DOSE-DEPENDENT EFFECTS OF ESTRADIOL ON WORKING MEMORY AND THE PHOSPHORYLATION OF cAMP RESPONSE ELEMENT-BINDING PROTEIN

By

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B.Sc., The University of Toronto, 2003

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

In

THE FACULTY OF GRADUATE STUDIES
(Neuroscience Program)

THE UNIVERSITY OF BRITISH COLUMBIA

JULY 2005

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Abstract

Estradiol can influence a variety of physiological and psychological processes. This hormone has been shown to influence learning and memory processes. The effects of physiological levels of estradiol on spatial working memory performance were explored in Chapter 1. In Experiment 1, daily systemic estradiol or oil injections were administered to adult, ovariectomized female rats approximately 4 hours prior to the testing on a win-shift version of the radial arms maze. A high dose of estradiol (5 μg estradiol benzoate) enhanced acquisition of the task whereas a low dose (0.3 μg estradiol benzoate) impaired working memory performance. Experiment 2 was conducted to examine site-specific influences of estradiol on spatial working memory in well-trained rats. Saline and two doses of estradiol cyclodextrin (0.1 μg and 0.9 μg estradiol/0.5 μL saline) were infused into the prelimbic region of the prefrontal cortex and dorsal hippocampus 40 minutes prior to testing on the win-shift task. The higher dose of estradiol facilitated working memory when infused into the prelimbic region, whereas lower doses of estradiol facilitated performance when infused into the hippocampus. Working memory performance was significantly impaired 24 hours after infusions of estradiol into the dorsal hippocampus relative to saline infusions. These data provide further evidence for the idea that estradiol can dose-dependently alter memory processes, and also suggest that the facilitation and impairment of working memory by estradiol is site- and time-specific. Chapter 2 sought to elucidate the role of long-term physiological levels of estradiol on the phosphorylation of cAMP response binding element (pCREB); a transcription factor related to cognitive processes. Ovariectomized rats were administered oil, high estradiol (5 μg estradiol benzoate) or low estradiol (0.3 μg estradiol
benzoate) for 17 consecutive days. Neither dose of estradiol significantly altered the number of pCREB-positive cells in the dorsal CA1, CA3 or in the prelimbic region relative to control levels. There was however a time of day effect, with animals perfused 40 minutes after the last injection exhibiting more pCREB-positive cells than animals perfused 4 hours after the last injection. Future research is required to clarify the role that estradiol plays in the activation of CREB.
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Acknowledgements

I would like to express gratitude to Dr. Liisa Galea for this amazing experience. Her support, guidance, and advice helped me grow as a student, researcher, and as a person. Thank-you so much for this opportunity; I will carry the skills I have learned at UBC with me always.

I would also like to thank Dr. Stan Floresco for his encouragement and his incredible assistance with the maze study.

I would like to thank Dr. Victor Viau for his helpful comments with the pCREB study and for graciously sharing his staining protocol.

A special thanks to Christine Mazzucco and Jas Kambo, I wouldn’t have made it without you guys!

I would like to thank Stephanie Lieblich, David Liang, Marie Tse, and Orsha Magyar for their excellent technical assistance.

I would like to dedicate this thesis to the memory of my late grandfather, Franco Falco, whose hard work, determination, and positive spirit has help shape the person I am today. 
*Ti amo Nonno e mi manchi tanto! Con Amore, la tua nipote.*
Dose-Dependent Effects of Estradiol on Working Memory and the Phosphorylation of cAMP Response Element-Binding Protein

Estrogens, including estradiol, estriol and estrone, are a family of steroid hormones that are produced mainly by the ovaries (Veiga et al., 2004). They can also be synthesized, albeit to a lesser degree, within the central and peripheral nervous systems and in tissues such as the liver, breasts, blood vessels, adipose tissue, and the skin (Baulieu, 1997; Dubey & Jackson, 2001; Plassart-Schiess & Baulieu, 2001). In adult humans and rodents, 17β-estradiol (estradiol) is the most abundant and most potent endogenous estrogen (Dubey & Jackson, 2001). Estradiol can be synthesized from testosterone via the enzyme aromatase, and from androstenedione via the conversion to estrone by aromatase, then to estradiol by 17β-hydroxysteroid dehydrogenase (Dubey & Jackson, 2001). The metabolism of estradiol results in both estrogenic (e.g. estriol) and non-estrogenic compounds, the latter of which are water-soluble and thus can be excreted (Dubey & Jackson, 2001).

There are two known estrogen receptors (ERs), ERα and ERβ, and several splice variants are associated with each receptor (for a review see Nilsson et al., 2001). Located in the cytoplasm and nucleus of target cells, activation of these receptors can result in alterations in genomic activity (Pfaff & McEwen, 1983; Green et al., 1986; Greene et al., 1986; Kuiper et al., 1996). ERα and ERβ are part of a superfamily of ligand inducible transcription factors, and consist of three functional domains that interact with one another (Evans, 1988). When estradiol binds to either ER, conformational changes occur within the ligand-binding domain of the receptor allowing the activated ER to translocate to the nucleus (i.e. if not already there) where receptor dimerization occurs (Evans, 1988;
Tsai & O'Malley, 1994). The DNA-binding domain of the ER will then target and bind to estrogen-responsive elements (EREs) in the promotor region of estrogen-regulated genes, and cofactors (i.e. coactivators, corepressors, cointegrators) will be recruited that either initiate or suppress transcription depending on the cellular environment (Pfaff & McEwen, 1983; Beato, 1989; Schwabe et al., 1993). The recruitment of co-activators such as steroid receptor co-activator 1 and cAMP binding protein is initiated via conformational changes occurring at the activation function 2 site located in the ligand-binding domain of the ER (Beato, 1989; Henttu et al., 1997; Klinge et al., 2004). The DNA-binding domains of ERα and ERβ are homologous, and hence both receptors can bind to EREs with similar affinity and specificity (Enmark et al., 1997). Conversely, activated ERs can target non-ERE AP1-binding sites in the promotor region of target cells to interact with fos/jun transcription factor complexes (Paech et al. 1997).

Examples of the genomic actions of estradiol include stimulation of prolactin, galanin, neurotensin, and tyrosine hydroxylase gene expression (Alexander et al., 1989; Gu et al., 1996; Watters & Dorsa, 1998; Shen et al., 1999), as well as oxytocin and serotonin receptor expression (Osterlund et al., 2000; Bale et al., 2001).

Estradiol can also exert rapid, non-genomic actions on cellular processes, and these influences are thought to rely predominantly on ERs located on the plasma membrane. These receptors either represent isoforms of ERα and ERβ, or are a different ER entirely (e.g. ERX) (Kelly & Levin, 2001; Abraham et al., 2004). When estradiol binds to and activates membrane-bound ERs, it quickly stimulates second messengers and cellular signalling pathways (Meyer & Habener, 1993; Kelly & Levin, 2001; Zhao et
The non-genomic actions of estradiol will be discussed in further detail in Chapter 2.

Estradiol significantly influences a myriad of psychological and physiological processes, although its traditional role has been in the mediation of reproduction. For example, estradiol initiates the formation of the corpora lutea, activates secondary sex characteristics of females at puberty, mediates the release of gonadotropin-releasing hormone, and is central to rodent sexual behaviour (Hilliard et al., 1966; Forleo et al., 1967; Komisaruk, 1974; Fitch & Denenberg, 1998). Estradiol also has a positive influence on the cardiovascular system (Dubey & Jackson, 2001) and skeletal system where it promotes bone production and has been linked to the prevention of osteoporosis (DeCherney, 1993; Drury, 1997). Furthermore, estradiol easily and rapidly diffuses into the brain where it exerts a significant influence over processes mediated by the central nervous system. For instance, estradiol has been implicated in neuroprotection: it shields the brain against the insults of ischemia, glutamate-induced neurotoxicity and hypoglycaemia; it prevents the accumulation of β-amyloid plaques and ATP depletion; and it reduces the generation of free radicals (Simpkins et al., 1997; Zhang et al., 1998; Wang et al., 2001; Fitzpatrick et al., 2002; Ritz et al., 2002; Eberling et al., 2004; Sribnick et al., 2004). Estradiol can also induce the expression of trophic factors such as brain-derived neurotrophic factor (Scharfman & Maclusky, 2005) and enhances the survival of adult generated neurons in the hippocampus (Ormerod et al., 2004).

Other influences of estradiol in the brain include the enhancement of signal transduction (Cordoba et al., 1997), locomotion (Beatty, 1979; Johnson & Stevens, 1983), limb coordination (Becker et al., 1982), sensory perception (Kow & Pfaff, 1983),
mood (Sherwin & Gelfand, 1985; Sherwin, 1988) and cognition (Sherwin, 1988).

Intriguingly, estrogen supplementation in menopausal women delays the onset and/or decreases the relative risk of developing Alzheimer’s disease, as well as ameliorating the associated cognitive deficits (Tang et al., 1996; Simpkins et al., 1997; Green et al., 1997). The effects of estradiol on cognition is relevant not only to those suffering from neurodegenerative disorders, but is also of relevance to the general population since estrogens have been implicated in alleviating cognitive decline in aged women and men (Kimura, 1995; Sherwin, 2003). Although the beneficial influences of estradiol supplementation are controversial, examination of the literature reveals that the ability of estradiol to enhance cognition include the duration of estradiol exposure, whether or not other hormones are also present, type of estrogen, route of estradiol administration and the type of cognition being evaluated (Hogevorst et al., 2000; Sherwin, 2005). Therefore, the aim of the following series of experiments was to further clarify the role of estradiol in learning and memory (Chapter 1), and to explore how physiological levels of estradiol alter transcription factors implicated in these processes (Chapter 2).
Chapter 1: Systemic and Local Administration of Estradiol Alters Working Memory in a Dose- and Site-Specific Manner

Adult levels of estradiol strongly influence a variety of learning and memory processes in both humans and rodents (e.g. Duff & Hampson, 2000; Korol, 2004). However the nature of estradiol’s influence varies along a number of parameters, including the dose of estradiol and the type of behaviour being assessed (Galea et al., 2001; Holmes et al, 2002; Sherwin, 2005). In rats for example, high levels of estradiol impair while low levels of estradiol facilitate spatial working memory, while the same doses of estradiol do not appear to influence spatial reference memory (Daniel et al., 1997; Warren & Juraska, 1997; Luine et al., 1998; Fader et al., 1999; Holmes et al., 2002). An intriguingly similar relationship exists in humans; across the menstrual cycle, high levels of estradiol are associated with impaired spatial ability and enhanced verbal and motor abilities as compared to performance when estradiol levels are low (Hampson & Kimura, 1988; Hampson, 1990; Hausmann et al., 2000; Lacreuse et al., 2001).

Estradiol can potentially modulate the coordination of multiple memory systems by acting at distinct neuroanatomical locations to affect different neurochemicals (Korol 2004). Research has shown that estradiol can directly influence learning and memory performance on tasks that involve the hippocampus and/or its projection sites, such as the prefrontal cortex (PFC; Keenan et al., 2001; Packard & Teather, 1997). The PFC and the hippocampus are connected via a unidirectional glutamatergic pathway that extends from the hippocampus to the medial aspect of the PFC (Swanson, 1981), and intact functioning of both structures are vital for working memory abilities (Floresco et al., 1997). Working memory can be defined as the maintenance of relevant information in a readily accessible
form while simultaneously processing and incorporating new information to guide
prospective action (Baddeley, 1986; Goldman-Rakic, 1987). Specifically, the
hippocampus is thought to convey spatial information and/or information concerning the
appropriate behavioural plan relevant to the navigational tasks subserved by the medial
PFC, and the PFC in turn organizes and provides multiple motor plans to drive the goal-
directed behaviour (Kolb, 1984; Ruit & Neafsey, 1988; Verwer et al., 1997; Izaki et al.,
2000; Vertes, 2002).

Estradiol has repeatedly been shown to affect working memory (e.g. Luine et al.,
1998; Holmes et al., 2002). For instance, spatial, non-spatial, and verbal working
memory abilities are enhanced in menopausal women receiving estrogen-replacement
therapy as compared to untreated women (Cohen et al., 1997; Duff & Hampson, 2000;
Keenan et al., 2001). In rodents, high levels of estradiol impair while low levels of
estradiol facilitate hippocampus-dependent spatial working memory (Holmes et al., 2002;
Daniel et al., 1997; Luine et al., 1998) and PFC-mediated non-spatial working memory
(Wide et al., 2004). However, estradiol-induced facilitations in performance were not as
pronounced in the non-spatial task as in the spatial task, and high levels of estradiol did
not impair non-spatial working memory when there was a short delay between the
acquisition and retrieval phases of the task (Wide et al., 2004). These data suggest that
estradiol may differentially modulate the functioning of the PFC and hippocampus in a
dose-dependent manner, and further implies that manipulation of estradiol levels could
have differential effects on working memory performance that relies on both structures.
One such task is the delayed spatial win-shift (SWSH) version of the radial arm maze.
Performance on this working memory task is disrupted following inactivation of the
hippocampus, PFC or disconnection between these two areas (Packard et al., 1990; Seamans et al., 1995; Floresco et al., 1997; Taylor et al., 2003). With this in mind, Experiment 1 was conducted to determine the effects of high and low physiological levels of systemic estradiol on working memory performance on the SWSh task.

As noted above, manipulation of systemic estradiol may differentially affect functioning of the PFC and the hippocampus. In light of this, it may be difficult to determine the particular brain region(s) that systemic estradiol is affecting to alter performance on the delayed SWSh task. To overcome this obstacle Experiment 2 assessed the effects of local infusions of different doses of estradiol into either the PFC or the hippocampus on performance on the SWSh task. Both the hippocampus and PFC contain estrogen receptors, with ERα and ERβ expressed in the hippocampus, while ERβ is almost exclusively expressed in the PFC (Shughrue et al., 1997; Krtizer, 2002; Zhang et al., 2002; Kalita et al., 2005). Surprisingly there are relatively few studies investigating the effects of local infusions of estradiol into the brain on learning and memory. These studies have focused on the hippocampus and hippocampus-dependent learning but not specifically working memory (Packard & Teather, 1997; Frye & Rhodes, 2002). Estradiol infused into the dorsal hippocampus prior to training facilitates active avoidance learning (Frye & Rhodes, 2002), and also enhances the retention of a water maze task twenty-four hours after local infusion (i.e. following acquisition) (Packard & Teather, 1997). Research has suggested that the dorsal hippocampus may exert a greater role over the PFC in mediating working memory performance when pre-test delays are extended beyond a few minutes (Lee & Kesner, 2003), thus it was expected that estradiol infusions into the dorsal hippocampus would cause greater alterations in behaviour than
infusions into the PFC would. Because dose-dependent alterations in working memory were observed in previous systemic studies (Holmes et al., 2002; Wide et al., 2004), it was hypothesized that similar dose-dependent changes in behaviour would occur when estradiol was infused into the dorsal hippocampus and/or the PFC.

Method

Experiment 1

Subjects

Twenty-four 3-month old female Long-Evans rats (Charles River, Quebec) weighing between 250 g and 300 g were used. The rats were housed individually in opaque cages and were maintained on a 12-hour light/dark cycle. They were given ad libitum access to water throughout the experiment. Prior to surgery, the rats were allowed to free feed on Lab Diet #5012 (Jamieson, Richmond, BC). Once the rats had recovered from surgery, they were food deprived to 90% of their free-feeding body weight, and food administration was adjusted 3g/week to account for growth. The treatment of animals was in concordance with the ethical standards of the Canadian Council for Animal Care.

Surgery

One week following arrival to the facilities, the rats were bilaterally ovariectomized utilizing aseptic procedures. They were anaesthetized via inhalation of halothane gas and oxygen (4% halothane for the induction, 2% for maintenance), and the level of anaesthesia was monitored throughout the surgeries and was adjusted as necessary. All rats were allowed to recover from surgery for 10 days before the commencement of food deprivation and training.
Hormone Replacement

Throughout the behavioural training, all rats received daily subcutaneous injections of either 0.1 mL sesame oil (OIL; Sigma, St. Louis, MO) or estradiol benzoate (EB; Sigma). Estradiol was administered at either a low dose of 0.3 µg EB/0.1 mL sesame oil (EB0.3) or a high dose of 5 µg EB/0.1 mL sesame oil (EB5). Previous studies have shown that these EB doses yield physiological serum levels of estradiol in the mid-low to high range: EB5 injections result in the high levels of estradiol observed during proestrus (e.g. ~60-90 pg/mL), and EB0.3 injections result in the low levels of estradiol observed during diestrus (e.g. ~20-30 pg/mL) (Butcher et al., 1974; Holmes et al., 2002). All injections occurred approximately 4 hours prior to testing. This time delay ensures maximal serum estradiol levels at testing (Woolley & McEwen, 1992). Rats were randomly assigned to one of the three treatment conditions: OIL, EB0.3, and EB5 (n=8 per group). EB/vehicle injections began on the first day of training and continued until cessation of the experiment. All injections occurred between 8:30 am and 9:30 am.

Apparatus

The rats were tested on an eight-arm radial maze that was elevated 71 cm from the ground, with arms 53 cm long x 10 cm wide. Removable metal barriers (25 cm x 15 cm) were used to block the arms from the centre platform during the training phase. The end of each arm contained a small food cup where the food reward was placed (Fruit Whirls; Glencourt Distributors, Calgary, AB). The maze was contained within a dimly lit room and was surrounded by extramaze cues that were kept constant throughout testing.
Procedure

Following recovery from surgery (approximately 1 week), the rats were handled five minutes daily for one week. During this time, the rats were also food deprived to 90% of their free-feeding body weights. Immediately after being handled, rats were habituated to the maze environment by being placed in the centre of the maze facing the back wall, and this “start” position was kept constant throughout the experiment. They were then allowed to explore all the arms of the unbaited maze for 10 minutes. Rats received 5 daily habituation sessions in total. In order to habituate the rats to the food reward, 2 Fruit Whirls were placed in each rat’s cage throughout the 5 days of maze habituation. Following the last habituation trial, training on the SWSh task commenced.

Each daily trial of the SWSh task was composed of 2 phases: the training phase and test phase, separated by a delay. During the training phase, 4 of the 8 arms of the maze were randomly selected and blocked with the metal barriers, and the remaining 4 arms were baited with ¼ of a Fruit Whirl. The rats were placed in the centre of the maze and were allowed to explore the maze for a total of 5 minutes, or until all 4 rewards had been retrieved. The rats were then removed from the maze, returned to their home cages, and placed in a quiet room for a 15-minute delay. Following the delay, the rats were returned to the centre platform of the maze for the testing phase of the trial. During this phase, all arms were open, but only the arms that were blocked previously contained the food reward. When all 4 rewards had been retrieved or 5 minutes had elapsed, the rats were removed from the maze and returned to their home cages. Rats received one such trial per day. Ethanol (70%) was used to clean the maze in between the training and test phases, as well as in between rats. The order in which the rats were tested was
randomized daily. In order to minimize the influence of intramaze cues, the maze was rotated every 3 days in a random direction. All testing occurred between 1 pm and 4 pm.

The following behaviours were recorded during the test phase: latency to enter the first arm, total duration of the test phase and number of errors committed. Errors were scored as entries into unbaited arms and were further subdivided into across-phase errors (APE), within-phase errors (WPE), and total number of errors. APE were defined as entries into arms that had been entered previously during training and WPE were defined as re-entries into any arm during the test phase. An arm entry was defined as a rat traversing more than half way down the length of an arm. Testing continued until 90% of the vehicle group reached criterion performance, as defined as no more than 1 error per day for 2 consecutive days. In total, all rats completed 17 days of training.

Data Analyses

A repeated-measures analysis of variance (ANOVA) test was conducted to analyze WPE and APE, with Treatment (EB0.3, EB5, or OIL) as the between-subjects factor and Training Day (days 1-17) and Error Type (APE, WPE) as the within-subjects factors. In order to evaluate whether the hormone treatment affected how quickly the rats learned the SWSH task, the number of days to criterion was analysed with an ANOVA with Treatment as the between-subjects factor. The latency per arm choice was analysed with a repeated measures ANOVA with Days as the within-subjects factor and Treatment as the between-subjects factor. Newman-Keuls post-hoc tests were employed with significance set at 0.05. All statistical analyses were conducted with Statistica version 6.1 statistical software (StatSoft, Inc., Tulsa, OK).
Results

*Administration of EB0.3 disrupts working memory, whereas EB5 improves acquisition of the SWSh task*

Analysis of the data from Experiment 1 revealed a significant three-way interaction of Days x Error Type x Treatment (F(32, 336)=1.49, p<0.04), although post-hoc tests revealed limited effects. EB5 rats made less WPE than OIL rats on Day 1 (p<0.02), and EB0.3 rats made more WPE than OIL rats on Day 4 of testing (p<0.001) (see Figure 1.1 A), but there were no other significant differences between groups. There were no significant differences between groups on the number of APE committed (see Figure 1.1 B). There was a significant Day x Error Type effect (F(16, 336)=1.83, p<0.03) indicating that over days, rats in all groups decreased the number of WPE, and a main effect of Error Type (F(1, 21)=39.89, p<0.001) with rats making more APE compared to WPE. There was a main effect of Days (F(16, 336)=5.57, p<0.001), indicating rats decreased the number of errors over the course of training. There was also a main effect of Treatment (F(2, 21)=4.14, p<0.03), with EB0.3 rats exhibiting an increase in the total errors committed over the 17 days of training as compared to OIL and EB5 rats (all p’s<0.05; see Figure 1.2). There were no other significant interaction effects.
Figure 1.1 A: Means and standard error of the means (SEM) of WPE across 17 days (EB0.3, n=8; EB5, n=8; OIL, n=8). EB5 females made significantly fewer WPE than OIL females on Day 1 of testing, whereas EB0.3 females made significantly more WPE than OIL females on Day 4 of testing.

Figure 1.1 B: Mean number (+SEM) of APE Across 17 Days (EB0.3, n=8; EB5, n=8; OIL, n=8). There were no significant differences between the 3 groups.
Figure 1.2: Mean (+SEM) total number of errors. EB0.3 (n=8) rats committed significantly more errors than OIL (n=8) and EB5 (n=8) rats.

A separate analysis conducted on the number of training days to reach criterion revealed a significant effect of Treatment (F(2, 17)=5.74, p<0.01). Post-hoc tests revealed that the EB5 group took significantly fewer days to reach criterion as compared to both the OIL and EB0.3 groups (p<0.01 for OIL, p<0.03 for EB0.3; see Figure 1.3).
Figure 1.3: Mean number (+SEM) of days to reach criterion (EB0.3, n=8; EB5, n=8; OIL, n=8). Rats in the EB5 group reached criterion significantly sooner than rats in either other groups.

Estradiol did not significantly alter motor activity and/or motivation

There was a significant main effect of day on latency per arm choice ($F(16, 336)=1.81, p<0.03$), suggesting that as training progressed latency spent per arm decreased. There were no other significant effects (main effect of Treatment $p<0.30$; interaction effect $p<0.57$), suggesting that the effects of EB injections were not due to alterations in motor or motivational factors (see Table 1.1).
Table 1.1: Mean (+SEM) latency per arm choice (sec) across all treatment groups. There were no significant differences between the groups.

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>MEAN LATENCY/ARM (SEC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OIL</td>
<td>16.19 (+1.4)</td>
</tr>
<tr>
<td>EB0.3</td>
<td>23.95 (+3.4)</td>
</tr>
<tr>
<td>EB5</td>
<td>20.88 (+4.7)</td>
</tr>
</tbody>
</table>

Experiment 2

Subjects

Seven animals from each of the treatment groups (OIL, EB0.3 and EB5) from Experiment 1 were utilized in this experiment. Moreover, 9 ovariectomized rats that had been trained for 17 days on the SWSh task without receiving injections of hormone or oil (non-injected) were also utilized. Once trained to criterion performance on the SWSh, the rats were allowed to free-feed for 10 days before undergoing cannula implantation surgeries. The rats were housed individually in opaque cages and were maintained on a 12-hour light/dark cycle. They were given ad libitum access to water, and were food deprived to 90 % of free-feeding body weight (adjusted 3g/week for growth) following recovery from brain surgery. The treatment of animals was in concordance with the ethical standards of the Canadian Council for Animal Care.

Surgery

The rats were deeply anaesthetized with 100 mg/kg ketamine hydrochloride and 7 mg/kg xylazine, and were prepared for surgery utilizing aseptic procedures. Rats had 23-gauge stainless steel guide cannulas implanted bilaterally into either the dorsal hippocampus (n=15) or the prelimbic region of the medial PFC (PL, n=15). The
assignment of rats to either surgical condition was such that each group contained an
approximately equal representation of animals from each of the previous treatment
conditions (OIL, EB0.3, EB5, non-injected). The stereotaxic coordinates were as
follows: for the PL region, AP= + 3.0 mm from bregma, ML= ± 0.7 mm from bregma,
and DV= -2.6 mm from dura; for the hippocampus, AP= -3.3 mm from bregma, ML= ±
1.9 mm from bregma, and DV= -2.0 mm from dura (Paxinos & Watson, 1998). The
cannulas were held in place by dental acrylic cement, and 30-gauge obdurators, that were
flush with the end of the guide cannulas, were utilized to ensure that the cannulas
remained free of debris. The animals were given 10 days to recover from surgery prior to
behavioural testing.

*Microinfusion Procedure*

The doses of estradiol utilized in Experiment 2 were chosen from previous studies
which have observed significant alterations in behaviour following intracranial infusions
(Packard & Teather 1997; Frye & Rhodes 2002). Estradiol cyclodextrin was employed
for the infusions as the encapsulation of estradiol within a cyclodextrin complex increases
the solubility and metabolism of the hormone, while retaining its ability to induce
physiological changes similar to un-encapsulated estradiol (Brewster et al., 1988; Hoon et
al., 1993).

Each rat received three bilateral infusions of saline vehicle or estradiol
cyclodextrin (0.1 and 0.9μg / 0.5μl; 45 mg estradiol/1 g complex; Sigma) in a
counterbalanced manner using a quasi-Latin square design. On infusion days, the
obdurators were removed and 30 g stainless steel injectors that extended 0.8 mm beyond
the end of the cannulas were inserted. Saline or estradiol cyclodextrin infusions were
delivered to the rats via a microinfusion pump (Harvard Apparatus, Holliston, MASS) equipped with 10 μL Hamilton microsyringes (VWR, Mississauga, ON) that delivered the solutions at a rate of 0.4 μL/minute. Following each infusion, the injectors remained in the cannulas for an additional minute to ensure diffusion of solution. All infusions occurred in the behavioural testing room between 9:30 am and 11 am.

Procedure

The same radial arm maze described in Experiment 1 was used for this experiment. Once the animals had recovered from surgery, they were re-trained on the SWSH task (see Experiment 1) until they had reached criterion performance of one error or less per day for 2 consecutive days. As soon as each individual rat reached criterion, the obdurators were removed and the animals received a mock infusion where the injectors were inserted but no solution was delivered. This was done to ensure that the cannulas were free of blood and debris when the infusion was delivered. The following morning, the animals received one of 3 counterbalanced infusions and were then placed in a quiet room for a 20 min delay. Following the delay, they underwent both the training and testing phase of the SWSH task, as described in Experiment 1. At the end of the test session, the obdurators were replaced and the animal was returned to its home cage.

Once an animal had received an infusion, it was re-trained daily (i.e. without receiving a central infusion) on the SWSH task until it again achieved criterion performance, after which the rat received another infusion test day. Once an animal had received all 3 infusions, it was allowed to free feed until it was perfused, which was approximately one week following its last testing day.
Histology

The rats were deeply anaesthetized with sodium pentobarbital (Somnotol), and were intracardially perfused using 4% paraformaldehyde. The brains were extracted and remained in the paraformaldehyde solution for 48 hours, after which they were transferred to a vial containing tris-buffered saline. The brains were then frozen, cut at 40 µm sections, and stained with cresyl violet. Placements were verified using a neuroanatomical rat brain atlas (Paxinos & Watson, 1998).

Data Analyses

Repeated-measures ANOVAs were conducted separately on either errors or latency per arm choice with Brain Area (PL, hippocampus) as the between-subjects factor, and Dose (saline, E0.1, E0.9), Error Type (WPE or APE), and Day (day of infusion vs. day after infusion) as within-subjects factors. A repeated-measures ANOVA was also conducted to examine whether there was a difference between the total number of errors prior to the first infusion day (i.e. baseline performance) and the total number of errors on the day of saline infusion. This analysis was included to examine whether or not the act of infusion altered performance. Because the same animals from Experiment 1 were utilized in this study, an analysis was needed to explore whether or not treatment condition from Experiment 1 affected performance on Experiment 2. Therefore, a repeated-measures ANOVA was employed that examined total number of errors on infusion days, with Treatment condition from Experiment 1 (OIL, EB0.3 and EB5) as the between-subjects factor, and Errors committed on the 3 test days as the within-subjects factor. Newman-Keuls post-hoc tests were conducted with significance set at 0.05. All
statistical analyses were conducted using Statistica version 6.1 statistical software (StatSoft, Inc., Tulsa, OK).

Results

Histology

Of the 15 animals receiving PL cannulas, 8 were deemed to have acceptable placements. Figure 1.4 A depicts cannula placement tips in the PL region. The other 7 animals exhibited cannula placements that extended more ventrally into the infralimbic area. Six animals in the hippocampus group had acceptable placements, with cannula tips mainly targeting the CA1 region (see Figure 1.4 B). The rest of the animals in this group had cannulas that had entered the thalamus ventrally or the corpus callosum dorsally, and therefore were excluded from analyses.
Figure 1.4 A and B: Figural representation of cannula placements in the PL region (1.4 A) and hippocampus (1.4 B). Black dots indicate targeted areas (adapted from Paxinos & Watson, 1998).
Intracranial saline infusions caused a moderate disruption in performance on the delayed SWSh task

Analysis of the numbers of errors committed on the day prior to the first infusion and on saline infusion test days revealed that saline infusions caused a moderate, but statistically significant increase in the number of errors relative to those made on the day prior to infusion as evidenced by a significant main effect of Day (F(1, 12)=9.07, p<0.01; see Figure 1.5) There was no significant main effect of Brain region (PFC or hippocampus, p≤0.25) nor was there an interaction effect (p≤0.49).

Figure 1.5: Mean number of total errors (+SEM) committed on the day prior to a rat’s first infusion (saline, E0.1 or E0.9) and on the day of saline infusion (n=14). Rats made significantly more errors following saline infusions.
Figure 1.6 A: Mean number of total working memory errors (+SEM) on the days of infusions in the PL group (n=8). Rats made significantly fewer errors when they received E0.9 than on the day of saline infusion.

Figure 1.6 B: Mean number of total working memory errors (+SEM) on the days of infusions in the hippocampus group (n=6). Rats made significantly fewer errors when they received E0.1 than on the days of saline infusions.
**Figure 1.7 A:** Mean number of total working memory errors (+SEM) on the days after infusions in the PL group (n=8). There were no significant differences in performance between the doses.

**Figure 1.7 B:** Mean number of total working memory errors (+SEM) on the days after infusions in the hippocampus group (n=6). Rats made significantly more errors on the days after both estradiol infusions as compared to the day after saline infusions.
E0.9 infused into the PL region and E0.1 infused into the hippocampus decreased the number of working memory errors relative to saline infusions

Analysis of the total number of errors committed following infusions into the PL region and hippocampus revealed a significant three-way interaction (Dose x Days x Brain Region; F(2, 24)=3.93, p<0.03). Within the PL group, post-hoc analyses revealed that on the day of infusion, animals made significantly fewer errors when they received E0.9 than when they received saline or E0.1 (p<0.05 for saline, p<0.02 for E0.1; see Figure 1.6 A). In addition, there were no differences among treatment conditions in the number of errors committed on the day following infusion test days (see Figure 1.7 A).

Post-hoc analyses conducted on the data from rats receiving infusions of estradiol into the hippocampus showed that on infusion test days, rats made significantly less errors with E0.1 as compared to saline and E0.9 (p<0.03, p<0.02, respectively; see Figure 1.6 B). When the performance of the hippocampus animals on the days after the infusions was analyzed, rats made significantly more errors following infusions of either E0.1 or E0.9 relative to the number of errors made on the day following saline infusions (p<0.005 for E0.1, p<0.004 for E0.9; see Figure 1.7 B).

There was also a significant three-way interaction of Dose x Error Type x Brain region (F(2, 24)=7.98, p<0.002). Post-hoc analysis revealed that in the hippocampus group, the animals made more APE on infusion test days when they received E0.9 versus the number of APE committed following saline infusions (p<0.003). There were no significant differences in WPE between the three infusions in the hippocampus group. Moreover, for rats receiving infusions of estradiol into the PL region, there were no significant differences in WPE or APE across the three infusions.
There were no other significant interactions (for Dose x Days x Error x Brain region p<0.06; Dose x Days x Error p<0.22; Days x Error x Brain region p<0.22; Day x Error p<0.86; Dose x Error p<0.08; Dose x Days p<0.13; Errors x Brain region p<0.22; Days x Brain region p<0.26; Dose x Brain region p<0.29). There was also a significant main effect of Error, with animals making more APE than WPE (F(1, 12)=31.33, p<0.0001). There were no other significant main effects (Days p<0.98; Dose p<0.80; Brain region p<0.60).

Motor activity and/or motivation were not altered by the infusions

Latency per arm choice was analyzed as a measure of motor activity and motivation. An ANOVA revealed that there were no significant main or interaction effects (Brain region, p<0.55; Days, p<0.48; interaction effect, p<0.98) (see Table 1.2). This suggests that infusions did not significantly affect motor activity and/or motivation.

Table 1.2: Mean (+SEM) for latency per arm choice (sec) across all treatment conditions. There were no significant differences between infusion doses or between brain regions.

<table>
<thead>
<tr>
<th>DOSE</th>
<th>DAY OF INFUSION</th>
<th>DAY AFTER INFUSION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PL Region</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SALINE</td>
<td>16.3 (+1.8)</td>
<td>18.9 (+2.5)</td>
</tr>
<tr>
<td>E0.1</td>
<td>16.6 (+2.6)</td>
<td>17.9 (+2.9)</td>
</tr>
<tr>
<td>E0.9</td>
<td>18.6 (+3.6)</td>
<td>16.7 (+2.8)</td>
</tr>
<tr>
<td><strong>Hippocampus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SALINE</td>
<td>14.3 (+2.0)</td>
<td>18.2 (+2.9)</td>
</tr>
<tr>
<td>E0.1</td>
<td>13.2 (+3.0)</td>
<td>15.1 (+3.4)</td>
</tr>
<tr>
<td>E0.9</td>
<td>16.1 (+4.1)</td>
<td>15.9 (+3.2)</td>
</tr>
</tbody>
</table>
Treatment condition during acquisition did not affect Experiment 2 performance

The possible influence of previous treatment condition during acquisition (i.e. OIL, EB0.3, EB5, or non-injected) was explored. For rats in the PL group, a repeated measures ANOVA revealed no significant main effects of Treatment condition (p<0.43) Day (p<0.10), or significant interaction effect (p<0.81). Similarly, for rats in the hippocampus group, there were no significant main effects of Treatment condition (p<0.84) or Day (p<0.49), nor was there a significant interaction (p<0.90). These data suggest that previous hormone treatment did not affect working memory performance in Experiment 2.

Discussion

The purpose of the current study was to investigate estradiol’s influence on hippocampal- and PFC-dependent spatial working memory. The results from Experiment 1 demonstrated that systemic administration of a high dose of estradiol (EB5) resulted in enhanced acquisition of the SWSH task relative to both control and low estradiol (EB0.3) treatments. Moreover, rats receiving low estradiol made more working memory errors overall than rats in either other treatment condition. When site-specific influences of estradiol were examined in Experiment 2, we found that E0.9 facilitated working memory when infused into the PL area and that E0.1 facilitated working memory when infused into the dorsal hippocampus, indicating that direct infusion of this hormone into either brain region can augment working memory, although at different doses at either site. Interestingly, there was also a delayed effect estradiol infusions into the hippocampus. Twenty-four hours following E0.1 and E0.9 infusions, animals in the hippocampus group made more errors compared to the day after saline infusion, possibly indicating that
genomic alterations in hippocampal functioning may have contributed to these impairments. Lastly, neither systemic injections nor central infusions of estradiol or vehicle significantly impacted the latency to enter each arm, suggesting that the preceding data are a result of estradiol influencing learning and memory processes and not motivational or motoric processes.

Experiment 1

High physiological levels of estradiol enhanced acquisition of the SWSh task

Rats in the EB5 group took significantly fewer days to reach criterion than rats in the EB0.3 and OIL groups. It is important to note that the facilitation of performance following treatment with EB5 was confined to the number of days to reach criterion, and not the total number of errors committed over the 17 days of training. Intriguingly, this facilitated acquisition in response to high estradiol is inconsistent with previous research that has demonstrated no influence of estradiol on acquisition of working memory tasks (Holmes et al., 2002; Wide et al., 2004). The discrepancy between studies may be due to a number of parameters, including task demands. For example, Holmes et al. (2002) utilized a hippocampus-dependent working/reference memory version of the radial arm maze. This task differs from the SWSh task used in the present study in that it does not utilize a delay, but does have an additional reference memory component. Wide et al. (2004) utilized a PFC-mediated delayed alternation task on a T-maze with delays between training and testing, but the delays were very short (i.e. 10 and 40 seconds) and the task was non-spatial in nature. Thus, data from Experiment 1 support the idea that the modulation of systemic estradiol over working memory functions is dependent on which
type of task that is being used, as well as the underlying neural circuits that are engaged under these conditions.

*Low systemic estradiol impaired working memory performance*

R rats administered a low dose of estradiol displayed increased working memory errors as compared to oil controls and rats receiving high estradiol. This is in contrast to previous research that exhibited that low estradiol facilitates working memory performance on hippocampus- or PFC-mediated working memory tasks (Daniel et al., 1997; Holmes et al., 2002; Wide et al., 2004). Once again, the incongruity between studies may reflect differential task demands that are associated with the neural circuitry subserving working memory on each task. Interestingly, the magnitude of dopamine release in the PFC is related to the accuracy of working memory performance on the SWSH task (Phillips et al., 2004), and there is evidence to suggest that long-term exposure to diestrus levels of estradiol results in low levels of dopamine in this brain area (Luine et al., 1998). Reduced dopamine in the PFC also implies low levels of D1 receptor activation, and hence impairments in working memory performance (Arnsten, 1988). Thus EB0.3 may have impaired working memory on the SWSH task by decreasing dopamine activity in the PFC.

*Experiment 2*

*Estradiol facilitated performance at different doses when infused directly into the PL region and dorsal hippocampus*

A higher dose of estradiol infused into the PL region of the PFC and a lower dose of estradiol infused into the hippocampus exerted similar facilitations in working memory performance relative to saline treatment. This finding reveals that estradiol can alter
working memory at different doses in different brain areas, and may also help to explain the different patterns of behaviour observed with systemic estradiol during the acquisition of the SWSh task. For example, the infusion data may reflect the relative contribution of high systemic estradiol (EB5) in the PFC and hippocampus as rats receiving this dose of estradiol exhibited enhanced acquisition of the SWSh task. Moreover, these data further support the conclusion that the influences of systemic estradiol on different types of cognition are dependent on the brain region(s) that this hormone may be acting on. For example, working memory is facilitated with systemic low doses of estradiol and is impaired with systemic high doses of estradiol in hippocampus-mediated tasks (Holmes et al., 2002). However, similar treatments do not show as pronounced an effect on a working memory task mediated by the PFC, and these effects appear to depend on the strength of the memory trace (Wide et al., 2004). In the present study, systemic administration of estradiol exerted moderate and dose-dependent effects on a task mediated by both neural structures, and also had differential effects when infused into either the PFC or the hippocampus.

In the PFC, a higher dose of estradiol was required to enhance working memory, suggesting that E0.1 was either too low to activate estrogen receptors, or that receptor activation induced by this dose was insufficient to alter working memory processes. The finding that E0.1 facilitated working memory performance in the hippocampus suggests that there is an optimal level of estrogen receptor activation in the hippocampus required for optimal working memory performance, with too much or too little activation resulting in null or impaired effects. Furthermore, this result may also indicate differing roles for estrogen receptors, as ERβ is the only ER in the PFC (Shughrue et al., 1997). Thus, these
data support the notion that estradiol's ability to alter behaviour is dependent on dose, neuroanatomical location on which the behaviour relies, and possibly estrogen receptor subtype.

It is notable that the lack of facilitation following infusions of E0.9 into the hippocampus is somewhat inconsistent with earlier research. Two previous studies have found that similar high doses of estradiol infused into the hippocampus facilitated active avoidance learning when administered prior to training (Frye & Rhodes, 2002), and enhanced the retention of the Morris water maze when infused after training (Packard & Teather, 1997). Thus, the present data, in combination with these previous studies further highlight the differential effects that estradiol may exert on cognition. Moreover these data suggest that the nature of these effects are dependent on a multitude of factors including dose of estradiol administered, the timing of the administration (i.e. pretraining, posttraining, pretest) and type of learning task being used (working memory, reference memory, conditioning).

*Estradiol protected against infusion-induced working memory impairments*

When discussing the improvements in performance of the SWSH task following infusion of estradiol into either the PFC or hippocampus, it is important to note that infusions of saline vehicle into both the PL region and the hippocampus caused a modest yet statistically significant increase in the number of errors made during the test phase relative to the number of errors made on the day prior to the first infusion, where no treatment was administered. This result implies that the act of infusion itself can have a somewhat disruptive effect on performance of the SWSH task. Thus, the improvements in performance relative to saline treatments following infusions of estradiol cyclodextrin
may have been due partially to neuroprotection against the disruptive actions of the infusion, albeit at different doses in different sites (i.e. E0.1 for the hippocampus rats, E0.9 in the PL rats). This is in concordance with the idea that estradiol is neuroprotective against a variety of insults, such as ischemia, glutamate-induced neurotoxicity, hypoglycaemia, the accumulation of β-amyloid plaques, ATP depletion, and free radicals (Simpkins et al., 1997; Zhang et al., 1998; Wang et al., 2001; Fitzpatrick et al., 2002; Ritz et al., 2002; Eberling et al., 2004; Sribnick et al., 2004). Although neuroprotection appears to occur mainly through the genomic actions of estradiol acting on ERβ (Barkhem et al., 1998; Simpkins et al., 1997), the protective influence of estradiol in the current study occurred 40 minutes following the infusions. This finding suggests that the actions of estradiol may be attributed to non-genomic actions of this hormone (McEwen, 2001). Indeed, estradiol-induced stimulation of the mitogen activated protein kinase pathway has been linked to neuroprotection and can occur in cultured hippocampal neurons within 30 minutes of hormone exposure (Singer et al., 1999; Kelly & Levin, 2001; Wade & Dorsa, 2003).

_Estradiol significantly impaired working memory 24 hours after infusion into the dorsal hippocampus_

Infusions of either E0.1 or E0.9 impaired working memory 24 hours following infusions into the hippocampus compared to saline infusions. This effect was selective to the hippocampus, as similar infusions into the PL region did not have the same effect. This delayed impairment indicates that estradiol may have altered hippocampal function through alterations in gene transcription (McEwen, 2001). These dissociable effects of estradiol infusions into the PL region versus the hippocampus may be related to estrogen
receptor distribution and function. Nuclear estrogen receptors, in particular ERα, are co-localized with GABAergic interneurons in the dorsal hippocampus (Weiland et al., 1997; Murphy et al., 1998; Hart et al., 2001). Although estradiol initially suppresses GABAergic activity (Rudick & Woolley, 2001), evidence suggests that estradiol may act through genomic mechanisms to subsequently enhance inhibitory tone. For example, elevated protein and mRNA levels of glutamate decarboxylase 65 and the cation chloride cotransporter NKCC1, both of which promote activation GABA_A receptor, are observed in the dorsal CA1 24 hours after systemic estradiol injection (Sun & Murali, 1999; Nakamura et al., 2004). GABA_A agonists infused directly into the hippocampus negatively impacts spatial working memory (McElroy & Korol, 2005). Therefore, estradiol cyclodextrin infusions into the hippocampus may have increased GABAergic activity over a 24-hour period via genomic mechanisms through ERα, which then resulted in impaired SWSh performance. Because ERβ is the exclusive estrogen receptor in the PFC (Shughrue et al., 1997; Krtizer, 2002), it is proposed that estradiol infusions into the PL region did not stimulate similar genomic mechanisms in this area as ERα stimulation did in the hippocampus. Indeed, previous research has revealed that anti-estrogens can act as agonists or antagonists depending on whether they act at ERα or ERβ (McDonnell et al., 1995; Barkhem et al., 1998), suggesting that stimulation of either receptor with the same substance results in distinct influences on cellular processes. Thus, differential genomic activity of each estrogen receptor may account for why no changes in working memory performance were observed 24 hours following estradiol infusions into the PL region.
Conclusions

The results of these experiments suggest that estradiol can influence both the acquisition of a spatial working memory task, as well as the expression of working memory in well-trained animals. Acquisition of the SWSh task was enhanced by long-term exposure to high estradiol, whereas low estradiol increased the number of working memory errors relative to oil control. Examination of site-specific influences of estradiol in well-trained rats revealed that estradiol protects against the adverse effects of infusions when administered 40 minutes prior to testing. Moreover, these effects were induced with a lower dose of estradiol cyclodextrin in hippocampus rats but with a higher dose in PL rats. Intriguingly both doses of estradiol cyclodextrin delivered to the hippocampus, but not the PL region, impaired working memory 24 hours later, suggesting an inhibitory effect of estradiol on working memory via gene transcription. These data suggest that estradiol has dose, time, and site-specific influences on working memory processes. Future research is required to explore whether estradiol is acting at nuclear or membrane-bound receptors, and whether or not these site-specific influences translate to other working memory tasks.
Chapter 2: Time Dependent Activation of pCREB in the Dorsal CA1 and Medial Prefrontal Cortex: Effects of Long-Term Estradiol Treatment

Estradiol, the most potent estrogen, has diverse influences beyond assisting reproduction. Numerous studies suggest that estradiol has neuroprotective effects and can benefit cognition in older men and women (Simpkins et al., 1997; Kavas et al., 1997; Sherwin, 2003). Estradiol may be mediating these effects either through gene transcription or through rapid non-genomic mechanisms. For example, estradiol can increase intracellular calcium concentration in cultured hippocampal neurons within 50 seconds, stimulate nitrous oxide formation within 40 seconds, and can vary the activity of GTPases within minutes (Stefano et al., 1999; Zhao et al., 2005). Estradiol can also rapidly activate G-coupled proteins (Kelly & Wagner, 1999), second messengers cAMP and calcium (Aronica et al., 1994), and a number of downstream kinases such as protein kinase A (PKA), PKB (or Akt), mitogen activated protein kinase (MAPK) and associated kinases ERK1/2, and calcium/calmodulin-dependent kinases (e.g. CaMKII) (Spaulding, 1993; Wade & Dorsa, 2003; Lee et al., 2004; Abraham et al., 2004). These hormone-induced alterations in cellular signalling do not rely on de novo synthesis of RNA or proteins (Nabekura et al., 1986; Aronica et al., 1994; Zhao et al., 2005), and have been shown to ultimately lead to the activation of cAMP response element-binding protein (CREB).

CREB is a leucine zipper transcription factor that binds to cAMP enhancer elements (CREs) in the promotor region of cAMP responsive genes (Meyer & Habener, 1993). CREB is activated via phosphorylation by various kinases (i.e. PKA, CaMK, MAPK) at Ser^{133} located in the kinase inducible domain or P-Box of the CREB molecule.
Phosphorylated CREB (pCREB) form dimers that bind to CRE sites on the promoter region of genes, which results in either increased or decreased transcription (Brindle et al., 1993; Meyer & Habener, 1993). Thus, through the rapid induction of protein kinases, estradiol can influence transcription in cells that lack estrogen response elements (i.e. sequences on the DNA of target cells that ligand-activated estrogen receptors recognize and bind to) (Pfaff & McEwen, 1983; Alexander et al., 1989). The effects of estradiol on pCREB and associated upstream signalling pathways occur very quickly, with effects noted within 10-30 minutes in vitro (Aronica et al., 1994; Wade & Dorsa, 2003; Zhao et al., 2005), and 15-30 minutes in vivo (Gu et al., 1996; Abraham et al., 2003; Abraham et al., 2004). In vivo, pCREB activation in response to high doses of estradiol has been observed in the hypothalamus, medial septum, bed nucleus of the stria terminalis, amygdala, and in the hippocampus (Gu et al., 1996; Zhou et al., 1996; Murphy & Segal, 1997; Carlstrom et al., 2001; Abraham et al., 2003; Wade & Dorsa, 2003; Lee et al., 2004; Abraham et al., 2004; Abraham & Herbison, 2005). Research with cultured hippocampal neurons has exhibited that estradiol-mediated increases in pCREB depend on the availability of extracellular calcium, PKA, CaMKII and MAPK (Murphy & Segal, 1997; Wade & Dorsa, 2003; Lee et al., 2004; Zhao et al., 2005), and with exception to the cAMP/PKA pathway, similar conditions are thought to mediate estradiol-induced pCREB expression in hippocampal neurons in vivo (Carlstrom et al., 2001).

Although controversy surrounds the roles of estrogen receptors (ERs) in the phosphorylation of CREB, many researchers agree that different kinds of ERs may be
involved. There is evidence that nuclear and cytoplasmic ERα and ERβ influence G-protein coupling, 2nd messenger cascades, rapid actions on MAPK/ERK signalling, and pCREB activation (Singer et al., 1999; Kelly & Levin, 2001; Song et al., 2002; Wade & Dorsa, 2003; Abraham et al., 2004). Conversely, estradiol may exert its actions on CREB via binding to plasma membrane ERs (Kelly & Levin, 2001). Estradiol acting at these receptors rapidly leads to the activation of G-proteins, calcium influx, cellular signalling pathways such as PKA and PKC, and may regulate gene expression indirectly via second messenger DNA binding proteins, such as CREB (Meyer & Habener, 1993; Kelly & Wagner, 1999; Kelly & Levin, 2001; Zhao et al., 2005). Lastly, some researchers suggest that estradiol may act directly on voltage-gated ion channels to alter calcium influx, which in turn activates CaMKs and pCREB expression (Zhou et al., 1996). Thus, there are multiple ways in which estradiol can activate CREB and CRE-associated gene transcription.

There appears to be a strong association between estradiol, pCREB, and processes occurring in and/or mediated by the hippocampus. For example, both estradiol and pCREB have been implicated in the formation and retention of long-term hippocampal-dependent learning and memory (Guzowski & McGaugh, 1997; Bourutchuladze et al., 1994; Packard & White, 1997) and in the survival of adult-generated neurons in the dentate gyrus (Nakagawa, 2002; Ormerod et al., 2004). Activation of pCREB and associated upstream kinases in the hippocampus are necessary steps preceding estradiol-induced stimulation of dendritic spines in CA1 pyramidal neurons (Murphy & Segal, 1997; Matus et al., 2000; Lee et al., 2004; Zhao et al., 2005). In vivo examination of estradiol’s influence on pCREB in the rat hippocampus has mainly involved the
utilization of supraphysiological doses of estradiol, and only one of these studies has examined the effects of long-term estradiol on pCREB activity (i.e. Carlstrom et al., 2001). Therefore, the purpose of the following experiment was to examine long-term exposure of physiological levels of estradiol on pCREB expression in the hippocampus and in one of its projection sites, the prefrontal cortex (PFC). Both the dorsal hippocampus and the medial PFC contain nuclear and non-nuclear ERs, with ERα and ERβ expressed in the hippocampus and ERβ expressed in the PFC (Shughrue et al., 1997; Blurton-Jones & Tuszynski, 2002; Zhang et al., 2002). Thus, it was hypothesized that estradiol would induce dose-dependent and time-dependent alterations in pCREB expression in both neural areas.

**Method**

*Subjects*

Thirty 3-month old female Long-Evans rats (Charles River, QC, Canada) weighing between 300 g and 350 g were used. The rats were housed individually in opaque cages and were maintained on a 12-hour light/dark cycle. They were given ad libitum access to food (Lab Diet #5012, Jameison, Richmond, BC) and water throughout the experiment. Animal protocols were in concordance with the ethical standards of the Canadian Council for Animal Care.

*Surgery*

One week following arrival to the facilities, the rats were bilaterally ovariectomized utilizing aseptic procedures. They were anaesthetized via inhalation of halothane gas and oxygen (4% halothane for the induction, 2% for maintenance), and the level of anaesthesia was monitored throughout the surgeries and was adjusted as
necessary. All rats were allowed to recover from surgery for 10 days before the commencement of experimental procedures.

Procedures

Once the rats had recovered from surgery, they were handled for five minutes daily over the course of seven days. They were then randomly assigned to a treatment group where each rat received daily subcutaneous injections of either 0.1 mL sesame oil (OIL; Sigma, St. Louis, MO, n=10) or estradiol benzoate (EB; Sigma), at doses of 0.3 μg EB/0.1 mL sesame oil (EB0.3, n=10) or 5 μg EB/0.1 mL sesame oil (EB5, n=10). The EB0.3 dose was chosen as it yields serum estradiol levels that are similar to those observed in animals during diestrus (~20-30 pg/mL), and the EB5 dose was chosen as it yields serum estradiol levels similar to those observed during proestrus (~60-90 pg/mL) (Butcher et al., 1974; Holmes et al., 2002). All injections occurred between 10 am and 11 am, and all rats received one injection per day, for a total of 17 injections.

On the 17th injection day, the rats were randomly assigned a time of perfusion: 40 min (40 MIN), 4 hours (4 HRS), or 24 hours (24 HRS) following their last injection. Rats were deeply anaesthetized with sodium pentobarbital (Somnotol), and were intracardially perfused with 120 mL 0.9 % saline and 120 mL of chilled 4 % paraformaldehyde in 0.1 M phosphate buffered saline. Prior to fixation, blood samples originating from the right atrium of the heart were collected into chilled Eppendorf tubes and were stored at 4°C for 24 hours. The samples were then centrifuged and the supernatant was removed and placed in a –20°C freezer until assay. The brains were extracted and remained in the paraformaldehyde solution for 4 hours, after which they were transferred to a vial containing potassium phosphate-buffered saline (KPBS). The
brains were then frozen and 20 μm sections were cut on the coronal plane through the prelimbic region (PL) of the medial prefrontal cortex (3.2 mm to 2.7 mm from Bregma) and through the dorsal CA1 (dCA1) and dorsal CA3 (dCA3) of the hippocampus (both areas, -2.92 mm to -3.6 mm from Bregma) (Paxinos & Watson, 1998). Every slice through these brain areas was collected into chilled KBS until further processing.

**Immunohistochemistry and Analyses**

For each rat, every 6th slice from the PL region, dCA1 and dCA3 were utilized for immunohistochemical procedures. Briefly, free-floating sections were incubated at room temperature (RT) in 0.3 % H₂O₂ for 10 minutes and then rinsed with KPBS. This was followed by a 5-minute incubation at RT in sodium borohydride and then a rinse in KPBS. Anti-rat pCREB primary antisera specific for CREB phosphorylated on Ser¹³³ (raised in rabbit, 1:4000; Upstate Biotechnology, Lake Placid, NY) and normal goat serum were added to the tissue and incubated at 4°C under constant agitation for 48 hours. The sections were then rinsed in KPBS and incubated at RT for 60 minutes in biotinylated (goat) anti-rabbit IgG secondary antiserum (Vector Laboratories, Burlingame, CA). The tissue was rinsed in KBS and incubated in an Avidin Biotin Complex (Vector) for 60 minutes at RT. Lastly, the sections were visualized for antibody labelling using diaminobenzidine (Sigma) and nickel ammonium sulphate (Fisher, Nepean, ON).

For quantification of pCREB-positive nuclei (pCREB+), images of each brain section were taken at a 40x objective with a Nikon Eclipse E600 light microscope (Nikon Corp., Mississauga, ON) that was equipped with a Nikon Digital Camera DXM1200 (Nikon). Nikon ACT-1 version 2.20 software (Nikon) was used to capture the images
onto a PC computer. All quantification of bilateral cell counts was conducted with Simple PCI version 5.1 imaging software (Compix Inc., Imaging Systems, Cranberry Township, PA). In the dCA1 and dCA3, 4 slices were analyzed per brain and pCREB+ cells were quantified along the entire length of the principle cell layer. pCREB was also examined in two sections containing the PL region, and cells were quantified across all layers of cortex. The area of each structure on each slice was also recorded, and thus all pCREB+ counts are expressed as per unit of area (mm$^2$). Lastly, cell counts in each brain area were summed, and statistical analyses were conducted on the mean number of cells/mm$^2$.

**Hormone Assays**

Blood samples were analyzed for estradiol content using a 17β-Estradiol Enzyme-Linked Immunosorbent Assay (ELISA) kit (Research Diagnostics, Flanders, NJ). On the evening prior to assay, the serum was defrosted overnight at 4°C. Because the ELISA estradiol kit was designed for analysis of human serum, extraction and enrichment of rat sera was performed (as adapted from Hany et al., 1999). Briefly, 1 mL of diethyl ether was added to 230 μL of serum. The solution was centrifuged for 5 minutes at 3800 rpm, placed in a −80°C freezer for 20 minutes, and the ether supernatant was decanted and evaporated to 100 μL. This procedure was repeated once, and the extracts were combined. 75 μL of steroid-free human serum was added to the extracts, which were then centrifuged for 5 minutes at 3800 rpm. The remaining ether was evaporated, and 50 μL of the final serum sample was utilized for analysis. The ELISA estradiol kit employed was based on the competition principle. That is, the serum samples competed with a fixed amount of estradiol conjugated with horseradish peroxidase for binding sites.
of a polyclonal estradiol antiserum coated onto the wells of the ELISA microplate during a two-hour incubation. This was then followed by a wash with assay buffer provided by the kit, then the addition of a substrate solution. A stop solution was then added to the samples to stop the competition reaction. The absorbance of each well was determined at 450±10 nm with a microwell reader. The sensitivity of the assay was 4.6 pg/mL. This assay has been shown to exhibit 100% cross-reactivity with estradiol, 0.2 % cross-reactivity with estrone, 0.05% cross-reactivity with estriol, and 0% cross-reactivity with the 17α isoform of estradiol. The intra-assay coefficient of variation was approximately 2 %.

Data Analyses

The number of pCREB+ cells/mm² was analyzed using a repeated-measures analysis of variance (ANOVA) with Brain Area (PL, dCA1, dCA3) as the within-subjects factor, and Time of perfusion (40 MIN, 4 HRS and 48 HRS) and Treatment (OIL, EB0.3 and EB5) as the between-subjects factors. Serum estradiol levels obtained from the ELISA analysis were analyzed with a factorial ANOVA with Treatment (OIL, EB0.3 and EB5) and Time of perfusion (40 MIN, 4 HRS and 48 HRS) as the between-subjects factors. Post-hoc analyses were performed using Newman-Keuls tests with significance set at 0.05. All statistical analyses were conducted using Statistica version 6.1 statistical software (StatSoft, Inc., Tulsa, OK).
Figure 2.1: Representative slices exhibiting pCREB activation in the dCA1, dCA3, and PL region at 40 minutes (A-C), 4 hours (D-F), and 24 hours (G-I) after the last injection.

Time-Dependent Alterations in pCREB Activation

Figure 2.1 depicts pCREB+ cells in representative samples of the dCA1, dCA3 and PL region, and immunolabeling revealed a nuclear staining pattern throughout the coronal sections. The total number of pCREB+ cells/mm² is presented in Figure 2.2 for the dCA1, Figure 2.3 for the dCA3, and Figure 2.4 for the PL region. Post-hoc tests revealed that in the PL region, animals perfused at 4 HRS exhibited significantly less pCREB+ cells/mm² than animals perfused at 40 MIN (p<0.02) (interaction of Brain Area x Time, F(4, 38)=9.51, p<0.00002). Animals perfused at 24 HRS did not significantly differ from animals perfused at 40 MIN (p≥0.45) or 4 HRS (p≥0.14) in the PL region. In the dCA1, post-hoc analyses revealed that there were a greater number of pCREB+ cells/mm² at 40 MIN than at both 4 HRS (p<0.0002) and 24 HRS (p<0.001), and
significantly more cells at 24 HRS than at 4 HRS (p<0.001). In the dCA3, there were fewer pCREB+ cells at 4 HRS than at both 40 MIN (p<0.01) and 24 HRS (p<0.048). The number of pCREB+ cells at 40 MIN and at 24 HRS in the dCA3 did not differ significantly from one another (p≤0.59). There were no other significant interactions (Brain Area x Treatment x Time p≤0.89; Brain Area x Treatment p≤0.57; Treatment x Time p≤0.35). There was a significant main effect of Brain Area (F(1, 19)=152.95, p<0.0001) and Time (F(2, 19)=40.4, p<0.0001) but no significant main effect of treatment (p≤0.14). Because a priori we wanted to determine whether or not physiological doses of estradiol could enhance pCREB levels compared to control, a Dunnett's test was performed. A non-significant trend was discovered when pCREB+ cells in EB0.3 rats were compared with OIL rats (p≤0.06), suggesting that EB0.3 may decrease the level of pCREB immunoreactivity relative to control.

Figure 2.2: Mean number of pCREB+ cells/mm² (+SEM) in the dCA1. Rats perfused at 40 MINS (n=6) exhibited significantly more pCREB+ cells than rats perfused at 4 HRS (n=12) and 24 HRS (n=12), and rats perfused at 24 HRS exhibited significantly more pCREB+ cells than rats perfused at 4 HRS.
**Figure 2.3:** Mean number of pCREB+ cells/mm² (+SEM) in the dCA3. Rats perfused at 4 HRS (n=12) exhibited significantly fewer pCREB+ cells than rats perfused at 24 HRS (n=12) and 40 MIN (n=6). Rats in the 24 HRS and 40 MIN groups did not significantly differ from one another.

**Figure 2.4:** Mean number of pCREB+ cells/mm² (+SEM) in the PL region. Rats perfused at 4 HRS (n=12) exhibited significantly fewer pCREB+ cells than rats perfused at 40 MINS (n=6). Rats in the 24 HRS (n=12) group did not significantly differ from rats in the 40 MIN or 4 HRS groups.
Hormone Analysis

EB5 females exhibited proestrus concentrations of serum estradiol (~60-90 pg/mL; Butcher et al., 1974; Piva et al., 1995) at all time points, EB0.3 females exhibited diestrous concentrations of serum estradiol (~20-30 pg/mL; Butcher et al., 1974; Piva et al., 1995) at the 4 HRS and 24 HRS time points, and OIL females exhibited levels of estradiol that were close to the sensitivity of the assay (see Table 2.1). There was a main effect of Treatment (F(2, 21)=120, p<0.0001), and post-hoc tests revealed that EB5 females had significantly higher plasma estradiol levels than both EB0.3 and OIL females (p’s<0.0001), and EB0.3 females exhibited significantly higher levels of plasma estradiol than OIL females (p<0.0003). Although not significant, there was a trend for rats in the EB5 group to exhibit elevated estradiol levels at all perfusion time points, and for EB0.3 rats to exhibit levels of estradiol that were less than EB5 rats but greater than OIL rats at all time points (interaction effect, p<0.06). Moreover, there was also a non-significant trend for animals perfused at 4 hours to have higher plasma estradiol levels than animals perfused at 24 hours or 40 minutes after the last injection (p<0.07).
Table 2.1: Mean (±SEM) of plasma estradiol levels in each group (pg/mL). EB5 females (n=10) exhibited significantly higher concentrations of serum estradiol than both EB0.3 (n=10) and OIL (n=10) females, and EB0.3 females exhibited significantly higher levels of serum estradiol than OIL females.

<table>
<thead>
<tr>
<th></th>
<th>40 MINS</th>
<th>4 HRS</th>
<th>24 HRS</th>
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<tbody>
<tr>
<td>OIL</td>
<td>5.5 (±0.5)</td>
<td>5.5 (±0.9)</td>
<td>4.5 (±0.5)</td>
</tr>
<tr>
<td>EB0.3</td>
<td>10 (±5.9)</td>
<td>30.5 (±4.5)</td>
<td>23.7 (±1.3)</td>
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<tr>
<td>EB5</td>
<td>68.7 (±6.3)</td>
<td>69.4 (±2.8)</td>
<td>53.6 (±9.2)</td>
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Discussion

The results of the current study suggest that long-term administration of physiological doses of estradiol do not significantly influence pCREB levels in either the dorsal CA1 or CA3 of the hippocampus or in the PL region of the PFC. Although not statistically significant, the direction of the means favoured a lowering response of pCREB activation with estradiol, particularly at the low dose of EB0.3. Differences in pCREB activation were found to depend on when the animals were perfused, regardless of condition. In the PL region, fewer pCREB+ cells were found to occur at 4 hours as compared to 40 minutes following the last injection. pCREB+ cells in the dCA1 were highest 40 minutes after the last injection, were decreased at 4 hours, and then increased again at 24 hours. In the dCA3, fewer cells were observed at 4 HRS as compared to 40 MIN and 24 HRS.
Physiological levels of estradiol do not influence the number of pCREB+ cells

Seventeen days of EB0.3 and EB5 injections produced similar levels of pCREB activation as OIL injections did at all three perfusion time points. This is in contrast to Carlstrom et al. (2001) who exhibited that 14 days of supraphysiological levels of estradiol enhanced pCREB activity in the CA1 24 hours after the last injection. However, with the exception of Carlstrom et al. (2001), previous studies exhibiting in vivo effects of estradiol on pCREB activation have utilized one injection of estradiol (Gu et al., 1996; Abraham et al., 2003; Abraham et al., 2004). There is precedent to suggest that the acute effects of estradiol on the brain are quite distinct from alterations induced by chronic injections of estradiol. For instance, acute administration of estradiol enhances spine density of hippocampal neurons and cell proliferation in the dentate gyrus (Gould et al., 1990; Miranda et al., 1999; Hao et al., 2003; Tanapat et al., 2005), whereas chronic injections of estradiol have no significant influence on either of these processes (Miranda et al., 1999; Tanapat et al., 2005). Differences between acute and chronic effects of estradiol may be related to alterations in the number/density of ERs. That is, long-term exposure to estradiol may have led to a downregulation of ERs, thus decreasing the number of sites that the hormone can act on to exert its rapid effects.

Indeed, both chronic injections and implants of estradiol have been shown to create a dose-dependent decrease in ER mRNA levels (Lauber et al., 1991; Medlock et al., 1991); an effect attributed to the long-lasting genomic actions of activated ERs (Evangelatou & Farrant, 1995). In the early phases of receptor downregulation (i.e. within 4 hrs), intracellular phosphodiesterase levels increase (Kobilka, 1992; Spaulding, 1993), which in turn can lead to decreased stimulation of protein kinases required for estradiol-
mediated pCREB activation such as PKA, CaMKII and MAPK (Murphy & Segal, 1997; Wade & Dorsa, 2003; Lee et al., 2004). The later phase of downregulation (i.e. after around 14 hours of exposure to the agonist) is mediated by a reduction in receptor biosynthesis, which can occur as a result of decreased transcription (Kobilka, 1992). Thus it is proposed that the multiple injections of estradiol in the current study may have led to the downregulation of ERs, which may have resulted in attenuated hormone-induced increases in pCREB and/or been the reason for a non-significant trend for a decrease in pCREB in response to chronic EB0.3.

Unlike the current study that utilized physiological doses of estradiol, Carlstrom et al. (2001) used a supraphysiological dose of estradiol and found enhanced pCREB in the hippocampus after long-term (14 day) treatment. The supraphysiological dose is often employed to optimize the detection of estradiol on cellular processes (Gu et al., 1996), but does not reflect the normal state of the animal. EB0.3 and EB5 may have produced serum estradiol levels that were too low to maximize these cellular responses, and hence may have exerted non-significant alterations in CREB phosphorylation. Alternatively, EB5 and/or EB0.3 may have influenced pCREB immunoreactivity within a timeframe that was sooner than what was measured in the current experiment. There are conflicting reports regarding how quickly estradiol can induce and maintain pCREB at levels exceeding control values. For instance, elevated concentrations of pCREB were observed in cultured hippocampal neurons within 10-20 minutes following the addition of estradiol (Wade & Dorsa, 2003; Zhao et al, 2005). While some researchers suggested that the magnitude of activated CREB returned to control values within 30 or 60 minutes (Wade & Dorsa, 2003; Zhao et al, 2005), others claimed that estradiol enhanced pCREB
in vitro for up to 1, 6, 12, and 24 hours after initial exposure to the tissue (Murphy & Segal, 1997; Lee et al., 2004). A similar controversy also exists within in vivo studies. In forebrain structures including the hypothalamus, medial septum, and bed nucleus of the stria terminalis, supraphysiological doses of estradiol increased pCREB immunoreactivity within 15 minutes of injection (Gu et al., 1996), and this increase was either sustained for 1 to 4 hours (Gu et al., 1996; Abraham et al., 2003; Abraham et al., 2004) or was reduced to control levels 1 to 4 hours later (Zhou et al., 1996). Hence, these studies argue that estradiol can alter pCREB levels very quickly, and that these alterations may not be sustained for extended periods of time. Although within the time limits previously shown to elicit pCREB activation following acute administration of estradiol, the perfusion time points in the current study may have occurred too late following the last estradiol injection, and thus may not have captured the rapid, short-term estradiol-induced elevations in pCREB immunoreactivity.

**Time-dependent alterations in pCREB immunoreactivity**

In the present experiment, there were time-dependent effects of pCREB activation, regardless of hormone condition. That is, there were more cells expressed in the dCA1, dCA3 and PL region 40 minutes following the last injection than at 4 hours. One possible explanation for this finding may be that the subcutaneous injections themselves altered the phosphorylation of CREB in a time-dependent fashion, perhaps through the activation of a stress response. Indeed, enhanced pCREB activation in response to a variety of acute stressors has been documented (Legradi et al., 1997; Chen et al., 2001; McCabe & Burrell, 2001; Shimizu et al., 2004). However it is not likely that stress played a role in the current results as the rats had been handled thoroughly prior to
injections, and their behaviour had habituated to the injections within the first few days of hormone/oil administration. Furthermore, the influence of stress on pCREB activation appears to be sex-dependent: Foot-shock stress elevated pCREB levels in the CA1 of male rats within one hour but did not alter pCREB immunoreactivity in the CA1 (or in the CA3) of female rats 1, 5, or 24 hours following the shock, even when the intensity of the shock was increased (Kudo et al., 2004). Moreover, rats perfused at 40 MIN exhibited similar pCREB activation as rats perfused at 24 HRS, also indicating that stress from the subcutaneous injections could not have influenced activation levels, as stress hormone (i.e. corticosterone) levels reach baseline approximately 90 minutes following acute stressors (McCormick et al., 2005; Vahl et al., 2005). Rather, this result suggests that circadian rhythms in biological processes may yield a significant influence on the processes mediating pCREB expression. Further research controlling for diurnal variations is required to fully elucidate these effects.

Conclusions

The results from the current study suggest that administration of physiological doses of estradiol to ovariectomized rats does not significantly alter pCREB immunoreactivity. Rather, time since last injection was a vital factor in determining the amount of pCREB+ cells. Although previous studies have found an increase in pCREB expression following an acute dose of estradiol, in the present study I did not observe an increase in pCREB after 17 days of continuous exposure to estradiol. Thus there are at least 3 possible explanations: long-term estradiol injections decreased ER levels in the PFC and hippocampus, which in turn reduced the influence of estradiol on pCREB; the time of perfusion was not quick enough to observe estradiol-mediated effects; or that
physiological levels of estradiol were too low to maximize the pCREB response. In order to fully investigate this hypothesis, a future study will be conducted to analyze the effects of acute injections of physiological levels of estradiol or oil on pCREB activation. Further studies are also needed to confirm whether or not long-term exposure to estradiol can enhance pCREB at earlier time points after the last injection (e.g. 10-30 minutes).
General Discussion

The current series of experiments have sought to examine the influence of estradiol on PFC- and hippocampus-dependent working memory processes (Chapter 1), and on pCREB expression in both the PFC and hippocampus (Chapter 2). High and low systemic doses of estradiol did not significantly alter working memory performance on the SWSh task, although EB5 rats did acquire the task sooner than OIL and EB0.3 rats. Different doses of estradiol cyclodextrin infusions into the dorsal hippocampus and prelimbic region of the PFC facilitated working memory and protected against infusion-induced working memory impairments in well-trained rats. These data indicate that estradiol has differential effects on working memory performance depending on the site of action and dose of estradiol, which may in turn explain why no systemic influences of estradiol were observed in Experiment 1. Rats also made significantly more working memory errors 24 hours following estradiol infusions into the dorsal hippocampus relative to performance 24 hours after saline infusions. The findings from Chapter 2 demonstrated that contrary to previous reports of acute estradiol treatment, 17 days of estradiol injections did not significantly alter pCREB expression in the dorsal CA1 and CA3 of the hippocampus or in the prelimbic region of the PFC, although there was a slight tendency for the low dose of estradiol to decrease pCREB expression. However, there was a time of day effect, with animals perfused at 40 MIN and 24 HRS exhibiting similar levels of pCREB expression, and animals perfused at 4 HRS exhibiting decreased levels of pCREB expression, regardless of condition.

The results from Chapter 1 support the claim that estradiol’s influence on learning and memory processes is dependent on the dose of estradiol, neuroanatomy subserving,
performance on the task, and route of estradiol administration. However, many questions remain to be answered. Given that we based our doses of estradiol cyclodextrin on previous reports (Packard & Teather, 1997; Frye & Rhodes, 2002), it is unknown whether or not these doses represent levels observed in the brains of intact and naturally cycling animals. Moreover, the relative contribution of specific ERs in working memory ability is unknown and this is potentially an important contributing factor given that ERβ, but not ERα, are found in PFC (Shughrue et al., 1997). Thus one potential study would be to use selective ERα and ERβ agonists infused into either the PFC or dorsal hippocampus prior to testing on the SWSh task. In order to examine the role of membrane-bound ERs in the rapid actions of estradiol on working memory performance, estradiol conjugated to bovine serum albumin, a complex that has been shown to act primarily on membrane-bound ERs (Zheng et al., 1996) could also be infused prior to testing. Lastly, the dose-dependent effects of estradiol cyclodextrin infusions should be examined in working memory tasks subserved by either the PFC or hippocampus in order to clarify whether the results in the current study are applicable to all working memory tasks.

The results from Chapter 2 indicate that chronic physiological levels of estradiol do not influence pCREB expression in the CA1, CA3 or PL region. This finding raises the possibility that chronic estradiol injections may have saturated the system, thereby leading to downregulation of ERs and as a consequence, reduced pCREB. Currently, I am exploring this hypothesis indirectly by administering an acute injection of estradiol benzoate (EB0.3 or EB5) or oil and perfusing the animals at the same 3 perfusion time points (40 MIN, 4 HRS, 24 HRS). Previous research has exhibited estradiol-related increases in pCREB in the hippocampus with supraphysiological doses within 1 hour of
injection (Abraham et al., 2004), thus it is proposed that rapid changes in CREB activation will also be captured with this acute paradigm. Moreover, intact cycling female rats at various stages in the estrous cycle should also be examined for pCREB activation in the hippocampus and PFC, as these animals exhibit natural fluctuations in physiological levels of estradiol.

Because we discovered a time of day effect on pCREB immunoreactivity, it has raised the possibility that circadian rhythms may mediate the activation of this transcription factor. In order to clarify this concern, a proper investigation of a possible circadian effect of pCREB activation could be conducted, or female rats housed in diverse light cycles could be utilized, which would result in animals with modified circadian rhythms. For instance, lengthening or shortening the light cycle results in alterations in the circadian oscillation of corticosterone and feeding and drinking behaviour (Ahlersova et al. 1992). Thus, by altering how much light and darkness the animals are subjected to, one can control for various time of day influences.

The results from the experiments in Chapters 1 and 2 have demonstrated that physiological levels of estradiol have diverse influences on the brain and subsequent behaviour. These results have also shed some new light on how estradiol can dose-dependently alter working memory ability by acting at distinct neuroanatomical locations. Future research is required to clarify if these findings extend to other cognitive systems, and what role estradiol plays in the activation of CREB and other transcription factors involved in learning and memory processes.
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