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Effects of Chronic Estradiol Treatment on Delayed Spatial Alternation and Differential Reinforcement of Low Rates of Responding

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Abstract

Estrogens have been shown to both enhance and impair cognitive function depending on several factors including regimen of hormone treatment, age of subject, and task attributes. In rodent models, estradiol tends to enhance spatial learning and impair response or cued learning, but effects on executive functions are less well-studied. In this experiment, spatial working memory and response inhibition were tested using delayed spatial alternation (DSA) and differential reinforcement of low rates of responding (DRL) tasks in ovariectomized rats that were given chronic estradiol via Silastic implants resulting in serum estradiol concentrations of 86.2 ± 8.2 (SEM) pg/ml. Rats were tested for 25 days DSA with variable delays of 0, 3, 6, 9, and 18 seconds between lever presentations, followed by 30 days on a DRL-15s operant schedule. Estradiol-replaced rats showed a significantly lower proportion of correct responses on the DSA task compared to vehicle-implanted ovariectomized animals. On DRL, estradiol-treated rats showed a lower ratio of reinforced to non-reinforced presses. These data suggest that chronic estrogen exposure may impair rats' abilities on measures of executive function including working memory and response inhibition.

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Keywords

estrogen; cognition; aging; DSA; DRL

Evidence from both laboratory animal studies and randomized controlled trials in women has indicated wide-ranging effects of estrogens on cognitive function (for review, see Sherwin, 2005). Estradiol can have either enhancing or impairing effects on learning and memory, and these effects appear to be task-dependent. In humans, beneficial outcomes generally include improved verbal and working memory (e.g. Gibbs & Gabor, 2003; Sherwin, 1988; Tanabe, Miyasaka, Kubota, & Aso, 2004), particularly on tasks that require active manipulation and integration of information such as reasoning, mental calculation, and reading comprehension (Manes et al., 2002; Owen et al., 1998). Activation of prefrontal cortex in humans, as measured with modern neuroimaging methods, is associated with performance in such working memory tasks (Berman et al., 1995; McCarthy et al., 1996). In addition, the prefrontal cortex appears to be activated during other cognitive activities such as decision-making, attention, planning, strategy shifting, and goal-directed behavior (Duncan & Owen, 2000; Goldman-Rakic, 1987b). Using a battery of cognitive tasks, Duff and Hampson (2000) compared postmenopausal women on hormone replacement therapy (HRT) to non-HRT users. Women on HRT scored significantly better on active working memory tasks such as Digit Ordering and Spatial Working Memory, tasks thought to involve a higher degree of prefrontal activation. Performance did not differ, however, between groups on Digit Span, a simpler working memory task that requires less manipulation and integration of information and is associated with less prefrontal activation.

Studies of hormone exposure in laboratory animals have also found variable effects of estrogen treatment on cognitive tasks. For example, in non-human primates, chronic estradiol administration in aged, ovariectomized rhesus monkeys improved performance compared to control treatment on a hippocampal-dependent spatial delayed recognition span test (Lacreuse, Wilson, & Herndon, 2002). However, in the same monkeys, estradiol did not alter performance on a modified Wisconsin Card Sort task, a prefrontally-mediated task that requires the animal to shift response strategies in order to receive food rewards, or on a delayed response task requiring working memory (Lacreuse, Chhabra, Hall, & Herndon, 2004). Interestingly, Rapp, Morrison, and Roberts (2003) found that cyclic estradiol replacement in ovariectomized rhesus monkeys reversed age-related impairments on a prefrontal cortex-sensitive delayed visual-spatial working memory task and on a hippocampus sensitive delayed non-matching to sample task, indicating that compared to constant treatments, cyclic estradiol treatment may improve performance on a broader array of tasks.

In rodents, hormone treatment improves performance on some cognitive tasks and produces impairments on other tasks (for reviews see Dohanich, 2003; Korol, 2004). Estradiol administration improves performance on tasks that are thought to tap hippocampus function, such as spatial working memory and spatial learning, but impairs performance on response tasks believed to engage striatal systems (Daniel & Lee, 2004; Korol & Kolo, 2002). For example, two days of estradiol treatment improved place learning but impaired response

learning on a 4-arm radial maze (Korol & Kolo, 2002). Similar estradiol-related impairments in response or cued learning, thought to engage the striatal system, have also been reported by other investigators (e.g. Daniel & Lee, 2004; Galea et al., 2001). Extensive findings demonstrate that estradiol improves performance in appetitive hippocampus-sensitive spatial memory tasks, such as the radial arm maze (Daniel, Roberts, & Dohanich, 1999; Fader, Johnson, & Dohanich, 1999) and delayed matching-to-position T-maze tasks (Gibbs, 1999, 2000). For example, improved choice accuracy on an eight-arm radial maze was observed in ovariectomized rats receiving chronic estradiol for 30 days prior to testing compared to vehicle controls (Daniel, Fader, Spencer, & Dohanich, 1997). However, Daniel and Lee (2004) found that rats receiving the same estradiol regimen were significantly impaired during acquisition of a dual solution Morris water maze task, which matches findings in intact, cycling rats using a dual solution land maze (Korol, Malin, Borden, Busby, & Couper-Leo, 2004). There is some evidence that dietary phytoestrogens have effects that are consistent with those of estradiol. In particular, a recent study found that ovariectomized rats given a high phytoestrogen diet performed better, relative to rats receiving minimal phytoestrogen diet, on a spatial memory task and an object placement task (Luine, Attalla, Mohan, Costa, & Frankfurt, 2006). In addition to these behavioral effects, rats on high phytoestrogen diet had higher prefrontal and hippocampal dendritic spine densities.

Many rodent studies of estradiol's effects on cognition are designed to tap hippocampal abilities using spatial learning and memory tasks (Daniel et al., 1997; Frick, Fernandez, & Bulinski, 2002; Gresack & Frick, 2006; Korol & Kolo, 2002; Markham, Pych, & Juraska, 2002; McElroy & Korol, 2005; Zurkovsky, Brown, Boyd, Fell, & Korol, 2007; Zurkovsky, Brown, & Korol, 2006). In contrast, the tasks typically utilized to assess estrogen's effects in human and non-human primate studies draw heavily upon prefrontal cortex function, though recent findings demonstrated in rats that direct infusions of rapidly metabolized estradiol into the prefrontal cortex decreased working memory errors in a win-shift version of a radial arm maze task (Sinopoli, Floresco, & Galea, 2006).

The effects of estradiol replacement on executive function, including prefrontal-mediated behaviors such as cognitive flexibility, response inhibition, and non-spatial working memory (Goldman-Rakic, 1987a; Miller & Cohen, 2001) have not been extensively studied in rodent models. Therefore, the goal of this study was to assess estradiol's effects on executive function using a rodent model. Rats were tested on delayed spatial alternation (DSA), an operant version of the delayed alternation working memory task (Shansky, Rubinow, Brennan, & Arnsten, 2006) and on a differential reinforcement of low rates of responding (DRL) task which assesses the both timing and inhibitory control (Popke, Mayorga, Fogle, & Paule, 2000). Three groups of rats were tested in this study. One group was ovariectomized and received long-term estradiol implants at the time of ovariectomy. The second group was ovariectomized and received cholesterol vehicle implants, serving as a control group. The third group consisted of animals receiving sham ovariectomies and cholesterol implants, serving as an intact control group that was included specifically to determine whether the chronically estradiol-replaced females differed significantly from normally cycling females.

Method

Animals and Exposure

Thirty young adult (90-day) ovariectomized or sham-ovariectomized female Long-Evans rats were obtained from Harlan (Indianapolis, IN) and pair-housed upon receipt. Surgeries were performed by the vendor. The rats were housed two per cage in standard plastic cages (45×24×20cm) with corncob bedding and were maintained on a 12hr reverse light/dark cycle (lights off at 8:30am). Many previous studies have used standard rodent diets in which soy is a major source of protein (Thigpen et al., 2004). Because the isoflavones in soy have been found to have significant estrogenic properties (A. M. Duncan et al., 1999; Setchell, 2001), the animals in this study were maintained on an AIN-93G soy-free diet to eliminate the possibility for estrogenic isoflavones to have confounding effects on the behavioral tasks.

After a one-week period of acclimation to the vivarium, rats were food restricted to and maintained at 85% free fed body weight on a standard soy free rat diet (AIN-93G; Harlan-Teklad, Madison, WI) and weighed daily. Food restriction can lead to irregular estrous cycling, however Long-Evans rats were shown to have normal estrous cycle patterns at 85% ad lib body weight (Tropp & Markus, 2001). Therefore, it is assumed that the sham-operated rats were cycling normally during testing.

All animals were implanted with a 1.5cm Silastic capsule (0.058" i.d., 0.077" o.d.) subcutaneously (Luine, Richards, Wu, & Beck, 1998) in the nape of the neck under halothane anesthesia one week after they were received from the vendor and two weeks before behavioral testing began. One end of the capsule was plugged with 0.25" silicone and dried overnight. They were then filled with 1cm of either cholesterol alone or a 10% cholesterol/estradiol mixture, and then plugged with 0.25" silicone on the open end. The diet was switched to the AIN-93G on the day of surgery. Three experimental groups consisting of 10 rats each were tested: 1) sham ovariectomized rats with cholesterol-only capsules, 2) ovariectomized rats with capsules packed with 10% 17 β estradiol in cholesterol, and 3) ovariectomized rats with cholesterol-only capsules.

A separate group of ovariectomized female rats were maintained under the same diet (AIN-93G) and housing conditions and used for blood collection at various time points during the study. Half of the rats were implanted with the cholesterol-only capsules and the other half received capsules with 10% estradiol in cholesterol. Circulating estradiol levels were monitored in these rats to document blood levels through the study period. Estradiol levels were analyzed from trunk blood collection at 1 month, 2.5 months, and 4 months from rats that were implanted but not tested. There were 10 total estradiol-implanted rats and 12 total cholesterol-vehicle implanted rats. Serum was analyzed using Coat-A-Count Estradiol radioimmunoassay kits (Diagnostic Products, Los Angeles, CA). Sensitivity for the radioimmunoassay kits was 8 pg/ml, with intra- and inter-assay coefficient of variance of less than 10%.

Apparatus

Behavioral testing was conducted in standard automated operant chambers (MedAssociates Inc., St. Albans, VT) housed in sound-attenuated wooden boxes. All of the 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 (P.J. Noyes Inc., Lancaster, NH) were dispensed through a pellet dispenser centered 2.5 cm above the floor on the operant panel. These pellets were soy-free purified rodent diet (45-mg Formula P). Located symmetrically on both sides of the pellet dispenser was 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 were 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. Experimental contingencies were programmed using the Med-State behavioral programming language (Med-Associates, Vermont).

Response shaping—All experimental animals were shaped to press the response levers by using an autoshaping program (Newland et al., 1986; Widholm, Villareal, Seegal, & Schantz, 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 on 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 had 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 2 to 3 days and there were no differences in number of days to criterion 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 was illuminated. This cycle of alternating levers terminated after 100 reinforcers were received or 60 min had elapsed. A performance criterion of 100 reinforcers for at least two consecutive sessions was established for this condition. All rats completed the lever-press training in two to three sessions.

Cued Alternation (CA), Non-cued Alternation (NCA), and Delayed Spatial Alternation (DSA)

After lever-press training, the rats were trained on a variable delay DSA task modified from Alber and Strupp (1996), which is 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 the initial training phase. Rats were trained to a criterion of one session above chance (>60% correct presses). Next, 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. Each rat was tested for 10 sessions on the non-cued spatial alternation task. 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 balanced within each session and a particular delay was not presented on more than three consecutive trials. Each animal was tested for 25 sessions.

Differential Reinforcement of Low Rates of Responding (DRL)

Following the completion of the DSA task, timing ability and inhibitory control were assessed using a DRL task. Only one lever, the right response lever, was used for the DRL condition. The lever remained extended throughout the session. The first two days of training consisted of a fixed-ratio 1 schedule in which every response was reinforced. Days 3 and 4 of training consisted of a DRL-5 second schedule in which reinforcement was contingent on a minimum at least 5 seconds separation between responses. If a response occurred within the 5-second window, the response timer was reset. Days 5 and 6 consist of a DRL-10 second schedule. Animals did not have to meet a performance criterion before moving on to the next stage of training. The rats were then tested for 30 days on a DRL-15 second schedule. Following completion of the 30 days of DRL-15s testing, rats were tested for 3 days on DRL extinction during which lever presses were no longer rewarded.

Statistical Analyses

For CA, cumulative errors across all sessions and days to criterion served as measures of learning, and were analyzed using one-way between-subjects ANOVA for group. For NCA, the overall proportion correct across the ten sessions served as the primary measure of learning, and was analyzed using a 3 (group) $\times$ 10 (day) mixed ANOVA where day was 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 (group) $\times$ 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 pattern analyses on DSA were 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. Win-stay and lose-stay errors were analyzed using one-way between-subjects ANOVA for group.

For DRL, the total number of responses and total number of reinforcers delivered were the primary measures of learning over 30 days of testing. The data were averaged to yield six blocks of five sessions each. Performance was also assessed by examining the ratio of reinforced to non-reinforced lever presses. The total number of responses, reinforcers delivered, and ratio of reinforced to non-reinforced lever presses were analyzed using separate mixed 3 (group) $\times$ 6 (block) ANOVAs where block (1–6) served as a repeated-measures factor.

Inter-response times (IRTs), which represent the delay between lever presses, were also examined for the DRL task. IRTs were divided into 2.5-s intervals (e.g., 0–2.5 s, 2.5–5.0 s, etc.) with any IRTs longer than 17.5-s falling in the last IRT interval. The IRT data from the first block (acquisition) and the last block (steady state) were analyzed separately using a 3 (group) $\times$ 8 (IRT interval) mixed ANOVA where IRT interval (1–8) was a repeated measures factor. For DRL extinction, the number of responses across the three days of testing were analyzed using a 3 (group) by 3 (day) mixed ANOVA where day was a repeated measures factor. IRT data were also collected during extinction and analyzed using a 3 (group) $\times$ 8 (IRT interval) mixed ANOVA with IRT interval as the repeated measures factor.

The reported Greenhouse-Geisser ϵ correction was less than 0.75 for all of the repeated measures factors, thus a Greenhouse-Geisser correction was used for all analyses involving a repeated measures factor (Maxwell & Delaney, 2003). In addition to the overall analyses, planned comparisons were also conducted to examine a priori hypotheses related to the treatment conditions. To determine if differences were present due to estrogen replacement, the ovx-estradiol rats were compared to those in the ovx-cholesterol group. To determine if the ovx-cholesterol rats differed from the intact rats, the sham group was compared to the ovx-cholesterol group. Likewise, to determine if the estradiol-replaced rats differed from intact rats, the sham group was compared to the ovx-estradiol-replaced group.

Results

Estradiol Levels

Mean blood estradiol levels in the estradiol-implanted rats were 86.2 ± 8.2 (SEM) pg/ml and mean blood estradiol levels for vehicle-implanted animals were 12.4 ± 1.9 (SEM) pg/ml. We did not see a decline of blood estradiol levels over the 4 month period of blood collection.

Cued and Non-Cued Alternation

For cued alternation, there were no significant group differences in total errors, $F(2, 27) = 1.67$, $p = .21$, or days to criterion, $F(2, 27) = 3.02$, $p = .07$, revealed in the overall analyses, although the effect of group on days to criterion approached significance. Planned comparisons for total errors between the ovx-estradiol and ovx-cholesterol group, $F(1, 18) = 3.24$, $p = .09$, the sham and ovx-cholesterol group, $F(1, 18) = 1.91$, $p = .18$, and the sham and ovx-estradiol group, $F(1, 18) = 0.28$, $p = .61$, were not statistically significant, (sham 262.8 ± 29.0 ; ovx-cholesterol 213.8 ± 20.4 ; ovx-estradiol 286.6 ± 34.9 errors). However, planned comparisons for days to criterion showed a significant difference between the ovx-

estradiol and ovx-cholesterol group, $F(1, 18) = 6.40, p = .02$, with the estradiol-treated animals taking longer to learn the task (see Figure 1). The planned comparisons between the sham and ovx-estradiol groups, $F(1, 18) = 0.65, p = .43$, and the sham and ovx-cholesterol groups, $F(1, 18) = 2.92, p = .11$, on days to criterion were not statistically significant.

For the non-cued alternation task, the overall analyses did not reveal a significant group difference on proportion correct, $F(2, 26) = 1.42, p = .26$, (sham $.80 \pm .01$; ovx-cholesterol $.82 \pm .01$; ovx-estradiol $.83 \pm .02$) or a significant group $\times$ day interaction, $F(9.30, 120.85) = 1.65, p = .11$. There were no significant differences for the planned comparison between the ovx-estradiol and ovx-cholesterol groups, $F(1, 18) = 0.24, p = .63$, the sham and ovx-cholesterol groups, $F(1, 17) = 2.10, p = .17$, or the sham and ovx-estradiol groups, $F(1, 17) = 2.14, p = .16$. The overall analysis revealed a significant main effect of day, $F(4.65, 120.85) = 43.58, p < .001$, showing that all rats improved across days of testing (data not shown).

Delayed Spatial Alternation

Comparison of treatment groups revealed no significant differences on proportion correct for the DSA task in the overall analysis, $F(2, 27) = 2.14, p = .14$. However, planned comparison of the ovx-cholesterol group and the ovx-estradiol group did reveal a significant difference, $F(1, 18) = 4.49, p = .048$, where estradiol-treated animals had a lower proportion correct (see Figure 2). Comparison of the sham and ovx-estradiol groups, $F(1, 18) = 2.29, p = .15$, and the sham and ovx-cholesterol groups, $F(1, 18) = 0.35, p = .56$, did not reveal significant differences. Although there were no significant group $\times$ delay, $F(4.89, 66.01) = 0.55, p = .73$; group $\times$ block, $F(3.58, 48.35) = 1.04, p = .39$; or group $\times$ delay $\times$ block, $F(7.69, 103.77) = 1.15, p = .34$, interactions revealed in the overall analysis, rats performed worse at longer delays and improved overall across blocks, revealed by a significant main effect of delay, $F(2.45, 66.01) = 206.86, p < .001$, and a significant main effect of block, $F(1.79, 48.35) = 125.91, p < .001$. Performance improved more rapidly across blocks for shorter delays as compared to longer delays, which was revealed by a significant block by delay interaction, $F(3.84, 103.77) = 11.07, p < .001$.

DSA Error Pattern Analyses

Win-stay errors were calculated as a way to assess whether rats were more likely to be influenced by recent reinforcement and return to the same lever where reinforcement was just received, rather than respond according to the alternation contingencies. No significant group effect was found for win-stay errors in the overall analyses, $F(2, 27) = 1.32, p = .29$. Likewise, the results from planned comparisons between ovx-estradiol vs. ovx-cholesterol groups, $F(1, 18) = 2.66, p = .12$, sham vs. ovx-cholesterol, $F(1, 18) = 0.11, p = .75$, and sham vs. ovx-estradiol groups, $F(1, 18) = 1.80, p = .20$, also revealed no significant differences.

Lose-stay errors, the tendency for rats to perseverate on the incorrect lever in spite of repeated non-reinforcement, were also analyzed. Lose-stay errors were calculated by calculating the number of errors committed on the same lever after the first error had been committed. For lose-stay errors, a near significant difference between groups was found in

the overall analysis, $F(2, 27) = 2.95, p = .07$. Planned comparison of the ovx-estradiol and ovx-cholesterol groups revealed that estradiol-treated animals made significantly more lose-stay errors than ovx-cholesterol animals, $F(1, 18) = 6.39, p = .02$, while the comparison of sham to ovx-cholesterol, $F(1, 18) = 0.71, p = .41$, and sham to ovx-estradiol, $F(1, 18) = 2.56, p = .13$, were not significant, (see Figure 3).

Differential Reinforcement of Low Rates of Responding

For DRL, there was an overall significant group difference in total lever presses, $F(2, 27) = 6.13, p < .01$ (see Figure 4). Planned comparisons showed that ovx-estradiol rats made significantly more lever presses than did ovx-cholesterol rats, $F(1, 18) = 11.43, p < .01$. Planned comparison between the sham and ovx-cholesterol groups revealed no significant difference, $F(1, 18) = 2.56, p = .13$. The planned comparison between the sham and ovx-estradiol groups on total presses also was not significant, $F(1, 18) = 3.99, p = .06$, although this effect did approach significance with the ovx-estradiol group making more total presses. There was not an overall significant group difference on reinforcers received, $F(2, 27) = 2.43, p = .11$. Planned comparisons, however, showed a significant difference between the ovx-cholesterol and ovx-estradiol groups, $F(1, 18) = 4.61, p = .046$, with ovx-estradiol animals receiving fewer reinforcers. Planned comparisons showed no significant difference between the sham and ovx-cholesterol group, $F(1, 18) = 0.20, p = .66$, or between the sham and ovx-estradiol group, $F(1, 18) = 6.09, p = .02$. There was an overall significant group difference on the ratio of reinforced to non-reinforced lever presses, $F(2, 27) = 4.89, p = .02$ (see Figure 5). The planned comparison between the ovx-cholesterol and ovx-estradiol groups revealed a significantly lower ratio for the ovx-estradiol group, $F(1, 18) = 8.24, p = .01$. The planned comparison between the sham and ovx-estradiol groups was also significant, $F(1, 18) = 6.30, p = .02$, with the ovx-estradiol group performing at a lower ratio than the sham group. The planned comparison between sham and ovx-cholesterol groups revealed no significant difference, $F(1, 18) = 1.24, p = .28$.

Rats in all groups appeared to learn, as measured by total lever presses, reinforcers received, and the ratio of reinforced to non-reinforced lever presses. The overall analyses revealed a main effect of block, $F(2.19, 58.98) = 51.12, p < .001$; $F(2.66, 71.86) = 64.28, p < .001$; and $F(2.74, 74.06) = 41.66, p < .001$, respectively, with total lever presses decreasing across block, reinforcers increasing, and the ratio of reinforced to non-reinforced increasing. There was a non-significant group $\times$ block interaction for all three measures, $F(4.37, 58.98) = 1.98, p = .10$; $F(5.32, 71.86) = 1.56, p = .18$; and $F(5.49, 74.06) = 1.29, p = .28$, respectively.

DRL Interresponse Times (IRTs)—Analysis of DRL IRTs during acquisition revealed a significant group difference, $F(2, 27) = 4.81, p = .02$ (see Figure 6). The planned comparisons between the ovx-cholesterol and ovx-estradiol groups showed a significant difference between ovx-cholesterol and ovx-estradiol groups, $F(1, 18) = 9.55, p < .01$, with ovx-estradiol animals averaging shorter IRTs. Planned comparisons also showed a significant difference between the sham and ovx-cholesterol groups, $F(1, 18) = 4.47, p = .049$, with the sham animals averaging shorter IRTs. There were no significant differences between the sham and ovx-estradiol animals, $F(1, 18) = 0.21, p = .66$. The analysis of acquisition IRTs revealed a significant main effect of IRT, $F(2.17, 58.69) = 49.77, p < .001$,

meaning that inter-response times differed between IRT bins for all animals (data not shown). There was no significant group $\times$ IRT interaction, $F(4.34, 58.69) = 2.17, p = .08$.

During steady state, there were no significant differences between treatment groups in the overall analysis, $F(2, 27) = 1.06, p = .34$, or on the planned comparisons between the ovx-cholesterol and ovx-estradiol groups, $F(1, 18) = 2.20, p = .16$, the sham and ovx-cholesterol groups, $F(1, 18) = 0.67, p = .42$, or the sham and ovx-estradiol groups, $F(1, 18) = 0.44, p = .52$. However, there was a significant main effect of IRT, $F(1.54, 41.62) = 33.23, p < .001$, again meaning that the number of lever presses differed significantly between IRT bins for all animals (data not shown). There was no significant group $\times$ IRT interaction $F(3.08, 41.62) = 0.82, p = .49$.

DRL Extinction—Overall, there was no significant effect of group on the number of presses during DRL extinction, $F(2, 27) = 2.71, p = .09$. However, the planned comparison between the ovx-estradiol and the ovx-cholesterol group did reveal a significant difference, $F(1, 18) = 6.38, p = .02$, with the ovx-estradiol group making more responses than did the ovx-cholesterol group during extinction (see Figure 7). There was not a significant difference for the planned comparison between sham and ovx-cholesterol, $F(1, 18) = 0.01, p = .92$, or sham and ovx-estradiol, $F(1, 18) = 2.73, p = .12$. The overall analysis of total presses during extinction also indicated a significant main effect of day, $F(2, 34.76) = 149.83, p < .001$, meaning that lever pressing decreased across the three days of extinction (data not shown). There was a non-significant group $\times$ day interaction, $F(2.57, 34.76) = 0.61, p = .59$.

Overall analysis of extinction IRTs revealed a main effect of IRT, $F(1.97, 53.17) = 121.05, p < .001$, with number of responses differing between IRT bins for all animals (data not shown). There was a non-significant group $\times$ IRT interaction, $F(3.94, 53.17) = 0.49, p = .74$.

Discussion

The cognitive tasks used in this study assessed several aspects of executive function, including working memory and inhibitory control in young adult female rats. The main focus was to assess the effect of chronic estradiol treatment initiated at the time of ovariectomy on these tasks. We were also interested in determining whether female rats treated chronically with estradiol differed significantly from intact, normally cycling females. Therefore, we included both an ovariectomized vehicle implanted group and a sham operated vehicle-implanted group for comparison with the estradiol-treated experimental group. The intact animals were not monitored in this study to confirm that they were cycling normally. While other published reports suggest small amounts of food restriction do not significantly alter estrous cycling in rats (McShane & Wise, 1996), in future studies it would be appropriate to monitor the estrous cycles of intact animals to confirm that the animals are, in fact, cycling normally with the degree of food restriction employed in our laboratory. In addition, a very recent study suggests that there may be behavioral effects associated with the cholesterol vehicle (McLaughlin, Harman, Hajo, Gomez, & Conrad, 2007). In light of these new findings it would be informative to include an additional control group with blank implants containing neither estradiol or cholesterol.

In general, there were few significant differences between the intact animals and the ovariectomized, estradiol-treated group. However, ovariectomized estradiol-treated rats performed significantly worse than did ovariectomized cholesterol-treated rats on the cued alternation (CA) training that preceded the delayed spatial alternation (DSA) task as well as on the DSA task itself. They also performed more poorly on the differential reinforcement of low rates of responding (DRL) task. Specifically, estradiol-treated rats took longer to master the CA training using days to criterion as the measure. On the DSA task they showed a lower proportion of correct lever presses. Estradiol-treated rats also showed a tendency to persist in pressing the incorrect lever in comparison to ovariectomized non-estrogen treated animals, suggesting impairment with perseveration, or the use of ineffective cognitive strategies as suggested by previous findings (Korol, 2004). On the DRL task, estradiol-treated rats had a lower ratio of reinforced to non-reinforced presses. Overall, estradiol-treated rats received fewer reinforcers and made more lever presses than did ovariectomized, vehicle treated rats.

DSA is a task that relies heavily upon the prefrontal cortex, a region involved in both memory and planning (Goldman-Rakic, 1987b). Delayed alternation has been used previously in examining estrogen status in cycling animals in relation to stress (Shansky et al., 2006) where high estradiol levels appeared to amplify animals' response to stress in impairing performance on the task. In the present study, we observed a pattern of effects on the DSA task that was consistent with an impairment of prefrontal cortex function. That is, performance of the estradiol-treated rats was impaired relative to rats without estradiol at both short and long delays, pointing to a key role of the prefrontal cortex in the impairment. Results from studies using rodents and primates have shown that prefrontal damage results in a similar temporal pattern of effects (Goldman-Rakic, 1987a; Levin, Schantz, & Bowman, 1992; Winocur, 1992; Winocur & Eskes, 1998). In contrast, with hippocampal damage performance is spared at relatively shorter delays but impaired at longer delays (Lee & Kesner, 2003; Zola-Morgan, Squire, & Amaral, 1989).

DRL is a task that tests the subjects' ability to withhold responding for a specific period of time, and thus is likely to involve the prefrontal cortex (Sokolowski and Salamone, 1994). With the exception of one early study (Lentz, Pool, & Milner, 1978), DRL tasks had not been used previously with rats receiving estrogen treatment. Briefly, Lentz et al. compared ovariectomized rats to intact rats and ovariectomized estrogen-replaced rats, finding that ovariectomized rats had an increased response rate relative to sham operated rats, but that estrogen-treated rats did not differ from intact rats on DRL efficiency (reinforcers/number of responses) and reinforcers received. In contrast, we found that rats on chronic estradiol replacement made more lever presses and received fewer food reinforcers, resulting in a lower ratio of reinforced to non-reinforced trials relative to either sham operated or ovariectomized vehicle-treated rats. A number of significant differences exist between the Lentz et al. study and the current one that could explain the disparate results. For example, the previous study used a DRL-20s task as opposed to the DRL-15s task used in the current study. The rat strains also differed between studies. Sprague-Dawley rats were used in the earlier study whereas Long Evans rats were used in the current study. These two strains have been shown to have differing behavioral responses on other tasks (Andrews et al., 1995). In addition, rats were housed individually in the previous studies but were pair-housed in the

current study. Differences in housing can also influence how rodents respond in behavioral tasks (Ferdman, Murmu, Bock, Braun, & Leshem, 2007). Finally, the estrogen-replaced rats in the Lentz et al. study had significantly higher estradiol levels than did those in the current study, which may also account for the behavioral differences.

The current study examined the effects of chronic estradiol on DSA and DRL. Other studies have found that behavioral and physiological measures may differ between chronic and acute estradiol administration. In aged animals, Markowska and Savonenko (2002) found that chronic estradiol administration did not improve working memory unless the rats were primed with repeated estradiol injections. Weekly injections of estradiol and progesterone was just as effective as chronic estradiol exposure for improving female rats' ability on the delayed matching-to-place spatial working memory task when given three months after ovariectomy (Gibbs, 2000). Thus we cannot draw conclusions about the effects of acute or periodic estradiol on executive function based on this study.

The underlying neurochemical mechanisms that mediate the effects of chronic estradiol treatment on DSA and DRL performance have not been investigated, but modulation of brain dopamine pathways is one possibility. Prefrontal dopamine plays an important role in both working memory and inhibitory control. For example, dopamine depletion in the mPFC was shown to impair the performance of rats on a DRL-30s task (Sokolowski & Salamone, 1994). Either too much or too little prefrontal dopaminergic activity has been implicated in producing working memory impairments, suggesting an inverted-U-shaped effect of dopamine activation in the PFC. Supranormal stimulation of dopamine D1 receptors via agonist injections in the prefrontal cortex reduced performance on a DSA task in rats (Zahrt, Taylor, Mathew, & Arnsten, 1997). Increased dopamine turnover in the PFC also impaired the performance of rats on a DSA task and monkeys on a delayed response task (Murphy, Arnsten, Goldman-Rakic, & Roth, 1996). Reductions in prefrontal dopamine produced impairments on delay response tasks such as DSA in rodents and non-human primates (Brozoski, Brown, Rosvold, & Goldman, 1979; Bubser & Schmidt, 1990; Goldman-Rakic, 1998; Sawaguchi & Goldman-Rakic, 1991).

Estrogen has also been shown to have the ability to modulate dopamine receptors in the prefrontal cortex. Hafner, Behrens, De Vry, and Gattaz (1991) found estrogen to modulate D2 receptors by causing a reduction of dopamine receptor affinity to sulpiride binding. Estrogen has also been shown to upregulate dopamine D1 receptor transcription in vitro (Lee & Mouradian, 1999). Evidence suggests dopamine D1 receptors to be important in modulating frontal executive functions. D1 receptors in particular are highly expressed in prefrontal cortex (Hurd, Suzuki, & Sedvall, 2001), and D1 receptor activation is known to be important for working memory in non-human primates (Goldman-Rakic, 1998). D1 receptor antagonists locally injected in the prefrontal cortex of rhesus monkeys impaired performance on a working memory oculomotor task in a dose-dependent manner (Sawaguchi & Goldman-Rakic, 1994). Other studies show that high levels of D1 receptor agonists impaired prefrontal spatial working memory performance in rats (Zahrt et al., 1997) and in aged monkeys (Cai & Arnsten, 1997). It is possible that changes in prefrontal dopamine D1 receptor expression in response to estradiol may explain effects on a spatial working memory tasks such as DSA.

Dopamine levels in the brain have also been shown to have a significant impact upon motivational behaviors (Berridge & Robinson, 1998). Changes in dopamine levels could cause reinforcers to have increased value (Frank & Claus, 2006), resulting in increased impulsivity and thus negatively influencing both DSA and DRL performance. Alternatively, increased activity levels due to estrogen exposure may impair the ability to withhold responses, again negatively influencing performance on both tasks. Slater and Blizard (1976) showed that estrogen administration to female rats increased open field activity. Running wheel activity also increases with estrogen administration (Gorzek et al., 2007; Morgan & Pfaff, 2002; Korol & Pruis, 2005). It is possible that activity levels may be mediated via estrogen's effects upon the striatum. Estradiol is known to modulate striatal dopaminergic function through effects on synthesis, release, and receptor sensitivity (Di Paolo, 1994; Becker, 2003). Ovariectomy has been shown to decrease spontaneous locomotor activity levels in rats and estrogen replacement to restore activity levels (Ohtani, Nomoto, & Douchi, 2001). Chronic estradiol also has been shown to increase D1 (Levesque & Di Paolo, 1989; Hruska & Nowak, 1988) and D2 (Le Saux, Morissette, & Di Paolo, 2006) receptor density in the striatum. Kritzer et al. (2007) examined several prefrontally mediated tasks and corresponding dopamine innervation by using gonadectomized male rats given estradiol or testosterone. Estradiol treated animals performed better than gonadectomized controls on the DRL task, however they took longer on acquisition of the spatial alternation task. Dopamine innervation was increased in the medial prefrontal area of estradiol treated rats relative to control rats.

Choline acetyltransferase (ChAT) activity has also been examined in basal forebrain cholinergic neurons after chronic estradiol administration (Gibbs & Pfaff, 1992; Gibbs, Wu, Hersh, & Pfaff, 1994) where ChAT levels were initially increased following estradiol exposure but were not maintained after longer estradiol treatment. These findings were consistent with another study where estradiol given to ovariectomized female rats increased ChAT expression in frontal cortex, hippocampus, and specific basal forebrain nuclei (Luine, 1985) following 10 days of estradiol exposure. Basal forebrain cholinergic activity has been shown to be vital to memory and attentional processes (Luine & Hearn, 1990; Tinkler & Voytko, 2005), and it is possible that changes in cholinergic function also could play a role in the DSA and DRL deficits we observed.

In humans, working memory is known to be affected by estrogen administration, but the results are dependent upon the type of working memory task used (Sherwin, 2005). Contrary to the findings in this study, estrogen has generally been reported to have positive effects on prefrontally mediated functions in humans (Duff & Hampson, 2000; Keenan, Ezzat, Ginsburg, & Moore, 2001). However, relatively few human studies have been double blind randomized control trials. A large number of studies using estrogen treatment compare women who are already on hormone replacement therapy to age-matched women not on HRT. Women on HRT are normally self-selected into the hormone replacement group and tend to be more highly educated, affluent, and healthier (Zec & Trivedi, 2002). All of these factors would predict better working memory performance on cognitive tests (Erickson et al., 2005; Erickson et al., 2007). Education and socio-economic status are highly correlated with health and cognitive performance (Reynolds & Ross, 1998). One of the randomized, double-blind, placebo-controlled ancillary cognitive studies of the Women's Health

Initiative found that women 65 years and older given conjugated equine estrogens either unopposed or combined with medroxyprogesterone acetate showed impaired cognitive function on a global test of cognitive function (the Modified Mini-Mental State Exam) compared to women given a placebo (Espeland et al., 2004). While there are conflicting reports regarding the risks and benefits in women of estrogens generally, and estradiol more specifically, more randomized double-blind studies that control for important covariates such as IQ, education, SES, general health, and tests in specific cognitive domains are needed in order to determine if estrogen replacement truly has beneficial effects on prefrontal function in women.

In summary, young adult ovariectomized rats treated chronically with estradiol showed deficits on several measures of executive function including working memory and inhibitory control when compared to ovariectomized rats that were not treated. These results differ from numerous rodent studies where estradiol has been shown to improve performance on behaviors such as place learning that are mediated hippocampally (Gibbs, 1999, 2000; Zurkovsky et al., 2006; Zurkovsky et al., 2007; Luine et al., 1998), but are consistent with studies showing that response learning, which is sensitive to striatal manipulations, is impaired by estradiol administration (e.g. Korol & Kolo, 2002). These results also appear to contrast with most findings in the human literature where women on HRT generally do better on tests of executive function than do age-matched women not on HRT. However, studies that more rigorously control for potential confounding variables such as differences in education and IQ between HRT and non-HRT users are needed. The findings suggest important areas for further research including using middle-aged and old rats to see if the same pattern of deficits in executive functions occurs in rats that receive estradiol treatment at these older ages. Such studies would more closely model estrogen replacement in humans and would provide important data on the utility of the rat as a model for women receiving estrogen replacement during or after menopause.

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Figure 1. Cued alternation days to criterion. * indicates a significant difference in the planned comparison ($p < .05$). Error bars represent *SEM*.

Figure 2. Mean proportion correct ($\pm$ SEM) during DSA. * indicates a significant difference in the planned comparison ($p < .05$).

Figure 3. Total lose-stay errors ($\pm$ SEM) across all test sessions of DSA. * indicates a significant difference in the planned comparison ($p < .05$).

Figure 4.

Total presses ($\pm$ SEM) across all test sessions of DRL. * indicates a significant difference in the planned comparison ($p < .05$). + indicates a near significant difference in the planned comparison ($p = .06$).

Figure 5. Ratio of Reinforced: Non-Reinforced lever presses ($\pm SEM$) averaged across all test sessions of DRL. * indicates a significant difference in the planned comparison ($p < .05$).

Figure 6. Mean inter-response time (IRT) during Acquisition ($\pm SEM$) for DRL. * indicates a significant difference in the planned comparison ($p < .05$).

Figure 7.

Average number of responses over three days of DRL Extinction ($\pm$ SEM). * indicates a significant difference in the planned comparison ($p < .05$).