EFFECTS OF CHRONIC ESTRADIOL TREATMENT ON ACQUISITION AND REACQUISITION OF WORKING MEMORY AND CELL PROLIFERATION IN THE DENTATE GYRUS

by

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ABSTRACT

Beyond its role in reproduction, estrogen exerts profound effects on cognition and behaviour as well as influencing the structural and electrophysiological properties of the brain. The present studies investigate the effect of chronic estradiol treatment on acquisition and reacquisition of the prefrontal cortex-dependent delayed non-matched to position T-maze task and on cell proliferation in the dentate gyrus of adult female rats.

Experiment 1 investigates the effect of estradiol on non-spatial working memory during the T-maze task. Ovariectomized (OVX) female rats were injected for 21d with estradiol benzoate (0.3, 5 or 10 μg/0.1 ml sesame oil) or vehicle (sesame oil, 0.1 ml). Approximately 2hr after each injection, animals were trained daily on the T-maze with an initial delay of 10s. Following a month with no estradiol treatment animals were injected for 21d with the same initial doses of estradiol and re-trained (reacquisition) at a 40s delay. Days to reach criterion (one error per day for three consecutive days), mean total errors (entries into previously baited arms), errors across blocks (3d per block), change in performance across training (acquisition subtracted from reacquisition), and latency to reach goal arm, were scored. Compared to OVX rats without estrogen administration, a dose of 0.3 μg of estradiol (low-to-medium physiological) significantly decreased the number of working memory errors during a 10s delay (acquisition), while doses of both 5 μg (high physiological) and 10 μg (supraphysiological) of estradiol significantly increased the number of working memory errors during a 40s delay (reacquisition).

Experiment 2 investigated the effect of chronic estradiol treatment on the proliferation of cells in the dentate gyrus of adult female rats. Ovariectomized (OVX) female rats were injected for 21d with estradiol benzoate (0.3, 5 or 10 μg/0.1 ml) or
vehicle (sesame oil; 0.1 ml). Four hours after the last hormone injection on day 21 animals were injected i.p. with bromodeoxyuridine (BrdU; 200mg/kg), a thymidine analogue and were perfused 24h later. Stereological counts of BrdU-labelled cells and dentate gyrus volume were correlated with serum estradiol levels. Results revealed that regardless of dose, estradiol treatment produced no significant change in the number of BrdU-labelled cells observed relative to vehicle-controls. Similarly, estradiol treatment produced no significant change in the number of pyknotic cells observed across treatment conditions relative to vehicle controls. However, chronic treatment with supraphysiological levels of estradiol (10μg) significantly increased dentate gyrus volume. There was no significant correlation between serum estradiol levels and number of BrdU-labelled cells, however DG volume correlated positively with serum estradiol dose.

These data demonstrate that chronic estradiol has a significant differential effect on acquisition and reacquisition of prefrontal cortex dependent working memory. While chronic estradiol did not significantly affect the number of BrdU-labelled cells, serum estradiol was positively correlated with DG volume.
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Effects of chronic estradiol treatment on acquisition and reacquisition of working memory and cell proliferation in the dentate gyrus

Researchers investigating the effects of estradiol on cognition have revealed discrepant effects on learning and memory processes (Berry, McMahun & Gallagher, 1997; Frye, 1995; Galea, Wide, Paine, Holmes, Ormerod &Floresco, 2001; Luine, Richards, Wu & Beck, 1998; Warren & Juraska, 1997; Wilson, Puolivali, Heikkenen & Riekkinen, 1999). These discrepancies may be related to the circulating levels of estradiol because there are large variations in doses of exogenous estradiol that have been used across studies (Luine & Rodriguez, 1994; Daniel, Fader, Spencer & Dohanich, 1997) and, in rodent studies using endogenous levels of estradiol, there are rapid dramatic changes in estradiol levels across the rodent estrous cycle (Butcher, Collins & Fugo, 1974). Additionally, estradiol differentially affects multiple memory systems that rely on the integrity of different brain regions (Galea et al., 2001). Two widely studied memory subtypes can be classified as working and reference memory. Working memory can be defined as the storage and manipulation of trial unique information necessary to guide prospective actions (Baddeley, 1986). Working memory function has been associated with the prefrontal cortex (PFC) and hippocampus (Goldman-Rakic, 1995; Floresco, Seamans & Phillips, 1997). Reference memory can be defined as trial independent long-term stable memory (Olton & Papas, 1979). Reference memory function has been associated with the caudate and hippocampus (Packard & White, 1990; Olton & Papas, 1979).
Estradiol affects working and reference memory processes (Galea et al., 2001; Holmes, Wide & Galea, 2002). Previous investigations using the spatial radial arm maze suggest that estradiol exerts differential dose-dependent effects on spatial working memory processes (Fader, Johnson & Dohanich, 1999; Galea et al., 2001; Holmes et al., in press; Luine et al., 1998). Low physiological levels of estradiol (15-30 pg/ml serum estradiol) reduce working memory errors, whereas high physiological levels of estradiol (40-90 pg/ml serum estradiol) increase the number of working memory errors relative to ovariectomized control rats (Galea et al., in press). A supraphysiological dose of estradiol (225 pg/ml of serum estradiol) does not significantly affect working memory performance on either the spatial radial arm maze or the spatial delayed win-shift task (Galea et al., 2001). This same supraphysiological dose however, does increase the number of reference memory errors in the spatial working/reference memory version of the radial arm maze, in which performance relies on the hippocampus (Galea et al., 2001). These findings suggest that there is a complex relationship between estradiol and working and reference memory: low levels facilitate and high levels impede working memory while supraphysiological levels impede reference memory for spatial tasks, in which performance relies in part on the hippocampus and the PFC.

Working memory is thought to rely, in part, on the PFC (Fuster, 1997; Goldman-Rakic, 1995). Lesions to the PFC cause impairments in performance on working memory tasks, such as delayed reaction tasks in both non-human primates and rats (de Brabander, Bruin, & Eden, 1991; Fuster, 1997; Granon et al., 1994; Izaki, Maruki, Hori & Nomura, 2001). Neuroimaging techniques such as functional magnetic resonance imaging (fMRI;
Cohen, et al 1997, Courtney Ungerleider, Keil, & Haxby, 1997), positron emission tomography (PET; Awh, et al., 1995; Schumaker et al., 1996), and single cell recordings (Fuster & Alexander, 1972; Funahashi, Inoue, & Kubota, 1994) further reveal activation of the PFC in working memory tasks across species. The PFC may be a site of action for estradiol's effects on working memory because there are a number of estrogen receptors in the PFC in both the human (Bixo, Backstrom, Winblad, & Anderson, 1995) and the rat (Handa, Hejna & Lorens, 1997; Shughrue, Lane & Merchenthaler, 1999).

Neurophysiological data reveals that estrogen replacement significantly increases regional cerebral blood flow in the human dorsolateral PFC, following performance of the Wisconsin Card Sorting task, a working memory task (Berman et al., 1995; Berman et al., 1997). Anatomically, the dorsolateral PFC of the primate has been presumed to be equivalent to the rat medial PFC, however, this is still an issue of debate (Kolb, 1984). There is additional mounting evidence that estradiol influences activation of the PFC and working memory in humans (Shaywitz et al., 1999), however, little work has been done with rodents.

**Estradiol, learning and the hippocampus**

Previous studies in rodents and primates have indicated that adult hippocampal lesions disrupt performance in spatial working memory (Olton & Pappas, 1979, Olton, 1982; Kesner, Dimattia, & Crutcher, 1987; Redish & Touretzky, 1997). A recent meta-analysis showed similar deficits in spatial working memory in humans with hippocampal damage (Kessels, de Haan, Kapelle & Postma, 2001). Spatial working memory is thought to rely on the hippocampus (for review see Kesner, Evans, & Hunt, 1987; Olton,
Becker, & Handelmann, 1980; Floresco et al., 1997) and PFC (Kessels, Postma, Wijinalda & de Haan, 2000; Floresco et al., 1997). Temporary lesions of the connections between the ventral hippocampus and the medial PFC increase working memory errors on the spatial delayed win-shift task (Floresco et al., 1997). Furthermore, PET studies in humans have shown significantly increased regional cerebral blood flow in both the PFC and the hippocampus during working memory tasks (Petrides, Alivisatos & Evans, 1995).

The hippocampus may be a sight for estrogen's effect on spatial working memory as estrogen receptors (ER), α and β, are located in the rodent hippocampus (Shughrue, Lane & Merchenthaler, 1997). Indeed, estradiol affects the structural and electrophysiological properties of the hippocampus (Becker, 1999; Desmond, Zhang & Levy, 2000; Hampson & Kimura, 1998; Ormerod & Galea, 2001; Packard, 1998; Tanapat, Hastings, Reeves & Gould, 1999; Woolley, 1999). High levels of estradiol are associated with a facilitation in long-term potentiation (LTP) as well as an enhancement in long-term depression (LTD) in the CA1 region of the hippocampus (Warren, Humpreys, Juraska & Greenough, 1995, Zamani, Desmond, Levy, 2000). Intriguingly, estradiol has very disparate effects on LTP depending on the subregion of the hippocampus. For example a high level of estradiol facilitates LTP in the CA1 region but impairs LTP in the dentate gyrus (Gupta et al., 2001). Estradiol also increases apical dendritic spine density in the CA1 and dentate gyrus region of the hippocampus but not the CA3 region (Miranda, Williams & Einstein, 1999; Woolley et al., 1990). There is additional evidence that estrogen use is associated with differential activation in the human brain. For example, women on estrogen replacement therapy (ERT) exhibit longitudinal activation of the parahippocampal gyrus and frontal gyrus during a figural
recognition task relative to control subjects (Maki & Resnick, 2000).

*Delayed Alternation T-maze*

Tasks which involve a delay are one prominent type of working memory task. The general design includes the display of a reward item, an enforced delay, followed by the presentation of a choice of objects (Fuster, 1997). The delay requires the temporal retention of information characteristic of working memory. Animals with PFC lesions show functional impairment in spatial delayed alternation tasks (Kolb & Whishaw, 1981, Nonneman & Corwin, 1981; Eichenbaum, Clegg & Feeley 1983). As the hippocampus appears to be involved preferentially in spatial working memory (Olton, 1979), we wanted to utilize a task in the present study in which performance did not rely on the integrity of the hippocampus but did rely on the PFC in order to determine whether estradiol affected working memory performance that relied primarily on the PFC. Thus because there are ERs in both the hippocampus and PFC, we wanted to determine whether there was a similar pattern in working memory performance with different doses of estradiol on a PFC-dependent task as there are in a hippocampus and PFC-dependent task. The non-spatial delayed alternation T-maze, adapted from Aultman and Moghaddam (2001) is a working memory task in which acquisition relies preferentially on the PFC and not on the hippocampus (Granon et al., 1994; Lipska et al., 2002). Therefore we used this task in the present study to assess the effect of estradiol on non-spatial PFC-dependent working memory.
Estradiol and neurogenesis

Neurogenesis exists in the hippocampus of all adult mammalian species studied, including humans (Eriksson et al., 1998; Gould et al., 1997; Ormerod & Galea, 2001). New cells are produced from dividing progenitor cells located in the subgranular zone of the hippocampal dentate gyrus. These daughter cells migrate into the granule cell layer, extend axons into the CA3 region (Stanfield & Trice, 1988), and 2-3 weeks after birth show similar morphology to granule cells residing in the granule cell layer (Cameron et al., 1993), including postsynaptic densities (Kaplan & Hinds, 1977). Protein markers of immature neurons begin to be expressed at 1 day after birth while protein markers of mature neurons begin to be expressed at 2-3 weeks after birth (Cameron et al., 1993). Moreover electrophysiological data suggest that these new neurons are functional in adulthood as they exhibit normal electrophysiological properties such as action potentials and passive membrane properties at 4 weeks but develop similar densities of ultrastructural properties as late as 5 months after birth (van Praag et al., 2002).

Bromodeoxyuridine (BrdU), a thymidine analogue, can be used to label new cells, in vivo, as this marker is incorporated into the DNA as cells divide, and labeled cells can then be visualized using immunohistochemistry (Dolbeare & Sheldon, 1994, Miller & Nowaskowski, 1988).

Neurogenesis can be influenced by changes in cell proliferation and/or cell survival. Various factors, including estrogen, influence both cell proliferation (the production of new neurons) and cell survival (the change in the number of new neurons surviving to maturity) in the dentate gyrus (Galea & McEwen, 1999; Banasr, Hery, Brezun & Daszuta, 2001; Ormerod & Galea, 2001; Tanapat et al., 1999, Gould, Tanapat,
An acute administration of a supraphysiological dose of estradiol results in a significant increase in the number of BrdU-labeled cells relative to vehicle controls (Banasr et al., 2001; Fowler, Liu, Quiment, & Wang, 2002; Ormerod & Galea, 2001; Ormerod, Lee & Galea, submitted; Tanapat et al., 1999). Previous research in our laboratory has revealed that an acute exposure to a supraphysiological estradiol significantly increases the number of BrdU-labelled cells 4h later but significantly suppresses the number of BrdU labeled cells 48 h later (Ormerod and Galea, 2001, Ormerod et al., submitted) relative to vehicle-controls. This study illustrates that estradiol mediates dynamic changes in cell proliferation. Recent research has shown that the estradiol-induced increase in cell proliferation is mediated by serotonin (Banasr et al., 2001), whereas the estradiol-induced decrease in cell proliferation is via its affects on adrenal steroids (Ormerod, Lee, & Galea, submitted). However, all of these studies investigating the effects of estradiol have utilized an acute supraphysiological level of estradiol. Given the nature of estradiol’s dose-dependent effects on LTP (Warren, Humphreys, Juraska & Greenough, 1995) and on hippocampal-dependent learning and memory (Holmes et al., 2002; Galea et al., 2001) it seems likely that there may be a dose-dependent effect of estradiol on cell proliferation.

Although the function of adult neurogenesis is not known, changes in neurogenesis have been linked to changes in hippocampal-dependent learning and memory (Gould, Beylin, Tanapat, Reeves, & Shors, 1999; Gould, Tanapat, Hastings & Shors, 1999; Ormerod et al., submitted; Shors et al., 2001; van Praag, Christie, Sejnowski, & Gage, 1999) and exposure to stress (Falconer & Galea, submitted; Gould et al., 1997, 1998; Holmes & Galea, 2002). For example: enhanced survival of new
neurons in the adult male dentate gyrus has been linked to hippocampal-dependent Morris Water Maze learning (Gould et al., 1999); elimination of new neuron production for 14d inhibits hippocampal-dependent trace eyeblink conditioning (Shors et al., 2001); and changes that enhance cell proliferation and survival such as physical activity also enhance hippocampal-dependent Morris water maze learning (Van Praag et al., 1999). As estradiol modulates neurogenesis, as well as learning and memory, we sought to determine whether different doses of chronic estradiol changed cell proliferation levels in the dentate gyrus of adult female rats. Past research has placed emphasis on the dynamic effects of acute doses of estrogen on cell proliferation. However, in most learning paradigms, the effects of estradiol on learning and memory are assessed using chronic exposure to estradiol. Thus the second objective of the current study was to examine the effects of chronic estradiol administration on cell proliferation in the dentate gyrus of female rats.

In the current studies we wanted to investigate the dose-dependent effects of chronic estradiol on: 1) performance on the delayed non-matched to position T-maze, a non-spatial working memory task in which performance preferentially relies on the PFC; and 2) cell proliferation in the dentate gyrus of adult female rats.

MATERIALS AND METHODS

Subjects

Female Long-Evans rats (Charles River, Quebec) between 225 –250g were used in the current study (n=36 for Experiment 1 and n=15 for Experiment 2). One week after their arrival, animals were anaesthetized under halothane (MTC Pharmaceuticals; 4%
flow rate for induction followed by a 2% flow rate for maintenance) and bilaterally
ovariectomized using aseptic techniques. Following surgery, animals were housed
individually in polyurethane cages and maintained on a 12:12 hr light/dark cycle
commencing at 0730 hrs, with water and food pellets (Lab Diet #5012, Jamieson,
Richmond, British Columbia) available ad libium until habituation. These animals were
fed food pellets with a total isoflavone, a phytoestrogen, level of 479 mg/kg (Jamieson,
Richmond, British Columbia). During training, rats were maintained at 90% of free
feeding body weight adjusted for growth (3g per week). Food deprivation commenced a
week following surgery at the start of habituation. All animals were cared for in
accordance to ethical guidelines set forth by the Canada Council for Animal Care and the
policies of the University of British Columbia. Every effort was made to minimize the
number of animals used per group.

Experiment 1

Apparatus

The apparatus used was a T-maze constructed from plywood. The main alley, 76
x 21 x 24 cm, was connected by two side arms, 94 x 15 x 24 cm. Each arm could be
closed off manually by a sliding door, 15 cm wide and 24 cm high. Careful consideration
was given to avoid providing the animals with any spatial cues. Thus, to minimize
hippocampal activation, we eliminated extramaze spatial cues by extending the length of
the walls of the maze, surrounding the maze with a uniform curtain, and randomizing the
maze's position daily (Aultman & Moghaddam, 2001; Hyde, Sherman & Denenburg,
2000). To further ensure spatial cues were minimized, during delays the animals were
returned to their cage and a cloth was draped over their cage to avoid visual cues.

**Design and Procedure**

**Habituation**

Habituation lasted 3d. At the start of habituation, animals were given food reward, fruit whirls (Generic Brand, Calgary AB) in their home cages. On Day 1, animals were placed on the maze in pairs for a period of 10 min. On Day 2 and 3, animals were placed on the maze individually for a period of 5 min. During each day of habituation one-quarter piece of fruit whirl was located at the end of each arm.

**Hormone preparation and replacement**

Animals were randomly assigned to one of three groups depending on their dose of estradiol or vehicle. There were three doses of estradiol given EB0.3 (0.3\(\mu\)g EB/0.1 ml sesame oil), EB5 (5\(\mu\)gEB/0.1ml sesame oil) and EB10 (10\(\mu\)gEB/0.1ml sesame oil) with a sample size of 7-8 per group. Estradiol benzoate (EB; Sigma Aldrich Chemicals) was dissolved in sesame oil (Sigma Aldrich Chemicals) over low heat. The solution was stored in a light insensitive container. Injections began on the first day of shaping, and continued daily for 21d. Throughout the training period all animals received subcutaneous injections of either estradiol benzoate (EB) or vehicle (VEH: 0.1 ml sesame oil; n= 16) approximately 2hr prior to testing. This time period was chosen to match the time period when a maximal amount of estradiol would be in the serum (Woolley & McEwen, 1993). Chronic EB5 injections produce physiological mean serum levels of estradiol of 102.2pg/ml, whereas chronic EB0.3 injections produce low physiological
levels of estradiol of 23.81 pg/ml (Holmes, et al., in press). EB5 level produced levels similar to proestrus level while EB0.3 levels produced levels similar to diestrus level (Shors, Pickett, Wood, & Paczynski, 1999; Viau & Meaney, 1991). Chronic EB10 injections produce approximately 225 pg/ml in serum estradiol, a supraphysiological level of estrogen.

**Shaping**

Shaping took place over 4d. Each animal was placed on the maze for ten alternating forced runs (for example Left, Right- L, R, L, R, L, R, L, R, L, R) per day. During each run, the animal only had access to one open arm, with the opposite arm being blocked. Once the animals retrieved the food reward it was returned to its home cage for a 20s intertrial interval (ITI). The arm previously visited was then blocked, restricting access during the next run to the alternate arm. For all runs the animals remained on the maze until its forepaws crossed the halfway point of the open arm or until 2 min elapsed. The shaping period continued until each animal freely alternated its entry into the open arms.

**Training**

Following the shaping period the animals began training, which continued for a period of 17d. Training consisted of five discrete delayed alternation trial-pairs per day. Each trial-pair consisted of a forced run followed by a choice run. An intratrial delay of either 10s or 40s separated each forced run from a choice run and an ITI (20s) separated each discrete trial-pair. During initial training (acquisition), animals were given a 10s
delay. Following a month of no hormone administration, the same animals were injected with the same doses and re-trained (reacquisition) using a 40s delay for 17 consecutive days. During the forced run, the rat had access to only one open arm, which was baited. Once the animal reached the reward it was removed from the maze, and placed in its home cage for the delay interval. Then, after a delay of 10 or 40s, the animal was returned to the maze for a choice run, where it had access to both arms, but was rewarded only if it chose the arm opposite to the previous forced run. Once it has consumed the reward, the animal was returned to its cage for a 20s ITI, after which a new trial-pair would begin. The daily sequence of forced runs was randomly selected in advance of the training. Each animal performed the same sequence of forced runs, but the sequence varied each day (for example, R, R, L, R, L).

**ACQUISITION**

(10s delay)

- 17d

**REACQUISITION**

(40s delay)

- 30d
- 17d

Daily injections of 0.3, 5 and 10 µg EB

**Figure 1.** Timeline of T-Maze Training
Experiment 2

Hormone Replacement

Animals were randomly assigned, according to dose, to one of three estradiol groups or the vehicle group. All animals received daily subcutaneous injections of either estradiol benzoate (EB) or vehicle (VEH: 0.1 ml sesame oil) for 21 consecutive days. There were three doses of estradiol given EB0.3 (0.3μg EB/0.1 ml sesame oil), EB5 (5μg EB/0.1ml sesame oil) and EB10 (10μgEB/01ml sesame oil) with a sample size of 3-4 animals per group.

Histological Procedures

Four hours following the last day of injections, day 21, animals were injected with bromodeoxyuridine (BrdU, 200 mg/kg i.p.), a marker of dividing progenitor cells and their progeny. Twenty-four hours later animals were given an injection of sodium pentobarbital (2ml/kg, i.p. Somnitol, MTC Pharmaceuticals) and transcardially perfused with 4% paraformaldehyde in 0.1 phosphate buffer (PB). As mitosis last approximately 16-24 hours in the rat, this time period allowed for one mitotic division (Cai, Hayes & Nowakowski, 1997; Cameron & McKay, 2001). Brains were extracted and post-fixed overnight in 20-30% sucrose at 4°C. The following day brains were sliced into 40μm sections through the entire hippocampus using a vibratome (Leica OTS 1000) at the level of the dentate gyrus in a bath of 0.1M PB. Sections were treated with 0.3% H2O2 for 20min, rinsed in PB, and mounted on to 3-aminopropylthriethoxysilane (3-AAS) treated slides.
**BrdU Labeling**

BrdU immunohistochemistry was performed as previously described (Ormerod & Galea, 2001). Briefly, cells were permeabilized with 0.05% Trypsin (Sigma Aldrich Chemicals) in Tris-HCl buffer (pH 7.5) containing 0.1% CaCl$_2$. DNA was denatured by applying 2N HCL for 30 minutes prior to application of 5.0% normal horse serum block (Sigma Aldrich Chemicals). Tissue was incubated with normal horse serum and left overnight in mouse monoclonal antibody against BrdU (1:400; Boeringer Mannheim) at 20°C. Biotinylated antibody anti-mouse IgG was applied (1:167, Vector Laboratories, Elite Kit) and tissue was incubated for 4hr at room temperature. Tissue was rinsed with 0.1 M PB (pH 7.4) in between steps. Tissue was reacted using an ABC reagent (Vector, elite Kit) with 0.02% 3’3’-diaminobenzidine (DAB; Sigma Aldrich Chemicals) and counterstained with cresyl violet, dehydrated with xylene and cover-slipped with Permount. Cresyl violet was used as a counter-stain in order to identify and digitize the area of the dentate gyrus.

**Stereology**

Slides were coded prior to analysis in order to blind the experimenter to the treatment conditions. Cells were counted in every 10th section throughout the extent of the dentate gyrus region to determine stereological estimates. BrdU-positive cells and pyknotic cells of the granule cell layer (GCL) of the dentate were counted using a Nikon Eclipse light microscope (under 100X oil objective). Criteria for establishing BrdU-positive cells included a dark stain and progenitor cell morphology (see Figure 2A). Criteria for establishing pyknotic cells were the absence of a nuclear membrane, pale or
absent cytoplasm and darkly stained spherical chromatin (Gould, Woolley & McEwen, 1991; see Figure 2B). Estimations of total numbers of BrdU-labeled cells throughout the GCL were made using a modified version of the optical fractioner method (West, Slomianka, & Gundersen, 1991). In the optical fractioner method a square section of the focal field is randomly mapped out and cells within this square are counted. This process is repeated multiple times in order to gain a sample population of cells. In the current study progenitor cells reside mainly in the GCL. Therefore the process is modified such that the section mapped out is the GCL. In order to estimate the total number of labeled cells in the GCL the cell count from this section is multiplied by the volume of the GCL. As the dentate is sectioned using systematic uniform random sampling one can integrate the areas to estimate the total GCL volume using Cavalieri's principles (Gunderson et al., 1988). First, the GCL area was measured using Analytical Imaging Station Software (AIS; Imaging Research Incorporated, Ontario, Canada). The volume is then estimated by multiplying the area of the 2-D section of the GCL by the number of sections and the thickness of the sections in the visual plane (Ormerod, Lee and Galea, submitted).

Figure 2. A) BrdU-labeled cell and B) a pyknotic cell in the dentate gyrus of the granule cell layer.
Hormone Assay

Serum hormone assays were performed as previously described (Ormerod & Galea, 2001). Briefly, blood samples were stored overnight at 4°C and then were centrifuged at 10g for 10 min. Serum estradiol was assayed using a Coat-A-Count kit (Diagnostic Products Corporation, Los Angeles, CA) modified for low expected levels of estradiol. Previous studies from our laboratory found that the intra-assay coefficient is less than 5% (Ormerod & Galea, 2000).

Statistical Analyses

**Experiment 1**: All behavioural variables were analyzed using a repeated-measures analyses of variance (ANOVA). Days to reach criterion, mean total number of working memory errors, and latency to reach goal arm were analyzed with dose (0.3μg, 5μg, 10μg) as the between-subjects factor and delay (10s, 40s) as the within-subjects factor. Additionally, latency to reach the goal arm was assessed by analysis of covariance (ANCOVA) with working memory errors as a covariate. The number of working memory errors were also grouped into Blocks (each Block consisted of 3d). The number of working memory errors was analysed with dose as the between-subjects factor and Blocks (1-5) as the within-subjects factor for all three doses. Change in performance across training sessions was also calculated by subtracting the performance during Block 5 of acquisition (10s delay) from the performance during Block 1 of reacquisition (40s delay). Change in performance at the start of training sessions was calculated by subtracting the performance during Block 1 of acquisition (10s delay) from the performance during Block 1 of reacquisition (40s delay). Change in performance was
analysed with dose (0µg, 0.3µg, 5µg, 10µg) as the between-subjects factor and delay (10s, 40s) as the within-subjects factor. Unless otherwise stated, all post hoc analyses were performed with the Newman-Keuls procedure and planned comparisons were performed using LSD. All statistical procedures set $\alpha = .05$. Although $p \leq .05$ will be referred to as statistically significant, these $p$ values reflect the probability of the effect occurring by chance.

**Experiment 2:** Number of BrdU-labelled cells, pyknotic cells, dentate gyrus volume and serum estradiol levels were analyzed using an ANOVA with dose (0.3, 5, 10) as the between-subjects factor. Correlation between serum estradiol levels and stereology/dentate gyrus volume were performed using Pearson’s product-moment correlation.

**RESULTS**

**Experiment 1**

Figure 3 displays the group means and standard errors of the mean for the total number of days to reach criterion. There was no significant effect of estradiol dose on days to reach criterion across all groups relative to vehicle controls (main effect of dose, $F(3, 32)=0.082, p\leq0.97$; main effect of delay, $F(1,32)=0.002, p\leq0.97$; and interaction effect, $F(3,32)=0.502, p\leq0.68$).
Figure 3. Group means (+SEM) for the total number of days to reach criterion (defined as no more than one error per day for three consecutive days).

EB0.3 rats made fewer working memory errors while both EB5 and EB10 rats made more working memory errors than VEH rats.

Figure 4 displays the groups means and standard error of the mean for the total number of working memory errors. There was an interaction effect ($F(3,32)=5.42$, $p<0.004$) and a main effect of delay ($F(1,32)=14.28$, $p<0.0006$) but no significant main effect of dose ($p<0.66$). Post-hoc analyses revealed that EB0.3 rats made significantly fewer working memory errors relative to VEH controls during the acquisition (10 s delay) phase ($p<0.04$; planned comparison). However, both EB5 and EB10 groups increased the number of working memory errors relative to VEH controls during the reacquisition (40 s delay).
delay) phase (p<0.006 and p<0.001, respectively).

Figure 4. Group means (+SEM) for the total number of working memory errors across conditions.

Figure 5 displays the group means and standard error of the mean for working memory errors across blocks of three trials at A) 10s delay and B) 40s delay. For the 10s acquisition data, there was a significant main effect of block (F(4,140)=8.408, p<0.000004), indicating that the number of working memory errors decreased as training progressed. There were no other significant main effect of dose or interaction effects (p<0.564, and p<0.492 respectively). A priori we expected a difference between EB0.3 and VEH rats and planned comparisons indicated that EB0.3 rats made significantly
fewer errors than VEH rats on Block 4 (p ≤ 0.032).

For the 40s reacquisition data, there was no significant effect of delay (F(4, 132) = 1.254, p ≤ 0.291), or interaction effect (F(12, 132) = 1.157, p ≤ 0.320). There was, however, a trend in that the lower doses of estradiol tended to make fewer errors (F(3, 33) = 2.772, p ≤ 0.057). Post-hoc analyses indicated that EB5 rats had significantly more errors on Block 3 (p ≤ 0.025) than vehicle-treated rats. EB10 rats made significantly more errors on Block 5 (p ≤ 0.014; Block 3 was a trend at p ≤ 0.015) than VEH rats.
Figure 5. Group means (±SEM) for the errors across blocks (1 block equals 3d) at A) 10s delay and B) 40s delay.
To determine whether there was any "savings" of information from acquisition to reacquisition mean working memory errors were compared across conditions during A) Block 1 of acquisition versus Block 1 of reacquisition and B) Block 5 of acquisition versus Block 1 of reacquisition. Comparing the Block 1s, the vehicle controls were the only group to show a significant difference (a decrease) between errors from Block 1 on reacquisition (40 s delay) versus acquisition (10s delay; p<0.001). Comparing Blocks 5 versus Block 1, the EB0.3 group was the only group that had a significant increase in working memory errors (p≤0.003). These data indicate that the vehicle group show the most benefit from previous training while the EB0.3 showed no savings.

To further assess "savings," difference scores were calculated across conditions by subtracting mean working memory errors at A) the start of training sessions (Block 1 of acquisition from Block 1 of reacquisition) and B) between training sessions (Block 5 of acquisition from Block 1 of reacquisition). Therefore a negative score indicates "savings," while a positive score indicates no "savings". Comparing Block 1 of acquisition from Block 1 of reacquisition, all conditions had a negative savings score and there was no significant difference across treatments in "savings" scores relative to vehicle controls (F(3,32)=0.298, p≤0.040; Figure 6A). Comparing Block 1 of acquisition from Block 1 of reacquisition, all conditions had a positive savings scores and there was no significant difference in "savings" score relative to vehicle controls (F(3,32)=0.128, p≤0.923; Figure 6B). Therefore, all groups exhibited savings at the start of re-training, however, no savings were exhibited across training sessions.
Figure 6. Group means (+SEM) for A) Block 1 reacquisition – Block 1 acquisition, B) Block 5 reacquisition – Block 1 acquisition
Figure 7 displays the group means and standard errors of the group means for latency to reach goal arm. There was significant main effect of dose ($F(3,32)=46.168$, $p<0.0002$), delay ($F(1,32)=13.604$, $p<0.0008$), and an interaction effect ($F(3,32)=15.336$, $p<0.000002$). Assessing mean working memory errors using motor as a covariate revealed a main effect of dose ($F(3,30)=3.213$, $p<0.037$), delay ($F(1,32)=14.28$, $p<0.006$), and an interaction effect ($F(3,32)=5.42$, $p<0.004$). Post-hoc analyses revealed that EB0.3 rats made significantly fewer working memory errors relative to VEH controls during the acquisition (10 s delay) phase ($p<0.04$; planned comparison). However, both EB5 and EB10 groups increased the number of working memory errors relative to VEH controls during the reacquisition (40 s delay) phase ($p<0.006$ and $p<0.001$, respectively).

![Graph showing latency to reach goal arm](image)

**Figure 7.** Groups means (±SEM) for latency to reach the goal arm at both the 10s (acquisition) and 40s (reacquisition) delays.
Experiment 2

Figure 8 displays the group means and standard errors of the mean for the number of BrdU-labelled cells. There was no significant main effect of dose on number of BrdU-labelled cells ($F(3,11)=1.88, p \leq 0.19$). Further there was no significant main effect of dose on the number of pyknotic cells ($F(3,11)=0.740, p \leq 0.550$; see Figure 9). There was a significant effect of dose on dentate gyrus volume ($F(3,10)=4.12, p \leq 0.038$; see Figure 10) with EB10 rats having larger volumes than vehicle-control rats ($p \leq 0.045$).
Figure 9. Group means (±SEM) number of pyknotic cells (±SEM) across all conditions.
Figure 10. The group means (±SEM) for the volume of the dentate gyrus across conditions.
There was a main effect of dose on serum estradiol level (F(3,11)=22.7, p<0.00005), with the EB5 and EB10 groups dose having a significantly higher level of estradiol relative to vehicle-controls (see Table 1 for group means).

There was no significant correlation between serum estradiol levels and the number of BrdU-labelled cells (r=-0.06, p<0.8), however there was a significant correlation between serum estradiol levels and dentate gyrus volume (r= 0.43, p<0.048, one-tailed), indicating that higher levels of chronic estradiol are associated with a larger dentate gyrus volume.

Table 1. Mean serum estradiol levels (pg/ml) across conditions.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum estradiol (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEH (n=4)</td>
<td>9.0 ± 3.1</td>
</tr>
<tr>
<td>EB0.3 (n=3)</td>
<td>28.3 ± 10.1</td>
</tr>
<tr>
<td>EB5 (n=4)</td>
<td>97.7 ± 16.5</td>
</tr>
<tr>
<td>EB10 (n=4)</td>
<td>269.3 ± 42.0</td>
</tr>
</tbody>
</table>
DISCUSSION

The results from Experiment 1 demonstrate a differential dose-dependent effect of estradiol on the acquisition versus reacquisition of PFC-dependent working memory in the non-spatial delayed matched to position T-maze task. During acquisition, at a 10s delay, low levels of estradiol [low physiological (EB0.3)] significantly reduced working memory errors relative to vehicle controls. During reacquisition, at a 40s delay, high levels of estradiol [high physiological (EB5) and supraphysiological (EB10)], significantly increased working memory errors relative to vehicle controls. This finding is consistent with previous research that has shown that low levels of estradiol facilitate working memory in various types of tasks (Fader, Hendrichson & Dohanich, 1998; Fader et al., 1999; Holmes et al., 2002; Luine et al., 1998). The effects of estradiol on working memory were independent of any effects of estradiol on motor output or motivational processes as there were no changes in mean working memory errors when using latency to reach goal arm as a covariate. There was no significant difference between days to reach criterion for all groups during both acquisition and reacquisition, thus suggesting that estradiol was not influencing acquisition or reacquisition per se but rather the number of working memory errors. To our knowledge, this is the first demonstration of a dose dependent effect of estradiol on acquisition versus reacquisition of a PFC-dependent non-spatial working memory task.

The results from Experiment 2 indicate that chronic estradiol treatment had no significant effect on cell proliferation or pyknosis in the dentate gyrus but did have a significant effect on dentate gyrus volume. These results are in contrast to previous studies using an acute dose of estradiol, whereby dynamic fluctuations in cell
proliferation are seen (Banasr et al., 2001; Galea & McEwen, 1999; Tanapat et al. 1999, Ormerod & Galea, 2001). Interestingly, previous research has found that there are no changes in cell proliferation in pregnant rats, and in pregnant rats high levels of estradiol are seen across the last trimester (approximately the last 7 days of gestation), which is at least partially consistent with our results (Banasr et al., 2001). However the pattern of cell proliferation levels across estradiol levels is similar to the pattern of working memory errors seen on a hippocampal/PFC task (Holmes, et al., in press). For example, low levels of estradiol (EB0.3) enhance both cell proliferation and working memory, while higher levels of estradiol (EB5) reduce both cell proliferation and working memory. This indicates that the impairment on acquisition during the spatial working/reference memory task may be due to estradiol’s influence on the hippocampus rather than the PFC. Recall that performance during acquisition in the present task does not appear to rely on the hippocampus (Lipska et al., 2002). Intriguingly, supraphysiological levels of estradiol (EB10) in the present study significantly increased dentate gyrus volume consistent with previous studies in female meadow voles (Galea, Perrot-Sinal, Kavaliers & Ossenkopp, 1999) and in previous pregnant and lactating rats (Galea et al., 2000).

Effects of estradiol on the acquisition of working memory

The present data demonstrate that during acquisition, a low dose of estradiol (EB0.3) significantly lowered the total number of working memory errors relative to vehicle controls. These data are consistent with previous studies in that low levels of estradiol are associated with facilitated acquisition of spatial learning via a reduction in working memory errors in rats (Daniel et al, 1997; Fader et al., 1999; Holmes et al., 2002;
Luine et al. 1998; O'Neal, Means, Poole, & Hamm, 1996; Wilson, et al., 1999). Note, however, that in the present study the significant decrease in working memory errors was fairly weak and may have been due to motor or motivational effects. Perhaps this indicates that there is a greater facilitation in working memory with low dose estradiol when the task relies more on the hippocampus and/or the interaction between the PFC and the hippocampus rather than when the task preferentially involves the PFC. It may also be that the present task is less sensitive to the effects of estradiol than the spatial radial arm maze.

In human studies a beneficial relationship has been found between estrogen replacement therapy and working memory amongst post-menopausal women using random clinical trials (Duff & Hampson, 2000; Keenan, Ezzat, Ginsburg & Moore, 2001; Hogervorst et al., 2000; Kimura, 1995, Rice, Graves, McCurry, & Larson, 1997; Sherwin, 1997). ERT typically restores estradiol levels to low or medium physiological levels compared to young women (Sherwin, 1997), thus the facilitation of working memory with ERT is through a "low" physiological dose of estradiol and is consistent with our present findings. Interestingly, on a novel spatial working memory task in which hidden pairs of coloured dots have to be matched amongst cards, women in the menses phase (low level estradiol) did not perform as well as women in other phases of the menstrual cycle (associated with higher levels of estradiol; Duff-Canning & Hampson, 2002). Hormone assays were not performed in that study, therefore these data maybe consistent with the present study, indicating that low (physiological) levels of estradiol are associated with a facilitation in working memory performance.

It is important to note, additionally, that clinical trials of ERT have lead to equivocal
results which may be due to differences in duration of dose, type of estradiol and socioeconomic background (Hogervorst, Williams, Budge, Riedel and Jones, 2000). Taken together, these data suggest, coupled with the previous findings with ERT, that there is a dose dependent effect of estradiol on working memory in humans as well as in rats.

In the present study a high physiological level (EB5) of estradiol had no significant effect on the number of working memory errors during acquisition relative to vehicle-controls. In contrast, previous studies from our laboratory have shown that high physiological levels of estradiol impaired acquisition of spatial working memory in which performance relies both on the hippocampus and PFC (Holmes et al., 2002). In the same task supraphysiological levels of estradiol have no significant effect on the acquisition of spatial working memory in rats (Galea et al., 2001), which is partially consistent with our data in that there was no significant difference between vehicle controls and EB10 during the acquisition phase of the present study.

Effects of estradiol on reacquisition and increased delay

The present results reveal that during reacquisition both EB5 and EB10 made significantly more errors relative to vehicle-controls. Furthermore, when comparing the difference score of Block 5 of acquisition with Block 1 of reacquisition, the low estradiol group (EB0.3) had a significantly higher difference score relative to vehicle-controls, indicating this group expressed less "savings" during reacquisition. In the present study the VEH group had significantly lower error scores during Block 1 of reacquisition compared to Block 1 of acquisition while estradiol groups were not significantly
different. This may suggest that estradiol does not have a beneficial effect on retention during extended delays. To our knowledge, no other studies to date have examined the role of estradiol in reacquisition of working memory tasks.

Delays are typically introduced in learning and memory tasks to increase the duration that information has to be retained, therefore making the task more difficult and more sensitive to differences in performance between experimental groups. In the spatial radial arm maze, a working/reference memory task, the introduction of a 1h delay before the final arm choice led to a very slight increase in the amount of errors in estradiol-relative to vehicle- treated animals (Luine & Rodriguez, 1994). In the spatial working memory task, the delayed win-shift task, the extension of the delay by 15 minutes also led to a slight increase in the number of working memory errors in estradiol-treated animals relative to vehicle controls (Galea, Wide, Paine, Holmes Ormerod & Floresco, unpublished data). These previous data are consistent with the present data in which high levels of estradiol (high physiological – EB5 and supraphysiological- EB10) significantly increased the number of working memory errors relative to vehicle controls during reacquisition of the task at a longer delay (see Figure 4).

Effects of chronic estradiol on cell proliferation

The present data demonstrate that the 21d of consecutive estradiol administration had no significant effect on cell proliferation in the dentate gyrus. To our knowledge this is the first study assessing the effects of chronic estradiol administration on cell proliferation. Previous work from our lab has shown that an acute supraphysiological dose of estradiol increases cell proliferation (Ormerod and Galea, 2001). However,
priming rats with one week of low level estradiol and progesterone, before the same acute supraphysiological dose of estradiol causes a significant suppression in cell proliferation relative to vehicle controls (Falconer and Galea, submitted). This difference in the system’s ability to respond to acute supraphysiological estradiol may be attributed to the interaction of progesterone and estrogen (Butcher et al., 1974) and/or to subchronic level of estradiol and progesterone. Another study found, consistent with the present study, that there was no significant differences in dentate gyrus cell proliferation in pregnant rats (who would have had high physiological levels of estradiol combined with other changes in hormone levels during the last week of pregnancy) relative to vehicle controls (Banasr et al., 2001).

It may not be surprising that acute versus chronic effects of estradiol would have disparate effects on cell proliferation in the adult female rats. Previous work in young and aged female rats has found that acute, but NOT chronic, estradiol influences spine density in granule neurons in the dentate gyrus (Miranda, Williams & Einstein, 1999). In the present studies we administered high levels of estradiol continually for at least 21d, and Miranda et al. (1999) found that continual replacement of estradiol (over 8 months) in young and older rats did not alter the spine density of granule neurons in the dentate gyrus relative to OVX control rats. Interestingly, in this previous report neither high nor low levels of chronic replacement affected spine density in young or aged females (Miranda et al., 1999). Thus, chronic versus acute levels of estradiol, have very disparate effects on the morphological properties (both spine density and cell proliferation) of the dentate gyrus. However, increases in dentate gyrus volume with chronic supraphysiological levels of estradiol suggest that another factor besides a change
in cell proliferation or spine density may be causing the increase in dentate gyrus volume. One possibility may be an increase in glial cells. Cultured glial cells of the rat central nervous system express estrogen receptors (Jung-Testa et al., 1992; Jung-Testas et al., 1998). Moreover, estradiol has been shown to stimulate morphological changes in both oligodendrocytes and astrocytes and increased synthesis of myelin basic protein and glial fibrillary acidic protein, markers of glial cells (Jung-Testa et al., 1992; Jung-Testas et al., 1994).

Role of the hippocampal-prefrontal cortical pathway in regulating working memory

Estradiol might influence different types of memory by acting differentially in distinct brain regions important for learning and memory. Brain regions involved in working memory include the PFC, hippocampus and their respective connections (for review see Laroche, Davis & Jay, 2000). The synapses of the hippocampal/PFC express forms of plasticity important for learning and memory (Laroch et al., 2000) including LTP (Jay, Burrette, & Laroche, 1996; Larouche, Jay & Thierry, 1990) and LTD (Takita et al., 1999). Spatial working memory is disrupted by lesions to the medial PFC (Brito & Brito 1990; Granon et al., 1994; Izaki et al, 2001; Kolb & Coie, 1996; Sanchez-Santed et al, 1997; Seamans, Floresco, & Phillips, 1995), the hippocampus (Olton, 1982; Jarrard, 1983; Seamans et al., 1995) and their respective connections (Floresco et al., 1997). Ventral CA1/subiculum neurons project mostly to the medial PFC, via a direct monosynaptic pathway, with few projections to the lateral PFC (Barbas & Blatt, 1995; Carmichael & Price, 1995; Goldman-Rakic, Selemon & Schwartz, 1984; Jay & Witter, 1991; Rosene & Van Hoesen, 1977). Disconnection lesions between the ventral
hippocampus and the medial PFC increase working memory errors on the spatial delayed win-shift task using male rats (Floresco et al., 1997). It is important though to note that lesion studies done using male rodents may not necessarily be extrapolated to female rodents. For example, Kolb and Coie (1996) have shown that medial PFC lesions increased the number of errors female rats made during performance of the Morris water maze and radial arm maze to a greater extent than male rats.

Distinct prefrontal regions may mediate subtypes of working memory. The dorsolateral PFC is believed to be involved in spatial working memory in primates (Goldman-Rakic, 1995; for review see Levy & Goldman-Rakic, 2000) and the ventrolateral PFC has been speculated to be involved in non-spatial working memory (Romaniski & Goldman-Rakic, 2002). However, there is speculation as to the function of discrete regions of the frontal cortex and other researchers believe that these regions are important for differential processing rather than domain specific information (Petrides, Alivisatos, & Frey, 20020; Ranier and Ranganath, 2002).

Distinct hippocampal regions may also mediate working memory as lesions of the CA1 region of the hippocampus increase working memory errors in the spatial working/reference memory version of the radial arm maze (Davis, Baranowski, Pulsinelli & Volpe, 1987; Kesner, Gilbert & Wallenstein, 2000) whereas lesions to the dentate gyrus increase the number of reference memory errors (Bouffard & Jarrard, 1988; Sutherland, Whishaw & Kolb, 1988). Furthermore, the ventral hippocampus has been shown to be involved in working memory processing (Burns, Annett, Kelley, Everitt & Robbins, 1996; Moser & Moser, 1998; Wilkerson & Levin, 1999).

Neurophysiological data reveals that ERT significantly increases regional cerebral
blood flow in both the hippocampus and the PFC during working memory tasks. In humans, the PFC is activated following performance of the Wisconsin Card Sorting task, a working memory task (Berman et al., 1995; Berman et al., 1997). Variations in hormone levels across the menstrual cycle are associated with changes in PFC activation following non-spatial working memory type tasks (Shaywitz et al., 1999). Furthermore both the parahippocampal gyrus and the frontal cortex exhibit longitudinal activation during spatial working memory tasks relative to control subjects (Maki and Resnick, 2000).

In male rats, working memory during acquisition of the present task was impaired by lesions to the PFC (Granon et al, 1994) but not by lesions to the ventral hippocampus (Lipska et al., 2002). Presumably in the present study the involvement of the hippocampus was limited by preventing the use of spatial cues. However, it is still questionable as to whether performance on the present task may be completely devoid of hippocampal involvement. In a previous hippocampal/PFC dependent task 5μg dose of estradiol significantly increased working memory errors (Holmes et al., 2002). Thus the increase in working memory errors at high physiological dose may be due to greater involvement of the hippocampus. The effects of hippocampal lesions on reacquisition of the delayed alternation T-maze task were not shown in the Lipska et al (2002) study, we cannot rule out involvement of the hippocampus in reacquiring the delayed alternation T-maze task (the 10s delay). Thus the impairment at high physiological doses may be the result of an hippocampal/PFC involvement. A discrepancy, however, is that in previous studies (Holmes et al., 2002) a supraphysiological dose of estradiol had no significant effect on a PFC-hippocampal dependent task. Future studies, with regionally specific
intracranial injections of estradiol will provide stronger evidence of PFC and hippocampus specific effects and may resolve these slight discrepancies.

Estrogen receptor regulation of working memory

Estrogen may be modulating behaviour by acting through distinct estrogen receptors (ER) in varying brain regions. There are at least two known types of nuclear ERs, α and the recently discovered β (Kuiper et al., 1996; Mosselman, Polman, & Dijkema, 1996). These receptors have distinct distribution patterns according to brain region, layer and cell type (Kritzer, 2002; Shughrue et al., 1997b; Shrugue & Mercenthaler, 2000). Interestingly, there are higher numbers of ERα in the ventral hippocampus, an area thought to be important for working memory, relative to the dorsal hippocampus (Shughrue & Merchenthaler, 1997; Shrugue & Mercenthaler, 2000). In contrast, β receptors show higher overall labeling throughout the dorsal and ventral hippocampus with weaker labeling in the dentate gyrus (Shugrue et al., 1997). Recent reports show greater quantities of ERα in the hippocampus than previously thought, perhaps due to the development of better antibodies (Hart, Patton & Wooley, 2001). The development of ER α and β knock-out mice (ERαKO and ERβKO respectively; Krege et al., 1998; LuBahn et al., 1993) has provided some evidence into the roles of these receptors in learning and memory. ERα has been speculated to be important in both amygdala-dependent emotional learning (Fugger, Foster, Gustafsson, & Rissman, 2000), and hippocampal-dependent spatial learning (Fuger, Cunningham, Rissman, & Foster, 1998). ERβ has also been speculated, in conjunction with the ERα, to be important for hippocampal-dependent spatial learning (Rissman et al., 2002). ERs have been found in
the PFC of humans (Bixo et al., 1995) as well as rats (Handa et al., 1997) and primates (Pau, Pau, & Spies, 1998). In rats the frontal cortices contain sparse expression of mRNAs for the ERα and β, with their main location being layer V of the cortex (Kritzer, 2002). Performance of knock-out mice on working memory tasks has yet to be addressed. Furthermore both the hippocampus and PFC may contain other types of ERs not yet discovered (such as the putative ERx — see below). Taken together, these data suggest that ER type and distribution may play a role in learning and memory.

Nuclear ERs, α and β, are high affinity ligand-inducible transcription factors that directly regulate the expression of target genes (McKenna & O’Malley, 2001). In particular, transcriptional activity of ERα is mediated by two activation sites, AF-1 and AF-2 (McDonnell & Norris, 2002; Tremblay, Tremblay, Labrie, & Giguere, 1999). Different cells require activation of different activation sites for transcription to occur (Meyer et al., 1989) and thus the same type of estrogen can have diverse effects on different brain regions. Further complicating the effects on cognition, different proteins involved in modulating the transcription apparatus, show preference for different activation sites on ER (McKenna et al., 1999). Included in this group are co-activators, which enhance the abilities of ER, such as cyclic AMP response element binding protein, (CREB), and repressors, which attenuate the abilities of ERs, such as Nuclear Receptor Co-repressor (NcoR) and the Repressor of Estrogen Action (REA; for review see McDonnell & Norris, 2002). Given the numerous agents that can be involved in estrogen mediated transcription, it is not surprising that there are differential effects of estrogen on learning and memory.

Interestingly, another regulator of ERα is ERβ. In cells in which both receptor
subtypes are expressed, the ERβ inhibits transcription of ERα (Hall & McDonnell, 1999). In terms of relevance to these studies, the ventral hippocampus, an area, important for working memory, has co-expression of the ERα and β (Shughrue, Scrimop, & Merchenthaler, 1998). Rissmen et al., (2002) have theorized that ERα leads to impairments of hippocampal dependent learning which may be blocked by ERβ as ERβ KO mice have impaired spatial learning. ERα and ERβ can occur as heterodimers (Cowley, Hoare, Mosselman, & Parker, 1997) and in these regions ERα may enhance and ERβ depresse gene transcription so differences in relative concentration may effect downstream transcription of factors important for learning and memory, such as CREB (for review see McEwen, 2001).

The rapid actions of estrogens on hippocampal electrophysiology suggests that mechanisms other than genomic receptors may mediate these effects (for review see Moore & Evans, 1999). ERs have been found in the plasma (Pappas, Gametchu, & Watson, 1995; Piestras & Szego, 1977; i.e. putative ER-X) and have fast acting, non-genomic effects on cell signalling pathways (for review see Toran-Allerand, Singh, & Setalo, 1999). Signal transduction leads to the activation of numerous pathways and proteins which estradiol, when coupled to the cascade, can enhance (McDonnell & Norris 2001). For example, chronic estrogen treatment leads to increases in Brain Derived Neurotrophic Factor (BDNF; Singh, Meyer, & Simpkins, 1995). BDNF is co-expressed with plasma ER (Toran-Allerand et al., 1992) and fluctuates over the rat estrous cycle (Gibbs, 1998). BDNF and estrogen coexpression results in convergence of their signalling pathways (Toren-Allerand et al., 1999). Other pathways estrogen may influence are second messenger pathways such as MAPK and cAMP that then indirectly
activate gene transcription through response elements such as CREB (McEwen & Alves, 1999). Phosphorylated CREB has been implicated in hippocampal-dependent learning of an inhibitory avoidance task, whereby high levels enhance and lower levels impair performance (Viola et al., 2000). Indeed, enhanced cell proliferation and cell survival results from the phosphorylation of CREB in dentate gyrus granule neurons (Nakawaga et al., 2002). Further, short- versus long- term treatment with estrogen differentially effects the expression of CRE-DNA and CREB in varying brains regions (Carlstrom, Understall, Cohen & Pandey, 2001).

_Estradiol modulates many neurotransmitter systems_

There is substantial evidence demonstrating interactions between estradiol and several neurotransmitter systems putatively important for learning and memory (for review see Fink et al., 1996; Luine et al., 1998; McEwen, Alves, Bulloch & Weiland, 1997; Woolley, 1998). The dose-dependent effect of estradiol observed in the present study may be in part due to alterations in the activity of neurotransmitter systems. In particular, the dopaminergic, cholinergic and glutamate systems are potential candidates for a mechanism via which estradiol can indirectly alter learning and memory.

Dopamine has been implicated in regulating working memory in both the PFC and hippocampus (Brozoski, Brown, Rosvold, & Goldman, 1979; Watanabe, Kodama & Hikisaka, 1997; Williams & Goldman-Rakic, 1995). Dopaminergic afferents originating in the brain stem innervate PFC pyramidal neurons and modulate their excitability (Williams & Goldman-Rakic, 1993, 1998). Selective chemical lesions to prefrontal dopamine afferents results in significant behavioural impairments in animals during
spatial working memory tasks (Brozoski et al., 1979; Kessler & Markowotsch, 1981; Nonneman & Corwin, 1981; Roberts et al., 1994; Simon, Scatton & Moal, 1980; Stam et al., 1989; Wilcott & Xuemei, 1990). Thus dopamine afferents may exert their effects on working memory through their actions on the PFC.

In adult male rats, D1 receptors are located predominantly in the frontal cortex (Lidow, Goldman-Rakic, Gallager & Rakic, 1991), but are also found in the dentate gyrus and CA1 layer of the hippocampus while D2 receptors are found heterogeneously throughout the brain (Cyr, Ghribi & Di Paolo, 2000). D1 receptors in the PFC play a dominant role in working memory in primate studies (Sawaguchi & Goldman-Rakic, 1994; Williams & Goldman-Rakic 1995). In a human visual-spatial delayed matching task administration of the D1/D2 receptor agonist, pergolide, facilitated learning, while a selective D2 receptor agonist, bromocriptine, had no affect on working memory (Muller, von Cramon & Pollman, 1998). Furthermore, the D1 receptor antagonist, SCH23390, but not the D2 receptor antagonist, haloperidol, impaired performance on the same task used in the present study, the non-spatial delayed alternation T-maze in rats (Aultman & Moghaddam, 2001). Dopamine receptors also modulate the activity of PFC pyramidal neurons (Henze, Gonzalez-Burgos, Urban, Lewis, & Barrionuevo, 2000) and appear to play a complex role in working memory processes. The ventral hippocampal D2 receptors and the PFC D1 receptors are important for working memory whereby activation facilitates and blockage impairs performance (Packard & White, 1989; Seamans, Floresco & Phillips, 1998; Wilkerson & Levin, 1999). Therefore a complex interaction exists between D1 and D2 receptors and dopamine levels have an inverted-U effect on working memory with high and low doses of dopamine impairing and medium doses
facilitating working memory (Zahrt, Taylor, Mathew & Arnsten 1997).

Estradiol modulates dopamine transmission, and it may be that estradiol affects working memory through its modulation of dopamine transmission. Estradiol dose-dependently modulates dopamine transmission in a number of neural sites including the nucleus accumbens and the striatum (Becker, 1990; McDermott, 1993). Findings suggest that dopamine clearance rates are affected by estrogen. High levels of estradiol are associated with a decreased affinity for the dopamine transporter while lower levels of estrogen are associated with augmented dopamine release (Disshon, Boja, & Dluzen, 1998; Thompson, 1999; Thompson & Moss, 1997). Further estrogen may modulate changes in dopamine metabolites. Following ovariectomy in rhesus monkeys a significant reduction has been seen in the PFC in tyrosine hydroxylase, an enzyme which reduces tyrosine to dopamine, and a significant increase in dopamine hydroxylase, an enzyme which breaks down dopamine (Kritzer and Kohama, 1998, 1999). Both of these reactions would lead to lower dopamine levels. Furthermore, dopamine release in the nucleus accumbens is heightened during the first stage of diestrous suggesting low to medium levels of estradiol enhances DA release (Thompson & Moss, 1997). Thus, both high and low levels of estradiol result in lower dopamine levels, while medium levels of estradiol result in higher dopamine levels. This suggests estradiol may mediate the inverted U effect of dopamine. Taken together, these data suggest that estradiol level may mediate its effects via its actions on dopamine transmission in distinct brain areas.

Administration of cholinergic antagonists appear to increase the number of working memory errors (Givens & Olton, 1995; Pilcher, Sessions, & McBride, 1997) possibly independent of interfering with reference memory errors (Okaichi, Oshima, &
Estradiol treatment can attenuate the impairment of scopolamine, a muscarinic receptor antagonist, on working memory (Fader et al., 1999) perhaps via directly increasing cholinergic activity in the hippocampus (Luine, 1985; Singh, Meyer, Millard & Simpkins, 1994). Furthermore, scopolamine blocks the facilitation by estradiol on performance in the Morris water maze in female rats (Packard & Teather, 1997). In addition, there are ERs on cholinergic neurons in the basal forebrain, including the PFC (Toran-Allerand, et al., 1992) and estradiol administration increases immunoreactivity of choline acetyltransferase in this region (Gibbs, Wu, Hersh, & Pfaff, 1994). In light of the fact that working memory performance on the working/reference memory task is dependent on the integrity of the basal forebrain, the PFC, and the hippocampus, estradiol may facilitate PFC-hippocampus processing, ultimately facilitating working memory performance (Fader et al., 1999).

In addition to dopamine, glutamate transmission in the PFC and hippocampus may be a physiological modulator of working memory (Verma & Moghaddam, 1996; Aultman & Moghaddam, 2001; Romanides, Duffy, & Kalivas, 1999). There are a high degree of N-methyl D-aspartate (NMDA) receptors in both the PFC and the hippocampus (Watanabe et al., 1997). Innervation of glutamate neurons with the PFC arises partly from hippocampal afferents (Floresco et al., 1997) and PFC neurons that respond to hippocampal stimulation are enhanced by NMDA agonists (Jay, Theirry, Wiklund & Glowinski, 1992). Non-competitive NMDA antagonists, ketamine and MK-801, cause performance impairments in the delayed alternation T-maze (Aultman & Moghaddam, 2001; Romanides et al., 1999) and other PFC sensitive tasks in rats (Verma,
Moghaddam, 1996; Wesierska, Macias-Gonalez, & Bures, 1990) and humans (Krystal et al., 1994). More specifically, using subregion specific injections of NMDA receptor antagonists, D-(-)2-amino-5-phosphonovaleric acid (APV), Lee & Kesner (2002) have revealed that NMDA receptors in CA1 are necessary for spatial working memory involving intermediate (<10s) delays. As stated earlier, ventral CA1 regions project afferents to the PFC and, in addition, the CA1 region has been implicated in working memory (Davis et al., 1987; Kesner et al., 2000). NMDA antagonists may exert their actions by attenuating dopamine transmission and can be blocked by D2 receptor antagonists (Verman & Moghaddam, 1996). Further, recall that ventral hippocampal D2 receptors and the PFC D1 receptors are important for working memory whereby activation facilitates and blockage impairs performance (Seamans, Floresco & Phillips, 1998; Wilkerson & Levin, 1999). Thus dopamine and glutamate may work in concert to exert effects on working memory (for review see Moghaddam & Sesack, 1996).

Acute supraphysiological estradiol increases NMDA receptor binding in area CA1 of the hippocampus following ovariectomy (Woolley, Weiland, McEwen, & Schwartzkroin, 1997) and concomitantly improves working memory performance in the spatial radial arm maze (Daniel & Dohanich, 2001). Acute estradiol (supraphysiological and high physiological) significantly decreases glutamate receptor concentration in the PFC, but not the hippocampus, relative to vehicle controls (Cyr et al., 2001; Cyr, Ghribi & DiPaolo, 2000). In contrast, 28d of chronic low physiological levels of estradiol facilitated working memory and was shown to have no effect on glutamate metabolite levels in the CA1, CA3 and DG regions of the hippocampus or the PFC (Luine et al., 1998). The expression of glutamate may therefore be related to the dose and duration of
estradiol. Further research is necessary, however, for a better understanding of the relationship between glutamate, estrogen and learning and memory.

Overall, estradiol may work to modulate any of these neurotransmitter systems and may influence working memory in conjunction with each other.

**Neurogenesis and Learning**

The role of adult neurogenesis in the dentate gyrus in hippocampal-dependent learning is supported by research showing that enriched environments and increased physical activity enhance both learning and memory and the number of new neurons in the dentate gyrus of adult rodents (Kemperman, Kuhn, & Gage, 1997; van Praag et al., 1999). Learning that is reliant on the hippocampus, such as trace eyeblink conditioning and spatial navigation in the Morris water maze, significantly enhances the survival of new neurons in the dentate gyrus of adult rats (Gould et al., 1999). When an anti-mitotic agent, methyloxymethanol acetate (MAM), is injected for 14d it leads to a significant reduction in the number of newly generated granule cells and coincident impairment in hippocampal-dependent trace eyeblink conditioning, suggesting that adult neurogenesis is critical for memory formation (Shors et al., 2001). Intriguingly, estradiol administered during days 6-10 after new cell birth during which time new cells are extending their axons (Hastings & Gould, 1999) significantly increases neurogenesis and spatial memory in the Morris water maze (Ormerod, Lee & Galea, submitted). Administration of estradiol at other time periods after cell birth (days 1-5 or day 10-15) did not influence either neurogenesis or memory. These studies may therefore pinpoint an active role of neurogenesis in the formation of hippocampal-dependent learning and
Mechanisms of estradiol on cell proliferation

Factors that affect cell proliferation either suppress or induce mitosis in precursor cells. The mechanism whereby estrogen regulates cell proliferation has yet to be determined. However, estrogen may be regulating neurogenesis by influencing the same mechanisms thought to influence learning and memory. The serotonin antagonist, p-chlorophenylalanine, prior to an acute dose of estrogen blocks the estrogen-induced facilitation of cell proliferation (Branasr et al., 2001). Additionally, the suppression in cell proliferation seen 48hr following estrogen administration is reversed in adrenalectomized animals (Ormerod et al., submitted). Other factors may be involved in estradiol's effects on neurogenesis. For example, high levels of NMDAr-activation, via NMDA administration, decreases cell proliferation and low level NMDAr-activation, via NMDA antagonists MK-801 or CGP37849, increases cell proliferation in the dentate gyrus of adults rats (Cameron, McEwen & Gould, 1995) and estradiol modulates NMDA receptor expression in the PFC and hippocampus (Cyr et al., 2001; Cyr, Ghribi & DiPaolo, 2000; Woolley, Weiland, McEwen, & Schwartzkroin, 1997). Furthermore, it has been recently determined that phosphorylated CREB, a protein that is differentially modulated by estrogen (Carlstrom et al., 2001), enhances neurogenesis (Nakawaga et al., 2002). In Experiment 2 chronic estradiol did not significantly affect cell proliferation in the dentate gyrus. Given that acute estradiol has dynamic effects on cell proliferation in the hippocampus (Banasr et al., 2001; Galea & McEwen, 1999; Tanapat et al., 1999; Ormerod & Galea, 2001) the differential effect of chronic estradiol on cell proliferation
may have been due to chronic estradiol's downregulation of ERα expression (Weiland, Orikasa, Hayashi & McEwen, 1997). In addition, estradiol differentially regulates ERβ expression (McEwen & Alves, 1999). ERβ is expressed in the subgranular zone of the hippocampus where progenitor cells reside (Merchenthaler et al., 1997). Furthermore ERβ has been localized on astrocytes in the subgranular zone (Garcia-Segura et al., 1999), and astrocytes have been identified as neural stem cell precursors (Seri et al., 2001). Thus during new cell birth ERβ may regulate ERα transcription in the areas in which they are both expressed (Hall & McDonnell, 1999). Overall, downregulation of ERα via ERβ and chronically high levels of estradiol may reduce the availability of ER and prevent its effects on putative mechanisms of action.
CONCLUSIONS

The results from Experiment 1 demonstrate that estradiol exerts a dose-dependent effect on acquisition and reacquisition of working memory in the PFC-dependent non-spatial delayed non-matching to sample T-maze. Chronic administration of low physiological doses of estradiol significantly decreases working memory errors during acquisition of T-maze task. In contrast, chronic administration of high doses of estradiol increase the number of working memory errors during reacquisition of the T-maze task. The results of Experiment 2 demonstrate that chronic estradiol treatment has no significant effect on cell proliferation or pyknosis in the dentate gyrus of adult female rats but chronic supraphysiological estradiol does increase dentate gyrus volume. To our knowledge this is the first demonstration of a differential dose-dependent effect of estradiol on acquisition versus reacquisition of a non-spatial working memory task and examination of chronic estradiol treatment on cell proliferation.
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