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Comparing the Effects of Various Estrogen Replacement Paradigms on Working Memory Performance in the Radial-Arm Maze

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COMPARING THE EFFECTS OF VARIOUS ESTROGEN REPLACEMENT PARADIGMS ON WORKING MEMORY PERFORMANCE IN THE RADIAL-ARM MAZE

A Thesis

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This manuscript is dedicated to my parents,

Dr. Gernot and Friederike Bohacek

Table of Contents

List of Figures

Abstract

The current study compared the impact of different paradigms of estradiol replacement on working memory performance. In adult ovariectomized rats, a vehicle-treated control group (*n*=10) was compared to three estradiol replacement paradigms: 1) continuous delivery via Silastic capsules (*n*=8); 2) cyclic replacement via two 10 µg injections on two out of every four days (*n*=10); 3) cyclic replacement via one 2 µg injection every four days (*n*=10). While treatment continued, animals were tested over 24 days in the 8-arm radial maze. After this acquisition period, various delay times were introduced between 4th and 5th arm choices. Treatments had no effects during acquisition or delay trials of 1 min, 10 min, and 3 hours. However, when a 5-hour delay was imposed, rats receiving estradiol via implants outperformed all other groups. These results indicate that long-term continuous estradiol replacement is more effective in enhancing working memory performance than the tested cyclic paradigms.

Comparing the Effects of Various Estrogen Replacement Paradigms

on Working Memory Performance in the Radial-Arm Maze

Estrogens, a group of female sex hormones (the most prevalent of which is estradiol), are produced mainly by the ovaries and play a crucial role in regulating reproductive functions in mammals (Becker, Breedlove, Crews, & McCarthy, 2002). In addition to its primary function as a reproductive hormone, estradiol also exerts remarkable effects on brain regions not classically associated with reproduction. Pronounced effects of estradiol on the hippocampus, a structure implicated in learning and memory, have been repeatedly reported (for review, see Woolley, 1998). In line with these findings, manipulations of estradiol levels have been demonstrated to affect behavioral measures of learning and memory in rodents, non-human primates and humans (Dohanich, 2002; Rapp, Morrison, & Roberts, 2003; Sherwin, 2003).

Estrogen and the Hippocampus

Many studies demonstrate that estrogen has profound effects on hippocampal structure and function. Specifically, dendritic spine density on CA1 pyramidal neurons in the hippocampus is lowered in ovariectomized rats, but can be restored to baseline of intact animals upon exogenous estradiol replacement (Gould, Woolley, Frankfurt, & McEwen, 1990; Woolley & McEwen, 1992). Accordingly, spine and synapse density also fluctuate over the female cycle (Woolley, Gould, Frankfurt, & McEwen, 1990; Woolley et al., 1992). Dendritic spines are protrusions with a thin neck and a bulbous head; they form the postsynaptic part of excitatory synapses and are believed to compartmentalize calcium influx in response to synaptic activity (Chittajallu, Alford, & Collingridge, 1998; Nimchinsky, Sabatini, & Svoboda, 2002). In line with these findings, estradiol replacement has been shown to increase presynaptic (synaptophysin and syntaxin) and postsynaptic (spinophilin) proteins in different regions of the hippocampus in

ovariectomized rats (Brake et al., 2001). Further, it has been demonstrated that estradiol replacement in ovariectomized animals increases NMDA receptor binding in the hippocampus (Weiland, 1992; Daniel & Dohanich, 2001). NMDA receptors are located mainly on the head of dendritic spines and are highly permeable to calcium, playing a crucial role in synaptic plasticity, e.g. long-term potentiation, a construct thought to represent a biological mechanism for memory formation (Bliss & Collingridge, 1993). Accordingly, in female rats during proestrus – when estradiol levels reach their peak – long-term potentiation is enhanced (Warren, Humphreys, Juraska, & Greenough, 1995), and similarly, estradiol replacement in ovariectomized animals also increases hippocampal long-term potentiation (Foy et al., 1999).

Estrogen and the Cholinergic System

Cholinergic neurons in the basal forebrain (septal nucleus, diagonal band nucleus) project afferent fibers to the hippocampus, constituting the septohippocampal pathway (Woolf, 1991). The cholinergic system has long been thought to play a role in learning and memory, and there is substantial evidence that the cognitive impairments associated with Alzheimer's disease are linked to a loss of cholinergic neurons (for review, see Francis, Palmer, Snape, & Wilcock, 1999). Interestingly, estradiol replacement in ovariectomized rats was shown to increase several markers of cholinergic function in the basal forebrain, such as choline acetyltransferase (ChAT) activity (Singh, Meyer, Millard, & Simpkins, 1994), number of ChAT containing neurons (Gibbs, Wu, Hersh, & Pfaff, 1994), and amount of acetylcholine release in the hippocampus (Gibbs, Hashash, & Johnson, 1997; Marriott & Korol, 2003). Because the septohippocampal projections constitute an important input pathway to the hippocampus, it has been argued that the modulating effects of estradiol on the cholinergic system could explain the impact of estradiol on learning and memory processes. Recent studies from our lab suggest that muscarinic M2

receptors in the hippocampus mediate the impact of estradiol on hippocampal NMDA receptor binding and on working memory performance (Daniel et al., 2001; Daniel, Hulst, & Lee, 2005).

Estrogen Acts Via Estrogen Receptors

The physiological effects of estrogen on the hippocampal structure and on the basal forebrain are mediated through different estrogen receptors (ER). "Classical" intracellular receptors (ERα and ERβ) are differentiated from newly detected membrane bound receptors (for reviews, see McEwen & Alves, 1999; Toran-Allerand, 2004). Estrogen binding to the classical receptor subtypes exerts its effects via comparatively slow genomic mechanisms, the receptor-ligand complexes dimerize and bind to either an estrogen response element (ERE) or to an activator protein 1 (AP-1) site where they trigger gene expression. The membrane bound receptors are thought to employ rapid nongenomic mechanisms of action via second messenger pathways, specifically cyclic adenosine monophasphate (cAMP) and mitogen-activated protein kinase (MAPK) pathways. Recently, the presence (and colocalization) of ERα and ERβ has been documented in the cortex, in the basal forebrain, and in all regions of the hippocampus (Li, Schwartz, & Rissman, 1997; Shughrue & Merchenthaler, 2000; Mehra, Sharma, Nyakas, & Vij, 2005).

Effects of Estrogen on Learning and Memory Performance

The physiological results demonstrating the impact of estrogen on brain structure and function discussed above are accompanied by a large body of behavioral studies analyzing the cognitive effects associated with fluctuating estrogen levels. Because the hippocampus is a structure known to play an important role in learning and memory (Eichenbaum & Cohen, 2001), it is natural to expect that the physiological impact of estrogen on this structure should modulate

cognitive performance. Interestingly, the behavioral data is very contradictory and task dependent (for review, see Dohanich, 2002).

One way to differentiate and categorize the equivocal findings is to distinguish between working memory and reference memory tasks. Working memory is here defined as a form of short-term memory for information that is currently but only temporarily useful, while reference memory is a form of long-term memory for information that yields a general principle which is useful over longer periods of time (see Dohanich, 2002). Within this framework, experiments from our and other laboratories have shown the differential effects of estradiol on working and reference memory using the radial-arm maze. In the working memory version of the radial-arm maze, all arms are initially baited and the food deprived animal can freely choose between arms. If an arm that has already been visited during that trial is entered again, the animal commits a working memory error. Variations of this task have consistently indicated improved performance of estradiol treated animals as compared to controls (Daniel, Fader, Spencer, & Dohanich, 1997; Luine, Richards, Wu, & Beck, 1998; Fader, Johnson, & Dohanich, 1999; Daniel et al., 2001). In contrast, for a reference memory task only certain (and always the same) arms of the radial-arm maze would be baited over several trials. Hence, a reference memory error is scored anytime the animal enters one of the arms that are never baited. Estradiol replacement in these tasks generally fails to improve or even impairs performance (Luine et al., 1998; Fader et al., 1999).

Working and reference memory can also be tested in the aversive water escape task, where the animal is put in a circular pool filled with opaque water and has to use extramaze cues to find an escape platform just beneath the water surface. For the reference memory task, the escape platform would be in the same spot every session/day, so the animal has to use extramaze cues to always find the platform in the same spot. For a working memory task, the animal has to

find the platform on the first trial and then find the platform again at the same spot on a later trial the same day, but the platform would be moved every day/session. Studies assessing the impact of estradiol on different water escape tasks have yielded contradictory results even within the framework of working versus reference memory (for review see, Dohanich, 2002). However, swim path analysis have raised the possibility that rats with high estradiol levels might employ different learning strategies than animals with low estradiol levels (Korol, 2004). This idea is well in line with modern theories of multiple memory systems that map onto distinct brain regions (McDonald & White, 1993; White & McDonald, 2002). Lesion studies have, for example, shown a clear dissociation between place and response learning which preferentially involve the hippocampus and striatum, respectively (Packard & McGaugh, 1996). Behaviorally, place and response learning can be dissociated in a T-maze with one (for example the right) arm baited. The animals can choose either an egocentric response strategy (always turn right) or an extramaze cue-dependent place strategy (always turn towards a specific location in the room). Consistent with the pronounced effects of estradiol on hippocampal structure and physiology, it has recently been demonstrated that estradiol differentially impacts strategy selection in the plus-maze (Korol & Kolo, 2002) and the water maze (Daniel & Lee, 2004). Specifically, high estradiol levels seem to bias female rats to use a place rather than a response strategy. According to modern theories of learning and memory, memory systems do not act independently, but rather seem to show complex patterns of competition and cooperation between different neural systems (White et al., 2002; Gold, 2004). Consequently, it is possible that striatum and hippocampus compete for participation in certain learning tasks, and estradiol could bias an animal towards using a hippocampus-based strategy, which might be advantageous or impairing according to the effectiveness of the chosen strategy for a given task (Korol, 2004). Applied to

the differentiation between working and reference memory, there is evidence that working memory might be dependent primarily upon the hippocampus, while reference memory seems to be striatum-dependent (Packard & White, 1990). Hence, it is possible that the estradiol-mediated enhancement of hippocampal structure and function might bias animals to use working memory strategies and potentially impair striatum-based reference memory performance.

Estrogen Replacement and its Role in Postmenopausal Women

Currently, there is an ongoing debate over the effectiveness of hormone replacement therapy (HRT) in postmenopausal women. HRT is typically administered in the form of daily pills containing either conjugated equine estrogen alone (CEE) or combined with medroxyprogesterone acetate (CEE+MPA). Until recently, accumulating experimental evidence suggested that the dramatic decline in ovarian hormones in women after menopause contributes to age-related cognitive decline and the increased incidence of Alzheimer's disease in elderly women, and that HRT can counteract these effects (for review, see Sherwin, 2003). HRT is highly effective in alleviating menopause-associated symptoms like acute climacteric symptom (hot flashes) and osteoporosis (loss of bone mass), but it has also become a promising tool for protection against postmenopausal cognitive decline (for review, see Prevelic, Kocjan, & Markou, 2005). Recently, however, this view has been challenged by the randomized, double-blind, placebo-controlled Women's Health Initiative Memory Study (WHIMS). A large sample of women aged 65 years and above received either CEE, CEE+MPA, or placebo. Unexpectedly, the study reported adverse effects of both HRT paradigms on cognitive function and an increased risk for Alzheimer's disease (Rapp et al., 2003; Espeland et al., 2004; Shumaker et al., 2004). Yet, these results were criticized for methodological inconsistencies with previous human and animal literature and many authors have concluded that the form (estradiol vs. conjugated equine

estrogen), route (oral, injections, transdermal), time (perimenopausal vs. postmenopausal) and type (chronic vs. cyclic) of hormone replacement therapy seem to be crucial factors for therapy success (Sherwin, 2005; Prokai-Tatrai & Prokai, 2005; Sohrabji, 2005; Gleason, Carlsson, Johnson, Atwood, & Asthana, 2005). For example, animal studies almost exclusively use synthetically produced estradiol, while the WHIMS trials used the clinically most commonly prescribed form, conjugated equine estrogen (CEE). CEE uses estrone as its main active ingredient, which is a less potent and pharmacologically distinct form of estrogen than estradiol. Fundamental differences in the pharmacology and effectiveness of these forms of estrogen preparations have recently been suggested, and there are some clinical trials that favor estradiol treatments over CEE administration (for review, see Gleason et al., 2005). With respect to time of HRT onset, recent evidence suggests that there is a critical time window following cessation of ovarian function during which estradiol replacement must be initiated to produce enhancing effects on working memory performance in middle-aged rats (Gibbs, 2000; Daniel, Hulst, & Berbling, 2006). The WHIMS trials, however, initiated HRT in women aged 65 years and above, which is more than a decade after ovarian hormones have declined during menopause.

In the current study, we wanted to focus on possible differences between chronic versus cyclic replacement paradigms. Data from animal studies have recently raised interesting questions concerning the effectiveness of chronic as compared to cyclic estradiol replacement. In the first study long-term hormone deprivation (14 – 20 months) caused a dramatic decline in spine density of dentate granule cells in ovariectomized rats (Miranda, Williams, & Einstein, 1999). However, long-term chronic estradiol implants (14 – 20 months) did not restore spine density in the dentate gyrus of the aged female rat if compared to ovariectomized controls. Interestingly though, acute injections of estradiol benzoate (two injections of 10 µg 24 hours

apart) did increase spine density in those animals that were long-term hormone-deprived as well as in those who received long-term chronic estradiol replacement. This opens the possibility that cyclic but not chronic estradiol treatment might exert positive effects on hippocampal physiology and hence maybe on memory function in aged animals.

In line with these findings, another experiment has demonstrated that long-term cyclic estradiol and progesterone replacement (~ 7 months) in aged rats proves at least as effective as chronic estradiol replacement in enhancing performance on a spatial delayed matching to position working memory task in the T-maze (Gibbs, 2000). In this study, the cyclic paradigm consisted of one 10 µg injection of 17*ß*-estradiol sc. once a week, followed by a 500 µg injection of progesterone after 48 hours. These studies yield some support to the hypothesis that cyclic estradiol replacement might be physiologically more beneficial than chronic replacement in aged animals, and behaviorally it seems to be at least as effective as chronic treatment on the examined tasks.

It must be mentioned though, that the use of progesterone in addition to estradiol adds another layer of complexity to these results. Generally, progesterone is believed to initially increase the effects of estradiol on the hippocampus, but to reduce them quickly thereafter (Woolley & McEwen, 1993). Although the role of progesterone in modulation of cognitive function needs to be further investigated, it is beyond the scope of the current investigation to include progesterone injections in the treatment schedule.

Different Paradigms of Estradiol Delivery

Two regimens of estradiol replacement dominate the behavioral as well as physiological animal literature. The most common replacement paradigm uses a two-day injection schedule of 10 µg of 17*ß*-estradiol benzoate s.c., 24 hours apart (Woolley et al., 1992; Woolley & McEwen,

1994; Sandstrom & Williams, 2001). The second frequently used paradigm is implantation of a Silastic capsule containing powdered estradiol – either pure or mixed with cholesterol – subcutaneously into the nape of the neck (Daniel et al., 1997; Luine et al., 1998; Bimonte & Denenberg, 1999). The fundamental pharmacokinetic differences between these treatment regimens are apparent: While injections result in a sharp peak of estradiol levels, Silastic implants produce steady levels over the course of the experiment. The cyclic injection paradigm of two 10 µg estradiol benzoate injections 24 hours apart has been demonstrated to cause sharp, supraphysiological estradiol peaks, with the second injection pushing estradiol titers up to about 150 – 200 pg/ml before they rapidly decline to physiological levels on days three and four (Woolley et al., 1994; Ziegler & Gallagher, 2005). In comparison, the most commonly used Silastic implants produce steady estradiol levels in the low- to mid-physiological range around 30 – 40 pg/ml (Singh et al., 1994; Luine et al., 1998; Fader et al., 1999; unpublished data from our lab).

In the intact female rat, the estrous cycle lasts four to five days. Typically it begins with two days of diestrus during which estradiol levels slowly increase to a level of about 15 – 30 pg/ml, followed by proestrus where estradiol levels gradually reach their peak (40 – 60 pg/ml), and during the final day – estrus – estradiol levels rapidly decline below the detectable limit (Isgor, Huang, Akil, & Watson, 2002; Becker et al., 2005). Consequently, neither the cyclic nor the chronic estradiol replacement paradigm mimic the in vivo fluctuations of estradiol observed in intact cycling animals.

With respect to mimicking the estradiol levels of naturally cycling animals, there is another interesting injection paradigm that has to our knowledge not yet been studied in cognition. Recent work in reproductive neuroscience has introduced a regimen of estradiol

replacement where one sc. injection of 2 µg of estradiol benzoate is given once every four days (Micevych, Eckersell, Holland, & Smith, 1996; Geary & Asarian, 1999; Asarian & Geary, 2002). This paradigm has proven successful in research on lordosis behavior, feeding and weight gain. The attractiveness of this replacement paradigm is that it resembles the natural estradiol levels during the rat estrous cycle more closely than any other treatment regimen mentioned above (Micevych et al., 1996; Asarian et al., 2002).

Different Estradiol Replacement Paradigms and Cognitive Function

Despite the pharmacokinetic differences discussed above, the enhancement of hippocampal structure by estradiol has been shown in naturally cycling animals (Woolley et al., 1990) as well as in response to the two-day cyclic injection paradigm (10 µg of estradiol benzoate; Woolley, 1998; Foy et al., 1999). Accordingly, this replacement paradigm increases working memory performance in the radial-arm maze as well as in the water maze (Daniel et al., 2001; Sandstrom et al., 2001). A recent study has revealed that the improved performance following this two day estradiol regimen is detectable for four days after the second injection (Sandstrom & Williams, 2004). This mirrors the estradiol-triggered increase in CA1 spine density reported in earlier experiments (Woolley et al., 1993) that lasts for about a week before returning to baseline.

In comparison, chronic estradiol replacement has not been assessed with regard to hippocampal spine density, but physiological effects on the basal forebrain and markers of acetylcholine activity have been repeatedly reported (Singh et al., 1994; Gibbs et al., 1994). Behaviorally, estradiol delivered continuously via Silastic capsules has yielded beneficial effects on spatial working memory performance in the radial-arm maze if delivered for several weeks at mid-physiological levels (30 - 40 pg/ml; Daniel et al., 1997; Luine et al., 1998; Fader et al.,

1999). However, a study using a chronic, high physiological dose of about 90 pg/ml found no memory enhancement (Luine & Rodriguez, 1994). Another study found that low physiologic estradiol levels produced by daily injections of 0.3 µg estradiol benzoate enhanced working memory, while higher doses impaired it (Holmes, Wide, & Galea, 2002). However, the use of daily injections is pharmacologically different from Silastic implants. The administered doses, 0.3, 1.0, and 5.0 µg, reportedly produced physiologically low (~ 24 pg/ml), mid-physiological (~ 38 pg/ml), and supraphysiological (~ 102 pg/ml) levels of serum estradiol, respectively.

Finally, the 2 µg cyclic one-day replacement paradigm introduced above has not been studied in cognition thus far. However, the authors who originally introduced this more physiologically realistic replacement paradigm and compared it to other doses and regimens of estradiol replacement, have reported some striking effects on a protein (cholecystokinin) relevant for reproductive behaviors (Micevych et al., 1996). Cholecystokinin (CCK) is an estrogen-regulated neuropeptide in the limbic-hypothalamic system and is associated with the expression of estrogen-induced lordosis behavior in cycling as well as in ovariectomized rats (for detailed review, see Micevych & Ulibarri, 1992). The relevant finding for the comparison of estradiol replacement paradigms is that the one-day 2 µg injection of estradiol benzoate induced expression of CKK in the amygdala as well as in the hypothalamus. Interestingly, it does so even more effectively than either a chronic estradiol replacement paradigm producing supraphysiologic estradiol levels, or an acute very high dose injection (50 µg). Hence, this study suggests that this physiologically more realistic estradiol replacement paradigm is not only behaviorally and neurologically effective, but that it might even have a more powerful impact on brain function than much higher doses of estradiol either chronic or acute. To our knowledge, there is only one study looking at estradiol replacement and cognition employing a similar

replacement paradigm. In this study, sc. injections of 4 µg of 17*ß*-estradiol were administered every fourth day and its effects on performance in a water maze reference memory task were assessed (El-Bakri et al., 2004). The authors reported an improvement in performance for the estradiol replacement group, which is somewhat surprising given that estradiol has most commonly been shown to impair performance in the water maze reference memory paradigm (for review, see Dohanich, 2002). Given the above mentioned effectiveness of the low dose cyclic replacement regimen in reproduction-associated neural circuits, it is tempting to speculate that a paradigm more closely resembling estradiol levels in the intact cycling animal might yield beneficial effects on learning and memory performance.

Aims and Hypothesis of the Current Investigation

The radial-arm maze has repeatedly proven to be a sensitive behavioral tool for assessing the impact of estradiol on working memory, and it yields reliable results over different paradigms of estradiol replacement, as discussed above (Daniel et al., 1997; Luine et al., 1998; Daniel et al., 2001). The aim of the current study was to investigate the impact of an estradiol replacement regimen that resembles the physiological estradiol levels of intact, cycling rats on working memory performance in the radial-arm maze. This cyclic one-day paradigm (one sc. injection of 2 µg estradiol benzoate every four days) was compared to a chronic replacement paradigm (Silastic capsule implant) which has a well-documented enhancing effect on acquisition and working memory on the radial-arm maze task (Daniel et al., 1997; Luine et al., 1998). Additionally, we also included a group of animals that received a very commonly used cyclic replacement paradigm that produces supraphysiological estradiol peaks (two injections of 10 µg of estradiol benzoate 24 hours apart, given on two out of four days). This paradigm is known to produce pronounced effects on hippocampal plasticity and has also been shown to

increase working memory performance in the radial-arm maze (Woolley, 1998; Daniel et al., 2001). Even though this cyclic paradigm has been shown to enhance working memory in the water escape task if given daily over the course of 10 consecutive days (Sandstrom et al., 2004), it has to our knowledge not been extended as a two-out-of-four-days injection paradigm over a longer time course. The present study, for the first time, tested the effectiveness of this "classic" replacement paradigm over an extended time period. Yet, the main hypothesis of the current work was that the cyclic, low dose estradiol replacement paradigm can induce working memory enhancements in the radial-arm maze.

Method

Subjects

Forty female Long-Evans hooded rats, approximately 4 months of age (adult female rats), were purchased from Harlan Sprague–Dawley (Indianapolis, IN). Rats were housed individually in a temperature-controlled vivarium under a 12-h light/dark cycle (lights on at 0700 h). One week after arrival all rats were ovariectomized while under anesthesia induced by injection of ketamine (100 mg/kg ip, Bristol Laboratories, Syracuse, NY) and xylazine (7 mg/kg ip, Miles Laboratories, Shawnee, KS). Rats were randomly assigned to one of the following four groups:

1) Blank implant + 2 µg of estradiol benzoate injections once every four days (n = 10; Cyclic E – 2µg x 1)
2) Blank implant + 10 µg of estradiol benzoate injections twice every four days (n = 10; Cyclic E – 10µg x 2)
3) Estradiol implant (containing 25% 17β-estradiol in cholesterol) + Blank injections (n = 10; Continuous E)
4) Blank implant + Blank injections (n = 10; OVX Control)

At the time of the ovariectomies, 5-mm Silastic capsules containing either 25% 17β-estradiol (Sigma Chemical, n = 10) diluted with cholesterol, or blank capsules (n = 30) were implanted subcutaneously on the dorsal aspect of the neck. Capsules of these dimensions and estradiol concentration maintain circulating estradiol levels in the low- to mid-physiological range (30 – 40 pg/ml; unpublished observations from our lab). In addition to the implants, all animals started receiving subcutaneous injections according to the group assignment at the time of surgery. For the estradiol injections, 2 µg and 10 µg of estradiol benzoate were diluted in 0.1 ml of cottonseed oil for the Cyclic E – 2µg x 1 and the Cyclic E – 10µg x 2 groups, respectively, whereas rats receiving blank injections were only pinched with an empty syringe. Vehicle injections of cottonseed oil alone were not given, because of potential interference of these injections with the Silastic capsules of the chronic treatment group. Rats in the Cyclic E – 2µg x 1 group received a 2 µg injection of estradiol benzoate on day one and a blank injection on day two; rats in the Cyclic E – 10µg x 2 group received 10 µg injections of estradiol benzoate on days one and two; animals in the Chronic E group and OVX Control group received blank injections on days one and two. None of the animals received injections on days three and four. All injections were given between 08:45 h and 09:15 h, approximately 3 - 6 hours before behavioral testing, when serum estradiol levels are known to reach their peak (Micevych et al., 1996; Asarian et al., 2002).

Behavioral Testing

Radial-Arm Maze Acquisition. One week after surgeries, animals were food restricted to maintain body weights at 90% of their free-feeding weights. Additionally, they received several Kellogg's Froot Loops which were later used as food rewards in the maze testing. After surgeries, when the animals had reached their target weight, radial maze training began for all

animals. The day before training, each rat was placed in the maze for a 15-minute acquisition period with Froot Loops sprinkled throughout the maze.

The maze was purchased from Lafayette Instruments (Lafayette, IN) and consisted of black metal floors and clear Plexiglas walls. The eight arms (10 cm wide x 70 cm long x 20 cm high) extended out from an octagonal center (33 cm across) and were separated by guillotine doors. The maze was located in the center of a 3 by 5 meter room. Fixed extramaze cues including lighting fixtures, a door and electrical outlets as well as large geometric shapes attached to walls were visible from the maze. To begin each trial, the rat was placed in the center compartment with all doors leading to the arms opened. Doors remained open throughout the trial. The rat was allowed to enter any of the eight arms. The experimenter, who was seated in the room at a fixed location approximately one meter from the maze, recorded arm choices. An arm choice was scored if the rat traveled halfway down the length of an arm. The animal was allowed to choose arms in any order until all arms had been visited or until 5 minutes had elapsed. A working memory error was scored if a rat reentered an arm previously visited. Arm-choice accuracy was measured by the number of correct arm choices before the first error. Maze training took place every day over the course of the experiment. Each animal received one trial per day across 24 days of acquisition.

Delay Trials. Because it has been shown that estradiol is effective in enhancing memory performance as working memory load increases (Bimonte et al., 1999), delay trials were conducted following the 24-day acquisition period. During these trials, delays at varying lengths were imposed between the fourth and fifth arm choices. Consequently, the animal had to remember – over an extended period of time - which arms had already been visited, which increases the demand placed on working memory. In order to prepare animals for the

introduction of such delays, rats underwent the same daily behavioral training as during acquisition for another treatment cycle (4 days), but after each fourth arm choice the animal was removed from the maze and put in a cage in a holding room for a delay time of one minute. Then the animal was returned to the maze until the four remaining, still baited arms had been visited or until 5 minutes had elapsed.

After this pre-training, another cycle (4 days) of one-minute delays was added to assure that animals had reached asymptote performance. Longer delays were then introduced. For 8 days (two consecutive cycles), the animals were tested on one daily trial with either a 10-minute or 3-hour delay interval in a counterbalanced fashion. As described for the training phase, the animal was removed from the maze after the fourth correct arm choice and placed in a holding cage in a different room for a period of 10 minutes or 3 hours. It was then returned to the center compartment of the maze with all arms open and allowed to choose arms in any order until all arms have been visited or until 5 minutes have elapsed. Finally, to further increase the memory load, one cycle (4 days) of a 5-hour delay period was added. As during acquisition, arm choice accuracy was measured by the number of correct arm choices before the first error. Additionally, errors made during the pre-delay and post-delay periods were scored separately. Post-delay errors were broken down into retroactive errors and proactive errors (Daniel et al., 2006). A retroactive error is the first reentry into an arm already visited prior to the delay. A proactive error is any reentry into an arm already visited in the post-delay period.

Blood Sampling and Hormone Assay

After behavioral testing had been completed, one last injection cycle (4 days) was carried out. To assess blood levels over the course of this last injection cycle, blood samples of at least two animals of each group were collected between 12:00 h and 15:00 h on each of these four

days. While under anesthesia induced by ketamine and xylazine (ip), jugular blood was collected and animals were sacrificed by decapitation.

Blood was collected from all animals in heparin-coated microtubes (Becton Dickinson and Company, New Jersey). The collected blood was immediately centrifuged and the plasma transferred into Eppendorf microtubes and stored for the hormone assay at -80˚C. Hormone assay was carried out using a commercially available ELISA kit (ultrasensitive estradiol, DRG Laboratories, Germany), in close adherence to the manufacturer's recommendations.

As additional measures of treatment efficacy, uteri were removed and weighed following sacrifice. Also, proper removal of the ovaries and intactness of Silastic capsules was assessed.

Statistical Analyses

Data collected from the 24-day acquisition trials were grouped into six four-day blocks and analyzed by a two-way ANOVA (treatment x four-day block) with repeated measures on block. Additionally, to assess effects of varying estradiol levels on performance in the cyclic replacement groups, separate repeated measures one-way ANOVAs, with cycle day as factor, were conducted for each cyclic group.

Data from the different delay trial intervals were analyzed with separate one-way ANOVAs, with group as factor. In addition, to assess the effects of varying estradiol levels in the cyclic replacement groups, separate one-way ANOVAs (cycle day) were conducted for each cyclic group.

A one-way ANOVA, with treatment as the factor, was used to test for differences in the mean uterine weight across the 4-day cycle. Separate one-way ANOVAs, with cycle day as factor, were used to test for differences in the uterine weight within each of the four treatment groups. Separate one-way ANOVAs, with cycle day as factor, were used to analyze fluctuations

in plasma estradiol levels for each cyclic estradiol injection group. If a significant main effect was revealed in any of these analyses ($p < 0.05$), then Duncan's multiple range post hoc test ($p < 0.05$) was applied. Further, partial eta-squared (η^2) was calculated to estimate the effect size for significant treatment effects.

Results

One rat from the Continuous E group was excluded from the experiment because it did not eat the food rewards until day 15 of acquisition training and spent most of the time in the center compartment of the radial-arm maze. Another rat from the Continuous E group was not included in the statistical analyses, because of unusually high plasma estradiol levels after sacrifice, indicating damage to the implanted Silastic capsule.

Acquisition Training

As illustrated in Figure 1, estradiol replacement had no effect on working memory performance as measured by number of correct arm choices before the first error during 24 days of acquisition in a radial-arm maze. There was a significant main effect of block ($F_{5,33} = 39.63$; $p < 0.001$, see Figure 1) indicating that the arm choice accuracy of all groups improved over time. There was no significant main effect for group and no interactive effect of treatment and block. Analyses of cycle day revealed no significant main effect of cycle day on number of correct arm choices before the first error within either the Cyclic E – 2µg x 1 group or the Cyclic E – 10µg x 2 group.

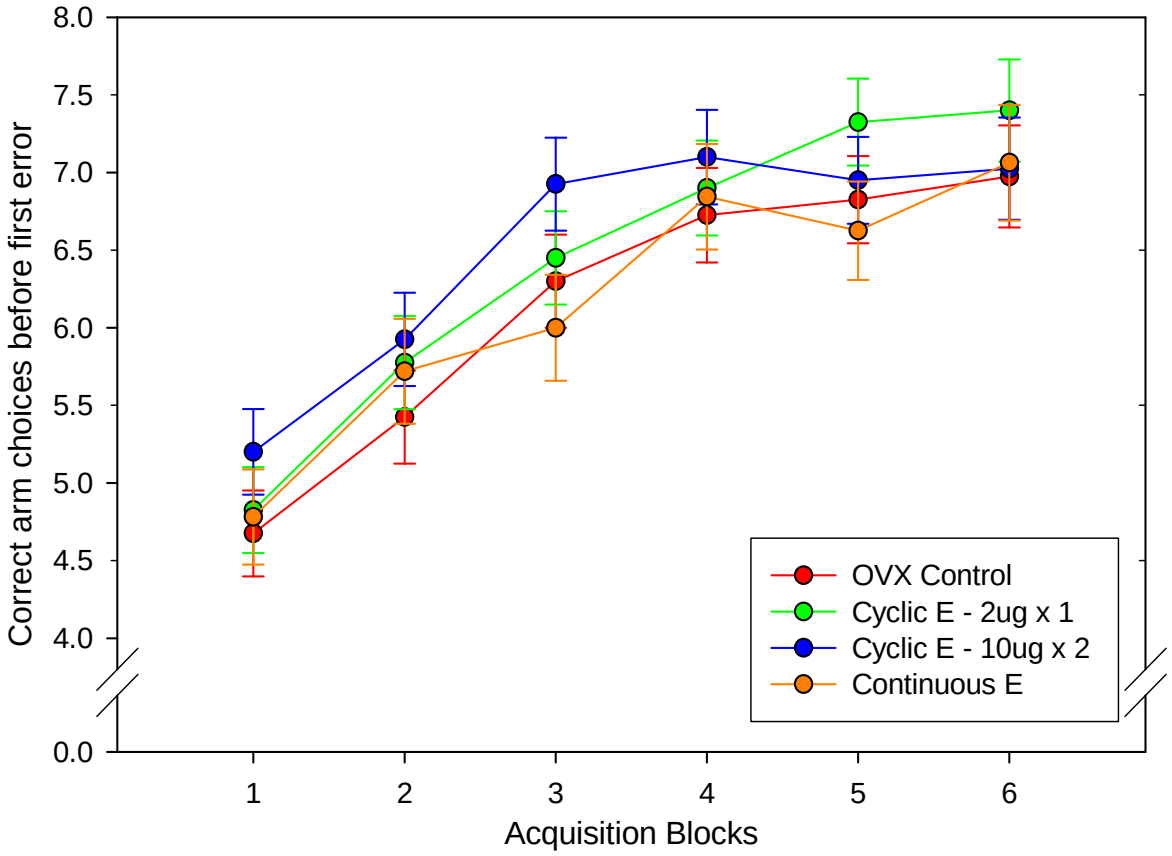

Fig. 1. Number of correct arm choices over 24 days of acquisition. There is no effect of treatment on the number of correct arm choices before the first error over the six four-day blocks of acquisition training.

Delay Trials

The first four days of delay testing with one minute delays between the fourth and fifth arm choices served as training trials and were not included in the analysis. The following section reports the data for one four-day cycle of each delay length, 1 minute, 10 minutes, 3 hours and 5 hours. The number of correct arm choices before the first error and the number of retroactive and proactive errors are presented.

Correct before the first error. Separate one-way ANOVAs on the different delay times revealed no significant group effect on number of correct arm choices on the 1-minute, 10-minute and 3-hours delays (see Figure 2). There was a significant effect of group on the 5-hour delay interval, when the memory load was highest ($F_{3,35} = 3.36$, $p < 0.05$, see Figure 2). Post hoc

analysis revealed that the Continuous E group made more correct arm choices before the first error than all other groups. The effect size (partial η^2) for the impact of estrogen treatment on performance in the 5-hour delay trials was found to be $\eta^2 = 0.229$, which is in the range of a large effect according to Tabachnick and Fidell (2006).

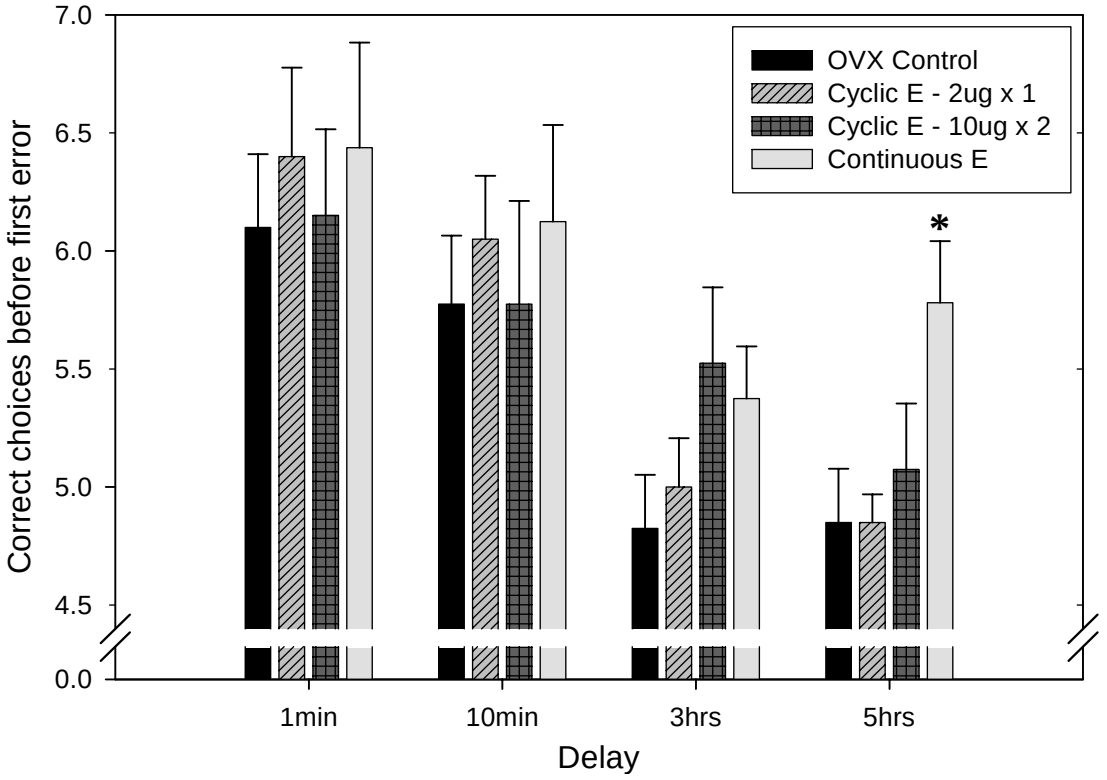

Fig. 2. Number of correct arm choices before the first error during all delay trials. *Asterisks* indicates a significant difference between all other groups at the 5-hour delay ($p < 0.05$).

Analyses of cycle day revealed no significant main effect of cycle day across all delay trials on number of correct arm choices before the first error within either the Cyclic E – 2µg x 1 group or the Cyclic E – 10µg x 2 group.

Retroactive errors. There was no significant effect of estradiol replacement on the number of retroactive errors on any of the delay intervals (see Figure 3). There was no significant effect for proactive errors on any delay interval for the different treatment groups (data not shown).

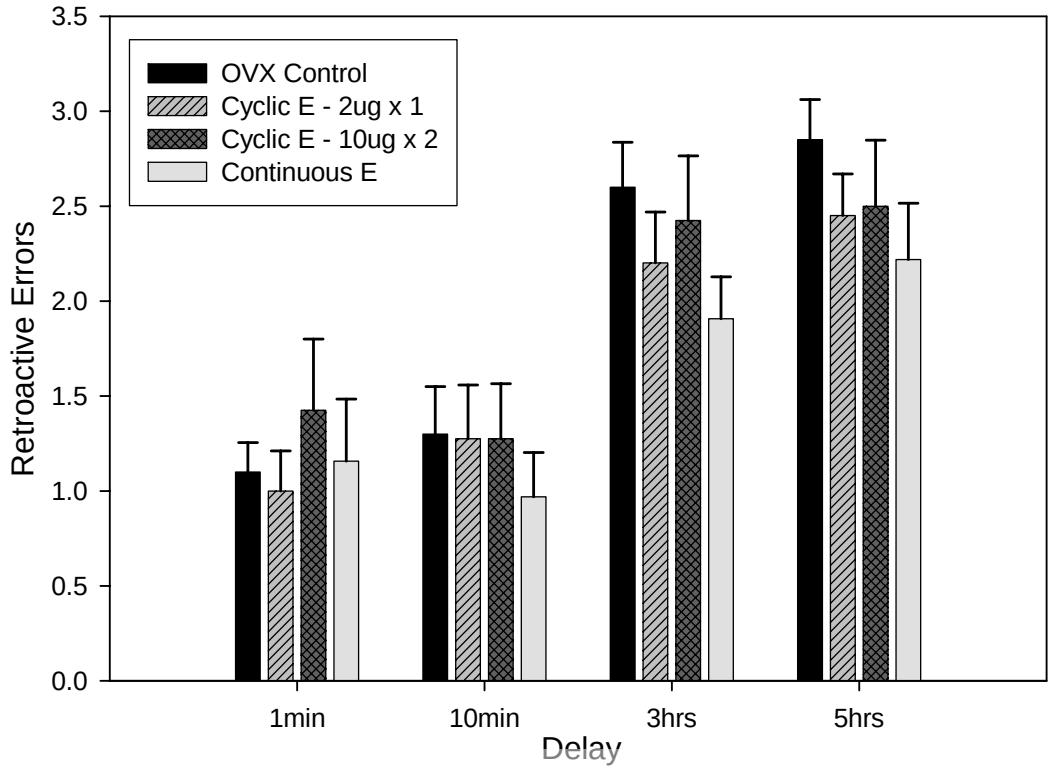

Fig. 3. Number of retroactive errors across four blocks of increasing delay lengths. There is no significant difference between groups across the different delay lengths.

Analysis of cycle day across all delays for retroactive errors revealed a significant main effect of cycle day for the Cyclic E – 2µg x 1 group ($F_{3,6} = 3.34$, $P < 0.05$, partial $\eta^2 = 0.271$, see Figure 4). Post hoc analyses revealed significantly fewer errors on Day 2 of the four-day cycle than on Day 3. There was no effect of cycle day in the other groups (data not shown).

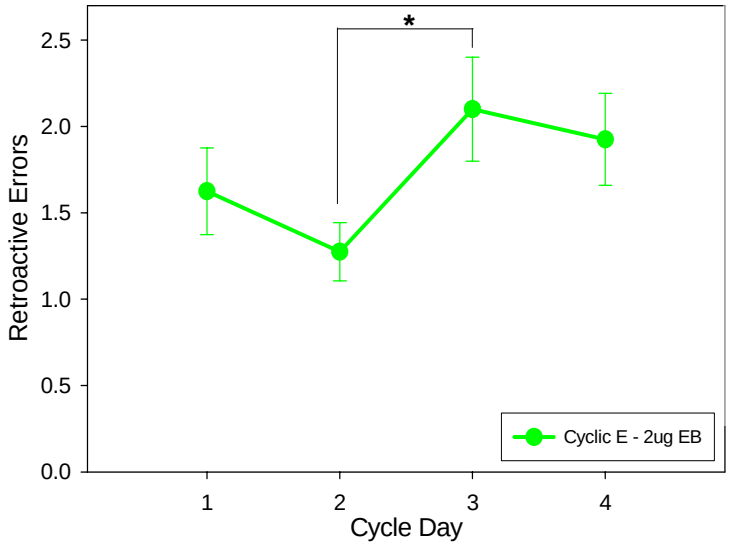

Fig. 4. Mean number of retroactive errors in the Cyclic E – 2ug x 1 group over all delay lengths on the four days of the injection cycle. *Asterisk* indicates a significant difference as result of Bonferroni post hoc test ($_P <$ 0.05)

Efficacy of Hormone Treatment

There was a main effect of hormone treatment on uterine weight ($F_{3,35} = 15.95$, $p < 0.001$, see Figure 5A). Post hoc analyses revealed that the uteri of animals in the control group weighed significantly less than those of all other replacement paradigm groups. Further, the uteri of the Cyclic E – 2µg x 1 group weighed significantly less than those of the Cyclic E – 10µg x 2 group and the Continuous E group. There was no significant uterine weight difference between the Cyclic E – 10µg x 2 group and the Continuous E group. Further, an analysis of uterine weight over the four days of the injection cycle revealed significant fluctuations of the uterine weight in the Cyclic E – 2µg x 1 group ($F_{3,6} = 4.928$, $p < 0.05$, see Figure 5B). No effects of cycle day were evident in any other group (data not shown).

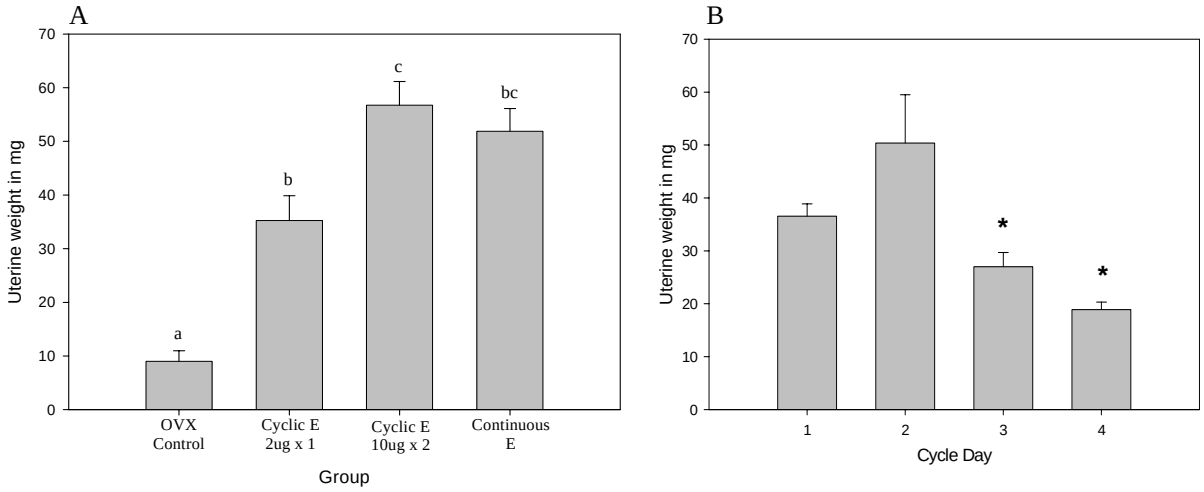

Fig. 5. Effect of estrogen replacement on uterine weight. (A) Mean uterine weight across days for each treatment group. Different letters represent significant differences between groups ($p < 0.05$) (B) Uterine weight for the Cyclic E – 2µg x 1 group across the four-day injection cycle. *Asterisks* indicate a significant difference from Cycle Day 2 as result of *Duncan's post hoc* test ($p < 0.05$).

Plasma Estradiol Levels

Blood from two animals was excluded from the analyses. One animal from the Continuous E group (sacrificed on Day 2) was excluded because its estradiol levels were unusually high (~ 140 pg/ml), indicating damage to the implanted Silastic capsule. Another

animal from the Cyclic E – 2ug x 1 group on day one was excluded because blood estradiol levels were too low (~ 8 pg/ml) three hours after injection, indicating a bad injection. Estradiol levels are summarized in Table 1. Data indicate successful hormone depletion of animals in the ovariectomized control group and steady mid-physiological levels of estradiol in the Continuous E group (~ 40 pg/ml) where estradiol was delivered continuously via Silastic capsules. The Cyclic E – 10µg x 2 group reached supra-physiological estradiol levels on days one, two, and three and data are comparable to those reported previously by Woolley and McEwen (1993). The Cyclic E – 2µg x 1 injection paradigm is shown here to produce low physiological levels when measured three hours after injection (~ 25 pg/ml), which is somewhat lower than previously reported by Asarian and Geary (2002), but still within the physiological range. Further, previous studies using the same paradigm have shown peak levels of estradiol at six hours after injection. The three hour time point was most likely too early to detect the peak levels following the 2 µg estradiol benzoate injection (Asarian et al., 2002). Cycle day analyses indicated significant fluctuations of estradiol across the four-day injection cycle for the Cyclic E – 2µg x 1 group ($F_{3,5}$ = 14.382, $p < 0.05$) and the Cyclic E – 10µg x 2 group ($F_{3,6}$ = 7.35, $p < 0.05$, see Table 1).

Table 1. Mean plasma estradiol levels in pg/ml per group over the four-day injection cycle.

Cycle Day	**Cyclic E – 2ug x 1** Mean ± SEM	**Cyclic E – 10ug x 2** Mean ± SEM	**Continuous E** Mean ± SEM	**OVX control** Mean ± SEM
1	25.51 ± 2.98[c]	176.56 ± 23.45[c]	41.52 ± 6.11	4.32 ± 1.50
2	14.96 ± 2.73[b]	144.51 ± 11.54[bc]	26.45 ± - [#]	5.75 ± 2.98
3	9.57 ± 0.28 [ab]	91.57 ± 28.04[ab]	44.08 ± 2.24	4.60 ± 2.40
4	2.99 ± 0.63[a]	35.57 ± 3.73[a]	41.44 ± 6.18	8.43 ± 1.31

Note. Different letters represent significant differences between days within the respective group ($p < 0.05$)
[#]only one animal was sacrificed at this time point

Discussion

The primary finding of the present work is that continuous but not cyclic estradiol replacement enhances working memory performance in ovariectomized adult female rats if the working memory load is sufficiently increased in a radial-arm maze memory task. Specifically, only during the longest delay period of 5 hours between the fourth and fifth arm choices, the continuous estradiol replacement group outperformed the ovariectomized control group and both cyclic replacement groups. No significant differences in working memory performance were observed between groups neither during acquisition training nor at the 1-minute, the 10-minute, and the 3-hours delay trials.

These results suggest that long-term continuous delivery of estradiol replacement at physiological levels (~ 40 pg/ml) is more effective in enhancing radial-maze performance than are the cyclic regimens tested in the current experiment. These findings are in line with earlier reports that already established the effectiveness of continuous estradiol replacement to increase working memory performance relative to ovariectomized control animals (Luine et al., 1998; Bimonte et al., 1999; for review, see Dohanich, 2002). However, two of the findings reported in the current work were unexpected, first the absence of an effect of any form of estradiol replacement as compared to ovariectomized controls during the acquisition period and during the short delay trials, and secondly, the ineffectiveness of the cyclic replacement paradigms as compared to the continuous replacement paradigm in the 5-hour delay trials.

The absence of enhanced performance during the acquisition period in the current study is in contrast to earlier reports from our and other laboratories that found enhanced performance during acquisition of the same maze task in animals treated with continuous estradiol replacement as compared to ovariectomized controls (Daniel et al., 1997; Luine et al., 1998;

Fader et al., 1999; Daniel et al., 2006). The only obvious methodological difference between these studies and our current work is the increased handling of the animals due to the injection paradigms. All rats were weighed every day to maintain their body weight at 90% of pre-ovariectomy free-feeding weight, and they were injected every two out of four days for the duration of the experiment. There is evidence to support the hypothesis that experience can interact with estrogen status to affect radial-arm maze performance. For example, we demonstrated in an earlier experiment that estrogen replacement enhances working memory performance in animals reared in isolated environments, not this effect of estrogen was not detected in animals reared in complex environments (Daniel, Roberts, & Dohanich, 1999). In this experiment all ovariectomized animals in the isolated environment performed well below our ovariectomized control animals in the current study. However, our control animals performed similarly as the animals in the complex environment condition (Daniel et al., 1999), although our rats were reared single caged in non-complex environments. Hence, increased handling might have had similar effects on these animals as the complex environment condition.

While it is well accepted that post-natal handling in rats has positive effects on memory performance throughout life, putatively via modulation of the stress response (Meaney, Aitken, vanBerkel, Bhatnagar, & Sapolsky, 1988), little is known about the effect of handling in adult rats. However, a reduced stress response in adults as a result of handling could have contributed to our results. Ovariectomized females receiving estradiol replacement respond to chronic stress with an enhanced working memory performance on the radial-arm maze, as compared to ovariectomized controls (Bowman, Ferguson, & Luine, 2002). In males, stress generally has impairing effects on memory performance, while females seem to be somewhat protected from this stress-associated impairment or even show enhanced performance on memory tasks in

response to chronic stress (for reviews, see Luine, 2002; Bowman, Beck, & Luine, 2003). The extensive handling might have rendered our animals less stressed when faced with the memory task as compared to other studies in which animals were not handled as much (Luine et al., 1994; Daniel et al., 1997; Luine et al., 1998; Fader et al., 1999; Daniel et al., 2006). Consequently, it is possible that the enhancing effects of estradiol on hippocampus-sensitive working memory performance repeatedly reported by different labs might be partially based on an interaction between stress and estradiol treatment. An absence of the stress response might have eliminated the effect of estradiol treatment during the acquisition period in the current experiment and only a sufficient increase in working memory load was able to tease out the differences between estradiol replacement and control animals. There has been another recently published study that also failed to detect estradiol induced enhancement in the radial-arm maze (Ziegler et al., 2005). These authors also used a cyclic injection paradigm, with two injections of estradiol given every two out of six days. Further, these authors used a pre-training protocol, and moreover the rats were used in a different behavioral experiment previous to radial-arm maze testing. Altogether, this exposed the animals to a large amount of handling and human contact, possibly reducing the stress response during memory testing. Nevertheless, a potentially modulating effect of handling on an estradiol-induced working memory enhancement at this point is only speculative and requires further investigation.

The ineffectiveness of the two cyclic replacement paradigms used was unexpected. Previously, short-term treatment with the Cyclic E – 10µg x 2 injection paradigm (72 and 48 hours before testing) has been shown to enhance working memory performance compared to ovariectomized control animals on the radial-arm maze with a 3-hour delay between fourth and fifth arm choices (Daniel et al., 2001), and on a delayed matching-to-place water escape task

(Sandstrom et al., 2001). Further, 10 µg injections of estradiol benzoate given daily over 10 consecutive days has also been reported to enhance performance of ovariectomized rats on a matching-to-place water escape task (Sandstrom et al., 2004). However, another study reports that over longer periods of daily 10 µg estradiol benzoate injections (> 30 days), no enhancement of working memory performance in the radial-arm maze was found (Galea et al., 2001). This is comparable to the findings reported in the current study where the cyclic 10 µg estradiol benzoate treatment was carried over almost two months of training, without significant effects on working memory performance. As shown in Table 1, the Cyclic E – 10µg x 2 injection paradigm produced supraphysiological levels on three out of the four cycle days, consequently leaving the animals almost constantly with supraphysiologically elevated estradiol levels. Therefore, our findings suggest that long-term supraphysiolgical levels of estradiol in ovariectomized animals do not enhance working memory performance, although short-term treatment with the same dose is effective behaviorally and physiologically (e.g. Woolley et al., 1994; Daniel et al., 2001)

The original hypothesis that physiological doses of cyclic estradiol injections would prove effective in enhancing working memory performance was not supported in the current experiment. During the five-hour delay trials only the continuous estradiol replacement paradigm significantly improved working memory performance compared to all other groups. The Cyclic E – 2µg x 1 injection paradigm has, to our knowledge, not been investigated in memory studies, although it has been shown to mimic estradiol levels over the four day estrous cycle in intact rats (Micevych et al., 1996; Asarian et al., 2002) and impact feeding behavior. Our blood analysis indicated somewhat lower levels than reported by these authors, but the estradiol levels reported here can be considered physiological and cyclic. Additionally, the uteri of the ovariectomized

animals treated with the Cyclic E – 2µg x 1 paradigm showed significant fluctuations across days, with the mean uterine weight being highest on Day 2 of the injection paradigm (~ 27 hours after the 2 µg estradiol benzoate injection, see Figure 5B). This indicates the physiological relevance of this low-dose cyclic injection paradigm. Interestingly, the number of retroactive errors averaged over all delay trials was shown to significantly fluctuate over the four-day cycle, with performance being better on Day 2 after injection as compared to performance on Day 3, which coincides with the increased uterine weight on Day 2. No effect of cycle day was revealed for any of the other treatment groups. However, the number of correct arm choices before the first error over all delay trials showed no significant fluctuations over the injection cycle.

In conclusion, the main finding of the current study is that chronic estradiol replacement enhances working memory performance in the radial-arm maze if the working memory load is sufficiently increased. The studied cyclic physiological and supraphysiological replacement paradigms do not enhance performance as compared to ovariectomized control animals. Further, we suggest a potential interaction between handling and estradiol replacement on working memory performance, especially during acquisition and during delay trials shorter than five hours. These results have possible implications with regard to hormone replacement therapy in postmenopausal women. Our data indicate that the regimen of estrogen administration may affect its efficacy. Further, the data suggest that continuous estradiol replacement such as provided by skin patches might prove more beneficial for maintaining cognitive function than cyclic treatments of physiological doses of estradiol.

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Appendix

Animal Subjects Approval Form

University of New Orleans

Institutional Animal Care and Use Committee (IACUC)

DATE: August 9, 2004

TO: Jill Daniel, Ph.D.
Assistant Professor

FROM: Gerald J. LaHoste, Ph.D.
Chairman

RE: *IACUC Protocol No.* **067**
Entitled: **Mechanism of Estrogen Action in the Hippocampus**

Your application for the use of animals in research (referenced above) has been approved for a three-year period beginning August 9, 2004 and expiring August 9, 2007.

Vita

Johannes Bohacek was born in Graz, Austria. He graduated from Bischoefliches Gymnasium (high school) in 2000. After 8 months of mandatory military service, he started the studies of Psychology at the Karl-Franzens Universitaet in Graz, Austria. He was awarded the 1st Diploma in Psychology in 2003. The academic year 2003 – 2004 he spent as an exchange student at the University of Arkansas at Little Rock, before being accepted to the Applied Biopsychology Ph.D. program at the University of New Orleans in 2004.