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Research Report

Effects of continuous and intermittent estrogen treatments on memory in aging female mice

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Abbreviations:
ANOVA, analysis of variance
ChAT, choline acetyltransferase
CEE, conjugated equine estrogens
Contin, continuous
E_2, estradiol
OVX, ovariectomy
PLSD, protected least significant difference
SEM, standard error of the mean
VEH, vehicle
WRAM, water-escape motivated 8-arm radial arm maze

ABSTRACT

The manner in which hormone therapy is given to postmenopausal women may significantly influence its ability to reduce age-associated memory loss. To test the hypothesis that a regimen that approximates the timing of estrogen surges in the natural cycle is more beneficial for memory than a regimen that provides continuous levels of estrogen, we examined the effects of continuous and intermittent estrogen regimens on spatial and object memory in aging female mice. Mice (18 months) were treated with 0.2 mg/kg 17β-estradiol (E_2) or vehicle (VEH) for 3 months following ovariectomy. A fast-acting water-soluble cyclodextrin-encapsulated E_2 was used to ensure metabolism within 24 h. Vehicle-treated mice received daily injections of 2-hydroxypropyl-β-cyclodextrin vehicle. The continuous estradiol group (Contin E_2) was injected daily with estradiol. The intermittent group (Twice/wk E_2) received estradiol every 4 days and vehicle on all other days. Mice (21 months) were tested in water-escape motivated 8-arm radial arm maze (WRAM) and object recognition tasks. During WRAM acquisition, the Twice/wk E_2 group committed significantly more reference memory errors than VEH and Contin E_2 groups, and tended to make more working memory errors than the VEH group. The Contin E_2 group did not differ from VEH on either WRAM measure. Additionally, the Twice/wk E_2 group tended to exhibit impaired object recognition. Thus, neither treatment improved spatial or object memory. Indeed, intermittent estradiol was detrimental to both types of memory. These results suggest that the timing of administration may play an important role in the mnemonic response of aging females to estrogen.

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1. Introduction

Both men and women experience cognitive deterioration with age. In women, however, the rate of this decline increases following menopause (Halbreich, 1995), as does the risk of Alzheimer's disease (Gao et al., 1998; Henderson, 1997; Mulnard et al., 2000; Paganini-Hill and Henderson, 1994). The dramatic reduction in estrogen and progesterone levels at menopause may contribute to the accelerated cognitive decline observed in older women. Prospective randomized controlled trials, as well as cross-sectional and longitudinal studies, have suggested that estrogen treatment may help maintain some types of memory (e.g., verbal, spatial, and figural memory) in healthy menopausal and postmenopausal women, and may also protect against cognitive decline in aging women (for review see Sherwin, 2002; also, Duff and Hampson, 2000; Duka et al., 2000; Resnick et al., 1998; although see Hogervorst, 1999; Kang et al., 2004). However, recent findings from the large, randomized, longitudinal Women's Health Initiative Memory Study suggest that long-term daily use of unopposed conjugated equine estrogens (CEE) impairs performance on tests of global cognitive function, and does not reduce the incidence of dementia or mild cognitive impairment (Espeland et al., 2004; Shumaker et al., 2004). The inconsistencies among clinical findings may be due, in part, to the fact that not all estrogen users are alike, differing in the age at which they initiate hormone therapy, as well as in the type (e.g. CEE vs. estradiol) and dose of their treatment. Furthermore, the extent to which estrogen therapy affects memory in menopausal women may depend on various other factors specific to the treatment, such as whether treatment is given in a cyclic or continuous manner (Gleason et al., 2005).

Although endogenous estrogen is normally released in a cyclic manner, with surges occurring just prior to ovulation and remaining at moderate levels during the luteal phase before dropping off for the remainder of the cycle (Sherwin, 2003), the majority of clinical studies have administered estrogen in a continuous daily manner (i.e., women take the same dose every day) which results in sustained levels of estrogen (e.g., Gleason et al., 2005; Thal et al., 2003). Cyclic regimens may be more beneficial to cognitive function because they would more closely approximate the fluctuating hormone levels to which women are naturally exposed during the menstrual cycle prior to menopause. However, testing the effectiveness of cyclic versus continuous treatment regimens in women is difficult given the health risks potentially associated with such treatment (WHI Steering Committee, 2004; Writing Group for WHI Investigators, 2002). As such, female rodents may be suitable models in which to study the effects of estrogen regimen on memory. First, like women, the rodent cycle involves fluctuating levels of estrogen and progesterone, although rodents do not experience the second lower peak of estrogen or the delayed peak of progesterone evident in the luteal phase in women because this phase is absent in the rodent cycle. In the rodent, surges of estrogen occur approximately every 4 days during the cycle (called the "estrous cycle"). Second, these fluctuations in mice become irregular in early middle-age (about 13–14 months) and then cease entirely at around 17 months (Frick et al., 2000; Nelson et al., 1982). Additionally, age-related mnemonic decline in female rats (Markowska, 1999) and mice (Frick et al., 2000) coincides with the loss of regular estrous cycling. Finally, age-related memory decline can be attenuated with estrogen (typically estradiol) treatment in female rodents. Specifically, continuous estradiol treatment regimens, utilizing silastic capsule implants or daily hormone injections, have been shown to improve spatial working memory, spatial reference memory, and nonspatial object memory in aging female rats (Foster et al., 2003; Gibbs, 2000a; Markham et al., 2002) and mice (Frick et al., 2002; Miller et al., 1999; Vaucher et al., 2002). Furthermore, in aging female rodents, continuous estrogen has numerous effects on brain structures that mediate learning and memory, such as the hippocampus. In aged female mice, estradiol treatment increases hippocampal levels of synaptophysin, a presynaptic protein and indicator of synaptic plasticity (Frick et al., 2002). Continuous estradiol also blocks the induction of long-term depression in hippocampal neurons (Foster et al., 2003) and increases hippocampal expression of the NMDAR1 glutamate receptor in aging female rats (Adams and Morrison, 2003; Adams et al., 2001).

It is somewhat surprising that continuous treatments improve hippocampal-dependent memory in aging rodents, given that chronic continuous treatment with estradiol can downregulate estrogen receptors (Brown et al., 1996) and levels of choline acetyltransferase (ChAT) and trkA receptors in the basal forebrain cholinergic neurons that project to the hippocampus (Gibbs, 1997). One might hypothesize that treatments that reproduce certain aspects of the estrous cycle would be more beneficial to the brain and memory because they would more closely approximate hormone fluctuations experienced by the brain during the natural cycle. Indeed, 2 weeks of intermittent estradiol plus progesterone injections administered to adult rats increased hippocampal ChAT activity, whereas continuous estrogen plus progesterone administration significantly decreased hippocampal ChAT activity (Gibbs, 2000b). This finding provides a neural basis for the hypothesis that intermittent estrogen treatment regimens should be more beneficial for hippocampal-dependent memory than continuous regimens. However, the few studies conducted thus far using various types of intermittent regimens have yielded equivocal results (Fernandez and Frick, 2004; Gibbs, 2000a; Markowska and Savonenko, 2002; Ziegler and Gallagher, 2005). For example, Gibbs (2000a) showed in aged female rats that chronic weekly injections of estradiol plus progesterone beginning 3 months after ovariectomy improved spatial working memory, and Markowska and Savonenko (2002) determined that priming aging female rats with estradiol injections enabled chronic continuous estradiol treatment beginning 6 months after ovariectomy to improve spatial working memory. However, more recently, using an oral estradiol preparation in middle-aged female mice that produced daily fluctuations in circulating estradiol levels, Fernandez and Frick (2004) found that chronic treatment improved object memory, but not spatial working or reference memory. Similarly, Ziegler and Gallagher (2005) found that an acute treatment regimen of two consecutive daily estrogen injections over a 6-day cycle had no effect on spatial working memory in middle-aged female rats. Together, these four

studies suggest that, while some types of intermittent treatments may improve memory, the effects of these treatments are highly dependent on methodological factors. The timing of hormone injections may be critical, and an intermittent regimen that more closely approximates the transient estrogen surge observed approximately every 4 days in young cycling females during proestrus may be more ideal for memory. Using a form of estrogen that is rapidly metabolized, such as water-soluble cyclodextrin-encapsulated estradiol, would be particularly helpful in this regard to more accurately reflect the fact that estradiol levels return to baseline within 24 h in the natural cycle. Further, directly comparing this type of estrogen regimen to a continuous regimen should shed light on the issue of whether intermittent treatments are more effective at reducing age-related memory decline than continuous treatments.

The present study tested the effects in aging female mice of chronic continuous and intermittent estradiol treatments on spatial working, spatial reference, and object memory as measured in radial arm maze and object recognition tasks. Mice were ovariectomized in middle-age (17.5 months), treated with estradiol for 3 months, and tested when they were aged (21 months). Continuous treatment was given using daily estradiol injections, whereas intermittent treatment used estradiol injections given every 4 days to approximate the timing of estradiol surges in young females. Although examining the effects of unopposed estrogen on memory in ovariectomized mice might have the greatest applicability to only a subset of postmenopausal women (i.e., those who have had an oophorectomy and/or hysterectomy), studying intermittent regimens is an important first step towards determining the utility of approximating the timing of hormone surges in replacement studies. If effective, future studies taking into account the timing of estrogen and progesterone surges would be even more informative to a broader population of menopausal women.

2. Results

2.1. *Subjects*

Sample sizes in each group at the start of the experiment were as follows: Vehicle (VEH, $n=10$), Continuous E_2 (Contin E_2,

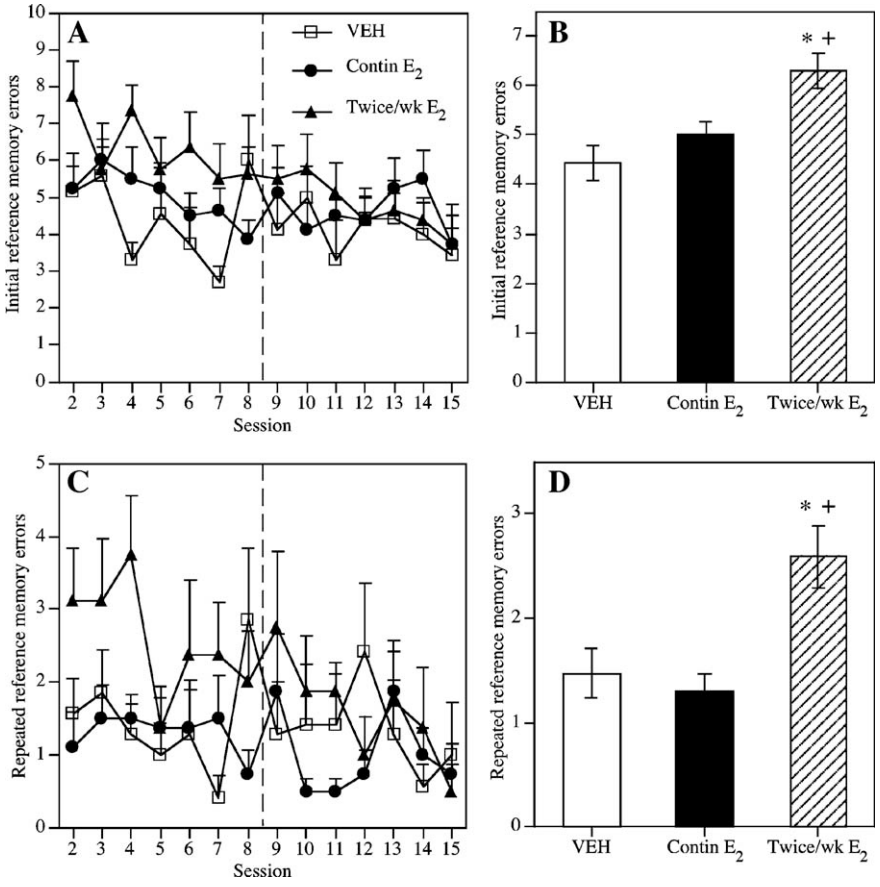

Fig. 1 – Initial and repeated reference memory errors committed by vehicle and estrogen-treated groups during WRAM testing. (A) The number of initial reference memory errors decreased across Sessions 2–15. (B) During acquisition (Sessions 2–8), the Twice/wk E_2 group committed significantly more initial reference memory errors than VEH ($^*p<0.05$) and Contin E_2 ($^+p<0.05$). (C) The number of repeated reference memory errors made during Sessions 2–15. (D) During acquisition (Sessions 2–8), the twice/wk E_2 group committed significantly more repeated reference memory errors than VEH ($^*p<0.05$) and Contin E_2 ($^+p<0.05$). In A and C, each point represents the mean (±S.E.M.) of each group for a single session. The dotted vertical line in A and C indicates the division between the first and last halves of testing. In B and D, each bar represents the mean (±S.E.M.) of each group in Sessions 2–8.

$n=12$), and Intermittent E_2 (Twice/wk E_2, $n=10$). Eight mice (two VEH, four Contin E_2, and two Twice/wk E_2) were euthanized during the 3 months prior to behavioral testing because of poor health (e.g., severe ulcerative dermatitis). In addition, two mice died of natural causes during the experiment: one VEH mouse died prior to water-escape motivated radial arm maze (WRAM) testing (and is excluded from all data analyses) and one Contin E_2 mouse died after the completion of behavioral testing but prior to uterus collection. (This animal is included in all behavioral analyses but is excluded from the uterine weight analysis.) Finally, due to prolonged inactivity in the testing arena, one VEH mouse was excluded from the object recognition analyses, but was included in the WRAM and uterine weight analyses. The resulting sample sizes for the WRAM analysis were as follows: VEH ($n=7$), Contin E_2 ($n=8$), Twice/wk E_2 ($n=8$). For object recognition, sample sizes were: VEH ($n=6$), Contin E_2 ($n=8$), Twice/wk E_2 ($n=8$). Sample sizes for the uterine weight analysis were: VEH ($n=7$), Contin E_2 ($n=7$), Twice/wk E_2 ($n=8$).

2.2. WRAM

The Twice/wk E_2 group made more initial reference memory errors than the VEH and Contin E_2 groups, particularly during the first half of testing (Figs. 1A and B). During Sessions 2–15, the number of initial reference memory errors decreased significantly among all groups ($F(13, 260)=1.88$, $p=0.03$, Fig. 1A). The main effect of Treatment during Sessions 2–15 was significant ($F(2, 20)=4.15$, $p=0.03$), and this was driven by significant group differences during Sessions 2–8 ($F(2, 20)=5.66$, $p=0.01$). Post hoc tests conducted on the Sessions 2–8 data revealed that the Twice/wk E_2 group committed significantly more initial reference memory errors than the VEH ($p=0.004$) and Contin E_2 groups ($p=0.03$; Fig. 1B). The Treatment × Session interaction was not significant at any point during testing.

Similar to initial reference memory errors, the Twice/wk E_2 group made more repeated reference memory errors than the VEH and Contin E_2 groups, particularly during the first half of testing (Figs. 1C and D). The significant main effect of Treatment during Sessions 2–15 ($F(2, 20)=4.83$, $p=0.02$) was again driven by group differences during Sessions 2–8 ($F(2, 20)=5.46$, $p=0.01$). Post hoc tests conducted on data from Sessions 2–15 (data not shown) and Sessions 2–8 (Fig. 1D) revealed that, in both instances, the Twice/wk E_2 group committed significantly more repeated reference memory errors than the VEH (Sessions 2–15: $p=0.04$; Sessions 2–8: $p=0.02$) and Contin E_2 (Sessions 2–15: $p=0.007$; Sessions 2–8: $p=0.006$) groups. The main effect of Session and the Treatment × Session interaction were not significant during any phase of testing.

More working memory errors were also noted in the Twice/wk E_2 group relative to the VEH group during the first half of testing (Fig. 2). The main effects of Session and Treatment were not significant at any point during testing, although the Treatment effect approached significance during Sessions 2–8 ($F(2, 20)=3.03$, $p=0.07$), with the Twice/wk E_2 group making more errors than VEH (Figs. 2A and B). The Treatment × Session interaction was not significant at any point during testing.

In the working memory load analysis, the number of working memory errors made in each trial increased from Trials 2–4 during all phases of testing (Sessions 2–15: $F(2, 40)=123.40$, $p=0.0001$; Sessions 2–8: $F(2, 40)=70.31$, $p=0.0001$; Sessions 9–15: $F(2, 40)=79.71$, $p=0.0001$; Fig. 2C). A one-way ANOVA used to analyze working memory errors within each trial across Sessions 2–8 indicated that the Treatment effect approached significance at low working memory loads (Trial 2) ($F(2, 20)=3.28$, $p=0.06$) with both estrogen-treated groups tending to commit more working memory errors than VEH.

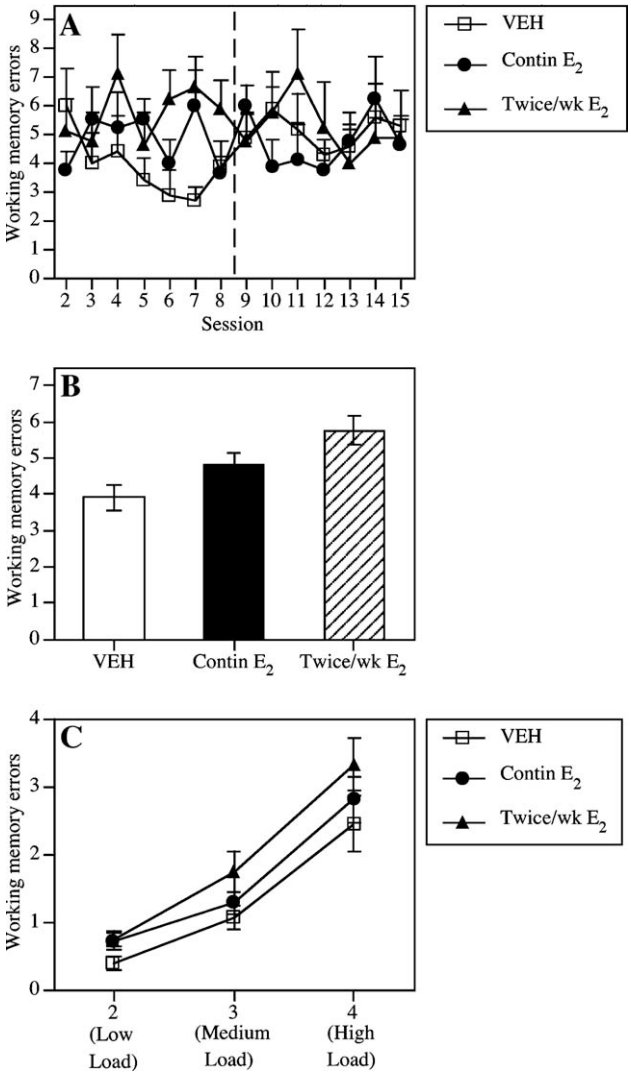

Fig. 2 – Working memory errors committed by vehicle and estrogen-treated groups throughout WRAM testing. (A) The number of working memory errors made during Sessions 2–15. Each point represents the mean (±S.E.M.) of each group for a single session. The dotted vertical line indicates the division between the first and last halves of testing. (B) During acquisition (Sessions 2–8), the Twice/wk E_2 group tended to commit more repeated reference memory errors than the VEH group. Each bar represents the mean (±S.E.M.) of each group averaged across Sessions 2–8. (C) During Sessions 2–8, the number of working memory errors committed by each group increased across Trials 2–4 with increased working memory load. The Twice/wk E_2 group tended to make more working memory errors than the VEH group particularly during Trials 2 and 3.

2.3. *Object recognition*

No effect of E_2 treatment was observed on general locomotor activity measured during the habituation phase of the object recognition task ($F(2, 19)=0.63$, $p=0.54$; Table 1). Furthermore, none of the groups showed a preference for one of the identical objects during the sample phase, suggesting no bias towards either corner of the testing arena in any group (Fig. 3A). Groups did not spend significantly more time with the object on the left side of the box (VEH: $t(5)=0.06$, $p=0.96$; Contin E_2: $t(7)=0.46$, $p=0.66$; Twice/wk E_2: $t(7)=1.42$, $p=0.20$), compared with the object on the right (VEH: $t(5)=-0.04$, $p=0.97$; Contin E_2: $t(7)=-0.42$, $p=0.69$; Twice/wk E_2: $t(7)=-1.40$, $p=0.21$). In addition, the time needed to accumulate 30 s of object exploration during the sample phase was not affected by estrogen treatment ($F(2, 19)=1.20$, $p=0.32$; Table 1), indicating that neither regimen affected activity level during the sample phase.

Only the VEH and Contin E_2 groups tended to prefer the novel object during the choice phase (Fig. 3B). There was a trend for these groups to spend more time exploring the novel object relative to chance (VEH: $t(5)=2.32$, $p=0.07$; Contin E_2: $t(7)=2.18$, $p=0.07$). The Twice/wk E_2 group, however, clearly did not spend more time with the novel object relative to chance ($t(7)=0.10$, $p=0.93$), suggesting no preference for the novel object. As in the sample phase, estrogen treatment did not affect activity level as indicated by the nonsignificant main effect of Treatment for time to accumulate 30 s ($F(2, 19)=1.05$, $p=0.37$; Table 1).

2.4. *Uterine weights*

Uterine weights differed significantly among the groups ($F(2,19)=9.49$, $p=0.001$). Post hoc analyses indicated that the Contin E_2 group ($p=0.004$), but not the Twice/wk E_2 group ($p=0.11$), had significantly increased uterine weights relative to VEH. The Contin E_2 group also had significantly increased uterine weights relative to the Twice/wk E_2 group ($p=0.01$, Fig. 4).

3. Discussion

The results of the present study indicate that treatment regimen influences the extent to which estrogen affects memory in aging female mice. A continuous treatment regimen, resulting from daily injections of a fast-acting estradiol over a 3 month period, had no effect on spatial working memory, spatial reference memory, or nonspatial object memory. In contrast, an intermittent treatment regimen, resulting from estradiol administration every 4 days, impaired spatial reference memory, and tended to impair spatial working and object memory, relative to non-estradiol treated mice.

The finding that a continuous treatment regimen did not affect performance on spatial and nonspatial hippocampal-dependent tasks is surprising in light of other studies which report that continuous estradiol treatment (resulting from silastic implants or daily injections) improves spatial working

Table 1 – Mean±SEM activity levels during object recognition testing

Group	Grid crossings	Elapsed time (s)	
	Habituation	Sample	Choice
VEH	87.83±14.22	228.29±33.17	344.75±80.44
Contin E_2	80.50±7.95	182.64±19.62	243.96±27.25
Twice/wk E_2	100.88±16.82	241.10±33.50	317.54±46.45

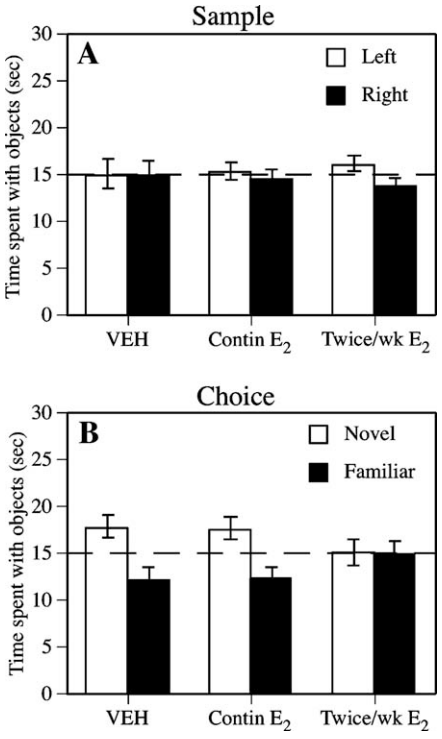

Fig. 3 – Time spent with the objects during the sample and choice phases of the object recognition task. (A) During the sample phase, none of the groups showed a preference for either identical object. (B) During the choice phase, only the VEH and Contin E_2 groups tended to prefer the novel object ($ps=0.07$) relative to chance (dashed line at 15 s). Each bar represents the mean (±S.E.M.) of each group.

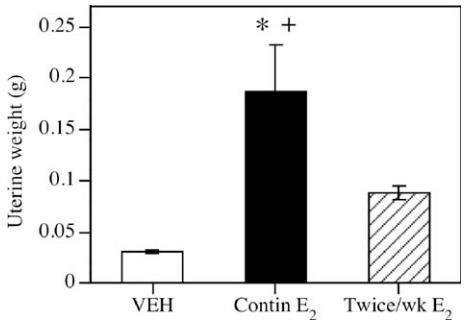

Fig. 4 – Uterine weights among vehicle and estrogen-treated subjects. Uterine weights of Contin E_2 group were significantly greater than those of VEH ($^*p<0.05$) and Twice/wk E_2 groups ($^+p<0.05$).

Table 2 – Effects of chronic-estradiol treatment on memory in middle-aged and aged female animals

Reference	Species	Age at ovariectomy	Age when treatment began	Type of estrogen	Route of administration	Duration of treatment prior to testing	Task	Result
Continuous								
Miller et al. (1999)	Mouse	24 months	24 months	17β-estradiol	Silastic implant	1 month	Spontaneous alternation T-maze	Improvement
Frick et al. (2002)	Mouse	Not ovariectomized	27–28 months	β-Estradiol-3-benzoate	Daily injection	5 days	Morris water maze	Improvement
Vaucher et al. (2002)	Mouse	22–24 months	22–24 months	Estratien-3,17-β-diol	Silastic implant	21 days	Object recognition	Improvement
Fernandez and Frick (2004)	Mouse	16–17 months	17–18 months	17β-estradiol	Daily oral	5 weeks	Object recognition	Improvement
							Water-motivated radial arm maze	Impaired reference memory
							Water-motivated radial arm maze	No effect working memory
Heikkinen et al. (2004)	Mouse	5 months	23 months	17β-estradiol	Minipellets	40 days	Win-stay radial arm maze	Improved reference memory
							Win-stay radial arm maze	No effect working memory
							Position discrimination T-maze	No effect
Gresack and Frick (present study)	Mouse	17.5 months	18 months	Cyclodextrin-encapsulated 17β-estradiol	Daily injection	3 months	Water-motivated radial arm maze	No effect reference memory
							Water-motivated radial arm maze	No effect working memory
							Object recognition	No effect
Gibbs (2000a)	Rat	13 months	13 months	17β-estradiol	Silastic implant	8–12 months	Delayed matching-to-position	Improvement
	Rat	13 months	16 months	17β-estradiol	Silastic implant	5–9 months	Delayed matching-to-position	Improvement
Markham et al. (2002)	Rat	14 months	15 months	17β-estradiol	Silastic implant	4 weeks	Morris water maze	Improvement
Markowska and Savonenko (2002)	Rat	13 months	21–22 months	17β-estradiol	Silastic implant	5 days	Delayed-nonmatching-to-position	No effect
Foster et al. (2003)	Rat	12–13 months	12–13 months	Estradiol benzoate	Silastic implant	21 days	Inhibitory avoidance	Impairment[a]
							Morris water maze	Improvement[a]
	Rat	17–18 months	17–18 months	Estradiol benzoate	Silastic implant	21 days	Inhibitory avoidance	Impairment[b]
							Morris water maze	Improvement[b]
Savonenko and Markowska (2003)	Rat	12–13 months	12–13 months	17β-estradiol	Silastic implant	6 days	T-Maze active avoidance	No effect
	Rat	20 months	20 months	17β-estradiol	Silastic implant	6 days	T-Maze active avoidance	No effect
Daniel et al. (2006)	Rat	12 months	12 months	17β-estradiol	Silastic implant	~5 months	Radial arm maze	Improvement
	Rat	17 months	17 months	17β-estradiol	Silastic implant	1 week	Radial arm maze	Improvement
	Rat	12 months	17 months	17β-estradiol	Silastic implant	1 week	Radial arm maze	No effect

Lacreuse et al. (2004)	Monkey	9 years	23–26 years[c]	Ethinyl estradiol	Daily oral[d]	Not applicable[e]	Modified Wisconsin Card Sort	No effect
Tinkler and Voytko (2005)	Monkey	Not specified	"Middle-aged"	17β-estradiol	Silastic implant	2 months	Delayed response Visuospatial attention	Improvement Improvement[f]
Intermittent treatment								
Gresack and Frick (present study)	Mouse	17.5 months	18 months	Cyclodextrin-encapsulated 17β-estradiol	Injection: Once every 4 days	3 months	Water-motivated radial arm maze Water-motivated radial arm maze Object recognition	Impaired reference memory No effect[g] No effect[g]
Gibbs (2000a)	Rat	13 months	16 months	17β-estradiol +progesterone	Injection: Weekly	5–9 months	Delayed matching-to-position	Improvement
Gibbs (2000a)	Rat	13 months	23 months	17β-estradiol +progesterone	Injection: Weekly	6–8 weeks	Delayed matching-to-position	No effect
Markowska and Savonenko (2002)	Rat	13 months	19 months	17β-estradiol	Injection (three consecutive days over a four day cycle) plus silastic implant	Injection: 16 days, Implant: 5 days	Delayed nonmatching-to-position	Improvement
Ziegler and Gallagher (2005)	Rat	14 months	~15 months	17β-estradiol benzoate	Injection: Two consecutive days over a 6-day cycle	Not applicable[h]	Radial arm maze	No effect
Lacreuse et al. (2002)	Monkey	9 years	21–24 years[c]	Ethinyl estradiol	Oral: Daily over a 28 day cycle[i]	Not applicable[j]	Delayed response Delayed nonmatching-to-sample Spatial-delayed recognition span	No effect No effect Improvement
Rapp et al. (2003)	Monkey	~22 years	~22 years[k]	Estradiol cypionate	Injection: Once every 3 weeks	6 weeks[l]	Delayed response Delayed nonmatching-to-sample	Improvement Improvement

[a] Low dose only.
[b] High dose only.
[c] Subjects received estrogen treatment at various points throughout their life prior to and during this study.
[d] Subjects switched between daily administration of ethinyl estradiol and placebo with each successive phase of testing during the modified Wisconsin card sort task.
[e] Treatment began after subjects learned first phase of task (i.e., initial discrimination).
[f] Placebo-treated subjects performed worse 2 months following treatment compared to their performance 1 week following treatment. Estradiol-treated subjects did not differ in their performance at 1 week and 2 months following treatment.
[g] Tended to impair.
[h] Treatment began at time of radial maze testing.
[i] In addition to ethinyl estradiol, subjects received either daily raloxifene or vehicle in alternating 28-day blocks.
[j] Treatment began at time of testing. Testing occurred for 9 consecutive months.
[k] Treatment began ~30 weeks after ovariectomy.
[l] Testing began 2 days after the second injection and treatment continued throughout testing.

and reference memory, as well as object recognition, in aging female rodents (Foster et al., 2003; Frick et al., 2002; Markham et al., 2002; Miller et al., 1999; Vaucher et al., 2002). Although the duration of estrogen treatment (ranging from 5 days to 1 month) prior to behavioral testing in these earlier studies could be considered chronic, estradiol in the present study was administered for a much longer period (3 months) before behavioral testing (See Table 2). Perhaps the prolonged duration of daily estradiol treatment can account for the discrepant findings. Sustained estradiol administration (8 days) downregulates estrogen receptors throughout the brain (Brown et al., 1996) and, furthermore, the extent of this downregulation becomes greater as the duration of estradiol administration increases (Brown et al., 1996). It is possible that estradiol administration over the course of several months in the present study significantly reduced estrogen receptor levels throughout the brain, thereby impeding a genomic mechanism through which estradiol could affect hippocampal function. In addition, continuous estradiol treatment (via silastic capsules) administered to 2-month-old female rats for more than a year (approximately 14–18 months) decreases hippocampal spine density (Miranda et al., 1999). A similar change in this study may have also contributed to an overall decrease in both the neural sensitivity to and mnemonic effectiveness of estradiol in the Contin E_2 group.

Alternatively, other methodological differences between previous reports and the present study may account for the discrepant findings. For example, we used a quickly metabolized form of estradiol, whereas other studies reporting beneficial effects in subjects even older than those used in the present study used slowly metabolized forms of 17β-estradiol or estradiol benzoate (e.g., Miller et al., 1999; Frick et al., 2002, refer to Table 2). In addition, estradiol in the present study was administered via daily injections rather than by silastic capsule or pellet implants. Although using injections enabled us to better simulate the timing of estradiol surges in the Twice/wk E_2 group, the stress associated with these daily injections may have interfered with the ability of estradiol to affect memory. This possibility may be illustrated by comparing this study to a previous report from our lab in which daily estradiol was given for 5 weeks prior to testing in the WRAM and object recognition tasks (Fernandez and Frick, 2004). In this study, 17β-estradiol was dissolved in ethanol and administered in drinking water to which mice had free access. Similar to the daily injections in the present study, oral estradiol produced daily peaks in estradiol levels (Fernandez and Frick, 2004). However, mice in the Fernandez and Frick study were left undisturbed in their cages during treatment, so were likely under considerably less handling stress than the mice in the present study. Whereas daily estradiol injections in the current study had no effect on object recognition, daily oral estradiol significantly improved object recognition at all three doses tested (Fernandez and Frick, 2004). This might suggest that the stress of daily handling and injections interfered with the ability of estradiol to improve object recognition. Nevertheless, the shorter duration of oral estradiol treatment might have also influenced the results as discussed above. Similar to the present study, oral estradiol treatment had no effect on spatial working memory in the WRAM (Fernandez and Frick, 2004), which may suggest that this type of memory is not amenable to improvement by chronic estradiol in aging female mice, regardless of route of administration. Because the object recognition task may be more sensitive than the WRAM to the effects of estradiol and/or stress, this task should be used in future work to directly compare the effects of daily injections and other chronic treatments (e.g. oral, capsules).

In addition, the age at ovariectomy may have contributed to discrepancies with previous studies. It has previously been suggested that the ability of estrogen to protect against neural and behavioral deficits resulting from hormone deprivation may decrease as the time from deprivation increases (Gibbs, 2000a; Daniel et al., 2006; Savonenko and Markowska, 2003; Yaffe et al., 1998). Prolonged hormone deprivation in adult female rats has been shown to reduce hippocampal dentate granule cell spine density (Miranda et al., 1999) and basal forebrain choline acetyltransferase mRNA (Gibbs, 1998). Although estrogen administration beginning shortly after estrogen loss (in this case, after ovariectomy) may prevent the occurrence of neural and mnemonic deficits, treatment may not be able to reverse these deficits after they have emerged. For example, continuous estradiol improves working memory in middle-aged rats when initiated immediately, but not 5 months, after ovariectomy (Daniel et al., 2006). In addition, intermittent hormone therapy enhances spatial working memory when initiated 3, but not 10, months after hormone deprivation (Gibbs, 2000a). Because irregular cycling can begin as early as 13 months (Savonenko and Markowska, 2003), it is possible that mice in the present study experienced declining serum estrogen levels for several months prior to ovariectomy and estradiol replacement. If hormone changes had already led to age-related neural dysfunction prior to ovariectomy, then this could explain why the continuous treatment regimen had no effect on spatial and object memory. This issue will need to be addressed in future work by monitoring cycling prior to ovariectomy in conjunction with conducting ovariectomies at younger ages (e.g. 13 months).

Finally, the lack of an effect of continuous estradiol may also be related to task difficulty. In the WRAM, young female mice learn to make significantly fewer working and reference memory errors as testing progresses (Gresack and Frick, 2003, 2004). Furthermore, estradiol has been shown to significantly reduce working memory errors in this task in young rats (Bimonte and Denenberg, 1999) and mice (Gresack and Frick, 2004). However, a significant reduction in errors was not observed during the course of testing in two of the three measures of the WRAM task in the present study. Previous studies have also shown that this task is difficult for aging rats and mice to learn (Bimonte et al., 2003; Fernandez and Frick, 2004), although we thought that this would make the WRAM an ideal task to use because the age-related deficits left ample room for improvement by estradiol. Further, the fact that this task can simultaneously test two kinds of memory afforded the advantage of detecting differential effects of estradiol on multiple types of memory. Unfortunately, the data may suggest that the current WRAM protocol is too difficult to allow for estradiol to modulate memory in aging mice. Reducing the task demand and/or using a higher dose of estradiol might help resolve this issue.

Interestingly, intermittent estradiol treatment impaired spatial reference memory and tended to impair spatial

working memory and object memory in the current study. These results contradict findings from other studies which have reported beneficial effects of intermittent regimens on memory (Table 2). For example, priming aging rats with three consecutive daily estradiol injections over a 4-day cycle enhances the ability of a continuous regimen to improve spatial working memory (Markowska and Savonenko, 2002). Also, weekly injections of estradiol plus progesterone improve spatial working memory in aging rats (Gibbs, 2000a). In rhesus monkeys, one estradiol injection every 3 weeks reverses age-related deficits in spatial working memory (Rapp et al., 2003). In contrast, one recent study which repeatedly administered estradiol injections in a manner known to increase hippocampal spine density (Gould et al., 1990) reported no improvement in middle-aged female rats on hippocampal tests of spatial working memory (Ziegler and Gallagher, 2005). Moreover, as previously mentioned, recent work from our lab demonstrated that oral estradiol administration producing daily estradiol fluctuations improved object recognition but not spatial memory in the WRAM (Fernandez and Frick, 2004).

Although the aforementioned data seem widely inconsistent, all utilized very different methods for approximating aspects of the natural cycle and for measuring memory, so the varying results are not too surprising. Importantly, none of the studies precisely modeled the estrogen and progesterone fluctuations inherent to the natural cycle. The current study attempted to approximate the timing of the estrogen surges observed in young rodents by administering estradiol once every 4 days. Because increases in hippocampal spine synapse density and LTP have been shown to accompany estrogen surges in cycling rats (Warren et al., 1995; Woolley et al., 1990; Woolley and McEwen, 1992), one might expect that estradiol given every 4 days would maintain this hippocampal plasticity. However, this regimen was clearly not beneficial for memory. Our regimen did not reflect the fact that low levels of estradiol are present most of the time during the natural cycle, which could, in part, have contributed to the negative findings. Alternatively, although estrogen-induced increases in hippocampal plasticity are generally believed to improve memory, this relationship has yet to be demonstrated directly. Some recent evidence indicates that estrogen regimens known to increase hippocampal plasticity do not benefit spatial memory (Frick et al., 2004; Ziegler and Gallagher, 2005). Perhaps the administration of estrogen without subsequent progesterone administration contributes to these null data, as well as to the impairments in the present study. Two consecutive daily injections of estradiol to ovariectomized rats significantly increases progesterone receptor isoform content in the hippocampus (Guerra-Araiza et al., 2003). This may render the brain more sensitive to remaining endogenous progesterone, an effect that could negatively impact hippocampal plasticity in light of the fact that progesterone reduces estrogen-induced increases in hippocampal spine density (Gould et al., 1990). Progesterone given 1 day following two consecutive estradiol injections significantly reduces hippocampal progesterone receptors (Guerra-Araiza et al., 2003), which could minimize any adverse effects of this hormone on estrogen-induced changes in hippocampal plasticity and memory. Indeed, a single injection of progesterone following two estradiol injections improved memory retention in a delay matching-to-place water maze task (Sandstrom and Williams, 2001). Thus, a regimen which more precisely mimics the timing of estrogen and progesterone fluctuations in the natural cycle may be more beneficial for spatial and object memory in the aged female.

In conclusion, the results of the present study are novel because they indicate that different long-term estradiol treatment regimens have discrepant effects on spatial and object memory in aging female mice. Continuous estradiol replacement had no effect on spatial and object memory, whereas intermittent estradiol replacement selectively impaired spatial reference memory. Despite these results, cyclic treatment regimens that are shorter in duration and that more accurately reflect the timing of ovarian hormone fluctuations should be explored before concluding that intermittent hormone treatment regimens are less beneficial than continuous regimens. Nevertheless, these data illustrate the importance of considering treatment regimen when devising estrogen therapies.

4. Experimental procedures

4.1. *Subjects*

Female ($N = 32$) C57BL/6 mice were obtained at 15–17 months from the National Institute on Aging colony at Harlan Sprague–Dawley (Indianapolis, IN). All mice were handled for 5 days (5 min/day) prior to behavioral testing. Up to five mice/cage were housed in a room with a 12:12 h light/dark cycle (lights on at 07:00). All behavioral testing occurred during the light phase of the cycle. Food and water were available *ad libitum* for the duration of testing. All procedures followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Care and Use Committee of Yale University.

4.2. *Ovariectomy*

All mice were ovariectomized at 17.5 months of age to ensure removal of endogenous sources of estrogen prior to estradiol replacement (Gresack and Frick, 2004). Approximately 40% of females still exhibit some form of estrous cycling at this age (Frick et al., 2000). Ovariectomy was necessary in the present study to eliminate any remaining cycling and allow for a more systematic examination of the effects of an exogenous surge in estrogen every 4 days. Mice were anesthetized with 2% isoflurane gas in 100% oxygen. Two incisions were made on the dorsal surface of the body, just above the pelvic bones. The ovary, oviduct, and tip of the uterine horn were isolated, clamped off and removed. The rest of the uterine horn was returned to the abdominal cavity. Mice were housed singly for 1 week, during which time pediatric acetaminophen (approximately 1 ml/100 ml of water) was added to each water bottle. After recovery, mice were re-housed with their original cagemates in groups of up to five.

4.3. *Estradiol administration*

Cyclodextrin-encapsulated 17β-estradiol (E_2; Sigma), in a dose of 0.2 mg/kg, was dissolved in physiological saline in a volume of 4 ml/kg. This dose has previously been shown to improve working memory in a water-escape motivated radial arm maze

and object recognition in young ovariectomized mice (Gresack and Frick, 2004). Cyclodextrin is a solubility-enhancing carrier for the estradiol which allows this hormone to be metabolized within 24 h (Pitha and Pitha, 1985; Taylor et al., 1989). The vehicle (VEH), 2-hydroxypropyl-β-cyclodextrin, contained the same amount of cyclodextrin as the 0.2 mg/kg dose of cyclodextrin-encapsulated E_2 and was dissolved in an equal volume of saline. Although estradiol levels produced by 0.2 mg/kg E_2 were not tested in this study, other preliminary work in our lab has shown that injecting this dose (i.p.) to ovariectomized 20-month-old mice, who were killed 2 or 22 h after injection, produces transiently high estradiol levels that return to baseline by 22 h (unpublished observations). Samples were analyzed by Cayman Chemical Company (Ann Arbor, MI) using an Estradiol EIA kit. Estradiol levels in vehicle-treated mice killed at 2 ($n=2$) or 22 ($n=3$) h after injection were 50.7 (±16.5) and 43.7 (±8.0) pg/ml, respectively. Although these values are somewhat high for ovariectomized females, all mice had experienced typical age-related weight gain (weighing from 25 to 37 g) and, thus, would have retained some estradiol in fat tissue (Deslypere et al., 1985; Rodriguez-Cuenca et al., 2005). Levels were 129.25 (±32.9) pg/ml 2 h after E_2 injection ($n=2$) and back to baseline levels of 56.6 (±12.3) pg/ml 22 h after injection ($n=2$). Thus, the increase in estradiol levels produced 2 h after injection was approximately two and a half-fold. Previous studies report estradiol levels during the mouse estrous cycle as fluctuating from approximately 20–80 pg/ml (Grasso and Reichert, 1996; Walmer et al., 1992) which represents a four-fold difference between proestrus and late estrus. As such, the 129.25 pg/ml level relative to the 50.7 pg/ml 2 h after injection likely represents an increase in the physiological range.

Control mice received daily intraperitoneal (i.p.) injections of VEH. Estradiol-treated mice received either daily or intermittent i.p. injections of E_2. The continuous group (Contin E_2) was injected daily with E_2. The intermittent group (Twice/wk E_2) received E_2 injections every 4 days and vehicle on all other days. This regimen was designed to approximate the timing of the natural estrogen surge occurring every fourth day of the estrous cycle. Injections began 2 weeks after ovariectomy, occurred for 3 months prior to behavioral testing, and continued throughout testing. Injections were given in the late afternoon. The present study was designed to test the cumulative effects of chronic treatment in the absence of acute effects on task performance. Previous work has shown that immediate, but not delayed (2 h), acute post-training injections of cyclodextrin-encapsulated E_2 improve spatial reference memory consolidation in a Morris water maze task (Packard and Teather, 1997). As such, E_2 was administered 2 h after training (22 h before testing the next day) so that memory on the following day could be examined in the absence of acute E_2 effects on memory consolidation.

4.4. WRAM

WRAM testing began at 21 months of age using the protocol described previously (Gresack and Frick, 2003, 2004). Briefly, an opaque central arena (diameter=44 cm) with eight clear Plexiglas arms radiating from the center (38×12 cm) was placed in a large tank filled with water (24±2 °C). The water level was approximately 9 cm below the top of the maze, precluding the use of the arms for escape. The water was made opaque with white non-toxic tempera paint. A variety of extramaze cues surrounded the tank. At the ends of four of the arms, escape platforms were submerged just beneath the water surface. The sequence of arms with platforms was randomized between mice but did not change within a mouse for all sessions. Platforms were never located in more than two consecutive adjacent arms. One arm was designated as a start arm for all mice and this arm never contained a platform. A five trial shaping procedure, completed 1 day prior to the first test session, was used to familiarize the mice with the platforms (see Gresack and Frick, 2003 for details). During testing, each mouse completed 4 trials per day for 15 consecutive days (each day was one session). At the start of trial 1, the mouse was released from the start arm and was given 120 s to locate a submerged platform. If the mouse did not find a platform in the allotted time, it was guided to the nearest one upon which it remained for approximately 15 s. The mouse was then removed from the platform, dried with a towel, and placed in a holding cage for a 30-s intertrial interval. During the intertrial interval, the located platform was removed from the pool, thus leaving three platforms in the maze. The mouse was then returned to the start arm for trial 2. The above procedure was repeated until all four platforms were found (one platform/trial). Mice were then returned to their home cages. Approximately 2 h after the final platform was found, mice were injected with VEH or E_2. The 15 day testing protocol made it necessary for mice in the Twice/wk E_2 group to receive estradiol during WRAM testing. During testing, the Twice/wk E_2 group received estrogen following the first day of training (Session 1) and then once every subsequent fourth day throughout testing (e.g. following Sessions 5, 9 and 13). This group received vehicle on all other days. We attempted to minimize potentially confounding effects of acute E_2 on performance by administering a rapidly metabolized form of estrogen 2 h after training (22 h prior to subsequent testing). As such, minimal amounts of estradiol were in the circulation during both the memory consolidation period 2 h following training and during testing the next day.

Within each trial, three different types of errors, working memory, initial reference memory, and repeated reference memory, could be committed (Gresack and Frick, 2003, 2004). Entries into arms from which a platform had been removed in a previous trial of the session were considered working memory errors (note that it was not possible to make a working memory error in trial 1). Initial reference memory errors were first entries into arms that never contained a platform. Repeated reference memory errors were repeated entries into arms that never contained a platform. The number of errors of each type was totaled for each session. In addition, the number of working memory errors committed in Trials 2–4 of each session was determined to allow working memory errors to be assessed as the working memory information to be remembered within the session (i.e. working memory load) increased (Gresack and Frick, 2003, 2004).

4.5. *Object recognition*

Object recognition testing began 3 days after the completion of WRAM testing. The protocol for object recognition was the

same as previously described (Gresack and Frick, 2004; Frick and Gresack, 2003). Briefly, testing occurred in a white, wooden open field chamber (58×58×46 cm high) located in a quiet room under dim halogen lighting. A video camera was mounted on the ceiling above the box and connected to a VCR, monitor, and computer in an adjacent room. The task takes advantage of the natural affinity of mice for novelty; mice that recognize a previously seen (i.e. familiar) object will spend more time exploring novel objects. To habituate the mice to the chamber, mice were initially placed in the empty open field box for 5 min. Locomotor activity was measured during habituation by recording the number of crossings of a 5×3 grid laid over the field on the computer monitor. The next day, mice completed the sample phase of the task. Mice were first re-habituated to the empty box for 1 min. Next, two identical objects were placed in the northwest (left) and northeast (right) corners of the box (approximately 5 cm from the walls). Mice remained in the chamber until they accumulated 30 s exploring the objects, at which point they were removed from the box and returned to their home cages. Object exploration was scored when the mouse's nose or front paws came in contact with the objects. Twenty-four hours later, the mice completed the choice phase, during which time one familiar object (identical to that which was presented during the sample phase) and one novel object were placed in the northwest and northeast corners of the box. The location of the novel object was counterbalanced across mice in each group. Mice remained in the chamber until they accumulated 30 s of object exploration. The box and objects were cleaned with 70% ethanol between mice. A video tracking system and custom-written computer program was used to record time spent exploring each object during sample and choice phases. Elapsed time (the time needed to accumulate 30 s of exploration) was also recorded during the sample and choice phases.

During object recognition testing, injections were given 2 h after the mouse was removed from the chamber and returned to its home cage. To avoid the potentially confounding effects of acute E_2 on memory consolidation in this task, the Twice/wk E_2 group did not receive E_2 at any point during testing. Mice in this group received E_2 1 day prior to habituation and then vehicle on the sample and choice days. They received their next E_2 injection 1 day after completing the choice phase.

4.6. *Uterine weights*

Vehicle and E_2 injections continued after the completion of object recognition until all mice were euthanized. Mice were euthanized 1–2 days following the final estradiol injection. All mice were sedated briefly with CO_2 and cervically dislocated. Uteri were removed and weighed (g) to measure the physiological effectiveness of chronic estrogen exposure.

4.7. *Data analysis*

Data from the WRAM were analyzed as described previously (Gresack and Frick, 2003). Working memory errors, initial reference memory errors, and repeated reference memory errors committed in the WRAM were analyzed separately using repeated-measures analyses of variance (ANOVA) with one between-subject (Treatment) and one within-subject (Session) factor (SuperANOVA, Abacus Concepts; Berkeley, CA, USA). Fisher's protected least significant difference (PLSD) post hocs were performed to delineate differences between groups. The effect of increasing working memory load on errors was first analyzed using repeated-measures ANOVA with one between-subject (Treatment) and one within-subject (Trials) factor. Working memory errors in individual trials were then analyzed using a one-way ANOVA with a between-subject (Treatment) factor.

Data from Session 1 of the WRAM were excluded from all analyses, as described previously (Gresack and Frick, 2003, 2004), because performance in this session does not accurately measure memory. In addition, it has previously been demonstrated that the greatest amount of learning in the WRAM task occurs during the first (i.e. Sessions 2–8), but not the last (i.e. Sessions 9–15), half of testing (Gresack and Frick, 2003). Thus, to examine the effects in the present study during task acquisition more closely, we also conducted separate ANOVAs on the first and second halves of testing.

Separate one-way ANOVAs without repeated measures were used to analyze grid crossings (locomotor activity) during habituation, as well as elapsed time during the sample and choice phases of the object recognition task. In addition, a preference for one object over another during the sample and choice phases was assessed using one-sample *t*-tests to determine whether time spent with an object (i.e. the left or right object in the sample phase; the novel or familiar object in the choice phase) differed significantly from the chance value of 15 s (Baker and Kim, 2002; Frick and Gresack, 2003). This type of *t*-test was used because time spent with one object is not independent from time spent with the other object (total exploration time must equal 30 s). Finally, a one-way ANOVA without repeated measures was performed on uterine weights.

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