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Chronic Estradiol Replacement Impairs Performance on an Operant Delayed Spatial Alternation Task in Young, Middle-aged, and Old Rats

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Abstract

The current study examined effects of chronic estradiol replacement on a prefrontally-mediated working memory task at different ages in a rodent model. Ovariectomized young, middle-aged, and old Long-Evans rats were given 5% or 10% 17 β estradiol in cholesterol vehicle via Silastic implants and tested on an operant delayed spatial alternation task (DSA). The two estradiol exposed groups did not perform as well as the vehicle control group did. Deficits were present at all but the longest delay, where all groups including the vehicle control group performed poorly. Surprisingly, there was not a significant effect of age or an age by estradiol interaction, despite the fact that old rats had longer latencies to respond after both correct and incorrect lever presses. These data confirm our earlier finding that chronic estradiol treatment has an impairing effect on working memory as measured on DSA task. However, contrary to expectations, young, middle-aged and old rats were similarly impaired by chronic estradiol treatment; there were no indications of differential effects at different periods of the lifespan. Also contrary to expectations, there were no indications of a decline in DSA performance with advancing age. Overall, the results demonstrate that chronic estradiol exposure causes deficits in the DSA performance of ovariectomized female rats, not only in young adulthood, but also at older ages analogous to those at which hormone replacement therapy is commonly prescribed in humans.

Keywords

estrogen; cognition; aging; DSA; working memory

Introduction

Cognitive decline is known to occur in humans with aging, including changes in memory (Albert, 1997), attention (Rogers and Fisk, 1991), and information processing speed (Cerella

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et al., 2006). These changes are most readily seen on tests of prefrontal function, including measures of working memory (Borella et al., 2008; Keenan et al., 2001; West, 1996). Working memory is a process that allows for temporary storage and on-line manipulation of information (Baddeley, 1996). A large body of research shows that the prefrontal cortex plays an important role in working memory not only in humans, but also in nonhuman primates and rodents (e.g. see Castner et al., 2004; D'Esposito, 2007; Dudchenko, 2004; Levy and Goldman-Rakic, 2000). In women, prefrontal functions including working memory appear to decline more rapidly after menopause (Halbreich et al., 1995), suggesting a role for gonadal hormones in mediating these functions.

Studies of the effects of HRT (unopposed estrogens or estrogens with progestin) on working memory in post-menopausal women have yielded variable and conflicting results. A number of observational studies suggest that estrogens are effective in reducing age-related decline in working memory (e.g. Carlson and Sherwin, 1998; Duff and Hampson, 2000; Keenan et al., 2001; Maki et al., 2001). However, contrary to the findings from observational studies, randomized trials suggest there may actually be negative effects of HRT on working memory (Grady et al., 2002; Maki et al., 2007; Resnick et al., 2006).

The impact of HRT on working memory has also been assessed in non-human primate models (Lacreuse, 2006), but, similar to human studies, the studies conducted to date have produced variable outcomes. Several investigators failed to find any effect of chronic or cyclic estrogen treatment on the delayed response task (Hao et al., 2007; Lacreuse et al., 2006; Voytko, 2000, 2002; Voytko et al., 2008), a test that measures visuospatial working memory and is mediated by the prefrontal cortex (Goldman-Rakic, 1987) or the delayed non-matching to sample task (Lacreuse et al., 2002), a task for which good performance at the longer delays used in these studies primarily relies upon the hippocampus and perirhinal cortex (Squire and Zola-Morgan, 1991). In contrast, one study reported improvements on both delayed response and delayed non-match to sample tasks in ovariectomized monkeys receiving cyclic estrogen treatment (Rapp et al., 2003).

Rodent models also have been used to help elucidate the effects of gonadal hormones on memory function. A very common model for studying human menopause in rodents is to ovariectomize female rats followed by hormonal manipulation and testing on behavioral tasks that tap different brain regions/memory systems (e.g. Korol and Kolo, 2002; Markham et al., 2002; Markowska and Savonenko, 2002; Zurkovsky et al., 2007). In general, these studies have shown that estradiol administration improves performance in maze tasks that involve place learning and engage hippocampal systems, but impairs performance on response tasks that are believed to engage striatal systems (Daniel et al., 1997; Daniel and Lee, 2004; Frick et al., 2002; Gresack and Frick, 2006; Korol and Kolo, 2002; Markham et al., 2002; McElroy and Korol, 2005; Zurkovsky et al., 2006, 2007).

Less is known about the impact of gonadal hormones on tasks that specifically engage the prefrontal cortex in rodents. While both the prefrontal cortex and the hippocampus are known to play important roles in working memory (Floresco et al., 1997; Goldman-Rakic, 1995), studies have identified specific tasks that rely primarily on the prefrontal cortex (e.g. Lee and Kesner, 2003; Winocur, 1992) and thus can be exploited to assess the role of gonadal hormones on prefrontally mediated aspects of working memory. In particular, the medial prefrontal cortex has been shown to be critical for accurate performance on operant versions of delayed matching to sample and delayed alternation (also referred to as delayed non-matching to sample) tasks (Izaki et al., 2008; Maruki et al., 2001; Sloan et al., 2006).

The majority of research on estradiol and working memory in rodents has utilized maze paradigms. This approach taps a rat's natural spatial foraging strategies that actively engage

the hippocampus (Floresco et al., 1997; Sandstrom and Williams, 1999; Wang and Cai, 2006). These maze-based working memory tasks typically use delays in the range of minutes to hours (Dudchenko, 2004; Hodges, 1996) and require the use of cue configurations to solve the tasks. Operant chamber-based working memory tasks such as delayed matching to sample or delayed alternation are very different in that they typically do not involve place cues and the delays between trials are measured in seconds rather than minutes to hours (Maruki et al., 2001; Sloan et al., 2006; Wang et al., 2008; Widholm et al., 2004). On operant working memory tasks using short delays (generally less than 15 seconds) inactivating or lesioning the prefrontal cortex impairs performance, whereas inactivating or lesioning the hippocampus is generally without effect (Maruki et al., 2001; Sloan et al., 2006). In contrast, inactivating or lesioning the dorsal hippocampus does disrupt performance on these tasks when longer inter-trial delays are employed (Izaki et al., 2008; Maruki et al., 2001). Together these studies provide evidence of a dissociation between prefrontal cortex and hippocampal involvement in working memory tasks, based upon the specific demands of the task and the length of the inter-trial delays that are used.

With these issues in mind, we recently used an operant version of the delayed spatial alternation (DSA) task with inter-trial delays ranging from 0–18 seconds to assess the impact of chronic estradiol treatment on the prefrontally-mediated component of working memory. Chronic treatment with a high physiologic dose of 17 β -estradiol via silastic implants resulted in impaired performance in ovariectomized young adult rats (Wang et al., 2008). However, this approach employing ovariectomized young female rats failed to consider the potential for interactions between hormone replacement and the aging process. Research has shown that working memory in prefrontally-mediated tasks is sensitive to aging (Gallagher and Rapp, 1997), and that the same hormone regimens can have different effects on brain (Frick et al., 2002; Singer et al., 1998) and behavior (Frick et al., 2002; Markowska and Savonenko, 2002; Rapp et al., 2003) in young adult versus aged adult animals (for a review see Frick, 2009). Thus, the goal of the current study was to expand upon our previous findings by characterizing the effects of chronic treatment with physiologic doses of 17 β -estradiol on the delayed alternation task in young, middle-aged, and old ovariectomized rats. We hypothesized that estradiol treatment would result in larger DSA deficits in older rats. A total of nine groups consisting of the three age groups and three 17 β -estradiol doses (vehicle, low, high) were tested on the operant delayed alternation task.

Methods

Animals and Hormone Exposure

A total of 144 female Long-Evans rats were obtained from Harlan (Indianapolis, IN). The rats were received in two cohorts of 72 animals each, spaced 6 months apart. Rats used in these procedures were maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Rats were housed in a temperature and humidity controlled room (22°C, 40–55% humidity) on a 12-hour reverse light-dark cycle (lights off at 0830h). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Urbana-Champaign and were in accordance with the guidelines of the Public Health Service Policy on Humane Care and Use of Laboratory Animals (National Institutes of Health, 2002) and the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2003). Each cohort consisted of 24 young (3 month old) virgin females and 48 middle-aged (12 month old) retired breeders. Half ($n = 24$) of the retired breeders in each cohort were housed until 18 months of age in the colony room at which time these rats were ovariectomized and tested (old age group). Each age group was divided into three estradiol treatment groups (vehicle, 5% estradiol, and 10% estradiol) with 8 animals per age per dose in each of the two cohorts, or a total of 16

animals in each dose group at each age (3, 12 or 18 months). Rats were pair-housed in standard plastic cages (45×24×20cm) with corncob bedding. All animals were maintained on an AIN-93G soy-free diet (Harlan-Teklad, Madison, WI) after surgery to avoid exposure to dietary estrogens and water was available *ad libitum*.

After a one-week period of acclimation to the vivarium, rats were ovariectomized and a Silastic capsule containing 5% or 10% 17 β -estradiol (Sigma, St. Louis, MO) or cholesterol vehicle was implanted subcutaneously at the nape of the neck under isoflurane gas anesthesia (VetEquip, Pleasanton, CA). One end of the Silastic implants (1.5 cm, 0.058" i.d., 0.077" o.d.) was plugged with 0.25" silicone and dried overnight before packing with 1 cm of the estradiol/cholesterol mixture or vehicle after which the other end was plugged with 0.25" silicone. Beginning one week after surgery, rats were food restricted to and maintained at 85% of their free fed body weights. After the rats had been food restricted for one week they were tested on a place vs. response dual solution T-maze task (data not provided). Operant testing began immediately following T-maze testing, or three weeks after the start of food restriction. Rats were tested six days per week during the dark phase of the light cycle in a darkened testing room.

Serum Estradiol Concentration Analysis

Blood samples were collected from the tail vein two months after the Silastic implants were inserted for serum estradiol analysis. Serum estradiol concentrations were determined by radioimmunoassay (RIA) using Coat-A-Count kits from Diagnostic Products Corporation (Los Angeles, CA). Sensitivity for the radioimmunoassay kits was 8 pg/ml, with intra- and inter-assay coefficients of variance of less than 10%.

Operant Testing

Apparatus—Behavioral testing was conducted once a day in standard automated operant chambers (MedAssociates Inc., St. Albans, VT) housed in sound-attenuated wooden boxes. All test chambers had the same features and dimensions: 21.6 cm tall with a 29.2 cm × 24.8 cm stainless-steel grid floor that rested just above a tray filled with corn cob bedding. Food pellets (Test Diet, Richmond, IN) were dispensed through a pellet dispenser centered 2.5 cm above the floor on the operant panel. Pellets used in the study were soy-free purified rodent diet (45-mg Formula P). Located symmetrically on both sides of the pellet dispenser were a pair of retractable response levers and a pair of stimulus cue lamps, one above each lever. The levers were 5.7 cm from midline and 7.0 cm above the floor and the cue lights are located 5.7 cm above the levers. Each chamber also contained a Sonalert tone generator, a white noise generator, and a house light located on the back wall that remained off during testing. Experimental contingencies were programmed using the Med-State behavioral programming language (Med-Associates, Vermont). Rats tested in the appetitively rewarded operant test were fed a measured amount of food, sufficient to maintain 85% free-fed weight, 30 minutes after behavioral testing was completed each day. Most rats consumed the total ration within 2 to 3 hours and thus were approximately 19 to 20 hours food-deprived when they entered the testing chamber each day. This feeding schedule was necessary to insure that the animals were motivated to work for the food rewards used in the cognitive test.

Response shaping—All rats were shaped to press the response levers by using an autoshaping program (Newland et al., 1986; Widholm et al., 2004). At the beginning of the session, both response levers were extended into the chamber. The white noise generator operated continuously during each session to mask extraneous noises. The cue light above the right response lever was illuminated for 15 seconds every 3 minutes according to a fixed-time 3-minute schedule (FT-3). Upon termination of the cue light stimulus, reinforcement was provided. Reinforcement consisted of the dispensing of a single food pellet concurrent with

the presentation of a 40-ms tone. Presses to either lever at any time during the autoshaping session resulted in immediate reinforcement. The FT-3 schedule terminated when 10 total lever presses occurred on either response lever during a session, at which point reinforcer delivery became contingent only on lever presses. Autoshaping test sessions terminated after 60 minutes elapsed or 100 reinforcers were delivered, whichever occurred first. Criterion for this condition was set at 100 lever presses within a single session. All rats reached criterion in two to three days and there were no differences in number of days between groups.

Lever-press training—Following autoshaping, all animals were exposed to a continuous reinforcement schedule in which the reinforced lever alternated following delivery of every fifth reinforcer. The purpose of this schedule was to strengthen the recently acquired lever press response and to prevent the rats from developing a lever or side preference. Each session began with a randomly determined extension of either the left or right lever into the test chamber and the illumination of the stimulus cue light above the extended lever. Single presses to the available lever resulted in reinforcement. After the receipt of the fifth consecutive reinforcer, the cue light was extinguished and the response lever was retracted. The opposite lever was then extended and the corresponding cue light illuminated. This cycle of alternating levers terminated after 100 reinforcers were received or 60 minutes had elapsed. A performance criterion of 100 reinforcers for at least two consecutive sessions was established for this condition. All rats reached criterion in two to three days. There were no differences in number of days between groups.

Cued Alternation (CA)—After lever-press training, the rats were trained on a variable delay delayed spatial alternation (DSA) task modified from Alber and Strupp (1996) and detailed in Widholm et al. (2004). The sequence began with cued alternation training in which a cue light indicated the correct lever on each trial. Each correct, cued lever press was reinforced. Levers were retracted and extended between trials. No delay was imposed between trials in this initial training phase. Rats were trained to a criterion of one session above chance (>60% correct presses).

Non-cued Alternation (NCA)—Following CA, a non-cued spatial alternation task was presented where the cue light no longer indicated the correct lever. Correct responses still consisted of alternating right and left lever presses with the levers retracting and extending between presses. The correct lever was always the lever opposite the one the rat had pressed on the previous trial. Each rat was tested for 10 sessions on the non-cued spatial alternation task.

Delayed Spatial Alternation (DSA)—The final phase was the DSA task in which variable delays of 0, 3, 6, 9, or 18 seconds were imposed randomly between trials. There were 40 trials at each delay and a total of 200 trials per session. Delays were randomly distributed within each session with the stipulation that a particular delay was not presented on more than three consecutive trials. Each animal was tested for 25 sessions.

Statistical Analyses—The data were analyzed via ANOVA using SPSS for Windows, Version 15.0. Estradiol dose, age, and cohort were included in the analyses as between subjects' factors and significance was set at $p < 0.05$. Tukey post-hoc analyses were used for subsequent comparisons.

For CA, cumulative errors across all sessions and days to criterion served as measures of learning, and each was analyzed using a three-way between-subjects ANOVA with age, estradiol dose, and cohort as the between subjects' factors. For NCA, the overall proportion correct across the ten sessions of testing served as the primary measure of learning, and was

analyzed using a 3 (dose) $\times$ 3 (age) $\times$ 2 (cohort) $\times$ 10 (day) mixed ANOVA with day as a repeated measures factor.

For DSA, the proportion correct across the 25 test sessions was first averaged across blocks of five test sessions to produce five 5-session test blocks. Proportion correct was then analyzed using a mixed 3 (dose) $\times$ 3 (age) $\times$ 2 (cohort) $\times$ 5 (block) $\times$ 5 (delay) repeated measures ANOVA with block (1 – 5) and delay (0, 3, 6, 9, 18 sec) serving as repeated measures factors. Response patterns were also examined to assess the rats' tendency to repeat a correct or incorrect response. A win-stay error was defined as an incorrect response on the same lever that had been correct on the previous trial. A lose-stay error was defined as an incorrect lever press on the same lever that had been incorrect on the previous trial and reflects perseveration. Win-stay and lose-stay errors were analyzed using three-way between-subjects ANOVA for estradiol dose, age, and cohort.

Results

Estradiol Blood Concentration

The serum estradiol concentrations in the treated groups were higher than those in the vehicle control group (Table 1). This was confirmed statistically by a significant main effect of estradiol dose ($F(2, 101) = 17.748, p < 0.001$). Tukey post-hoc analyses found both the 5% and 1210% estradiol groups to have serum estradiol concentrations (pg/ml) significantly higher than the cholesterol control group ($p < 0.05$). However, the 5% and 10% estradiol groups did not differ significantly from each other ($p > 0.05$). The old animals, particularly in the vehicle and 10% estradiol groups appeared to have somewhat lower serum estradiol concentrations, but the main effect of age was not significant ($p = 0.087$). Serum estradiol concentrations achieved in the 5 and 10% estradiol groups were similar to concentrations that have been reported in intact, cycling rats at proestrus (Bridges and Byrnes, 2006; Strom et al., 2008).

Cued Alternation Training (CA)—Two measures, days to criterion and total errors to criterion, were used to assess learning during CA, the first of the two training stages prior to DSA testing. The 5% estradiol dose appeared to improve performance on the CA task in middle-aged rats, but impaired performance on the CA task in aged rats. This was revealed by a significant age $\times$ estradiol interaction for the total number of errors prior to reaching criterion ($F(4, 177) = 4.487, p = 0.002$). Tukey post-hoc analyses by estradiol group at each age found the middle-aged 5% estradiol replaced group committed fewer errors than did both the middle-aged vehicle and 10% estradiol replaced groups ($p < 0.05$, Figure 1). In contrast, the old 5% estradiol replaced group committed more errors than did the old cholesterol replaced control group ($p < 0.05$), and the old 10% estradiol replaced group ($p = 0.063$, Figure 1). Tukey post-hoc analyses across age within each estradiol treatment group found the old 5% estradiol-replaced group committed more errors than both the young and middle-aged 5% estradiol replaced groups ($p < 0.05$); whereas the old 10% estradiol group did not differ from the young or middle-aged 10% groups ($p > 0.05$, Figure 1). There were no effects of estradiol treatment or age on the number of days to reach the criterion of $>60\%$ correct.

The rats were tested in two cohorts spaced about 6 months apart, and, thus, cohort was included as a factor in the statistical analyses. Overall, there was very little impact of cohort on the experimental results. The one exception was an effect of cohort in the young rats during CA training, with rats in cohort two performing better than rats in cohort one. This effect was revealed by significant age $\times$ cohort interactions on both measures of learning (for days to criterion $F(2, 177) = 4.909, p = 0.009$ and for total errors to criterion $F(2, 117) = 3.324, p = 0.039$). Tukey post-hoc analyses confirmed that significant differences between cohorts were present in the young rats ($p < 0.05$), but not in the middle-aged or old rats ($p > 0.05$). Young rats in cohort 2 took fewer days to reach criterion and made fewer errors than did young rats

in cohort 1 ($p < 0.05$, data not shown). This difference did not carry over to NCA training, nor was there evidence of cohort effects the DSA task itself.

Noncued Alternation Training (NCA)—Proportion correct was used as a measure of learning on NCA, the second of the two training stages that preceded DSA testing. NCA performance was influenced by estradiol treatment, with the 10% estradiol replaced group exhibiting a lower proportion of correct responses than the cholesterol replaced group. This effect was revealed by a significant main effect of estradiol treatment on proportion correct for NCA training ($F(2, 117) = 4.837, p = 0.01$), and Tukey post-hoc analyses revealed that the 10% estradiol replaced group performed more poorly on this phase of training than the cholesterol replaced control group ($p < 0.05$), whereas the 5% estradiol-replaced group did not differ from the control group or the 10% group ($p > 0.05$, Figure 2). Unlike the results for CA training, there was not a significant estradiol group by age interaction, indicating a similar effect of estradiol across age groups on NCA training. Analyses also revealed a significant age $\times$ day interaction ($F(11.002, 643.622) = 1.899, p = 0.037$). However, the importance of this effect is unclear given that Tukey post-hoc analyses comparing proportion correct for the three age groups on each day of training failed to find any significant differences between age groups on any of the individual days of testing. Analyses also revealed a significant effect of day ($F(5.501, 643.622) = 130.854, p < 0.001$). As expected, the proportion of correct responses increased across testing days in all groups (data not shown).

Delayed Spatial Alternation (DSA)

Proportion correct

Estradiol treatment influenced the proportion correct on the DSA task, significantly impairing performance across testing blocks and across delays. This was revealed by a main effect of estradiol group ($F(2, 117) = 9.737, p < 0.001$), as well as by estradiol group $\times$ block, ($F(3.678, 215.162) = 4.257, p = 0.003$) and estradiol group $\times$ delay ($F(4.978, 291.208) = 3.666, p = 0.003$) interactions. Tukey post-hoc analyses for estradiol group differences at each testing block revealed significant differences between the cholesterol replaced control group and both the 5% and 10% estradiol replaced groups for blocks 2 through 5 ($p < 0.05$, Figure 3a), with the cholesterol control group performing better at all blocks. Tukey post-hoc analyses for estradiol group differences at each delay revealed significant differences between the cholesterol replaced control group and the 10% estradiol replaced group at the 0 second delay, and between the cholesterol replaced control group and both the 5% and 10% estradiol replaced groups at the 3, 6, and 9 second delays ($p < 0.05$, Figure 3b). No significant group differences were found at the 18-second delay.

There were no significant estradiol treatment by age interactions, but there was a significant age $\times$ block $\times$ delay interaction ($F(13.008, 760.930) = 2.230, p = 0.005$). Analyses of the block $\times$ delay interactions were significant for all three age groups ($p < 0.05$). All age groups improved across blocks of testing at the 3, 6, and 9 second delays but not at the 18 second delay (data not shown). The old rats performed better than the young and middle-aged rats at the 6 and 9 second delays during early blocks of testing (Figure 4a and 4b). However, post hoc comparisons were significant only at the 6 second delay ($p < 0.05$).

Win-stay and lose-stay errors

Both win-stay and lose-stay errors were increased in estradiol-treated groups. This was supported by significant main effects of estradiol group for both types of errors ($F(2, 117) = 9.084, p < 0.001$ for win-stay errors and $F(2, 117) = 9.230, p < 0.001$ for lose-stay errors). ANOVA also revealed a estradiol group $\times$ block $\times$ age interaction on lose-stay errors ($F(9.585, 280.360) = 2.075, p = 0.029$). For win-stay errors, Tukey post-hoc analyses of the main effects found the cholesterol replaced group committed significantly fewer win-stay and lose-stay

errors than both the 5% and 10% estradiol replaced groups ($p < 0.05$, Figure 5a and 5b). No significant age, age $\times$ estradiol group, or estradiol group $\times$ age $\times$ block effects were uncovered, but there was a significant main effect of block on win-stay errors ($F(3.807, 222.718) = 128.690$, $p < 0.001$). As expected, the number of win-stay errors committed decreased across testing block in all groups (data not shown).

Further analysis of the 3-way interaction for lose-stay errors included analysis of the estradiol $\times$ block interactions at each age and the block $\times$ age interactions at each estradiol dose. The estradiol $\times$ block analyses revealed a significant effect of estradiol on lose-stay errors for the middle-aged animals only ($p < 0.05$). Tukey post-hoc analyses found that for middle-aged animals, the cholesterol replaced control group committed fewer lose-stay errors than both the 5% and 10% estradiol replaced groups did in blocks 3 through 5 of testing ($p < 0.05$, data not shown). The analyses of the block $\times$ age interactions for each estradiol dose revealed a significant effect for the 10% estradiol replaced rats only, ($p < 0.05$). Tukey post-hoc analyses found the young 10% estradiol rats to commit more lose-stay errors than the old 10% estradiol rats in block 1 only ($p < 0.05$, data not shown).

Unexpectedly, lose-stay errors were impacted by age, with old rats making fewer of this type of error. This was revealed by a main effect of age for lose-stay errors ($F(2, 117) = 3.469$, $p = 0.034$). Tukey post-hoc analyses found the old rats to make fewer lose-stay errors than the middle-age rats ($p < 0.05$, Figure 6). Similar to win-stay errors, a significant main effect of block was also revealed ($F(2.396, 280.360) = 276.406$, $p < 0.001$); with the number of lose-stay errors committed decreasing across testing blocks in all groups (data not shown).

Latency to respond following correct and incorrect responses

There were no effects of estradiol treatment on latency to respond. However, old rats had longer latencies to respond. Repeated measures ANOVA revealed a main effect of age on latency to respond following either a correct ($F(2, 117) = 33.846$, $p < 0.001$), or an incorrect response ($F(2, 117) = 21.708$, $p < 0.001$). Tukey post-hoc analyses found old rats to have longer latencies to respond following either a correct or an incorrect response than did young and middle age rats ($p < 0.05$). Significant main effects of block were also present for latency to respond following either a correct ($F(2.219, 259.574) = 8.883$, $p < 0.001$), or an incorrect response ($F(2.174, 254.411) = 28.338$, $p < 0.001$). Latencies following both correct and incorrect responses decreased across blocks of testing.

Discussion

The current study expanded on our previous findings by exploring the effects of chronic exposure to physiologic doses of estradiol replacement on an operant version of the DSA task in ovariectomized young, middle-aged, and old rats. Previously, we had examined the effects of chronic exposure to a high physiologic dose of estradiol on this same task in ovariectomized young adult female Long Evans rats (Wang et al., 2008). In that study, rats with Silastic implants containing 10% estradiol had a clear deficit in proportion correct on the DSA task relative to control rats with cholesterol vehicle implants. In the present study, a similar impairing effect of estradiol was observed in rats with either a 5 or a 10% estradiol implant. Specifically, the estradiol-treated rats performed significantly worse than did rats with cholesterol implants in all but the first block of testing, and at all but the longest (18 sec) delay, where all rats were performing close to chance. The lower proportion correct in estradiol-treated groups was the result of increases in both win-stay and lose-stay errors relative to the control group, indicating that the poorer performance of estradiol-treated rats was the result of an overall increase in the error rate, rather than an increased propensity to make a particular type of error. There were also some subtle effects of estradiol treatment in the two training

stages that preceded DSA testing, suggesting that estradiol replacement may have impaired not only DSA performance, but also the acquisition of the basic alternation response.

It is important to note that the rats in this study were tested in a single day on a place vs. response dual solution T-maze task prior to DSA testing. While there is a possibility that this prior experience may have influenced performance on the subsequent DSA task, this seems unlikely given that the rats in the previous study described above did not undergo the T-maze testing, but showed very similar estradiol-related deficits on the DSA task (Wang et al., 2008). Whether or not a single day of testing would modify estradiol effects on DSA in middle-aged and old rats is unknown.

The blood estradiol levels in both of the estradiol-treated groups tested in this study were significantly higher than levels in the vehicle control group. However, the blood estradiol levels in the two exposure groups did not differ significantly from each other. The results of the DSA task reflect this with both estradiol-treated groups showing a similar degree of impairment relative to control. Although we only assessed serum estradiol levels at one time point (2 months after implant) in the current study, in an earlier study we sampled rats with similar estradiol implants 1, 2.5 and 4 months after the capsules were implanted and did not see a significant decline in blood estradiol levels over that time period (Wang et al., 2008).

It is important to note that the circulating estradiol levels measured in the vehicle implanted control rats were higher than would be expected after ovariectomy. Commercial RIA kits—which were designed for use with human samples—have been shown to produce discrepant results when used to measure estradiol in rat sera, possibly due to interactions of the immunochemical reagents with the rat serum matrix (Strom et al., 2008). Because of the problems we encountered with the samples from this study, we recently compared the Coat-a-Count RIA kit utilized in this study (Siemens #TKE21, Los Angeles, CA) with an estradiol double antibody RIA kit available from the same company (Siemens # KE-2D1, Los Angeles, CA). We found serum estradiol levels from ovariectomized female rats with cholesterol implants similar to those used in the current study to be below the level of detection with the double antibody kit, while these same serum samples run in parallel using the Coat-a-Count RIA resulted in serum estradiol levels very similar to those reported for the cholesterol implanted rats in Table 1 (Unpublished data). Unfortunately, we do not have serum left from the current study that can be reanalyzed using the double antibody kits. However, given the results from our follow-up study, we are confident that the OVX rats tested in this study had very low serum estradiol levels, and that the 5% and 10% estradiol replaced groups received physiologically relevant, albeit similar, levels of estradiol replacement.

The effects of estradiol on working memory have been found to be variable and complex, likely due to the fact that working memory has many components and has been assessed using a variety of different tasks that tap different brain pathways/memory systems. Most human studies have found hormone replacement in postmenopausal women to improve verbal learning and memory (for review, see Rice et al., 1997), as well as other prefrontally mediated aspects of working memory (Duff and Hampson, 2000). However, recently the WHISCA has reported conflicting results, finding that women taking hormone replacement had impaired verbal working memory relative to women not on hormone replacement (Resnick et al., 2006). Most of the women in the WHI began HRT a number of years after menopause, but recently a smaller randomized, double blind trial reported similar negative effects of HRT on verbal working memory in younger, recently postmenopausal women (Maki et al., 2007). Studies using older female non-human primates have also reported that estrogen status of the animal has disparate cognitive effects, improving some cognitive functions but not others. Estrogens appear to improve or have no effect on performance on the prefrontally mediated delayed response working memory task (Rapp et al., 2003; Roberts, Gilardi et al., 1997) and also to mildly

improve performance on the more hippocampal delayed non-matching to sample recognition memory test (Lacreuse et al., 2000; Rapp et al., 2003). These findings are in contrast to our results showing impaired performance on a prefrontally mediated working memory task in a rodent model.

Very few rodent studies have assessed the effects of estradiol treatment on prefrontal tasks in rodents. Our own research showed estradiol to impair performance on prefrontally mediated tasks in young ovariectomized females (Wang et al., 2008), a finding that is further supported by the current results. Wide et al. (2004) also found that chronic estradiol treatment impaired performance on a T-maze version of the DSA task. McGaughy and Sarter (1999) found that ovariectomized rats did better than sham-operated rats on an attentional task that is sensitive to lesions of either the prefrontal cortex (Muir et al., 1996) or the medial forebrain cholinergic input to the prefrontal cortex (McGaughy et al., 2002). In contrast, Barnes et al. (2006) reported that attention was impaired in ovariectomized rats and that estradiol treatment ameliorated the deficit. However, unlike our studies or the McGaughy and Sarter study, the rats in Barnes study were ovariectomized after rather than before acquisition of the task. In summary, while there are some studies to the contrary (e.g. Barnes et al., 2006), the effects of estradiol on prefrontally-mediated tasks appear to be mostly negative. This differs from the findings of numerous rodent studies where estradiol has been shown to improve performance on behaviors such as place learning, which are more hippocampally mediated (Gibbs, 2000; Korol and Kolo, 2002; Luine et al., 1998; Zurkovsky et al., 2006, 2007).

The rats in the current study were maintained on a soy free diet, whereas most other rodent studies have used soy-based diets containing phytoestrogens. The degree to which estrogens from the diet interact with the experimental estrogen treatments to alter behavioral outcomes is unknown and would be difficult to determine without studies specifically designed to address this question. However, it is well-established that the amount of soy phytoestrogens in rodent diets varies widely depending on the supplier and the lot number of the feed (Thigpen et al., 2004). These differences could be one factor contributing to the variability of the results that have been observed across studies. Although, in a recent experiment, two days of systemic estradiol treatments produced robust impairments in response learning in young adult rats on phyto-free diets and these were similar to impairments seen in rats fed standard rodent chow (Pisani et al., 2009).

The extent to which the use of a soy free diet may have contributed to the negative effects of estradiol reported here in comparison to the positive effects reported on cognitive tasks in many other rodent studies is difficult to determine. However, these are not the only studies to report impairing effects of estrogen treatment on cognitive functioning. For example, the results are consistent with studies showing that response learning, which is sensitive to striatal manipulations, is impaired by estradiol administration either systemically or directly to the striatum (Korol and Kolo, 2002; Zurkovsky et al., 2007). Collectively, the literature suggests that the effects of estrogens on cognition are complex, improving some functions and impairing others.

The pattern of effects seen in this study is suggestive of altered dopamine function in the prefrontal cortex (Kritzer et al., 2007; Verma and Moghaddam, 1996). Dopamine is important for accurate performance on prefrontally-mediated working memory tasks in humans and non-human primates (Luciana et al., 1998), as well as in rodents (Bubser and Schmidt, 1990; Luine et al., 1998), with reductions in prefrontal dopamine producing impairments on short delay delayed response tasks, including DSA, in both rodents and nonhuman primates (Brozoski et al., 1979; Bubser and Schmidt, 1990; Goldman-Rakic, 1998; Sawaguchi and Goldman-Rakic, 1991). Prefrontal D1 receptor activation has also been implicated in accurate DSA performance in rodents (Vijayraghavan et al., 2007; Zahrt et al., 1997). Estradiol treatment has been shown

to reduce prefrontal dopamine levels (Luine et al., 1998). Thus, the deficits in DSA performance observed in our studies could be mediated by a loss of tonic dopaminergic neurotransmission and disrupted D1 activation in the prefrontal cortex.

Interestingly there was no differential effect of estradiol treatment in young, middle-aged and old rats. All three age groups were similarly impaired by chronic estradiol treatment. There also were not any negative effects of age, itself, on DSA performance. In fact, old rats actually performed somewhat better than young or middle-aged rats at the 6 and 9-second delays across the first three blocks of testing. The old rats also made fewer lose-stay errors than the young and middle-aged rats did. The lack of an age effect is at odds with prior studies, showing that working memory in prefrontally-mediated tasks is sensitive to aging (Gallagher and Rapp, 1997). We had hypothesized that older rats would do more poorly on the DSA task and that estradiol treatment would result in larger DSA deficits in older rats.

There are several possible explanations for the lack of an age effect. It may be that the operant form of the DSA task used in this study is relatively insensitive to aging or that the age at which these rats were tested (18–20 months) was not old enough to see a clear age-related decline in function. Recently we tested 22–24 month-old female Long Evans rats on the same DSA task and found that these older rats did not perform as well as did young and middle-aged rats, particularly at the shortest delays and in the later blocks of testing (Neese et al., in prep). This supports the latter contention. Consistent with our results, Dunnett et al., (1988, 1990) also did not find an age-induced deficit in rodents (<21 months) on operant tests of working memory (delayed matching and nonmatching to sample) at short delays (<10 seconds: Dunnett et al., 1988, 1990).

It is important to note that the young rats used in this study were nulliparous whereas the middle-aged and old rats were retired breeders. Several studies have linked reproductive experience to improved cognitive function (Pawluski et al., 2006a, 2006b). However, these effects are usually reported shortly after pup weaning (Pawluski et al., 2006a), or in rats that have given birth to a single litter (Pawluski et al., 2006b). Thus, it seems unlikely that differences in parity between the young and middle-aged or old rats are responsible for the lack of age-related differences in DSA performance.

Another possibility is that young rats may attend more to nonessential visual cues making memory or attentional tasks more difficult for them than for older animals. However, the rats in this study were tested in darkened testing chambers. Thus, extraneous visual cues are unlikely to have played a significant role in performance.

Finally, the rats in the current study were tested during the early part of the dark phase of the light-dark cycle, whereas many studies test animals during the light phase of the cycle (e.g. Galea et al., 2001; Gresack and Frick, 2006; Korol and Kolo, 2002). Research suggests an interaction between age, time of day and delay on performance of tasks that include delays. For example, Winocur and Hasher (1999) found that aged rats tested early in the dark phase of the cycle performed better than those tested late in the dark phase in an operant delayed alternation task (Winocur and Hasher, 1999), although young rats still performed better than old rats, on average.

In summary, it is likely that several factors contributed to the lack of any age-related decrements in DSA performance in the current study. First, operant versions of the DSA task, particularly those employing short delays, appear to be relatively resistant to age-related decline. Second, the “aged” rats in this study were still relatively early in the aging process (18–20 months of age) at the time of testing, and third, the rats were tested during a stage of the light-dark cycle that would be likely to optimize the performance of the older animals on the task.

In conclusion, this study found that chronic estradiol replacement produced impairments in an operant version of the DSA task, independent of age. Given that the task employed short intertrial delays (less than 20 seconds), these results suggest prefrontal disruption (Maruki et al., 2001; Sloan et al., 2006). Dopamine neurotransmission and subsequent prefrontal D1 receptor activation have been implicated in accurate DSA performance (Vijayraghavan et al., 2007; Zahrt et al., 1997). Given that chronic estradiol treatment has been found to reduce basal levels of dopamine in the prefrontal cortex, the observed deficits in DSA performance could be mediated by an overall loss of tonic dopaminergic neurotransmission and disrupted D1 activation in the prefrontal cortex. Future studies should begin to address these potential underlying neurochemical mechanisms for the cognitive deficits.

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Figure 1.

Total errors made during cued alternation in cholesterol, 5% estradiol and 10% estradiol treated rats. The middle-aged 5% estradiol replaced group committed fewer errors than did both the middle-aged vehicle and 10% estradiol replaced groups (*5% Estradiol to Cholesterol and 10% Estradiol, $p < 0.05$). The old 5% estradiol replaced group committed more errors than did the old cholesterol replaced control group (**Cholesterol to 5% Estradiol, $p < 0.05$). The old 5% estradiol-replaced group committed more errors than both the young and middle-aged 5% estradiol replaced groups (^Old 5% estradiol to both young and middle-aged 5% estradiol, $p < 0.05$).

Figure 2.

Proportion correct during noncued alternation training in cholesterol, 5% estradiol and 10% estradiol treated rats. The 10% estradiol replaced group performed worse on this phase of training than the cholesterol replaced control group, whereas the 5% estradiol-replaced group did not differ from the control group or the 10% group (*10% estradiol to cholesterol, $p < 0.05$).

Figure 3a.

Proportion correct during DSA across 5-day blocks of testing in cholesterol, 5% estradiol and 10% estradiol treated rats. The cholesterol control group performed better than both the 5% and 10% estradiol replaced groups for blocks 2 through 5 (*Cholesterol to both 5% estradiol and 10% estradiol, $p < 0.05$).

Figure 3b.

Proportion correct during DSA testing across five delays in cholesterol, 5% estradiol and 10% estradiol treated rats. The cholesterol replaced control group performed better than the 10% estradiol replaced group at the 0 second delay (**Cholesterol to 10% estradiol, $p < 0.05$), and both the 5% and 10% estradiol replaced groups at the 3, 6, and 9 second delays (*Cholesterol to both 5% estradiol and 10% estradiol, $p < 0.05$).

Figure 4a.

Proportion correct during DSA testing at the 6-second delay across 5-day blocks of testing in young, middle-aged and old rats. The old rats performed better at the 6-second delay during the first three 5-day blocks of testing (*Old to both young and middle-aged rat, $p < 0.05$).

Figure 4b.

Proportion correct during DSA at the 9-second delay across 5-day blocks of testing in young, middle-aged and old rats. Although post hocs were not statistically significant, the figure indicates that the old rats performed better during the first three 5-day blocks of testing.

Figure 5a.

Total win-stay errors committed during DSA testing in cholesterol, 5% estradiol and 10% estradiol treated rats. The cholesterol replaced group committed significantly fewer win-stay errors than both the 5% and 10% estradiol replaced groups (*Cholesterol to both 5% estradiol and 10% estradiol, $p < 0.05$).

Figure 5b.

Total lose-stay errors committed during DSA testing in cholesterol, 5% estradiol and 10% estradiol treated rats. The cholesterol replaced group committed significantly fewer lose-stay errors than both the 5% and 10% estradiol replaced groups (*Cholesterol to both 5% estradiol and 10% estradiol, $p < 0.05$).

Figure 6. Total lose-stay errors committed during DSA testing in young, middle-aged and old rats. The old rats made fewer lose-stay errors than the middle-age rats (**Old to middle-aged only, $p < 0.05$).

Table 1

Estradiol blood levels at 2 months after ovariectomy and implant. Values are in mean $\pm$ S.E. pg/m (n).

	Young	Middle Age	Old
Vehicle	38.7 $\pm$ 4.9 (14)	36.8 $\pm$ 5.4 (12)	19.0 $\pm$ 2.6 (16)
5%	69.4 $\pm$ 15.9 (10)	51.4 $\pm$ 9.4 (6)	60.8 $\pm$ 9.6 (15)
10%	75.3 $\pm$ 10.3 (12)	76.8 $\pm$ 11.7 (11)	63.2 $\pm$ 7.3 (13)