THE EFFECTS OF ESTROGENS AND HORMONE REPLACEMENT THERAPY ON ADULT HIPPOCAMPAL NEUROGENESIS AND COGNITION IN YOUNG AND MIDDLE-AGED FEMALE RATS

by

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ABSTRACT

In women, age-associated decline in cognitive functioning is associated with the onset of menopause, which is the cessation of ovarian functioning and leads to dramatic reduction in circulating levels of ovarian hormones including estradiol. Estrogens have been implicated as possible therapeutic agents for improving cognition in postmenopausal women and have been linked to neurodegenerative disorders such as Alzheimer's disease. However, the utility of replacement with estrogens has recently been questioned in the literature. The experiments in this thesis aimed to determine the effects of replacement with different estrogens on hippocampus-dependent learning and memory and hippocampal neurogenesis in female rats, and whether these effects were dependent on different factors, including length of exposure, type of estrogens, dose of estrogens, type of memory system examined, age of subjects, and previous reproductive experience. The main findings of the experiments presented in this thesis are that hormone replacement therapy and estrone negatively impact hippocampus-dependent learning and memory (Chapters 2 and 4; Barha and Galea, in press; Barha et al., 2010), whereas other estrogens can improve hippocampus-dependent learning and memory (Chapter 4; Barha et al., 2010). Additionally, hormone replacement therapy alters hippocampal neurogenesis and decreases new neuronal activation in the dentate gyrus, which may account for impairments seen in memory functioning (Chapter 2; Barha and Galea, in press). Naturally occurring estrogens also differentially increase cell proliferation in the dentate gyrus in adult and middle-aged female rats (Chapters 3, 5; Barha et al., 2009; Barha and Galea, 2011), and this effect is dependent on previous reproductive experience in middle-aged females (Chapter 5; Barha and Galea, 2011). Thus, taken together the results from these experiments suggest that some estrogens increase while other estrogens decrease hippocampal neurogenesis and hippocampus-dependent learning and memory. These findings have important implications for determining which alternative forms of estrogens to incorporate into hormone therapy treatments in the

future. Furthermore, the findings from this thesis provide new insights into our understanding of the mechanisms and function of adult neurogenesis in the female rat.

PREFACE

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3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β- hydroxysteroid dehydrogenase
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
APP	Amyloid precursor protein
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
CA	*Cornu Ammonis*
CEE	Conjugated equine estrogen
CORT	Corticosterone
DAB	4',6-diamidino-2-phenylindole
DCX	Doublecortin
DG	Dentate gyrus
E_1	Estrone
E_2	Estradiol
E_3	Estriol
EB	Estradiol benzoate
EC	Entorhinal cortex
ER	Estrogen receptor
FSH	Follicle stimulating hormone
GABA	γ-Aminobutyric acid
GCL	Granule cell layer
GnRH	Gonadotropin releasing hormone

GPR30	G-protein receptor 30
HRT	Hormone replacement therapy
HT	Hormone therapy
HPG	Hypothalamic-pituitary-gonadal
I.P.	Intraperitoneal
IEG	Immediate early gene
KCC2	Potassium, chloride cotransporter
LTP	Long term potentiation
LH	Luteinizing hormone
MAM	Methylazoxymethanol
NaCl	Sodium chloride
NDS	Normal donkey serum
NeuN	Neuronal nuclei
NHS	Normal horse serum
NIH	National institute of health
NKCC1	Sodium, potassium, chloride cotransporter
NMDA	N-methyl-D-aspartic acid
OD	Optical density
OFT	Open field test
OVX	Ovariectomy
NRS	Normal rabbit serum
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
P_4	Progesterone
PVA-DABCO	Polyvinyl alcohol-1,4-Diazabicyclo[2.2.2]octane

RCT	Randomized control trial
RME	Reference memory errors
ROI	Region of interest
SD	Sprague-Dawley
SEM	Standard error of the mean
SGZ	Subgranular zone
SOX2	Sex-determining region Y-box
SVZ	Subventricular zone
TBS	Tris buffered saline
VEGF	Vascular endothelial growth factor
WHIMS	Women's health initiative memory study
WME	Working memory errors
W/RME	Working/reference memory errors

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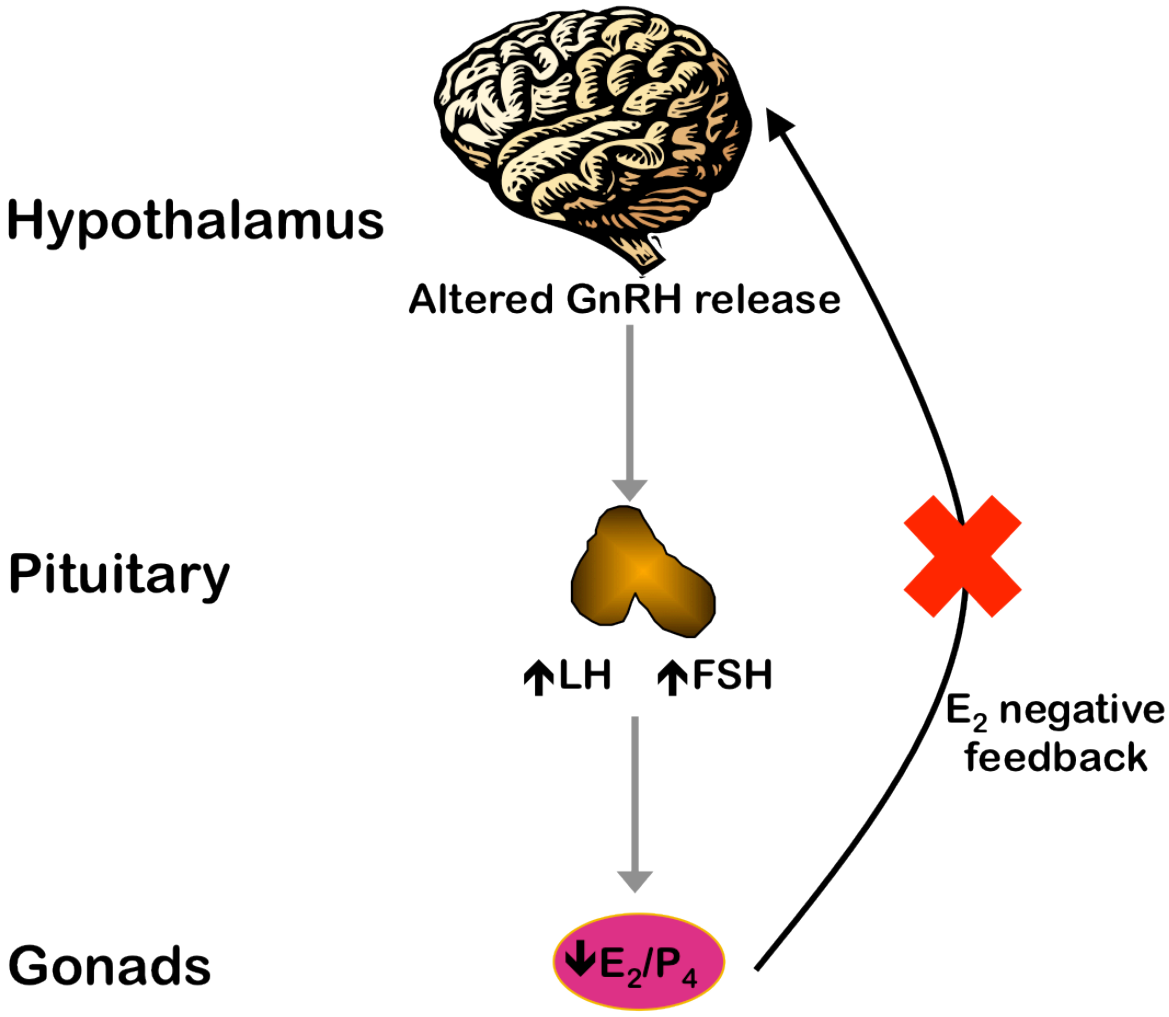
1. GENERAL INTRODUCTION

The ageing population is rapidly increasing worldwide. In Canada, the proportion of the population that is over the age of 65 was approximately 14.4% in 2011, and is expected to increase to 24% by the year 2041, and by the year 2017 there will be more senior citizens living than children (Statistics Canada: www.statcan.gc.ca). Interestingly a greater proportion of females (8.0%) are over 65 than males (6.4%; Statistics Canada). Age-associated memory impairment is seen in approximately 38% of older people (Hanninen, et al., 1996) and reductions in distinct domains of cognitive domains are seen as young as 50-60 years of age (Albert, et al., 1987,Hanninen, et al., 1996). Impairments are typically isolated to episodic, declarative, and working memory, whereas memory domains such as semantic and procedural memory remain largely intact (Buckner, 2004). The functioning of learning and memory systems that depend on the hippocampus are also considerably altered with age, including spatial learning and memory.

Evidence suggests that age-associated cognitive decline may be related to gonadal hormones. In some women the cessation of ovarian function and subsequent decline in ovarian hormones (including estrogens) that occurs during menopause is associated with poorer cognition and increased incidence of neurodegenerative diseases (Sherwin, 2005). Menopause occurs at approximately 50 to 51 years of age and thus with life expectancy increasing to over 80 years, women are living more than a third of their lives in a hypoestrogenic state. Menopause is the cessation of menstrual cycles and is caused by alterations at each of level of the hypothalamic-pituitary-gonadal (HPG) axis (Figure 1.1).

Figure 1.1: Alterations in the hypothalamic-pituitary-gonadal axis associated with menopause. Abbreviations: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; E_2, estradiol; P_4, progesterone.



At the level of the gonads there is a loss of ovarian follicles causing a dramatic decline in the production of estradiol (E_2). This lack of E_2 negative feedback leads to increases in luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary. Furthermore, in the hypothalamus, aging leads to alterations in the temporal pattern of neural signaling of GnRH (gonadotropin-releasing hormone) neurons (Downs and Wise, 2009). Postmenopausal women have reduced performance in tasks requiring memory for the acquisition and early

retrieval of new information and working memory, which are mediated by the hippocampus and prefrontal cortex (Sherwin and Henry, 2008). Therefore it has been speculated that ovarian hormones (estrogens and progesterone) may have the most profound influence on these brain structures and the types of memory mediated by them.

It has long been thought that the cognitive decline seen in postmenopausal women is related to the loss of circulating levels of ovarian hormones, and that replacing back these lost hormones may restore memory function or at least prevent further decline (Hogervorst, et al., 2000). The majority of studies support this idea, although not all studies have found that giving back estrogens in the form of hormone replacement therapy (HRT) is beneficial for cognition (see Section 1.1 below). Estradiol, the most potent estrogen, is a potent regulator of learning and memory and of hippocampal plasticity (Barha and Galea, 2010,Daniel, 2006,Frick, 2009) in humans, nonhuman primates, and rodents. Past research in this area has shown a strong but complex relationship between estrogens and cognition, with many profound alterations in neuroplasticity in the hippocampus coinciding with estrogens' influence on cognition. The relationship between estrogens and cognition is immensely complex and depends on many factors, each of which requires unique consideration. These factors include the brain structures and memory systems being recruited by the cognitive task (Bimonte and Denenberg, 1999,Galea, et al., 2001), length of exposure to estrogens (Luine, et al., 1998), timing of testing in relation to treatment (Daniel, et al., 1997,Gresack and Frick, 2004), the dose of estrogens (Holmes, et al., 2002,Wide, et al., 2004), type of estrogens (Luine, et al., 2003), and the age of the subjects.

The experiments presented in this thesis focus of the hippocampus. The hippocampus is a temporal lobe structure that is critical for spatial, contextual, and relational memory formation (Eichenbaum, 2004). Many of the cognitive domains that show decline with age and with

menopause are subserved by this brain structure (Buckner, 2004). Importantly, the hippocampus shows a remarkable degree of plasticity throughout the lifespan and is highly responsive to steroid hormones such as estrogens and glucocorticoids (Galea, 2008,Galea, et al., 2006,Galea, et al., 2008,McEwen and Milner, 2007). The dentate gyrus of the hippocampus is the site of ongoing neurogenesis throughout the lifespan. The series of experiments presented in this thesis delve into the effects of replacement with different estrogens on hippocampus-dependent learning and memory and hippocampal neurogenesis in both adult and middle-aged female rats. Chapter 2 explores the effects of the most prescribed hormone replacement therapy, Premarin, on hippocampus-dependent learning and memory and hippocampal neurogenesis, as well as new neuronal activation (Barha and Galea, in press). Chapter 3 examines the effects of acute treatment with different naturally occurring estrogens on cell proliferation in the dentate gyrus of the hippocampus of adult female rats, and whether these effects are dependent on dose and length of exposure (Barha, et al., 2009b). In Chapter 4, the functional impact of acute administration of different estrogens is examined, as the effects of different doses of estrogens on hippocampus-dependent contextual fear conditioning and hippocampus-independent cued fear conditioning are shown (Barha, et al., 2010). Chapter 5 investigates whether the effects of different estrogens on hippocampal cell proliferation in middle-aged female rats are dependent on previous reproductive experience (Barha and Galea, 2011). This introduction will outline previous literature regarding the effects of estrogens on cognition in both women and female rodents and on hippocampal neurogenesis in female rodents.

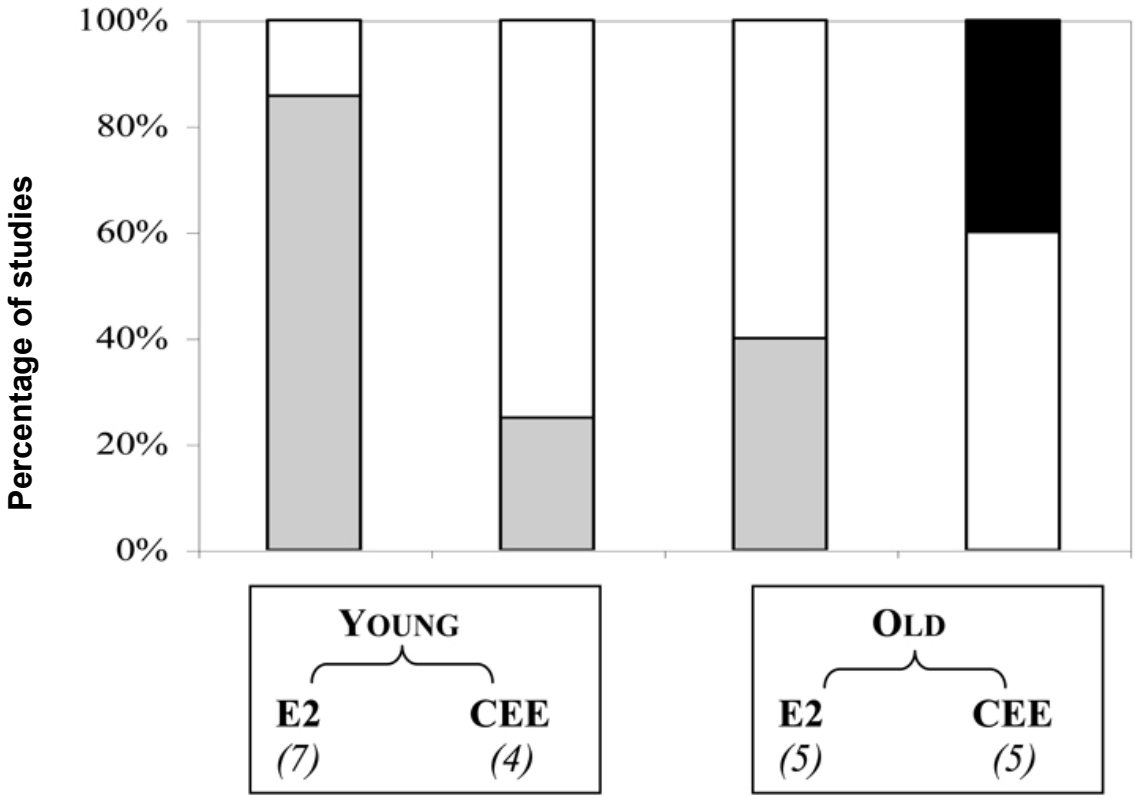
1.1 WOMEN'S COGNITIVE HEALTH AND HORMONE REPLACEMENT THERAPY

There are three main types of naturally occurring classical estrogens: estrone (E_1), estradiol (α- and β-E_2) and estriol (see Section 1.2 for a description of the different estrogens).

Estrogens have well known effects on reproductive behaviors and associated brain regions; however, estrogens also influence non-reproductive behaviors such as cognition and associated cognitive regions of the brain such as the hippocampus. The role of estrogens in eliciting and maintaining reproductive behaviors by influencing associated brain regions are well established in the literature (McEwen, 2002). Recently the effects of estrogens on non-reproductive behaviors, such as cognition, have moved to the forefront of research (Spencer, et al., 2008). In humans estrogens are strongly related to certain types of cognition, with fluctuations in performance on spatial tasks seen across the menstrual cycle (Hampson, 1995). For example, performance on verbal fluency tests is positively correlated with estradiol levels whereas performance on spatial tasks is negatively correlated with estradiol levels (Hampson, et al., 2005,Maki, et al., 2002). Furthermore, aging and the menopausal period are associated with a decline in cognition in women (Berent-Spillson, et al., 2012,Greendale, et al., 2009), and as such hormone replacement therapy (HRT) has been implicated as a possible therapeutic agent for ameliorating age-related cognitive decline in postmenopausal women. Many studies have investigated the utility of HRT to restore cognitive function and/or prevent further decline. Results from randomized, placebo-controlled trials in humans examining the effects of HRT on cognitive functioning are mixed, with studies showing negative, positive or neutral results (Henderson, 2010,Hogervorst and Bandelow, 2010,Sherwin, 2009). Discrepant results could be due to important methodological differences including age of women, type of memory tests given, and the type of HRT. A meta-analysis suggested that discrepant findings about the effectiveness of HRTs might be related to the type of estrogen being given to women, with HRTs containing 17β-estradiol having more positive effects on different aspects of cognition and dementia risk than HRTs containing conjugated equine estrogens (CEE) (Hogervorst, et al., 2002,Ryan, et al., 2008). Overall it seems that estradiol-based HRTs used short-term in younger

menopausal women either enhance or have neutral effects on cognition including verbal memory (Gleason, et al., 2006,Joffe, et al., 2006,Phillips and Sherwin, 1992,Rasgon, et al., 2005,Sherwin, 1988,Tierney, et al., 2009); whereas treatment with CEE in both younger and older menopausal women has negative or neutral effects on cognition including verbal memory (Maki, et al., 2007,Rapp, et al., 2003,Resnick, et al., 2009; see Figure 1.2).

Figure 1.2: Effects of hormone replacement therapy (HRT) on specific cognitive function in non-demented menopausal women as a function of age and type of HRT. Twenty-one randomized control trials (RCTs) were performed over the last 20 years using estradiol (E2) or CEE (conjugated equine estrogens) opposed or not with progestins and administered either to "young" menopausal women (*i.e.* younger man 65) or to "old" women of 65 years and more. The number of RCTs under each condition is indicated between brackets. Black, grey, and white bars correspond respectively to the % of RCTs where HRT was found to have a negative, a positive, or no effect, on at least one cognitive task. The studies on global cognitive functioning (MMSE) or those with ultra-low-dose estradiol were not considered.



Furthermore, the Women's Health Initiative Memory Study (WHIMS), an ancillary study to the Women's Health Initiative and the largest randomized controlled trial conducted thus far, did not find evidence of a beneficial effect of HRT on cognition or risk for dementia in women and found a slight decrease in global cognitive functioning with HRT-use (Espeland, et al., 2004,Rapp, et al., 2003,Shumaker, et al., 2003). Importantly, the participants in the WHIMS were given a HRT containing CEE trade named Premarin, the most common type of HRT prescribed in the United States (Wysowski, et al., 1995). Despite the large number of participants, many important methodological issues have been raised with the WHIMS in recent years. Specifically the women in the WHIMS were between 65 and 79 years of age, suffered from many serious health issues, and had been without significant ovarian functioning for more

than 15 years, which may have greatly impacted the ability of HRT to influence the brain. Furthermore, the type of HRT given to participants is of central concern. Premarin, the CEE given to these women, is a natural mixture of at least 10 different estrogenic compounds that are isolated from pregnant horse urine and formulated to represent an average standardized estrogenic activity, containing many substances other than just estrogens (Bhavnani, et al., 2000). Furthermore, in addition to the estrogens also produced in humans (17β-estradiol, estrone, 17α-estradiol), CEE contains ring B unsaturated equine estrogens that are produced exclusively in the horse (Bhavnani, et al., 2000). As stated above, Premarin is the most prescribed HRT in the United States with one in four women taking Premarin in the 1990's (Wysowski, et al., 1995). The slight reduction in global cognitive functioning seen in the participants of the WHIMS study persists and remains after therapy is stopped (Espeland, et al., 2010). Furthermore, when domain-specific cognitions were assessed, a negative impact of Premarin treatment was seen on verbal memory and spatial rotational ability, although these deficits were only seen short-term (Coker, et al., 2010). Studies have also shown that Premarin use is associated with greater hippocampal atrophy in post-menopausal women compared to non-users (Resnick, et al., 2009). Clinically, publication of the negative results of the WHIMS has led to a dramatic reduction in women using HRT around the world, with reduction rates ranging between 40-80% (Burger, et al., 2012).

As stated earlier, evidence suggests that the effectiveness of HRTs in ameliorating cognitive decline in postmenopausal women is highly dependent on the specific estrogenic compound being used. For example, women receiving a HRT containing 17β-estradiol performed three standard deviations higher in tests of verbal memory and had higher cerebral brain metabolism than women receiving CEE (Silverman, et al., 2011). Additionally, in a cohort of women at risk for developing Alzheimer's disease, use of 17β-estradiol improved verbal

memory performance compared to women receiving CEE (Wroolie, et al., 2011). In postmenopausal women higher endogenous levels of estrone (the main estrogen in Premarin) are associated with lower cognitive performance (Yaffe, et al., 1998), and higher circulating estradiol levels are associated with less cognitive decline (Bittner, et al., 2011,Lebrun, et al., 2005). Thus evidence further suggests that CEE-based HRTs do not confer the same benefits on cognition and dementia risk early in menopause as do estradiol-based HRTs.

Age of subjects also plays an important role in determining the effectiveness of HRTs (see Figure 1.2). According to an updated meta-analysis conducted by Ryan et al. (2008) based on the work of Hogervorst et al. (2000), 80% of randomized control trials conducted in menopausal women younger than 65 years of age found a beneficial effect of estradiol-based HRTs on cognition, whereas only 40% of trials found a beneficial effect in older menopausal women (over 65). Importantly, 20% of trials in young menopausal women found a beneficial effect with CEE HRT and 40% of trials found a detrimental effect on cognition in older menopausal women with CEE (Ryan, et al., 2008). These results further support the need to investigate the effects of different estrogens on cognition in both younger and older menopausal subjects.

Many reasons have been proposed to explain the different effects HRT-use has on cognitive functioning in women, including the critical period hypothesis (Daniel, 2012,Gibbs, 2010), the healthy cell bias hypothesis (Brinton, 2008), and the formulation of HRT. The critical period or the window of opportunity hypothesis of HRT-use claims that the ability of estrogens to influence cognition greatly decreases as time without endogenous estrogens increases (Daniel, 2012). Therefore, HRT-use must commence temporally close to the onset of menopause in order to be beneficial; otherwise HRT will not have an effect on cognitive functioning. According the healthy cell bias hypothesis, negative effects on cognition are seen

when HRT is begun after neurodegenerative decline has already commenced, in other words once cells and the brain are unhealthy (Brinton, 2008). Both of these hypotheses indicate that HRT should begin as close to menopause as possible and that the age of subjects is crucial in determining the effects on cognition.

Recently, researchers have begun to investigate potential neurobiological mechanisms underlying the effects of Premarin (CEE) on cognition using rodent models of menopause. Oral treatment with Premarin for two weeks decreases ERα, but has no significant effect on ERβ, levels in the hippocampus of long-term (48 days) ovariectomized female rats (Jin, et al., 2005). Importantly, in this study treatment with a 17β-estradiol-based therapy had the opposite effect with increases seen in ERβ and no effect on ERα. The few studies previously conducted examining the effects of Premarin on cognition using middle-aged female rats found beneficial effects (Acosta, et al., 2009,Acosta, et al., 2010,Engler et al., 2011). Cyclic treatment of middle-aged female rats with Premarin (2 days on, 2 days off) slightly improved reference memory performance and acquisition of a delay-match-to-sample plus maze task compared to oil controls, potentially by increasing the number of choline acetyltransferase positive cells in the basal forebrain (Acosta, et al., 2009). Interestingly, these beneficial effects are only seen in surgically, not transitionally, menopausal rats, and are associated with lower levels of androstenedione, the principal steroid produced by the postmenopausal ovary (Acosta, et al., 2010). It is important to note that because of the nature of the cyclic hormone treatment schedule used in these two papers, it is not clear whether rats were behaviorally tested with Premarin in circulation, further complicating a direct comparison of results between studies. Recently, Engler-Chiurazzi et al. (2011) found that tonic administration of a low dose of Premarin via osmotic pumps slightly impaired spatial working memory and reference memory retention in middle-aged female rats, whereas higher doses slightly enhanced spatial reference

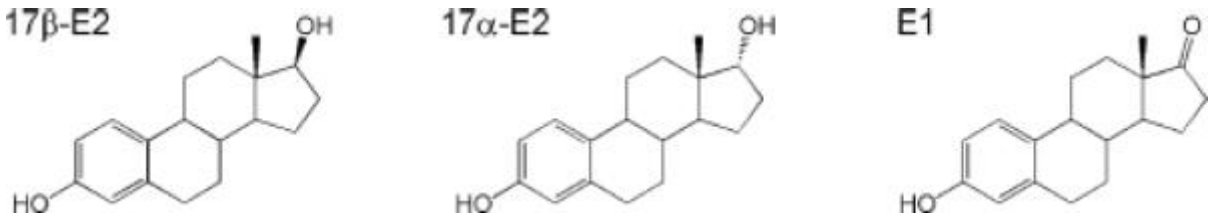
and working memory. The discrepant results seen in studies with humans and rats indicate that the relationship between the HRT Premarin and cognition depends on multiple factors including age of the subjects, route and schedule of administration, dose, and the learning and memory task. And although this line of research looking at neural mechanisms underlying the effects of HRT has only recently begun, these findings emphasize the need for further research into how different types of estrogens and Premarin influence the brain, particularly the hippocampus, and cognition in women and female rats in order to develop more effective therapies in the treatment of symptoms associated with menopause in women. To date no study has examined the effects of Premarin treatment on hippocampal neurogenesis alone or in combination with learning and memory and this is examined in Chapter 2.

1.2 DIFFERENT TYPES OF NATURALLY OCCURRING ESTROGENS

There are three principal forms of estrogens: estrone (E_1), estradiol (E_2) and estriol. Furthermore, there are two naturally occurring optical isomers of estradiol, 17β-estradiol and 17α-estradiol. High circulating levels of both 17β-estradiol and estrone are found in the body, with 17β-estradiol being the prevalent form before menopause and estrone being the prevalent form after menopause (Rannevik, et al., 1995). 17α-estradiol has been found at low but measurable levels in pre- and post-menopausal women (Adlercreutz, et al., 1974) and originates primarily from the adrenal glands and in the past was considered to be transcriptionally and biologically inactive (Toran-Allerand, et al., 2005). Figure 1.3 shows the biosynthetic pathways of these three estrogens, although not all pathways have been fully characterized.

Figure 1.3: (a) Chemical structure of three naturally occurring classical estrogens. (b) Catabolic pathways, with associated enzymes, for androgens, estrogens, and other pregnenolone derivatives. There is suggestion that 17α-estradiol can be converted from estrone and may be further transformed into 17β-estradiol, though evidence for this has yet to be found in humans. Some enzyme names are abbreviated: 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD). Adapted from Toran-Allerand et al., 2005.

a)



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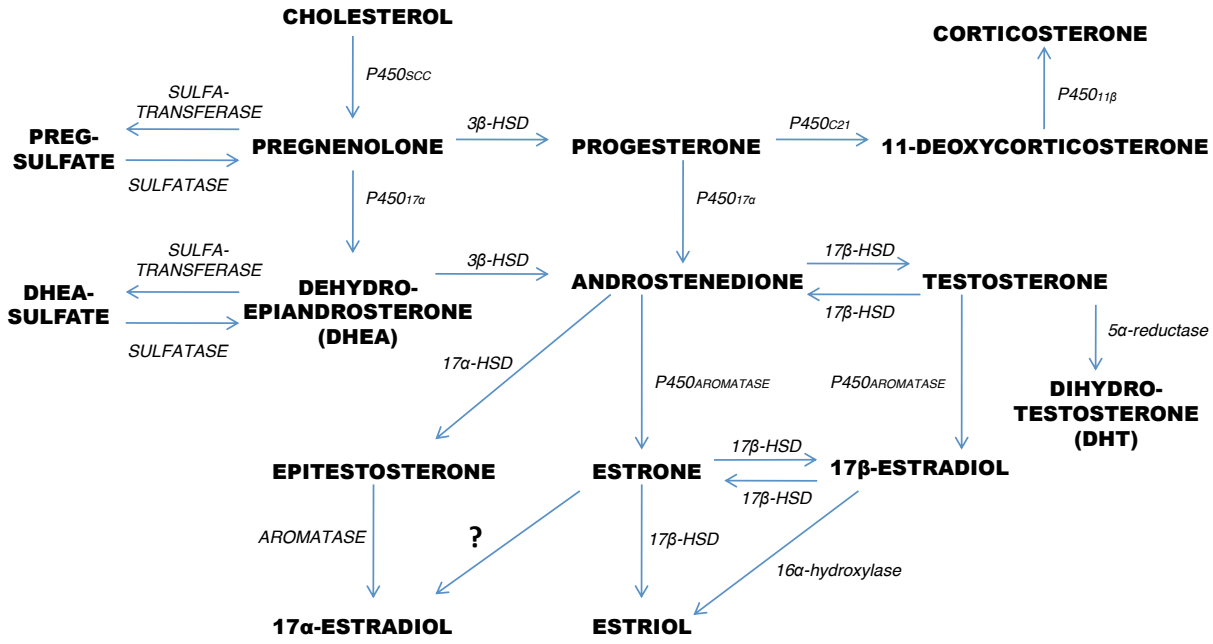


17β-estradiol is the most potent of the estrogens and binds to the classical estrogen receptors (ERα and ERβ) with greater affinity than both estrone and 17α-estradiol. Estrone and 17α-estradiol both have a 40-fold lower affinity for ERα than 17β-estradiol, and estrone binds to ERβ with a greater affinity than 17α-estradiol but with a much lower affinity than 17β-estradiol (Perez, et al., 2005). 17α-estradiol has been theorized to be the preferred ligand of ER-X, a plasma membrane-associated ER (Toran-Allerand, 2005).

Endogenous levels of 17α-estradiol in the mouse hippocampus are much greater than levels of estrone and 17β-estradiol (Toran-Allerand, 2005). Furthermore, levels of 17α-estradiol are greater after gonadectomy and adrenalectomy, suggesting that 17α-estradiol may also be derived from local synthesis (Toran-Allerand, et al., 2005). Although very little work has been done looking at the biological effects of 17α-estradiol as it has been considered biologically inactive for many years, previous work has shown that this estrogen can rapidly induce dendritic spine formation in the CA1 region of the hippocampus (MacLusky, et al., 2005) and enhance visual and place memory (Luine, et al., 2003).

The vast majority of studies examining the effects of estrogens on brain and behavior have solely used 17β-estradiol, and although 17β-estradiol and estrone can be enzymatically interconverted, these estrogens can differentially affect neuroprotection (Bhavnani, et al., 2003,Budziszewska, et al., 2001). Notably, the CEE HRT Premarin is composed of approximately 50% sulfated estrone, 0.56% sulfated 17β-estradiol, and 3.7% sulfated 17α-estradiol (Kuhl, 2005), indicating that many of Premarin's cognitive effects may be attributed to estrone. However, it is imperative to compare the effects of all three of these estrogens as they are all naturally occurring in women and may differentially influence the brain and behavior, therefore rendering some of these estrogens as more promising alternatives for therapeutic use

in treating menopausal symptoms in the hopes of circumventing some of the adverse effects associated with current therapies.

1.3 THE RODENT MODEL OF MENOPAUSE

The effects of replacement with different estrogens on the structure and function of the hippocampus across the lifespan is an area of research that has come to the forefront in recent years, particularly in lieu of the rapidly aging world population. The experiments described in the present thesis examine how HRT and naturally occurring estrogens influence hippocampus-dependent learning and memory and hippocampal neurogenesis (see Section 1.6 for description) using a rodent model of menopause. Whereas reproductive failure and menopause in humans is the result of the loss of ovarian follicles, reproductive failure in rats occurs during middle-age but at a time when very few changes occur in the ovary. Rats undergo a transition in mid-life that is referred to as 'estropause', which involves irregular, prolonged estrous cycles that eventually lead to acyclicity (Chakraborty and Gore, 2004). Once acyclic, rats first enter a stage called persistent estrus in which estradiol levels are chronically high and then enter a stage called persistent diestrus in which estradiol levels are low. Thus, rats and women share some characteristics of reproductive senescence such as gradual cessation of reproductive cycles and reduced fertility, but differ in the effects of aging on levels of ovarian hormones, with rats maintaining high levels of estradiol while women have very low levels after reproductive senescence. Therefore, in order to model the substantial reduction in levels of estradiol seen with menopause, rats are ovariectomized (OVX). Ovariectomy involves the bilateral removal of the ovaries. Different ages of rats have been used for the surgical model of menopause. In the present thesis adult females at 3 to 4 months of age and middle-aged females at 12 to 13 months of age were ovariectomized. Estrogens can differentially influence the hippocampus in young

versus middle-aged rats. For example, dendritic spine density on granule cells in the dentate gyrus increase after estradiol treatment in aged females but not in young adult females (Miranda, et al., 1999), whereas the opposite is found in the CA1 region with estradiol increasing spine density in young adult females but not in aged females (Adams, et al., 2001). Therefore, it is important to investigate the effects of estrogens in both ovariectomized adult and older rats as the ability of the brain to respond the estrogens may depend on age. Chapters 2, 3, and 4 make use of the surgical model of menopause using adult female rats and Chapter 5 uses middle-aged female rats.

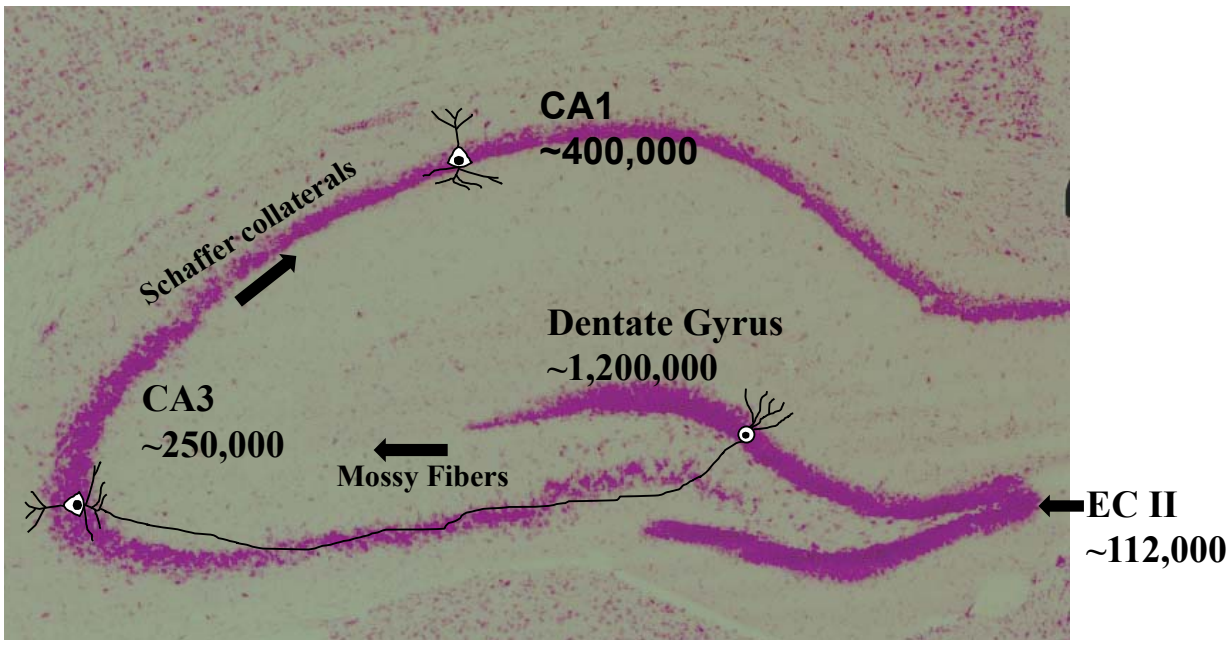
1.4 THE HIPPOCAMPAL FORMATION

The hippocampus is a temporal lobe structure that is critical for spatial, contextual, and relational memory formation (Eichenbaum, 2004). The hippocampus shows a remarkable degree of plasticity throughout the lifespan and is highly responsive to steroid hormones such as estrogens and glucocorticoids (Galea, 2008,Galea, et al., 2008,McEwen and Milner, 2007). The hippocampal structure consists of three principal subfields: cornu ammonis 1 (CA1), cornu ammonis 3 (CA3), and the dentate gyrus (see Figure 1.4a). Although each region of the hippocampus is believed to make a unique contribution to the memory-encoding function of this brain structure, together they connect to form what is referred to as the trisynaptic circuit or loop (Goodrich-Hunsaker, et al., 2008). Afferent input from the entorhinal cortex arrives at the granule cells of the dentate gyrus via the perforant path; information is then propagated along the mossy fiber tract to the pyramidal cells of the CA3. The CA3 pyramidal neurons synapse onto CA1 pyramidal cells via the Schaffer collaterals. Intriguingly, in addition to the traditional trisynaptic view of information processing in the hippocampus, information can also be shared between the CA3 and dentate gyrus in the opposite direction of the trisynaptic circuit along a

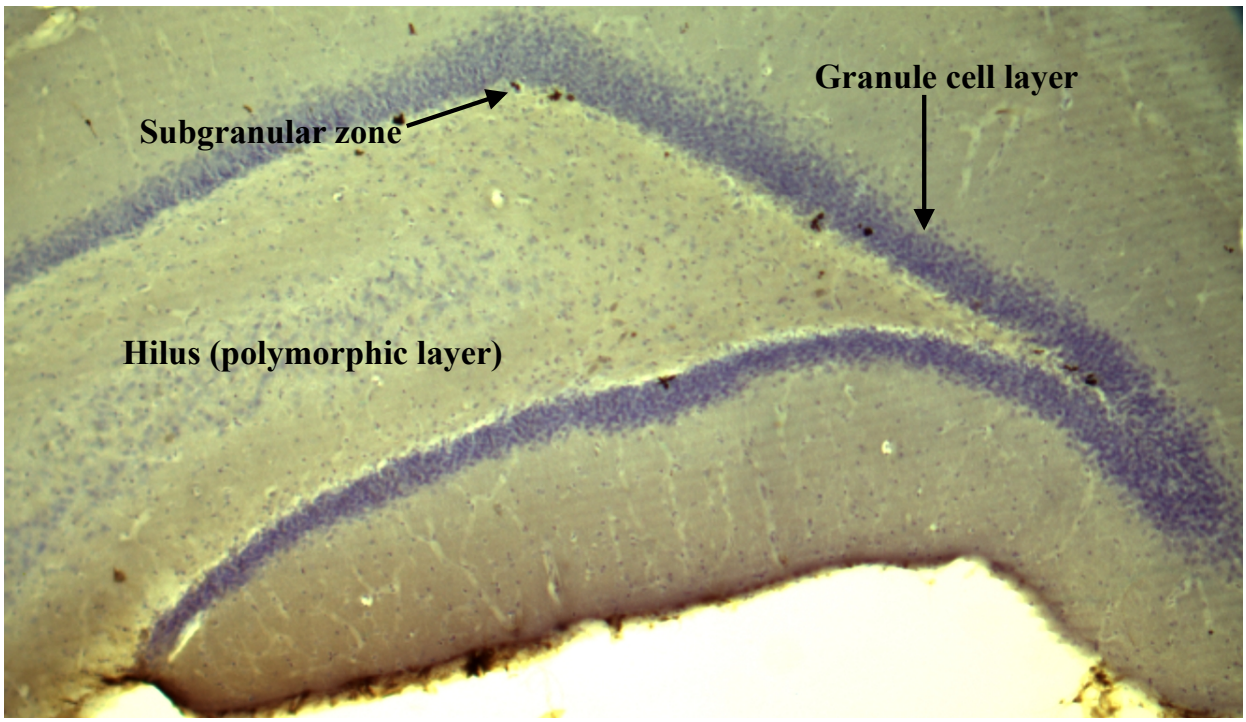
back projection from CA3 pyramidal cells to hilar cells to granule cells (Scharfman, 2007). Each of the three subfields of the hippocampus shows remarkable neuroplastic alterations in response to steroid hormones, including estrogens. For example, estradiol increases dendritic spine density in the CA1 region of the hippocampus (Woolley and McEwen, 1992,Woolley and McEwen, 1994), activates pyramidal CA3 neurons as assessed by increased Fos expression (Rudick and Woolley, 2000), and increases the proliferation of progenitor cells in the dentate gyrus (Ormerod, et al., 2003,Tanapat, et al., 2005,Tanapat, et al., 1999). The dentate gyrus is a three-layered cortex, with an outer molecular layer, below which is the tightly packed granule cell layer, and a polymorphic layer called the hilus (see Figure 1.4b). Between the hilus and the granule cell layer is a narrow region called the subgranular zone (~50μm wide), which contains progenitor cells that retain the ability to divide producing new neurons in the adult mammalian brain. These adult-generated granule neurons are involved in hippocampus-dependent learning and memory and also respond to steroid hormones (Barha, et al., 2009a,Barha and Galea, 2010). A reduction in the level of progenitor cell proliferation is seen in the aging brain and may be related to age-associated memory decline (Kuhn, et al., 1996).

Figure 1.4: (a) Coronal section of the dorsal hippocampus. Afferent input from the entorhinal cortex (EC) is serially processed in the hippocampus via the trisynaptic circuit. The ~112,000 pyramidal cells of layer II of the EC send input via the perforant path to the ~1,200,000 granule cells of the dentate gyrus. The axons of the granule cells send information to the ~250,000 CA3 pyramidal cells along the mossy fiber tract. CA3 pyramidal cells innervate the ~400,000 CA1 pyramidal cells via Schaffer collaterals (West et al., 1991). Cell numbers obtained from one side of the brain in rats. (b) A detailed view of the dentate gyrus.

a)



b)



1.5 HIPPOCAMPUS-DEPENDENT LEARNING AND MEMORY AND ESTROGENS

The hippocampus is implicated in many different forms of learning and memory and many of the effects that estrogens have on cognition may be via influences on this brain structure. The hippocampus contains the classical estrogen receptors (Herrick, et al., 2006,Weiland, et al., 1997): ERα (Koike, et al., 1987) and ERβ (Kuiper, et al., 1996). Both types have been found throughout the dentate gyrus (Herrick, et al., 2006,for review see McEwen and Milner, 2007,Weiland, et al., 1997). ERβ is the predominate form expressed in the hippocampus (Shughrue and Merchenthaler, 2000). Aging leads to dramatic reductions in the expression of both ERα and ERβ in the hippocampus, with a more pronounced reduction seen in the CA3 region (Mehra, et al., 2005). Interestingly, ovariectomy (>3 months) decreases ERβ levels in the brain without influencing ERα levels (Rose'Meyer, et al., 2003). Treatment with a

17β-estradiol based therapy after ovariectomy (approximately 3 months) increases ERβ levels, with no significant effect on ERα, in the cortex and the hippocampus above and beyond levels seen in intact rats (Jin, et al., 2005). In the same study, treatment with an estrone-based therapy (Premarin) decreased ERα levels, with no effect on ERβ, in the cortex and the hippocampus (Jin, et al., 2005). Together, these studies indicate that the hippocampus is a target of estrogens.

Physiological levels of estrogens as seen during the different phases of the estrous cycle alter spatial learning and memory (Frye, 1995,Galea, et al., 1995,Korol, et al., 2004,Warren and Juraska, 1997). When endogenous levels of estrogens are high, rodents performed more poorly on the standard Morris water maze than when endogenous levels of estrogens are low (Frye, 1995,Galea, et al., 1995,Warren and Juraska, 1997). Furthermore, proestrous rats (high levels of estrogens) prefer the use of an allocentric place strategy (hippocampus-dependent) over an egocentric response strategy, while the reverse pattern is seen in estrous rats (low levels of estrogens) (Korol, et al., 2004). These findings indicate that spatial ability and strategy use are altered with ovarian steroid levels.

There are multiple memory systems in the brain. Working memory can be defined as the manipulation and retrieval of trial-unique information to guide prospective action (Baddeley, 2003b). On the other hand, reference memory is long-term stable memory (Olton and Papas, 1979). Estradiol has dissociable effects on these two memory processes, with high levels impairing spatial working and reference memory, and low levels facilitating spatial working memory but not affecting reference memory (Galea, et al., 2001,Holmes, et al., 2002). Administration of low exogenous 17β-estradiol to young ovariectomized females improves spatial working memory (Fader, et al., 1999,Holmes, et al., 2002,Luine, et al., 1998) (for review see Frick, 2009), whereas administration of high levels of 17β-estradiol impair spatial working memory (Holmes, et al., 2002). Reference memory is impaired with high levels of 17β-estradiol

(Daniel, et al., 1999,Frye, 1995,Galea, et al., 2001,Warren and Juraska, 1997,Wilson, et al., 1999) but not significantly affected by low levels of 17β-estradiol (Berry, et al., 1997,El-Bakri, et al., 2004,Holmes, et al., 2002). Thus, taken together this research suggests that there are dose-dependent effects of 17β-estradiol on spatial working and reference memory.

Contextual fear conditioning relies on the integrity of the hippocampus and amygdala. Female rats in proestrus and female rats treated with high doses of 17β-estradiol have impaired contextual fear conditioning compared to females with low levels of 17β-estradiol (Gupta, et al., 2001,Markus, 1997). In humans, women have greater context memory during the early follicular phase of the menstrual cycle when estrogen levels are physiologically low compared to the midfollicular phase when estrogen levels are physiologically high and compared to men (Milad, et al., 2006). Importantly, the effects of other estrogens, such as estrone and 17α-estradiol, on this type of hippocampus-dependent memory have not been examined but will be in Chapter 4.

In addition to dose, age of the subject also plays a role in the estrogenic effects on hippocampus-dependent learning and memory. Ovariectomy impaired spatial reference memory in young (4 months old) female rats, but did not have an influence in middle-aged (16 months old) and aged (24 months old) female rats (Talboom, et al., 2008). Furthermore, 17β-estradiol replacement restored the ovariectomy-induced impairment in reference memory in younger rats, enhanced performance in middle-aged females but did not influence performance in aged females (Talboom, et al., 2008). Interestingly, the effect of 17β-estradiol on spatial discrimination is also dependent on age and dose, with ovariectomy increasing retention in young (6 to 7 months old) female rats, a low dose of 17β-estradiol increasing retention in middle-aged (12 to 13 months old) female rats, and a high dose of 17β-estradiol increasing retention in aged (17 to 18 months old) female rats (Foster, et al., 2003). The modulation of

17β-estradiol's effects on hippocampus-dependent learning and memory by age may be partly mediated by the cholinergic system, as 17β-estradiol treatment prevented disruption of this system in middle-aged females but was ineffective in aged females (Savonenko and Markowska, 2003). As mentioned previously, 17β-estradiol's ability to influence the morphology of the dentate gyrus is also dependent on age, as 17β-estradiol treatment does not influence spine density of granule cells in young (3 months old) female rats but does increase spine density in middle-aged (14 months old) female rats back to levels seen in their younger counterparts (Miranda, et al., 1999). Therefore, it seems that age does alter the responsiveness of the hippocampus to respond to estrogens. Together these findings emphasize the complex relationship between estrogens and hippocampus-dependent learning and memory.

Although the vast majority of rodent research has examined the effects of only 17β-estradiol on hippocampus-dependent learning and memory, two studies have looked at the effects of other forms of estrogens. Farr et al. (2000) found that direct infusion of estrone into the hippocampus enhanced retention of footshock avoidance in the T-maze in ovariectomized mice. Furthermore, 17α-estradiol treatment rapidly enhanced consolidation or encoding of hippocampus-dependent object placement memory in young ovariectomized female rats (Luine, et al., 2003). However, no study has directly compared the effects of these naturally occurring estrogens on cognition or neuroplasticity. In Chapter 4 I will compare the effects of 17β-estradiol, estrone and 17α-estradiol on hippocampus-dependent learning.

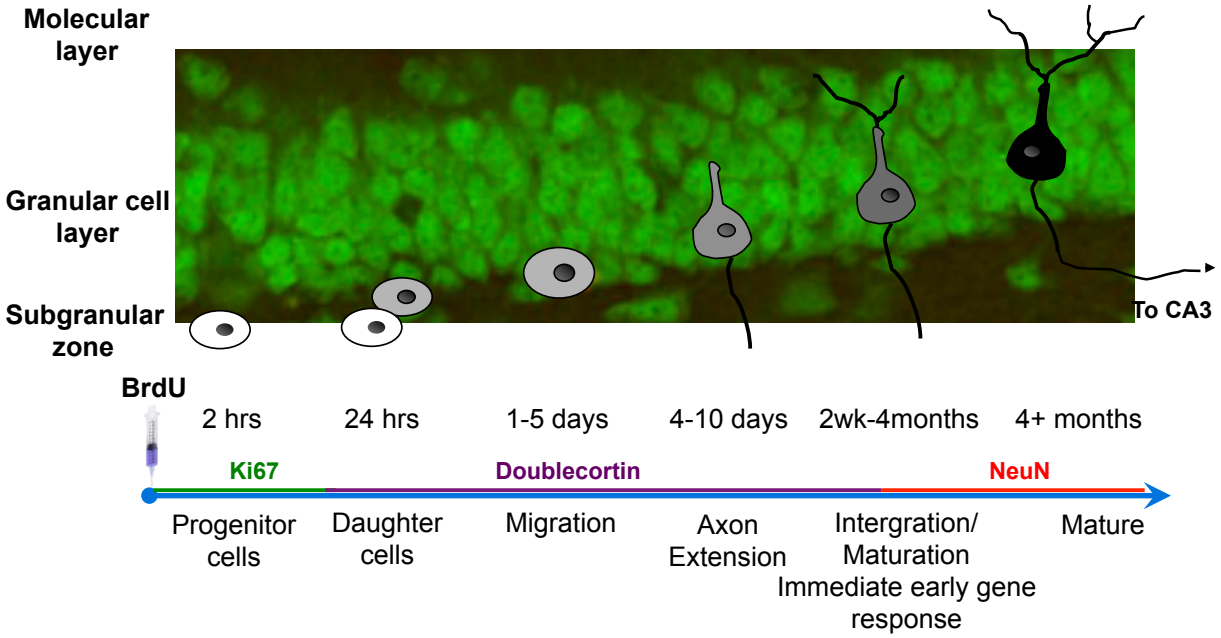
1.6 HIPPOCAMPAL NEUROGENESIS

The production and maturation of new neurons in adulthood, adult neurogenesis, has been found in nearly all mammalian species studied to date, including humans (Amrein, et al., 2007,Amrein, et al., 2004,Eriksson, et al., 1998,Galea and McEwen, 1999,Gould, et al.,

1997,Gould, et al., 2001,Huang and Sato, 1998,Lavenex, et al., 2000). Adult neurogenesis has been confirmed in at least two main areas: the subventricular zone (new cells from this area migrate to the olfactory bulbs along the rostral migratory stream) and the dentate gyrus of the hippocampus (see Figure 1.4b). The subgranular zone of the dentate gyrus contains progenitor cells that retain the ability to divide with the majority of the resulting daughter cells becoming mature granule cells and a smaller portion becoming glial cells (Gage, 2000,Seaberg and van der Kooy, 2003,van der Kooy and Weiss, 2000). Many of the new neurons die within 2 weeks of being produced (Cameron, et al., 1993) but exposure to different stimuli, including steroid hormones, can alter the survival rate (Barker and Galea, 2008,Epp, et al., 2007,Spritzer and Galea, 2007).

Neurogenesis is comprised of at least four processes: cell proliferation, differentiation, migration, and cell survival (Figure 1.5). Different factors can have effects on one or more of these processes and the number of new neurons can be increased either by enhancing cell proliferation and/or by enhancing the survival of new neurons (Barha and Galea, 2010). Furthermore, it is possible to increase the number of cells surviving without influencing cell proliferation, as well as increase the number of cells proliferating without influencing cell survival (Malberg, et al., 2000,Olson, et al., 2006). For example, chronic treatment with antidepressants increases cell proliferation without independently influencing cell survival (Malberg, et al., 2000), and exposure to an enriched environment increases cell survival without influencing cell proliferation (Olson, et al., 2006). Therefore, it is important to study the regulatory mechanisms of both cell proliferation and cell survival in order to fully understand how adult neurogenesis is modulated in the hippocampus.

Figure 1.5: Cartoon depiction of the maturational timeline of adult neurogenesis in the dentate gyrus.



Newly synthesized cells are typically identified by using either exogenous thymidine analogues (for example 5-bromo-2-deoxyuridine; BrdU) that are incorporated into the cell's DNA during the synthesis stage of mitosis, or endogenous markers (for example Ki-67) that label actively dividing cells at all stages of mitosis except G_O. BrdU has a bioavailability time of approximately 2 hours (Packard, et al., 1973); therefore it will incorporate into cells synthesizing new DNA during this time. Cell proliferation is assessed by perfusing animals 30 minutes to 25 hours (one complete cell cycle; 24.7 hours in the adult rat (Cameron and McKay, 2001) after a single injection of a DNA synthesis marker (such as BrdU). Cell survival is assessed by perfusing animals more than 25 hours after an injection of a DNA synthesis marker. The use of BrdU is associated with a few important caveats that require consideration (Taupin, 2007). For example, many studies inject animals multiple times over hours and/or days in order to label a greater number of dividing cells. This protocol labels multiple populations of cells,

therefore the age of BrdU-labeled cells cannot be easily determined, making interpretation of results more difficult as some treatments can affect cell proliferation and cell survival independently (see above). Thus, the use of a single injection of BrdU is preferred in many circumstances as it allows for a more accurate determination of the age of labeled cells. Though the majority of studies looking at adult neurogenesis use BrdU, direct comparison of results is often difficult because many factors can influence findings, including the number of BrdU injections given, the timing between injections, the dose of BrdU given, and the timing of injections in relation to administration of any treatment (for review see Gould and Gross, 2002,Leuner and Gould, 2010,Taupin, 2007). For example, Westenbroek et al. (2004) found that exposure to chronic stress for 21 days increased cell survival in female rats when BrdU was administered on days 3 to 7 of stress; on the other hand, cell survival was decreased in female rats when BrdU was administered on days 13 to 16 of stress (Kuipers, et al., 2006). Another important consideration associated with the use of BrdU is that incorporation of BrdU into dividing cells does not necessarily provide an index of neurogenesis as the cells could be either neurons or glial cells. Therefore, the phenotype of BrdU-labeled cells should be determined by co-labeling BrdU with endogenous neuronal markers, such as doublecortin (DCX) or NeuN (see Figure 1.5). DCX is a microtubule-associated protein expressed in immature neurons 1 to 21 days old (Brown, et al., 2003). NeuN, neuronal nuclei, is a transcriptional factor that is expressed in mature neurons (Mullen, et al., 1992).

Neural progenitor cells exist in the germinal region of the dentate gyrus, the subgranular zone, and generate neurons in the presence of a permissive neurogenic microenvironment. Interestingly, progenitor cells from the spinal cord transplanted into the subgranular zone differentiate into neurons (Shihabuddin, et al., 2000). Evidence suggests that the maintenance and proliferation of neural progenitor cells is dependent on the vascular niche

as angiogenesis and neurogenesis are correlated (Palmer, et al., 2000,Shen, et al., 2004). Clusters of progenitor cells are found close to blood vessels and next to endothelial cells (Palmer, et al., 2000), which provide trophic support for neurogenesis (Leventhal, et al., 1999). Furthermore, microglial cells are key components of the neurogenic niche as activated microglia synthesize transforming growth factor-β which promotes neurogenesis (Battista, et al., 2006). The neurogenic environment is also rich with growth factors that promote neurogenesis such as vascular endothelial growth factor, fibroblast growth factor-2, and insulin growth factor-1 (Jordan, et al., 2007).

Although hippocampal neurogenesis persists across the lifespan, the rate of neurogenesis dramatically declines with age as there are lower levels of neural progenitor proliferation, neuronal differentiation, and neuronal survival compared to younger rats (Heine, et al., 2004,Kuhn, et al., 1996,Leuner, et al., 2007,Rao, et al., 2006). This reduction in hippocampal neurogenesis is seen by middle-age and levels remain fairly constant thereafter (Rao, et al., 2006). The cause for the age-associated reduction in neurogenesis is still under debate. It is possible that the population of neural stem cells is reduced in older animals; therefore, accounting for reduced cell proliferation. However, this may not be the case as the level of sex-determining region Y-box 2 (Sox2), a transcription factor that is a marker for neural stem cells, is not reduced in the subgranular zone of older rats but the proliferative activity in these older rats is reduced, suggesting that neural stem cells do exist in the older brain but that they may enter a quiescent state (Hattiangady and Shetty, 2008). Other research has shown a reduction in the level of a specific subpopulation of neural stem cells while other subpopulations remain intact (Lugert, et al., 2010). Furthermore, the reduction of cell proliferation in the older brain is not due to an alteration in the length of the synthesis-phase of the cell cycle (Olariu, et al., 2007). It is possible that reduced cell proliferation and increased quiescent progenitor cells in

the subgranular zone may be related to reduced telomerase activity and shorter telomere length in progenitor cells in the aged brain (Ferron, et al., 2009).

There is strong evidence that reduced neurogenesis in the aged dentate gyrus is related to alterations in the neurogenic microenvironment. Basal glucocorticoids are increased with age (Sapolsky, 1992), and removal of the adrenals in aged rats increases cell proliferation and prevents the age-associated reduction in neurogenesis (Cameron and McKay, 1999,Montaron, et al., 2006,Montaron, et al., 1999), indicating that adrenal steroids are a strong negative regulator of hippocampal neurogenesis. Furthermore, there are vascular changes in the aged hippocampus, including impaired angiogenesis and increased permeability of the blood brain barrier, that lead to alterations in compounds that regulate neurogenesis and that may account for reduced hippocampal neurogenesis. Levels of growth factors that increase hippocampal neurogenesis, such as vascular endothelial growth factor, fibroblast growth factor-2, and insulin growth factor-1, are decreased with aging (Shetty, et al., 2005) and there are increased levels of inflammatory cytokines in the brain that downregulate neurogenesis (Njie, et al., 2012). Together these findings indicate serious alterations in the neurogenic niche, which could lead to quiescent stem cells but also indicate that altering the neurogenic niche can promote neurogenesis.

1.7 FUNCTION OF ADULT GENERATED NEURONS

It is important to note that species differences exist in the timeline of new neuronal development; therefore, the species in which results were found is stated. Within 2 weeks following birth, newly adult-generated neurons born in the subgranular zone migrate into the granule cell layer, and extend axons that synapse onto pyramidal cells of the CA3 region and dendrites into the molecular layer 4-10 days after birth in mice and rats (Hastings and Gould, 1999,Markakis and Gage, 1999,Zhao, et al., 2006). Between 2 and 4 weeks after birth, these

newly generated immature neurons display elaborate dendritic arborizations with dendritic spines and begin to morphologically resemble developmentally generated mature granule cells in mice (Zhao, et al., 2006). Physiologically however, newly generated immature neurons and mature neurons differ at this time point. During the first three weeks after birth, immature neurons in the dentate gyrus of mice receive excitatory GABAergic synaptic inputs and are depolarized, whereas GABA typically acts as an inhibitory neurotransmitter for mature neurons (Ge, et al., 2006). The ability of GABA to excite or inhibit neurons is dependent on the type of chloride transporter expressed by the neuron. Immature neurons express high levels of the chloride importer transporter NKCCl, which allows cells to have a high internal concentration of chloride allowing GABA to depolarize the neuron (Ge, et al., 2006). At approximately 2 to 3 weeks of age there is a decrease in NKCCl transporter levels and an increase in the chloride exporter KCC2, which causes a decrease in internal chloride concentration and consequently GABA hyperpolarizes the neurons (Ge, et al., 2006). These newborn immature neurons show enhanced excitability compared to mature neurons as they have a lower threshold for long-term potentiation (LTP), a cellular model of learning and memory, between 2 to 4 weeks of age in both rats and mice (Ge, et al., 2007,Schmidt-Hieber, et al., 2004). This enhanced excitation continues for neurons 4 to 6 weeks of age in mice as they show larger LTP amplitudes and are sensitive to glutamate as the enhanced LTP is dependent on the NR2B subunit of the NMDA receptor (Ge, et al., 2007). Thus, newly generated immature neurons in mice are more likely to be excited than neighboring mature neurons and may be preferentially recruited and activated by behavioral experiences, such as spatial memory retrieval involving the hippocampus (Kee, et al., 2007). It is important to note that the majority of these findings originate from studies conducted in mice. Although both species have similar levels of cell proliferation, rats have twice as many new neurons surviving compared to mice and a greater proportion of new cells die in mice

(Snyder, et al., 2009). Furthermore, neuronal protein markers such as NeuN are expressed at an earlier time point in rats than mice, new neurons are activated earlier in rats, and the surviving neurons are less likely to be activated by stimuli in mice than rats (Snyder, et al., 2009). Together these results indicate that new neurons mature about 1 to 2 weeks earlier in rats than mice, are much more likely to be recruited for learning and memory, and adult hippocampal neurogenesis is more important for hippocampus-dependent learning and memory in rats than mice.

The maturation timeline of newly generated neurons in the older brain has received very little attention in the literature. However, evidence does exist that indicates that neurons generated in the aged mouse dentate gyrus mature and form dendritic spines at the same rate as neurons generated in the young adult. So it seems that despite considerable alterations in the dentate gyrus microenvironment in older rodents, the neurogenic niche still permits surviving neurons to reach maturity and complexity comparable to that seen in the young adult brain. For example, fully mature neurons born in 10 month old mice show similar density of dendritic spines compared to mature neurons born in 2 month old mice (Morgenstern, et al., 2008). Interestingly, a study looking at the maturation process of newly generated neurons using aged rats instead of mice found delayed early maturation in the older brain (Rao, et al., 2005).

At the behavioral level, findings from many studies suggest that hippocampal neurogenesis is involved in hippocampus-dependent learning and memory (Leuner, et al., 2006a). Newly generated neurons may be preferentially activated (as assessed by IEGs) over more mature granule cells into the memory system during spatial learning (Clark, et al., 2011,Kee, et al., 2007,Sandoval, et al., 2011), indicating that even the addition of a small number of neurons could impact the function of the dentate gyrus. In particular, the dentate gyrus is responsible for pattern separation during memory formation (Leutgeb, et al.,

2007,McHugh, et al., 2007,Rolls and Kesner, 2006), and recently studies have shown that adult neurogenesis within the dentate gyrus is critical for pattern separation (Aimone, et al., 2009,Clelland, et al., 2009).

There may be an optimal number of new neurons that are beneficial for hippocampal function with too few or too many new neurons interfering with the proper functioning of the dentate gyrus. Jessberger et al. (2007), found that the decrease in cognitive performance that follows status epilepticus, prolonged and continuous seizures, may be attributable to the increase in hippocampal neurogenesis that is seen following seizures. Furthermore, traumatic brain injury, which impairs cognitive functioning, also increases cell proliferation in the dentate gyrus (Richardson, et al., 2007). Importantly, treatment with progesterone restores cognitive functioning after traumatic brain injury and also reduces the injury-induced increase in cell proliferation (Barha, et al., 2011b). Studies such as these suggest that a neurogenic homeostasis exists in the dentate gyrus and deviations in this can result in impairments in hippocampal functioning. Indeed, computer models of the function of adult neurogenesis indicate this relationship (Aimone, et al., 2009,Butz, et al., 2006,Wiskott, et al., 2006).

Strong support for a functional role for hippocampal neurogenesis in some forms of hippocampus-dependent learning and memory comes from studies that experimentally reduce or block adult neurogenesis through antimitotic agents such as methalazoxymethanol, focal irradiation, or genetic manipulation. Reducing neurogenesis with these techniques impairs certain types of hippocampus-dependent learning (trace conditioning, contextual fear conditioning, nonmatch to sample, pattern separation) and leaves hippocampus-independent learning intact (Clelland, et al., 2009,Saxe, et al., 2006,Shors, et al., 2001,Shors, et al., 2002,Winocur, et al., 2006). Snyder et al. (2005) has also shown that irradiation does not interfere with spatial learning but does disrupt long-term spatial reference memory.

Furthermore, ablation of newly generated neurons in mice at least 4 weeks of age after learning, indicates that these new neurons are required for spatial reference memory and contextual fear conditioning (Arruda-Carvalho, et al., 2011). There are many issues associated with the techniques that are used to reduce neurogenesis which have been reviewed elsewhere (Epp, et al., 2010,Leuner, et al., 2006a), but it is clear that multiple studies have found that at least some types of hippocampus-dependent learning are disrupted by reduced hippocampal neurogenesis. Furthermore, a study by Deng et al. (2009) provided evidence that immature adult-born neurons (1 to 4 weeks old) in the dentate gyrus of mice are important for long-term reference memory retention, possibly because of their lower threshold for long-term potentiation and greater excitability. In particular, these immature neurons may be important in pattern separation performed by the dentate gyrus (Clelland, et al., 2009) and are particularly malleable in response to spatial learning (Epp, et al., 2007).

Previous studies have found a complicated relationship between spatial working memory and hippocampal neurogenesis. Reducing adult hippocampal neurogenesis with irradiation or antimitotic agents has led to impairments in working memory in the radial arm maze (Iwata, et al., 2008), the Morris water maze (Winocur, et al., 2006), and object location recognition task (Mustafa, et al., 2008). However, reduction in neurogenesis has also led to enhancements in working memory in the radial arm maze (Saxe, et al., 2007), or had no significant effect in the delayed-nonmatched-to-place task (Hernandez-Rabaza, et al., 2009). Although an increase in hippocampal neurogenesis is in general associated with enhanced spatial reference memory, this is not always the case as evidence also suggests that an optimal range of neurogenesis exists and increases above this point do not necessarily translate into benefits for hippocampal function (for review see Koehl and Abrous, 2011).

Further evidence for the involvement of hippocampal neurogenesis in learning and memory can be found in the age-associated cognitive decline literature. Hippocampus-dependent memory performance predicts levels of hippocampal neurogenesis in aged rodents (for review see Drapeau and Nora Abrous, 2008), further indicating a role for neurogenesis in age-related memory decline. Furthermore, aged rats that are impaired in spatial reference learning and memory had lower levels of cell proliferation and survival compared to age-matched unimpaired rats (Drapeau, et al., 2003). However, not all studies find this positive correlation between memory functioning and level of hippocampal neurogenesis (Bizon, et al., 2004,Lee, et al., 2012). Furthermore, many treatments that improve hippocampus-dependent learning and memory in older animals also increase adult hippocampal neurogenesis, such as exercise (van Praag, et al., 2005).

It is well established that encoding of long-term memories requires protein synthesis. Interestingly, in older rodents there is a reduction in levels of protein synthesis in brain regions involved in learning and memory including the dentate gyrus of the hippocampus (Smith, et al., 1995). This reduction in overall protein synthesis may be related to the memory impairments seen with aging. In general a reduction in transcription of genes involved in mitochondrial function, plasticity, and synaptic function is seen with age, while an increase is seen in the expression of genes involved in the stress and immune systems (Bishop, et al., 2010). Specifically related to memory function, an age-related reduction is seen in the activity of many immediate early genes (IEGs). IEGs encode transcription factors that regulate the expression of target genes, as well as effector proteins that influence the cellular function at the synapse of cells (Kubik, et al., 2007). *Zif268* is a type of IEG that functions as a transcription factor for genes required for hippocampus-dependent long-term memory. Furthermore, expression of *zif268* in the dentate gyrus is required for long-term memory, as disruption of *zif268* leads to the

absence of late long-term potentiation (Jones, et al., 2001). Importantly, mRNA and protein levels of *zif268* in the hippocampus are decreased in older rodents (Desjardins, et al., 1997,Yau, et al., 1996), indicating that reductions in this IEG may be related to spatial memory impairments seen in aged rodents. Additionally, new adult-generated neurons are preferentially recruited and activated, as indexed by IEG expression, by spatial learning compared to developmentally-generated neurons in the dentate gyrus (Kee, et al., 2007). Importantly, Snyder et al. (2009) show that *zif268* expression in newly generated neurons in response to spatial learning and memory peaks at 3 weeks of age in rats. Therefore, functional incorporation of immature newly generated neurons into the existing hippocampal circuitry can be indexed by assessing IEG expression in new neurons at different developmental stages. In Chapter 2 of this thesis, I examine IEG expression in new neurons of two different ages after exposure to Premarin.

1.8 HIPPOCAMPAL NEUROGENESIS AND ESTROGENS

As stated above, estrogens can greatly impact hippocampus function across the lifespan, and has been proposed as a potential therapeutic treatment for alleviating age-associated cognitive decline in women. One potential mechanism through which estrogens could influence hippocampus-dependent learning and memory is hippocampal neurogenesis. ERs have been colocalized with progenitor cells within the subgranular zone of the dentate gyrus in adult rats (Isgor and Watson, 2005,Mazzucco, et al., 2006), indicating that estrogens could directly modulate cell proliferation in the dentate gyrus through interaction with ERα and/or ERβ. Indeed, Mazzucco et al. (2006) found a small percentage of newly dividing cells that expressed mRNA for ERs, with a greater percentage expressing ERβ in ovariectomized female rats. On the other hand, in intact male rats the majority of newly dividing cells expressed mRNA for ERα

and ERβ (Isgor and Watson, 2005), potentially indicating that different levels of ERs are expressed on progenitor cells in males and females. The modulation of adult hippocampal neurogenesis by estrogens has been assessed by examining how neurogenesis levels are altered with endogenous fluctuations in estrogens, such as across the estrous cycle, and with aging, as well as by administration of exogenous estrogens following bilateral ovariectomy.

Estrous cycle

The female rodent estrous cycle lasts for 4-5 days, during which time 17β-estradiol levels increase slowly during the diestrous phase until the day of proestrus when 17β-estradiol rises and falls quickly. 17β-estradiol levels are lowest during the estrous phase, which follows proestrus. This naturally occurring fluctuation in gonadal hormones influences hippocampal neurogenesis as adult female rats have 50% more cells proliferating during proestrus compared to diestrous females, estrous females or intact male rats (Tanapat, et al., 1999). Importantly, the higher number of proliferating cells during proestrus does not result in lasting changes in cell survival, as the difference in BrdU-labeled cells found between proestrus and estrus is no longer seen 21 days after BrdU labeling (Tanapat, et al., 1999). These estrous cycle effects on hippocampal neurogenesis may be species specific, as a study conducted with female C57BL/6 mice failed to find a significant increase in cell proliferation during proestrus (Lagace, et al., 2007); however, this may have been due to methodological issues. Furthermore, reproductively inactive female meadow voles have higher levels of cell proliferation than reproductively active female meadow voles (Galea and McEwen, 1999,Ormerod and Galea, 2001). These findings from intact cycling female rodents emphasize the powerful mitogenic effects of 17β-estradiol in the dentate gyrus.

Aging

Despite the persistence of hippocampal neurogenesis throughout the lifespan, the level of hippocampal neurogenesis dramatically decreases with age in many species (Klempin and Kempermann, 2007) and may contribute to age-related reductions in learning and memory (Drapeau, et al., 2003,Montaron, et al., 2006). As stated earlier, the age-related reduction in neurogenesis may be related to many factors such as cell cycle kinetics, progenitor cell availability and hormone/growth factor levels (for review see Jessberger and Gage, 2008). In female rats, aging is also associated with alterations in circulating 17β-estradiol levels as reproductively senescent female rats undergo "estropause", which involves irregular, prolonged estrous cycles that eventually lead to acyclicity (for review see Chakraborty and Gore, 2004).

Kuhn et al. (1996) first reported an age-related decrease in cell proliferation in the dentate gyrus between 6 and 21 month old female rats. This reduction in the level of cell proliferation occurs by middle-age (10-12 months old) and does not linearly continue as the rat ages as comparable levels of neurogenesis (cell proliferation and cell survival) are seen in middle-aged (10-12 months old) and older rats (20-24 months old) (Rao, et al., 2005,Rao, et al., 2006). However it is important to note that these studies did not take into account the number of new proliferating cells in each of the age groups and therefore could not determine the percentage of newly proliferating cells that survived. Kempermann et al. (1998) found a higher number of cells surviving in 18 month old females compared to 6 month old female C57BL/6 mice; however the percentage of surviving cells, once the rate of proliferation is taken into account, did not differ between the two age groups. Therefore, it seems that the ability of new cells to survive and differentiate into a neuronal phenotype remains intact in old age, suggesting that treatments that increase the number of proliferating cells in the dentate gyrus may have beneficial effects on hippocampal morphology and functioning.

One potential way in which cell proliferation could be enhanced in older female rats is to replace the age-related loss in estrogens. Very few studies have investigated the effects of estradiol treatment on hippocampal neurogenesis in older female rats. Unlike in younger ovariectomized female rats (Barha, et al., 2009b,Ormerod, et al., 2003,Tanapat, et al., 2005,Tanapat, et al., 1999), acute treatment with a high dose of 17β-estradiol does not increase cell proliferation in 12 month old virgin ovariectomized female rats (Barha and Galea, 2011,Chiba, et al., 2007). However, longer-term treatment with 17β-estradiol and daily intake of soy extract, a naturally occurring compound with weak estrogenic activity, does increase cell proliferation in 22 month old long-term ovariectomized female rats (Perez-Martin, et al., 2005). Furthermore, chronic (10 days and 60 days) injections of 17β-estradiol increase cell proliferation but have no effect of cell survival in middle-aged 10-12 month old C57BL/6 female mice (Saravia, et al., 2007). In Chapter 5, I examined the ability of different estrogens to influence cell proliferation in middle-aged female rats.

Ovariectomy

The bilateral removal of ovaries minimizes circulating endogenous gonadal hormones 24 hrs after surgery (Woolley and McEwen, 1993), decreases spine density in the hippocampus 7 days after surgery (Woolley and McEwen, 1993), and decreases estrogen receptor β, but not α, density in the brain 3 months after surgery (Rose'Meyer, et al., 2003). Short-term (6-7 days) ovariectomy reduces cell proliferation levels in adult female rats compared to sham-operated female rats in proestrus (Tanapat, et al., 1999). Conversely, long-term (3-4 weeks) ovariectomy does not seem to reduce cell proliferation (Green and Galea, 2008,Lagace, et al., 2007,Tanapat, et al., 1999) or short-term (1 week) cell survival (Tanapat, et al., 2005), suggesting the presence of a compensatory mechanism through which levels of neurogenesis are returned to post-ovariectomy levels over time. In support of this Zhao et al. (2005) found that circulating levels

of estradiol in the periphery increased as time since ovariectomy increased, likely via extragonadal sources. Long-term ovariectomy could also result in subsequent increases in a different population of proliferative cells to compensate for the initial decrease in cell proliferation.

Neurosteroids and adult neurogenesis

Steroid hormones are synthesized in steroidogenic tissues, including the gonads, the adrenal gland, adipose tissue, brain and the placenta, from the precursor cholesterol. The brain has the ability to synthesize steroid hormones *de novo* from locally synthesized cholesterol independent of peripheral circulating hormones (Naftolin, et al., 1996). Recently it has been suggested that postnatal neurogenesis may be dependent to a certain degree on local *de novo* estrogen synthesis in the hippocampus (Fester, et al., 2006). Fester et al. (2006) showed a reduction in cell proliferation after inhibiting synthesis of estrogens by hippocampal cells from postnatal day 5 rats *in vitro*, although it is not clear whether the tissue used was from male or female rats. Furthermore, a large quantity of 17β-estradiol is synthesized *de novo* and released by hippocampal neurons at levels reported to be higher than in serum in the adult male (Hojo, et al., 2004). Despite these studies, the necessity of *de novo* synthesis of 17β-estradiol for adult hippocampal neurogenesis *in vivo* is yet to be established and recent evidence suggests limited local production of estradiol in the hippocampus of adult gonadectomized male or female rats (Barker and Galea, 2009).

Ovariectomy plus replacement with estrogens

The effects of estrogens on adult hippocampal neurogenesis are complex and depend on many factors including sex of subject, the hormonal status of the subject, length of exposure, dose, and the type of estrogens given (for review see Barha, et al., 2009a,Galea, 2008,Galea, et al., 2006,Galea, et al., 2008). The reduction in cell proliferation that occurs with short-term (1

week) ovariectomy can be rescued with a single acute injection of 17β-estradiol (10 μg; (Tanapat, et al., 1999)); however this single injection is ineffective in altering cell proliferation 4 weeks after ovariectomy (Tanapat, et al., 2005). These findings could potentially reflect a reduction in the ability of progenitor cells in the dentate gyrus to respond to estradiol as time since ovarian cessation increases, reminiscent of the critical period hypothesis (refer to Section 1.1). This could be due to the reduction in ERβ density in the brain that is seen 3 months post-ovariectomy (Rose'Meyer, et al., 2003) and/or could be related to the healthy cell bias hypothesis (refer to Section 1.1). As predicted by both the critical period and healthy cell bias hypotheses, the decreased ability of the hippocampus to respond to estrogens as the length of time since ovariectomy increases is also seen in hippocampus-dependent learning and memory (Bohacek and Daniel, 2010,Daniel, et al., 2006,Gibbs, 2000). Acute short-term exposure (30 minutes to 2 hours) to a high level of 17β-estradiol (10 μg) increases cell proliferation in ovariectomized female rats compared to controls (Barha, et al., 2009b,Barker and Galea, 2008,Mazzucco, et al., 2006,Tanapat, et al., 2005,Tanapat, et al., 1999). Furthermore, exposure to estradiol benzoate, a slower metabolizing estradiol, initially enhances cell proliferation within 4 hours but subsequently suppresses cell proliferation within 48 hours in ovariectomized female rats, with the suppressive effect dependent partly on adrenal steroids (Ormerod, et al., 2003).

Many studies have investigated the effects of acute administration of 17β-estradiol on cell proliferation, however far fewer studies have looked at the effects of chronic administration of 17β-estradiol. Chronic cyclic treatment every four days for 20 days or chronic continuous treatment for 21 days with 17β-estradiol did not significantly alter cell proliferation in adult ovariectomized female rats compared to controls (Tanapat, et al., 2005). On the other hand, Barker and Galea (2008) found an increase in cell proliferation with repeated treatment with 17β-estradiol benzoate for 15 days. The contrasting results of these two studies may be due to

important methodological differences. For example, Barker and Galea (2008) administered estradiol benzoate, which is a conjugated form of 17β-estradiol, via a single subcutaneous injection each day for 15 days resulting in a pulsatile exposure to estradiol, whereas Tanapat et al. (2005) gave 17β-estradiol via silastic pellets resulting in continuous exposure to high plasma levels of 17β-estradiol (see Barker and Galea, 2008 for further discussion). Interestingly, Barker and Galea (2008) found an effect of chronic 17β-estradiol treatment on cell proliferation in female rats, but not in male rats.

Recent studies have examined the effects of repeated 17β-estradiol treatment on cell survival independent of its effects on cell proliferation. In castrated male rats repeated 17β-estradiol benzoate administration (15 or 29 days) does not influence cell survival in the dentate gyrus (Barker and Galea, 2008,Spritzer and Galea, 2007), although testosterone treatment does increase cell survival (Spritzer and Galea, 2007). However, 15 days of 17β-estradiol benzoate administration does decrease cell survival (as measured by the number of surviving BrdU-ir cells 16 days after injection) in ovariectomized female rats (Barker and Galea, 2008). Studies conducted in meadow voles indicate that the effects of 17β-estradiol on cell survival in the hippocampus may depend on the particular stage of cell maturation in which the cells are exposed to estradiol. In male meadow voles 17β-estradiol increases cell survival only if given during the 'axon extension phase' of the cell maturation cycle (Ormerod, et al., 2004). However, in female meadow voles 17β-estradiol enhances cell survival when given at all time points (Ormerod and Galea, unpublished results), suggesting that 17β-estradiol may have a more ubiquitous affect in the female hippocampus. Furthermore, recent data suggests that chronic (20 days) treatment with 17β-estradiol increases cell survival, while chronic estrone decreases cell

survival, when hormone treatment is begun 24 hours before newly divided cells are labeled (McClure, et al., accepted).

Together these studies indicate that 17β-estradiol impacts different aspects of hippocampal neurogenesis in the young adult female. However, as mentioned above, there are other naturally occurring estrogens such as estrone and 17α-estradiol that may differentially influence the hippocampus. Furthermore, the influence of Premarin, the most prescribed HRT in the United States, on hippocampal neurogenesis has not been investigated. HRTs differ widely in their cognitive and neuroprotective effects, which may be due to the different composition of estrogens (17β-estradiol, estrone and 17α-estradiol) in these therapies. Ultimately findings from these studies may lead to the development of new therapeutic advances in the treatment of cognitive decline in postmenopausal women.

1.9 THESIS OVERVIEW AND OBJECTIVES

The experiments described in this thesis investigate the effects of different forms of estrogens on hippocampus-dependent learning and memory and hippocampal neurogenesis using a rodent model of menopause in adult and middle-aged female rats. The objectives of the present thesis are as follows:

1. To determine the effects of long-term treatment with the hormone replacement therapy Premarin on hippocampus-dependent spatial learning and memory and hippocampal neurogenesis in adult female rats (Chapter 2). The experiment described in this chapter examined the effects of Premarin, a conjugated equine estrogen formulation that is the most prescribed hormone replacement therapy to menopausal women in North America, on the hippocampus. In particular, the effect of Premarin on spatial working and reference memory performance in the working/reference memory version of the radial arm maze, and on

neurogenesis and new neuronal activation in the dentate gyrus was investigated. The specific hypotheses were that Premarin would impair spatial working and reference memory, and that performance would correlate with alterations in both hippocampal neurogenesis and new neuronal activation.

2. To determine the effects of three main forms of estrogens on hippocampal cell proliferation in adult female rats, and whether these effects are dependent on time and dose (Chapter 3). The experiment described in this chapter determined the effects of 17β-estradiol, 17α-estradiol, and estrone at three different doses on hippocampal cell proliferation after 30 minutes and 4 hours of hormone exposure. The specific hypotheses were that different doses of each estrogen would increase cell proliferation in a time-dependent manner, with effects seen after 30 minutes but not after 4 hours.

3. To determine the effects of short-term treatment with three main forms of estrogens on hippocampus-dependent contextual fear conditioning in adult female rats, and whether these effects are dependent on dose (Chapter 4). The experiment described in this chapter determined the behavioral effects of the three estrogens used in Chapter 1. In particular, the effects of three different doses of the estrogens on hippocampus-dependent contextual fear conditioning and hippocampus-independent cued fear conditioning were examined. These behavioral effects were correlated with potential alterations in synaptic proteins. The specific hypotheses were that 17β-estradiol and 17α-estradiol would dose-dependently enhance, whereas estrone would impair contextual fear conditioning. Furthermore, these behavioral effects would correlate with alterations in synaptophysin expression in the hippocampus.

4. To determine the effects of three main forms of estrogens on hippocampal cell proliferation in middle-aged female rats, and whether these effects are dependent on previous reproductive experience (Chapter 5). The experiment described in this chapter

determined the effects of 17β-estradiol, 17α-estradiol, and estrone on hippocampal cell proliferation in multiparous and nulliparous middle-aged female rats. The specific hypotheses were that 17β-estradiol and 17α-estradiol would increase cell proliferation in multiparous, but not nulliparous, middle-aged female rats.

2 THE HORMONE THERAPY, PREMARIN, IMPAIRS HIPPOCAMPUS-DEPENDENT SPATIAL LEARNING AND MEMORY AND REDUCES ACTIVATION OF NEW GRANULE NEURONS IN RESPONSE TO MEMORY IN FEMALE RATS.[1]

2.1 INTRODUCTION

Neurodegenerative diseases and cognitive decline associated with aging affect millions of people every year and may be related to gonadal hormone levels. For example, some studies have shown that older men and women with lower levels of gonadal hormones have poorer cognition and increased incidence of neurodegenerative diseases (Baum, 2005,Veiga, et al., 2004). Furthermore, natural menopause, the cessation of ovarian function and subsequent reduction in gonadal hormones (i.e. 17β-estradiol) that occurs around 50 to 51 years of age in women, and surgical menopause are associated with a decline in certain domains of cognition (Hogervorst and Bandelow, 2010,Hogervorst, et al., 2000,Sherwin and Henry, 2008). It is important to note that normal aging impairs certain cognitive domains, including long-term memory and working memory, while other cognitive domains remain intact, such as vocabulary memory (Buckner, 2004).

Estrogens, such as 17β-estradiol and estrone, have been suggested as a possible therapeutic agent for improving cognition in postmenopausal women. Indeed certain types of hormone therapy (HT) improve cognition and reduce the incidence of Alzheimer's disease in postmenopausal women (Hogervorst, et al., 2000,Ryan, et al., 2008,Seshadri, et al., 2001). The effects of HTs on cognition vary dramatically across studies in humans, with some studies

[1] Barha, C.K. and Galea, L.A. (in press) The hormone replacement therapy, Premarin, impairs hippocampus-dependent spatial learning and memory and reduces activation of new granule neurons in response to memory in female rats. Neurobiology of Aging. Accepted July 10, 2012.

finding improvements and other studies findings impairments. There may be many reasons for these discrepancies including the healthy cell bias hypothesis (Brinton, 2008), the critical period hypothesis (Gibbs, 2010), and the formulation of HT.

The healthy cell bias of estrogen action hypothesis purports that discrepant results of HT to improve cognition may be related to the health status of females and cells, with positive effects of estrogens seen in healthy women and cells and negative effects seen in unhealthy women and cells (Brinton, 2005). A key component of this hypothesis is that timing of estrogen treatment is critical, with beneficial outcomes seen when treatment is begun before neurodegenerative decline commences or before aging negatively influences cells. Studies outcomes are dependent on estradiol's ability to enhance mitochondrial function in the healthy cell, maintain mitochondrial bioenergetics, and prevent dysregulation of intracellular calcium homeostasis (Brinton, 2008).

Another important determinant of the effectiveness of HT on cognition relates to the critical period hypothesis that states that the ability of estrogens to influence cognition greatly decreases as the time without endogenous estrogens increases (Gibbs and Gabor, 2003). Gibbs (2000) showed that treatment with 17β-estradiol prevented impairments in spatial learning when treatment was initiated immediately or 3 months after ovariectomy but not if initiated 10 months after ovariectomy. Furthermore, 17β-estradiol enhanced spatial working memory performance when treatment began immediately, but not 5 months, after ovariectomy (Daniel, et al., 2006). The importance of timing for initiating HT may be related to the loss of basal forebrain cholinergic function, the decline of which is accelerated after ovariectomy (Gibbs, 2010). Importantly, clinical studies in women also support the critical period hypothesis (for review see Maki, 2006,Rocca, et al., 2011,Sherwin, 2009). Together, these studies support the existence of

a critical period of time within which HT must be commenced in order to confer beneficial effects of plasticity and behavior.

In addition to the healthy cell bias and the critical period hypotheses, the formulation of HT may be important. A meta-analysis suggested that HTs containing 17β-estradiol had more positive effects on certain aspects of cognition than HTs containing conjugated equine estrogens (CEE) (Hogervorst, et al., 2000,Ryan, et al., 2008). Furthermore, the Women's Health Initiative Memory Study (WHIMS), the largest randomized controlled trial conducted thus far, did not find evidence of a beneficial effect of the HT Premarin on cognition or risk for dementia in women (Espeland, et al., 2004,Rapp, et al., 2003,Shumaker, et al., 2003). Importantly Premarin, the most common type of HT prescribed in the United States in the 1990's (Wysowski, et al., 1995), is comprised of CEEs and has at least 10 different estrogenic compounds but over 50% are sulphated forms of estrone. Despite the large number of participants, many important methodological issues have been raised with the WHIMS in recent years. Specifically the women in the WHIMS suffered from many serious health issues, were between 65 and 79 years of age, and had been without significant ovarian functioning for more than 15 years, which may have greatly impacted the ability of HT to influence the brain (for review see Henderson, 2006,Sherwin and Henry, 2008). Furthermore, the type of HT given to participants is of central concern, as CEE-based HTs, such as Premarin, do not confer the same benefits on cognition and dementia risk early in menopause as do 17β-estradiol-based HTs (Hogervorst, et al., 2000,Ryan, et al., 2008). Indeed, Premarin treatment has been associated with greater hippocampal atrophy in post-menopausal women compared to placebo treatment (Resnick, et al., 2009).

Estrone and 17β-estradiol, two main estrogens used in HTs, influence both brain and behavior. Experimental studies have shown that 17β-estradiol has potent neuroprotective properties (Chen, et al., 2006), and can greatly increase neuroplasticity in the hippocampus

(Barha, et al., 2010,Barha and Galea, 2010,Barha and Galea, 2011,Barha, et al., 2009b,Frick, et al., 2010). Importantly, 17β-estradiol, at certain doses, can have positive effects on learning and memory (Barha and Galea, 2010). The relationship between 17β-estradiol and spatial performance is complex with many moderating variables, including dose of 17β-estradiol, length of treatment, type of memory system under investigation, age of animals, route of administration, memory demand, and strategy use (Daniel, 2006,Frick, 2009,Korol, 2004). Estrone can also influence neuroplasticity (Barha and Galea, 2011,Barha, et al., 2009b,Bhavnani, et al., 2003,Budziszewska, et al., 2001) but impairs hippocampus-dependent memory at certain doses (Barha, et al., 2010). Furthermore, in middle-aged female rats Premarin can have positive or negative effect on spatial memory depending on higher dose and type of exposure (cyclic or tonic; Acosta, et al., 2009,Engler-Chiurazzi, et al., 2011).

The hippocampus mediates many forms of learning and memory (Sutherland, et al., 1983) and shows a remarkable degree of plasticity throughout the lifespan (Galea, et al., 2008). In adulthood progenitor cells in the dentate gyrus of the hippocampus retain the ability to proliferate into daughter cells that can become neurons in most mammalian species including humans (Eriksson, et al., 1998,Gould, et al., 1997). Evidence suggests that adult hippocampal neurogenesis is involved in hippocampus-dependent learning and memory, as inhibition of neurogenesis severely impairs some forms of hippocampus-dependent learning and memory (Clelland, et al., 2009,Koehl and Abrous, 2011). Furthermore, findings suggest adult-generated neurons are functional and are activated by spatial learning and memory (Epp, et al., 2011a,Kee, et al., 2007). Short-term treatment with 17β-estradiol and estrone increase cell proliferation, whereas longer-term treatment with 17β-estradiol reduces cell survival (Barker and Galea, 2008) but no study has examined the influence of Premarin on neurogenesis in the hippocampus or on activation of new neurons in response to memory retrieval.

Therefore, the aim of the present study was to determine the effects of the hormone therapy, Premarin, on hippocampus-dependent spatial reference and working memory in the radial arm maze, hippocampal neurogenesis and activation of new neurons in response to memory using a rodent model of surgical menopause. Ovariectomized female rats were chronically treated with different doses of Premarin and tested on the spatial working/reference memory version of the radial arm maze. Hippocampal neurogenesis and activation of new neurons that were 34 days old in response to the radial arm maze, via immediate early gene expression was assessed after 33 days of hormone treatment. Based on the negative effects of lower doses of Premarin on cognition (Engler-Chiurazzi, et al., 2011,Espeland, et al., 2004,Rapp, et al., 2003,Shumaker, et al., 2004) we hypothesized that Premarin would dose-dependently impair spatial memory, alter hippocampal neurogenesis and decrease activation of new neurons in response to spatial memory.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Forty-five adult female Long-Evans rats, weighing 225- 250 g (4 months of age), were used in this study. Ovariectomized rats were purchased from Charles River Canada (St-Constant, Quebec, Canada). Briefly, rats were anesthetized using a combination of Ketamine and Xylazine and ovaries were bilaterally removed through a dorsal midline incision, which was closed with wound clips. Rats arrived at the Department of Psychology at the University of British Columbia five days following surgery. Rats were individually housed in opaque polyurethane bins (48 x 27 x 20 cm) with aspen chip bedding and were given Purina rat chow

containing 0.1-0.15% isoflavones by weight (lab diet 5012) and tap water *ad libitum*. Rats were maintained under a 12h: 12h light/dark cycle (lights on 07:30h). Two days after arrival, wound clips were removed from the dorsal incision on each rat. Following this rats were handled daily for 5 days for 2 min before testing commenced (Barha, et al., 2009b,Pawluski, et al., 2006a) in order to habituate the rats to the experimenters and general procedures. This amount of handling was much less than the level of handling that has previously been shown to negate estradiol's influence on cognition (Bohacek and Daniel, 2007). Thirty rats were assigned to be behaviorally tested, and the remaining fifteen rats were assigned to be cage controls (see below for further details). All experiments were conducted in accordance with the ethical guidelines set by the Canada Council for Animal Care and were approved by the University of British Columbia Animal Care Committee. All efforts were made to reduce the number and the suffering of animals.

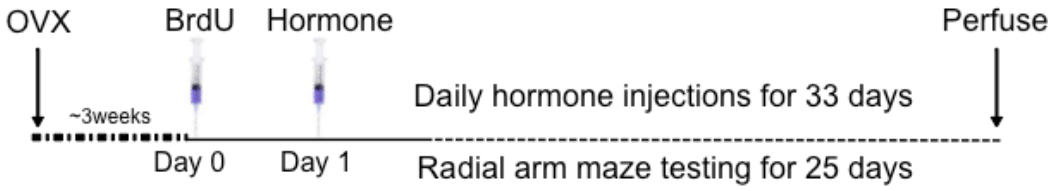
2.2.2 Procedures

2.2.2.1 Hippocampus-dependent reference and working version of the radial arm maze

See Figure 2.1 for experimental timeline. Nine days after arriving in the facility, rats were habituated on the spatial working/reference memory version of the radial arm maze (Barha, et al., 2007,Olton and Papas, 1979). The spatial working/reference version of the radial arm maze was used as it allows for the simultaneous assessment of working memory and reference memory. Working memory is defined as the manipulation and retrieval of trial-unique information that is used to guide prospective action (Baddeley, 2003a) and reference memory is defined as long-term stable memory (Olton and Papas, 1979). The wooden radial arm maze was elevated 80 cm from the floor, with eight-arms (53 cm long x 10 cm wide) projecting at equal angles from an octagonal center platform (36 cm in diameter). The maze was placed in a dimly

lit room with multiple extra-maze cues that were not moved throughout the duration of the experiment. In order to minimize the use of intra-maze cues, the radial arm maze was randomly rotated twice a week. Rats were tested between 12 pm and 4 pm daily, 2-4 h after injection (see below). All rats were introduced to the food reward (Kellogg's Froot Loops Cereal, Battle Creek MI) in their home cages for 3 days while habituating to the maze.

Figure 2.1: Timeline of procedure.



Beginning on the first day of habituation, rats were food deprived to 90% of their body weight and maintained at this for the duration of the experiment. Rats were allowed weight gain of 3 grams/week to account for natural growth.

Rats were habituated to the maze for a total of 20 min over 3 days (Pawluski, et al., 2006a,Pawluski, et al., 2006b). In order to accomplish this, rats were placed on the center platform and were allowed to freely explore the maze for the allotted amount of time. Each rat was randomly assigned a pattern of baited and non-baited arms (four baited arms out of eight possible arms) that remained constant for the duration of the experiment. Following habituation, rats were shaped to move along the entire arm to reach the baited well at the end of the arm. The assigned arms were each baited with 4 quarters of Froot Loop placed at equidistant intervals along the arm. Each of the 3 shaping days began by placing a rat on the

center of the platform with access to all arms, and ended when 10 min had elapsed or the rat had entered all 4 baited arms. An arm was considered entered when the front forepaws of the rat had crossed halfway down the length of the arm. Testing sessions, one per day, began 1 day after the end of shaping and consisted of the rat being released on the center of the platform and remaining on the maze until all baited arms were entered or until 10 min had elapsed. This occurred only three times over the course of testing, all on the first day of testing (2 rats in the group given a low dose of Premarin and 1 rat give a high dose of Premarin). Rats were tested for a total of 25 days over the course of 5 weeks (5 days of testing with 2 days off each week). During each testing session, rats could make three types of errors: 1) reference memory errors (RME) defined as entries into non-baited arms, 2) working memory errors (WME) defined as repeat entries into baited arms, and 3) working/reference memory errors (W/RME) defined as repeat entries into non-baited arms. Total number of RME, WME, and W/RME, number of days taken to reach criterion (defined as no more than 2 errors per day for 2 consecutive days), and total number of choices to criterion were calculated. Furthermore the total number of RME, WME, and W/RME committed across blocks of 5 testing days were calculated (i.e. each block of trials consisted of 5 days using the procedures of (Bannerman, et al., 2008,Barha, et al., 2007,Park, et al., 2008,Pawluski, et al., 2006a,Spritzer, et al., 2011).

2.2.2.2 5-Bromo-2-deoxyuridine (BrdU) administration

The day after the final shaping session and twenty-four hours before the first testing session all rats were given a single intraperitoneal injection of 5-Bromo-2-deoxyuridine (BrdU; Sigma, St. Louis, MO) dissolved in 0.9% saline (200 mg/kg) at approximately 11 am. BrdU is an exogenous thymidine analogue that incorporates into cells undergoing DNA synthesis (S-phase) within two hours of administration (Packard, et al., 1973). Depending on the amount of time elapsed between injection and perfusion of the animal, BrdU can be used to assess cell

proliferation or cell survival in the granular cell layer of the dentate gyrus (for further discussion see Barha, et al., 2011b,Taupin, 2007). In the present study, because we perfused the rats 34 days after BrdU administration, the number of BrdU-ir cells was used as a marker of the survival of newly synthesized cells.

2.2.2.3 Premarin administration

Three weeks after ovariectomy and twenty-four hours following BrdU injection, rats were randomly assigned to one of three treatment groups (n=15) and received daily subcutaneous injections of either vehicle (Control, 0.10 ml sesame oil), or Premarin (liquid, injectable form; distributed by Wyeth Pharmaceuticals ETC) at either a low dose (10 μg/0.10 ml sesame oil) or a high dose (20 μg/0.10 ml sesame oil). The 20 μg high dose of Premarin used in the present study is based on the most common dose of Premarin prescribed to women (0.625 mg per day; Acosta, et al., 2009). The low dose of 10 μg was chosen as this dose of estrogens influences hippocampus-dependent learning and memory and hippocampal neurogenesis (Barha and Galea, 2010,Frick, et al., 2010). All rats received a daily hormone injection for 33 days. Thirty rats (n=10/group) were tested on the radial arm maze, two to four hours after the hormone or vehicle injection, for a total of 25 days over the course of 5 week (i.e. 5 testing days per week with 2 days off per week). The remaining 15 rats were cage controls and remained undisturbed other than daily injections (n=5/group) and weekly cage changing. Therefore, we had 3 groups of 10 rats each that were tested on the radial arm maze (Control, Low Premarin, High Premarin), and 3 groups of 5 rats each that were kept as cage controls (Control Cage Control, Low Premarin Cage Control, High Premarin Cage Control).

2.2.2.4 Open field test

On day 16 of testing, 3 hours after RAM testing, anxiety-like behavior and locomotor activity of the rats was assessed during a 5 min session in the open field apparatus, which was a

120 cm x 120 cm square arena divided into 16 squares of equal dimensions (each square was 30 cm x 30 cm) with 40 cm high walls. Rats were individually placed in the center of the arena and activity was recorded and scored by a video-tracking system (ANYmaze; Stoelting Co., Wood Dale, IL). An observer blind to the groups scored the distance travelled and number of line crossings in the central region (60 cm x 60 cm; defined as the four squares in the center of the open field apparatus), the peripheral region (defined as the 12 squares along the sides of the open field apparatus), and the entire arena. A line was considered crossed or a zone entered when a rat's forepaws had both completely crossed over the line. The apparatus was cleaned with 70% ethanol between rats. An increase in the number of central crossings is considered indicative of decreased anxiety-like behavior, whereas an increase in total crossings is considered an index of locomotor behavior (Prut and Belzung, 2003).

2.2.2.5 Tissue processing

Two hours following the final 25th radial arm maze testing session (34 days after BrdU injection), rats were deeply anaesthetized with an overdose of sodium pentobarbital and perfused with 0.9% saline followed by 4% formaldehyde. Brains were removed and post-fixed in 4% formaldehyde at 4°C for 24 hours. Intact brains were transferred to 30% sucrose in phosphate buffered saline (PBS) for cryoprotection. Ten series of 40 μm sections were collected throughout the rostral-caudal extent of the hippocampus using a Leica SM2000R freezing sliding microtome (Richmond Hill, Ontario, Canada).

2.2.2.6 Immunohistochemistry

Every 10th slice was either processed for BrdU, double labeled with fluorescent markers for BrdU and NeuN (a marker of mature neurons) to allow for phenotyping of BrdU-ir cells, or double labeled with fluorescent markers for BrdU and the immediate early gene product zif268 (Egr-1) to assess activation of new surviving cells.

BrdU immunohistochemistry was performed as described previously (Barha, et al., 2011a,Barha and Galea, 2011,Barha, et al., 2011b,Epp, et al., 2011b). Briefly, sections were pretreated in 0.6% hydrogen peroxide at room temperature for 30 min, rinsed four times in 0.1M PBS, and then were incubated in 2N hydrochloric acid at 37°C. This was followed by incubation with the primary antibody (1:200 mouse anti-BrdU; Roche, Toronto, ON, Canada), 3% normal horse serum, and 0.1% Triton-X for 24 h at 4°C. The tissue was then incubated with the secondary antibody (1:100 anti-mouse IgG; Vector Laboratories, Burlington, ON, Canada) for 4 h, followed by incubation in an ABC solution (Vector) for 1.5 h. BrdU-labeled cells were visualized with diaminobenzidine (DAB; Sigma, Oakville, ON, Canada).

A second series of brain tissue was double-labeled for BrdU and the mature neuronal protein NeuN as described previously (Barha, et al., 2011a). All procedures were performed in the dark. The tissue was incubated in 0.1M PBS containing mouse anti-NeuN (1:250; Chemicon) and 0.3% Triton-X for 24 h, then in 0.1M PBS containing donkey anti-mouse Alexa 488 (1:200; Invitrogen Molecular Probes) for 18 h. The tissue was fixed in 4% PFA for 10 min, rinsed twice in 0.9% NaCL for 10 min each, and incubated in 2N hydrochloric acid at 37°C. In order to visualize BrdU labeling, tissue was first incubated for 24 h at 4°C in rat anti-BrdU (1:500; ABD Serotec), then incubated in donkey anti-rat Cy3 (1:500; Invitrogen Molecular Probes) for 24 h at 4°C.

Another series of tissue was double-labeled for BrdU and the immediate early gene zif268 (Epp, et al., 2011b). *Zif268* is an immediate early gene that functions as a transcription factor for target genes required for hippocampus-dependent long-term memory. Tissue was incubated in primary antibody rabbit anti-zif268 (1:1000; Egr-1 SC-189, Santa Cruz Biotechnology, Santa Cruz, CA, USA) with 4% normal donkey serum and 0.03% Triton-X for 24 h. This was followed by incubation with the secondary antibody (1:200 anti-rabbit IgG;

Vector Laboratories, Burlington, ON, Canada) for 24 h and then in streptavidin Alexa 594 (1:200; Invitrogen Molecular Probes) for 24 h. The tissue was fixed in 4% PFA for 10 min, rinsed twice in 0.9% NaCL for 10 min each, and incubated in 2N hydrochloric acid at 37°C. In order to visualize BrdU labeling, tissue was first incubated for 24 h at 4°C in mouse anti-BrdU (1:500; Roche), then incubated in donkey anti-mouse Alexa 488 (1:250; Invitrogen Molecular Probes) for 18 h at 4°C.

An additional series of tissue was double-labeled for the immature neuronal marker doublecortin (DCX) and the immediate early gene product zif268 (Epp, et al., 2011b). This was done to examine immature neurons that had been produced and developed under Premarin (unlike the BrdU/zif268 cells that had developed only under Premarin). Doublecortin is a microtubule-associated protein that is transiently expressed in proliferating progenitor cells and immature neurons temporal expression of doublecortin ranges from 2 hours to 3 weeks after birth (Brown, et al., 2003). Tissue was incubated in primary antibody rabbit anti-zif268 (1:1000; Egr-1 SC-189, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and primary antibody goat anti-DCX (1:500; SC-8066, Santa Cruz Biotechnology, Santa Cruz, CA, USA) with 4% normal donkey serum and 0.03% Triton-X for 24 h. Tissue was then incubated in a secondary solution containing donkey anti-rabbit Cy3 (1:500; Jackson ImmunoResearch, West Grove, PA, USA) and donkey anti-goat Alexa 488 (1:250; Invitrogen Molecular Probes) for 18 h.

2.2.2.7 Cell counting

An experimenter blind to treatment conditions conducted all cell quantifications. BrdU-immunoreactive (-ir) cells were counted in every 10^{th} section throughout the entire granule cell layer (GCL), including the subgranular zone (SGZ) using a Nikon microscope at 1000x magnification. Cells in the hilus were counted separately for many reasons: 1) to account for

potential changes in the blood-brain barrier permeability by hormone treatment; 2) cells in the hilus are considered ectopic; and 3) cells in the hilus give rise to a different population of cells. Cells were considered to be BrdU-ir if they were intensely stained and exhibited medium round or oval cell body morphology (Cameron, et al., 1993,Ormerod and Galea, 2001,Ormerod, et al., 2004). The volume of the granule cell layer and hilus of each counted section was measured using image software (ImageJ, NIH) and the total volume was estimated using Cavalieri's principle (Gundersen and Jensen, 1987). The total number of BrdU-ir cells was estimated by multiplying the total number of cells counted by 10 (Barha, et al., 2011b,Eadie, et al., 2005,Kronenberg, et al., 2003). An Olympus epifluorescent microscope at 400x magnification was used to determine the percentage of BrdU-ir cells that co-expressed NeuN or zif268. The percentage of double-labeled cells was obtained by arbitrarily selecting 50 (for NeuN) or 100 (for zif268) BrdU-ir cells per brain from at least five sections, equally distributed between dorsal and ventral sections of the GCL. In order to estimate the total number of mature neurons surviving for 34 days the total number of BrdU-ir cells was multiplied by the percentage of BrdU/NeuN cells (Brandt, et al., 2010,Epp, et al., 2011a,Ramirez-Rodriguez, et al., 2009).

2.2.2.8 Blood collection and radioimmunoassay

Blood was taken at the time of perfusion from the chest cavity. Blood samples were stored overnight at 4°C and centrifuged at 10 X g for 15 mins. Briefly all samples were run in duplicate using commercially available 17β-estradiol and estrone radioimmunoassay kits from Diagnostic Systems Laboratories (Webster, Texas). The sensitivity for the ultra-sensitive 17β-estradiol kit is 2.2 pg/mL and the antibody has is highly specific for 17β-estradiol with 2.40% cross-reactivity with estrone. The sensitivity for the estrone kit is 1.2 pg/mL and has 1.25% cross-reactivity with 17β-estradiol. Average intra- and inter-assay coefficients of variation were less than 10% for both kits.

2.2.3 Data analyses

One-way analysis of variance (ANOVA)'s with treatment (Control, Low Premarin, and High Premarin) as the between-subjects variable were conducted on the number of days taken to reach criterion (defined as making no more than two errors per day for two consecutive days), and the number of total errors, reference memory errors, and working memory errors committed before reaching criterion. Separate repeated-measures ANOVAs were conducted on the number of each of the error types (RME, WME, W/RME, and total errors) committed across 5 blocks of 5 testing days with treatment (Control, Low Premarin, and High Premarin) as the between-subjects variable and blocks (1-5) as the within-subjects variable. Differences between treatment groups in total time in seconds taken to enter first arm and first correct baited arm were analyzed with repeated-measures ANOVAs with day as the within-subjects variable. Ten rats that were tested in the radial arm maze were excluded from neurogenesis analyses because BrdU was not expressed in the brains (1 control rat, 5 low Premarin rats, 4 high Premarin rats).

Repeated-measures ANOVAs were conducted on the volume of the dentate gyrus and on the density of surviving BrdU-ir cells with treatment (Control, Low Premarin, and High Premarin) and experience (tested on radial arm maze, cage controls) as the between-subjects variables and region (GCL and hilus) as the within-subjects variable. The percentages of BrdU-ir cells co-labeled with NeuN and the total number of new mature neurons were analyzed separately with factorial ANOVAs with treatment and experience (tested on radial arm maze, cage controls) as the between-subjects factors. Repeated-measures ANOVA were conducted on the percentage of BrdU/zif268-ir cells or DCX/zif268-ir cells with treatment as the between-subjects factor as this analysis was only conducted on the rats that underwent behavioral testing and region (dorsal and ventral GCL) as the within-subjects variable. Furthermore, improvement in radial arm maze performance was assessed by determining the difference score between

reference memory errors and working memory errors committed in the first block of testing and committed in the last block of testing. Improvement in reference memory and working memory were correlated with the percentage of DCX/zif268 and BrdU/zif268 in the dorsal and ventral GCL. In order to assess the relationship between activation of 34 day old surviving cells and performance on the radial arm, Pearson's correlations were determined between percent BrdU/zif268-ir in the dorsal GCL and the ventral GCL and errors of each type committed on the final day of testing, number days taken to reach criterion, and number of each error type committed before criterion performance is achieved. Furthermore the percentage of DCX/zif268-ir in the dorsal and ventral GCL was correlated with total number of each error committed across all testing days.

Repeated-measure ANOVAs were conducted on the total number of line crossings, total distance traveled, and time spent immobile with OFT area (central, peripheral) as the within-subjects variable and treatment as the between-subjects variable. To determine the effects of hormone therapy on hormone levels, separate one-way ANOVAs were conducted levels of 17β-estradiol and estrone. Post-hoc comparisons utilized the Neuman-Keuls test, while *a priori* comparisons used the Dunnett's test. All statistical procedures were set at $\alpha = 0.05$ unless otherwise specified.

2.3 RESULTS

2.3.1 Radial arm maze

2.3.1.1 The groups given low and high doses of Premarin required more days and made more errors before reaching criterion compared to controls.

A one-way ANOVA conducted on the total number of errors made before reaching criterion (defined as making no more than two errors per day for two consecutive days) showed a significant main effect of treatment ($F(2, 26) = 3.63$, $p = 0.041$; see Figure 2.2b), with the groups given low and high doses of Premarin making significantly more errors (p's = 0.021 and 0.028 respectively) than control rats. A one-way ANOVA conducted on the number of days taken to reach criterion showed a tendency for a main effect of treatment ($F(2, 26) = 2.82$, $p = 0.07$). *A priori* we were interested in hormone effects on the number of days and errors rats made to reach criterion, due to established effects of estrogens on radial arm maze performance (Bimonte-Nelson, et al., 2010). Furthermore we expected a negative effect of Premarin on cognition based on previous published reports in women (Hogervorst, et al., 2000,Ryan, et al., 2008). *A priori* comparisons revealed that the groups given low and high doses of Premarin took significantly more days to reach criterion compared to controls (p's = 0.045 and 0.040 respectively; see Figure 2.2a).

In order to further explore the memory impairment seen with Premarin, the total number of reference memory errors and total number of working memory errors made before reaching criterion were analyzed. There was a strong tendency for a main effect of treatment for total number of reference memory errors committed before reaching criterion ($F(2, 26) = 3.20$, $p = 0.057$). *A priori* comparisons revealed that both the low and high doses of Premarin increased the number of reference memory errors made before reaching criterion compared to control (p's

= 0.031, 0.034 respectively; see Figure 2.2c). A similar pattern was seen when examining the total number of working memory errors made before reaching criterion, with a tendency for a main effect of treatment ($F(2, 26) = 3.04$, $p = 0.065$). *A priori* comparisons revealed that both the low and high doses of Premarin increased the number of working memory errors made before criterion was reached compared to control (p's = 0.028, 0.049 respectively; see Figure 2.2d).

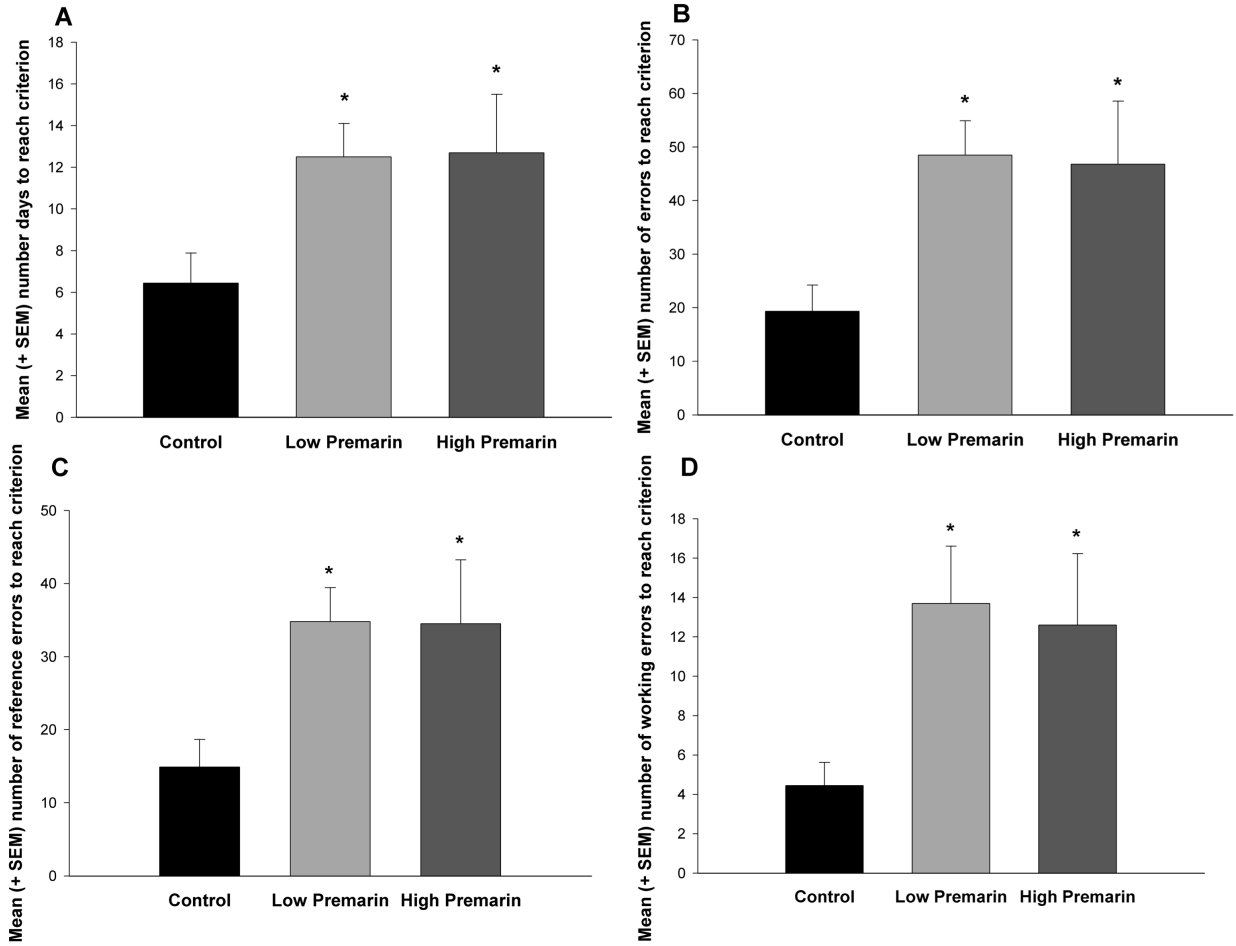
No statistical differences were seen between groups in time in seconds taken to enter first arm ($F(2, 26) = 1.42$, $p = 0.260$; see Table 2.1), and time in seconds taken to enter first correct baited arm ($F(2, 26) = 1.09$, $p = 0.351$; see Table 2.1), indicating that there were no significant motivational/motor differences between treatment conditions in this task. There was, however, a significant main effect of day for each of these variables [time to enter first arm: ($F(24, 624) = 7.44$, $p = 0.001$); time to enter first correct baited arm ($F(24, 624) = 6.11$, $p = 0.001$)], indicating that as days progressed the time in seconds taken to enter an arm and time taken to enter a correct baited arm decreased.

Table 2.1: Group means (± SEM) for locomotor and motivational variables in the radial arm maze and circulating hormone (estrone and 17β-estradiol) levels.

	Time in seconds enter first arm	Time in seconds enter first correct arm	Estrone levels (pg/ml)	17β-estradiol levels (pg/ml)
Control	14.43 ± 1.67	16.46 ± 1.87	33.07 ± 1.88	5.30 ± 0.32
Low Premarin	23.11 ± 2.48	26.65 ± 2.95	39.23 ± 2.32*	8.46 ± 0.39#
High Premarin	25.72 ± 1.66	30.24 ± 2.11	39.58 ± 3.43*	7.80 ± 0.54#

* indicates significantly different from control group.
# indicates significantly different from control group.

Figure 2.2: (a) Mean (+ SEM) number of days taken to reach criterion (2 or less errors per day for 2 consecutive days). Mean (+ SEM) number of (b) total errors, (c) reference memory errors, (d) working memory errors committed before reaching criterion. The low and high doses of Premarin increased the number of days taken to reach criterion (both p's < 0.05), the number of total errors (both p's < 0.03), the number of reference errors (both p's < 0.03), and the number of working errors (both p's < 0.05) committed before reaching criterion compared to control rats. * indicates significantly different from control group.



2.3.1.2 The low dose of Premarin initially impaired working memory compared to controls.

Figure 2.3 displays (a) number of reference memory errors, (b) number of working memory errors, (c) number of working/reference memory errors, and (d) number of total errors across 25 days in 5 blocks of 5 days. As expected there was a significant main effect of blocks for number of reference memory errors ($F(4, 104) = 16.27$, $p = 0.001$; see Figure 2.3a), number of working memory errors ($F(4, 104) = 11.46$, $p = 0.001$; see Figure 2.3b), number of working/reference memory errors ($F(4, 104) = 22.22$, $p = 0.001$; see Figure 2.3c), and total errors ($F(4, 104) = 27.74$, $p = 0.001$; see Figure 2.3d), indicating that as the days progressed there were fewer errors of each type committed irrespective of treatment and this will not be discussed further.

Treatment groups did not significantly differ in the number of reference memory errors across blocks [interaction treatment x blocks: $F(8, 104) = 1.56$, $p = 0.145$)], nor was there a main effect of treatment ($F(2, 26) = 1.81$, $p = 0.183$).

Treatment groups significantly differed in the number of working/reference memory errors made across the five blocks of testing [interaction treatment x blocks: ($F(8, 104) = 1.98$, $p = 0.051$] but no significant main effect of treatment was found ($F(2, 26) = 1.73$, $p = 0.197$). The control group made significantly fewer working/reference memory errors than the low and high Premarin groups in the first block of testing (both p's < 0.002; see Figure 2.3c).

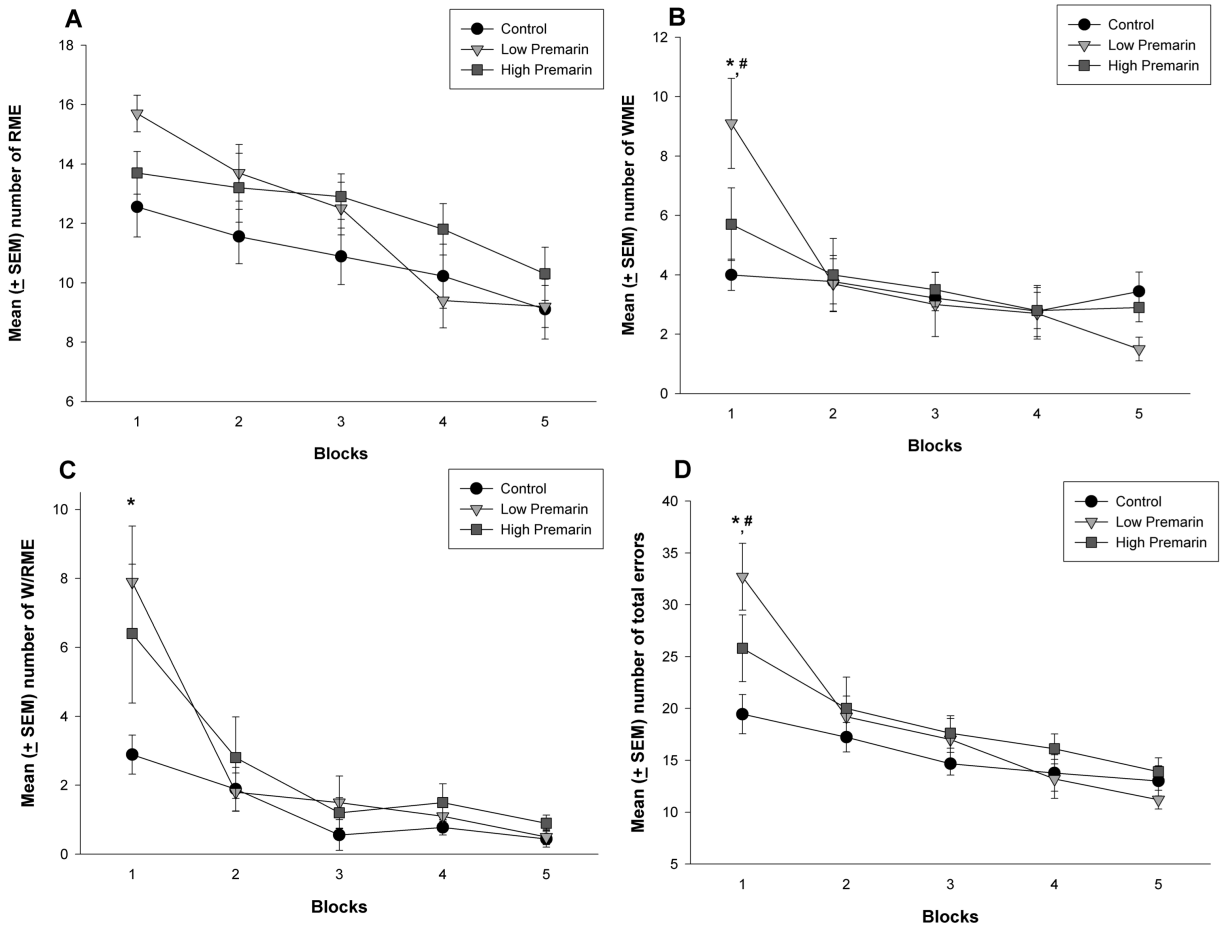
When examining number of working memory errors across the five blocks of testing, a significant two-way interaction between treatment and blocks was found ($F(8, 104) = 3.20$, $p = 0.003$; see Figure 2.3b). Post-hoc tests revealed that the low Premarin group made significantly more working memory errors across the first block of testing compared to controls and high Premarin ($p = 0.001$ and 0.002 respectively) but not across any other block (p's > 0.618). The high Premarin group did not significantly differ from controls during any of the blocks of testing

days (all p's > 0.118). There was no significant main effect of treatment ($F(2, 26) = 0.24$, $p = 0.791$).

When examining number of total errors made across the five blocks of testing, a significant two-way interaction between treatment and blocks was found ($F(8, 104) = 3.18$, $p = 0.003$; see Figure 2.3d) but no significant main effect of treatment was found ($F(2, 26) = 1.58$, $p = 0.225$). Post-hoc analyses revealed that the low Premarin group made significantly more total errors during the first block of testing compared to controls ($p = 0.001$) and high Premarin ($p = 0.005$). The high Premarin group made more total errors during the first block of testing compared to controls ($p = 0.026$).

In order to examine differences between treatment groups in improvements in performance, difference scores for each error type between the first block and last block of testing were determined. A one-way ANOVA conducted on the difference scores for working memory errors showed a significant main effect of treatment ($F(2, 26) = 8.43$, $p = 0.002$), with the low Premarin group improving significantly more than the control group ($p = 0.001$) and the high Premarin group ($p = 0.011$). The high Premarin group did not differ from the control group ($p = 0.211$). A significant main effect of treatment ($F(2, 26) = 6.48$, $p = 0.005$) was seen in improvement scores for total errors, with the low Premarin group improving significantly more than the control group ($p = 0.004$) and the high Premarin group ($p = 0.032$). The high Premarin group did not differ from the control group ($p = 0.208$). Groups did not significantly differ in improvements in reference memory ($F(2, 26) = 3.09$, $p = 0.062$) or working/reference memory ($F(2, 26) = 2.25$, $p = 0.125$).

Figure 2.3: Mean (+ SEM) number of (a) reference memory errors (RME), (b) working memory errors (WME), (c) working/reference memory errors (W/RME), and (d) total errors across 5 blocks of 5 days of testing in rats given oil control, low dose of Premarin, or high dose of Premarin. Groups did not significantly differ in RME across blocks. The low Premarin group made significantly more WME than the control group ($p = 0.0002$) and the high Premarin group ($p = 0.003$) in block 1. The low and high Premarin groups both made significantly more W/RME than the control group in block 1 (both p's < 0.002). The low Premarin group made significantly more total errors than both the control group ($p = 0.0001$) and the high Premarin group ($p = 0.006$) in block 1. The high Premarin group made significantly more total errors than the control group ($p = 0.03$) in block 1. * indicates significantly different from control group, # indicates the low and high Premarin groups differ significantly.



2.3.2 Hippocampal neurogenesis

2.3.2.1 Treatment with Premarin did not alter dentate gyrus volume.

Results indicate that treatment groups tested in the radial arm maze and cage controls did not differ in the volume of the granule cell layer or the hilus (three-way interaction effect between treatment, experience and region: $F(2, 29) = 2.26$, $p = 0.123$; see Table 2.2). As expected there was a significant main effect of region with greater hilar volumes than granule cell layer volumes ($F(1, 29) = 744.79$, $p < 0.001$). No other significant two-way interactions or main effects were found (all p's > 0.258).

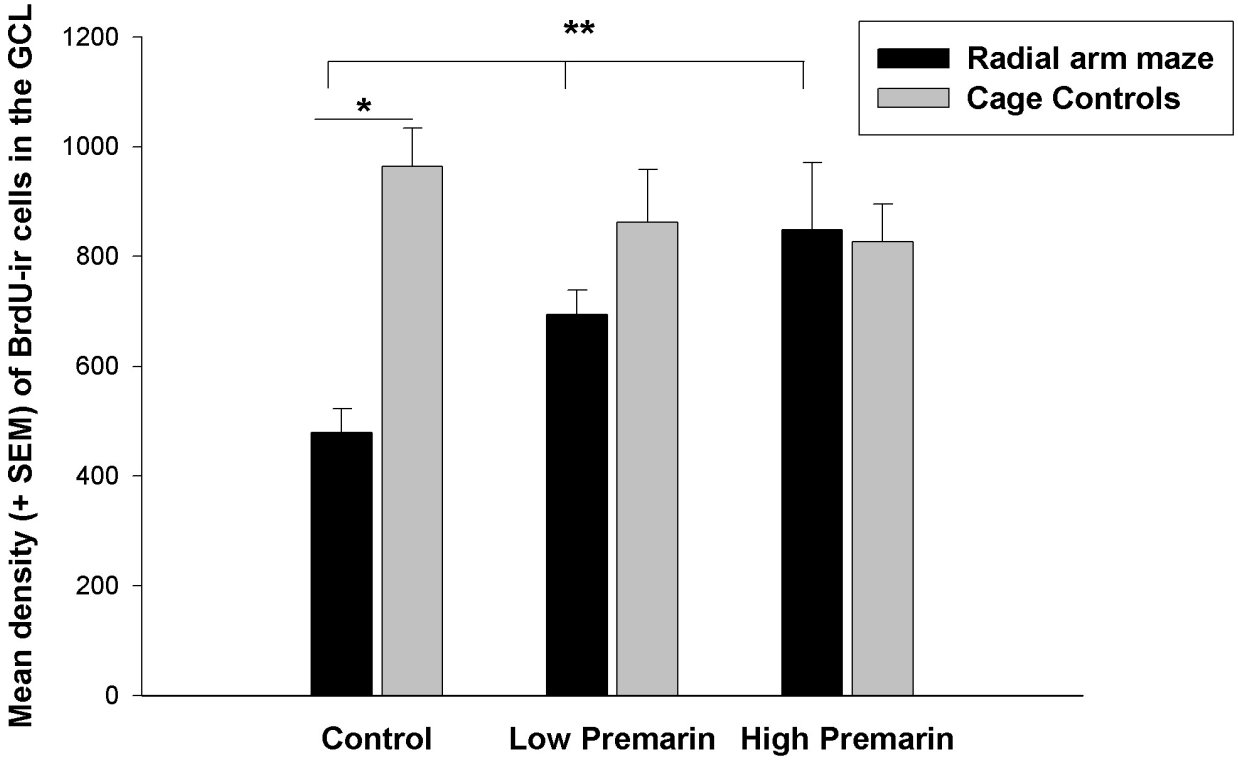
Table 2.2: Total volume of the granule cell layer and the hilus (± SEM) and the mean density of BrdU-ir cells (± SEM) in the hilus of groups behaviorally tested in the radial arm maze and cage control groups.

	GCL (± SEM)	Hilus (± SEM)	BrdU-ir cells Hilus
Radial Arm Maze			
Controls	2.98 ± 0.09	6.47 ± 0.31	58.44 ± 6.14
Low Premarin	3.06 ± 0.17	6.10 ± 0.31	97.16 ± 9.63
High Premarin	2.93 ± 0.08	6.79 ± 0.21	79.45 ± 12.77
Cage Controls			
Controls	3.05 ± 0.20	6.33 ± 0.43	42.26 ± 9.04
Low Premarin	3.25 ± 0.11	6.90 ± 0.17	72.50 ± 8.93
High Premarin	3.47 ± 0.14	6.62 ± 0.59	59.71 ± 7.08

2.3.2.2 The groups given low and high doses of Premarin had higher levels of cell survival in the granule cell layer compared to controls tested in the radial arm maze.

The density of BrdU-ir cells in the granule cell layer for all treatment groups tested in the radial arm maze and cage controls are shown in Figure 2.4. A repeated-measures ANOVA conducted on density of BrdU-ir cells surviving for 34 days revealed a significant three-way interaction between treatment, experience and region ($F(2, 29) = 6.36$, $p = 0.005$). Post-hoc analysis showed that in rats tested in the radial arm maze, the low and high doses of Premarin both increased the density of BrdU-ir cells in the GCL compared to controls (p's = 0.007, 0.001 respectively). Cage control treatment groups did not differ in density of BrdU-ir cells in the GCL (all p's > 0.179). Comparing between experience groups only oil control groups differed from each other ($p = 0.001$, with the oil cage controls having a greater density of BrdU-ir cells), but there was no effect of experience between the rats given the high dose of Premarin ($p = 0.765$) or the low dose of Premarin ($p = 0.125$). All groups did not differ in the density of BrdU-ir cells in the hilus (all p's > 0.961; see Table 2.2). Significant two-way interactions were found between treatment and experience ($F(2, 29) = 5.06$, $p = 0.013$), and region and experience ($F(1, 29) = 14.68$, $p = 0.001$). Significant main effects of experience ($F(1, 29) = 7.65$, $p = 0.009$) and region ($F(1, 29) = 559.80$, $p = 0.001$) were also found.

Figure 2.4: Mean (+ SEM) density of BrdU-ir cells surviving in the granule cell layer of the dentate gyrus of rats tested in the radial arm maze and cage control rats not tested in the radial arm maze. The low and high doses of Premarin increased the density of BrdU-ir cells compared to controls in rats behaviorally tested in the radial arm maze (both p's < 0.007). Cage controls rats did not differ in the density of BrdU-ir cells. Oil control cage control rats had significantly higher density of BrdU-ir cells compared to oil control radial arm maze rats. ** indicates significantly different from radial arm maze control group, * indicates significant difference between radial arm maze control group and cage controls control group.



2.3.2.3 Premarin increased the number of new neurons in the granule cell layer in rats undergoing behavioral testing.

The percentage of BrdU-ir cells that co-express the neuronal marker NeuN and the total number of new neurons in the granule cell layer for all groups is shown in Table 2.3. Treatment groups that underwent behavioral testing and cage controls did not differ in the percentage of double-labeled BrdU/NeuN cells [interaction treatment x experience: $F(2, 29) = 0.56$, $p = 0.579$].

A factorial ANOVA conducted on the total number of new neurons in the granule cell layer showed an interaction between treatment and experience ($F(2, 29) = 3.03$, $p = 0.050$). Post-hoc analysis showed that in rats tested in the radial arm maze the high dose of Premarin significantly increased ($p = 0.030$) and the low dose of Premarin tended to increase ($p = 0.066$) the number of new neurons compared to controls. Cage control treatment groups did not differ (all p's > 0.189). Within treatment groups only oil control groups differed from each other ($p = 0.003$); high Premarin groups did not differ ($p = 0.357$), nor did low Premarin groups ($p = 0.311$) differ from each other.

Table 2.3: The percentage of BrdU-ir cells colabeled with NeuN (±SEM) and the total number of new neurons (±SEM) in the granule cell layer of groups behaviorally tested in the radial arm maze and cage control groups.

	% BrdU/NeuN	Total number of new neurons
Radial Arm Maze		
Controls	83% ± 1.95	1198 ± 135.21^
Low Premarin	84% ± 2.53	1787 ± 162.18#
High Premarin	82% ± 0.95	2027 ± 297.98*
Cage Controls		
Controls	86% ± 2.23	2479 ± 124.80^
Low Premarin	83% ± 2.44	2329 ± 264.38
High Premarin	80% ± 2.00	2314 ± 265.70

* indicates significantly different from and # indicates tendency to be different from controls tested in the radial arm maze.
^ indicates groups significantly different from each other—controls tested in the radial arm maze significantly different from controls that are cage controls.

2.3.2.4 The low dose of Premarin decreased the activation of 34 day old surviving cells in the granule cell layer.

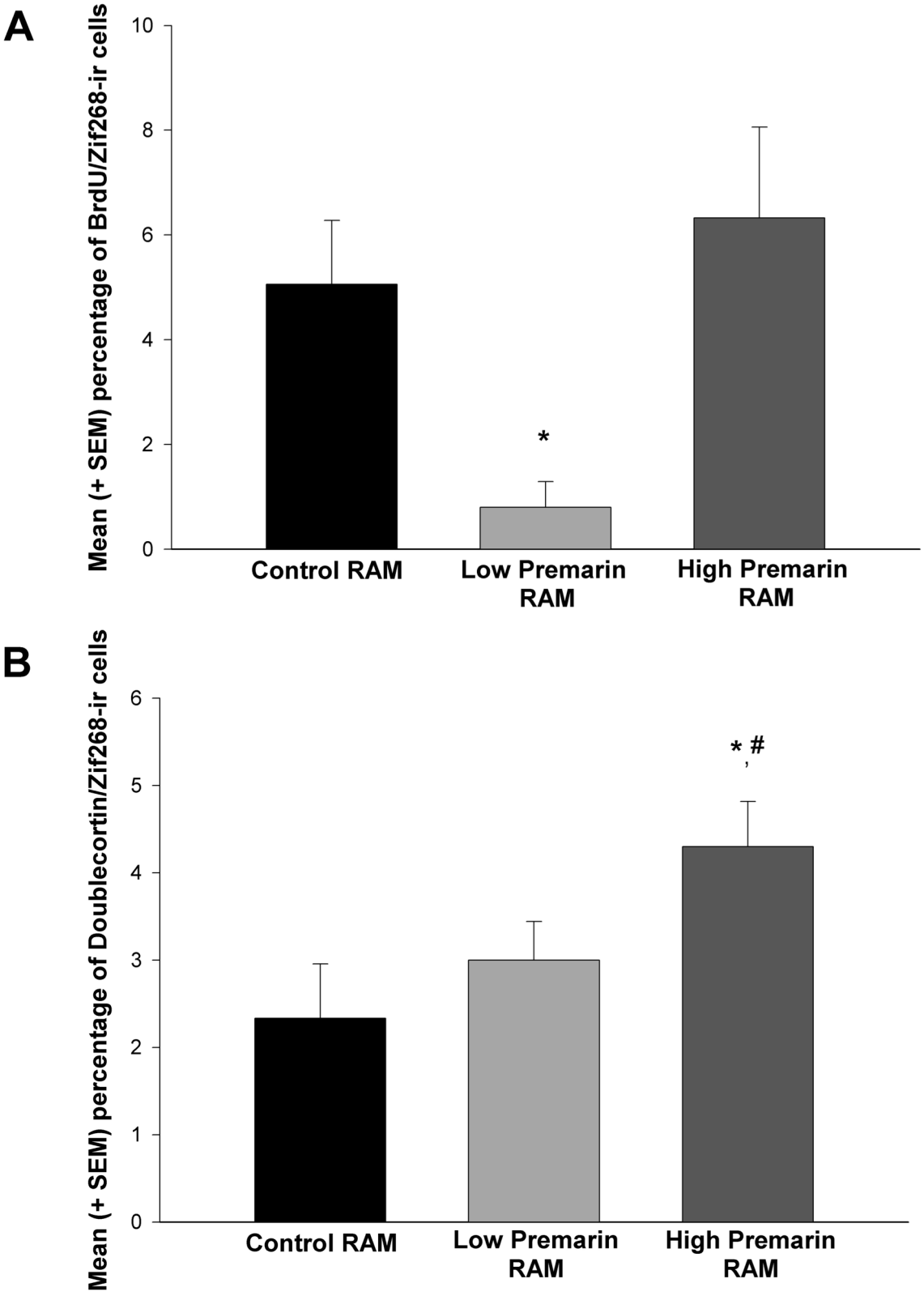
The percentage of BrdU-ir cells that co-express the immediate early gene zif268 in response to RAM testing in the granule cell layer for all groups is shown in Figure 2.5a. A repeated-measures ANOVA conducted on the percentage of double-labeled cells found in the dorsal GCL and the ventral GCL (within-subjects variable) showed a significant main effect of treatment ($F(2, 15) = 4.12$, $p = 0.038$), with the low dose of Premarin significantly decreasing the percentage of BrdU-ir cells co-labeled with the immediate early gene product zif268

compared to controls and high Premarin groups (p's = 0.033 and 0.034 respectively). Control rats did not differ from high Premarin (p = 0.656). An interaction between treatment and region was not found ($F(2, 15) = 0.94$, $p = 0.411$), nor was a main effect of region ($F(1, 15) = 3.80$, $p = 0.070$).

2.3.2.5 The high dose of Premarin increased the activation of immature neurons in the granule cell layer.

The percentage of DCX-ir cells that co-express the immediate early gene product zif268 in response to RAM testing in the granule cell layer for all groups is shown in Figure 2.5b. A repeated-measures ANOVA conducted on the percentage of DCX/zif268 double-labeled cells found in the dorsal GCL and the ventral GCL (within-subjects variable) showed a significant main effect of treatment ($F(2, 25) = 3.62$, $p = 0.042$). Post-hoc analysis found that the high dose of Premarin significantly increased the percentage of DCX/zif268 cells compared to controls (p = 0.039), and tended to increase compared to the low dose of Premarin (p = 0.097). Control rats did not differ from low Premarin (p = 0.385). An interaction between treatment and region was not found ($F(2, 25) = 2.37$, $p = 0.114$), nor was a main effect of region ($F(1, 25) = 0.37$, $p = 0.550$).

Figure 2.5: (a) Mean (+ SEM) percentage of BrdU-ir cells co-labeled with the immediate early gene zif268 in the granule cell layer of the dentate gyrus of rats tested in the radial arm maze. The low dose of Premarin significantly decreased the percentage of BrdU-ir co-labeled with zif268 compared to control rats ($p = 0.04$) and rats given the high dose of Premarin ($p = 0.03$). (b) Mean (+ SEM) percentage of doublecortin expressing cells co-labeled with the immediate early gene zif268 in the granule cell layer of the dentate gyrus of rats tested in the radial arm maze. The high dose of Premarin significantly increased the percentage of DCX-ir co-labeled with zif268 compared to control rats ($p = 0.04$) and tended to increase compared to rats given the low dose of Premarin ($p = 0.09$). * indicates significantly different from control group, # indicates tendency to be different from low Premarin group.

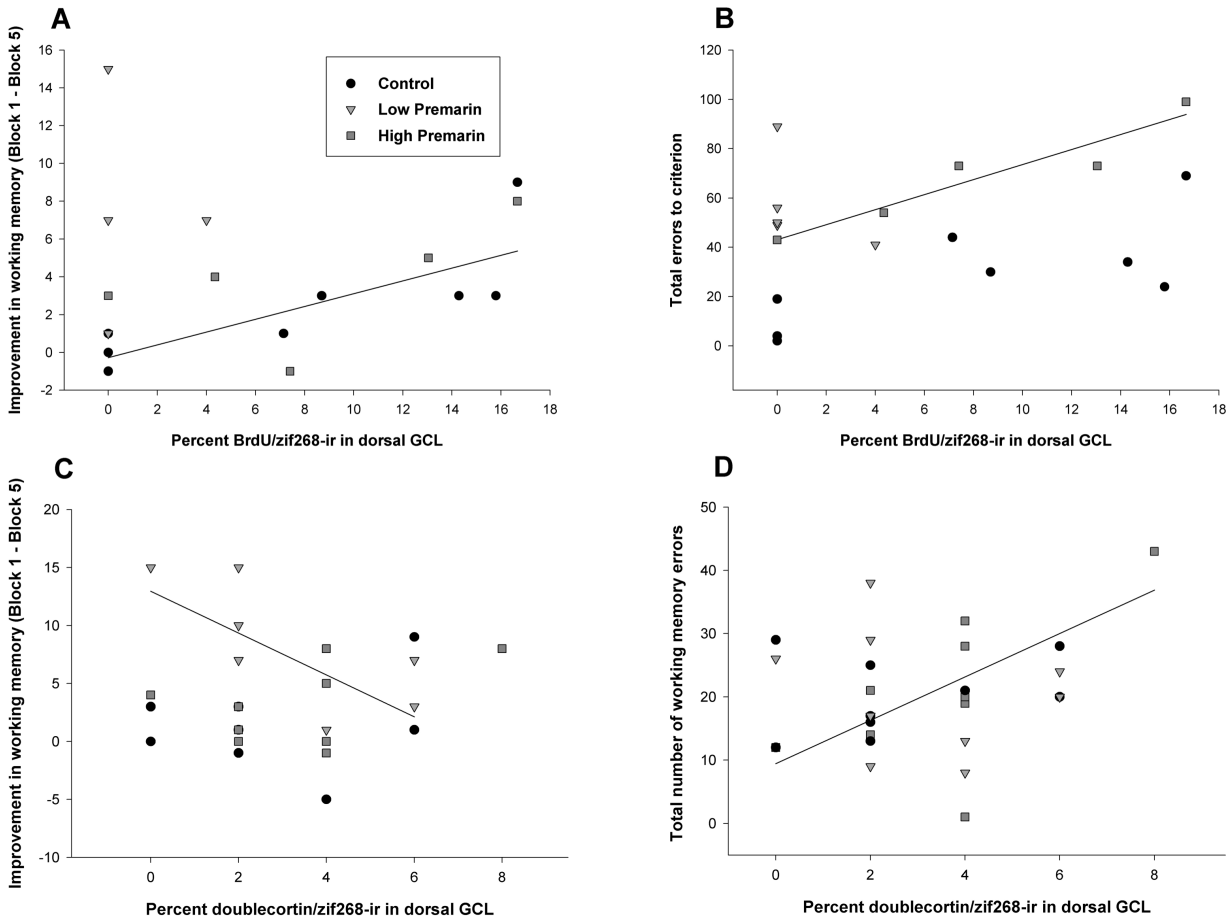

A
Mean (+ SEM) percentage of BrdU/Zif268-ir cells
10
8
6
4
2
0
*
Control RAM
Low Premarin RAM
High Premarin RAM
B
Mean (+ SEM) percentage of Doublecortin/Zif268-ir cells
6
5
4
3
2
1
0
*,#
Control RAM
Low Premarin RAM
High Premarin RAM

2.3.2.6 Higher levels of activation of new neurons of different ages are correlated with decreased performance in the radial arm maze in Premarin treated groups and with increased performance in the control group.

The percentage of BrdU/zif268-ir cells in the dorsal GCL of the control group was positively correlated with improvement in working memory [r = 0.80, p = 0.017; see Figure 2.6a] and reference memory [r = 0.70, p = 0.055] as indexed by the difference score between errors of each type committed in block 1 and block 5. Significant correlations were not seen in the percentage of BrdU/zif268 in the dorsal GCL and improvement in working memory or in reference memory in the low and high Premarin groups (all p's > 0.22). The percentage of BrdU/zif268-ir cells in the dorsal GCL of only the high Premarin group was positively correlated with total reference memory errors made on the final day of testing [r = 0.92, p = 0.025], number of days taken to reach criterion [r = 0.89, p = 0.043], and total number of errors [r = 0.95, p = 0.014; see Figure 2.6b] and working memory errors [r = 0.93, p = 0.026] committed before reaching criterion. Correlations between these measures and percentage of BrdU/zif268 in the ventral GCL were not seen for any group (all p's > 0.15).

The percentage of DCX/zif268-ir cells in the dorsal GCL was negatively correlated with improvements in working memory [r = -0.69, p = 0.039; see Figure 2.6c] and working/reference memory [r = -0.69, p = 0.039] in the low Premarin group only. The percentage of DCX/zif268-ir cells in the dorsal GCL was positively correlated with total number of working memory errors committed across testing [r = 0.63, p = 0.050; see Figure 2.6d] in the high Premarin group. Behavioral measures were not correlated with percentage of DCX/zif268-ir cells in the control group (all p's > 0.20).

Figure 2.6: Correlations between the percent activation of 34 day old BrdU-ir cells surviving and percent activation of immature neurons in the dorsal granule cell layer and spatial learning and memory variables. Percent BrdU-ir cells co-labeled with zif268 were positively associated with improvements in working memory (block 1 errors minus block 5 errors) in the control group only (a) and with total number of errors committed before reaching criterion in the high Premarin group only (b) (r = 0.80 and 0.95 respectively). Percent doublecortin-ir cells co-labeled with zif268 were negatively correlated with improvements in working memory in the low Premarin group only (c) and were positively correlated with total number of working memory errors committed across testing (d) (r = -0.69 and 0.63 respectively).



2.3.4 Premarin did not influence anxiety or locomotor activity as assessed by the open field test.

A repeated-measure ANOVA was conducted on the number of line crossing with OFT area (central, peripheral) as the within-subjects variable and treatment as the between-subjects variable. No significant treatment group differences were found in the number of line crossing made in the central or peripheral areas of the OFT [interaction: ($p = 0.87$); main effect of group: ($p = 0.82$)]. All rats made significantly more line crossings in the peripheral area compared to central area [main effect of area: ($F(1, 26) = 853.03$, $p = 0.0001$)]. There were no significant differences between treatment groups in the total number of line crossings, total distance traveled, and time spent immobile (all p's > 0.66 for main effect of group).

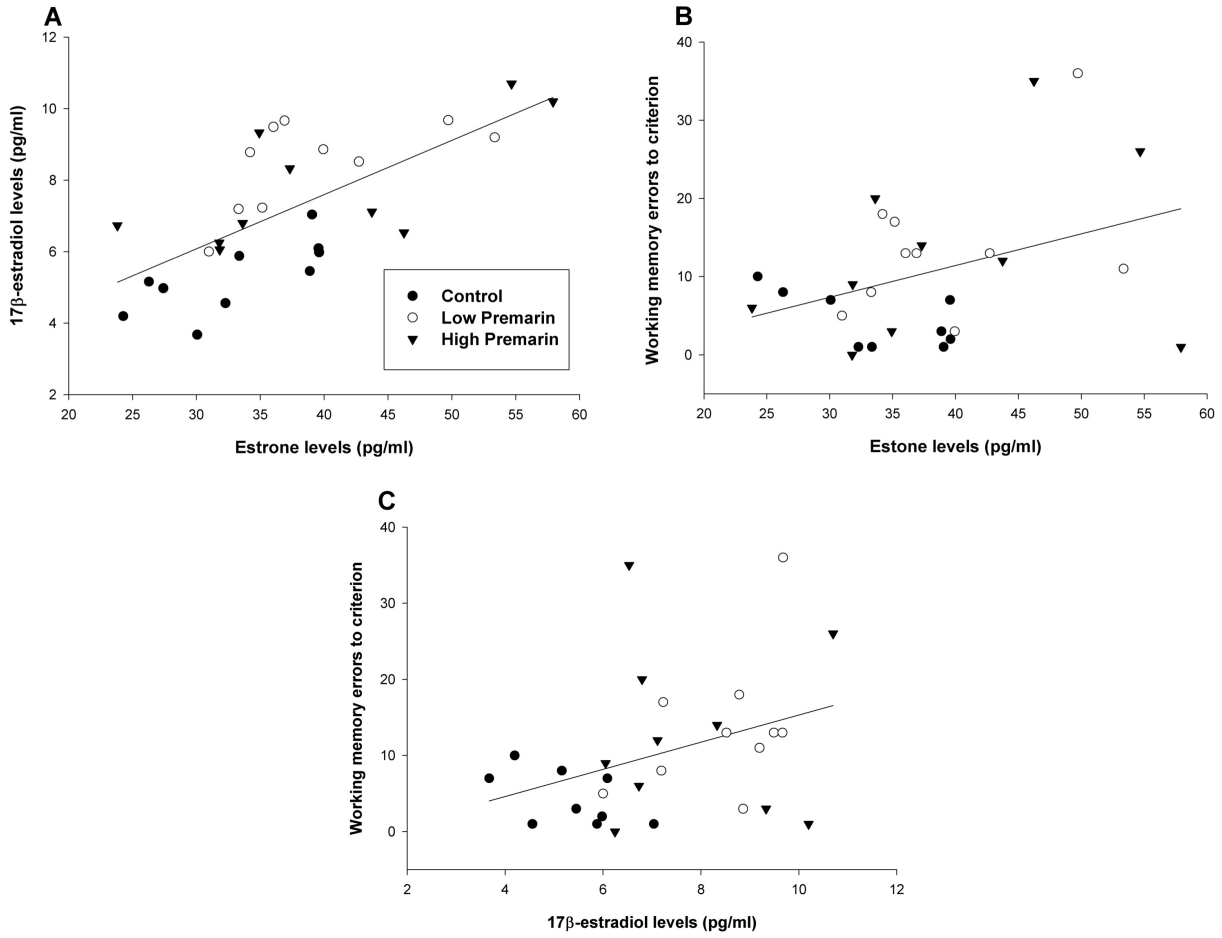
2.3.5 Premarin treatment increased levels of 17β-estradiol and estrone

The levels of 17β-estradiol and estrone in blood collected from the right atrium of the heart at the time of perfusion of all rats are shown in Table 2.1. A one-way ANOVA on serum levels of 17β-estradiol found a significant main effect of treatment ($F(2, 27) = 15.20$, $p = 0.001$; see Table 2.1), with low and high doses of Premarin increasing levels of 17β-estradiol compared to controls (p's = 0.001 and 0.001). The groups given the low and high doses of Premarin did not differ from each other in level of 17β-estradiol ($p = 0.286$). Furthermore we expected higher levels of estrone in rats given Premarin at either dose compared to non-treated ovariectomized female rats based on previously published reports (Engler-Chiurazzi, et al., 2011). *A priori* comparisons revealed that the groups given low and high doses of Premarin had higher levels of estrone in perfusion blood compared to controls (p's = 0.045 and 0.040 one-tailed; see Table 2.1).

Estrone and 17β-estradiol levels were positively correlated when all rats were included [$r(30) = 0.68$, $p = 0.001$; see Figure 2.7a] or only when rats treated with Premarin were included

[r(20) = 0.64, p = 0.002]. Estrone and 17β-estradiol levels were positively correlated with total working memory errors committed before reaching criterion [r = 0.37, p = 0.050; r = 0.36, p = 0.050, respectively; see Figure 2.7 b, c].

Figure 2.7: Pearson product-moment correlations between estrone and 17β-estradiol levels and working memory errors committed before reaching criterion. (a) A positive correlation was seen between estrone and 17β-estradiol levels (r = 0.68). (b) Higher levels of estrone were associated with higher numbers of working memory errors to criterion (r = 0.37). (c) Higher levels of 17β-estradiol were associated with higher numbers of working memory errors to criterion (r = 0.36).



2.4 DISCUSSION

The results from the present study demonstrate that treatment with the hormone therapy Premarin for 33 days impairs hippocampus-dependent spatial learning and memory by increasing the number of days and errors (reference and working) committed to reach criterion performance in a rodent model of surgical menopause. This effect was not due to treatment changes in motoric or motivational processes, as we saw no significant differences in latency to reach the first goal arm. Furthermore, treatment with a low dose of Premarin impaired spatial working memory during the first week of testing. Both doses of Premarin increased neurogenesis in the animals that underwent behavioral testing and the low dose of Premarin reduced activation of new neurons in response to spatial memory retrieval, providing a potential mechanism through which the low dose of Premarin impaired spatial working and reference memory. Here we show for the first time that while chronic exposure to Premarin increased neurogenesis in the hippocampus, these new neurons expressed decreased activation in response to spatial memory retrieval in surgically menopausal rats.

2.4.1 Premarin impairs spatial reference and working learning and memory.

The present study found that Premarin treatment for 33 days impaired spatial reference and working memory performance on the hippocampus-dependent spatial version of the radial arm maze in ovariectomized female rats. This is consistent with a number of studies that have shown detrimental effects on cognition in women using CEEs (Espeland, et al., 2004,Gleason, et al., 2006,Maki, et al., 2007,Rapp, et al., 2003,Ryan, et al., 2008,Shumaker, et al., 2004,Shumaker, et al., 2003,Wroolie, et al., 2011). The few studies previously conducted examining the effects of Premarin on cognition using middle-aged female rats found beneficial effects (Acosta, et al., 2009,Acosta, et al., 2010,Engler-Chiurazzi, et al., 2011). Cyclic treatment of middle-aged female rats with Premarin (2 days on, 2 days off) improved reference

memory performance and acquisition of a delay-match-to-sample plus maze task compared to oil controls (Acosta, et al., 2009). Interestingly these beneficial effects are only seen in surgically, not transitionally, menopausal rats (Acosta, et al., 2010). Consistent with the current study, Engler-Chiurazzi et al. (2011) found that tonic administration of a low dose of Premarin (12 μg) via osmotic pumps impaired spatial working memory and reference memory retention in middle-aged female rats. Furthermore, it is interesting to note that the WHIMS study used tonic administration of Premarin and found a negative effect on cognition, consistent with the present study and that of Engler-Chiruazzi et al. (2011). Taken together these results indicate that the relationship between hormone therapy and cognition likely depends on multiple factors including age of the subjects, route of administration, dose, learning and memory task, and also the schedule (tonic or cyclic) of administration.

Results from randomized, placebo-controlled trials in humans examining the effects of HT on cognitive functioning are mixed (Henderson, 2010,Hogervorst and Bandelow, 2010). Discrepant results could be due to important methodological differences including age of women, type of memory tests given, and the type of HT. Overall it seems that 17β-estradiol-based HTs in younger postmenopausal women enhance cognition (Gleason, et al., 2006,Joffe, et al., 2006,Phillips and Sherwin, 1992,Rasgon, et al., 2005,Sherwin, 1988,Tierney, et al., 2009); whereas treatment with CEE in both younger and older postmenopausal women has more negative, neutral, or in a few cases short-term (2 months) positive effects on cognition (Gleason, et al., 2006,Hogervorst, et al., 2009,Maki, et al., 2007,Resnick, et al., 2009,Wroolie, et al., 2011). Interestingly in postmenopausal women higher endogenous levels of estrone are associated with lower cognitive performance (Yaffe, et al., 1998), which was the same relationship seen in the present study.

As seen in the current study, treatment with Premarin results in physiological circulating levels of estrone (Barha, et al., 2010). These levels of estrone are positively correlated with total number of working memory errors committed before reaching criterion, indicating that higher levels of circulating estrone are related to impairments in spatial working memory. Interestingly, we saw a similar relationship between 17β-estradiol levels and spatial working memory, as increased levels of 17β-estradiol were associated with increased number of working memory errors committed before reaching criterion performance. These relationships may be related to the ratio of estradiol/estrone, as 17β-estradiol levels were strongly correlated with estrone levels. These findings are not in complete agreement with previous studies in postmenopausal women that found higher circulating estradiol levels were associated with less cognitive decline, although it is important to note that these levels of estradiol in women would not considered high compared to younger women (Bittner, et al., 2011,Drake, et al., 2000,Lebrun, et al., 2005). Furthermore, higher serum 17β-estradiol levels were associated with enhanced spatial reference memory in female rats (Talboom, et al., 2008).

In the present study, both the low and high doses of Premarin increased the number of days, working and reference memory errors to reach criterion performance. Criterion-based measures assess differences in rates of acquisition and learning (Knowlton, et al., 1989,Park, et al., 2008); therefore, both doses of Premarin impaired spatial learning (both working and reference memory) in this task. However, it should be noted that differences between groups were not seen in total numbers of reference memory errors or working memory errors, potentially suggesting that Premarin most negatively affects initial learning and not memory function. This warrants further investigation.

2.4.2 Premarin-induced increases in hippocampal neurogenesis coincide with spatial memory impairments.

In the present study chronic treatment with both low and high doses of Premarin increased the number of new neurons in the dentate gyrus. Although, to our knowledge, no study has examined the effect of Premarin on hippocampal cell survival and neurogenesis, our findings are partially consistent with other studies examining the effects of Premarin on neuroplasticity. Premarin protects cultured neurons against toxic insults (Brinton, et al., 2000,Diaz Brinton, et al., 2000), and increases survival of different types of neurons (Browne, et al., 2009,Zhao and Brinton, 2006,Zhao, et al., 2003) in vitro. In our study we found that although Premarin increased the number of new neurons in the dentate gyrus this was not related to better learning, indicating that new neurons born into a Premarin-rich environment may not have been functioning optimally (see below and section 2.4.3).

At first glance it may seem counterintuitive that Premarin enhanced hippocampal neurogenesis without conferring a positive effect on spatial memory performance. However, increases in hippocampal neurogenesis do not necessarily equate to enhancements in memory function (Epp and Galea, 2009,Epp, et al., 2009). For example increases in neurogenesis are seen in the brains of Alzheimer's disease patients and following seizures, and both conditions present with severe impairments in hippocampus-dependent memory (Jessberger, et al., 2007,Jin, et al., 2004). Furthermore, reduction in neurogenesis has also led to enhancements in working memory in the radial arm maze (Saxe, et al., 2007) or no effect on spatial learning (Snyder, et al., 2005). However other treatments such as exercise or enriched environments increase both neurogenesis and spatial learning and memory. These studies collectively suggest that there is an optimal level of neurogenesis that corresponds to better learning and memory (Koehl and Abrous, 2011) and increases above this point do not necessarily translate into

benefits for hippocampal function (Koehl and Abrous, 2011), as evidenced in the current study. Furthermore, new neurons born under different conditions (i.e. pathological or non-pathological) can dramatically differ in terms of their contribution to the functioning of the hippocampus (i.e. Jakubs, et al., 2006 and see below for discussion).

Interestingly higher levels of cell survival were seen in cage control rats compared to the control-treated rats tested on the radial arm maze. These differences may be related to either food restriction, and/or spatial learning. It is important to note that in addition to not being trained on the radial arm maze, cage controls were also not food restricted. Food restriction has a beneficial effects on cognition, certain parameters of the aging process (Masoro, 2000), and increases neurogenesis in the hippocampus (Lee, et al., 2000). However, food restriction also increases basal corticosterone levels (Patel and Finch, 2002) and in general chronic high levels of corticosterone reduces hippocampal neurogenesis (Brummelte and Galea, 2010,Pawluski, et al., 2009,Schoenfeld and Gould, 2012). However, it is not clear why food restriction would only decrease neurogenesis in the control and not Premarin-treated rats. Exposure to spatial learning can decrease hippocampal neurogenesis compared to cage controls (Aztiria, et al., 2007,Epp, et al., 2009,Epp, et al., 2011a). Thus, in the present study the reduction in neurogenesis with behavioral testing could have been due to the extended behavioral training paradigm (Aztiria, et al., 2007), increased task difficulty (Epp, et al., 2009), and the fact that female rats were used (Aztiria, et al., 2007). In any case, whatever the cause of the reduction in neurogenesis in the behaviorally tested control group it is interesting that treatment with Premarin prevented this reduction in cell survival. These findings warrant further investigation.

It is important to note that in the present study hormone treatment was begun three weeks following ovariectomy. Previous literature in humans and rodents has indicated that a critical window of opportunity exists after ovariectomy or menopause for HT to exert beneficial

effects and must be started temporally close to surgical or natural menopause (Gibbs, 2010,Rocca, et al., 2011,Sherwin, 2009). Indeed, Tanapat, et al. (2005) found that acute treatment with 17β-estradiol 1 week, but not 4 weeks, after ovariectomy increased cell proliferation in the dentate gyrus, suggesting that progenitor cells loose the ability to respond to acute 17β-estradiol 4 weeks after ovariectomy. However, the results of the present study indicate that newly divided cells can still respond to steroid hormones after 3 weeks of estrogen deprivation, as treatment with Premarin 3 weeks after ovariectomy was able increase the number of BrdU-ir cells surviving compared to controls. This may be related to the healthy cell bias hypothesis, as the female rats used in the present study were 4 months of age when ovariectomy occurred. Therefore, the cells in the dentate gyrus were "healthy" in that they had not yet been exposed to negative influences associated with aging.

2.4.3 Premarin treatment altered activation of new neurons, depending on dose and age of neurons. Higher levels of activation of new neurons of different ages were correlated with impaired spatial performance in the Premarin-treated groups but enhanced performance in the control-treated group.

In the present study we found that the low dose of Premarin decreased the percentage of 34 day old BrdU/zif268-ir cells whereas the high dose of Premarin increased the percentage of DCX/zif268 (immature neurons 1 to 21 days old). These results suggest that different doses of Premarin differentially influence populations of neurons of different ages and maturational stages. Furthermore, it is important to note that the DCX/zif268 cells were produced and matured under Premarin while the BrdU/zif268 cells had only matured under Premarin's influence, thus showing that the differences in activation levels may be due to timing of exposure to Premarin.

Interestingly, we also found that both doses of Premarin increased hippocampal neurogenesis that corresponded to impaired memory performance. It is possible that these new cells produced and/or surviving in a Premarin-exposed environment are not functioning appropriately. In support of this, we found that activation of new neurons (34 day old cells and immature neurons) in the dorsal GCL in the Premarin treated groups are correlated with impairments in spatial working and reference memory performance in the radial arm maze. On the other hand, higher levels of activation of 34 day old cells in the dorsal GCL in the control group are associated with improved spatial working and reference memory performance. Thus control rats showed activation of new neurons being associated with improvements in spatial performance, whereas Premarin-treated rats showed activation of new neurons being associated with impairments in spatial performance, suggesting that new neurons behave differently in different environments. Indeed environment matters, as the environment into which new neurons are born and develop is important in determining the electrophysiological and perhaps behavioral function of these new neurons (Jakubs, et al., 2006,Jessberger, et al., 2007). A pathological environment can increase the production and survival of neurons but can also lead to aberrant integration of these neurons into the existing neural circuitry, which may contribute to any functional impairment seen (i.e. Barha, et al., 2011b,Jessberger, et al., 2007). Studies have shown that new neurons produced in normal or enriched environments (such as after running exercise) have different synaptic properties than new neurons produced in pathological environments like after seizures (Jakubs, et al., 2006,Wood, et al., 2011). It is also important to note that we saw these correlations only in the dorsal, and not ventral, dentate gyrus. This is of particular interest as the dorsal hippocampus is associated with spatial memory while the ventral hippocampus is associated more with emotional behavior (Fanselow and Dong, 2010).

Importantly we found that treatment with the low dose of Premarin reduced the activation of newly surviving mature neurons and impaired spatial reference and working memory, whereas the high dose of Premarin increased activation of immature neurons and still impaired performance. Together these results suggest that a curvilinear relationship may exist between new neuronal activation and spatial reference and working memory performance, with levels too low and too high being detrimental to learning and memory or that new neurons surviving in a 'pathological' environment may respond inappropriately to spatial memory retrieval. Very high levels of activation may lead to disrupted activity patterns in the hippocampal circuitry and could contribute to learning and memory impairments particularly in pathological environments. For example cognitive impairments seen under conditions of chronic neuroinflammation are associated with overexpression of the immediate early gene Arc in the dentate gyrus (Rosi, et al., 2006). The disrupting effect of high levels of activation on learning and memory performance may be related to the hypothesis that the dentate gyrus uses sparse coding of information by relatively few cells in order to facilitate orthogonalization of information, a key function attributed to the dentate gyrus (McNaughton, et al., 1996). The low expression level of immediate early genes in the dentate gyrus under normal learning conditions is consistent with the role of sparse coding in the functioning of the dentate gyrus (Chawla, et al., 2005).

2.5 CONCLUSIONS

Neurodegenerative diseases and age-associated cognitive decline have been linked to decreases in gonadal hormone levels in women. In recent years the utility of hormone therapy has been questioned. Using a rodent model of surgical menopause, the present study

demonstrates that the prevalent hormone therapy Premarin, a conjugated equine estrogen, impairs hippocampus-dependent spatial reference and working memory, and points to alterations in activation of new neurons in the dentate gyrus as a potential mechanism through which Premarin is impairing learning and memory. These results are particularly important in lieu of recent findings that estrone, the main estrogenic component of Premarin, does not confer the same beneficial cognitive effects as other estrogens. Our results also show that alterations, either increase or decrease, in activation of new neurons in the dentate gyrus may impair hippocampus-dependent learning and memory, suggesting the existence of an optimal level of activation and neurogenesis in the adult brain. Furthermore, we found that higher levels of neuronal activation in the dorsal granule cell layer were associated with impairments in performance in the Premarin-treated rats, and with enhancements in performance in control rats. Although interventions such as hormone therapy may increase hippocampal neurogenesis, they may also cause aberrant integration of these new neurons into existing circuitry that may further exacerbate cognitive impairments. Our results suggest the possibility that a pathological neurogenic environment in the dentate gyrus develops from treatment with Premarin. Further research is required into assessing the characteristics of new neurons developing in this estrone-rich pathological environment.

3 DIFFERENT FORMS OF ESTROGEN RAPIDLY UPREGULATE CELL PROLIFERATION IN THE DENTATE GYRUS OF ADULT FEMALE RATS.[2]

3.1 INTRODUCTION

Estrogens are known to exert significant structural and functional effects in the hippocampus of adult rodents. For example, fluctuations in endogenous and exogenous estrogen levels in adult female rats have been shown to influence dendritic morphology of the CA1 region of the hippocampus (Woolley and McEwen, 1993), neurogenesis in the dentate gyrus (for review see Galea, 2008), and performance in hippocampus-dependent learning and memory tasks (Holmes, et al., 2002). However, despite the natural occurrence of several different forms of estrogen, most studies, including the ones mentioned above, have only examined the influence of 17β-estradiol on hippocampal structure and function.

There are three principal forms of estrogen: estrone (E_1), 17β-estradiol (17β-E_2), and estriol (E_3). 17β-E_2 is the most potent form of estrogen and is found at higher levels than E_1 in young pre-menopausal women (Rannevik, et al., 1995). After menopause, the ratio of 17β-E_2 and E_1 reverses as the levels of 17β-E_2 drop dramatically and levels of E_1 remain fairly undisturbed (Rannevik, et al., 1995). E_1, the predominate form of estrogen found in post-menopausal women (Rannevik, et al., 1995), originates primarily from the ovaries, adrenal cortex and adipose tissue, with 25% of circulating E_1 being converted from 17β-E_2 via enzymes in the family 17-hydroxysteriod dehydrogenases in the ovaries. Despite the fact that these estrogens can be interconverted, E_1 and 17β-E_2 have been shown to have some differential

[2] A version of this chapter has been published. Barha, C.K., Lieblich, S.E. and Galea, L.A.M. (2009) Different forms of oestrogen rapidly upregulate cell proliferation in the dentate gyrus of adult female rats. Journal of Neuroendocrinology. 21: 155-166.

effects on neuroprotection (Bhavnani, et al., 2003,Budziszewska, et al., 2001). E_1 is used in many hormone replacement therapies (HRTs), and is the primary estrogen in Premarin, the most popular HRT and the HRT used in the controversial NIH-controlled Women's Health Initiative study (Shumaker, et al., 2003). Despite the fact that it is routinely used in HRTs, very little research has focused on E_1. However these few studies indicate that E_1 is neuroprotective against several different insults (Bhavnani, et al., 2003,Budziszewska, et al., 2001) and can alter estrogen receptor (ER) subtypes independently of the effects of 17β-E_2. Recently, Jin et al. (2005) found that treatment with E_1 (Premarin) led to decreased levels of ERα but did not affect ERβ levels, while treatment with 17β-E_2 increased ERβ but did not affect ERα levels in the hippocampus and cortex of adult female rats. Given the tissue and region specific expression of ER receptors in the brain (Shughrue, et al., 1997), the diverse behaviors they regulate (Kudwa, et al., 2006,Rhodes and Frye, 2006) and that estrogens differentially affect ER subtypes, these different forms of estrogen could have profound and distinct effects on neural plasticity and/or behavior.

There are two naturally occurring optical isomers of estradiol, 17β-estradiol (17β-E_2) and 17α-estradiol (17α-E_2). 17β-E_2 is considered more active than 17α-E_2 because it binds to ERα and ERβ with approximately 40-fold higher affinity then 17α-E_2 (Perez, et al., 2005). However, 17α-E_2 has recently been theorized to be the preferred ligand of ER-X, a novel plasma membrane-associated ER (Toran-Allerand, et al., 2002) as the rapid (less than 30 minutes) effects of 17α-E_2 have been associated with estrogens non-genomic, non-classical pathways. Though the biosynthetic pathways of 17α-E_2 have not entirely been elucidated, it is known that 17α-E_2 is synthesized from aromatization of epitestosterone by the enzyme cytochrome P450 aromatase (Finkelstein, et al., 1981) in many sites including the brain. The endogenous content of 17α-E_2 has recently been found to be greater in the brain than 17β-E_2 and E_1 in intact and

gonadectomized male and female mice suggesting that this estrogen is locally synthesized in the brain (Toran-Allerand, et al., 2005). Furthermore, despite the majority of research using the 17β-E_2 form, 17α-E_2 has been shown to induce spines in the CA1 region of the hippocampus (MacLusky, et al., 2005), enhance visual and place memory (Luine, et al., 2003,Rhodes and Frye, 2006), induce sexual behavior in female rodents (Rhodes and Frye, 2006), and to be neuroprotective (Levin-Allerhand, et al., 2002,Simpkins, et al., 2004). Therefore, it is possible that 17α-E_2 could have effects on other forms of neuroplasticity, such as neurogenesis, in the hippocampus.

Adult neurogenesis in the dentate gyrus of the hippocampus consists of at least two processes: cell proliferation (production of new cells) and cell survival (cells surviving to maturity). The number of new neurons can be increased either by enhancing cell proliferation and/or by enhancing the survival of new neurons. Furthermore, it is possible to increase the number of cells surviving without influencing cell proliferation, as well as increase the number of cells proliferating without influencing cell survival. For example, chronic exposure to antidepressants upregulate cell proliferation but has no independent effect on cell survival (Malberg, et al., 2000), whereas exposure to an enriched environment upregulates cell survival, but has no independent effect on cell proliferation (Olson, et al., 2006). Research has shown that increased cell survival is related to better hippocampus-dependent learning and memory (Nilsson, et al., 1999,van Praag, et al., 2005) and that the inhibition of neurogenesis has been shown to severely impair hippocampus-dependent learning and memory (Shors, et al., 2001,Snyder, et al., 2005). A complex relationship exists between cell proliferation and hippocampus-dependent learning in the literature (Dupret, et al., 2007,Pham, et al., 2005). Taken together, these studies suggest that hippocampal neurogenesis contributes to certain forms of learning and memory.

The effects of 17β-E_2 on hippocampal neurogenesis in adult female rodents are well established in the literature. Short-term ovariectomy decreases cell proliferation in the dentate gyrus in young adult female rats, whereas high levels of 17β-E_2 (10μg) upregulate the ovariectomy-induced decrease in cell proliferation (Tanapat, et al., 1999). Furthermore, exposure to estradiol benzoate for 4 hours increases, whereas exposure for 48 hours, decreases cell proliferation in adult female rodents (Ormerod, et al., 2003). Both ERα and ERβ are involved in the 17β-E_2-induced increase in cell proliferation as both ERα and ERβ agonists enhance cell proliferation in the dentate gyrus of young ovariectomized female rats (Mazzucco, et al., 2006). Though research has established the effects of 17β-E_2 on cell proliferation, the effects of other estrogens such as 17α-E_2 and E_1 on neurogenesis have not been investigated.

The aim of the present study was to determine the effects of 17β-E_2, 17α-E_2, and E_1 at various doses on cell proliferation at various time points. Young ovariectomized female rats approximately 3 to 4 months old were injected with either 17β-E_2, 17α-E_2, E_1, or estradiol benzoate (EB; a benzoated form of 17β-estradiol) at one of three doses. In experiment 1, rats were exposed to the hormone for 4 hours and in experiment 2 rats were exposed to the hormone for 30 minutes prior to 5-bromo-2-deoxyuridine injection to label proliferating cells and their progeny. The two time points were chosen because of previous literature and because of the potential rapid effects of some of these ligands. We hypothesized that 17β-E_2 and 17α-E_2 would dose-dependently increase cell proliferation at 30 minutes and that only EB would dose-dependently increase cell proliferation at 4 hours due to slower metabolism of the benzoated form of estradiol.

3.2 MATERIALS AND METHODS

3.2.1 Animals

One hundred and forty four adult female Sprague-Dawley rats, weighing between 200 and 250 grams, purchased from the University of British Columbia Animal Care Centre (Vancouver, BC, Canada) were used in the study. Rats were initially housed in pairs in opaque polyurethane bins (48 x 27 x 20cm) with aspen chip bedding and were given Purina rat chow and tap water *ad libitum*. Rats were maintained in a 12h:12h light/dark cycle (lights on at 7:30 a.m.). Beginning the day after arrival, rats were handled every other day for 5 minutes. All experiments were conducted in accordance with the ethical guidelines set by the Canada Council for Animal Care and were approved by the University of British Columbia Animal Care Committee. All efforts were made to reduce the number and the suffering of animals.

3.2.2 Procedures

3.2.2.1 Surgery

Approximately 1 week after arrival, all females were bilaterally ovariectomized using aseptic procedures. Rats were placed in a chamber and anesthetized with Isoflurane, which was delivered at an induction flow rate of 5% (flow rate of O_2 was approximately 1.5%). Rats were then maintained on a flow rate of 2.5-3% in order to sustain a stable respiratory rate and were given an injection of Lactated Ringer's solution (100ml/kg, s.c.) and an injection of a nonsteroidal anti-inflammatory analgesic (Anafen, MERIAL Canada; 5ml/kg). Following surgery, rats were placed singly into a clean, sterile opaque polyurethane bin and kept warm until recovery from anesthetic was complete. A topical antibacterial ointment was externally applied to the incision (Flamazine, Smith & Nephew, Canada). Rats were weighed daily to

monitor recovery from surgery. The rats were given 7 days to recover before any experimental manipulations were initiated.

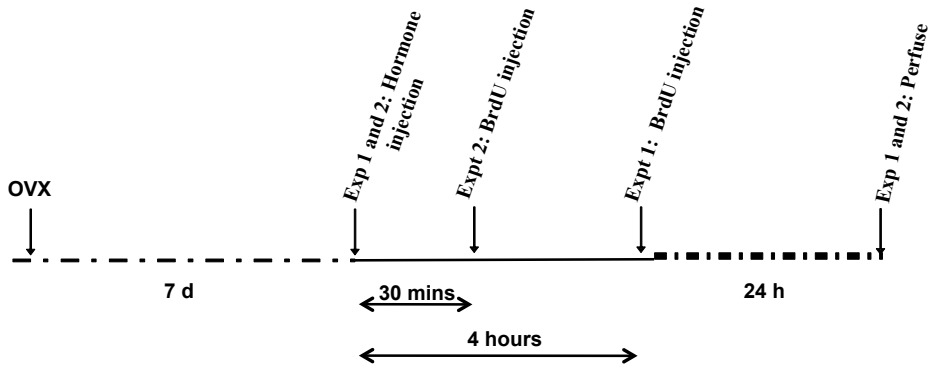
3.2.2.2 Drug Treatment

Approximately 1 week after ovariectomy surgery rats were randomly assigned to one of 13 treatment groups (n = 5-8 per group) and received a single subcutaneous (s.c.) injection of either: vehicle (Control; 0.10 mL sesame oil), estradiol benzoate (EB), 17β-estradiol (17β-E_2), 17α-estradiol (17α-E_2), or estrone (E_1) at one of three doses: low (0.30μg/0.10 mL sesame oil), medium (1μg/0.10 mL sesame oil), or high (10μg/0.10 mL sesame oil). These doses of estrogens were chosen based on previous studies investigating spatial learning and memory and hippocampal neurogenesis. A high dose of 10μg was chosen because it results in circulating levels of E_2 seen on the morning of proestrus (Viau and Meaney, 1991) and has been shown to enhance cell proliferation 2 hours (Tanapat, et al., 2005,Tanapat, et al., 1999) and 4 hours (Ormerod, et al., 2003) after injection and to suppress cell proliferation 48 hours (Ormerod, et al., 2003) after injection. We chose a medium dose of 1μg as previous studies have shown this dose of 17β-E_2 slightly increases cell proliferation (Tanapat, et al., 2005) and impairs working memory on the spatial working/reference memory version of the radial arm maze (Holmes, et al., 2002). On the other hand, a 0.3μg dose of 17β-E_2 has been shown to facilitate working memory and not affect reference memory (Holmes, et al., 2002), and has been shown to result in circulating levels of E_2 found during diestrus (Viau and Meaney, 1991). Effort was made to ensure that groups did not differ in post-operative body weights because ovariectomy is known to lead to increases in weight.

All rats received a single s.c. injection of 0.10 mL of either hormone or vehicle. All hormone injections were given between 9 am and 11 am. Previous studies have shown an increase in hippocampal cell proliferation 4 hours after a single injection of 10μg of EB

(Ormerod, et al., 2003). Thus, in Experiment 1, female rats (n= 69) received a single i.p. injection of 5-bromo-2-deoxyuridine (BrdU; Sigma, St. Louis, MO) (200 mg/kg), 4 hours after the hormone injection. BrdU is a thymidine analogue that incorporates itself into the DNA of cells during the synthesis phase of the cell cycle, which will label proliferating cells and their progeny. To determine whether estrogens influence cell proliferation at a time point earlier than 4 hours, another set of female rats (n = 75) received a single i.p. injection of BrdU (200 mg/kg) 30 minutes after the hormone injection in Experiment 2. All rats (Experiment 1 and 2) were perfused 24 hours after BrdU administration in order to determine the number of newly divided cells after one complete mitotic division (Cameron and McKay, 2001). For a timeline of experiments 1 and 2 see Figure 3.1. Estradiol has been shown to alter blood-brain barrier permeability, which in turn could potentially alter availability of BrdU; however, treatment with estradiol for at least 3 weeks is required in rats before alterations in blood-brain barrier permeability occur (Ziylan, et al., 1990).

Figure 3.1: Time line of BrdU injections and perfusions for Experiments 1 and 2. To test whether different forms of estrogen influence cell proliferation in the dentate gyrus, rats were injected with hormone between 9 am and 11 am, injected with BrdU 4 hours later (Experiment 1), and perfused 24 hours later. To determine whether these estrogens could influence cell proliferation at an earlier time point, rats were injected with hormone between 9 am and 11 am, injected with BrdU 30 minutes later (Experiment 2), and perfused 24 hours later. OVX = ovariectomy.



3.2.2.3 Drug Preparation

Estrogens (Sigma Aldrich Chemicals, Canada) were dissolved in sesame oil (Sigma Aldrich Chemicals, Canada) over low heat to a concentration of 0.30μg, 1μg or 10μg of hormone per 0.10 mL oil. Estrogen solutions were stored in opaque containers at room temperature. BrdU (Sigma Aldrich Chemicals, Canada) solution was prepared just prior to injection by dissolving BrdU in freshly prepared 0.9% saline heated to 40°C containing 0.7% 1N NaOH to a concentration of 20 mg BrdU/mL saline.

3.2.2.4 Histology

All histological procedures were based on previous work (Mazzucco, et al., 2006,Ormerod, et al., 2003). Rats were deeply anaesthetized with a lethal dose of sodium pentobarbital and then perfused with 4% paraformaldehyde within 24 hours to assess cell proliferation following BrdU injection. A 24-hour survival time post-BrdU injection was followed to allow for one mitotic division (Cameron and McKay, 2001). Following extraction, brains were stored at 4°C in 4% paraformaldehyde for 24 hours, before being transferred to 30% sucrose for a minimum of 72 hours. Brains were sliced in 40 μm sections through the entire extent of the hippocampus in a bath of TBS (pH 7.4) using a vibratome (Leica VT1000S; Leica Microsystems, Inc., Richmond Hill, ON, Canada). The sections were stored at -20°C in PBS

antifreeze sterile culture plates. Sections were washed 3x for 10 minutes each with TBS and then stored in sterile culture plates filled with TBS for the 24 hours prior to BrdU immunohistochemistry processing. BrdU-ir cells were counted on peroxidase-treated sections.

3.2.2.5 BrdU Immunohistochemistry

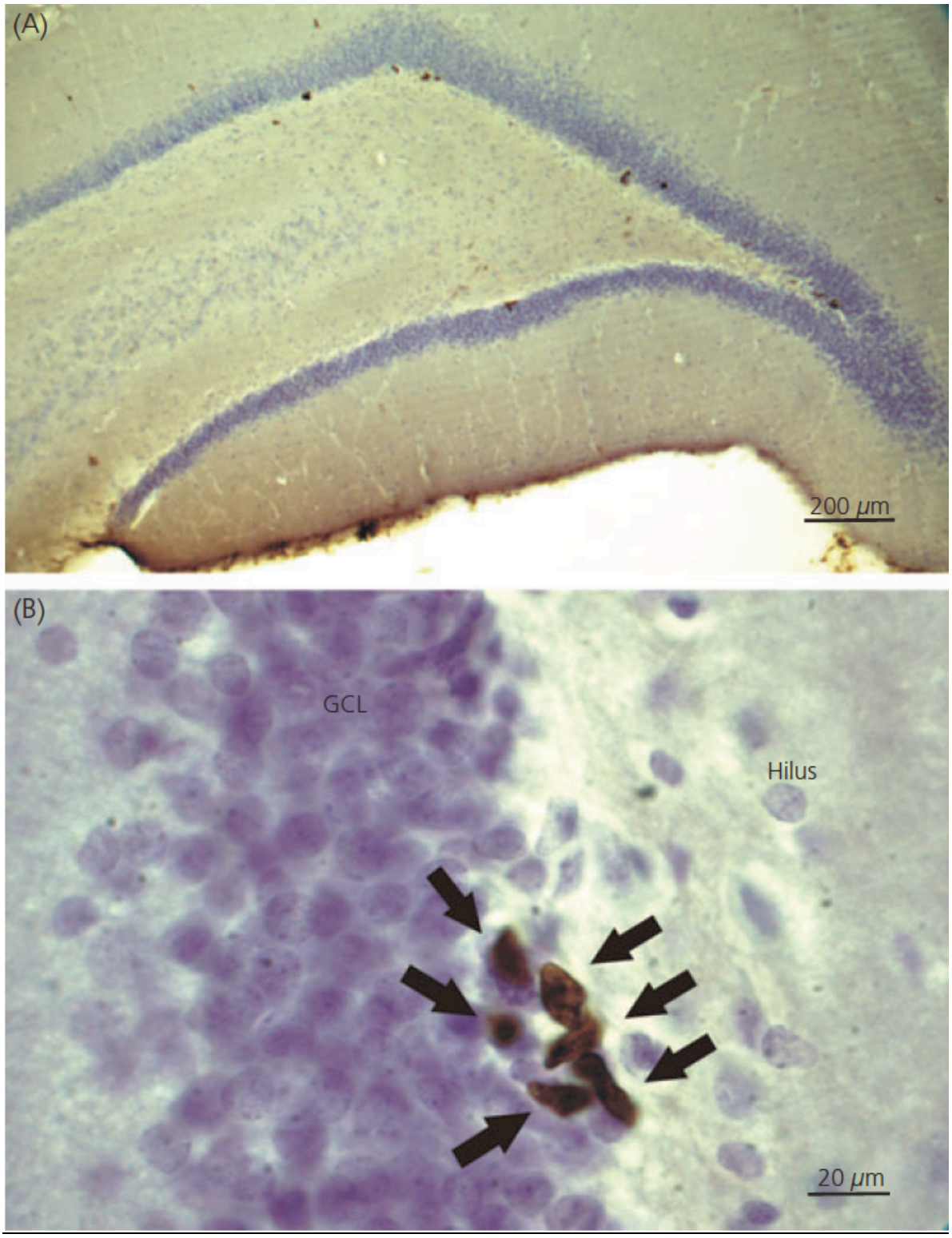
Between all steps, free-floating sections were rinsed 3x for 10 minutes each in TBS (0.1 M tris-phosphate buffer in 0.9 % saline; pH 7.4) unless stated otherwise. Sections were incubated in 0.6% H_2O_2 for 30 minutes at room temperature**.** DNA was denatured by applying 2N HCl for 30 min at 37°C. Sections were blocked with 3.0% normal horse serum (NHS; Vector Laboratories, Burlington, ON, Canada) and 0.1% Triton-X for 30 min and then incubated overnight in mouse monoclonal antibody against BrdU (1:200 + 3% NHS + 0.1% Triton-X; Boehringer Mannheim, Laval, Quebec, Canada) at 4°C. Twenty-four hours later, sections were incubated in mouse secondary antisera (1:200 + 3% NHS + 0.1% Triton-X; Vector Laboratories, Burlington, ON, Canada) for 4 hours at room temperature. Sections were incubated in avidin-biotin horseradish peroxidase complex (ABC Elite Kit; 1:50; Vector Laboratories, Burlington, ON, Canada) for 90 minutes. Sections were reacted in 0.02% diaminobenzidine (DAB; Sigma Aldrich Chemicals) with 0.0003% H_2O_2 for approximately 5 minutes. The sections were mounted on super frost slides (Fisher Scientific, Edmonton, AB, Canada) and dried overnight. The sections were counterstained with cresyl violet acetate (Fisher Scientific, Edmonton, AB, Canada), dehydrated and coverslipped with Permount (Fisher Scientific, Edmonton, AB, Canada).

3.2.3 Data Analyses

For both experiments all slides were coded prior to any quantitative analysis by an experimenter blind to the conditions.

To estimate cell numbers, total BrdU-immunoreactive (-ir) cells were counted under 100x objective on every 10th section (approx. 11-12 sections per rat). Cells were counted throughout the dentate gyrus separately for the granule cell layer (GCL), which included the subgranular zone (defined as approximately the 50 μm band between the GCL and the hilus), and the hilus. Total cell counts were calculated by multiplying the number of BrdU-ir cells per animal by 10. BrdU-ir cells are counted in the hilus and compared to counts in the granule cell region for a variety of reasons: (1) to determine whether any effects are attributable to generalized effects on blood brain permeability and not specifically to the treatments; (2) progenitor cells in the hilus give rise to a different population of cells that are mainly glial cells compared to progenitor cells in the subgranular zone which give rise to cells that are mainly neurons (Cameron, et al., 1993); and (3) new neurons in the hilus are considered ectopic (Scharfman, et al., 2007). Cells were considered BrdU-ir if they were intensely stained and exhibited medium round or oval cell bodies (Cameron, et al., 1993,Mazzucco, et al., 2006; see Figure 2). The areas of the GCL+SGZ and hilus were measured using the digitizing program Image J (National Institutes of Health, Bethesda, Maryland) to verify that volumes of structures counted did not differ between groups. Estimates of GCL+SGZ and hilar volumes were made using Cavalieri's principle (Gundersen and Jensen, 1987) by multiplying the aggregated areas by the distance between sections (400 μm) and the hilus was expected to have a larger volume than the GCL (Brummelte, et al., 2006,Ormerod, et al., 2003).

Figure 3.2: Photomicrograph of representative a) dentate gyrus 24 hours after BrdU injection and b) BrdU-ir cells (black arrows) in the subgranular zone of the dentate gyrus 24 hours after BrdU injection. Scale bar = 200µm in (a) and 20µm in (b).



3.2.4 Statistical Analyses

To ensure that groups did not differ in post-operative body weights, a one-way ANOVA with group (Control, EB-Low, EB-Med, EB-High, 17β-E_2-Low, 17β-E_2-Med, 17β-E_2-High, E_1-Low, E_1-Med, E_1-High, 17α-E_2-Low, 17α-E_2-Med, 17α-E_2-High) as the between-subjects factor was run for Experiment 1 and 2. For both experiments, analysis of the volume of the dentate gyrus, and total number of BrdU-ir cells were calculated using repeated-measures ANOVAs with group (Control, EB-Low, EB-Med, EB-High, 17β-E_2-Low, 17β-E_2-Med, 17β-E_2-High, E_1-Low, E_1-Med, E_1-High, 17α-E_2-Low, 17α-E_2-Med, 17α-E_2-High) as the between-subjects factor and region (GCL+SGZ, hilus) as the within-subjects factor. Data were further analyzed using the Newman-Keuls post-hoc test, or a priori tests subject to a Bonferroni correction. All statistical procedures were set at $\alpha = 0.05$ unless otherwise stated.

3.3 RESULTS

3.3.1 Experiment 1: Exposure to estrogens for 4 hours

Results indicate that groups did not differ in post-operative body weight at the time of BrdU administration ($F(12,56)=1.77$, $P<0.68$).

Results demonstrate there were no significant differences between groups in the volume of the GCL or the hilus (main effect of group: $P\leq0.15$; interaction effect: $P\leq0.39$; see Table 3.1). As expected, there was a significant main effect of region with greater hilar volumes than GCL+SGZ volumes ($F(1, 56)=650.90$, $P<0.001$). Because there were no group differences in volumes of either GCL+SGZ or hilus, the total number of BrdU-ir cells was reported in Experiment 1.

Table 3.1: Mean ± SEM volume (mm^3) of the granule cell layer and the hilus: exposure to estrogens for 4 hours (Experiment 1)

Group	Granule cell layer (mm^3)	Hilus (mm^3)
Control	2.34±0.12	5.83±0.41
Estradiol Benzoate		
Low	2.55±0.12	6.26±0.24
Medium	2.85±0.16	6.09±0.57
High	2.76±0.27	6.16±0.56
17β-estradiol		
Low	2.50±0.25	6.00±0.65
Medium	2.15±0.20	5.08±0.71
High	2.68±0.35	5.73±0.53
Estrone		
Low	2.92±0.22	6.02±0.36
Medium	2.91±0.25	6.77±0.54
High	2.39±0.16	5.48±0.41
17α-estradiol		
Low	2.50±0.24	4.36±0.33
Medium	2.34±0.18	5.29±0.48
High	2.30±0.12	5.17±0.56

There were no significant differences between groups in GCL volumes or hilus volumes.

Low and high doses of EB increase cell proliferation after 4 h compared to controls

At the 4 h time point post-hoc tests revealed that the EB-Low group ($P<0.001$) and the EB-High group ($P<0.001$) had significantly more total BrdU-ir cells in the GCL+SGZ compared to the control group (interaction between group and region: $F(12, 56)=2.20$, $P<0.05$; see Figure 3.3), but not in the hilus (both P's≥ 0.99; see Table 2). There were no other differences between groups in total number of BrdU-ir cells in the GCL (all P's≥ 0.15) or in the hilus (all P's≥ 0.89; see Table 3.2). Significant main effects of group ($F(12, 56)=2.32$, $P<0.05$) and region ($F(1, 56)=792.63$, $P<0.001$) were also found.

Figure 3.3: Mean (+ SEM) total number of BrdU-ir cells in the granule cell layer of the dentate gyrus at 4 hours (Experiment 1). Rats given a low dose (0.3µg) and a high dose (10µg) of estradiol benzoate had significantly more BrdU-ir cells in the GCL 24 hours after BrdU injection compared to control rats (both *P*'s<0.001). Asterisks indicate ***$P < 0.001$ versus control group.

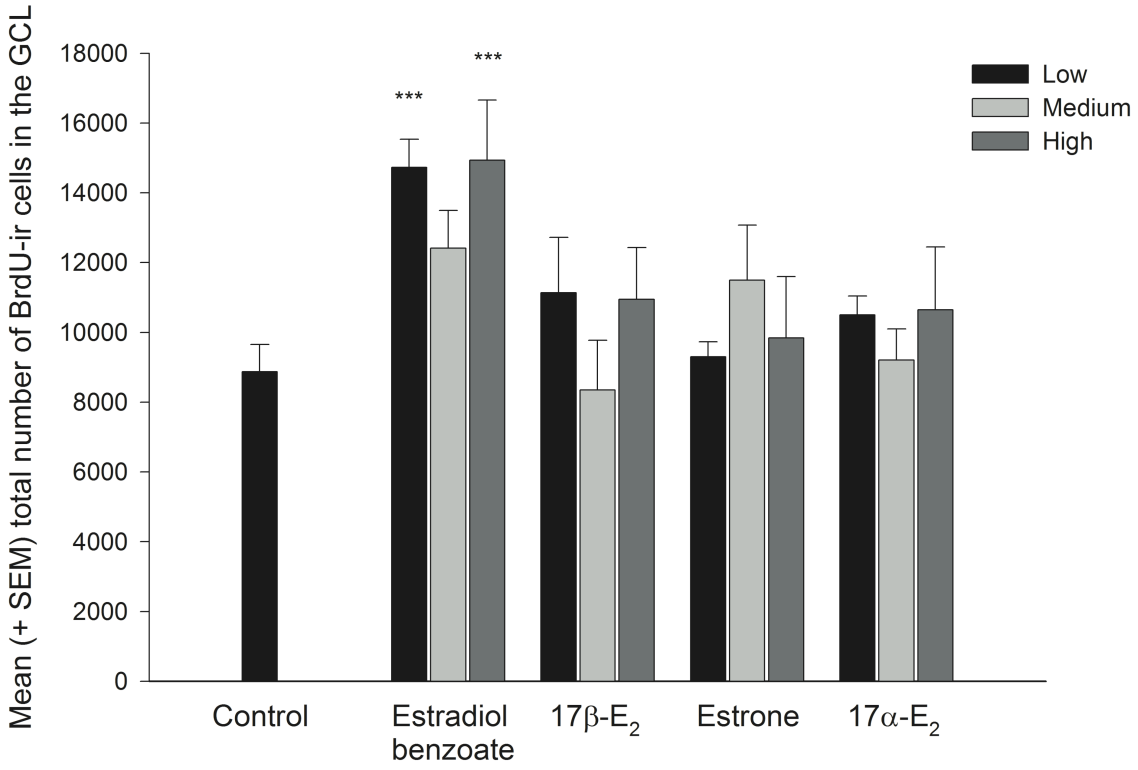


Table 3.2: Mean ± SEM total number of BrdU-ir cells in the hilus: exposure to estrogens for 4 hours (Experiment 1)

Group	Hilus
Control	791.43±122.97
Estradiol Benzoate	
Low	1540.00±213.04
Medium	1020.00±104.56
High	1485.00±139.98
17β-estradiol	
Low	1185.60±255.84
Medium	784.00±161.91
High	1240.00±276.04
Estrone	
Low	1320.00±192.35
Medium	1588.00±219.96
High	970.00±98.35
17α-estradiol	
Low	1375.00±281.00
Medium	1210.00±246.24
High	1126.67±277.16

There were no significant differences between groups in total number of BrdU-ir cells in the hilus.

3.3.2 Experiment 2: Exposure to estrogens for 30 minutes

Results indicate that groups did not differ in post-operative body weight at the time of BrdU administration ($F(12,62)=1.20$, $P<0.30$).

There were no significant differences between groups in the volume of the GCL or the hilus (main effect of group: $P\leq0.56$; interaction effect: $P\geq0.82$; see Table 3.3). As expected, there was a significant main effect of region with greater hilus volumes than GCL volumes ($F(1, 62)=732.97$, $P<0.001$). Because there were no group differences in volumes of either GCL+SGZ or hilus, the total number of BrdU-ir cells was reported in Experiment 2.

Table 3.3: Mean ± SEM volume (mm^3) of the granule cell layer and the hilus: exposure to estrogens for 30 minutes (Experiment 2)

Group	Granule cell layer (mm^3)	Hilus (mm^3)
Control	2.70±0.11	5.92±0.48
Estradiol Benzoate		
Low	2.91±0.15	5.60±0.70
Medium	2.51±0.18	4.92±0.29
High	2.68±0.23	5.98±0.45
17β-estradiol		
Low	2.75±0.19	6.20±0.48
Medium	2.88±0.18	5.85±0.48
High	2.54±0.16	4.91±0.24
Estrone		
Low	2.90±0.20	5.85±0.51
Medium	2.51±0.19	5.45±0.40
High	2.45±0.25	5.36±0.66
17α-estradiol		
Low	2.50±0.14	5.46±0.34
Medium	2.57±0.15	5.44±0.31
High	3.14±0.09	6.25±0.19

There were no significant differences between groups in GCL volumes or hilus volumes.

Low and high doses of 17β-E_2 and E_1 and the medium dose of 17α-E_2 increase cell proliferation after 30 min compared to controls

At the 30 min time point, post-hoc tests comparing each group to the control group revealed that the 17β-E_2-Low group (P<0.001), 17β-E_2-High group (P<0.001), E_1-Low group (P<0.001), E_1-High group (P<0.01), 17α-E_2-Medium group (P<0.001), and 17α-E_2-High (P<0.05) had significantly more total BrdU-ir cells in the GCL, but not in the hilus (all P's≥0.88, see Table 3.4; interaction between group and region: (F(12, 62)=4.45, P<0.001; see Figure 3.4). All other groups did not differ from controls in total number of BrdU-ir cells in the

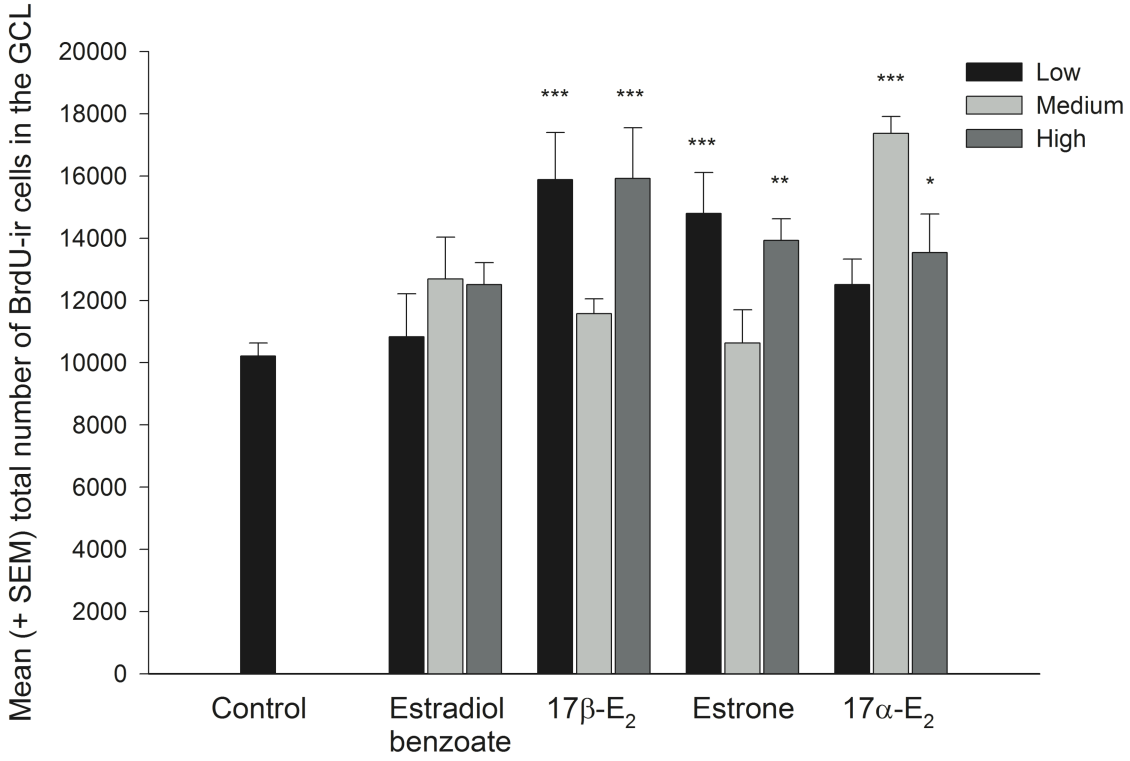
GCL (all *P*'s≥0.16) or in the hilus (all *P*'s≥0.99; see Table 3.4). Significant main effects of group ($F(12, 62)=5.04$, $P<0.001$) and region ($F(1, 62)=1811.97$, $P<0.001$) were also found.

Table 3.4: Mean ± SEM. total number of BrdU-ir cells in the hilus: exposure to estrogens for 30 minutes (Experiment 2)

Group	Hilus
Control	1476.00±118.99
Estradiol Benzoate	
Low	1113.33±149.28
Medium	1144.00±177.47
High	1451.43±172.15
17β-estradiol	
Low	1728.00±154.87
Medium	1412.00±147.05
High	1656.00±240.28
Estrone	
Low	1744.00±257.79
Medium	1082.50±180.45
High	1608.00±229.22
17α-estradiol	
Low	1380.00±121.98
Medium	2086.67±438.99
High	1784.00±160.42

There were no significant differences between groups in total number of BrdU-ir cells in the hilus.

Figure 3.4: Mean (+ SEM) total number of BrdU-ir cells in the granule cell layer of the dentate gyrus at 30 minutes (Experiment 2). Rats given a low dose (0.3μg) and a high dose (10μg) of 17β-estradiol (E_2) (both *P*'s<0.001) and of estrone (*P*<0.001 and *P*<0.01, respectively) had significantly more BrdU-ir cells in the GCL 24 hours after BrdU injection compared to control rats. Rats given a medium dose (1.0μg) and a high dose (10μg) of 17α-estradiol (*P*<0.001 and *P*<0.05, respectively) had significantly more BrdU-ir cells in the GCL 24 hours after BrdU injection compared to control rats. Asterisks indicate **P* < 0.05 versus control group; ***P* < 0.01 versus control group; ****P* < 0.001 versus control group.



3.4 DISCUSSION

As expected, the results from the present study demonstrate that different forms of estrogen rapidly increase the number of newly-divided cells in the dentate gyrus after a 30-min

exposure in a dose-dependent manner in young, ovariectomized female rats. Of the four estrogens tested, only EB was able to dose-dependently increase cell proliferation after a 4 h exposure compared to control rats. Interestingly, 17β-E_2 (at 30 min), E_1 (at 30 min), and EB (at 4 h) all increased cell proliferation with the same dose-dependent curve, with the low and high doses increasing cell proliferation and the medium dose not having a significant effect compared to the control group. On the other hand a different pattern was seen with 17α-E_2 as the medium and high, but not the low, doses of 17α-E_2 significantly increased cell proliferation at 30 min.

3.4.1 Low and high, and not medium, doses of 17β-E_2 and EB increase cell proliferation at different time points

Exposure to a single acute injection of 0.3 or 10 μg of 17β-E_2 for 30 min and of EB for 4 h led to increased number of total BrdU-ir cells compared to oil-injected control ovariectomized female rats. These findings are consistent with and further extend previous work that found an increase in cell proliferation 4 h after administration of 10μg of EB (Mazzucco, et al., 2006,Ormerod, et al., 2003) and 2 h after administration of 10μg of 17β-E_2 (Tanapat, et al., 2005,Tanapat, et al., 1999). Previously, Tanapat et al. (2005) found that the dose of 1μg of 17β-E_2 did not significantly increase cell proliferation compared to control females. We replicated this result and have also shown that a lower dose of 0.3μg increases cell proliferation. To our knowledge, this is the first time that a dose as low as 0.3μg of 17β-E_2 has been shown to influence cell proliferation in female rodents. This same dose facilitates spatial working memory (Holmes, et al., 2002) and is thought to represent physiological levels of estradiol during diestrus (Viau and Meaney, 1991).

Estradiol benzoate significantly increased cell proliferation after the 4 h exposure, but not after 30 min of exposure. The benzoate group that is conjugated to the 17β-E_2 molecule functions to slow down the metabolism of the hormone. Thus, it is likely that EB requires more

time to enter the circulation and is slower to reach the brain. Our results indicate that 30 min is not enough time to allow a sufficient amount of subcutaneously injected EB to significantly affect cell proliferation in the dentate gyrus. However, our results and previous literature indicate that 4 h is enough time for EB to affect hippocampal function (Holmes, et al., 2002) and morphology (Mazzucco, et al., 2006,Ormerod, et al., 2003). Future studies should attempt to address this issue by measuring levels of estradiol in the hippocampus after subcutaneous injections of EB at different time points.

3.4.2 Estrone and 17α-E_2 increase cell proliferation after 30 min, and not 4 h, of exposure

Administration of E_1 increased cell proliferation in a similar manner as administration of 17β-E_2, with low and high doses increasing the total number of BrdU-ir cells after a 30 min exposure to the hormone. Interestingly 17α-E_2, the optical isomer of 17β-E_2, also increased cell proliferation but with a different dose profile than 17β-E_2. Although both the medium and high doses of 17α-E_2 increased cell proliferation, the medium dose increased proliferation to the greatest extent. These differences between the estrogens in the dose-dependent increase in cell proliferation may in part be mediated by variations in activation of estrogen receptors or through recruitment of different mechanisms. For example, membrane-associated estrogen receptors have been shown to have greater sensitivity for 17α-E_2 (Toran-Allerand, et al., 2002). Furthermore, E_1 and 17β-E_2 influence ER subtypes differently as chronic E_1 treatment decreases ERα levels, but not ERβ levels, whereas chronic 17β-E_2 treatment increases ERβ levels, but not ERα levels, in the hippocampus (Jin, et al., 2005). Furthermore, both E_1 and 17β-E_2 reduce cell death induced by glutamate toxicity, but with differing potencies depending on the type of cell line under investigation (Bhavnani, et al., 2003). Thus, these studies suggest that different estrogens can modulate the sensitivity and expression of ERs.

In accordance with our results, previous literature has shown that both 17α-E_2 and 17β-E_2 influence hippocampal function and morphology but at different doses. Luine et al. (2003) found that rapid enhancement of visual and place memory within 4.5 h could be achieved by either 17α-E_2 or 17β-E_2, with the former being a considerably more potent enhancer of place memory. Furthermore, a lower dose of 17α-E_2 is required to increase pyramidal spine synapse density in the CA1 region of the hippocampus 30 min after administration compared to 17β-E_2 (MacLusky, et al., 2005).

It is possible that the effects of 17α-E_2 on cell proliferation may be due to its metabolic conversion to 17β-E_2. However, this is unlikely for at least three reasons: (i) the activity of 17α-hydroxysteroid dehydrogenase, the enzyme responsible for interconverting these two hormones, is relatively low in female rats (Stenberg, 1976); (ii) the pattern of dose-dependent increase of cell proliferation is different from what is seen with 17β-E_2; and (iii) 17α-E_2 has very limited affects on peripheral organs in stark contrast to the large effects seen with 17β-E_2 (Lundeen, et al., 1997). This latter finding further supports the idea that the effects of 17α-E_2 on cell proliferation are not occurring through a conversion into 17β-E_2 in the periphery. This could also potentially explain why 17α- and 17β-E_2 are increasing cell proliferation at different doses.

Estrone is interconverted to 17β-E_2 via 17β-hydroxysteroid dehydrogenase. In humans, 25% of circulating E_1 is directly converted from 17β-E_2 within the ovaries. Therefore, it is feasible in the present study that E_1 was converted into 17β-E_2 and the subsequent increase in cell proliferation was actually stimulated by 17β-E_2. This could partially explain why E_1 increased cell proliferation at the same doses as 17β-E_2, despite it being a weaker estrogen.

3.4.3 Possible explanations for the dose-dependent increase in cell proliferation

The dose-dependent increase in cell proliferation seen with the different estrogens is intriguing. It is possible that varying concentrations of the administered estrogens may recruit

different mechanisms through which to act, for example, these different estrogens may affect different populations of cells, and/or may recruit different ER receptors. In the present study, we found that the medium dose of 17α-E_2 increased cell proliferation 30 min after injection, but the same dose of 17β-E_2 and E_1 was ineffective in this regard. One potential reason for this could be that 17α-E_2 is exerting its effects on cell proliferation through a different mechanism. For example, 17α-E_2 may be activating on a membrane-bound receptor (Toran-Allerand, et al., 2002) and this type of receptor may require a different dose of estrogen to substantially alter cell proliferation in the dentate gyrus. 17α-E_2 has a high affinity for ER-X, a plasma membrane-associated ER, whereas, on the other hand, a 100x greater amount of 17β-E_2 is required to activate this receptor (Toran-Allerand, et al., 2002).

It should also be noted that the U-shaped response curves are not without precedent in the field of endocrinology. For example, very low and very high estradiol levels are associated with impaired spatial working memory and medium levels of estradiol are associated with enhanced spatial working memory (Galea, et al., 2001,Holmes, et al., 2002). Furthermore, very low and very high levels of adrenal steroids are associated with increased cell death in the hippocampus while medium levels are associated with reduced cell death (for review see Joels, 2007). As well, dopamine activation of the D1 receptor in the prefrontal cortex has been shown to mediate working memory with an inverted U-shape (for review see Floresco and Magyar, 2006).

3.4.4 Possible role of classical genomic estrogen receptors in the effects of different estrogens on cell proliferation

There are two identified estrogen receptor subtypes-α (ERα) (Koike, et al., 1987) and β (ERβ) (Kuiper, et al., 1996), and also several isoforms and splice variants of each subtype. Interestingly, ERs have been located directly on progenitor and daughter cells in the subgranular

zone (Isgor and Watson, 2005,Mazzucco, et al., 2006) and both ERα and ERβ agonists increased cell proliferation in female rats (Mazzucco, et al., 2006). Interestingly, 17β-E_2 binds to the ERs with the greatest affinity and is the most potent form of estrogen (Kuiper, et al., 1997). Estrone and 17α-E_2 appear to have similar binding affinities for ERα; however, E_1 binds to ERβ with a much greater affinity than 17α-E_2 (Kuiper, et al., 1997). Therefore, it is possible that the different effects that these estrogens are having on cell proliferation are mediated by differences in their binding affinities for ERα and ERβ. Furthermore, it should be noted that all females in the present study were ovariectomized for 1 week prior to hormone manipulations. Ovariectomy alters the brains ability to respond to estrogen treatment by significantly decreasing ER density in the hippocampus (Rose'Meyer, et al., 2003). Interestingly, levels of 17α-E_2 in the brain do not decrease following ovariectomy unlike levels of 17β-E_2 (Toran-Allerand, et al., 2005), suggesting that levels of ER-X are at least maintained, if not actually increased, following ovariectomy. As well, ovariectomy without hormone replacement decreases cell proliferation in a time-dependent manner, as a decrease in cell proliferation is seen 1 week but not 4 weeks after surgery (Green and Galea, 2008,Tanapat, et al., 2005,Tanapat, et al., 1999). Therefore, it is possible that our findings could be different in intact female rats.

3.4.5 Possible role of nonclassical, nongenomic estrogen receptors in the effects of different estrogens on cell proliferation

Importantly, non-nuclear ERα and ERβ protein expression has been identified in the same corresponding areas as their nuclear counterparts in the hippocampus of the adult male and female rat (Kalita, et al., 2005). To date, ERs have been located at the plasma membrane, in the mitochondria, and in the cytoplasm (for review see Levin, 2005). Recent research has concentrated on the nongenomic actions of estrogens, which are defined as rapid (onset within minutes) effects that usually begin at the plasma membrane and result in the activation of signal

transduction pathways (for review see Vasudevan and Pfaff, 2008). Two potential novel membrane ERs that could be involved in the rapid effects of estrogens are ER-X (Toran-Allerand, et al., 2002) and the G-protein receptor, GPR30 (Raz, et al., 2008). Recently GPR30 has been localized to the cell surface (Filardo, et al., 2007) and is found throughout the hippocampus, including the dentate gyrus (Brailoiu, et al., 2007).

Interestingly plasma membrane ERs have been shown to be particularly sensitive to 17α-E_2 (Toran-Allerand, et al., 2002,Wade, et al., 2001). Furthermore, many of the rapid effects of 17α- and 17β-E_2 (within hours) effects are believed to be independent of classical genomic effects (Luine, et al., 2003,MacLusky, et al., 2005). The rapidity of these effects is the key evidence that supports the hypothesis that the estrogens examined in this study are possibly acting through plasma membrane receptors to activate nongenomic mechanisms. Furthermore, 17α-E_2 only binds weakly to nuclear ERs and only transiently binds to the estrogen-responsive element (Clark, et al., 1982,Lubahn, et al., 1985), further supporting the idea that the effects of this estrogen are not dependent on gene transcription. The results of the present study also support this idea as the effects of 17β-E_2, E_1, and 17α-E_2 on cell proliferation are seen after 30 min of exposure to the hormones and not after 4 h. It should also be noted that BrdU, which was injected 30 min after the hormone, actively labels dividing cells within 2 hours of injection (Packard, et al., 1973). Therefore, any effect that the estrogens had on stimulating cell proliferation in the present study occurred within 2.5 h, which is still within the timeframe for nonclassical, nongenomic effects.

3.4.6 Different estrogens rapidly increase cell proliferation after 30 min of exposure but not after 4 h: role of cell cycle kinetics

The rapid increase in the total number of BrdU-ir cells in the dentate gyrus that was seen 30 min after administration of 17β-E_2, E_1, and 17α-E_2 was not seen 4 h after administration of

these estrogens. One potential reason for this could be the activation of a compensatory mechanism that downregulates cell proliferation. As discussed by Nowakowski and Hayes (2008), the two-population model describes one potential way to view neurogenesis in the dentate gyrus. This model hypothesizes that two discrete populations of proliferative cells exist in the adult dentate gyrus: the progenitor, steady-state population and a replenishing stem cell population. The characteristics of this model lead to the hypothesis that any modification to one of these populations must be followed by another modification in the other population in order to compensate for the modification to the first population. For example, if a mitogenic factor such as estradiol was introduced into the replenishing stem cell population resulting in an increase in proliferation in this population, then some sort of compensatory mechanism would be activated in the steady-state population, or visa versa, in order to restore the proliferative population constant. In support of this model, administration of 17β-E_2 has been shown to reduce proliferation of adult neural stem cells that were increased by epidermal growth factor (Brannvall, et al., 2002) and EB initially increases and then suppresses cell proliferation 48 h after administration (Ormerod, et al., 2003). Therefore, it appears that 17β-E_2 can work through a compensatory mechanism to reduce elevated levels of cell proliferation.

One potential way that estradiol could reduce cell proliferation is by altering the cell cycle kinetics of one of the proliferative cell populations in the dentate gyrus. Brannvall et al. (2002) found that the subsequent reduction in cell proliferation by 17β-E_2 was due to an increase in the cyclin-dependent kinase inhibitor, p21. p21 is a member of the Cip/Kip family of cell cycle regulators that inhibit the activity of various cyclin-dependent kinases (Sherr and Roberts, 1999), resulting in cell cycle arrest by preventing Rb phosphorylation (Harper, et al., 1993). Importantly, high levels of p21 have been found within the subgranular zone, the area where progenitor cells reside, in the dentate gyrus (Pechnick, et al., 2008). In addition, p21-null mice

show increased levels of cell proliferation in the subgranular zone compared to wild-type mice, suggesting that p21 restrains neurogenesis in the dentate gyrus (Pechnick, et al., 2008). In addition to this, Wright et al. (2005) suggested that, at high enough levels, estradiol can block proliferation through a unique function of the ER that is nonmitogenic and dependent on dose. Wright et al. were able to prevent the estradiol-mediated induction of p21 by inhibiting transcription by administering actinomycin D, providing evidence for p21 being a transcriptional target of estrogen and the ER. Interestingly, the time frame that would be required for the transcription of the p21 protein by estradiol via the ER fits with the time course used in Experiment 1 of the present study. By 4 h, the estrogens would have had enough time to rapidly increase (within 30 min; see results of Experiment 2) proliferation of progenitors in one population and also be able to induce the transcription of p21, thereby potentially halting certain progenitor cells from a different population from entering the S-phase. Thus, this would result in no net increase in the total number of BrdU-ir cells that were labeled at 4 h after hormone injection. Future experiments should concentrate on further elucidating the ability of different estrogens at different doses to stimulate p21.

3.4.7 Functional significance of estrogen treatment and possible role of hippocampal neurogenesis

Although controversial, adult hippocampal neurogenesis has been shown to contribute to different types of learning and memory subserved by the hippocampus. Experimentally reducing adult neurogenesis has been shown to transiently disrupt performance on various hippocampus-dependent tasks (Saxe, et al., 2006,Shors, et al., 2002). Additionally, engaging in a hippocampus-dependent learning and memory task results in increased levels of cell survival (Ambrogini, et al., 2000,Epp, et al., 2007,Leuner, et al., 2004b). Interestingly, training on a hippocampus-dependent learning and memory task during a discrete period of the cell

maturation cycle can enhance cell survival in male rats (Epp, et al., 2007). Furthermore, treatment with EB during this same time period also enhances cell survival and is associated with enhanced hippocampus-dependent memory in male meadow voles (Ormerod, et al., 2004). Hippocampus-dependent learning and memory has also been linked to levels of cell proliferation (Dupret, et al., 2007,Pham, et al., 2005). However, it is important to note that increased levels of cell proliferation and/or survival are not synonymous with increased levels of learning and memory. Despite numerous examples of positive correlations between adult neurogenesis and learning or memory (Nilsson, et al., 1999,van Praag, et al., 1999), high levels of adult neurogenesis can also be associated with poor hippocampus-dependent learning (Jessberger, et al., 2005). This suggests that there is an optimal level of cell proliferation and/or cell survival that is associated with better hippocampus-dependent learning and memory and that very low or very high levels of cell proliferation or survival may not be beneficial to hippocampus functioning.

With this in mind, it is not surprising that chronically treating ovariectomized female rats with the same high dose of EB used in the present study impairs hippocampus-dependent reference memory. However chronic administration of the same low dose of EB given in the present study facilitates hippocampus-dependent working memory (Galea, et al., 2001,Holmes, et al., 2002). Further experiments are required to determine the functional significance of an acute estrogen-induced increase in cell proliferation because research indicates a complex link between hippocampal neurogenesis and hippocampus-dependent learning and memory. In addition, estrogens are also known to alter synaptogenesis (for review see Woolley, 2007) and, thus, estrogens may modulate learning and memory in addition to or independent of its effects on neurogenesis in the hippocampus.

3.5 CONCLUSIONS

The present study demonstrates that cell proliferation in the dentate gyrus of the hippocampus in young ovariectomized female rats is differentially and dose-dependently rapidly increased, after 30 min of exposure, by 17β-E_2, E_1, and 17α-E_2, but not by EB. Interestingly, only EB increased cell proliferation after 4 h of exposure to the hormone. Therefore, it seems that the effects of these estrogens on neurogenesis are rapid and may be independent of the classical, genomic pathway. Furthermore, it is possible that, because these estrogens are having effects at different doses and they bind to estrogen receptors with different affinities, they may be working through different mechanisms. Importantly, we have shown that a very low dose of these estrogens is as effective in increasing cell proliferation as a high dose. This could have very important therapeutic implications because it may be possible to use lower doses of estrogen in hormone replacement therapies. Furthermore, the results of the present study also show that 17α-E_2 is biologically active and we have added to the growing list of its estrogenic effects. Our data also suggest that 17α-E_2 is as potent as 17β-E_2 in influencing the hippocampus. Although further studies are required, it may be possible to use 17α-E_2, as well as lower doses of estrogens, as neuroprotective agents.

4 LOW DOSES OF 17A-ESTRADIOL AND 17B-ESTRADIOL FACILITATE, WHEREAS HIGHER DOSES OF ESTRONE AND 17A- AND 17B-ESTRADIOL IMPAIR, CONTEXTUAL FEAR CONDITIONING IN ADULT FEMALE RATS.[3]

4.1 INTRODUCTION

Gonadal hormones influence hippocampus-dependent learning and memory (for review see Daniel, 2006,Frick, 2009). In particular estradiol has equivocal effects on cognition, with some studies showing improvements (e.g., Bimonte and Denenberg, 1999,Daniel, et al., 1997,Luine and Rodriguez, 1994), whereas other studies either show no effect (e.g., Berry, et al., 1997,Fader, et al., 1999,Luine, et al., 1998) or impairments with estradiol (e.g., Frye, 1995,Warren and Juraska, 1997,Wilson, et al., 1999). These differential effects are likely based on dose and the different types of cognitive tasks used in which performance depends on the integrity of different brain regions. Both endogenous and exogenous estrogen levels influence hippocampus-dependent learning and memory. For example, female rats in proestrus, when endogenous plasma levels of estrogen are highest, show impaired performance on different hippocampus-dependent tasks compared to female rats in estrus, when endogenous plasma levels of estrogen are lowest (Frye, 1995,Markus and Zecevic, 1997,Warren and Juraska, 1997). Intriguingly, however, rats during proestrus are more likely to use a spatial strategy when solving certain tasks (Korol, et al., 2004). In addition, exogenous administration of estradiol to ovariectomized female rats shows a dose-dependent relationship between estradiol and memory. Low but physiological levels of exogenous estradiol enhance performance (Fader, et al.,

[3] A version of this chapter has been published. Barha, C.K., Dalton, G.L. and Galea, L.A. (2010) Low doses of 17α-estradiol and 17β-estradiol facilitate, whereas higher doses of estrone and 17α- and 17β-estradiol impair, contextual fear conditioning in adult female rats. Neuropsychopharmacology. 35: 547-559.

1999,Holmes, et al., 2002,Luine, et al., 1998), whereas administration of high exogenous pharmacological or physiological levels of estradiol impair performance (Galea, et al., 2001,Holmes, et al., 2002) on a hippocampus-dependent spatial working/reference memory version of the radial arm maze task. Therefore, it is necessary to investigate dose-dependent effects of estradiol on different types of learning and memory tasks that are subserved by different memory systems.

Fear conditioning relies on the integrity of the amygdala and contextual fear conditioning relies on the integrity of the hippocampus and amygdala (Phillips and LeDoux, 1992). Estradiol influences hippocampus-dependent contextual fear conditioning, but not hippocampus-independent cued fear conditioning (Gupta, et al., 2001,Markus and Zecevic, 1997), indicating that estradiol may mediate hippocampus-dependent conditioning, but has less impact on amygdala-dependent fear conditioning. Physiologically high levels of estradiol influence contextual fear conditioning negatively, as female rats in proestrus or treatment with high estradiol reduces the amount of freezing after conditioning compared with females in estrus (Altemus, et al., 1998,Gupta, et al., 2001,Markus and Zecevic, 1997), whereas ovariectomy increases freezing behavior in response to a context associated with shock (Gupta, et al., 2001). Interestingly, a similar relationship between estrogen and contextual fear conditioning is seen in humans. Milad et al. (2006) found that women had greater context memory in the early follicular phase (low physiological estrogen levels) of the menstrual cycle compared to the midfollicular phase (high physiological estrogen levels) and men. Collectively, these studies indicate a function for estradiol in contextual fear conditioning, although to date, it is not known whether a dose-dependent effect of estradiol exists for contextual fear conditioning as it does for other hippocampus-dependent tasks or whether contextual fear conditioning is influenced by other types of estrogens.

17β-estradiol is the most potent form of estrogen and is the most prominent estrogen in young pre-menopausal women, whereas estrone is a weaker estrogen that is the most prominent estrogen in postmenopausal women (Rannevik, et al., 1995). Interestingly, estrone is the main component of Premarin, the most popular hormone replacement therapy given to menopausal women. A recent meta-analysis found that Premarin does not have as many cognitive-enhancing properties as other hormone replacement therapies that use different types of estrogen, such as 17β-estradiol (Hogervorst, et al., 2000,Ryan, et al., 2008). Furthermore, Premarin can alter estrogen receptor (ER) subtypes independently of the effects of 17β-estradiol. Jin et al. (2005) found that treatment with Premarin led to decreased levels of ERα, but did not affect ERβ levels, whereas treatment with 17β-estradiol increased ERβ, but did not affect ERα levels in the hippocampus and cortex of adult female rats. Given that ERs in the brain show tissue and region-specific patterns of expression (Shughrue, et al., 1997), and the diverse behaviors they regulate (Kudwa, et al., 2006,Rhodes and Frye, 2006), it is plausible that different forms of estrogen could have profound and distinct effects on neural plasticity and/or behavior. Despite the fact that estrone is centrally active (Barha, et al., 2009b), and is the main component of the HRT Premarin, to our knowledge only one study has directly examined the effects of estrone on learning (Farr, et al., 2000). Thus, in this study we sought to determine whether there were differential effects of the estrogens, estradiol and estrone, on contextual fear conditioning.

In addition to estrone, there are naturally occurring optical isomers of estradiol: 17α-estradiol and 17β-estradiol. Although 17β-estradiol binds to ERα and ERβ with an approximately 40-fold higher affinity (Perez, et al., 2005), 17α-estradiol may be the preferred ligand of ER-X, a plasma membrane-associated ER (Toran-Allerand, et al., 2002). In addition, 17α-estradiol has been shown to affect the structure and function of the hippocampus by rapidly increasing spine density in the CA1 region of the hippocampus (MacLusky, et al., 2005),

enhancing place memory (Luine, et al., 2003,Rhodes and Frye, 2006), and increasing cell proliferation in the dentate gyrus of adult female rats (Barha, et al., 2009b). Therefore, it is possible that 17α-estradiol could have effects on hippocampus-dependent contextual fear conditioning.

Estradiol's effects on behavior may be linked to its ability to alter the number and properties of synapses in the hippocampus. Specifically, 17β-estradiol increases the number of spines and synapses in the CA1 field of the hippocampus (Woolley, et al., 1990,Woolley and McEwen, 1992), and synaptophysin, a presynaptic protein that is a critical component of synaptic vesicle exocytosis, in the hippocampus (Frick, et al., 2002,Murphy and Segal, 1996,Pozzo-Miller, et al., 1999,Stone, et al., 1998). Importantly, the increase in synaptophysin expression in the whole hippocampus is associated with enhanced spatial reference memory (Frick, et al., 2002). Interestingly, contextual fear conditioning is reliant on an intact, functioning dorsal hippocampus as seen from lesion and immediate early gene studies (Barrientos, et al., 2002,for review see Kubik, et al., 2007). Therefore, it is of interest to see whether different forms of estrogen affect synaptophysin expression in the dorsal hippocampus after training in the contextual fear conditioning task.

This study aimed to determine the effects of 17β-estradiol, estrone, and 17α-estradiol at different doses on acquisition of hippocampus-dependent contextual fear conditioning and hippocampus-independent cued fear conditioning. Adult ovariectomized female rats were injected with either a low, middle, or high dose of one of the three hormones or vehicle, and later tested for contextual and cued fear conditioning. Levels of synaptophysin, a synaptic vesicle-associated integral membrane protein and a general marker of presynaptic nerve endings, were also assessed in the dorsal hippocampus of rats to identify a potential synaptic correlate of hormonal effects on contextual fear conditioning. We hypothesized that low doses

of estradiol would facilitate, and high and middle doses of all three hormones would impair contextual fear conditioning, but have no significant effect on cued fear conditioning. Furthermore, we expected higher levels of synaptophysin expression in rats given estrogen treatment compared with controls.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Eighty-one adult female Sprague–Dawley rats, weighing 200–250 g, obtained from the University of British Columbia Animal Care Centre (Vancouver, BC, Canada), were used in the study. Rats were initially housed in pairs in opaque polyurethane bins (48 x 27 x 20 cm) with aspen chip bedding and were given Purina rat chow and tap water *ad libitum*. Rats were maintained under a 12h: 12 h light/dark cycle (lights on 07.30 h). Beginning the day after arrival, rats were handled every other day for 5 min. All experiments were conducted in accordance with the ethical guidelines set by the Canada Council for Animal Care and were approved by the University of British Columbia Animal Care Committee. All efforts were made to reduce the number and the suffering of animals.

4.2.2 Procedures

4.2.2.1 Surgery

Approximately 1 week after arrival, all females were bilaterally ovariectomized using aseptic procedures. Rats were placed in a chamber and anaesthetized with isoflurane, which was delivered at an induction flow rate of 5% (the flow rate of O_2 was approximately 1.5%). Rats

were then maintained on a flow rate of 2.5–3% to sustain a stable respiratory rate and were given an injection of lactated ringer's solution (100 ml/kg, s.c.) and an injection of a nonsteroidal anti-inflammatory analgesic (Anafen, MERIAL Canada Inc., Baie d'Urfe´, Quebec, Canada; 5 ml/kg). After surgery, rats were placed singly into a clean, sterile opaque polyurethane bin and kept warm until recovery from anesthetic was complete. A topical antibacterial ointment was externally applied to the incision (Flamazine, Smith & Nephew, St Laurent, Quebec, Canada). Rats were weighed daily to monitor recovery from surgery. The rats were given 7 days to recover before any experimental manipulations were initiated.

4.2.2.2 Apparatus

Conditioning and testing occurred in two identical observation chambers (30.5 x 24 x 21 cm; Med Associates, St Albans, VT, USA), consisting of aluminum (side walls) and Plexiglass (ceiling, hinged front door, and rear wall). The chambers were enclosed within sound-attenuating boxes located in a brightly lit and quiet room. A video camera was positioned above each chamber to record subject's behavior for video scoring. The floor of each chamber consisted of 19 stainless steel rods spaced 1.5 cm apart that were connected to a shock generator and scrambler for the delivery of an unconditioned stimulus footshock. Each chamber was illuminated by a single 100mA houselight positioned in the top center of one wall. In the left corner of the same wall a speaker connected to a programmable audio generator (ANL-926, Med Associates) was located. On the wall opposite to the houselight, a speaker and two 100mA stimulus lights (2.5 cm diameter) were located 7 cm above the floor. Ventilation fans in each box supplied background noise (70 dB, A scale).

Each chamber was positioned on a load-cell platform that recorded chamber displacement in response to a subject's motor activity (Med Associates). The output from each load cell was set to a gain (vernier knob 8) that was optimized for detecting freezing behavior,

and the output was digitized and acquired online using Threshold Activity software (Med Associates). The load cell activity was digitized at 5Hz, resulting in one observation per rat every 200ms. Freezing is a defensive fear response that consists of an immediate suppression of behavior that is accompanied by immobility, shallow breathing, increased heart rate, and pilo-erection (Barrientos, et al., 2002). Freezing was quantified by calculating the number of observations for each rat that had a value less than the freezing threshold, allowing for exclusion of behaviors such as grooming, sniffing, and head turning. The same freezing threshold was used for each animal throughout the duration of the experiment. Importantly, an observation was only considered to be freezing if it was a part of at least 5 continuous observations that fell below the threshold; therefore, freezing was only scored if the animal was immobile for at least 1 s (Maren, 1998). The threshold output of freezing was verified by comparing with results from video scoring of an animal's behavior. The amount of time spent actively exploring the chamber, self-grooming, and rearing were quantified by calculating the number of load cell values above appropriate thresholds for each measure. These thresholds were determined by comparing load cell output with an observer's ratings of these behaviors.

In order to assess the effects of different forms of estrogen on contextual fear conditioning as well as on cued fear conditioning, animals were exposed to both a conditioning context (Context A, for context-associated fear) and a novel environment (Context B, for cue-associated fear). Context A consisted of a standard operant chamber as described above with an illuminated houselight, bare aluminum and Plexiglass walls, and a "strawberry" car air freshener used as an odor cue. Context B was a standard operant chamber that was illuminated by the two stimulus lights, had stripped and dotted inserts covering the Plexiglass walls, the stainless steel rods on the floor were covered by a smooth, white plastic insert, and a "vanilla" air freshener

provided an odor cue. Each animal was exposed to both Context A and Context B as described below.

4.2.2.3 Drug treatment

Approximately 1 week after ovariectomy, rats were randomly assigned to one of 10 treatment groups (n = 7-9 per group) and received a single s.c. injection of either vehicle (Control, 0.10 ml sesame oil), 17β-estradiol, estrone, or 17α-estradiol. The estrogens were given at one of three doses: low (0.30 μg/0.10 ml sesame oil), middle (1 μg/0.10 ml sesame oil), or high (10 μg/0.10 ml sesame oil). These doses were chosen based on earlier studies investigating spatial learning and hippocampal neurogenesis (Barha, et al., 2009b,Holmes, et al., 2002,Ormerod, et al., 2003,Tanapat, et al., 2005,Tanapat, et al., 1999). A high dose of 10 μg 17β-estradiol was chosen because it results in circulating levels of estradiol observed on the morning of proestrus (Viau and Meaney, 1991) and has been shown to enhance cell proliferation 30 minutes (Barha, et al., 2009b), 2 h (Tanapat, et al., 2005,Tanapat, et al., 1999), and 4 h (Barha, et al., 2009b,Ormerod, et al., 2003) after injection. However, this same dose produces superphysiological levels of estradiol shortly after administration (Woolley and McEwen, 1993). We chose a middle dose of 1 μg as previous studies have shown this dose of 17β-estradiol slightly increases cell proliferation (Tanapat, et al., 2005) and impairs working memory on the spatial working/reference memory version of the radial arm maze (Holmes, et al., 2002). On the other hand, a 0.3 μg dose of 17β-estradiol facilitates working memory (Holmes, et al., 2002), increases cell proliferation 30 minutes after injection (Barha, et al., 2009b), and results in circulating levels of estradiol found during diestrus (Viau and Meaney, 1991).

The doses chosen for 17β-estradiol were also used for 17α-estradiol and estrone allowing for direct comparison of effects between estrogens. It is well established that the low and middle doses of 17β-estradiol are physiological levels as reviewed above, but that the high

dose is pharmacological 30 minutes after administration (Woolley and McEwen, 1993). We found using radioimmunoassay that estrone levels range from 27.57 to 108.96 pg/mL across the estrous cycle. We have also established that the low, middle and high doses of estrone correspond roughly to 85.59, 108.82, and 516.32 pg/ml 30 minutes after administration. Thus, this indicates that the low and medium doses of estrone produce physiological levels, whereas the high dose is pharmacological. It is not at the moment possible to determine the levels of 17α-estradiol by a commercially available radioimmunoassay kit. However, using a different technique Toran-Allerand et al. (2005) have shown that 17α-estradiol is undetectable in serum, but is found in the brain of adult mice. We chose the same doses for 17α-estradiol and estrone to directly compare all three doses with all three estrogens. Furthermore, we have used these same doses of all three estrogens in an earlier paper examining the effects of these estrogens on cell proliferation in the dentate gyrus of adult female rats (Barha, et al., 2009b). All rats received a single s.c. injection of 0.10 ml of either hormone or vehicle. All injections were given between 8:30 and 9:00 a.m. and hormones were given 30 minutes before conditioning to assess the effects of hormone administration on memory acquisition 24 h later. Earlier studies have shown that these same estrogens can influence hippocampus structure and function within 30 minutes of exposure (Barha, et al., 2009b,Luine, et al., 2003,MacLusky, et al., 2005). Effort was made to ensure that groups did not differ in post-operative body weights because ovariectomy is known to lead to increased body weight.

Estrogens (Sigma-Aldrich Chemicals, Oakville, ON, Canada) were dissolved in sesame oil (Sigma-Aldrich Chemicals) over low heat to a concentration of 0.3, 1 or 10 μg of hormone per 0.10 ml oil. Estrogen solutions were stored in opaque containers at room temperature.

4.2.2.4 Behavioral Procedure

This experiment was conducted over 2 days, conditioning day and testing day. On day 1, the conditioning day, rats were injected with hormone or vehicle by one experimenter. Thirty minutes later, rats were transported to the testing room in their home cages on a four-wheeled cart by a different experimenter. Rats were then placed in the Context A operant chamber. Three minutes after placement into the chamber, rats were given three presentations of the tone CS (4kHz, 80dB, 30 seconds) each co-terminating with a footshock (2 seconds, 1.0mA) with 60 second intershock intervals. Sixty seconds after the third and final tone/shock pairing (after 3 minutes of exposure to Context A), rats were immediately returned to their home cage and colony room. To assess whether there were group differences in pain sensitivity, we recorded freezing behavior during the minute after each shock presentation. To assess contextual fear conditioning, 24 hours after conditioning rats were transported back to the testing room in their home cages on a four-wheeled cart by the same experimenter as the day before. The same procedure was followed as the conditioning day to activate the representation of the context. Rats were placed back into Context A for eight minutes and freezing behavior was assessed. After the eight minutes, rats were immediately returned to the colony room in their home cages. To assess cued fear conditioning, rats were transported approximately 1 hour later (Winocur, et al., 2006) to the testing room in a clean, unused, transparent cage (48 x 27 x 20 cm) by a different experimenter along a completely novel route without a cart. Rats were placed into the novel Context B and three minutes later were presented with three tones (4kHz, 80dB, 30 seconds) 60 seconds apart. A timeline of the experiment is presented in Table 4.1. Time spent freezing to the tone (cue) was measured during each tone presentation. In addition, time spent freezing for 3 minutes before the first tone presentation was also recorded as an index of

whether the groups differed in how much they recognized the new Context B as the old Context A.

Table 4.1: Experimental Procedure

		Conditioning		Testing: Contextual Fear Conditioning		Testing: Cued Fear Conditioning		
Hormone injection	→ ***30 mins later***	**Context A**: 3-mins exploration, 3 pairings of 30-sec tone and 2-sec 1.0mA shock	→ ***24 hrs later***	**Context A**: 8-mins in conditioning chamber (no tone or shock)	→ ***1 hr later***	**Context B**: 3-mins in novel chamber, 3 presentations of 30-sec tone (no shock)	→ ***4hr later***	Perfusion and extraction of brains for synaptophysin analysis

4.2.2.5 Immunohistochemistry

A random subset of rats (n=4) from each group were perfused 4 hours after exposure to Context B (cued fear conditioning) to assess levels of synaptophysin in the hippocampus. Briefly, rats were deeply anaesthetized with a lethal dose of sodium pentobarbital and then perfused with 4% paraformaldehyde. After extraction, brains were stored at 4 ºC in 4% paraformaldehyde for 24 h before being transferred to 30% sucrose for a minimum of 72 h. Brains were sliced into 30µm sections through the entire extent of the hippocampus in a bath of Tris-buffered saline (TBS) (pH 7.4) using a vibratome (Leica VT1000S; Leica Microsystems, Inc., Richmond Hill, ON, Canada). The sections were stored at -20 ºC in phosphate-buffered saline (PBS) antifreeze in sterile culture plates. Sections were washed 3x for 10 min each with TBS and then stored in sterile culture plates filled with TBS for the 24 h before immunohistochemistry processing.

All histological procedures were based on modification of previous work (Burton, et al., 2007). Between all steps free-floating sections were rinsed 3x for 10 min each in PBS (0.1 M

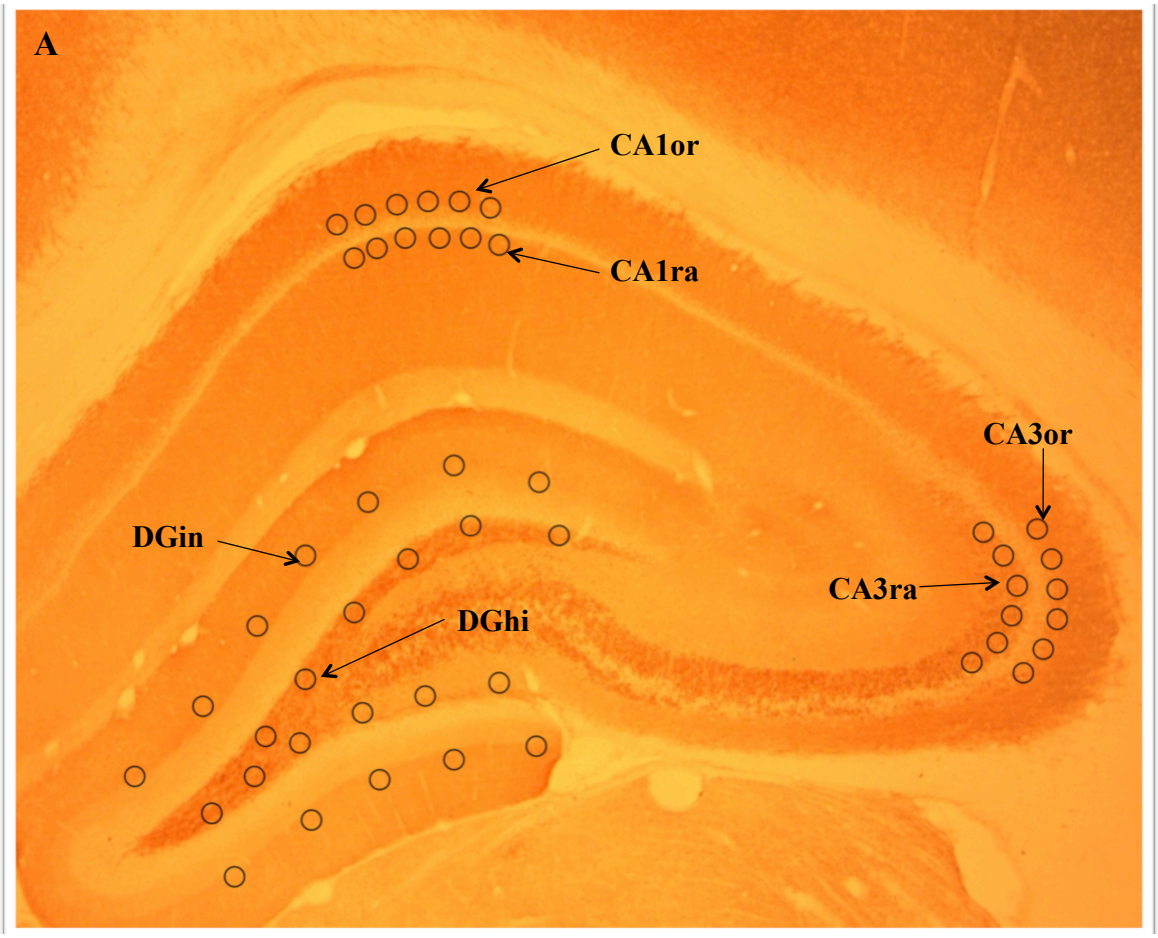
sodium phosphate buffer in 0.9% saline; pH 7.4) unless stated otherwise. Sections were blocked with 5% normal horse serum (NHS; Vector Laboratories, Burlington, ON, Canada) and 3% Triton-X (Boehringer Mannheim, Laval, QC, Canada) for 15 min and then incubated for 2 h in mouse monoclonal antibody against synaptophysin (1:200; Sigma-Aldrich Chemicals) at room temperature. Sections were then incubated in mouse secondary antisera (1:200; Vector Laboratories) for 2 h at room temperature. Sections were then incubated in avidin-biotin horseradish peroxidase complex (ABC Elite Kit; 1:50; Vector Laboratories) for 90 min. Sections were reacted in 0.01% diaminobenzidine (DAB; Sigma-Aldrich Chemicals) with 0.0003% H_2O_2 for approximately 5 min. The sections were mounted on super frost slides (Fisher Scientific, Edmonton, AB, Canada), dried overnight, dehydrated, and then coverslipped with Permount (Fisher Scientific).

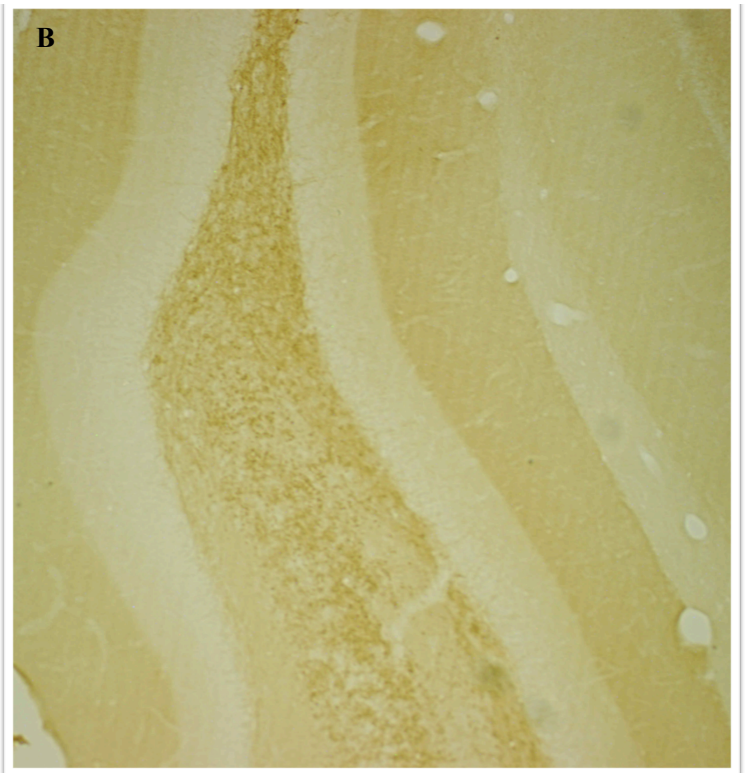
4.2.2.6 Quantitative Image Analysis

Quantitative densiometric assessment of synaptophysin protein was carried out at 40x magnification by using SimplePCI image analysis system Ver.5 (Compix, Cranberry Township PA, USA). The light intensity used was adjusted so that the range of gray level intensities for each slide was 0-255 (black to white). Gray levels were measured on the photomicrographs by placing open circles with diameters of 100μm along different regions of the hippocampus (see Figure 4.1a). We followed procedures of Gao et al. (2006). Specifically, optical density (OD) of synaptophysin was assessed in each of the hilus and inner molecular layer of the dentate gyrus (DGhilus and DGin), the stratum radiatum of the CA3 and CA1 (CA3ra and CA1ra), and the stratum oriens of the CA3 and CA1 (CA3or and CA1or). The background gray level for each section was obtained from the mean of eight circles placed within the corpus callosum to control for variations in staining and illumination. The mean gray levels for each hippocampal region of interest (ROI) were obtained by averaging the gray levels of all circles within that region. Then

the mean OD was obtained by subtracting each mean gray level from 255 (the maximum white intensity). Then the mean OD for each ROI was obtained by subtracting the mean reference OD from the corpus callosum from the mean OD for each ROI on each section (Gao, et al., 2006).

Figure 4.1: A) Representative photomicrograph of a dorsal hippocampus section showing the placement of open circles used for densiometric analysis. 12 circles each with diameter of 100 µm were placed along each of the DGin and DGhi, and 6 circles were placed along each of the CA3ra, CA3or, CA1ra, CA1or. All hippocampal sections were viewed at 40x magnification. B) Photomicrograph of dentate gyrus under 400X magnification contrasting the signal integrity of the synaptophysin expression in the hilus to what is seen in the granule cell layer.





4.2.3 Data Analyses

To assess the effect of hormone treatment on freezing and any differences between groups in pain sensitivity to the shock on conditioning day, the total amount of time in seconds spent freezing after each shock presentation was calculated and analyzed using a one-way ANOVA, with group (Control, 17β-estradiol-Low, 17β-estradiol-Middle, 17β-estradiol-High, Estrone-Low, Estrone-Middle, Estrone-High, 17α-estradiol-Low, 17α-estradiol-Middle, 17α-estradiol-High) as the between-subjects factor. Total amount of time in seconds spent freezing in Context A (contextual fear conditioning) and Context B (cued fear conditioning) on testing day were each analyzed using a one-way ANOVA, with group as the between-subjects factor. Total amount of time spent freezing before (to assess recognition of the different context) and during the three tone presentations in Context B (cued fear conditioning) on testing day were each analyzed using a one-way ANOVA, with group as the between-subjects factor. Owing to

equipment malfunction, data from one control animal was not included in the analysis of cued fear conditioning. Differences in OD of synaptophysin expression were analyzed using repeated-measures ANOVA, with group as the between-subjects factor and region (DGhilus, DGin, CA3ra, CA3or, CA1ra, and CA1or) as the within-subjects factor. Spearman's rank correlations were conducted between OD of synaptophysin expression in each region and total amount of time in seconds spent freezing during contextual fear conditioning testing in Context A. Data were further analyzed using the Newman-Keuls post-hoc test. All statistical procedures were set at $\alpha = 0.05$ unless otherwise stated.

4.3 RESULTS

4.3.1 Treatment with estrogens did not influence sensorimotor activity of rats during conditioning.

The amount of time spent in locomotion, grooming, and rearing during conditioning 30 minutes after hormone administration are shown in Table 4.2. Groups did not differ in the amount of time spent in motion [$F(9,64) = 0.77$; $p < 0.70$] or in the amount of time spent grooming [$F(9,64) = 1.22$; $p < 0.30$]. Groups did differ in the amount of time spent rearing [$F(9,64) = 4.85$; $p < 0.0001$]. Post-hoc tests indicate that only the high dose of estrone decreased the time spent rearing compared to control ($p < 0.05$).

Table 4.2: Total (±SEM) amount of time in seconds spent in locomotion, grooming, and rearing on the conditioning day in ovariectomized female rats.

Group	Locomotion (sec)	Grooming (sec)	Rearing (sec)
Control	152.04 ± 12.08	41.51 ± 6.53	157.76 ± 6.32
17β-Estradiol			
Low	163.54 ± 17.46	54.57 ± 9.26	150.54 ± 19.54
Medium	151.97 ± 14.54	37.49 ± 6.08	192.63 ± 10.98
High	181.63 ± 14.94	44.40 ± 5.81	115.06 ± 20.97
Estrone			
Low	170.00 ± 11.85	40.17 ± 6.75	144.89 ± 32.66
Medium	162.74 ± 7.23	39.17 ± 11.65	200.46 ± 13.84
High	171.31 ± 12.93	62.20 ± 7.90	80.14 ± 10.06*
17α-Estradiol			
Low	161.11 ± 14.51	47.23 ± 6.80	118.51 ± 27.66
Medium	159.13 ± 12.00	41.03 ± 2.56	181.58 ± 10.58
High	185.78 ± 12.72	50.78 ± 4.38	104.55 ± 15.64

Groups did not significantly differ in locomotion or grooming. Rats administered high dose of estrone spent less time rearing than control rats. *$p < .05$ versus control.

4.3.2 All groups exhibit immediate postshock freezing and estrone tended to alter the amount of postshock freezing during conditioning.

The percentage of total time spent freezing during the three postshock minutes during conditioning for all groups is shown in Table 4.3. The results indicate that all groups showed immediate freezing after presentation of the shock and that groups tended to differ in the amount of time spent freezing after presentation of the shock [$F(9,71) = 1.86$, $p = 0.07$]. A closer look at Table 4.3 suggests that a lower percentage of freezing after the shocks is seen in the group given a middle dose of estrone compared to controls (15.97% vs. 36.81%) and a higher percentage of freezing after the shocks is seen in the group give a high dose of estrone compared to controls

(53.53% vs. 36.81%). Therefore, because groups tended to differ in initial response to shock, we used this factor as a covariate in all subsequent analyses.

Table 4.3: Total (±SEM) percentage of freezing during the three postshock minutes on the conditioning day in ovariectomized female rats.

Group	Total Percentage
Control	36.81 ± 7.41
17β-Estradiol	
Low	29.53 ± 5.54
Medium	25.11 ± 7.99
High	36.90 ± 8.52
Estrone	
Low	31.64 ± 10.42
Medium	15.97 ± 5.34
High	53.53 ± 4.49
17α-Estradiol	
Low	39.94 ± 9.40
Medium	24.89 ± 5.50
High	39.42 ± 6.47

Groups exhibit immediate postshock freezing and did not significantly differ in postshock freezing during conditioning.

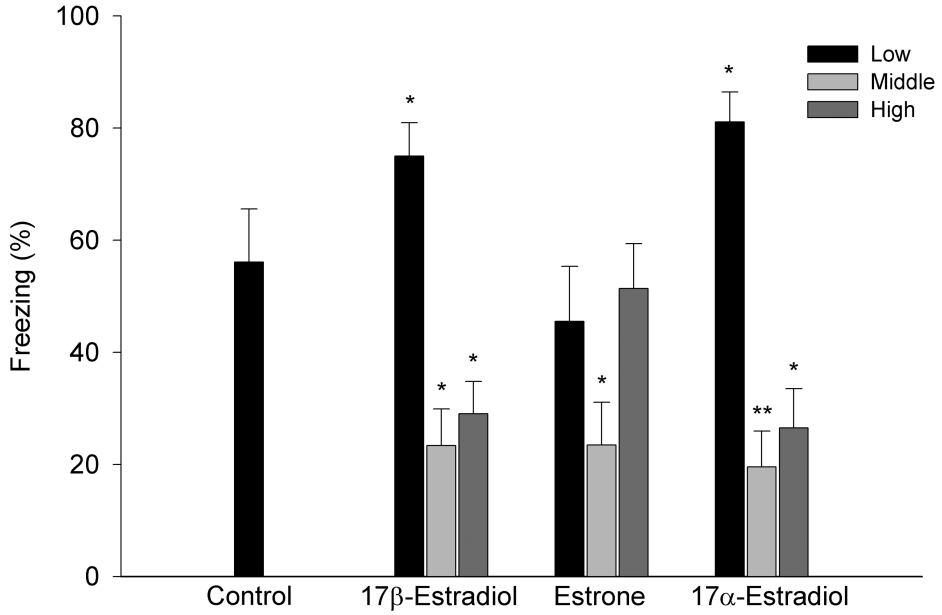
4.3.3 Low physiological doses of 17β-estradiol and 17α-estradiol increase, whereas high pharmacological doses decrease, contextual fear conditioning and the middle dose of all three estrogens dramatically decreases contextual fear conditioning.

The percentage of time spent freezing during the contextual fear conditioning test for all groups is shown in Figure 4.2. Groups tended to differ in the amount of time spent freezing after presentation of the shock on the conditioning day; therefore, an analysis of covariance was conducted on the percentage of total time spent freezing across the eight minutes of contextual

fear conditioning with total time spent freezing during the conditioning day as a covariate. The analysis yielded a significant main effect of the covariate [$F(1,70) = 9.28$, $p < 0.01$], and a main effect of Group [$F(9,70) = 8.61$, $p < 0.0001$]. Post-hoc tests revealed that high doses of 17β-estradiol and 17α-estradiol decreased the percentage of time spent freezing ($p < 0.04$ and $p < 0.03$, respectively), and middle doses of 17β-estradiol, estrone and 17α-estradiol decreased the percentage of time spent freezing compared to control (all p's < 0.03). In contrast, the low dose of 17α-estradiol increased the percentage of time spent freezing compared to control ($p < 0.04$). On the basis of earlier studies (Holmes, et al., 2002), we expected to find an enhancement in contextual fear conditioning with the low dose of 17β-estradiol; therefore, an *a priori* comparison was conducted and the low dose of 17β-estradiol was found to increase the percentage of time spent freezing compared to control ($p < 0.05$).

Figure 4.2: A) Total (+SEM) percentage of freezing during the eight minute contextual fear conditioning test (Context A) in ovariectomized female rats when tested 24 hours after conditioning. Rats given a low dose (0.3 μg) of 17β-estradiol and 17α-estradiol had higher levels of freezing compared to controls (both p's < .05). Rats given a middle dose (1.0 μg) of 17β-estradiol, estrone, and 17α-estradiol and a high dose (10 μg) of 17β-estradiol and 17α-estradiol had lower levels of freezing compared to controls (all p's < .05). B) Total (+SEM) percentage of freezing during the presentation of the three tones during the cued fear conditioning test (Context B) in ovariectomized female rats when tested one hour after contextual fear conditioning test. Groups did not differ in freezing to the tone during the cued fear conditioning test compared to controls. Asterisks indicate *p < 0.05 versus control; **p < 0.01 versus control.

A


100
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Freezing (%)
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**
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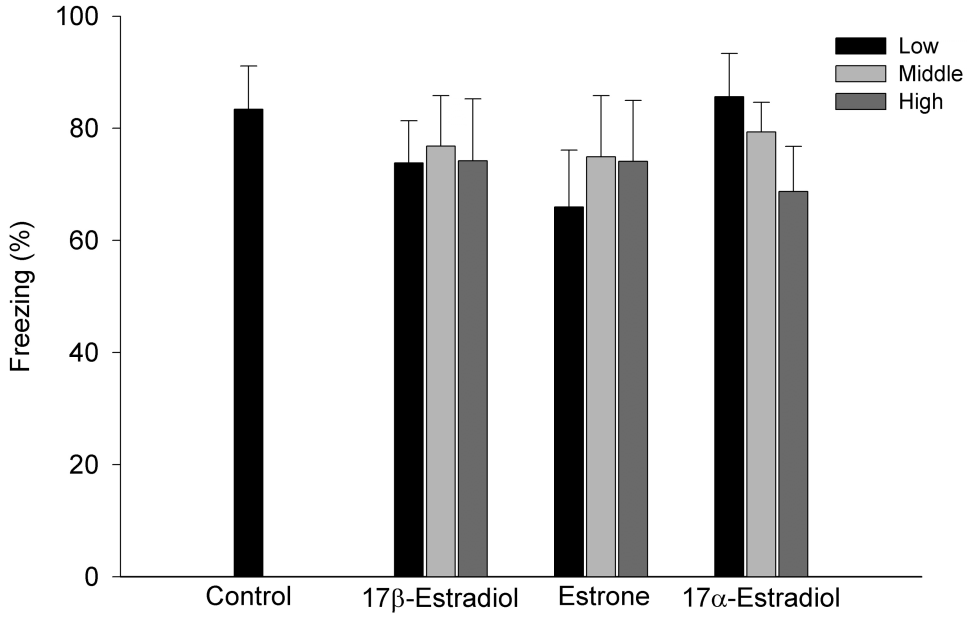
Control
17β-Estradiol
Estrone
17α-Estradiol

B


100
80
60
40
20
0
Freezing (%)
Low
Middle
High
Control
17β-Estradiol
Estrone
17α-Estradiol

4.3.4 Different doses of different estrogens do not influence cued fear conditioning.

To determine whether groups differed on their recognition of Context B as being distinct from Context A, we measured freezing in the first three minutes of the cued fear conditioning test, before the tone presentation. Importantly, groups did not differ in the percentage of freezing at baseline, the first three minutes of the cued fear conditioning test [$F(9,70) = 0.87, p = 0.56$; see Table 4.4], indicating that all groups viewed Context B as being distinct from Context A. The percentage of time spent freezing during the presentation of the three tones during the cued fear conditioning test for all groups is shown in Figure 4.2. An analysis of covariance was conducted on the percentage of total time spent freezing during the presentation of tones during the cued fear conditioning test with time spent freezing on the conditioning day as a covariate. A main effect of the covariate was not found [$F(1,69) = 3.50$, $p = 0.07$] nor was a main effect of Group found [$F(9,69) = 0.57$, $p = 0.82$], indicating that groups did not differ in the amount of time spent freezing during the presentation of the tones during the cued fear conditioning test in Context B.

Table 4.4: Total (±SEM) percentage of freezing during the three minute baseline during the cued fear conditioning test (Context B) in ovariectomized female rats.

Group	Total Percentage
Control	27.43 ± 7.05
17β-Estradiol	
Low	13.46 ± 4.83
Medium	26.52 ± 7.82
High	15.86 ± 6.20
Estrone	
Low	20.49 ± 10.81
Medium	20.86 ± 6.69
High	21.08 ± 5.76
17α-Estradiol	
Low	31.44 ± 9.96
Medium	25.89 ± 7.39
High	13.06 ± 6.16

Groups do not significantly differ in freezing durations during baseline indicating that Context B was viewed as a novel environment.

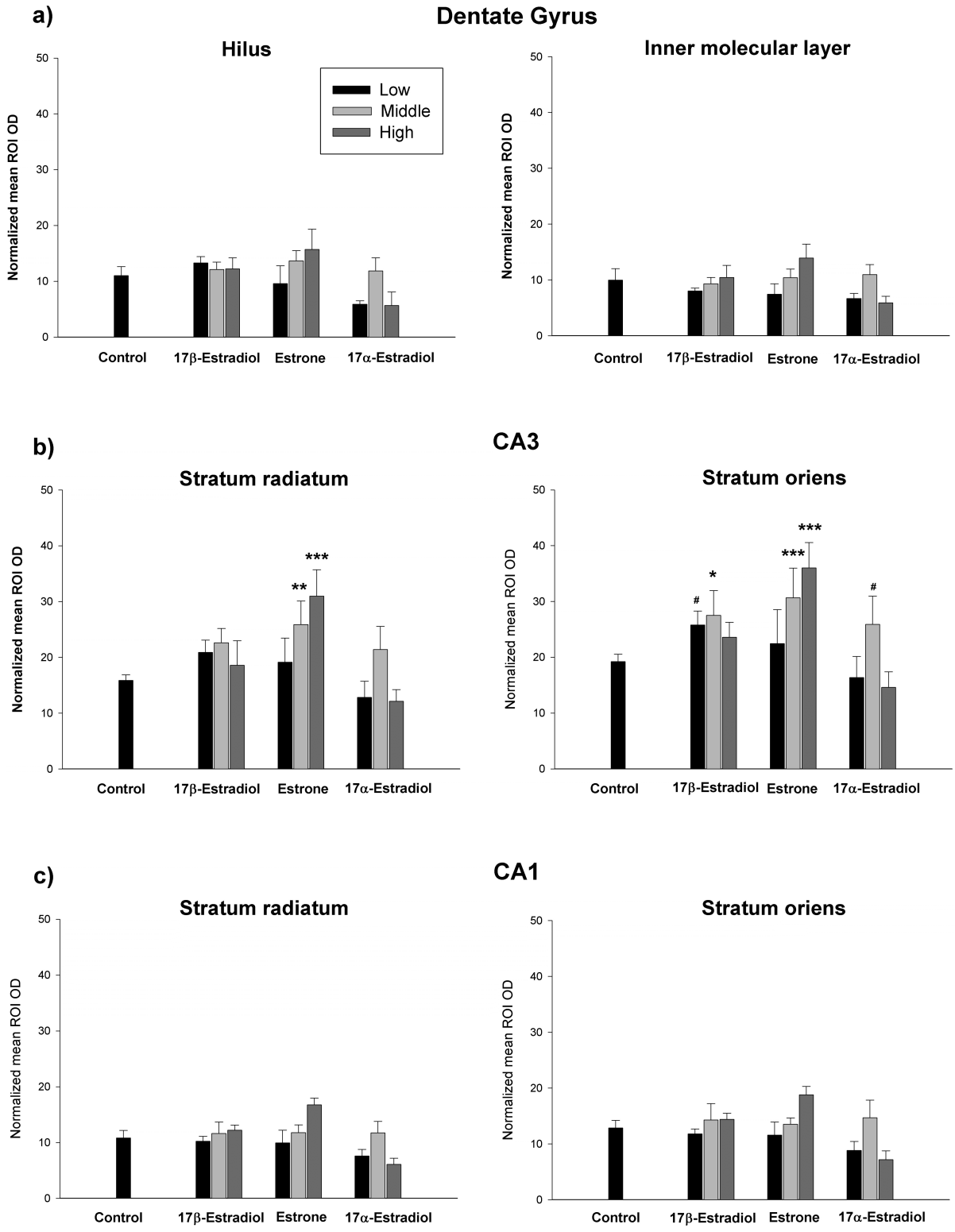
4.3.5 The middle and high dose of estrone and the middle dose of 17β-estradiol increase synaptophysin in only the CA3 region of the hippocampus.

Intense and consistent staining for synaptophysin throughout the CA1, CA3, and DG was observed in all groups with very low levels of staining observed in the pyramidal cell layers or the granule cell layer as expected (see Figure 4.1 a and b). As section thickness can influence OD, we also determined section thickness across the groups. Section thickness did not differ between groups, with thickness ranging between 11.50 and 14.00 μm [$F(9, 32) = 1.10$, $p < 0.40$; data not shown]. Repeated-measures ANOVA conducted on OD of synaptophysin expression across treatment groups found a significant interaction between group and region [$F(45,145) = 1.57$, $p < 0.03$; see Figure 4.3]. Further analyses showed that in the CA3 striatum oriens region,

the high dose of estrone and the middle dose of 17β-estradiol and estrone increased synaptophysin expression compared to control (all p's < 0.02). The low dose of 17β-estradiol tended to increase synaptophysin expression in this same area (p < 0.08). Only the high and middle doses of estrone increased synaptophysin levels in the CA3 striatum radiatum region (both p's < 0.002). Estrogens did not statistically influence synaptophysin expression in the dentate gyrus (all p's > 0.77) or the CA1 (all p's > 0.44) compared to control.

No significant correlations were found between synaptophysin expression in the DGhilus, DGin, CA3ra, CA3or, CA1ra, or CA1or regions and total amount of time in seconds spent freezing during the contextual fear conditioning test (all p's > 0.13).

Figure 4.3: Average normalized optical density for each region of interest in ovariectomized female rats approximately 30 hours after hormone treatment and 5 hours after contextual fear conditioning test. Rats given a high dose of estrone and a middle dose of 17β-estradiol and estrone had increased synaptophysin expression in the CA3 striatum oriens region (p < 0.0001, p < 0.02, and p < 0.0001, respectively). Rats given a low dose of 17β-estradiol and a middle dose of 17α-estradiol tended to have higher synaptophysin expression in the CA3 striatum oriens (p = 0.08 and p = 0.11, respectively). Rats given a high and a middle dose of estrone had higher synaptophysin expression in the CA3 striatum radiatum (p < 0.0001 and p < 0.002, respectively). Asterisks indicate *p < .05 versus control; **p < .01 versus control; ***p < .0001 versus control. # indicates tendency p < .11.


a)
Dentate Gyrus
Hilus
Inner molecular layer
Low
Middle
High
Normalized mean ROI OD
Control
17β-Estradiol
Estrone
17α-Estradiol
b)
CA3
Stratum radiatum
Stratum oriens
c)
CA1
Stratum radiatum
Stratum oriens

4.4 DISCUSSION

The results from this study demonstrate that different forms of estrogen dose dependently influence the acquisition of hippocampus-dependent contextual fear conditioning in adult, ovariectomized female rats. Specifically, a low dose of 17β-estradiol and 17α-estradiol enhanced contextual fear conditioning, whereas middle and high doses of these estrogens impaired contextual fear conditioning. In contrast, estrone impaired contextual fear conditioning at a middle dose (resulting in high physiological levels), whereas a low and high dose of estrone did not significantly influence contextual fear conditioning. Although these estrogens affected hippocampus-dependent contextual fear conditioning, they did not affect hippocampus-independent cued fear conditioning, indicating that the effects of 17β-estradiol, estrone, and 17α-estradiol on these tasks are limited to those involving the hippocampus. Interestingly, 17β-estradiol (middle dose) and estrone (middle and high doses) increased synaptophysin levels in the CA3 region of the hippocampus, and were not correlated with the behavioral effects, suggesting synaptophysin expression was disassociated from learning and memory performance in this task.

4.4.1 Different forms of estradiol influence hippocampus-dependent contextual fear conditioning in a dose-dependent manner

Administration of a low 0.3 μg dose of 17β-estradiol and 17α-estradiol 30 minutes before training enhanced contextual fear conditioning performance 24 hours after hormone injection, whereas administration of a high 10 μg dose of 17β-estradiol and 17α-estradiol impaired contextual fear conditioning. These findings are consistent with and further extend earlier work showing that high physiological and pharmacological levels of exogenous or endogenous estradiol impair contextual fear conditioning after administration of 10 μg dose of estradiol benzoate, a conjugated form of 17β-estradiol (Gupta, et al., 2001) and during proestrus

(Markus and Zecevic, 1997). This study is the first demonstration that a low physiological dose of estradiol (17α and β) facilitates contextual fear conditioning. In addition, we found that a middle (1.0 μg) dose of 17β-estradiol, estrone, and 17α-estradiol impairs contextual fear conditioning compared to ovariectomized controls. This 1.0 μg dose has been shown to produce physiological levels of estradiol and estrone similar to levels seen during the range of proestrus (Holmes, et al., 2002,Shaikh, 1971; present study). To our knowledge, the effects of the low and middle doses of these estrogens and the high dose of estrone and 17α-estradiol on fear conditioning have not previously been investigated.

The pattern of effects seen in the present study with the different doses of 17β-estradiol support findings using other hippocampus-dependent cognitive tasks (Galea, et al., 2001,Holmes, et al., 2002). Specifically, acute treatment with the low physiological dose of 17β-estradiol increases, whereas the middle physiological and high pharmacological doses disrupt working memory and reference memory performance, respectively, on the radial arm maze (Galea, et al., 2001,Holmes, et al., 2002). Furthermore, pharmacological levels of estradiol benzoate resulting from a 40 μg dose (but not 10 μg and 20 μg) enhance associative learning in trace eye-blink conditioning, in which performance depends on the hippocampus and cerebellum (Leuner, et al., 2004b). These same types of curvilinear relationships of hormonal effects are not unprecedented in the literature. For example, very low and very high physiological levels of adrenal steroids are associated with increased cell death in the hippocampus, whereas intermediate levels are associated with decreased cell death (Joels, 2007). In addition, dopamine activation of the D1 receptor in the prefrontal cortex mediates working memory performance with an inverted U-shape (Floresco and Magyar, 2006). Furthermore, physiologically low and pharmacologically high levels of 17β-estradiol and estrone increase hippocampal cell proliferation, while medium levels do not have an effect

(Barha, et al., 2009b). Overall, low levels of 17β-estradiol enhance acquisition of different types of hippocampus-dependent cognitive tasks, and may have less consistent effects on recall (for review see Luine, 2008).

The effects of 17α-estradiol on learning and memory have not been afforded as much attention in the literature as the more potent optical isomer 17β-estradiol. One study reported that 17α-estradiol rapidly enhances acquisition of spatial memory in a similar manner as 17β-estradiol (Luine, et al., 2003), similar to findings in the present study. Interestingly 17α-estradiol is a more potent inducer of CA1 pyramidal spine synapse density than is 17β-estradiol (MacLusky, et al., 2005) and the greatest increase in spine density was seen 30 minutes after hormone injection (MacLusky, et al., 2005) along the same timeline used in the present study. Although 17α-estradiol was once considered biologically and functionally inactive (Toran-Allerand, et al., 2005), it rapidly influences hippocampus-dependent learning (Luine, et al., 2003; present study), upregulates hippocampal cell proliferation (Barha, et al., 2009b) and spine density (MacLusky, et al., 2005) in a dose-dependent manner. Together, these findings indicate that 17α-estradiol is biologically active and may be a potent and viable alternative for hormone replacement therapy.

4.4.2 Estrone impaired contextual fear conditioning

Although different doses of 17α-estradiol and 17β-estradiol both facilitated and impaired contextual fear conditioning, estrone did not facilitate contextual fear conditioning at any dose and dramatically impaired contextual fear conditioning at the middle dose. This is in agreement with conclusions reached in a meta-analysis conducted by Ryan et al. (2008), indicating that hormone replacement therapies consisting primarily of estrone do not have a beneficial effect on cognition in post-menopausal women and may even enhance risk for dementia, whereas therapies using 17β-estradiol are more consistently associated with improved cognition. In the

present study, the low physiological and high pharmacological doses of estrone did not influence contextual fear conditioning. This may be partly related to the lower affinity estrone has for the estrogen receptors, potentially rendering this estrogen less functionally active. Although estrone can be converted to 17β-estradiol through the enzyme 17β-hydroxysteroid dehydrogenases, we do not believe this was the mechanism through which estrone influenced contextual fear conditioning in the current study for three important reasons: (1) the oxidative pathway converting 17β-estradiol into estrone is favored over the reverse reaction (estrone into 17β-estradiol) in rodent tissue (Martel, et al., 1992); (2) the effects of estrone on behavior in this study occur very rapidly (within 30 minutes); and (3) the pattern of results is different for estrone and 17β-estradiol. The results from the present study are not in complete agreement with those of Farr *et al*. (2000), who found that direct infusion of estrone into the hippocampus enhanced footshock avoidance learning in a similar manner as 17β-estradiol in ovariectomized mice. However, this may be due to different modes of hormone administration, as well as due to the different type of task used. Furthermore, the results from the present study are also not in complete agreement with a recent study that found that chronic cyclic (2 days on, 2 days off) treatment with Premarin enhanced spatial working memory and spatial reference memory in long-term ovariectomized middle-aged female rats (Acosta, et al., 2009). However, many discrepancies between these two studies exist. For example, different strains of rat, different ages, and different post-ovariectomy times were used in these studies and all of these factors can profoundly influence the ability of estrogens to modulate neuroplasticity in the hippocampus (Barha, et al., 2009b,Miranda, et al., 1999,Tanapat, et al., 2005,Tanapat, et al., 1999). Furthermore, although Premarin largely consists of estrone, it also contains other forms of estrogen including 17β-estradiol and 17α-estradiol. In the present study we gave an acute injection of purified estrone thus making direct comparison between these studies problematic.

Our results in combination with other studies indicate that therapies using certain doses of estrone may not have the same cognitive-enhancing benefits as other forms of estrogen. Our results further emphasize the need for research conducted in both animals and humans focusing on the effects of different doses of estrone and other estrogens on cognition across the lifespan.

4.4.3 Different estrogens did not influence amygdala-dependent cued fear conditioning

Exposure to different forms of estrogens at different doses did not significantly affect hippocampus-independent cued fear conditioning, consistent with past literature examining behavior across the estrous cycle (Markus and Zecevic, 1997). Different Pavlovian fear conditioning paradigms depend on different brain structures and systems. In particular, cued fear conditioning with a tone does not require an intact hippocampus, whereas contextual fear conditioning does (Kim and Fanselow, 1992,Phillips and LeDoux, 1992). The amygdala is important for both contextual and cued based fear learning (Phillips and LeDoux, 1992), with both the lateral and central nuclei of the amygdala implicated in cued fear conditioning (Maren, 2008). Therefore, it is perhaps not surprising that exposure to estrogens, which influence the hippocampus also influence contextual fear conditioning, but not cued fear conditioning. However, in contrast with our results, Galea et al. (2001) found that pharmacologically high levels of estradiol disrupted acquisition of the conditioned place-preference task, an associative learning task in which performance is reliant on the integrity of the basolateral amygdala. There are a number of differences between these two tasks that may account for these discrepant findings (Galea, et al., 2001; this study), such as the different types of motivation underlying each task, aversive versus appetitive, and the fact that performance on each task relies to different extents on different nuclei of the amygdala. It may be that aversive learning is more natural and ethologically valid and, therefore, naturally occurring hormones, such as estrogen, would be expected to have a greater influence on tasks that involve this type of motivation.

There is evidence that the different nuclei of the amygdala can respond differentially to estradiol (Osterlund, et al., 1998,Schiess, et al., 1988,Womble, et al., 2002), which could contribute to discrepancies between studies. For example, different nuclei of the amygdala differentially contain ER subtypes (ERα and ERβ; Osterlund, et al., 1998) and estradiol reduces excitatory postsynaptic potential (EPSP) in the basolateral amygdala, but increases EPSP occurrence in the medial nucleus of the amygdala (Schiess, et al., 1988,Womble, et al., 2002). Furthermore, infusion of estradiol into the medial amygdala has antianxiety and antidepressant effects in ovariectomized female rats (Frye and Walf, 2004). Therefore, it may be that estradiol has different effects on amygdala-based learning depending on the nuclei being recruited by the task and/or the different tasks being performed.

4.4.4 Estrone and estradiol increase synaptophysin expression in the CA3 region of the dorsal hippocampus

Synaptophysin is a presynaptic protein that is a critical component of synaptic vesicle exocytosis and has been identified as a molecular correlate of learning and memory. In the current study increases in synaptophysin levels were only seen in the CA3 region of the hippocampus after administration of the middle and high doses of estrone and the middle dose of 17β-estradiol compared to controls. Our results are consistent with a study showing that 8 days of treatment with 17β-estradiol increased synaptophysin expression in the CA3 region of the hippocampus *in vitro* (Rune, et al., 2002). In contrast, short-term (2 days) treatment with 10 μg estradiol benzoate led to increases in synaptophysin expression in only the CA1 region of the dorsal hippocampus in ovariectomized female rats (Brake, et al., 2001). Estradiol benzoate is a conjugated form of 17β-estradiol and is metabolized at a much slower rate. Therefore, discrepancies between our results and those found by Brake et al. (2001) could reflect a number of differences between the studies (different estrogens, timing, etc.). Interestingly, long-term (4

weeks) treatment with 17β-estradiol increased the ovariectomy-induced reduction in synaptophysin expression in the CA1 region (no other region was examined) of the whole hippocampus in ovariectomized female rats(Sharma, et al., 2007), indicating that longer-term treatment with estradiol may affect other regions of the hippocampus.

In this study estrogens increased synaptophysin expression only in the CA3 region of the hippocampus. The CA3 region of the dorsal hippocampus is preferentially associated with acquisition, whereas the CA1 region is associated with retrieval of contextual fear memory (Lee and Kesner, 2004). Furthermore, studies suggest that it is the NMDA receptors in the CA3 region, particularly in the dorsal hippocampus, that are involved in the rapid, one-trial encoding of complex associations between contextual stimuli (Cravens, et al., 2006,Rajji, et al., 2006).

The changes in synaptophysin levels seen in the current study do not consistently reflect behavioral changes, as the middle dose of 17β-estradiol and estrone impaired contextual fear conditioning and the high dose of estrone did not affect contextual fear conditioning, but all three treatments increased synaptophysin levels in the CA3 region. Furthermore, we did not find any significant correlations between synaptophysin levels and amount of freezing. This is in contrast with one study, which found that increases in synaptophysin expression with chronic 17β-estradiol were associated with enhanced spatial reference memory in older mice (Frick, et al., 2002). In the present study synaptophysin expression was assessed 4 hours after testing and 30 hours after acute hormone treatment. Thus, it is possible that in this study a consistent association between contextual fear conditioning performance and synaptophysin expression in the hippocampus may be found if other time points were examined. Furthermore, it has been previously shown that training in a hippocampus-dependent task interferes with the ability of estradiol to increase spine density in the dorsal hippocampus (Frick, et al., 2004). Therefore, our

results could also be reflecting an interference of training on estrogen's ability to enhance synaptophysin.

4.5 CONCLUSIONS

The present study demonstrates that acquisition of hippocampus-dependent contextual fear conditioning is influenced by 17β-estradiol, estrone, and 17α-estradiol in a dose-dependent manner, with a low dose of 17β-estradiol and 17α-estradiol enhancing and a middle or high dose of either form of estradiol impairing contextual fear conditioning. Interestingly, impairments in contextual fear conditioning were seen with a middle dose of estrone. These results are particularly important in lieu of recent findings that estrone-based hormone replacement therapies are ineffective in preventing cognitive decline in post-menopausal women. Our results emphasize the need for further systematic research into the effects of different doses of estrone in both human and animal models of menopause. Furthermore, we found that estrone and 17β-estradiol, but not 17α-estradiol, upregulated synaptophysin expression in the CA3 region of the hippocampus, which was uncoupled from the behavioral findings. Importantly, we have shown that a low physiological dose of both 17β-estradiol and 17α-estradiol can enhance hippocampus-dependent learning and memory. This could have therapeutic importance, as it may be possible to use lower doses of estrogens in hormone replacement therapies. Our results also suggest that 17α-estradiol is as potent as 17β-estradiol in influencing cognition. Therefore, future research is required to determine whether it may be possible to use 17α-estradiol, as well as lower doses, in hormone replacement therapies potentially circumventing some of the adverse effects associated with current therapies.

5 MOTHERHOOD ALTERS THE CELLULAR RESPONSE TO ESTROGENS IN THE HIPPOCAMPUS LATER IN LIFE[4]

5.1 INTRODUCTION

The female brain is highly plastic, with numerous factors influencing this plasticity throughout the lifespan. One such important factor is reproductive experience or motherhood. Although virgin female rats are neophobic to rat pups after gestation and parturition, female rats display maternal behaviors that permanently alter the response to pups. This maternal memory is rapid, permanent, and results in lasting altered neural responses to pups and hormone levels (Kinsley, et al., 2008). Motherhood is also associated with numerous profound functional and structural alterations in the hippocampus, including enhanced spatial memory after weaning, reduced dendritic arborization in the CA1 and CA3 regions after weaning, and a reduction in hippocampal neurogenesis shortly after parturition (Kinsley, et al., 2008,Pawluski, et al., 2009). Many of these alterations seen with reproductive experience are long-lasting with enhancements in spatial memory persisting into old age (Gatewood, et al., 2005).

Reproductive senescence and aging in women have long been associated with cognitive decline, with a higher prevalence of dementia seen in elderly women than in elderly men (Baum, 2005). Disease progress and manifestation may be partly due to reduced levels of estrogen that accompany the postmenopausal period in women (Baum, 2005). Estrogen can have a positive influence on the function and structure of the brain, and in particular the hippocampus, and as such hormone replacement therapy (HRT) has been implicated as a

[4] A version of this chapter has been published. Barha, C.K. and Galea, L.A. (2011) Motherhood alters the cellular response to estrogens in the hippocampus later in life. Neurobiology of Aging. 32(11): 2091-2095.

possible therapeutic agent for ameliorating age-related cognitive decline in postmenopausal women. HRT is controversial as a therapeutic agent due to the failure of a large-scale NIH study to find a positive effect on cognition and/or dementia (Shumaker, et al., 2003). However, a recent meta-analysis indicates that the cognitive-enhancing ability of HRT is dependent on age of subject and the type of estrogen in the HRT (Hogervorst, et al., 2000,Ryan, et al., 2008). Estrogens are comprised of estrone, estradiol and estriol, and Premarin, the HRT used in the large-scale NIH study, is comprised of mainly estrone. Different types of estrogen differentially alter hippocampus-dependent learning and memory and neurogenesis in rodents (Barha, et al., 2010,Barha, et al., 2009b,Galea, et al., 2008).

Adult neurogenesis exists in the dentate gyrus of the hippocampus in most mammalian species studied including humans. Hippocampal neurogenesis is required for some hippocampus-dependent learning and memory tasks (Winocur, et al., 2006). Furthermore, different types of estrogen influence both hippocampal neurogenesis and hippocampus-dependent learning and memory in adult female rodents (Galea, et al., 2008). A dramatic reduction in hippocampal neurogenesis is seen with aging in rodents (Kuhn, et al., 1996), and may be related to age-dependent impairments in hippocampus-dependent memory (Drapeau, et al., 2003). Although estradiol increases hippocampal cell proliferation in young female rats, it does not significantly influence hippocampal cell proliferation in reproductively senescent virgin female rats (Chiba, et al., 2007). However, high levels of life-time estrogen exposure have been linked to decreased age-associated cognitive decline (Ryan, et al., 2009), therefore it is possible that exposure to high levels of estrogen seen during pregnancy may modulate the ability of the dentate gyrus to respond to estrogen therapy later in life. Therefore in the present study we examined whether different types of estrogens (17β-estradiol, estrone, and 17α-estradiol) could influence hippocampal cell proliferation in middle-aged female rats and whether

this was dependent on previous reproductive experience. We found that previous reproductive experience altered the ability of the hippocampus to upregulate cell proliferation in middle-aged female rats.

5.2 MATERIALS AND METHODS

Fifty-one 13 to 14 month old middle-aged female Sprague-Dawley rats were used in this study. Multiparous retired breeder rats (n = 23) were purchased from Harlan Laboratories (Indianapolis) and virgin rats (n = 28) were purchased from Charles River (Quebec). Transport time was approximately 24 hours for both groups. All experiments were conducted in accordance with the ethical guidelines set by the Canada Council for Animal Care and were approved by the University of British Columbia Animal Care Committee. All efforts were made to reduce the number and the suffering of animals. Approximately 2 months after arrival, 41 females were bilaterally ovariectomized as previously described (Barha, et al., 2009b); the remaining females were sham-operated. 1 week after ovariectomy multiparous and virgin females were assigned to 1 of 4 treatment groups (n = 4-6 per group) and received a single subcutaneous injection of either: vehicle, or 10 μg dose of 17β-estradiol, estrone, or 17α-estradiol. This dose results in pharmacological levels of estradiol and estrone in adult female rats (Barha, et al., 2010) and is slightly lower than the dose of Premarin, a popular HRT that is typically prescribed to women.

All female rats (n= 51) received a single intraperitoneal injection of 5-bromo-2-deoxyuridine (BrdU; Sigma, St. Louis, MO) (200 mg/kg), 30 min after the hormone injection. BrdU is a thymidine analogue that incorporates itself into the DNA of cells during the synthesis phase of the cell cycle which will label proliferating cells and their progeny. All rats were

perfused with 4% paraformaldehyde 24 hours after BrdU administration in order to determine the number of newly divided cells after one complete mitotic division. All histological procedures are based on Barha et al. (2009). Briefly brains were sliced into 40 μm sections throughout the entire extent of the hippocampus using a vibratome before BrdU immunohistochemistry processing. To estimate cell numbers, total BrdU-immunoreactive (-ir) cells were counted under 100x objective on every 10th section (approx. 11-12 sections per rat). Cells were considered BrdU-ir if they exhibited intense staining and round or oval morphology (Barha, et al., 2009b) and were counted throughout the dentate gyrus separately for the granule cell layer (GCL), which included the subgranular zone (defined as approximately the 50 μm band between the GCL and the hilus), and the hilus. Total cell counts throughout the entire dentate gyrus were estimated by multiplying the number of BrdU-ir cells per animal by 10 (as cells were counted on every 10^{th} slice) in accordance with previous studies (Barha, et al., 2009b).

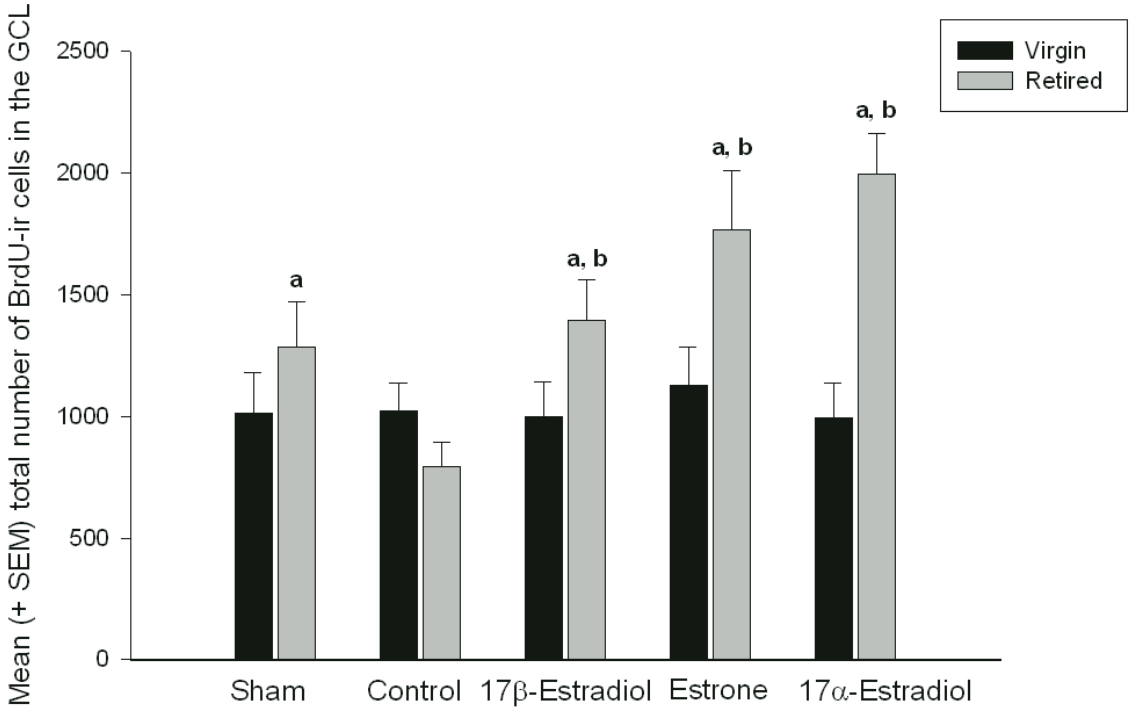
As noted above, multiparous and virgin females were obtained from two different vendors. Previous work has shown altered hypothalamic-pituitary-adrenal function in male Sprague-Dawley rats obtained from different vendors (Turnbull and Rivier, 1999). Therefore in order to address possible vendor effects adrenocorticotropic hormone (ACTH) and corticosterone (CORT) levels were assessed in the present study. Blood was taken at the time of perfusion from the chest cavity and cannot be considered true basal levels but resting levels of CORT and ACTH. Blood samples were stored overnight at 4°C and centrifuged at 10 x *g* for 15 min. Briefly all samples were run in duplicate using commercially available RIA kits for ACTH and rat corticosterone (MP Biomedicals; Solon, OH, USA). The average intra-assay coefficient of variation for all assays was below 10%. For ACTH the antibody cross-reacts 100% with human $ACTH_{1\text{-}39}$ and $ACTH_{1\text{-}24}$, but not with β-endorphin, β-MSH, or β-lipotropin (all < 0.8%).

The minimum detectable ACTH concentration was 5.7 pg/ml. For CORT the antiserum cross-reacts 100% with corticosterone, 0.34% with deoxycorticosterone, 0.10% with testosterone, 0.10% with cortisol, but does not cross-react with progesterone and estrogens (<0.01%). The minimum detectable CORT concentration was 0.63 μg/dl. In order to further explore possible vendor differences in cell proliferation, cell proliferation levels of multiparous and virgin females were compared between the two control groups: OVX and sham-operated rats.

5.3 RESULTS

Increased cell proliferation in response to an acute subcutaneous injection of 17β-estradiol, estrone, and 17α-estradiol was dependent on reproductive experience. The total number of BrdU-ir cells did not differ in ovariectomized middle-aged virgin female rats treated with any of these estrogens compared to oil control rats or sham-operated control rats (all p's > 0.99; Figure 5.1). However 17β-estradiol, estrone, and 17α-estradiol significantly increased cell proliferation in ovariectomized age-matched multiparous rats (had given birth to at least 4 litters before reproductive failure) compared to OVX control rats ($p < 0.005$, $p < 0.0002$, $p < 0.0002$, respectively; Figure 5.1). Interestingly multiparous sham-operated control rats had significantly more total number of BrdU-ir cells compared to multiparous OVX controls ($p < 0.03$), but fewer total BrdU-ir cells compared to multiparous rats given estrone ($p < 0.006$) and 17α-estradiol ($p < 0.0002$). Within hormone treatment, multiparous rats given estrone and 17α-estradiol had significantly more total BrdU-ir cells compared to virgin rats given these estrogens ($p < 0.0006$ and $p < 0.0001$, respectively), indicating that estrone and 17α-estradiol are having greater effects in multiparous rats than 17β-estradiol.

Figure 5.1: Increased cell proliferation in the dentate gyrus after estrogen replacement in multiparous females but not in virgin females. An acute, high dose of 17β-estradiol, estrone, and 17α-estradiol significantly increased cell proliferation in multiparous middle-aged reproductively senescent female rats but not in age-matched virgin females. **a** denotes significantly different from multiparous control group, **b** denotes significantly different from virgin group given the same hormone treatment.



Importantly resting ACTH and CORT levels did not differ between OVX control groups (ACTH: $p = 0.57$; CORT: $p = 0.40$; see Table 5.1) or between sham-operated control groups (ACTH: $p = 0.86$; CORT: $p = 0.20$). Importantly virgin OVX controls and multiparous OVX controls did not differ in cell proliferation ($p < 0.53$), nor did virgin sham-operated controls and multiparous sham-operated controls ($p < 0.28$). Therefore obtaining multiparous and virgin

Sprague-Dawley female rats from two different vendors did not significantly influence basal rates of cell proliferation or resting ACTH and CORT levels.

Table 5.1: Mean ± SEM adrenocorticotropic hormone (ACTH) and corticosterone (CORT) levels in multiparous and virgin female rats.

Group	Adrenocorticotropic hormone (pg/ml)	Corticosterone (μg/dl)
Multiparous female rats		
OVX control	309.32±15.99	42.58±5.65
Sham-operated control	266.38±20.76	25.75±3.14
Virgin female rats		
OVX control	386.27±69.48	57.00±13.23
Sham-operated control	334.72±23.27	48.87±4.98

5.4 DISCUSSION

Taken together, these results indicate that previous reproductive experience can alter the ability of progenitor cells in the dentate gyrus of the hippocampus to respond to estrogen replacement in middle-age in female rats. Interestingly all three forms of estrogen at a 10 μg dose increased cell proliferation in multiparous middle-aged female rats, with 17α-estradiol having the greatest effect. Somewhat surprisingly estrone, which may be responsible for the inability of HRTs to improve cognition in postmenopausal women (Ryan, et al., 2009,Ryan, et al., 2008), also increased cell proliferation in the current study. However it is important to note that increased levels of cell proliferation do not necessarily coincide with improvements in cognition (Galea, et al., 2008). In fact doses of estrone that increase cell proliferation in adult

female rats (Barha, et al., 2009b) either impair or have no effect on hippocampus-dependent learning and memory (Barha, et al., 2010). It may be that these newly divided cells will become ectopic and/or will have altered functional properties if they become neurons. For example new neurons created after seizures are ectopic, have more restricted electrophysiological properties, and contribute to learning deficits (Jakubs, et al., 2006,Jessberger, et al., 2007). It remains to be seen if alterations in cell proliferation in response to various estrogen treatments coincide with improvements in cognition in aged females.

It is important to note that the phenotype of these new cells was not assessed in the present study. Although 10-60% of newly divided cells express neuronal proteins within 1 day of proliferation (Brown, et al., 2003,Cameron, et al., 1993,Plumpe, et al., 2006), a substantial amount (30-70%) of these newly divided cells will die (Cameron and McKay, 2001,Dayer, et al., 2003,Sun, et al., 2004). Therefore attempting to phenotype newly generated cells (within 24 h) does not provide an accurate assessment of adult neurogenesis. Future studies that allow a longer time window for newly generated cells to mature are required in order to address whether exposure to different forms of estrogen alters neurogenesis and whether this is dependent on previous reproductive experience.

This change in ability to respond to estrogens later in life with reproductive experience may be related to differing hormone exposure or greater enrichment (due to pup exposure) across the lifespan of the female. This is consistent with epidemiological results which show an association between high levels of estrogen exposure throughout the lifespan and decreased risk for cognitive decline and Alzheimer's disease (Veiga, et al., 2004). Results from studies in rodents have strongly supported this finding, as previous reproductive experience reduces the age-related decline in spatial memory and also reduces levels of amyloid precursor protein (APP), a marker of neurodegeneration and cognitive decline, in the hippocampus (Gatewood, et

al., 2005). Furthermore, previous reproductive experience increases brain-derived neurotrophic factor (BDNF) levels, which is involved in neuronal plasticity and hippocampus-dependent spatial memory (Allen and Dawbarn, 2006), in the hippocampus in middle-aged rats (Macbeth, et al., 2008). Interestingly many of estradiol's effects in the hippocampus are mediated by BDNF (Scharfman and MacLusky, 2006). Therefore it is possible that the increase in cell proliferation induced by different types of estrogen in the current study may be related to elevated BDNF levels in the multiparous females compared to the virgin females. Additionally, estrogen's beneficial effect on cognition and neuronal health in older brains could be related to estradiol's ability to normalize the age-related increase in L-type voltage-gated calcium channel current (Brewer, et al., 2009). Therefore previous reproductive experience, which is associated with altered hormone exposure and greater enrichment, can result in higher levels of plasticity in the middle-aged brain and may protect the brain from the deleterious effects of aging in females.

5.5 CONCLUSIONS

Previous reproductive experience renders the middle-aged reproductively senescent female brain more responsive to different forms of estrogen compared to the virgin brain. The results from the present study emphasize the need for consideration of hormonal events, such as pregnancy and/or enrichment from exposure to pups, which occur throughout the lifespan in order to fully understand the effects of HRT on cognition and the brain. Findings from this study will advance our understanding of how different forms of estrogen mediate hippocampal neurogenesis in aged rats, and indicate that 17α-estradiol is a more potent inducer of cell proliferation than 17β-estradiol. Findings such as these may ultimately lead to the development

of new therapeutic advances in the treatment of symptoms associated with menopause in women.

6. GENERAL DISCUSSION

The experiments in this thesis aimed to investigate how different types of naturally occurring estrogens and the most prescribed hormone replacement therapy, Premarin, can differentially influence various forms of hippocampus-dependent learning and memory and adult neurogenesis in the hippocampus. The main findings presented in this thesis are: (1) Chronic treatment with Premarin impaired hippocampus-dependent spatial working and reference learning and memory, increased adult hippocampal neurogenesis, and altered activation of newly generated neurons in the dentate gyrus in response to memory, which may account for impairments seen in memory functioning (Chapter 2: Barha and Galea, in press); (2) Three naturally occurring estrogens, 17β-estradiol, estrone, and 17α-estradiol, differentially increased cell proliferation in the dentate gyrus in adult female rats in a time- and dose-dependent manner. Low and high doses of 17β-estradiol and estrone increased cell proliferation, while the medium and high doses of 17α-estradiol increased cell proliferation (Chapter 3: Barha, et al., 2009); (3) 17β-estradiol and 17α-estradiol dose-dependently enhanced or impaired, while estrone impaired or had no significant effect on hippocampus-dependent contextual fear conditioning. Estrone also increased levels of the presynaptic protein synaptophysin 24 hours after hormone treatment, but levels did not correlate with memory performance (Chapter 4: Barha, et al., 2010); (4) In middle-aged female rats, the effect of estrogens on cell proliferation were dependent on previous reproductive experience, as estrogens significantly increased cell proliferation in multiparous but not nulliparous middle-aged females (Chapter 5: Barha and Galea, 2011). Taken together, the experiments in this thesis demonstrate that the influence of estrogens and hormone replacement therapy on the hippocampus depend on numerous factors, including dose and type of estrogens given, duration of treatment, the age of the subjects, type of memory system investigated, and previous reproductive experience.

6.1 ESTROGENS DIFFERENTIALLY INFLUENCE HIPPOCAMPUS-DEPENDENT LEARNING AND MEMORY IN OVARIECTOMIZED ADULT FEMALE RATS

The data presented in Chapter 2 of the present thesis shows that chronic treatment with two different doses of the hormone replacement therapy Premarin impairs hippocampus-dependent spatial working and reference learning and memory (Barha and Galea, in press). Although these findings are consistent with a number of studies that have shown detrimental effects of Premarin on cognition in postmenopausal women (Ryan, et al., 2008), the few studies conducted in middle-aged female rats have mainly found beneficial effects (Acosta, et al., 2009,Acosta, et al., 2010,Engler-Chiurazzi, et al., 2011). Taken together, these results indicate that the relationship between Premarin and cognition likely depends on multiple factors including age of the subjects, route of administration, dose, learning and memory task, and also the schedule (tonic or cyclic) of administration.

Zhao and Brinton (2006) have shown that of the three classical estrogens found among the 10 estrogenic compounds identified in Premarin, low levels of 17β-estradiol and 17α-estradiol showed greater *in vitro* neuroprotective efficacy against neurodegenerative insults associated with Alzheimer's disease than did estrone. Interestingly, data from Chapter 4 provides behavioral support for this as treatment with 17β-estradiol and 17α-estradiol either enhanced or impaired hippocampus-dependent contextual fear conditioning, whereas estrone only impaired learning (Chapter 4: Barha, et al., 2010). Although at the moment it is not entirely clear which components of Premarin are contributing to the impairments seen in cognitive performance, estrone seems a likely candidate.

Previous work using the same version of the radial arm maze has shown that treatment with the same high dose of a conjugated form of 17β-estradiol (estradiol benzoate) also impaired spatial reference memory in female rats (Galea, et al., 2001). Treatment with lower

doses (1 and 5 μg) of estradiol benzoate that produce physiologically high levels of 17β-estradiol in the blood impair spatial working memory on this same task (Holmes, et al., 2002). Similar to our results, Engler-Chiurazzi (2012) have recently shown that continuous treatment with a 8 μg/day dose of estrone given via osmotic pumps impairs spatial working memory, but not spatial reference memory, in the Morris water maze in ovariectomized middle-aged female rats. Only two other studies have examined the influence of 17α-estradiol on learning and memory. Both studies found that 17α-estradiol was a more potent enhancer of spatial object placement memory than 17β-estradiol (Inagaki, et al., 2010,Luine, et al., 2003). Therefore, it seems that 17α-estradiol is a potent enhancer of learning and memory. Furthermore, the two forms of estradiol dose-dependently enhance hippocampus-dependent learning, whereas estrone impairs, supporting the hypothesis that estradiol-based replacement therapies are more beneficial for cognition than estrone-based replacement therapies.

6.2 ESTROGENS DIFFERENTIALLY INFLUENCE HIPPOCAMPAL PLASTICITY

Data from Chapters 2, 3, and 5 indicate that Premarin and different estrogens have effects on distinct components of hippocampal neurogenesis. Acute treatment with 17β-estradiol and estrone increases cell proliferation in the same dose-dependent manner, with a low dose (0.3 μg) and a high dose (10 μg) increasing and a medium dose (1.0 μg) having no effect on proliferation in the granule cell layer of ovariectomized adult female rats after 30 minutes of exposure. On the other hand, acute treatment with 17α-estradiol increased cell proliferation with a different dose-dependent curve with the medium and high doses increasing cell proliferation (Chapter 3: Barha, et al., 2009). Treatment with a high dose (10 μg) of all three estrogens increased cell proliferation in multiparous middle-aged female rats after 30 minutes of exposure

compared to ovariectomized controls, with estrone and 17α-estradiol having the greatest effect as only these two estrogens increased cell proliferation above levels seen in sham-operated multiparous rats (ovaries were not removed) (Chapter 5: Barha, et al., 2011). Chronic, 33 days of treatment with both a 10 μg and 20 μg dose of Premarin increased the total number of new neurons surviving for 34 days (Chapter 2: Barha and Galea, in press). These higher levels of cell proliferation and cell survival after hormone treatment are not synonymous with better learning. This is interesting in light of the relationship between higher levels of cell proliferation and survival and neurodegenerative disorders such as Alzheimer's disease (Jin, et al., 2004), suggesting more cells and neurons are not always beneficial for behavioral functioning (see Sections 6.4 and 6.5 for further discussion).

These are the first studies showing that estrone, 17α-estradiol, and Premarin treatment can influence components of hippocampal neurogenesis, and no other studies have directly compared the effects of the three naturally occurring estrogens. Previous studies have examined the effects of 17β-estradiol and its conjugated form, estradiol benzoate, on hippocampal neurogenesis. For example, Barker and Galea (2008) found that 15 days of treatment with estradiol benzoate (10 μg) decreased survival of 16 day old cells in female rats and slightly increased cell proliferation, with no effect seen in male rats. On the other hand, we have recently shown that 20 days of treatment with 17β-estradiol (10 μg) significantly increases, whereas estrone significantly decreases, the total number of 19 day old new neurons in the dentate gyrus of female rats trained on a spatial task (McClure, et al., accepted). It is important to note that hormone treatment was begun 24 hours prior to when newly dividing cells were labeled in the study by McClure et al. (accepted), so that new neurons were produced and survived in a hormone rich environment. On the other hand, in the study by Barker and Galea (2008) hormone treatment was begun 24 hours after newly dividing cells were labeled, so that new

neurons only survived in a hormone rich environment. By labeling dividing cells before beginning hormone treatment, the assessment of the effects of hormone treatment on cell survival independent of any effects on cell proliferation is achieved. Alternatively, in the study by McClure et al. (accepted) the effects of hormone treatment on initial cell proliferation cannot be teased apart from the effects on cell survival. Therefore, these contradictory results regarding the effects of 17β-estradiol may be related to this difference as the new neurons were produced under different hormonal environments. Furthermore, Barker and Galea (2008) gave estradiol benzoate, a slower metabolizing conjugated form of 17β-estradiol, to behaviorally naïve rats; whereas, McClure et al. (accepted) gave unconjugated 17β-estradiol to rats trained in the Morris water maze. Regardless, it is interesting that estrone decreased neurogenesis because Premarin, which is composed of 50% estrone, increased cell survival (Chapter 2). However, the effects of the many other active estrogenic compounds in Premarin cannot be ruled out and may have accounted for, alone or in combination, the increase seen in cell survival. Furthermore, reminiscent of the study conducted by Barker and Galea (2008), Premarin treatment began 24 hours after newly dividing cells were labeled (with BrdU), therefore, allowing for the assessment of the effects of hormone treatment on cell survival independent of any effects on cell proliferation. It is possible we would have obtained different results if BrdU was given after Premarin treatment had begun. Importantly, treatment with Premarin did not influence neurogenesis when given to behaviorally naïve rats (cage controls), suggesting the relationship between Premarin and hippocampus neurogenesis is a complex one.

Treatment with the medium doses of 17β-estradiol and estrone and with the high dose of estrone significantly increased the expression of the presynaptic protein synaptophysin in the CA3 region of the hippocampus (Chapter 4: Barha, et al., 2010). However, levels of synaptophysin did not correlate with hippocampus-dependent memory performance, suggesting

a disassociation between synaptic plasticity and memory performance in this task. This is intriguing as most studies that have found increases in spine density and synaptic proteins in the hippocampus with 17β-estradiol treatment use behaviorally naïve animal. On the other hand, we investigated the effects of estrogens on synaptophysin in behaviorally experienced rats, indicating that hormone treatment may be interacting with factors associated with training in a hippocampus-dependent learning and memory task, a relationship also seen by Frick, et al. (2004). We did not see a consistent increase in synaptophysin with 17β-estradiol, as only one dose had an effect in only one region of the hippocampus. Despite a lack of significant correlations between synaptophysin and memory performance, it is of some interest that two of the three effects seen in synaptophysin levels were in groups showing impairments in memory retrieval (medium doses of 17β-estradiol and estrone). Therefore, similar to the results that increases in hippocampal neurogenesis occur in rats with impaired spatial working and reference memory (Chapter 2: Barha and Galea, in press), increases in presynaptic proteins in the CA3 region are seen in rats with impaired contextual fear conditioning.

6.3 WHICH ESTROGENS ARE OPTIMAL FOR HORMONE REPLACEMENT THERAPY?

The experiments presented in this thesis endeavored to compare the effects of three main, naturally occurring classical estrogens on hippocampus-dependent learning and memory and cell proliferation in the dentate gyrus of both adult and middle-aged female rats. This was done in order to better understand how estrogens mediate hippocampal function and neuroplasticity and to aid future work in determining which of these estrogens are likely candidates for use in alleviating age-associated memory decline in postmenopausal women. In general, data presented in Chapter 4 indicate that estrone does not have a positive effect on

hippocampus-dependent contextual fear conditioning regardless of dose, whereas 17β-estradiol and 17α-estradiol do have positive effects depending on dose. Further research is required to determine whether chronic treatment with a lower dose of 17β- and α-estradiol could actually enhance spatial working and reference memory. Therefore, it seems that although 17β-estradiol and 17α-estradiol are better candidates for enhancing cognitive performance than estrone, the effects are dependent on many mediating factors, including the dose of hormone given and the type of memory system investigated.

Previous studies in women indicate that in general treatment with 17β-estradiol-based hormone replacement therapies in younger postmenopausal women enhance cognition when started close in time to ovarian cessation and used short-term (Gleason, et al., 2006,Joffe, et al., 2006,Phillips and Sherwin, 1992,Rasgon, et al., 2005,Sherwin, 1988,Tierney, et al., 2009). On the other hand, treatment with estrone-based therapies in both younger and older postmenopausal women have either negative or neutral effects on cognition (Gleason, et al., 2006,Maki, et al., 2007,Resnick, et al., 2009,Wroolie, et al., 2011). Higher endogenous levels of estrone are associated with lower cognitive performance (Yaffe, et al., 1998), whereas higher levels of 17β-estradiol are associated with better cognitive performance (Bittner, et al., 2011,Drake, et al., 2000,Lebrun, et al., 2005) in postmenopausal women. In female rats higher serum levels of 17β-estradiol after exogenous treatment correlated with better spatial reference memory, however endogenous levels of 17β-estradiol did not correlate with performance (Talboom, et al., 2008). Circulating levels of exogenous 17β-estradiol after treatment with Premarin correlated with impaired spatial working learning and memory (Chapter 2: Barha and Galea, in press). Furthermore, higher circulating levels of estrone after treatment with Premarin were related to increased spatial working memory errors and 17β-estradiol and estrone levels

were positively correlated (Chapter 2: Barha and Galea, in press). Therefore, it is possible that the positive relationship between 17β-estradiol levels and working memory errors seen in this study may be related to the higher levels of estrone seen in these rats. Taken together, the data indicate that the relationship between circulating levels of 17β-estradiol and cognitive performance depends on many factors including type of memory system, source of hormone (exogenous vs endogenous, treatment with hormone alone vs treatment with hormone in Premarin formulation), and presence of estrone. However, it is important to keep in mind that absolute values of these hormones will be different between studies; therefore, direct comparisons should be done with caution. Overall, it does seem that endogenous levels of 17β-estradiol are associated with better cognitive performance.

Unfortunately, circulating levels of 17α-estradiol have yet to be determined in human or rodent studies that examine cognitive performance. This is partially because a radioimmunoassay kit, which is typically used to accurately measure levels of specific hormones in serum, is not commercially available for 17α-estradiol. However, the case for the use of 17α-estradiol as a treatment for cognitive decline is strong. 17β-estradiol and to a lesser extent estrone, have been the endogenous estrogens of greatest interest in the fields of neuroendocrinology and neuroprotection. 17α-estradiol, the optical isomer of 17β-estradiol, has been considered less biologically active due to a weaker binding affinity for the classical estrogen receptors (Toran-Allerand, et al., 2005). Although the effects of 17α-estradiol are still grossly understudied, recent studies have begun to show that 17α-estradiol is as equally neuroprotective against different brain insults as 17β-estradiol (Simpkins and Dykens, 2008). This form of estrogen has also been purported as a potential therapeutic agent for the treatment of Alzheimer's disease, as treatment with 17α-estradiol decreases the levels of the amyloid β-

precursor protein in a mouse model of Alzheimer's disease (Levin-Allerhand, et al., 2002). Importantly, 17α-estradiol also prevented neuron loss in the CA1 region of the hippocampus, a hallmark of Alzheimer's disease, in a mouse model of Alzheimer's disease which shows AD-typical neuron loss (Manaye, et al., 2011).

It has been proposed that 17α-estradiol is produced by the aromatization of epitestosterone (optical isomer of testosterone) (Finkelstein, et al., 1981), at least in the placenta of pregnant women (Higuchi and Villee, 1970) and possibly in the brain (MacLusky, et al., 1994). The biosynthetic pathways of 17α-estradiol have not been fully characterized (see Figure 1.3b) nor have the sites in the body and brain in which synthesis of this hormone may occur. In addition to epitestosterone, it has been suggested by Toran-Allerand et al. (2005) that estrone may also be a precursor for 17α-estradiol. Interestingly, higher levels of both endogenous 17α-estradiol and estrone are found in the hippocampus compared to 17β-estradiol, whereas higher levels of 17β-estradiol are found in the ovary of mice (Toran-Allerand, et al., 2005). Furthermore, levels of 17α-estradiol are not influenced by ovariectomy, castration, and/or adrenalectomy, suggesting that this estrogen may be derived from local synthesis in the brain (Toran-Allerand, et al., 2005).

In addition to the effects of 17α-estradiol on neuroprotection, this estrogen has been shown to be more potent than 17β-estradiol in some regards. For example, 17α-estradiol was found to be a more potent enhancer of hippocampus spatial memory, as acute administration of lower doses of this estrogen enhanced object placement memory compared to 17β-estradiol when given 30 minutes before (Luine, et al., 2003), immediately after but not 45 minutes after training on this task (Inagaki, et al., 2010). Furthermore, 17α-estradiol was a more potent regulator of CA1 pyramidal spine synapse density than 17β-estradiol as lower doses were

required to increase spine density (Maclusky, et al., 2005). The increase in CA1 spine density was greater 30 minutes after administration of either estrogen compared to 4.5 hours after administration (Maclusky, et al., 2005), a finding that parallels the time-dependent effects of these estrogens on cell proliferation in the dentate gyrus (Chapter 3: Barha, et al., 2009). Treatment with 17α-estradiol, a less feminizing estrogen, has also been shown to present no adverse health risks when given to postmenopausal women in a Phase 1 clinical trial (Dykens, et al., 2005). When one considers the data concerning the effects of 17α-estradiol on brain and behavior, including the results presented in this thesis, the potential efficacy of this particular estrogen become apparent. Further research is required in postmenopausal women in order to substantiate the positive effect 17α-estradiol has on cognition.

6.4 WHAT IS THE RELATIONSHIP BETWEEN NEUROGENESIS AND LEARNING AND MEMORY AFTER TREATMENT WITH ESTROGENS?

All three estrogens increase cell proliferation in the dentate gyrus, although these effects are dependent on dose, age of subjects, and previous reproductive experience (Chapter 3: Barha, et al., 2009; Chapter 5: Barha and Galea, 2011). I found that estrone and 17β-estradiol increase cell proliferation at the same doses, whereas 17α-estradiol increases cell proliferation at different doses in adult female rats. 17α-estradiol is also the most potent regulator of proliferation in multiparous middle-aged female rats. Importantly, effects of estrogens on cell proliferation were not consistently related to alterations in hippocampal function. For example, exposure to the low and high doses of 17β-estradiol for 30 minutes led to increased number of proliferating cells seen 24 hours later (Chapter 3). However, treatment with the same low dose of 17β-estradiol for 30 minutes before fear conditioning led to enhancements in contextual memory 24 hours later but treatment with the high dose led to impairments (Chapter 4).

Furthermore, low and high doses of estrone increased cell proliferation but had no significant influence on contextual fear conditioning, while treatment with the medium dose of 17α-estradiol increased cell proliferation but severely impaired contextual memory. These results suggest that the effects of estrogens on hippocampal function are independent of effects on cell proliferation. This is perhaps not entirely surprising as these newly divided cells are have not yet formed synapses or extended an axon, therefore an increase in the number of daughter cells will not likely influence hippocampal functioning assessed at this early stage of neural development. A small portion of these daughter cells may already express the endogenous marker for immature neurons; however, these young immature neurons at 24 hours of age are too young to have any appreciable influence on neural circuitry involving the dentate gyrus.

Treatment with Premarin lead to increases in the total number of neurons surviving for 34 days but impaired spatial working and reference learning by increasing criterion-performance measure. This may seem counterintuitive as more neurons should relate to better learning and memory as indicated by some of the literature (Drapeau, et al., 2003,Saxe, et al., 2006,Snyder, et al., 2005,Winocur, et al., 2006; see Section 6.5 below for further discussion). For example, exposure to a complex enriched environment, physical exercise, or cognitive enhancers like ginseng increase both hippocampal neurogenesis and hippocampus-dependent functioning (Qiao, et al., 2005,van Praag, 2008). However, more new neurons are not always beneficial for cognitive performance. Alterations in different stages of neurogenesis in the hippocampus are seen in the brains of patients with Alzheimer's disease, with decreases in stem cells but increases in proliferating progenitor cells and new immature neurons (Jin, et al., 2004,Perry, et al., 2012). Furthermore, increases in hippocampal neurogenesis are seen following seizures (Parent, 2007). Both of these disease states also present with severe impairments in hippocampus-dependent memory functioning, and it has been suggested these impairments are

related to the abnormally high levels of neurogenesis (Jessberger, et al., 2007,Parent and Lowenstein, 2002). Therefore, it seems that a physiologically adaptive and optimal range exists for hippocampal neurogenesis and addition of new neurons within this range may confer a beneficial influence on spatial learning and memory. Alternatively, the addition of new neurons outside this range may lead to impairments in memory function.

Additionally, it is possible that these new neurons produced under pathological conditions may dramatically differ in how they function compared to new neurons produced under non-pathological conditions. The neurogenic environment into which new neurons are born and develop is important in determining the electrophysiological, and perhaps, behavioral function of these new neurons (Jakubs, et al., 2006,Jessberger, et al., 2007). A pathological environment can increase the production and survival of neurons but can also lead to aberrant integration of these neurons into the existing neural circuitry, which may contribute to any functional impairment (i.e. Barha, et al., 2011b,Jessberger, et al., 2007). Studies have shown that new neurons produced in normal or enriched environments (such as after running exercise) have different synaptic properties than new neurons produced in pathological environments like after seizures (Jakubs, et al., 2006,Wood, et al., 2011). Therefore, it may be that new cells proliferating into and/or surviving in a neurogenic environment in the dentate gyrus that have been exposed to treatments that increase hippocampal neurogenesis but also impair memory functioning, such as Premarin and estrone, may be functioning in an aberrant fashion. For examples, these cells may be making aberrant connections, may be ectopic, have altered electrophysiological properties, or may not be activated by appropriate stimuli.

It is possible that treatment with Premarin is causing abnormal alterations in the neurogenic niche of the dentate gyrus and the high number of new neurons surviving in this environment may be functioning inappropriately and could be leading to the impairments in

spatial learning and memory seen in these rats (Chapter 2: Barha and Galea, in press). We began to examine this possibility by looking at whether these new neurons either produced or surviving in a Premarin-rich environment are activated, as indexed by the expression of the immediate early gene product zif268, by memory retrieval. This was done by determining the percentage of new cells that either only survived or were produced and survived under the influence of Premarin (BrdU-ir and DCX-ir cells, respectively in Chapter 2) that co-expressed the immediate early gene product zif268. Results indicate that treatment with the low dose of Premarin decreased activation of BrdU-ir cells, and that treatment with the high dose of Premarin increased activation of the younger doublecortin-ir cells. Importantly, activation of the older BrdU-ir cells was positively correlated with improvements in spatial learning in the control rats only. Interestingly, increased activation of BrdU-ir cells and doublecortin-ir cells was related to impaired performance in the Premarin-treated rats only, suggesting that although these new neurons exposed to Premarin are being activated by memory retrieval they are also related to memory impairments unlike the activation in control rats, which was related to memory improvements. Therefore, this indicates that Premarin-exposed new neurons may be functioning inappropriately in response to spatial memory. Further research is required in order to determine how these new neurons exposed to Premarin differ compared to new neurons in the control rats not exposed to Premarin.

Although the activation of new neurons was only assessed in Chapter 2, it is possible that treatment with estrogens at certain doses that lead to impairments in hippocampal functioning may also alter some intrinsic property of these new neurons. Hippocampal regions CA1 and CA3 are hyperexcitable during the proestrous stage of the estrous cycle when estrogens are high, and the rate of excitability is positively correlated with serum estradiol levels (Scharfman, et al., 2003). Treatment with 17β-estradiol also increases excitability in the dentate

gyrus (Kim, et al., 2006). The susceptibility to seizures also increases during times of the female reproductive cycle when estrogens are high (Woolley and Schwartzkroin, 1998). We have seen that chronic (20 days of treatment) with 17β-estradiol does increase the percentage of new surviving cells that co-express zif268, whereas estrone does not significantly increase activation compared to ovariectomized controls (McClure, et al., accepted). Importantly, in this study the newly dividing cells were labeled 24 hours after hormone treatment had begun, therefore these new cells were born into a hormone-rich environment, which may have influenced their development. Further research is required to determine how cells chronically exposed to 17α - estradiol fare under these conditions.

The dissociation between new neuron number and learning and memory performance after treatment with estrogens suggests that other mechanisms may be supporting the estrogenic effects on cognition. One potential mechanism through which estrogens may be exerting their effects on learning and memory is dendritic spine number and morphology. For example, it is well established in the literature that high levels of endogenous and exogenous 17β-estradiol increase the number of dendritic spines and synapses on the pyramidal cells in the CA1 stratum radiatum region of the dorsal hippocampus (for review see Woolley, 2007). Female rats in late proestrus have 30% higher density of apical dendritic spines (Woolley, et al., 1990), and 32% more synapses (Woolley and McEwen, 1992) than rats in estrus. Furthermore rats in proestrus have a higher proportion of mushroom-shaped spines, which are larger more mature types of spines associated with stable memory, compared to rats in estrus (Gonzalez-Burgos, et al., 2005). Ovariectomy gradually decreases dendritic spine density in the CA1 region over the first six days after surgery (Woolley and McEwen, 1993) and this reduction can be reversed with 48 hours of treatment with 17β-estradiol (Woolley and McEwen, 1992, 1993). Increases in progesterone levels following 17β-estradiol lead to a rapid decrease in spine density (Woolley

and McEwen, 1993). These effects of ovariectomy and 17β-estradiol treatment do not influence spine density in the CA3 region or the dentate gyrus of young adult females (Woolley and McEwen, 1992, 1993, Miranda, et al., 1999). However, in older 16 to 20 month old reproductively senescent female rats, short-term treatment with 17β-estradiol leads to increases in spine density on granule cells of the dentate gyrus (Miranda, et al., 1999).

6.5 THE RELATIONSHIP BETWEEN HIPPOCAMPAL NEUROGENESIS AND HIPPOCAMPUS-DEPENDENT LEARNING AND MEMORY IS NOT STRAIGHTFORWARD

Acute treatment with three naturally occurring classical estrogens, 17β-estradiol, estrone, and 17α-estradiol, increased cell proliferation in a dose-dependent manner in ovariectomized adult female rats (Chapter 3: Barha, et al., 2009). Treatment with these same doses of these three estrogens also dose-dependently influenced contextual fear conditioning (Chapter 4: Barha, et al., 2010). However, a straightforward relationship between cell proliferation and memory cannot be deduced by comparing the results from these two studies. For example, treatment with a medium and a high dose of 17β-estradiol impaired contextual fear conditioning, but only the high dose increased cell proliferation. Furthermore, the low and high doses of estrone increased cell proliferation but did not influence contextual fear conditioning. Together these results indicate that cell proliferation in the dentate gyrus of female rats does not relate to hippocampus-dependent learning and memory. Although previous studies have found a relationship between cell proliferation and the expression of learning and memory performance dependent on the hippocampus, it is not known whether cell proliferation is required for learning and memory. Epp and Galea (2009) found that lower levels of cell proliferation in the dentate gyrus was related to more efficient memory processing involving the hippocampus, as

male rats with lower cell proliferation were more likely to adopt a hippocampus-dependent place strategy to complete the Morris water task. However, it is not clear whether lower levels of cell proliferation led rats to adopt a place strategy or whether some other variable associated with using a place strategy actually led to a reduction in cell proliferation. On the other hand, female rats that adopted a place strategy had increased levels of cell proliferation; however, this may be related to higher estradiol levels as rats in proestrus were more likely to be place strategy users (Rummel, et al., 2010). Pham et al. (2005) showed that acquisition of an association between a shock and context (dependent on the amygdala) and not the recall of the context (dependent on the hippocampus), leads to a reduction in cell proliferation of male rats. Furthermore, an increase in cell proliferation is seen with training on a hippocampus-dependent task, but only once performance has reach an asymptotic level, suggesting that learning is not reliant on cell proliferation (Dupret, et al., 2007). Therefore, it seems that although cell proliferation may be altered by exposure to hippocampus-dependent learning and memory, a direct relationship between level of proliferation and learning and memory is not tenable in the literature. However, an increase in the number of cells proliferating may lead to a net increase in the number of new immature neurons that are surviving, as the majority of new cells will develop a neuronal phenotype (Brown, et al., 2003,Cameron, et al., 1993). Therefore, although cell proliferation itself may not be directly related to learning and memory, alterations to this pool of cells may lead to increases in immature neurons and the number of neurons surviving, which has been shown to be involved in hippocampal functioning.

Results from Chapter 2 of this thesis indicate that higher levels of cell survival are related to impairments in hippocampus-dependent spatial working and reference memory. This may seem counterintuitive and contradictory to the prevailing belief in the literature towards the role that hippocampal neurogenesis plays in learning and memory. However, more neurons do

not always equate to better learning and memory. For example, hippocampus-dependent learning regulates hippocampal neurogenesis, as training in tasks involving the hippocampus increases survival of young immature neurons of discreet ages (Epp, et al., 2009,Epp, et al., 2007,Gould, et al., 1999,Leuner, et al., 2004a,Leuner, et al., 2006b), and increases cell death of new neurons of other ages (Dupret, et al., 2007). Therefore, learning regulates neurogenesis by selectively adding and removing new young neurons depending on their age suggesting that an optimal level of neurogenesis in the hippocampus is required for learning and memory. Increases above this optimal level can result in learning and memory impairments. Furthermore, aged male rats with higher levels of neurogenesis performed worse on the Morris water task (Bizon, et al., 2004). Higher levels of hippocampal neurogenesis are also seen in many different neuropathological diseases that present with impaired cognitive performance.

The impairments that may accompany increases in survival of adult generated new neurons may relate to the interference that these new neurons could potentially introduce into the neural circuitry. Young immature neurons are more excitable and more responsive to neural inputs than older neighboring mature granule cells (for review see Deng, et al., 2010). The unique physiological characteristics of adult born neurons allow these neurons to be preferentially activated and recruited by spatial training (Epp, et al., 2011a,Kee, et al., 2007,Stone, et al., 2011,Trouche, et al., 2009), which in turn allows them to make distinct contributions to learning and memory. Furthermore, neuronal activity is able to induce immediate early gene expression in new adult-born neurons as young as 2 weeks old in rats, and 3 to 4 weeks old in mice (Snyder, et al., 2009), indicating that new neurons are participating in the neural circuitry at a very young age.

The dentate gyrus is purported to engage in pattern separation, which involves separating or orthogonalizing similar events; while pattern completion, associating two events as the same

despite variation and/or completing partial patterns, is attributed to the CA3 region (Schmidt, et al., 2012). To facilitate the orthogonalization of inputs, the dentate gyrus utilizes sparse coding of information, as evidenced by the fact that only 2-4% of granule cells are active in any particular environment (Schmidt, et al., 2012). Input from the entorhinal cortex is serially processed in the hippocampus via the trisynaptic circuit (see Figure 1.4a). Data accumulated from numerous sources indicate that one granule cell can innervate 15 CA3 pyramidal cells and one CA3 pyramidal cell can receive convergent input from 72 granule cells, a single CA3 neuron can get up to inputs from 6000 other CA3 neurons and 5500 CA3 neurons can innervate a single CA1 cell (Witter, 2010). Due to these as well as other intrinsic properties, the dentate gyrus is well equipped to undertake the process of pattern separation. Pattern separation is required for proper hippocampal functioning because it prevents interference from occurring between memories, as the dentate gyrus separates incoming information and encodes events dissimilarly which then allows the CA3 to store memories using distinct neurons far apart so that memory recall does not elicit activation of incorrect neurons (Deng, et al., 2010). Recent studies have shown that neurogenesis is required for pattern separation (Clelland, et al., 2009,Pan, et al., 2012,Sahay, et al., 2011). However, the specific role that newly-generated neurons in the adult dentate gyrus have in pattern separation is still under debate. For example, Aimone et al. (2009) proposed that immature neurons are more likely to be activated in multiple contexts or environments, therefore new immature neurons are involved in pattern integration by linking inputs that occur temporally close in nature. Thus, increases in immature neurons in the dentate gyrus may impair hippocampus learning and memory by increasing pattern integration substantially and consequently decreasing pattern separation to a greater degree, ultimately increasing interference between different memories and impairing memory retrieval.

6.6 POTENTIAL MEDIATING FACTORS IN THE EFFECTS OF DIFFERENT ESTROGENS ON THE HIPPOCAMPUS

The results presented in this thesis point out many factors that mediate the effects of estrogens on hippocampal neuroplasticity and function, including the dose of estrogens (Chapters 2, 3, 4), timing of when estrogens given (Chapter 2, 3), and previous reproductive experience (Chapter 5). These factors to different extents have been shown to influence how estrogens influence the hippocampus.

Three different doses were chosen to investigate how estrogens influence cell proliferation and contextual fear conditioning in adult female rats: a low dose of 0.3 μg, a medium dose of 1.0 μg, and a high dose of 10 μg. These doses were chosen based on work done with 17β-estradiol, the most potent and most studied estrogen (Holmes, et al., 2002,Ormerod, et al., 2003,Tanapat, et al., 2005,Tanapat, et al., 1999). Treatment with a 10 μg dose of 17β-estradiol results in circulating levels of estradiol observed on the morning of proestrus (Viau and Meaney, 1991) and produces superphysiological levels 30 minutes after administration (Woolley and McEwen, 1992). Treatment with a 0.3 μg dose of 17β-estradiol results in estradiol levels seen during diestrus (Viau and Meaney, 1991). I found that endogenous estrone levels range between 27.57 and 108.96 pg/ml across the estrous cycle in female rats, and that treatment with the 0.3 μg and 1.0 μg doses of estrone results in circulating levels that fall within this range (85.59 and 108.82 pg/ml) and therefore, are physiological doses, whereas a 10 μg dose results in superphysiological levels (516.32 pg/ml; Barha, et al., 2010). The same doses for each of the estrogens were used in order to facilitate direct comparison of effects between estrogens.

Estrogens were given to female rats 30 minutes before proliferating cells were labeled (Chapters 3, 5) and before fear conditioning training commenced (Chapter 4). Results indicate that estrogens were able to enter the circulation after subcutaneous injection and rapidly

influence the brain. It is important to note that these estrogens are unconjugated and therefore, are more quickly metabolized than conjugated forms. A 30 minute time point was chosen because we were interested in the rapid effects of these estrogens and previous studies have shown that both 17β-estradiol and 17α-estradiol can impact hippocampus-dependent learning and memory (Luine, et al., 2003) and synaptic plasticity (Maclusky, et al., 2005) at this time point. Maclusky et al. (2005) found that both types of estradiol increased spine density in the CA1 region of the hippocampus 30 minutes after injection but this increase was much lower 4.5 hours after injection, although circulating levels of estradiol continued to increase during this time. These results are somewhat similar to our finding that the increase seen in cell proliferation in the dentate gyrus 30 minutes after treatment with estrogens was no longer evident 4 hours later (Chapter 3: Barha, et al., 2009). Therefore, it seems that length of exposure does matter, as initial rapid effects on the hippocampus are followed by down-regulation of the response possibly through some sort of compensatory mechanism.

Research in both humans and rodents show that the ability of estrogens to influence the central nervous system decreases as time since ovarian cessation increases (Daniel, 2012). This is known in the literature as the critical period or window of opportunity hypothesis. For example, treatment with 17β-estradiol prevents impairments in spatial learning when treatment is initiated immediately or 3 months after ovariectomy, but not if initiated 10 months after ovariectomy (Gibbs, 2000). Therefore, it seems that the brain and cognitive functioning become insensitive to exogenous estrogens with long-term ovarian loss. To avoid this, estrogen treatment is typically begun within a few weeks of ovariectomy in rats. Furthermore, an extended hormone deprivation period (> 3 months) may decrease the health status of the hippocampus and hippocampal neurons. As stated by the healthy cell bias hypothesis, treatment with estrogens can be detrimental to brain and behavior if treatment is begun after

neurodegenerative decline commences or after the healthy status of cells is compromised (Brinton, 2005). The increased amount of time without endogenous estrogens may compromise or even accelerate cellular dysfunction.

Another important factor that has been shown to mediate the effects of estrogens on hippocampal cell proliferation is previous reproductive experience, with multiparous but not nulliparous rats responding to estrogens in middle-age (Chapter 5: Barha and Galea, 2011). This increased responsiveness to estrogens in older age with previous parity could be related to levels of estrogen receptors in the hippocampus. Both ERα and ERβ levels are reduced with age in female rats (Mehra, et al., 2005,Waters, et al., 2011), but only ERβ increased with estrogen treatment in aged rats (Waters, et al., 2011). Genetic variants in ERα and ERβ genes are associated with age-associated cognitive decline in non-demented women (Yaffe, et al., 2009,Yaffe, et al., 2002). Furthermore, middle-aged female rats with previous reproductive experience have increased levels of ERα in some areas of the brain compared to middle-aged virgins (Byrnes, et al., 2009). ERβ levels may also be increased in multiparous rats but this has yet to be tested. This increased responsivity to estrogens in reproductively experienced rats in middle-age may have some intriguing implications for the treatment of cognitive decline associated with normal aging and with dementia. In many cases use of hormone replacement therapy for a short period at the time of menopause has been shown to reduce risk for cognitive decline and dementia later in life (Bagger, et al., 2005,Rocca, et al., 2011,Whitmer, et al., 2011), although potential influence of reproductive experience was not directly examined in any study. Interestingly, women with children present with an increased risk for dementia and an earlier age of onset of Alzheimer's disease (Beeri, et al., 2009,Colucci, et al., 2006,Corbo, et al., 2007,Sobow and Kloszewska, 2004). This may be related to the increased responsivity of the

hippocampus we see with previous reproductive experience. It remains to be seen whether the effectiveness of hormone replacement therapy is dependent on parity in women.

6.7 LIMITATIONS AND FUTURE DIRECTIONS

Unconjugated versions of three naturally occurring estrogens, 17β-estradiol, estrone and 17α-estradiol, were chosen for inclusion in this thesis. However, Premarin the hormone replacement therapy used in Chapter 1 is a formulation of more than 10 estrogenic substances and although these three estrogens are found within this formulation, they are in their sulfated form. Sulfated estrogens have different absorption rates in the body than unconjugated estrogens and the pharmacokinetics of these estrogens may also be different (Bhavnani, 1998). Estrogen sulfates can be absorbed directly by the body but after hydrolysis of the sulfates unconjugated estrogens are more readily absorbed (Bhavnani, 1998). Unconjugated estrogens are more rapidly cleared from the circulation, therefore after absorption these estrogens are sulfated and circulate in this form. It is believed that the sulfate conjugated estrogens in circulation act like a reservoir from which the biologically active unconjugated estrogens are being produced (Bhavnani, 2003). Additionally, the majority of women take Premarin orally while we gave Premarin to rats via subcutaneous injection, further complicating direct comparisons between our results and studies conducted in women. The oral route of administration results in first pass effects (metabolism by the liver) and leads to a whole host of alterations in circulating levels of other substances, including cholesterol, sex hormone binding globulin and angiotensin (Bhavnani, 2003). Subcutaneous injection circumvents first pass effects, resulting in higher bioavailability. Therefore, directly extrapolating results from rodent studies to humans and vice versa should be done with caution as effects depend on conjugation status of estrogens and route of administration.

As stated previously, Premarin contains at least 10 estrogens in their sulfate ester form: the ring B saturated estrogens (estrone, 17β-estradiol, 17α-estradiol; see Figure 1.3a) and the ring B unsaturated estrogens (equilin, 17β-dihydroequilin, 17α-dihydroequilin, equilenin, 17β-dihydroequilenin, 17α-dihydroequilenin, and Δ^8-estrone) (Kuhl, 2005). These estrogens are all biologically active in their unconjugated form and can interact with the classical estrogen receptors to some degree, with 17β-estradiol and 17β-dihydroequilin having the greatest and similar binding affinity for both ERs (Bhavnani, 2003). All other estrogens had greater affinity for ERβ compared to ERα. Studies indicate that the ring B unsaturated estrogens may have greater effects on certain variables than the ring B saturated estrogens (17β-estradiol, estrone and 17α-estradiol: the estrogens studied in this thesis). For example, all 7 ring B unsaturated estrogens showed greater antioxidant activity compared to 17β-estradiol, estrone and 17α-estradiol (Bhavnani, et al., 2001). Furthermore, Δ^8-estrone and equilin, which are found naturally in horses but not in women or rats, were the most potent neuroprotectors against glutamate excitotoxicity (Zhao and Brinton, 2006). Behaviorally, Δ^8-estrone but not equilin enhanced spatial working and reference memory (Talboom, et al., 2010). Taken together, these studies suggest that the effects of Premarin on hippocampal plasticity and function may also be attributable to estrogens other than the three naturally occurring estrogens studied in this thesis. However, many of those other estrogens found in Premarin are not naturally found in humans or rodents. Therefore, studying the effects of 17β-estradiol, estrone and 17α-estradiol in female rats is more ecologically and biologically relevant. Future experiments should focus on investigating the effects of other non-endogenous estrogens on cognition for inclusion as therapeutic agents in women. Furthermore, more work is required to determine the

pharmacokinetics of these estrogens, in particular of 17α-estradiol as very little is known about this estrogen, in both women and rats.

In women prescribed hormone replacement therapy, a progestin is typically also included if women have an intact uterus. The WHIMS actually found greater negative effects on mild cognition impairment and risk for dementia in women taking Premarin plus medroxyprogesterone acetate (a synthetic form of progesterone) compared to women taking Premarin alone (Espeland, et al., 2004, Rapp, et al., 2003, Shumaker, et al., 2003). Recent studies have implicated the use of medroxyprogesterone acetate in the negative effects seen in the WHIMS study. For example, postmenopausal women given Premarin in combination with medroxyprogesterone acetate performed worse on a working memory task compared to women given Premarin combined with micronized progesterone (Sherwin and Grigorova, 2011). Furthermore, treatment with medroxyprogesterone acetate alone or in combination with Premarin impairs hippocampus-dependent spatial reference and working memory in middle-aged female rats (Braden, et al., 2011,Lowry, et al., 2010). Progesterone treatment alone or in combination with 17β-estradiol has been shown to improve hippocampus-dependent learning and memory in healthy (for review see Bimonte-Nelson, et al., 2010) or injured brains (for review see Stein, 2011). Therefore, future research should determine how progesterone might mediate the effects of different estrogens on hippocampus-dependent learning and memory.

The studies presented in this thesis utilize a model of surgical menopause, which involves surgical bilateral removal of the ovaries in both adult and middle-aged female rats in order to dramatically reduce circulating levels of estrogens as is seen in women after menopause. Rodents do not naturally undergo menopause, their ovaries continue to produce estrogens late into life (Chakraborty and Gore, 2004). Therefore, surgical removal of the ovaries is necessary. In humans, the cognitive impact of surgical menopause that typically occurs earlier

in life than natural menopause is different than what is seen with natural menopause (Nappi, et al., 1999). Furthermore, the effectiveness of hormone replacement therapy on cognitive performance depends on the etiology of menopause-induced ovarian hormone loss, with enhancements seen in surgically menopausal rats and impairments seen in transitionally menopausal rats (Acosta, et al., 2010). Only about 13% of women undergo surgical menopause (Acosta, et al., 2010); therefore, transitional menopause models in female rats may allow for greater translational value for the naturally menopausal woman.

Results from Chapter 5 indicate that progenitor cells within the dentate gyrus of nulliparous and multiparous female rats differ in their ability to respond to acute treatment with estrogens (Barha and Galea, 2011). In this case multiparous rats were female rats that had been used for breeding purposes for the vast majority of their lives and were retired from breeding after successfully giving birth to at least 4 litters. For them, reproductive experience involved sex, pregnancy, giving birth, and mothering the pups until weaning. The nulliparous rats were not exposed to any of these events throughout their lifespans. Therefore, the effect of estrogens only seen in the multiparous females could be due to any of these factors alone or in combination. Previous studies have shown that pregnancy or pup-exposure ('mothering') alone do not account for enhancements seen in spatial reference and working memory with reproductive experience (Pawluski, et al., 2006a,Workman, et al., 2012). Interestingly, acute and chronic sexual experience increased hippocampal cell proliferation and cell survival in male rats (Leuner, et al., 2010), although female rats have not yet been tested. Therefore, many aspects of the reproductive experience may account for the greater responsivity to estrogens in terms of neurogenesis in the hippocampus. Further research is required to tease apart the contributions of each aspect of the complex maternal experience. Additionally, nulliparous women are rarely

actual virgins and therefore, future studies should factor this into their designs as sexual experience has been shown to influence hippocampal neurogenesis.

The experiments presented in this thesis indicate that the relationship between hippocampus-dependent learning and memory and neurogenesis in the dentate gyrus is complex. Increases in cell proliferation in the dentate gyrus do not correspond to alterations in contextual fear conditioning (Chapter 3: Barha. et al., 2009; Chapter 4: Barha, et al., 2010). On the other hand, increases in survival of new neurons are seen in rats with impaired hippocampus-dependent spatial working and reference memory. One important limitation with this work is that it is correlational. To establish a role for hippocampal neurogenesis in estrogens' effects on learning and memory, studies must extend beyond correlating levels of neurogenesis with performance by directly investigating the effect of depletion of adult-born neurons on cognitive ability with or without estrogen treatment. Previous studies that have depleted or reduced neurogenesis in the dentate gyrus via many different methods, including treatment with anti-mitotic agents, irradiation, and genetic knock-down models, indicate that neurogenesis is required from some but not all hippocampus-dependent tasks. For example, the ablation of adult neurogenesis using anti-mitotic agents in rats impairs performance on a hippocampus-dependent trace conditioning task but not in a hippocampus-independent delay conditioning task (Shors, et al., 2001,Shors, et al., 2002). Some studies have shown impairments in contextual fear conditioning after reductions in neurogenesis (Saxe, et al., 2006,Warner-Schmidt, et al., 2008,Winocur, et al., 2006), while other studies have not (Deng, et al., 2009,Dupret, et al., 2008,Shors, et al., 2002,Zhang, et al., 2008). Similarly complex results are seen with the Morris water task and the radial arm maze, with some studies finding impairments and other studies findings no significant effect (for review see Koehl and Abrous, 2011). Many reasons for these discrepant results have been proposed, including side effects associated with ablation methods,

level of knockdown, species and strain differences, and experimental design differences. One of the more intriguing reasons that has been proposed for data discrepancies is that the behavioral paradigms used in most of these studies do not directly assess the function of the dentate gyrus but rather use tasks dependent on the functioning of the hippocampus as a whole (Deng, et al., 2010). Although pattern separation to some degree is involved in most of these tasks, such as the Morris water task, radial arm maze and contextual fear conditioning, it is very possible that these tasks are not dentate gyrus-specific enough to observe alterations in performance after alterations in neurogenesis. It is also possible that some of these hippocampus-dependent tasks do not require the dentate gyrus as the entorhinal cortex also projects directly to areas CA3 and CA1 (Scharfman, 2007,Witter, 2010). Evidence for this idea is seen in a study conducted by Nakashiba et al. (2008) in which the pathway between entorhinal cortex and CA1 region of the hippocampus was found to be sufficient for learning in the Morris water maze. Correlational studies presented in this thesis were performed using the radial arm maze task and the contextual fear conditioning task. Results may have been more consistent if a more dentate gyrus-mediated task was used that more heavily relied on pattern separation rather than tasks that assess more global hippocampal function. Therefore, it is possible that the function of new adult-generated neurons in the dentate gyrus in learning and memory may be more subtle than previously thought, and can be best delineated in terms of the function of the dentate gyrus specifically.

6.8 CONCLUSIONS

The experiments presented in this thesis showed that three naturally occurring estrogens differentially influence hippocampus-dependent learning and memory in adult females as well as hippocampal neurogenesis in adult and middle-aged females. These effects were shown to be

dependent on numerous factors, including dose and previous reproductive experience. As well, estrone, a main component of the most prescribed hormone replacement therapy, impaired hippocampus-dependent learning and memory. Future experiments should expand upon these findings and aim to understand the role that hippocampal neurogenesis may be playing in the effects of estrogens on cognition. Importantly, the experiments in this thesis also showed that 17α-estradiol is a very potent estrogen with great therapeutic potential. Thus far only one study in humans has attempted to investigate 17α-estradiol in a phase I clinical trial that assessed the safety of this hormone (Dykens, et al., 2005). Future clinical studies are required to determine whether 17α-estradiol is a viable neuroprotective therapeutic agent for use in the prevention and treatment of various neurodegenerative diseases. Overall, this thesis suggests that hormone replacement therapies in the future should be tailored to different populations of women, such as multiparous and nulliparous women, and that 17β-estradiol and 17α-estradiol are likely estrogenic therapeutic alternatives to estrone-based therapies in post-menopausal women.

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