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Assessment of estradiol influence on spatial tasks and hippocampal CA1 spines: Evidence that the duration of hormone deprivation after ovariectomy compromises 17 β -estradiol effectiveness in altering CA1 spines

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Abstract

Two pulses of 17 β -estradiol (10 μ g) are commonly used to increase hippocampal CA1 apical dendritic spine density and alter spatial performance in ovariectomized (OVX) female rats, but rarely are the measures combined. The goal of this study was to use this two-pulse injection protocol repeatedly with intervening wash-out periods in the same rats to: 1) measure spatial ability using different tasks that require hippocampal function and 2) determine whether ovarian hormone depletion for an extended 10-week period reduces 17 β -estradiol's effectiveness in elevating CA1 apical dendritic spine density. Results showed that two injections of 10 μ g 17 β -estradiol (72 and 48 hrs prior to testing and timed to maximize CA1 apical spine density at behavioral assessment) corresponded to improved spatial memory performance on object placement. In contrast, two injections of 5 μ g 17 β -estradiol facilitated spatial learning on the water maze compared to rats given two injections of 10 μ g 17 β -estradiol or the sesame oil vehicle. Neither 17 β -estradiol dose altered Y-maze performance. As expected, the intermittent two-pulse injection protocol increased CA1 apical spine density, but ten weeks of OVX without estradiol treatment decreased the effectiveness of 10 μ g 17 β -estradiol to increase CA1 apical spine density. Moreover, two pulses of 5 μ g 17 β -estradiol injected intermittently failed to alter CA1 apical spine density and decreased basal spine density. These results demonstrate that extended time without ovarian hormones reduces 17 β -estradiol's effectiveness to increase CA1 apical spine density. Collectively, these findings highlight the complex interactions among estradiol, CA1 spine density/morphology, and task requirements, all of which contribute to behavioral outcomes.

Keywords

Estrogen; Hippocampus; CA1; Dendritic Spines; Spatial Learning; Spatial Memory; Ovariectomy; Y-maze; water maze; object placement

Introduction

Estrogen-mediated behavioral sensitization was first described from experiments examining brain regions that were historically known for their role in reproduction, i.e. hypothalamus (McEwen, 1981; Parsons et al., 1982; Pfaff and McEwen, 1983). These studies provided the foundation for a series of landmark discoveries in the early 1990s regarding estradiol actions on hippocampal CA1 spine density. Specifically, Woolley and colleagues (1990a) discovered that CA1 dendritic spine density naturally fluctuates across the female estrous cycle with peaks during proestrus when estradiol levels are highest. Subsequent studies showed that replacement with estradiol and other estrogens in ovariectomized (OVX) females increases CA1 spine density (Gould et al., 1990; Silva et al., 2000; Woolley and McEwen, 1992; Woolley and McEwen, 1993) through NMDA receptor mechanisms (Woolley and McEwen, 1994; Woolley et al., 1997). These findings triggered many studies to investigate a link between estradiol levels and hippocampal function.

Despite the abundance of investigations studying estrogen's effects on cognition, a complex and incomplete story remains (for review, see Daniel, 2006). Some effects can be attributed to estrogen dose (Bimonte-Nelson et al., 2006; Fernandez and Frick, 2004; Foster et al., 2003; Holmes et al., 2002; Sinopoli et al., 2006), route of administration (Garza-Meilandt et al., 2006; Iivonen et al., 2006; Zurkovsky et al., 2007), type or complexity of task (Bimonte and Denenberg, 1999; Fader et al., 1999; Galea et al., 2001; Korol and Kolo, 2002), age of the subject (Foster et al., 2003; Savonenko and Markowska, 2003; Talboom et al., In Press), and other methodological issues (Leuner et al., 2004). Equivocal outcomes are still observed in studies that measure hippocampal-dependent spatial ability and implement estradiol injection timelines similar to those described by Woolley and colleagues to alter CA1 dendritic spine density (two injections of 17 β -estradiol, 24 hours apart, Woolley and McEwen, 1992; Woolley and McEwen, 1993; Woolley and McEwen, 1994; Woolley et al., 1997). Indeed, some studies report that estradiol improves spatial memory, an effect that corresponds with the timing of estradiol-mediated CA1 spine induction (Sandstrom and Williams, 2001; Sandstrom and Williams, 2004), while others show no significant effect (Chesler and Juraska, 2000; Markham et al., 2002; Ziegler and Gallagher, 2005).

Variability in the literature may arise from the duration between OVX and the start of estradiol treatment. Most studies investigating mechanisms of estradiol action on CA1 spines are typically completed within a week following OVX (MacLusky et al., 2005; Woolley and McEwen, 1992; Woolley et al., 1997; Woolley, 1998). In contrast, most studies investigating cognition begin estradiol treatment at least a week or more following OVX and quantification of CA1 spine density is not performed (Chesler and Juraska, 2000; Korol and Kolo, 2002; Sandstrom and Williams, 2001; Sandstrom and Williams, 2004; Bimonte-Nelson et al., 2006; Talboom et al., In Press). One concern is that conjugated equine estrogen treatment starting within four days after OVX increases CA1 spine density, whereas this treatment started ten days after OVX may be ineffective, according to electron microscopy techniques (Silva et al., 2003). These findings draw into question whether CA1 spine density is sufficiently altered in behavioral studies with extended ovarian hormone depletion. Moreover, Silva and colleagues (2003) used conjugated equine estrogen, which contain biologically active estrogens that are not found in rats or humans (Bhavnani, 1998; Dey et al., 2000). Another concern is that testing parameters may mask estradiol-induced elevations in CA1 spines. For instance, daily handling, such as that which might occur with the procedural aspects of testing, can negate estradiol's influence on CA1 spine density (Bohacek and Daniel, 2007). Water maze testing can also mask estradiol's effect on CA1 spine density (Frick et al., 2004) and alter CA3 synapses (Sandi et al., 2003). Therefore, careful consideration must be paid to the timeline between OVX and estradiol treatment, as well as conditions that may influence CA1 spine

density, before conclusions can be made regarding CA1 spine density as a mechanism underlying the behavioral outcomes.

The purpose of this study was to compare the dose-dependent effects of 17 β -estradiol in the same OVX female rats across a battery of well-described hippocampal-dependent, spatial tasks and then assess hippocampal CA1 spine density in these rats. Tasks included variations of the Y-maze (Conrad et al., 1996; McLaughlin et al., 2005; McLaughlin et al., 2007), water maze (Jarrard, 1983; Morris, 1981; Morris et al., 1982), and object placement (Beck and Luine, 2002; Ennaceur et al., 1997). All rats underwent OVX and then started on a two-pulse schedule of 17 β -estradiol injections (0, 5, 10 μ g, s.c.) at 72 and 48 hours prior to behavioral testing. This estradiol regimen has been shown to induce increases in CA1 dendritic spine density at a time that corresponds with our behavioral testing time point. After completing a behavioral task, rats were given a wash-out period prior to the next two-pulse injection schedule and behavioral test cycle, a procedure also used by others to evaluate estradiol's effects on behavior (Sandstrom and Williams, 2004). After nearly ten weeks, half the vehicle-treated rats were then injected with two pulses of 17 β -estradiol (10 μ g, Woolley and McEwen, 1992; Woolley and McEwen, 1993), while the remaining rats received the same 17 β -estradiol dose as they had received previously during behavioral assessment. Rats were not tested on any behavioral task following the last injection cycle that proceeded brain removal to allow us to test the hypothesis that ovarian hormone depletion for 10 weeks reduces 17 β -estradiol's effectiveness in elevating CA1 spine density.

Methods

The Arizona State University Institutional Animal Care and Use Committee approved all subjects and procedures, in addition to following the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Science, National Research Council, 1996). All efforts were made to minimize the number of animals used and their pain and suffering.

Subjects

Twenty-four female Sprague-Dawley rats, weighing 225–250g at the time of arrival, were purchased from Charles River Labs. Rats were pair-housed in closed chambers (21–22° C) under a 12/12 light-dark cycle (lights off at 6:00AM), with access to food and water *ad libitum*. All rats were weighed at different time points throughout the study. Rats were initially divided into three experimental groups for behavioral testing according to hormone injection: sesame oil (O), 5 μ g 17 β -estradiol (5E), and 10 μ g 17 β -estradiol (10E). Following the completion of behavioral testing, a fourth experimental condition was introduced by taking half of the rats in the sesame oil (O) group and administering two acute 10 μ g 17 β -estradiol injections prior to brain removal (O+10E). The project timeline is described in Figure 1.

Surgery

The OVX procedure was the same as that previously described (McLaughlin et al., 2005). Briefly, rats were anesthetized with a ketamine cocktail mixture (10 ml ketamine, 5 ml xylazine, 2 ml acepromazine, 3 ml 0.9% NaCl) and received bilateral OVX through a small incision on the abdomen. The skin was secured with wound clips and rats were given one week to recover.

Hormone Administration

About one week after OVX, wound clips were removed and hormone injections began. Two acute injections of estradiol or vehicle were given 72 and 48 hours prior to behavioral testing, as described by Woolley and McEwen (1997). Increases in CA1 apical spine density are seen after two injections given at this temporal regimen, which corresponded to when our designated

behavioral test was given. It should be noted that 10 μ g injections of 17 β -estradiol likely yield greater serum estradiol levels than observed in a cycling female rat (Scharfman et al., 2007; Sims et al., 1996; Woolley and McEwen, 1993), although these levels dramatically decrease within 24 hours of injection (Sims et al., 1996; Woolley and McEwen, 1993). The 5 μ g dose most likely yields serum estradiol levels within/slightly above the range of that found in a cycling, proestrous rat (Scharfman et al., 2007).

After completion of each behavioral task, rats experienced a “wash-out” period, free of hormone injections (similar to that described by Sandstrom and Williams, 2004; Ziegler and Gallagher, 2005) before the next set of injections and behavioral testing. Wash-out periods ranged from 9–12 days and also served as a control to prevent carry-over effects from testing (McIlwain et al., 2001). After the final behavioral assessment, all groups experienced one last wash-out period. Seventy-two and 48 hours prior to brain removal, rats received their previously assigned injection dose except the oil group, which was further subdivided into two conditions: rats that received two oil injections prior to decapitation (O) and rats that received two 10 μ g 17 β -estradiol injections prior to decapitation (O+10E). The latter group represented OVX females free of hormone replacement for nearly 10 weeks.

Handling

All rats were briefly handled 4–5 days a week to reduce any stress created between the experimenter and injections and to decrease potential anxiety for behavioral testing. For the handling procedure, rats were held for approximately 1 minute.

Golgi

Golgi staining was conducted according to FD Rapid Golgistain™ Kits (FD NeuroTechnologies, Baltimore, MD) on unperfused brain tissue. All brains were removed and processed at the same time (72 hours following the last injection). The staining procedure was the same as described previously by our lab (Bellani et al., 2006; Conrad et al., 2007; Kleen et al., 2006; McLaughlin et al., 2005; McLaughlin et al., 2007).

Histology

To be included in histological quantification, hippocampal cells met the following criteria: 1) the cell body and dendrites were fully impregnated, 2) the cell was relatively isolated from surrounding neurons, and 3) the cell was located in the CA1 region of the hippocampus. CA1 neurons were traced using a Camera Lucida Drawing tube and dendritic properties were quantified using Scion Image Microcomputer Imaging Device Program (Scion Corporation, Frederick, MD, USA). CA1 spine densities were quantified on the most lateral apical tertiary dendrite and the most lateral basal secondary dendrite, similar to previously described protocols (Gould and Allan, 1990; McLaughlin et al., 2005; Woolley et al., 1997). In addition, spines were categorized by shape and separated into spine heads and headless spines because we previously found treatment effects based upon spine shape (McLaughlin et al., 2005). All spine data are represented per 10 μ m segment.

CA1 spine density

Successful staining of the CA1 region provided 3–5 neurons, which were averaged to obtain one value for each rat, and separate analyses were conducted for apical and basal dendritic properties. For CA1 spine density, the number of rats/group were: O (n = 4), 5E (n = 6), 10E (n = 4), O+10E (n = 4).

CA1 spine shape

Spines were categorized based upon shape and represented as a ratio of heads to headless spines. All spines were quantified at 1250x magnification. At this high magnification, we were unable to clearly discern spine shape from the staining obtained in three rats. Subsequently, these rats were excluded from the data analysis, which resulted in the following final number of subjects: O (n = 3), 5E (n = 5), 10E (n = 4), O+10E (n = 3).

Y-Maze

Previous research from our lab suggests that the traditional Y-maze task may be more sensitive to detecting changes in spatial memory of male rats relative to female rats (Conrad et al., 1996; Conrad et al., 2003; Kleen et al., 2006; McLaughlin et al., 2005; McLaughlin et al., 2007; Wright and Conrad, 2005; Wright et al., 2006; Wright and Conrad, 2008). Therefore, to make the task more challenging in this study, we modified the procedure to include longer delays (6 and 24 hours) than previously used (4 hours, Conrad et al., 1996; McLaughlin et al., 2007).

The Y-maze was constructed of black Plexiglas and consisted of three identical exploratory arms ($50 \times 16 \times 32$ cm) that were randomly assigned as start, novel, and other. During training, a rat was placed in the start arm and allowed to explore the start and other arm for 15 minutes. Following an intertrial interval, the rat was placed back into the maze for a five minute testing session, where it had access to all three arms. The first procedure used included a six-hour inter-trial interval between training and testing and the second procedure included a 24-hour inter-trial interval between training and testing. It should also be noted that both Y-maze testing procedures were separated by the wash-out period, where rats were free of hormone injections. Moreover, behavioral testing occurred two weeks apart and new spatial cues (by using fabrics and curtains) were provided to avoid carry over effects from the first testing procedure. Behavior was videotaped and arm entries were quantified after completion of the experiment. An entry was defined as the entrance of both front paws into an arm.

Intact spatial memory was defined as a preference for the novel arm, which was determined by comparing the percent of entries into the novel arm to those made in the other, previously explored arm. Difference scores of entries into the novel minus the other arm were also computed, with positive scores reflecting preference for the novel arm and negative scores reflecting preference for the other arm. Total entries were calculated to assess potential differences in motivational factors and locomotor activity among the groups. All rats completed both Y-maze procedures: O (n = 8), 5E (n = 8), 10E (n = 8).

Morris Water Maze

The water maze consisted of a circular pool with a diameter of 188 cm. The water was opaque with black non-toxic tempura paint (Black F-24, Rich Art Color Co., Inc., New Jersey). Water temperature was maintained at approximately 21–23° C. Water maze testing occurred in a separate room than Y-maze testing and had novel extra maze cues specific to the room. The maze was divided into four quadrants, with a 10 cm black circular escape platform placed at a fixed location approximately 2 cm under the water surface during the training sessions.

Water maze training included 4 trials per day for 2 days based upon a version used previously (Wright and Conrad, 2008). Rats swam for a maximum of 90s or until they located the escape platform. If a rat failed to locate the platform within 90s, it was guided to the platform where it remained for 30s. Rats started each trial at four different points equidistant around the pool, while the escape platform remained at the same position within a quadrant. Rats were tested in squads so that all groups completed trial 1 before starting the next trial. Following the last trial on day 2, the escape platform was removed and rats completed a 60s probe trial. A rat's

swim path was tracked by the Ethovision tracking system (Noldus Information Technology Inc., VA, USA) during all trials.

Behavior analysis in the water maze included latency to the platform, swim distance, and total speed across the eight training trials. For the probe trial, when the platform was removed, use of a spatial strategy was assessed by the total amount of time spent in the target compared to the opposite quadrant and by the percent distance swam in the target quadrant compared to the opposite quadrant. Total distance traveled and swim speed were also measured during the probe trial to elucidate potential differences in locomotor activity and/or strategy. All rats completed water maze training and testing: O (n = 8), 5E (n = 8), 10E (n = 8).

Open Field and Object Placement

Open field testing served as an evaluation of locomotion and anxiety levels, and permitted rats to habituate to the testing apparatus that will be used for object placement. All rats received one 10-minute session in the open field (Day 1). The open field consisted of a black box made of Plexiglas (61 × 61 × 38 cm). The Ethovision tracking system (Noldus Instruments) recorded exploratory activity within the box and separated the open field into inner and outer zones for analyses, according to our designations. The outer region represented 15 cm distance from the periphery of the open field and the inner region was the remaining innermost portion of the arena.

Object placement testing occurred 24 hours after open field testing and was similar to paradigms previously described (Beck and Luine, 2002; Luine et al., 2006; Wallace et al., 2006). Rats were placed in a corner of the open field apparatus and the starting position was counter-balanced among groups. Two objects were located equidistant from the corners of the box and along the wall opposite to the rat's starting position. The objects were identical, 24 cm high, and weighted (.88 kg) so that rats could neither climb nor knock them over during exploration. Each object, as well as the apparatus, was cleaned after every trial to avoid odor cues within the apparatus. Object placement testing consisted of a 3-minute session where a rat could explore the objects, a four-hour inter-trial interval, and then another 3-minute session of object exploration. During the latter session, one of the familiar objects was moved to a new corner location, equidistant from the walls and opposite of where the rat was placed at the beginning of the trial (the same objects were used for both 3-minute sessions). The starting location and object displaced were counterbalanced among groups. The task was repeated a second time after a wash-out period using the same procedures as that described above; however, new spatial cues were placed in the room and new objects were used. The two new objects were again identical, and were 26 cm high and weighed 1.0 kg.

Object exploration was defined as a rat facing the object within 3 cm and actively sniffing or attentively touching the object (Luine et al., 2006). Total exploration time of the apparatus during open field was measured to represent overall activity level. In addition, the time spent in each zone and fecal boli were counted as an anxiety index (Hill and Gorzalka, 2006; Lund et al., 2005) because estradiol benzoate can influence anxiety during open field testing (Morgan and Pfaff, 2001). All rats completed the open field task for both tests: O (n = 8), 5E (n = 8), 10E (n = 8). For object exploration, a rat in the vehicle (O) group did not sufficiently explore the objects during the first exposure trial (less than 20 seconds total, Fernandez and Frick, 2004) and was removed from data analyses, resulting in the following number of subjects: O (n = 7), 5E (n = 8), 10E (n = 8).

Body Weight

Rats were weighed weekly throughout the study, resulting in 10 different weight collections. Consequently, weights were grouped into blocks of 3 (with initial body weight remaining as

its own measure). All rats were included in body weight analyses: O ($n = 8$), 5E ($n = 8$), 10E ($n = 8$).

Statistical Analyses

ANOVAs were used for parametric data, followed by Newman-Keuls posthoc tests, when $p \leq 0.05$. Additionally, t-tests were used for planned comparison analyses when an a priori hypothesis was present (α set at $p \leq 0.05$). Wilcoxon tests were computed for nonparametric data.

Results

Morphology

CA1 Dendritic Spines: Apical Region—Rats treated with $10\mu\text{g}$ 17β -estradiol (10E) had the most CA1 apical spines compared to all other groups (O, 5E, O+10E; Figure 2A). A one-way ANOVA revealed a significant effect of hormone on CA1 apical spine density, $F(3, 11) = 20.97$, $p < 0.01$. Post hoc analyses showed that 10E-treated rats expressed significantly greater CA1 apical spine density compared to O, 5E, and O+10E-treated rats. In addition, rats in the O+10E group had significantly more CA1 spines than rats in the O or 5E groups. O- and 5E-treated rats displayed similar CA1 apical spine density.

The same group pattern was observed for CA1 apical spine shape, with rats in the 10E group having significantly more CA1 apical spine heads than all other groups (O, 5E, O+10E; Fig. 2B). A one-way ANOVA showed a significant effect of hormone on CA1 spine head shape, $F(3,11) = 27.31$, $p < 0.01$. Post hoc results showed that 10E treated rats expressed significantly more CA1 spine heads compared to O, 5E, and O+10E- treated rats. Moreover, O+10E-treated rats had significantly more CA1 spine heads than O or 5E- treated rats. This dose-dependent effect on CA1 spine shape was also shown by an ANOVA demonstrating a significant main effect of hormone on the ratio of CA1 apical spine heads to headless spines, $F(3,11) = 32.38$, $p < 0.01$. Both the O- and 5E groups had a similar number of CA1 spine heads and a similar spine shape ratio (Fig. 2C).

CA1 Dendritic Spines: Basal Region—Rats treated with $5\mu\text{g}$ 17β -estradiol (5E) displayed the lowest mean CA1 basal spine density compared to rats in the other conditions (O, O+10E, 10E; Fig. 3A). A one-way ANOVA showed a significant effect of hormone on CA1 basal spine density, $F(3,11) = 4.29$, $p < 0.05$ and post hoc results found that rats in the 5E group had significantly decreased CA1 basal spine density compared to all other groups (O, O+10E, 10E). There were no other significant differences.

CA1 basal spine shape analyses found rats in the 5E group also had significantly fewer CA1 basal spine heads compared to rats in either the 10E or O+10E groups (Fig 3B). A one-way ANOVA revealed a significant effect of hormone on CA1 basal spine heads $F(3,11) = 6.22$, $p < 0.01$ and a significant effect of hormone on the ratio of CA1 basal heads to headless spines, $F(3,11) = 5.41$, $p < 0.05$. Post hoc results showed that rats treated with 5E had fewer CA1 basal spine heads and a lower ratio of heads to headless spines compared to rats treated with 10E. However, while 5E rats had significantly fewer CA1 basal spine heads compared to the O+10E group, the spine shape ratio was similar between these two groups (Fig. 3C). There were no other significant differences between the groups.

CA1 Dendritic Arborization—All groups expressed similar CA1 dendritic arborization properties. A one-way ANOVA on CA1 total dendritic length and number of branch points for the apical and basal region failed to detect any significant effects (data not shown).

Behavioral Assessments

Y-maze—A one-way ANOVA failed to detect significant differences across the hormonal conditions in the total entries made into all three arms during the full five minutes of testing for both the 6hr and 24hr ITI procedures. Consequently, additional analyses were conducted to determine the optimal minutes to detect spatial memory and to avoid potential habituation. A mixed factor ANOVA with minute as a repeated measure showed a significant effect of minute for the 6hr ITI procedure, $F(4,84) = 34.16$, $p < 0.0001$, and the 24hr ITI procedure, $F(4,84) = 36.11$, $p < 0.0001$. Similar to previous findings from our lab (Conrad et al., 2003; McLaughlin et al., 2005; Wright et al., 2006), rats made the most entries during minute 1 compared to all other minutes ($p < .01$ for 6hr ITI and $p < 0.05$ for 24hr ITI). Subsequently, minute 1 was used for the following Y-maze analyses, as done previously.

For the first minute of testing in the 6hr ITI procedure, all groups made similar total entries into all three arms (average entries were 4.3 to 5.0). In addition, all groups displayed positive difference scores (ranging from 3.1 to 17.7) with a one-way ANOVA failing to detect any group differences. Wilcoxon matched-pairs tests failed to detect a preference for the novel arm within any group, despite entries into the novel arm that appeared to be greater than entries into the other arm for the O and 5E groups ($p = 0.11$ for both O and 5E; Fig. 4A).

For the first minute of testing in the 24hr ITI procedure, all groups made similar total entries into all three arms (average entries were 3.6 to 4.3). A one-way ANOVA was performed on the difference score and failed to detect any group differences, despite positive difference scores (ranging from 10.4 to 14.4). Similar to the results found with the 6hr Y-maze paradigm, a Wilcoxon matched-pairs test failed to detect a preference for the novel arm within any group ($p \geq 0.11$, Fig. 4B). Lastly, a repeated measures ANOVA for hormone treatment on the two Y-maze versions was performed on the difference scores during minute 1 entries, but failed to approach significance.

Water maze—All groups acquired the platform location as supported by decreased latency to locate the platform and decreased swim distance across the 8 training trials (Fig. 5A, B). A mixed factor ANOVA, with trial as the repeated measure, showed a significant effect of trial for latency $F(7, 147) = 20.00$, $p < 0.01$ and distance swam to reach the platform, $F(7, 147) = 22.32$, $p < 0.01$. Post hoc analyses revealed that rats swam significantly longer and covered more distance to find the platform during trial 1 and 2 compared to all other trials.

Rats treated with 5E showed facilitated acquisition during trial 2 of training, as supported by a significant interaction between hormone and trial for distance to the platform $F(14, 147) = 2.74$, $p < 0.01$, and latency to the platform, $F(14, 147) = 2.46$, $p < 0.01$. Posthocs for the distance measure showed that during trial 2, rats in the 5E group swam significantly less distance than rats in the 10E group ($p < 0.05$), and both of these groups swam significantly less distance to the platform than rats in the O group $p < 0.001$ for O vs. 5E, $p < 0.05$ for O vs. 10E, Fig. 5A). Posthocs for the latency measure revealed that during trial 2, rats in the 5E group found the platform faster than rats in the 10E ($p < 0.05$) and O groups ($p < 0.01$; Fig. 5B). The escape latency of the 10E and O groups approached, but did not reach statistical significance ($p = 0.07$).

Swim speed changed across training with rats swimming the fastest during trial 2. An ANOVA, with trial as a repeated measure, found that rats swam faster during trial 2 compared to trials 5, 7, and 8. Since trials 5, 7, and 8 contribute to Day 2's performance, trials were then grouped to represent two separate blocks for speed analyses on Day 1 and Day 2, respectively. An ANOVA revealed a significant main effect of hormone, $F(2, 21) = 3.43$, $p = 0.05$, and a significant repeated effect of block, $F(1, 21) = 6.85$, $p < 0.05$. Posthoc analyses showed that

in general rats swam slower during Day 2 of training. Moreover, 5E rats swam significantly slower compared to 10E rats during trials on Day 2 (data not shown).

During the probe trial, all groups (O, 5E, 10E) showed localized swim patterns in the target quadrant. An ANOVA, with quadrant (target and opposite) as a repeated measure, revealed a significant effect of quadrant for percentage of distance, $F(1, 21) = 253.44$, $p < 0.01$. Rats in all three conditions (O, 5E, 10E) covered a greater percentage of distance in the target quadrant than the opposite quadrant (Fig. 6A). Time spent in the target quadrant was also greater than the opposite quadrant, as shown by a significant effect of quadrant for latency, $F(1, 21) = 304.09$, $p < 0.01$ (data not shown). Moreover, swim speed was similar between all groups during the probe trial and showed significant effects of quadrant on swim speed, $F(1, 21) = 14.14$, $p < 0.01$. Specifically, all groups (O, 5E, 10E) swam faster in the opposite quadrant compared to the target quadrant (Fig. 6B).

Open Field—Separate ANOVAs of data from the two tests were performed. A one-way ANOVA for distance covered in the inner zone during the second test was significant, $F(2, 21) = 3.38$, $p = 0.05$. Rats in the 10E group traveled greater distances exploring the inner zone compared to O and 5E groups (Table 1). While a similar pattern was observed for distance covered in the inner zone during the first test, it was not statistically significant. No other effects were significant.

Object Placement—An ANOVA including the results from test 1 and 2 as a repeated measure revealed no effects on total object exploration time, indicating that rats in all hormonal conditions (O, 5E, 10E) explored the objects similarly during the first trial. During testing in the second trial when one object was moved to a new location, rats preferred exploring objects in the new location, $F(1,20) = 18.15$, $p < 0.001$. The hormone and object location interaction approached significance, $F(2, 20) = 3.08$, $p = 0.068$. Given the past findings of both phytoestrogen's (Luine et al., 2006) and estradiol's (Luine et al., 2003) enhancing effect on object exploration, we conducted planned comparisons on the time spent exploring the objects in the new versus old location within each hormone dose. Moreover, distance covered in the open field significantly differed by test period (Table 1). Consequently, planned comparisons were conducted on tests 1 and 2 separately. For both test 1, $t(7) = 4.95$, $p < 0.005$, and test 2, $t(7) = 4.13$, $p < 0.005$, rats in the 10E group spent significantly more time attending to the object in the new location compared to the old location. The analyses comparing the 5E and O groups did not reach significance.

Physiological Measures—For fecal boli counts, one-way ANOVAs failed to detect differences among groups across open field or object placement testing (data not shown). The majority of the rats did not defecate during open field or object exploration.

A history of both low (5E) and high (10E) estradiol injections decreased body weight gain by the end of the study. A mixed-factor ANOVA, with block as a repeated measure, showed a significant effect of block $F(2, 42) = 478.53$, $p < 0.01$ and a significant interaction between block and hormone $F(4,42) = 3.03$, $p < 0.05$. While all groups had similar weights at the beginning of the study, rats in the 5E and 10E groups gained less weight compared to those in the O group after 10 weeks (Table 1).

Discussion

The findings support our hypothesis that extended OVX without estradiol replacement reduced the sensitivity of the hippocampus to estradiol-induced increases in CA1 apical dendritic spine density. Although two acute injections of 17β -estradiol ($10\mu\text{g}$ each) increased CA1 apical spine density and spine heads in rats sacrificed after 10 weeks of ovarian hormone absence, this

increase failed to reach the same levels as observed in rats treated with two injections of 10 μ g 17 β -estradiol repeatedly over the ten week period. Moreover, we demonstrated that two pulses of 5 μ g 17 β -estradiol failed to increase CA1 apical spine density and even decreased CA1 spine density in the basal dendritic region, thereby producing different morphological effects on CA1 spines than two pulses of 10 μ g 17 β -estradiol. These same rats were tested on a battery of tasks that are well described for their usefulness in measuring spatial ability and requiring hippocampal function. We found that the two pulse, intermittent estradiol injection regimen produced different effects across the tasks. The 10 μ g 17 β -estradiol dose (10E) facilitated performance on object placement, whereas 5 μ g 17 β -estradiol (5E) enhanced water maze acquisition. All groups performed similarly on the Y-maze. Combined, these findings extend the literature on the effects of estradiol on CA1 spine properties and spatial ability in females and allow us to suggest plausible mechanisms.

Effects of intermittent and acute estradiol treatment on hippocampal CA1 morphology

OVX female rats with a history of ovarian hormone deprivation had a decreased response in CA1 apical dendritic spine density and spine heads after acute 10 μ g 17 β -estradiol (OVX + 10E) injections compared to OVX female rats with a history of intermittent 10 μ g 17 β -estradiol (10E) injections. Rats with a history of this 10 μ g 17 β -estradiol intermittent injection regimen showed a 1.5 fold increase in CA1 apical spine density compared to OVX and rats injected intermittently with 5 μ g of 17 β -estradiol (5E). However, ovarian hormone-depleted rats with an oil history followed by acute 10 μ g 17 β -estradiol injections showed only a 50% increase in CA1 apical spine density compared to oil and 5 μ g 17 β -estradiol treated rats. Past reports have shown OVX reduces CA1 apical spine density (Gould et al., 1990; Wallace et al., 2006). Moreover, our measures of CA1 apical spine heads showed a similar pattern with intermittent 10 μ g 17 β -estradiol (10E) injections enhancing CA1 apical spine heads compared to both OVX and to 10 μ g 17 β -estradiol after prolonged OVX. The present findings suggest that there is a critical window in which the optimal effects of estradiol replacement on CA1 apical dendritic spine density and spine heads occurs.

A previous report indicated that a critical window existed between OVX and the start of estrogen treatment using conjugated equine estrogen for increasing CA1 apical spine density (Silva et al., 2003). In that report, twelve days of ovarian hormone absence prevented conjugated equine estrogen from increasing CA1 spine density. In our current study, we detected increased CA1 apical spine density and spine heads with intermittent injections of 10 μ g 17 β -estradiol after ovarian hormone absence ranging from 9 to 12 days. Critical differences between these studies are that Silva and colleagues used conjugated equine estrogen administered orally, whereas we used 17 β -estradiol given subcutaneously. Conjugated equine estrogen includes a variety of compounds that are hormonally active (Bhavnani, 1998; Dey et al., 2000), but very little research has investigated their cognitive effects. Moreover, serum estradiol levels could have differed substantially. Another difference is that cognitive testing could have interacted with estradiol treatment in our paradigm, to produce cross-sensitization effects. Importantly, the variety of techniques using electron microscopy (Silva et al., 2003), nissl stain (Silva et al., 2003) and Golgi (current study) confirm the overall finding that the CA1 region shows a truncated ability to increase CA1 apical spine density and spine heads when estrogen (10 μ g 17 β -estradiol or conjugated equine estrogen) is administered after prolonged OVX.

Estradiol influenced both apical and basal CA1 spine properties in region-specific ways. The 10 μ g 17 β -estradiol injection regimen increased CA1 apical spine density without altering CA1 basal spine density. In contrast, the 5 μ g 17 β -estradiol injection regimen did not alter CA1 apical spine density, but decreased CA1 basal spine density. The significance of the reduction of CA1 basal spine density with the intermittent 5 μ g 17 β -estradiol injection regimen is unclear,

but the region-specific effects may reflect different influences from synaptic afferents (Amaral and Lavenex, 2007) and hence, different neurobiological substrates.

The reduced sensitivity of 10 μ g 17 β -estradiol to increase CA1 apical spine density and heads following an extended period of ovarian hormone deprivation supports the critical period hypothesis, suggesting that a critical window exists in which to achieve the optimal neurobiological (Silva et al., 2003) and neurobehavioral (Daniel et al., 2006; Savonenko and Markowska, 2003; Sherwin, 2007) effects of estrogens. While we found that extended ovarian hormone absence may desensitize the brain to estradiol, these findings may represent a continuum of declining sensitivity to estrogen, starting from the moment of ovariectomy. This may be especially relevant to the Women's Health Initiative Memory Study, an ancillary study of the Woman's Health Initiative, undertaken to determine whether hormone replacement therapy enhanced global cognitive function in woman 65 and older (Shumaker et al., 2003; Zec and Trivedi, 2002). The study was abruptly halted when, in contrast to expectations, estrogen therapy using conjugated equine estrogen increased the risk of dementia and other negative outcomes in postmenopausal women (Shumaker et al., 2003; Shumaker et al., 2004). However, many of the women were in menopause for years before the onset of hormone therapy. Given that our data and others suggest that the brain responds to estrogens differently after an extended absence from ovarian hormones, future research should determine whether the effectiveness of estrogen replacement therapy on cognitive function is influenced by menopause duration.

Effects of intermittent estradiol treatment on hippocampal function

The estradiol effects were task-specific; 10 μ g 17 β -estradiol (10E) facilitated object placement, 5 μ g 17 β -estradiol (5E) facilitated water maze acquisition, and none of the treatment conditions (O, 5E, 10E) influenced Y-maze performance.

For object placement, 10E rats spent more time exploring the object in the novel location, suggesting intact spatial memory. Indeed, the effect was not likely influenced by motivation or anxiety since total object exploration time and fecal counts, respectively, were similar among treatment groups. One caveat is that 10E rats showed increased locomotion within the inner zone during the open field compared to O and 5E groups, which was used as an index of anxiety (Bowman et al., 2002). However, preference for the outer zone was recently suggested to reflect thigmotaxia, not less anxiety per se (Ennaceur et al., 2006). Moreover, increased exploration of the inner zone may reflect estradiol-induced increases in locomotor activity in general (Scimonelli et al., 1999; Steiner et al., 1981). Importantly, these findings are consistent with reports showing that proestrous rats (high estrogen levels) perform better on object placement than diestrous rats (lower estrogen levels, Frye et al., 2007). In addition, rats given chronic 17 β -estradiol via drinking water (Fernandez and Frick, 2004) or a high phytoestrogen diet (Luine et al., 2006) show enhanced performance on object recognition or object placement. Therefore, our results support previous findings of estrogen effects on object placement.

In the water maze, 5 μ g 17 β -estradiol treatment facilitated acquisition during the second training trial. The 5E group covered less distance to the platform compared to 10E, and both groups swam shorter distances than the vehicle condition. While the 5E group displayed slower swim speeds during day 2 of testing compared to 10E, differences in motivation and motor ability are unlikely because distance covered was consistent with latency to escape. This nonlinear effect for the 5E rats to outperform the 10E and vehicle groups on spatial acquisition is consistent with other reports using a different treatment regimen with estradiol benzoate (Xu and Zhang, 2006) or low-dose tonic or cyclic estradiol treatment in young (El-Bakri et al., 2004) and middle-aged OVX female rats (Bimonte-Nelson et al., 2006; Fernandez and Frick, 2004). At the end of the training session in our study, we gave a probe trial to assess navigation strategies, which showed that all treatment groups learned the platform location. Thus, several

parameters in the current study support the interpretation that 5µg 17β-estradiol facilitated spatial learning compared to 10µg 17β-estradiol and oil treatment without confounds from locomotion or motivation and that all treatment conditions utilized spatial strategies.

For the Y-maze, we failed to detect significant group differences in exploration or arm preference, regardless of inter-trial interval (6hr or 24hr). A lack of an effect cannot be attributed to ineffective estradiol administration as we detected changes in body weight with estradiol dose for both 5µg and 10µg 17β-estradiol, which is consistent with past reports (Geary et al., 1994). We also confirmed the effectiveness of estradiol administration on CA1 apical spine density. Moreover, we were able to detect estradiol effects on the other spatial tasks using object placement and the water maze. Therefore, the Y-maze may not be sensitive to estradiol actions using the two-pulse injection paradigm using 5µg or 10µg 17β-estradiol doses.

Interpretation of Estradiol's Mechanism of Action

The divergent outcomes in the current study cause one to ask why we observed different behavioral results when all of these tasks require hippocampal function. Lesion studies support the interpretation that the Y-maze (Conrad et al., 1996), water maze (Jarrard, 1993; Morris et al., 1982), and object placement (Ennaceur et al., 1997) tasks require an intact hippocampus and functional spatial ability. However, estrogen actions on the brain and behavior may be facilitatory or inhibitory of hippocampal function (Korol, 2004) and morphology (Woolley et al., 1990b). An important explanation for the differences across the current tasks is that each maze includes specific and unique testing parameters, which may subsequently activate different neural circuits. For example, interactions between estradiol and circulating glucocorticoids were likely affected by both maze demands and perceived aversiveness (Conrad, 2005; Zurkovsky et al., 2007). Therefore, task requirements, the activation of multiple brain structures, and interactions with other hormonal systems should be considered in the interpretations of the current data.

For object placement, optimal performance was observed under conditions that increased CA1 apical spine density and the number of spine heads. CA1 apical spines receive input from hippocampal CA3 neurons via the Schaffer collaterals (Amaral and Lavenex, 2007) and the medial perforant path (Hunsaker et al., 2007). Given the recent reports that CA3 neurons within the trisynaptic pathway that includes the CA1 region are crucial for memory acquisition of one trial learning (Nakashiba et al., 2008; Nakazawa et al., 2003), we suggest that these pathways may be important contributors for estradiol's influence on object placement. Moreover, increased CA1 apical spine heads may reflect synaptic strengthening following learning, a process that has been observed in the dentate gyrus (Eyre et al., 2003) and CA1 region (Diamond et al., 2006). Object placement depends on innate novelty-seeking of a rat and the CA1 region is associated with novelty processing (Martin and Clark, 2007). In addition, object placement depends on input from the prefrontal cortex (Ennaceur et al., 1997), with decreased prefrontal cortex spine density corresponding with impaired object placement (Seib and Wellman, 2003; Wallace et al., 2006). Since estradiol modulates CA1 spines, a contributing mechanism for enhanced exploration of an object in a novel location after high doses of estradiol may include an estradiol-induced increase in CA1 apical dendritic spine density/heads. While estrogens undoubtedly influence other areas of the hippocampus and brain functions, these data offer potential insights for the influence of estradiol on CA1 spine density and object placement performance.

Unlike object placement, performance on the water maze and Y-maze showed a lack of concordance with estradiol-induced changes in CA1 spines. In the water maze, 5E facilitated spatial acquisition under conditions that decreased CA1 basal spine density and no treatment influenced Y-maze performance. A similar divergence is observed for the ratio of CA1 basal spine heads to headless spines. An important difference between object placement and the

water maze is that the latter relies on a rat's motivation to escape and is aversive (Conrad, 2005; Conrad, 2006; Hodges, 1996; Pietersen et al., 2006), with enhanced arousal and activation of the amygdala (Akirav et al., 2001). Subsequently, estradiol may influence spatial learning and memory differently on such an emotionally arousing task, perhaps through interactions with the stress system. Rubinow et al., (2004) suggest that ovarian hormones interact with aversiveness on the water maze, such that rats in proestrus show enhanced learning when the task is less aversive (water temperature at 33 degrees C), while rats in estrus show enhanced learning when the task is more aversive (water temperature at 19 degrees C). These data support the idea that estradiol interacts with task requirements and emphasize more complex interactions with other systems in mediating performance on these tasks.

Conclusion

This experiment revealed the novel finding that a temporal component may exist to observe the optimal effects of estradiol on CA1 apical spine density and shape in OVX female rats. In addition, the task-dependent influence of estradiol supports the idea that estradiol levels and task requirements interact to affect performance (Korol, 2004) and that some of these effects also rely on brain regions besides the hippocampus. Taken together, our data suggest that estradiol may influence object placement and water maze acquisition, but not Y-maze performance, with object placement being influenced through mechanisms that may involve CA1 spine morphology.

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Figure 1. Experimental Timeline

Rats were ovariectomized (OVX) and 8 days later, injections started as indicated by the solid arrows. Injections were timed to occur 72 and 48 hours prior to behavioral testing (Test), indicated by the open arrows, to maximize testing at the point when CA1 dendritic apical spine density should be elevated with 17β -estradiol treatment. Rats were injected (s.c.) with the same 17β -estradiol dose (sesame oil vehicle = O, $5\mu\text{g} = 5\text{E}$, or $10\mu\text{g} = 10\text{E}$) throughout all the behavioral testing conditions to produce three hormonal conditions. Wash-out periods without injections occurred between each task and ranged from 9 to 12 days. The testing order is listed for all behavioral tasks. After behavioral tests ended, rats were given injections without behavioral testing and brains were removed for Golgi processing 48 hours after the last injection. For the last two injections, the sesame oil group was divided into those that received the vehicle (O) and those that received $10\mu\text{g}$ 17β -estradiol after prolonged OVX in the absence of ovarian hormones (O + 10E) to produce four conditions for the morphological assessment.

Figure 2. CA1 Apical Dendritic Spine Properties

Rats in the 10E group had significantly more CA1 apical spines, followed by rats in the O+10 group (A). O and 5E-treated rats had statistically similar data. This same pattern was repeated for CA1 apical spine heads (B) and spine shape ratio (C). Data points represent group means $\pm$ S.E.M. Statistical significance is indicated by groups with different letters. Legend: O = Sesame Oil Injections, 5E = 5µg 17β-Estradiol, 10E = 10µg 17β-Estradiol, O + 10E = Sesame Oil injections during behavioral testing and 10µg 17β-estradiol for the last two injections prior to Golgi processing.

Figure 3. CA1 Basal Spine Properties

Rats in the 5E group had significantly lower CA1 basal spine density compared to all other groups (A). 5E-treated rats also had significantly less CA1 basal spine heads compared to 10E and O+10E treated rats (B). Interestingly, the CA1 basal spine shape ratio was similar between 5E, O, and 10+E groups, while rats in the 10E group had a significantly greater ratio of heads to headless spines (C). Data points represent group means $\pm$ S.E.M. Statistical significance is indicated by groups with different letters. * $p < 0.05$, 5E vs. all other groups. Legend: O = Sesame Oil Injections, 5E = 5μg 17β-Estradiol, 10E = 10μg 17β-Estradiol, O + 10E = Sesame Oil injections during behavioral testing and 10μg 17β-estradiol for the last two injections prior to Golgi processing.

Figure 4. Y-Maze Arm Choice (6 and 24 hr)

All groups preferred the novel and other arms equally during the 6 hr version of the Y-maze (A). There appeared to be a tendency for all groups to prefer the novel arm over the other, previously explored arm, during the 24 hour ITI task; however, no significant effects were detected (B). Data points represent group means $\pm$ S.E.M. Legend: O = Sesame Oil Injections, 5E = $5\mu\text{g}$ 17β -Estradiol, 10E = $10\mu\text{g}$ 17β -Estradiol, ITI = Inter-Trial-Interval.

Figure 5. Water Maze Acquisition

A) Distance to Escape on the Water Maze. Rats in the 5E group showed facilitated acquisition by swimming shorter distances to reach the platform during trial 2 compared to 10E and O-treated rats. The 10E group swam shorter distances to reach the platform than O-treated rats. B) Latency to Escape on the Water maze. Rats in the 5E group escaped to the platform faster during trial 2 compared to 10E and O-treated rats, and the 10E and O-treated rats were statistically similar to each other. Data points represent group means $\pm$ S.E.M. The parallel lines represent the separation of day, with trials 1–4 occurring on day 1 and trial 5–8 occurring on day 2 of training. ** $p < 0.05$ represents statistical difference among all three doses (5E < 10E < O), * $p < 0.05$ represents statistical difference for 5E versus 10E and O. Data points represent group means $\pm$ S.E.M. Legend: O = Sesame Oil Injections, 5E = 5µg 17β-Estradiol, 10E = 10µg 17β-Estradiol, ITI = Inter-Trial-Interval.

Figure 6. Water Maze Probe Trial

All groups swam a greater percentage of distance in the target quadrant compared to the opposite quadrant (A). In addition, groups swam faster searching for the platform in the opposite quadrant compared to the target quadrant (B). Data points represent group means $\pm$ S.E.M. * $p < 0.05$. Legend: O = Sesame Oil Injections, 5E = 5 μ g 17 β -Estradiol, 10E = 10 μ g 17 β -Estradiol, ITI = Inter-Trial-Interval.

Figure 7. Object Placement Preference

Within group analyses revealed that during both test 1 (A) and test 2 (B) only rats in the 10E group preferred spending time with objects in a new location compared to a familiar (old) location. Data points represent group means $\pm$ S.E.M. * $p < 0.05$. Legend: O = Sesame Oil Injections, 5E = 5 μ g 17 β -Estradiol, 10E = 10 μ g 17 β -Estradiol, ITI = Inter-Trial-Interval.

Table 1**Open Field and Physiological Assessments**

Open Field (OF)	Oil (0)	5E	10E
Time in OF Inner Zone test 1 (sec)	39 ± 11	45 ± 7	49 ± 6
Time in OF Outer Zone test 1 (sec)	561 ± 11	555 ± 7	551 ± 6
Time in OF Inner Zone test 2 (sec)	21 ± 6	28 ± 8	45 ± 11
Time in OF Outer Zone test 2 (sec)	579 ± 6	572 ± 8	555 ± 11
Distance in OF Inner Zone test 1 (cm)	348 ± 81	450 ± 63	539 ± 77
Distance in OF Outer Zone test 1 (cm)	3400 ± 270	3385 ± 177	3659 ± 204
Distance in OF Inner Zone test 2 (cm)	223 ± 67 ^a	243 ± 72 ^a	459 ± 85 ^b
Distance in OF Outer Zone test 2 (cm)	3044 ± 176	3089 ± 138	3401 ± 163
Physiological Measures	Oil (0)	5E	10E
Body Weight at Start of Study (gm)	221 ± 3	223 ± 4	216 ± 3
Body Weight at the End of Study (gm)	370 ± 13 ^a	357 ± 4 ^b	350 ± 6 ^b

Treatments with different letters indicate statistically significant difference for that row