ± 0.4), WT + E_2 (left: 2.9 ± 0.6, right: 3.3 ± 0.7), βERKO + E_2 (left: 2.8 ± 0.5, right: 3.0 ± 0.8), WT + DPN (left: 2.2 ± 0.6, right: 2.5 ± 0.5), βERKO + DPN (left: 2.2 ± 0.3, right: 2.2 ± 0.3).

Administration of E_2 or DPN to WT, but not βERKO, mice significantly increased the percentage of time spent investigating the displaced object (vs. the non-displaced object as a function of the total time exploring both objects during testing) over that of vehicle administered control mice (Figure 2). During the test period, there were main effects of genotype ($F_{1,124} = 16.15$, $P< 0.01$) and hormone condition ($F_{2,124} = 4.12$, $P< 0.0.2$), and an interaction between these variables to influence the percentage of time spent investigating the displaced object ($F_{2,124} = 3.06$, $P< 0.05$). The time mice spent with each object (mean ± sem in seconds) during testing is as follows: WT + vehicle (displaced: 3.8 ± 0.5, non-displaced: 4.9

± 0.8), βERKO + vehicle (displaced: 3.0 ± 0.4, non-displaced: 5.0 ± 0.6), WT + E_2 (displaced: 3.4 ± 0.4, non-displaced: 2.5 ± 0.5), βBERKO + E_2 (displaced: 4.0 ± 0.9, non-displaced: 5.7 ± 1.0), WT + DPN (displaced: 2.7 ± 0.4, non-displaced: 1.8 ± 0.3), βERKO + DPN (displaced: 2.7 ± 0.3, non-displaced: 2.6 ± 0.4).

4. Discussion

Our hypothesis that actions at ERβ may be important for cognitive performance in hippocampally- and cortically-mediated cognitive tasks was supported. Administration of E_2, or the ERβ selective SERM, DPN, significantly increased the percentage of time spent investigating the novel or displaced object over that of vehicle-administered WT mice. A similar pattern of effects for E_2 and DPN were not seen in βERKO mice, despite E_2 and DPN administration producing similar increases in 3α,5α-THP levels in the hippocampus. This implies that actions at ERβ may underlie the effects of E_2 and/or DPN (and perhaps 3α,5α-THP) to enhance performance in these tasks.

The present findings that post-training E_2 (0.1 mg/kg, SC) enhanced performance in the object recognition task confirm and extend previous research utilizing this behavioral assay. In the object recognition task, pre-training or post-training E_2 (0.2 mg/kg) improved performance of young adult, ovx mice when they were tested 48-hrs later (Gresack and Frick, 2004, 2006b). Post-training injection of E_2 also improved object recognition memory of young adult, ovx rats (Luine et al., 2003; Walf et al., 2006). Notably, these effects of E_2 are observed with different E_2 regimen, rodent species, and training/testing paradigms. Regarding the latter, in some object recognition protocols, mice are trained until they reach a set total investigation time, which ensures consistent duration of exposure to the objects across individuals during training (Gresack and Frick, 2004, 2006b). Given this longer time spent in contact with training stimuli, it is then possible to examine and demonstrate effects of E_2, 24 to 48 hours later, when E_2 levels are on the decline and may be less of a concern for effects on performance during the test phase. The protocol that we employ provides rodents the opportunity to investigate target stimuli for a fixed training phase duration (180 secs), which typically involves a much shorter duration of exposure to the stimuli during training than do these other approaches. Mice are then tested four hours later. This delay is utilized based upon previous findings from our laboratory demonstrating that this interval is sufficient to reveal effects of endogenous and/or post-training administered steroids (Frye et al., 2007; Walf et al., 2006; 2007). A shorter interval between training and testing produces E_2 levels that are similarly elevated during consolidation and testing. However, the latter factor limits our interpretation of E_2 to effects on cognitive performance, rather than learning/memory. In the present study, there were apparent differences in the time mice spent exploring the objects during training in the object recognition and object placement tasks, which would not be expected because order of exposure to these tasks was counter-balanced. Despite mice having these differences in time spent with objects during training before treatment, mice in the same treatment groups spent comparable duration with the objects during testing. To take into potential differences between groups in total time spent exploring both objects during testing (which were not observed in the present study), data during testing are analyzed as a function of time spent with the target object (i.e. novel object, displaced object) compared to both objects. Indeed, similar enhancements in performance were observed in both tasks following E_2 or DPN administration to WT mice.

The object recognition and placement tasks were selected for use in this experiment because they provide multiple indices of hippocampal function and each task taps into different types of memory encoded by the hippocampus. The object placement task requires intact hippocampal function (Ennaceur et al., 1997). There is some controversy on the neural substrates for object recognition (Mumby, 2001), but performance in this task may involve both the hippocampus and the prefrontal cortex (Baker and Kim, 2002, Clark, Zola and Squire,

2000). E_2 and DPN to WT mice similarly improved performance in both tasks in the present study. Considering the present findings that post-training E_2 enhanced performance in both the object recognition and object placement tasks, in conjunction with previous findings that E_2 enhances CA1 dendritic spine density (Frick et al., 2004, Woolley and McEwen, 1992, 1993), long-term potentiation (Foy et al., 1999, Warren et al., 1995), and neurogenesis in the dorsal hippocampus (Tanapat et al., 1999), effects of E_2 on hippocampal structure and/or function may underlie some of the behavioral changes observed in the present experiment.

A lack of effect in βERKO mice may indicate that non-mnemonic processes may have interfered with their performance in this task (i.e. aberrant brain ontogeny or developmental differences or other compensatory processes associated with their mutation). Although βERKO mice can show increased anxiety and poorer cognitive function (Krezel, Dupont, Krust, Chambon, and Chapman, 2001; Rissman et al., 2002; Walf and Frye, 2006), we found that WT and βERKO mice similarly explored the novel stimuli utilized in both of these tasks. Given these effects, we do not expect that differences in motivation and/or neophobia underlies effects on performance in either the object recognition or object placement tasks utilized in this study. Thus, the lack of beneficial cognitive effects of E_2 among βERKO mice in the object recognition and object placement task did not appear to be associated with baseline deficits.

Because of concerns regarding interpretation of studies relying exclusively on knockout mice, we also utilized the ERβ selective agonist, DPN, to test the hypothesis that ERβ is involved in object recognition and/or object placement performance. Post-training administration of DPN had effects similar to E_2 to enhance performance in these tasks among ovx WT, but not βERKO, mice. Systemic administration of DPN has effects within 30 mins that are maintained for up to 12 hours to increase expression of nuclear ER (Lund et al., 2005). Furthermore, other studies have demonstrated that DPN can enhance performance in object recognition tasks of ovx rats (Walf et al., 2006). An important question is how ERβ may mediate some of the effects of E_2 on performance. One possibility is that some of the effects of ERβ may be secondary to actions at ERα. Indeed, we (Frye et al., 2007) and others (Luine et al., 2003) have demonstrated that propyl-pyrazole-triol (PPT), which binds with 400-fold greater affinity for ERα and demonstrates minimal binding to ERβ (Stauffer, Coletta, Tedesaco, Nishiguchi, Carlson, Sun, Katzenellenbogen, Katzenellenbogen, 2000), can also enhance performance in the object placement task compared to vehicle or DPN. Similarly, post-training administration of E_2 or coumestrol (a SERM with greater affinity for ERβ than ERα, but one that is not as specific for ERβ as DPN) enhanced performance in the inhibitory avoidance task of ovx rats, unlike DPN or vehicle post-training (Rhodes and Frye, 2006). Indeed, co-administration of DPN with 17α-E_2 (which has greater affinity for ERα than ERβ) improved performance in this task (Rhodes and Frye, 2006). That there may be interactions between ERα and ERβ need to be investigated further (Toran-Allerand, 2004). In the present study, differences between WT mice administered E_2 and DPN in either task were not observed, suggesting that residual effects of ERα were not likely. Furthermore, residual effects of ERβ were not supported as there was little evidence for differences between WT and βERKO mice administered E_2. Future studies comparing effects of E_2, ERβ- and ERα- SERMs may reveal the interactions between these isoforms for the functional effects observed.

There are a number of non-ER actions that should be considered. First, rapid, non-ER mediated effects of E_2 have been observed (Mhyre and Dorsa, 2006; Sheldahl et al., 2007; Deecher, Swiggard, Frail, and O'Connor, 2003). Second, findings from *in vivo* and *in vitro* models have suggested the intriguing possibility is that actions of E_2 at nuclear ER and via rapid membrane actions may integrate for their functional effects (Vasudevan et al., 2005) and this may be extended to effects on learning and memory processes. Third, the effects of 3α,5α-THP, which has trophic actions, neuroprotective effects in models of neural injury, and can enhance cognitive performance (Ciriza et al., 2004; Frye et al., 2007; Garcia-Estrada et al., 1993; He et

al., 2004; Rhodes et al., 2004; Vongher & Frye, 1999; Walf et al., 2006), should also be considered. Indeed, E_2 increases activity of metabolism enzymes necessary for 3α,5α-THP synthesis and can increase 3α,5α-THP levels in the hippocampus (Cheng & Karavolas, 1973; Frye & Rhodes, 2005; Vongher & Frye, 1999). The present data, that E_2 and DPN increased hippocampal (but not cortical) levels of 3α,5α-THP and improved performance in tasks mediated by the hippocampus, substantiate further investigation of the role of 3α,5α-THP in some of the mnemonic effects observed in E_2 and/or SERM-administered rodents. Thus, there are many possible ER and non-ER mechanisms that may underlie the mnemonic effects of E_2.

In studies using aging rats, there is evidence of different underlying mechanisms for cognitive processes. For example, ER binding in the hippocampus declines among middle-aged female rats (Wilson, Rosewell, Kashon, Shughrue, Merchenthaler and Wise, 2002), yet E_2 increases dentate spine density in aged rats (Miranda, Williams and Einstein, 1999), synaptophysin levels in middle-aged (Fernandez and Frick, 2004) and aged mice (Frick et al., 2002), and nerve growth factor levels in middle-aged mice (Fernandez and Frick, 2004). These findings imply that some cognitive effects of E_2 in younger and aged individuals may involve different mechanisms. Given the potential beneficial role of E_2 on cognition and the direct relevance for the aging population, the question of how E_2 has actions for cognition will be the subject of much ongoing investigation by our lab and others.

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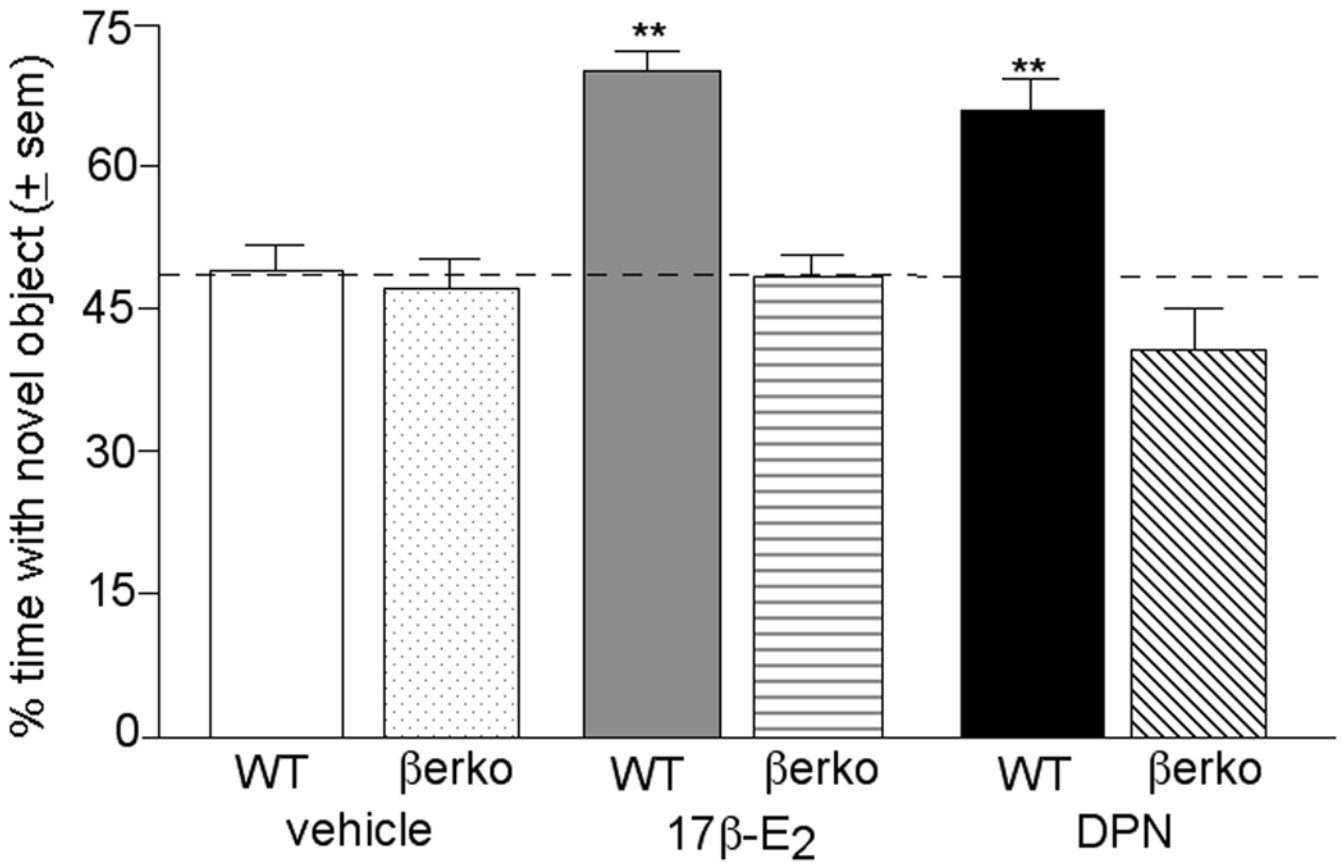

Figure 1.

Mean (± sem) percentage of time spent exploring the novel object by vehicle-administered WT or estrogen receptor β knockout (βERKO) mice, 17β-E_2-administered WT or βERKO mice, or DPN-administered WT or βERKO mice. ** indicates a significant ($P \leq 0.05$) interaction between genotype and hormone condition. Chance levels of responding are considered when mice spend 50% of the total exploration time investigating each object, as indicated by dashed line.

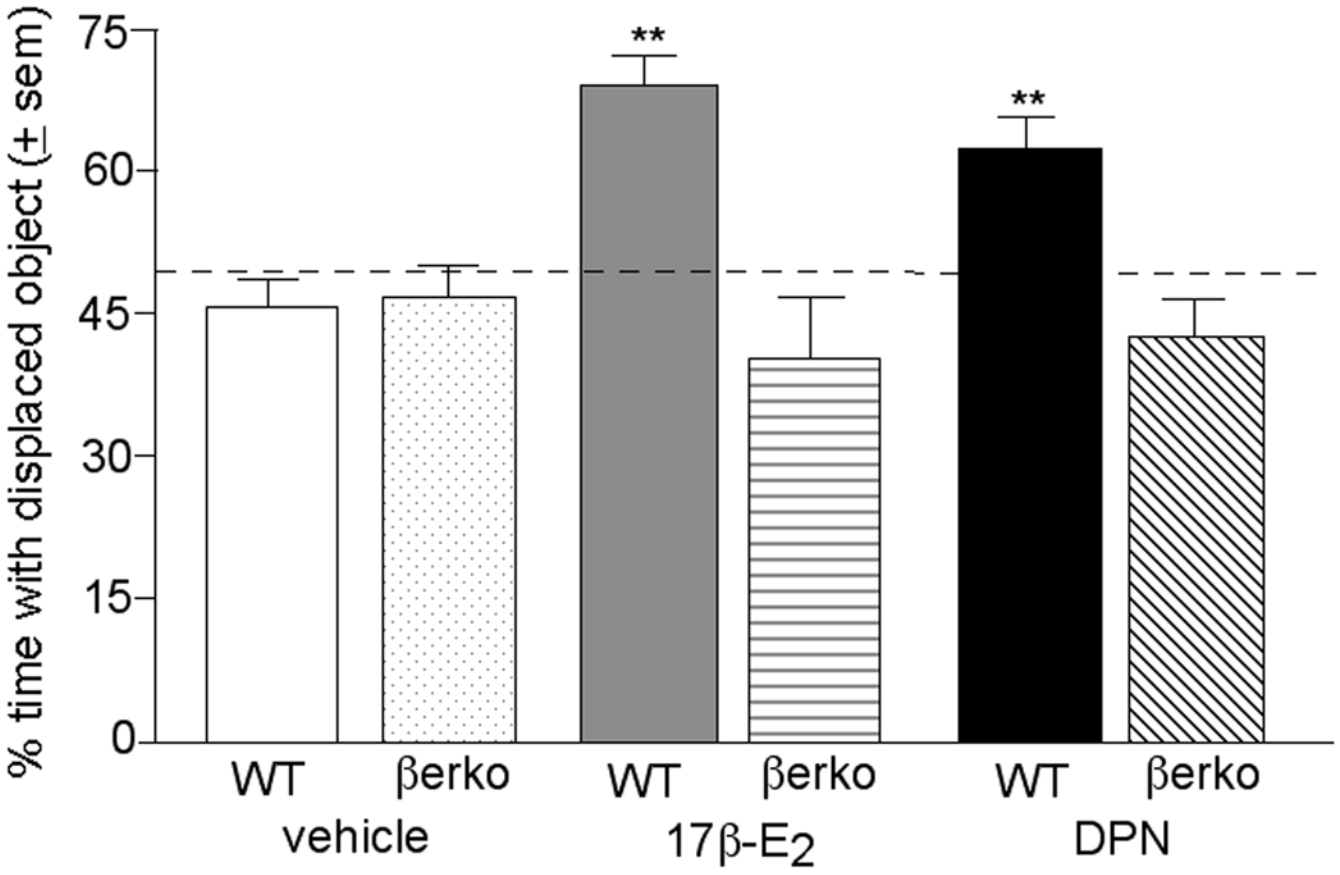

Figure 2.

Mean (± sem) percentage of time spent exploring the displaced object by vehicle-administered WT or estrogen receptor β knockout (βERKO) mice, 17β-E_2-administered WT or βERKO mice, or DPN-administered WT or βERKO mice. ** indicates a significant ($P \leq 0.05$) interaction between genotype and hormone condition. Chance levels of responding are considered when mice spend 50% of the total exploration time investigating each object, as indicated by dashed line.

Table 1

Mean (± sem) prefrontal cortex concentrations of estradiol (E_2), progesterone (P_4) and 3α,5β-THP of ovariectomized wildtype (WT) or estrogen receptor β knockout (βERKO) mice administered subcutaneous vehicle (far left), 17β-E_2 (middle), and DPN (far right).

	CONDITION					
	vehicle		17β-E_2		DPN	
	WT	βERKO	WT	βERKO	WT	βERKO
n=	17	15	15	12	23	17
E_2 (pg/g)	1.5 ± 0.4	1.4 ± 0.3	9.2 ± 1.5*	10.6 ± 2.0*	-	-
P_4 (ng/g)	2.9 ± 0.5	3.5 ± 0.6	4.4 ± 0.9	2.5 ± 0.9	4.6 ± 0.6	6.0 ± 1.0
3α,5α-THP (ng/g)	3.1 ± 0.6	4.0 ± 0.7	5.4 ± 0.8	3.2 ± 1.0	3.8 ± 0.8	4.8 ± 1.1

* indicates a significant ($P \leq 0.05$) effect of E_2 or DPN, which is attributable to higher levels vs. vehicle.

Table 2

Mean (± sem) hippocampal concentrations of estradiol (E_2), progesterone (P_4) and 3α,5α-THP of ovariectomized wildtype (WT) or estrogen receptor β knockout (βERKO) mice administered subcutaneous vehicle (far left), 17β-E_2 (middle), and DPN (far right).

	CONDITION					
	vehicle		17β-E_2		DPN	
	WT	βERKO	WT	βERKO	WT	βERKO
n=	17	15	15	12	23	17
E_2 (pg/g)	1.4 ± 0.4	1.5 ± 0.5	9.3 ± 1.1*	11.9 ± 1.9*	-	-
P_4 (ng/g)	3.9 ± 0.8	2.0 ± 0.5	5.2 ± 0.8	5.5 ± 1.1	5.8 ± 0.8	8.3 ± 0.9
3α,5α-THP (ng/g)	3.9 ± 1.0	4.1 ± 1.1	8.6 ± 1.9*	6.9 ± 2.2*	8.9 ± 1.6*	4.6 ± 1.1*

*indicates a significant ($P \leq 0.05$) effect of E_2 or DPN, which is attributable to higher levels vs. vehicle.