SEX DIFFERENCES IN THE NEUROGENIC AND BEHAVIOURAL EFFECTS OF REPEATED ESTRADIOL ADMINISTRATION IN THE ADULT RAT

by

Jennifer Marie Barker

B.Sc., The University of Toronto, 2001

M.Sc., The University of Toronto at Scarborough, 2004

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ABSTRACT

Estradiol affects neurogenesis in the hippocampus of adult female mammals, but relatively little is known about how estradiol affects cells in the male brain, or how repeated estradiol administration affects either sex. I show in this thesis that repeated estradiol affects cell production and neuron survival in the dentate gyrus of female, but not male rats. Specifically, estradiol administered to female rats increased cell proliferation, decreased the number of young neurons, and decreased the number of dying cells. This difference was not due to differential uptake of estradiol, as the administration of estradiol resulted in concentrations of estradiol in the serum, hippocampus, amygdala, and prefrontal cortex that were similar between males and females. The function served by the new neurons in the hippocampus remains controversial, but evidence suggests they may play a particularly important role in modulating performance in hippocampus-dependent tasks. I used a hippocampus-dependent task, contextual fear conditioning, to determine whether the effects of estradiol on different aspects of neurogenesis - or lack thereof - could be related to its effects on learning and memory. I found a consistent sex difference, with males spending more time freezing than females regardless of treatment. Furthermore, I found that repeated estradiol reduces the amount of time spent freezing in response to a novel context after training in females but not in males. Collectively my results suggest that repeated estradiol influences hippocampal structure and function in female but not male rats. Furthermore the production and survival of adult-generated neurons are regulated differently in males and females

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which has strong implications for any potentially therapeutic manipulations of these cells.

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ABBREVIATIONS

- ActC3: activated caspase-3
- BDNF: brain-derived neurotrophic factor
- BrdU: 5-bromo-2-deoxyuridine
- DCX: doublecortin
- DG: dentate gyrus
- EB: estradiol benzoate
- ER: estrogen receptor
- ERE: estrogen response element
- FJ: Fluoro-Jade B
- GABA: gamma-aminobutyric acid
- GCL: granule cell layer
- MAM: methylazoxymethanol
- NDS: normal donkey serum
- PBS: phosphate-buffered saline
- SGZ: subgranular zone

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A version of each data chapter presented has been published (Chapters 2 and 3) or submitted for publication (Chapter 4). The work described in this dissertation was initiated by Dr. Liisa A.M. Galea (supervisor), based in part on work on neurogenesis in adult meadow voles done primarily by Dr. Brandi Ormerod as a graduate student in the laboratory of Dr. Galea. All experimental work, statistical analysis, interpretations of results and writing of the manuscripts presented herein were done by J.M. Barker, with supervision and editorial feedback provided by Dr. Galea.

Chapter 2, *Repeated estradiol administration alters different aspects of neurogenesis and cell death in the hippocampus of female, but not male, rats.* This manuscript was based on previous studies done by Dr. Ormerod in meadow voles demonstrating sex differences in the effects of short-term estradiol on neurogenesis in the hippocampus of adult meadow voles. This work was undertaken to determine whether the effects of longer-term estradiol administration also differed between the sexes. All experimental work, statistical analysis, interpretations of results and writing of the manuscript was done by J.M. Barker, with supervision and editorial feedback provided by Dr. Galea. This work resulted in a publication of the same title (Barker & Galea, 2008. Neuroscience 152:888-902).

Chapter 3, Sex and regional differences in estradiol content in the prefrontal cortex, amygdala and hippocampus of adult male and female rats. This manuscript was

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Chapter 4, *Males show stronger contextual fear conditioning than females after context pre-exposure*. This manuscript was conceived and designed by J.M. Barker in discussions with Dr. Galea. All experimental work, statistical analysis, interpretations of results and writing of the manuscript was done by J.M. Barker, with supervision and editorial feedback provided by Dr. Galea. This work has resulted in a publication of the same title (Barker & Galea, 2009. Physiol Behav doi:10.1016/j.physbeh.2009.10.014).

1 INTRODUCTION

Male and female mammals differ from each other in a variety of measures. The reproductive system particular to each sex in mammals is typically a result of the genes present in a particular individual, which usually determine whether an animal will produce ovaries or testes and determine its gonadal sex. The gonads ultimately generate particular hormones; and rogens are the predominant hormones produced by testes, and estrogens and progesterone are the predominant hormones produced by the ovaries. These hormones subsequently can affect a variety of structures, including the brain, and exposure to these hormones results in an increased likelihood of expressing reproductive behaviours typical of each sex. However, the biological sex of an animal – and the predominant gonadal hormones produced – can affect behaviours outside of reproduction, including performance on learning and memory tasks. Furthermore, sex and sex hormones influence the incidence, progression and symptoms of neuropsychiatric or neurodegenerative disease. For example, women are more prone than men to suffer depression (Gutiérrez-Lobos et al., 2002) and bipolar II disorder (Curtis, 2005), or develop Alzheimer's Disease (Baum, 2005). Men show an earlier onset of schizophrenia and more negative symptoms of the disease than women, whereas women with schizophrenia display more affective symptoms (Leung and Chue, 2000). Men and women also respond differently to antidepressants (Bies et al., 2003) and antipsychotic medications (Leung and Chue, 2000). Similar levels of overall pathology related to Alzheimer's Disease are more likely to result in dementia in

women than in men (Barnes et al., 2005). These sex differences may be related to gonadal hormone levels. For example, women are more vulnerable to depression at times when estradiol is typically low or decreasing (Harsh et al., 2009), and administration of estradiol can alleviate symptoms of depression (Studd and Panay, 2009). Furthermore, estradiol administration reduces both affective and general psychopatholocial symptoms of schizophrenia in women (Kulkarni et al., 2008). In cases of Alzheimer's Disease, estradiol can regulate levels of β -amyloid protein, and protect neurons against damage resulting from β -amyloid protein deposition and excitotoxicity (Pike et al., 2009). Thus sex and sex hormones can influence a variety of factors beyond reproduction, and it is important to understand how the sexes differ in behaviours and in their response to various treatments.

It is important to note that, although sex differences in behaviour of intact animals are often attributed to difference in gonadal hormone levels, these hormones – estradiol included – are not unique to one sex. Males produce estradiol by aromatization of testosterone, and many of the biological effects of testosterone in males are a result of estradiol (for reviews, see Nelson and Bulun, 2001; Simpson et al., 2005). Both sexes have measurable levels of estradiol in their serum and estrogen receptors (ERs) in various tissues, though the ratios, concentrations, and localization may differ between the sexes (e.g. Yokosuka et al., 1997; Zhang et al., 2002; Pérez et al., 2003; Simpson et al., 2005, Isgor and Watson, 2005 Mazzucco et al., 2006). Furthermore, estradiol affects neurons in both the male and female brain, though not necessarily in the same manner. For example, estradiol increases the

synaptic protein spinophilin in females but has the opposite effect in males (Lee et al., 2004); and estradiol increases spine synapse density in the female, but not male, hippocampus (Leranth et al., 2003; MacLusky et al., 2005). Such results highlight the importance of examining both males and females when attempting to elucidate the effects of gonadal hormones, and suggest that there are differences in the adult brain that account for the different effects of estradiol. Thus when attempting to determine the effects of a particular hormone on the brain or behaviour, it is important to study its effects in both sexes to determine which, if any, effects are indeed sex-specific and which may be applicable to both sexes.

Estradiol in males and females

One step in determining the potential effects of estradiol in males and females is determining whether cells or tissues have the capacity to respond to the presence of estradiol. The presence (or absence) of estrogen receptors (ERs) is obviously an important clue as to which cell or tissue types are expected to respond to estradiol. There are two main types of ERs that are typically responsible for the effects of estradiol: ER α and ER β . The classical, genomic mechanism by which estradiol affects cells involves the binding of estradiol to ERs found most usually in the cytoplasm of cells (Nelson and Bulun, 2001). These activated ERs then migrate to the nucleus, dimerize, and bind to estrogen response elements (EREs) of the genome. This binding subsequently results in transcription of downstream DNA, and ultimately protein production. In addition to this mechanism, estradiol can also

activate the MAPK/ERK and PKA pathways by binding to ERs associated with the cell membrane (Evinger and Levin, 2005), and also appears to activate a distinct transmembrane, G protein-coupled receptor (Qiu et al., 2003; Prossnitz et al., 2008). The effects of estradiol binding to ERs are dependent on a variety of other factors, including the specific type of cell and the cofactors that are present and the receptor involved. Both ERs are activated by estradiol, but the specific EREs to which they bind differ and produce different effects within a cell and ultimately on behaviour (Rissman, 2008). Both ERs are found in the hippocampus and amygdala, and ERß is found in the prefrontal cortex (Shughrue et al., 1997). Perhaps surprisingly, receptor subtype localization is similar between males and females in the hippocampus (ERα: Weiland et al., 1997), amygdala and prefrontal cortex (ERα and $ER\beta$: Simerly et al., 1990; Kritzer, 2002). However, there is data to suggest subtle sex differences in ER localization within cell subtypes in the hippocampus (Isgor and Watson, 2005; Mazzucco et al., 2006). In general, however, the distribution of ERs in the hippocampus is similar between the sexes (Kalita et al., 2005), and estradiol itself is present in the hippocampus of intact rats of both sexes (Chapter 3; Henderson et al., 1979; Bixo et al., 1986; Hojo et al., 2004). The presence of estradiol in the hippocampus is of particular interest in this thesis, as I will investigate the effects of estradiol on hippocampus structure and function (Chapter 2 and 4).

There is evidence that estradiol affects the structure and function of the hippocampus differentially in males and females. For example, estradiol added to rat hippocampal slices increases the spike amplitude of the field potential recorded

from the CA1 region after Schaffer collateral stimulation in tissue of male, but not female, origin (Teyler et al., 1980). Estradiol promotes cell survival in the hippocampus when administered during the axon-extension phase of cell maturation in males (Ormerod et al., 2004), but in females may promote survival when administered at any maturation stage (Ormerod et al, 2003). In addition, estradiol increases CA1 spine synapse density in female (MacLusky et al., 2005) but not in male rats (Leranth et al., 2003). Although estradiol protects both male and female rats against neurodegeneration caused by ischemia, an animal model of stroke (for reviews, see McCullough and Hurn, 2003; Merchenthaler et al., 2003; Gibson et al., 2006), this neuroprotective effect is stronger in males (Heyer et al., 2005). In the present thesis I examined the effects of estradiol on cell proliferation, survival, and death in the hippocampus (Chapter 2), the estradiol concentration in a variety of brain regions (Chapter 3), and on performance in a fear conditioning task (Chapter 4) in both males and females.

Adult neurogenesis in the hippocampus

In Chapter 2, I explore the effects of estradiol in both males and females on adult hippocampal neurogenesis. A striking feature of the hippocampus of the adult mammalian brain is its capacity for neurogenesis throughout life. Although the brain for decades was thought incapable of self-renewal, in the 1960s Joseph Altman produced evidence that dividing and young cells were present in the adult brain (e.g. Altman and Das, 1965; Altman, 1966). At that time, however, there was little

evidence that these new cells were actually neurons. New techniques have since become available - including detection of particular endogenous proteins in individual cells, electron microscopy, and single-cell electrophysiological recordings that have allowed the determination of cellular phenotype and conclusively demonstrated that new neurons are generated and become functional in the adult brain. In the adult mammalian brain, both the subventricular zone and the subgranular zone of the dentate gyrus within the hippocampus generate new neurons. These new neurons subsequently mature and ultimately integrate themselves into existing circuitry as mature neurons, the subventricular cells in the olfactory bulb (Carlén et al., 2002; Petreanu and Alvarez-Buylla, 2002; Belluzzi et al., 2003) and the subgranular cells in the granule cell layer of the dentate gyrus (van Praag et al., 2002; Kempermann et al., 2004). Although cells generated in these regions share a number of characteristics, they are regulated by different factors and involved in different behaviours (e.g. Banasr et al., 2004; Lee et al., 2008; Niidome et al., 2008). I will focus in this thesis on the new cells generated in the hippocampus. This structure both provides the neural foundation for the formation of memory for spatial, contextual and relational information (Eichenbaum 2004), and is compromised in neurodegenerative and neuropsychiatric diseases that show sex differences in disease incidence and treatments (including Alzheimer's Disease and depression).

Most of the cells generated in the adult hippocampus are not stem cells, which divide with a relatively low frequency and an unlimited number of times, but

instead are progenitor cells, dividing relatively frequently but a limited number of times, and restricted to production of glial or neuronal precursors (Gage, 2000; van der Kooy and Weiss, 2000; Seaberg and van der Kooy, 2003). The oldest and currently most common method of identifying newly synthesized cells involves the use of thymidine analogues that can be detected with specific antibodies. Of these, the one most commonly used in recent neurogenesis research has been 5-bromo-2deoxyuridine (BrdU; Packard et al., 1973; Miller and Nowakowski, 1988; Hayes and Nowakowski, 2000; Kee et al., 2002). The number of times a thymidine analogue is administered, the timing between subsequent administrations, and the dose used can be modified to suit particular experimental goals. A single injection of a relatively high but non-toxic dose of BrdU (200-300 mg/kg in rats: Cameron and McKay, 2001), followed by tissue collection shortly (30 min to 4 h) after BrdU administration can be used to determine the total number of cells synthesizing DNA at a particular time. Collecting tissue after completion of one cell cycle (24.7 h in the adult rat subgranular zone Cameron and McKay, 2001) allows for examination of daughter cells produced at the time of BrdU administration.

BrdU is available for incorporation into cells in the process of synthesizing their DNA for a maximum of 2 h after injection (Packard et al., 1973; Nowakowski et al., 1989; Boswald et al., 1990; Cameron and McKay, 2001), so multiple injections spaced up to two hours apart over the estimated length of one cell cycle should label every cell proliferating in the brain. However, multiple injections decrease the accuracy of the estimated birthdate of any particular cell, as multiple injections will

label a heterogeneous population of cells and may increase the possibility of BrdU toxicity (Taupin, 2007). If administered over days and followed by a short period before tissue collection, multiple injections may result in labelling of proliferating, immature, and maturing cells. If an experimental treatment differentially affects cell proliferation and cell survival, multiple BrdU injections can prevent accurate description of treatment effects. BrdU is permanently incorporated into the DNA of each cell undergoing DNA synthesis at (and about) the time of administration, and can thus be used to examine those cells at much later time points, up to years later (e.g. Eriksson et al., 1998; Dayer et al., 2003; Kempermann et al., 2003). It can be detected by applying antibodies against BrdU that have a fluorescent dye molecule attached to them (or applied via a secondary antibody against the first), and exciting the dye molecules with a specific wavelength of light, causing labelled cells to emit visible fluorescence.

Although thymidine analogues have proven useful in identifying when and where new cells are produced, exclusive use of these compounds does not necessarily permit identification of cellular phenotype. They can be combined with morphological stains to identify, for example, BrdU cells that are in a particular stage of mitosis or are in the process of dying (e.g. pyknotic). Alternatively, cell proliferation and neuronal production can also be quantified using endogenous proteins found in specific cell types, or present at particular stages of cell maturation or stages of the cell cycle. For example, the Ki-67 antigen is normally present in all cells throughout most of the cell cycle, from the early G1 phase up until the cell has

exited the cell cycle and entered G0, and can thus be used to quantify cell proliferation (Scholzen and Gerdes, 2000; Kee et al., 2002; Schmidt et al., 2003). Doublecortin (DCX) is an endogenous microtubule-associated protein present in immature, migrating neurons (Francis et al., 1999; Gleeson et al., 1999; Kempermann et al., 2003; Rao and Shetty, 2004). Such endogenous markers can both provide information about the phenotype of a particular cell and allow for a rough estimate of a labelled cell's stage of maturation. The use of endogenous proteins rather than injected compounds allows avoidance of the major potential problems involved in injection of markers. For example, it allows examination of cells collected from wild animals or post-mortem human tissue, for which injection of exogenous compounds would be unsuitable for practical, theoretical, or ethical reasons (Boonstra et al., 2001; Kee et al., 2002; Rao and Shetty, 2004; Eisch and Mandyam, 2007). Another concern may be whether group differences observed in labelled cell numbers are real, or due to differences in the permeability of the bloodbrain barrier to BrdU, especially if BrdU is administered after experimental treatment has begun (Taupin, 2007). As endogenous proteins are already present in the brain, effects on the permeability of the blood-brain barrier do not affect the ability to detect them.

Combining a variety of techniques to detect new neurons and measure their properties has led to the description of specific characteristics of these cells. New neurons extend axons to the CA3 pyramidal cell field, reaching the target area within 10-12 days after cell division (Stanfield and Trice, 1988; Hastings and Gould, 1999;

Markakis and Gage, 1999; Zhao et al., 2006). These new cells become surrounded with synaptic markers and form spines (Zhao et al., 2006). Immature neurons generated in the adult hippocampus are depolarized by currents of very small amplitude (Schmidt-Hieber et al., 2004) and GABA exposure makes young neurons more excitable, rather than inhibiting neuronal activity as it does in mature neurons (Wang et al., 2000; Ambrogini et al., 2004). Thus long-term potentiation, a putative cellular mechanism for learning in the hippocampus, is more easily induced in young immature neurons than it is in older neurons (Wang et al., 2000; Ambrogini et al., 2004). The effect of these adult-generated cells on the processing of new information may be time-limited, as around 2 weeks after division, individual neurons start to become less readily excitable (Espósito et al., 2005). Within only 1-2 months the new neurons are electrophysiologically comparable to mature granule neurons (van Praag et al., 2002). Nevertheless, new neurons, because they are more excitable when young, may function to enhance plasticity and enhance the efficiency of the hippocampus. Indeed recent evidence suggests that new neurons are preferentially recruited during a hippocampus-dependent task (Kee et al., 2007).

Neurogenesis and cognition

The hippocampus is important for the formation of particular types of memory (Jarrard, 1993; Sanders et al., 2003; Shors, 2004; Rolls and Kesner, 2006), so it seems plausible that the addition of new, readily excitable neurons to such a structure could have a profound influence on performance in a variety of learning

and memory tasks. One of the most convincing indications that this may be the case is that reduced neurogenesis in the hippocampus, using a variety of methods (MAM, irradiation, and inducible knockouts), reduces performance in certain forms of hippocampus dependent learning and memory. Methylazoxymethanol (MAM) administered systemically reduces the number of dividing cells in the hippocampus and impairs hippocampus-dependent trace conditioning but not delay conditioning that does not require an intact hippocampus (Shors et al., 2001; Shors et al., 2002). Several studies using irradiation to reduce neurogenesis in the adult hippocampus have revealed that contextual fear conditioning and non-match to sample (both hippocampus-dependent tasks) are impaired when the number of new cells is reduced (Winocur et al., 2006). Selective removal of dividing progenitor cells by genetic manipulation also impairs performance in contextual fear conditioning (Saxe et al., 2006). Although reduction of neurogenesis does not impair hippocampusdependent spatial learning in the short term (Shors et al., 2002; Snyder et al., 2005; Saxe et al., 2006), it does disrupt long-term spatial memory (Snyder et al., 2005). This again suggests that the new cells are important for hippocampus-dependent learning, but perhaps only required for encoding spatial information in the long term. There is also evidence that the newly-generated cells may serve specific functions during learning or recall of certain tasks, distinct from the function served by mature granule cells. For example, both cell proliferation and total cell number in the dentate gyrus is negatively correlated with a rat's locomotor activity in response to exposure to novelty (Lemaire et al., 1999). The hippocampus is involved in the suppression of attention to irrelevant stimuli (Gray and McNaughton, 1982; Feldon

and Weiner, 1992; Thinus-Blanc et al., 1996), so the new cells may serve an important role in the recognition of novelty and in producing an appropriate response in a novel context. Adult-generated cells are also preferentially active in neuronal networks activated during testing in a spatial memory task (Kee et al., 2007). This suggests that these cells may serve a function in the encoding or recall of spatial information that is distinct from that served by more mature granule neurons. It is also possible that the young cells may be important for encoding information about the timing of specific memories (Aimone et al., 2006). The dentate gyrus allows the differentiation of similar memories by production of distinct, nonoverlapping representations in the CA3 region (McNaughton and Morris, 1987; Treves and Rolls, 1992, 1994; Bakker et al., 2008). However, memories that occur temporally close to each other do remain associated (Shum, 1998; Brown and Schopflocher, 1998; Burt et al., 2003). Immature neurons in the dentate gyrus are electrophysiogically distinct from mature cells (van Praag et al., 2002; Schmidt-Hieber et al., 2004; Song et al., 2005), but do receive and supply synaptic inputs before fully mature (Hastings and Gould, 1999; Esposito et al., 2005; Zhao et al., 2006). As the set of newlygenerated neurons available at the time of encoding would be similar for memories formed in close temporal proximity, their involvement in encoding could help tie memories together in time (Aimone et al., 2006). Which of these proposed functions is served by adult-generated neurons remains to be determined. In chapter 4, I explore the functional consequences of manipulations of systemic estradiol concentrations in both males and females on contextual fear conditioning. Successful contextual fear conditioning requires the hippocampus (Fanselow, 2000;

Rudy and O'Reilly, 2001; Sanders et al., 2003) and estradiol and sex of subject influence contextual fear conditioning (Maren et al., 1994; Markus and Zecevic, 1997; Altemus et al., 1998; Gupta et al., 2001; Wiltgen et al., 2001). These features make it a useful task to examine when considering the functional consequences of the effects of estradiol on cells in the male and female hippocampus.

Estradiol and adult neurogenesis in the hippocampus

Naturally occurring fluctuations in gonadal hormones transiently influence hippocampal neurogenesis in adult females (for review see Galea et al., 2008). For example, a sex difference in cell proliferation favouring females has been found after a short survival period (2 days), though by day 14 after BrdU administration (cell survival) this sex difference is no longer evident (Tanapat et al., 1999). Adult female rats have 50% greater levels of cell proliferation in the dentate gyrus during proestrus (the high estrogen stage) compared to adult female rats in either the estrous or diestrous stage when estrogen levels are much lower, and ovariectomy dramatically reduces cell proliferation (Tanapat et al., 1999). Replacing ovariectomized females with high estradiol (10 μ g), which results in circulating levels of estradiol seen on the morning of proestrus (Viau and Meaney, 1991; Sohrabji et al., 1994), eliminates this ovariectomy-induced reduction in cell proliferation (Tanapat et al., 1999; Banasr et al., 2001; Ormerod and Galea, 2001; Mazzucco et al., 2006). The number of BrdU-labeled cells in an adult female rat hippocampus 1-2 weeks after BrdU injection is also highest if BrdU is administered during proestrus,

but the number of those cells still present at 21 days is independent of the estrous phase in which the cells were labelled (Tanapat et al., 1999). Furthermore, ovariectomy does not appear to affect cell proliferation over the long-term (Tanapat et al., 2005; Lagace et al., 2007; Green and Galea, 2008). This may suggest that endogenous fluctuations in estradiol have effects on cell survival as the higher proliferation rate in females than in males suggests that the overall survival rate of new cells generated in females is actually lower than that in males (Tanapat et al., 1999). The effects of estradiol over the long-term may also differ from those in the shorter term. In reproductively-active female meadow voles, which have a persistently high endogenous level of estradiol, cell survival 5 weeks after ³Hthymidine injection was lower than that in reproductively inactive female voles (with persistently low endogenous estradiol levels; Ormerod and Galea, 2001). Furthermore, acute exposure to estradiol initially enhances cell proliferation (within 4 hours) but subsequently suppresses cell proliferation within 48 hours in the dentate gyrus of adult female rats and meadow voles (Ormerod and Galea, 2001; Ormerod et al., 2003). Conversely administration of estradiol for 3 days does not alter cell proliferation in the dentate gyrus of adult female meadow voles, although the direction of means favours a suppression (Fowler et al., 2003). Thus the length and timing of exposure to estradiol can significantly influence its effects of hippocampal cell proliferation in the adult female rodent. The effects of long-term administration of estradiol on adult neurogenesis are not well known and the effects of estradiol on adult hippocampal neurogenesis in the male rodent have not been systemically studied and are studied in Chapter 2.

Hippocampus-dependent cognition: sex differences and effects of estradiol

The performance of males and females, both human and non-human, differ on a variety of learning and memory tasks (for review, see Shors et al., 2000). In general, males tend to outperform females on tasks that are considered hippocampus-dependent (for reviews, see Galea et al., 1996; Luine, 2008) or amygdala-dependent (Fernandes et al., 1999; Aguilar et al., 2003; Lopez-Aumatell et al., 2008), whereas females tend to outperform males in working memory tasks that depend on the prefrontal cortex (Eals and Silverman, 1994; McBurney et al., 1997; Ghi et al., 1999). Male deer mice and meadow voles learn to find the hidden platform in a Morris water task faster than females when gonadal hormones levels are relatively high (Galea et al., 1994; Galea et al., 1995). Intact male mice learn anxiety- or fear-inducing tasks faster and with stronger retention than females (Fernandes et al., 1999; Aguilar et al., 2003; Lopez-Aumatell et al., 2008). As I was interested in linking sex differences in hippocampal neurogenesis to those in cognition, I chose to focus on a hippocampus-dependent task.

For this thesis, contextual fear conditioning was selected as a useful behavioural measure to determine the functional consequences of manipulations of systemic estradiol concentrations in both males and females (Chapter 4). Contextual fear conditioning is dependent on the integrity of the hippocampus and amygdala, and can be learned in a single trial (Vazdarjanova and McGaugh, 1999; Huff and Rudy, 2004). Performance in a contextual fear conditioning task is related

to ovarian hormone levels, as females in proestrus spend less time freezing than females in estrus (Markus and Zecevic, 1997). Furthermore, ovariectomized rats freeze more than estradiol-treated females, but to the same extent as intact males (Gupta et al., 2001).

Typical contextual fear conditioning paradigms involve allowing rats to explore a conditioning chamber and then administering a mild footshock. The subsequent association of the conditioning context with the footshock results in trained rats freezing when re-exposed to the training context (e.g. Vazdarjanova and McGaugh, 1999; Gupta et al., 2001; Jasnow et al., 2006). This task is typically considered dependent on the hippocampus, because damage to the hippocampus in general reduces the amount of freezing in response to re-exposure to the training context. However, the specific effects of hippocampal damage depend on the time of damage in relation to training, how damage is caused, and the lesion site (Rudy et al., 2004). For example, in the dorsal hippocampus, electrolytic damage (Maren et al., 1997), blockade of NMDA receptors (Young et al., 1994; Bast et al., 2003), or blockade of muscarinic cholinergic receptors (Gale et al., 2001; Wallenstein and Vago, 2001) prior to training produces a behavioural deficit. However, excitotoxic lesions of the dorsal hippocampus prior to training do not impair contextual fear conditioning (Frankland et al., 1998; Richmond et al., 1999; Cho et al., 1999; Rudy et al., 2002), although any damage shortly after training consistently impairs contextual fear conditioning (Kim and Fanselow, 1992; Maren et al., 1997; Frankland et al., 1998; Anagnostaras et al., 1999). This may be because the hippocampus

forms a representation of the context which is then associated by the amygdala with a stimulus such as footshock, a process which competes with the formation of associations between individual features of the context and a footshock (Rudy et al., 2004). Under this model, the hippocampus both forms a representation of the context and inhibits formation of associations between individual features and a footshock. Affecting the ability of the hippocampus to acquire a conjunctive representation of the context while leaving intact its ability to inhibit feature-based associations impairs contextual fear conditioning (Young et al., 1994; Maren et al., 1997; Gale et al., 2001; Wallenstein and Vago, 2001; Bast et al., 2003). However, when the hippocampus is prevented from functioning at all, so that it can neither store a contextual representation nor impair the formation of associations with individual features, then behavioural deficits are not observed (Frankland et al., 1998; Richmond et al., 1999; Cho et al., 1999; Rudy et al., 2002).

The amygdala, on the other hand, seems to act as a storage site for the association between the contextual representation within the hippocampus and the footshock. The typical fear conditioning procedure fails to result in substantial freezing in response to the context if the time given to explore the context prior to shock is very short (Fanselow, 1986). A modification of this procedure provides pre-exposure to the training context without the administration of shock, with an immediate footshock delivered in the training context on a subsequent training day (Huff et al., 2005). It is important to note that, unlike the traditional contextual fear conditioning paradigm, the context pre-exposure procedure absolutely requires the hippocampus for successful training. Damage to the hippocampus blocks the initial

formation of the contextual representation prior to footshock, the reactivation of that representation immediately prior to footshock, and the recognition of the context during testing (Rudy et al., 2004). However, as with any fear conditioning paradigm the amygdala is important for successful conditioning. Inactivation of the basolateral amygdala either before or immediately after context pre-exposure, or immediately before (but not after) shock training, impairs performance on this task (Wilensky et al.,2000; Huff and Rudy, 2004; Rudy et al., 2004). However, inhibition of protein synthesis in the basolateral amygdala immediately after pre-exposure to the context has no effect on freezing behaviour after training (Huff and Rudy, 2004), but impairs memory for cue-shock associations (Schafe and LeDoux, 2000; Huff and Rudy, 2004). Taken together, this suggests that the basolateral amygdala both is critical for the acquisition of a context-shock association, and modulates memory consolidation in the hippocampus (Rudy et al., 2004). Nevertheless, the hippocampus is required for this task, and performance should be sensitive to the state of the dentate gyrus in particular. This is because a vital part of the task initial, automatic encoding of a context during pre-exposure – depends on the dentate gyrus. Although connections directly from the entorhinal cortex to the CA3 region of the hippocampus are important for retrieval, the mossy fiber pathway from the dentate gyrus to CA3 is vital for initial encoding of contextual information (Lassalle et al., 2000; Lee and Kesner, 2004). Pre-exposure to a context in the absence of aversive cues thus dissociates the involvement of the amygdala (involved in formation of the context-shock association) from that of the dentate

gyrus (processing contextual information for encoding). Thus, performance on this task should therefore be sensitive to changes within the dentate gyrus.

Experimental outline

Given the links presented above between estradiol, adult hippocampal neurogenesis, learning and memory, I performed a set of experiments aimed at determining the effects of repeated estradiol treatment in rats. Because there is reason to believe that both males and females should be affected by estradiol treatment, but perhaps in different ways, I used both males and females throughout. In the work described in Chapter 2, I administered estradiol to gonadectomized rats over 15 days and examined measures of cell proliferation, neurogenesis, and cell death in the hippocampus. In Chapter 3, I describe the estradiol concentrations in both the serum and brain of animals treated with the same estradiol administration protocol. Chapter 4 describes work aimed at determining whether estradiol affects performance on a fear conditioning task that is particularly sensitive to hippocampal function. Finally, in Chapter 5 I summarize the findings of this work and discuss the implications and possibilities for future studies.

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2 REPEATED ESTRADIOL ADMINISTRATION ALTERS DIFFERENT ASPECTS OF NEUROGENESIS AND CELL DEATH IN THE HIPPOCAMPUS OF FEMALE, BUT NOT MALE, RATS¹

Estradiol has been shown to have multifaceted neuroprotective effects: estradiol enhances neurotrophic factors (Gibbs, 1999; Scharfman et al., 2005), enhances hippocampal neurogenesis (Ormerod et al., 2003a), and suppresses cell death (Wise et al., 2001). There are sex differences in the efficacy with which estradiol protects neurons (Murray et al., 2003; Heyer et al., 2005), suggesting that sex can play a significant role in expression of hormonal neuroprotection.

Neurogenesis continues throughout life in specific regions of the mammalian brain, including the dentate gyrus of the hippocampus. Neurogenesis includes the related processes of cell proliferation (production of new cells) and cell survival (cells surviving to maturity). In adult rodents, there are sex differences in the amount of hippocampal cell proliferation and cell survival (Galea and McEwen, 1999; Westenbroek et al., 2004), and in the effects of reproductive status and stress on neurogenesis in the hippocampus (Galea and McEwen, 1999; Falconer and Galea, 2003; Westenbroek et al., 2004). For example, female meadow voles show seasonal changes in hippocampal cell proliferation, but no such changes exist in male meadow voles (Galea and McEwen, 1999). In addition, the effects of both

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acute and chronic stress on hippocampal neurogenesis differ between male and female rats (Falconer and Galea, 2003; Westenbroek et al., 2004). Although one study failed to find evidence for a significant sex difference in basal rates of hippocampal cell proliferation in mice, the direction of the means favored females (Lagace et al., 2007) and they did not examine females in proestrus against males, which is the only time a sex difference is observed in laboratory rats (Tanapat et al., 1999). Generally, when sex differences exist, gonadal hormones regulate these differences. Although most of the literature to date has focused on the effects of estradiol on cell proliferation in *female* rodents (Tanapat et al., 1999; Ormerod and Galea, 2001; Ormerod et al., 2003b; Ormerod et al., 2003a; Tanapat et al., 2005), estradiol can also affect the male brain (Nelson and Bulun, 2001; Ormerod et al., 2004; Veiga et al., 2005). Thus, in the present experiment we examined the influence of estradiol on adult hippocampal neurogenesis in both male and female rats.

Acute estradiol enhances cell proliferation and decreases cell death in the dentate gyrus of adult female rats (Tanapat et al., 1999; Banasr et al., 2001; Ormerod et al., 2003a). Fewer studies have examined the effects of repeated or chronic estradiol treatment on hippocampal neurogenesis. Chronic estradiol administration does not affect cell proliferation in the dentate gyrus of adult female rats (Tanapat et al., 2005). However, estradiol administration doubles the number of new neurons in the dentate gyrus of male meadow voles, although only when estradiol is administered throughout the axon extension phase of these new neurons

(Ormerod et al., 2004). The effects of repeated or chronic administration in particular are important to establish because clinical applications of estradiol, including estrogen replacement therapy for postmenopausal women, typically involve long-term administration rather than a single, acute dose. To date, no studies have examined the effects of repeated administration of estradiol on cell survival in adult female rodents. Furthermore, there have been very few studies directly comparing neurogenesis between the sexes (Galea and McEwen, 1999; Tanapat et al., 1999; Lagace et al., 2007) and no studies examining sex differences in the effects of estradiol on neurogenesis in the hippocampus. If we are to use our knowledge of how adult neurogenesis is regulated to develop strategies to repair neuron loss in neurodegenerative diseases, it seems reasonable that we should also test neurogenic regulatory factors, such as gonadal hormones, in both male and female rats. Cell survival can be measured directly, as the survival of cells labeled during cell division, or indirectly, using markers of cell death. The former measure allows for determination of a specific timeline in the life cycle of a relatively new cell, whereas the latter allows for a measure of overall cell death, including both new neurons and older cells. In the present study, we examined the effects of repeated administration of estradiol on cell proliferation, survival of new cells, and cell death in the dentate gyrus of gonadectomized adult male and female rats. Cell proliferation was quantified as the number of cells containing Ki-67, an endogenous protein present in actively cycling cells (Kee et al., 2002; Schmidt et al., 2003; Zacchetti et al., 2003). Cell survival was quantified as the number of cells containing bromodeoxyuridine (BrdU), a thymidine analog that incorporates itself into the DNA

of cells during the synthesis phase of the cell cycle. The survival of young neurons was further quantified as the number of cells with processes containing doublecortin (DCX), an endogenous microtubule-associated protein present in immature neurons (Gleeson et al., 1999; Rao and Shetty, 2004). Cell death was measured by counting pyknotic (dying) cells, counting cells expressing activated caspase-3, an effector caspase that is activated in cells in the early stages of apoptosis (Porter and Jänicke, 1999), or by counting the number of cells immunoreactive with Fluoro-Jade B, an anionic fluorescein derivative that labels degenerating neurons (Schmued et al., 1997; Schmued and Hopkins, 2000). We chose repeated administration of estradiol as, to date, no studies have investigated the effects of repeated estradiol administration on cell survival or cell death in male and female rodents. We chose to give 15 d of exposure as 15 days is commensurate with the time when the majority of new cells are beginning to express mature neuronal markers (Ormerod et al., 2004, Cameron et al., 1993). We expected that estradiol would decrease cell death and alter neurogenesis (cell survival and cell proliferation), and that there would be sex differences in these effects.

Experimental procedures

All experiments were conducted in accordance with the Canadian Council on Animal Care guidelines regarding appropriate treatment of animals and were approved by the University of British Columbia. Every effort was made to minimize the number of animals used per group and to minimize the suffering of animals used throughout all experimental procedures.

Experimental subjects

Ten male and ten female adult (80-90 days old) Sprague-Dawley rats (Charles River Canada, Québec, Canada) were kept on a 12-hour light-dark cycle (lights on at 0700), singly housed in opaque polyurethane bins (48 x 27 x 20 cm) with aspen chip bedding and Purina rat chow and tap water ad libitum.

Procedure

All subjects (n = 5 per group) were gonadectomized under halothane anaesthesia one week after their arrival in the colony. Briefly, all rats were anaesthetized using 2-bromo-2-chloro-1,1,1,-trifluoroethane (halothane, MTC Phrmaceuticals, Cambridge, Canada) using an initial flow rate of 4% and a maintenance flow rate of 2% during surgery, All females were bilaterally ovariectomized through bilateral flank incisions, and all males were bilaterally castrated through an incision in the scrotal sac. Eight days after surgery (Day 0), all rats received an i.p. injection of BrdU (200 mg / kg: Sigma-Aldrich, Oakville, Ontario, Canada) dissolved in 0.9% saline. Injecting rats with BrdU prior to estradiol administration allows the examination of the effects of estradiol on the survival of new cells independently of its effects on cell proliferation. Beginning 24 hours after BrdU injections (Day 1), rats received s.c. injections of either estradiol benzoate (EB, dissolved in 0.1 mL sesame oil) or sesame oil (vehicle, 0.1 mL) each day for 15 consecutive days. The male rats used were approximately 1.5X the size of the females (mean male body mass: 450 g; mean female body mass: 282 g), so to maintain similar EB dose per unit body mass, and thus present the brain with a

similar plasma concentration of EB, females were given 10 μ g EB per injection, while males were given 15 μ g EB per injection. All animals were injected between 1000 h and 1230 h each day. A dose of 33 μ g/kg EB was used to match previous studies investigating the effects of estradiol on hippocampal neurogenesis, spine density and sexual behaviour in the female rodents (e.g. Woolley and McEwen, 1993; Tanapat et al., 1999; Rössler et al., 2006). Castrated male rats have recently been given a similar dose of EB as adults (Spritzer and Galea, 2007).

Tissue processing

On the day following the last injection (Day 16), rats were anaesthetized using an overdose of sodium pentobarbital (Euthanyl, MTC Pharmaceuticals, Cambridge, ON), and perfused via cardiac puncture with 0.9% saline followed by 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2). The brains were removed and post-fixed for 24 hours in 4% formaldehyde in phosphate buffer, and stored in 0.1 M phosphate-buffered saline (PBS, pH 7.4) until sectioning. Brains were sectioned into 40 µm coronal slices using a vibrating microtome VT1000S (Leica) and stored in 0.2% sodium azide in PBS until staining. For Ki-67, BrdU, cresyl violet and activated caspase-3 staining procedures, every tenth section through the entire hippocampus was stained and counted; for DCX and Fluoro-Jade staining, every twentieth section was used. For all staining with fluorescent antibodies, stained sections were mounted on plain glass microscope slides and coverslipped using PVA-DABCO.

Cell proliferation was quantified as the number of cells containing Ki-67 (Ki-67+ cells), an endogenous protein present in actively cycling cells (Fig. 2.2a). New cell survival was quantified as the number of cells containing BrdU (BrdU+ cells) 16 days after BrdU administration (Fig. 2.2b); BrdU was injected prior to estradiol administration, to focus on the influence of estradiol on survival independent of its well-known effects on cell proliferation. Young neuron production was quantified as the number of cells containing DCX (DCX+ cells), an endogenous protein present in migrating neurons (Fig. 2.2c). Cell death was measured by counting pyknotic cells (Fig. 2.2d), and by counting cells expressing activated caspase-3 (ActC3+ cells), an effector caspase that is activated in cells in the early stages of apoptosis (Fig. 2.2e), or immunoreactive with Fluoro-Jade B dye, which stains degenerating neurons (Fig. 2.2f).

Measures of cell death

Pyknotic cells were visualized with cresyl violet staining. For cells containing activated caspase-3, the selected sections were rinsed three times in 0.1 M PBS for 10 minutes each, then incubated with rabbit anti-activated-caspase-3 IgG primary antibody (AbCam, Cambridge, MA) in 0.3% Triton X-100 in PBS (1:200) at 4°C for 48 hours. Sections were again tinsed three times in PBS, then incubated with donkey anti-rabbit FITC-conjugated IgG secondary antibody (Jackson Immunoresearch, West Grove, PA) in 0.3% Triton X-100 in PBS (1:200) at 4°C for 4 hours.

To observe degenerating neurons, tissue was rinsed three times in PBS, then mounted on slides and left to air-dry for 48 hours at room temperature. The slides were soaked in 0.06% potassium permanganate for 15 minutes, followed by soaking for 2 minutes in deionized water. The slides were then soaked in 0.0004% Fluoro-Jade solution (Chemicon, Temecula, CA) in 0.1% acetic acid, rinsed three times in deionized water, dried rapidly, cleared in xylene, and coverslipped with Permount (Fisher Scientific Canada, Ottawa, ON).

Measures of cell proliferation and new cell survival

To observe cells containing Ki-67, sections were rinsed three times in PBS, then incubated with mouse anti-Ki-67 IgG primary antibody (Vector Laboratories, Burlington, ON) in 0.3% Triton X-100 in PBS (1:200) at 4°C for 48 hours. The sections were again rinsed three times in PBS, then incubated in donkey anti-mouse Cy3-conjugated IgG secondary antibody (Jackson Immunoresearch, West Grove, PA) in 0.3% Triton X-100 in PBC (1:200) at 4°C for 2 hours.

To observe cells containing BrdU (new cell survival), sections were rinsed three times in PBS, then incubated in 2 N hydrochloric acid for 30 minutes at 37°C and rinsed three times in PBS. The tissue was incubated with 3% normal donkey serum in 0.3% Triton X-100 in PBS for 30 minutes, then with mouse anti-BrdU IgG primary antibody (Roche, Laval, QC) in 0.3% Triton X-100 in PBS (1:200) at 4°C for 48 hours. The sections were again rinsed three times in PBS, then incubated with donkey anti-mouse Cy3-conjugated IgG secondary antibody (Jackson

Immunoresearch, West Grove, PA) in 0.3% Triton X-100 in PBS (1:200) at 4°C for 10 hours.

To observe cells containing DCX (young neuron production), sections were rinsed three times in PBS, then incubated in 0.05% Triton X-100 in sodium citrate buffer (pH 6.0) for 30 minutes at 85°C. The tissue was allowed to cool to room temperature, then incubated with 3% normal donkey serum in 0.3% Triton X-100 in PBS for 30 minutes, then with rabbit anti-DCX IgG primary antibody (Cell Signaling Technologies, Danvers, MA) in 0.3% Triton X-100 in PBS (1:200) at 4°C for 18 hours. The sections were again rinsed three times in PBS, then incubated with donkey anti-rabbit Alexa488-conjugated IgG secondary antibody (Invitrogen Canada, Burlington, ON) in 0.3% Triton X-100 in PBS (1:200) at room temperature for 18 hours.

Cell counting

All slides were coded prior to analysis so the experimenter was blind to treatment condition and sex. Cells containing Ki-67 (Ki-67+), BrdU (BrdU+), or active caspase-3 (active caspase-3+), or having a pyknotic morphology were counted throughout the granule cell layer, including the subgranular zone (SGZ, defined as an area 50 μ m wide adjacent to the inner edge of the granule cell layer, e.g. Palmer et al., 2000) and hilus, provided their entire nucleus was visible (Fig. 2.1). Cells with long processes and containing DCX (DCX+) or cells that were immunoreactive with Fuoro-Jade B dye (FJ+) were counted throughout the granule

cell layer, including the SGZ but not the hilus. All counting was done using a fluorescent E600 microscope (Nikon) either under brightfield (pyknotic cells) or fluorescent illumination (Ki-67+, BrdU+, DCX+, active caspase-3+, or FJ+ cells). Stereological estimates of the total number of cells in the dentate gyrus were generated by the exclusion of cells touching the uppermost focal plane of each slice and multiplying the total counts by 10 (Gundersen et al., 1988; West et al., 1991).

Figure 2.1: Bright-field photomicrograph showing the subfields of the dentate gyrus of the hippocampus. Cells were counted in the hilus and the granule cell layer (GCL), which included the subgranular zone (SGZ). Cells expressing Ki-67, BrdU, or activated caspase-3, or cells expressing a pyknotic morphology, were counted throughout the GCL (including the SGZ) and the hilus.



The area of the granule cell layer (including the SGZ) and hilus was measured in cresyl-stained sections using the digitizing software Act-1 (Nikon), and the volume for each area was calculated separately using Cavalieri's principle (Gundersen et al., 1988). Because there were differences between groups in dentate gyrus volume (Table 2.1), density scores for all cell counts were generated by taking the stereological estimates of cell number in either the granule cell layer (GCL) or hilus and then dividing this by the total volume of the respective region (GCLor hilus) to generate cell counts per mm³.

Table 2.1: Effects of sex and chronic estradiol treatment on dentate gyrus volume. The granule cell layer (GCL) and hilus in females were generally smaller than in males, and the hilus was larger than the granule cell layer in all groups. Data are shown as group mean \pm standard error of the mean. ^astatistically significantly different from the same area in males receiving the same treatment, p < 0.05; ^bstatistically significantly different from hilus volume, p < 0.05.

<u>Sex</u>	<u>Treatment</u>	Total GCL	<u>Total hilus</u>
		volume (mm ³)	volume (mm ³)
Female	Oil	$3.80 \pm 0.23^{a, b}$	7.36 ± 0.34 ^a
	Estradiol benzoate	3.72 ± 0.29 ^b	7.33 ± 0.50^{a}
Male	Oil	4.79 ± 0.23^{b}	9.27 ± 0.26
	Estradiol benzoate	4.29 ± 0.20^{b}	8.84 ± 0.57

Phenotyping of cells containing BrdU

To determine the phenotype of cells containing BrdU, sections were rinsed three times in PBS. The tissue was blocked in 0.1 M PBS/1% NDS/3% Triton X-100 for 30 min at room temperature, then incubated with mouse anti-NeuN (1:200, Chemicon, Temecula, CA) primary antibody in 0.3% Triton X-100 in PBS at 4°C for 48 hours. The tissue was rinsed in PBS, and then incubated with fluorescent donkey anti-mouse Alexa 488 (1:200, Invitrogen, Burlington, ON) secondary antibody for 4 hrs at room temperature in the dark; the stained tissue was kept in the dark for the remainder of the staining procedure. The tissue was rinsed three times in PBS, incubated in 4% formaldehyde at room temperature for 10 min, then incubated in 2N HCl at 37°C for 30 min. The tissue was rinsed in PBS again and blocked in 0.1 M PBS/1% NDS/3% Triton X-100 for 30 min at room temperature, then incubated in rat anti-BrdU primary antibody (1:200, Oxford Biotechnology, Oxfordshire, UK) at 4°C for 10 hours. The tissue was rinsed in PBS, incubated in donkey anti-rat Cy3-conjugated secondary antibody (1:200, Jackson Immunoresearch Laboratories, West Grove, PA) for 4 hrs at room temperature. Using a fluorescent microscope, 25 BrdU-labelled cells in each of 3 animals per treatment group were scored for co-expression with NeuN, indicating a neuronal phenotype (Mullen et al., 1992). Cell phenotype was verified on a subset of BrdUlabelled cells with confocal microscopy (Zeiss, Fig. 2.2g).

Figure 2.2: Representative photomicrographs of cells in the dentate gyrus of adult rats after 15 days of estradiol administration. (A) To quantify cell proliferation, we counted cells containing Ki-67. To quantify young cell survival, we counted cells containing (B) BrdU or (C) DCX 16 days after BrdU injection. To quantify overall cell death, we counted cells (D) expressing a pyknotic morphology, (E) containing active caspase-3 or (F) that were immuno-reactive with a Fluoro-Jade stain. (G) Tissue was also double-labelled with fluorescent antibodies against BrdU (red) and NeuN (green) to determine whether BrdU-labelled cells were neurons.

A <u>1</u>	Dμm	D	<u>10µm</u>
Hilus	,	Hilus	
<u> </u>		N	×
		El	
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B 10	Dμm	E	10µm
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đ	GCL	F	<u>10µm</u>
C 20	Dμm	G	20 µ m
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Hormone assays

Blood was collected from the right atrium just prior to perfusion on day 16. Whole blood was allowed to clot at 4°C for 24 hours, and then spun in a rotating centrifuge (8000 rpm for 8 minutes). The serum was collected and stored at -20°C until processing using a commercial colorimetric enzyme-linked immunosorbent assay (ELISA) kit (RDI, Flanders, NJ; cross-reactivity with other estrogens < 3%, intra-and inter-assay CV < 10%; analytical sensitivity 16 pg/mL). One millilitre of diethyl ether was added to 230 μ L of serum in borosilicate glass tubes to extract out the steroid hormones. The tubes were then centrifuged at 4°C (5 minutes at 1000 *g*) and frozen at -80°C for 20 minutes. The ether fraction was decanted, and then dried down under nitrogen at 37°C. The resulting crystalline hormones were re-dissolved in 75 μ L of stripped human serum and analyzed using the ELISA kit.

Data analyses

The densities of pyknotic cells and activated caspase-3+, BrdU+ and Ki-67+ cells were each analyzed using repeated-measures ANOVA, with sex (male, female) and treatment (oil, estradiol) as between-subjects factors, and area (granule cell layer, hilus) as the within-subjects factor. DCX+ and FJ+ cells were only counted in the GCL, so the densities of these cells were analyzed using two-way ANOVA, with sex and treatment as between-subjects factors. Data were further analyzed using the Newman-Keuls post-hoc test, or *a priori* tests subject to a Bonferroni correction. Spearman rank order correlations were used to correlate serum estradiol levels with histological measures for both treatment groups within each sex.

Results

Male rats have larger dentate gyrus volumes than female rats

Overall, as expected, the volumes of the granule cell layer and hilus of the hippocampus were larger in males than in females, and the hilus was larger than the granule cell layer, but there were no other significant effects (main effect of sex: F1.16 = 15.6, p = 0.001; main effect of area: $F_{1.16}$ = 819, p < 0.001; all other effects except for sex x area: p > 0.40; Table 2.1). There was a significant sex x area interaction effect in volume ($F_{1.16}$ = 11.0, p = 0.004), post-hoc tests revealed that males had larger hilar volume (p < 0.001) than females and a trend for a larger granule cell layer than females (p = 0.058; Table 2.1). In addition, total dentate gyrus (DG) volume, as determined by adding together the GCL and hilus volume for each animal was calculated and analyzed with an ANOVA. DG volume was larger in males than in females ($F_{1.16}$ = 15.8, p = 0.001) but there was no significant main effect of estradiol treatment, and no significant sex x treatment interaction effect (all p's > 0.40). To account for the sex differences in dentate gyrus volumes, cell counts were analyzed as total counts or as densities (total cells per unit volume, with cells in the GCL and SGZ divided by GCL+SGZ volume, and cells in the hilus divided by hilus volume).

Estradiol increases the density of proliferating cells in the granule cell layer of female, but not male, rats

There was a significant main effect of area ($F_{1,16}$ = 266; p < 0.001), indicating that there was a higher density of Ki-67+ cells in the granule cell layer than in the hilus (see Table 2.4 for counts of cells in the hilus). There were no other significant main or interaction effects on the density of Ki-67+ cells in the dentate gyrus ($p \ge 1$ 0.17). Although the ANOVA revealed no significant sex x treatment interaction effect ($F_{1,16}$ = 1.08; p = 0.31), a priori tests indicated that estradiol treatment increased the density of Ki-67+ cells in the granule cell layer of female (p = 0.027) but not male rats (p = 0.73; Fig. 2.3). An ANOVA on the total number of Ki-67+ cells found similar results to those found with density; there was a higher number of Ki-67+ cells in the granule cell layer than in the hilus (main effect of area: $F_{1,16} = 230$; p < 0.001), but no other significant main or interaction effects on the number of Ki-67+ cells in the dentate gyrus (p > 0.23). Within each sex, the effects of estradiol treatment on the total number of Ki-67+ cells were in the same direction as the effects on the density of Ki-67+ cells in the granule cell layer, but the *a priori* tests revealed a slight trend for estradiol treatment to increase Ki-67+ cells in females (Table 2.2; males: p = 0.62; females: p = 0.07).

Figure 2.3: Repeated estradiol benzoate administration increased cell proliferation in the granule cell layer of the dentate gyrus in adult female but not adult male rats, as indicated by the density of cells expressing Ki-67 per unit volume of the granule cell layer (including the subgranular zone). Bars show group mean + standard error of the mean. * statistically significantly different (p < 0.05) from oil-injected controls.



Table 2.2: Total counts of Ki-67+ and BrdU+ cells were lower in the hilus than in the granule cell layer (GCL) (p < 0.01). Treatment with estradiol benzoate (EB) affected subsets of cells in females, but not in males. Data are shown as group mean ± standard error of the mean. * statistically significantly different from oil-injected controls (p < 0.05).

<u>Cell type</u>	<u>Sex</u>	<u>Treatment</u>	<u>Total number of</u>	Total number of
			cells in GCL	<u>cells in hilus</u>
Ki-67+	Female	Oil	8603 ± 1017	146 ± 36
		EB	11290 ± 1548	157 ± 44
	Male	Oil	11696 ± 1480	328 ± 63
		EB	10993 ± 1472	234 ± 85
BrdU+	Female	Oil	2885 ± 355	939 ± 141
		EB	* 2035 ± 406	1037 ± 150
	Male	Oil	2824 ± 240	1068 ± 154
		EB	2416 ± 113	1210 ± 166
DCX+	Female	Oil	11377 ± 1693	n/a
		EB	7855 ± 1735	n/a
	Male	Oil	11887 ± 2041	n/a
		EB	8533 ± 2602	n/a
BrdU+/NeuN+	Female	Oil	2614 ± 344	n/a
		EB	* 1218 ± 95	n/a
	Male	Oil	2247 ± 462	n/a
		EB	1735 ± 331	n/a

Table 2.3: Total counts of pyknotic cells and ActC3+ cells were lower in the hilus than in the granule cell layer (GCL) (p < 0.01). Treatment with estradiol benzoate (EB) affected subsets of cells in females, but not in males. Data are shown as group mean ± standard error of the mean. * statistically significantly different from oil-injected controls (p < 0.05).

<u>Cell type</u>	<u>Sex</u>	<u>Treatment</u>	Total number of	<u>Total number of</u>
			<u>cells in GCL</u>	<u>cells in hilus</u>
Pyknotic	Female	Oil	120 ± 19	108 ± 22
		EB	* 66 ± 8	* 38 ± 8
	Male	Oil	180 ± 19	118 ± 23
		EB	152 ± 25	116 ± 21
ActC3+	Female	Oil	3192 ± 436	918 ± 278
		EB	* 2206 ± 378	732 ± 190
	Male	Oil	2830 ± 248	830 ± 107
		EB	2754 ± 671	708 ± 150
FJ+	Female	Oil	104.0 ± 16.0	n/a
		EB	* 40.0 ± 15.5	n/a
	Male	Oil	92.0 ± 13.6	n/a
		EB	90.0 ± 23.8	n/a

Table 2.4: Densities of pyknotic cells and Ki-67+, BrdU+, or ActC3+ cells were much lower in the hilus than in the granule cell layer (p < 0.05; compare to Figures 2.2-2.4). Data are shown as group mean ± standard error of the mean.

Cell type	<u>Sex</u>	Treatment	Density of cells in
			<u>hilus (per mm³)</u>
Ki-67+	Female	Oil	20.1 ± 5.0
		Estradiol benzoate	21.0 ± 5.5
	Male	Oil	35.5 ± 6.7
		Estradiol benzoate	28.0 ± 10.7
BrdU+	Female	Oil	127.4 ± 16.9
		Estradiol benzoate	142.4 ± 19.8
	Male	Oil	116.1 ± 18.3
		Estradiol benzoate	142.0 ± 26.7
Pyknotic	Female	Oil	14.3 ± 2.4
		Estradiol benzoate	5.0 ± 0.8
	Male	Oil	12.5 ± 2.2
		Estradiol benzoate	13.7 ± 2.9
ActC3+	Female	Oil	123.8 ± 34.9
		Estradiol benzoate	101.8 ± 25.2
	Male	Oil	89.9 ± 12.3
		Estradiol benzoate	83.3 ± 19.8

Estradiol decreases the survival of BrdU+ cells in the granule cell layer of female, but not male, rats

There was a significant main effect of area ($F_{1.16} = 200$; p < 0.001), indicating that there was a higher density of BrdU+ cells in the granule cell layer than in the hilus (see Table 2.3 for counts of cells in the hilus). There were no other significant main or interaction effects on the density of BrdU+ cells in the dentate gyrus (region x treatment interaction effect: $F_{1.16} = 4.27$, p = 0.06; all other effects p > 0.16). Although the ANOVA revealed no significant sex x treatment interaction effect (F_{1.16} = 2.12; p = 0.16), a priori tests indicated that estradiol treatment decreased the density of BrdU+ cells in the granule cell layer of female (p = 0.005) but not male rats (p = 0.80; Fig. 2.4a). An ANOVA on the total number of BrdU+ cells in the dentate gyrus found similar results to those found with density; there was a higher number of BrdU+ cells in the granule cell layer than in the hilus (main effect of area: $F_{1.15}$ = 105; p < 0.001). There was also an area x treatment interaction effect ($F_{1.16}$ = 6.76; p = 0.02), which *post hoc* testing revealed to be due to an estradiol-induced decrease in the number of BrdU+ cells in the granule cell layer (p = 0.003) but not in the hilus (p = 0.66). There were no other significant main or interaction effects on the number of BrdU+ cells in the dentate gyrus ($p \ge 0.20$). Within each sex, a priori tests revealed that estradiol significantly decreased the number of BrdU+ cells in the granule cell layer of females (p = 0.009) but not that of males (p = 0.18; Table 2.2).

There were no significant main or interaction effects on the density of DCX+ cells in the granule cell layer (all effects p > 0.23). *A priori* tests did not indicate any
effect of estradiol treatment on the density of DCX+ cells in the granule cell layer of female (p = 0.22) or male rats (p = 0.61), although the direction of the means may suggest effects similar to those found in BrdU+ cells (Fig. 2.4c). An ANOVA on the total number of DCX+ cells in the granule cell layer found similar results to those found with density (Table 2.2; ANOVA: all effects p > 0.12; *a priori* comparison within males: p = 0.24, *a priori* comparison within males: p = 0.29).

Figure 2.4: Repeated estradiol administration (A) decreased the survival of cells labelled with BrdU in the granule cell layer and subgranular zone of the dentate gyrus in adult female but not adult male rats and (B) decreased the production of mature neurons (BrdU+/NeuN+ cells) in the granule cell later in adult female but not adult male rats, but (C) did not have any statistically significant effect on the production of young neurons (cells with processes containing DCX; n = 3-4 in each group), as indicated by the density of labelled cells per unit volume of the granule cell layer. Bars show group mean + standard error of the mean. * statistically significantly different (p < 0.05) from oil-injected controls.





Estradiol decreases cell death in the dentate gyrus of female but not male rats

Overall, females also had lower densities of pyknotic cells than males, estradiol decreased the density of pyknotic cells (Fig. 2.5a), and the density of pyknotic cells was higher in the granule cell layer than the hilus (main effect of sex: $F_{1.16} = 7.4$; p = 0.01; main effect of treatment: $F_{1.16} = 4.4$; p = 0.05; main effect of area: $F_{1.16}$ = 61; p < 0.001; see Table 2.4 for counts of cells in the hilus). In addition, the sex x treatment effect approached significance (p = 0.08), and from Figure 2.5 it is apparent that the main effect of estradiol reducing the density of pyknotic cells is driven by the data from the females. A priori tests confirmed that estradiol reduced the density of pyknotic cells in the granule cell layer of female (p = 0.02) but not male rats (p = 0.60; Fig. 2.5a). An ANOVA on the total number of pyknotic cells in the dentate gyrus found similar results as those found with density; females had fewer pyknotic cells than males, estradiol decreased the number of pyknotic cells, and there were more pyknotic cells in the granule cell layer than in the hilus (main effect of sex: $F_{1.16} = 14.6$; p = 0.001; main effect of treatment: $F_{1.16} = 6.33$; p = 0.02; main effect of area: $F_{1.16}$ = 9.39; p = 0.007), and no significant interaction effects (p > 0.14). Within each sex, a priori tests revealed that estradiol decreased the number of pyknotic cells in the granule cell layer of females (p = 0.03) but not that of males (p =0.23; Table 2.3).

Figure 2.5: Repeated estradiol administration decreased cell death in the granule cell layer of the dentate gyrus and subgranular zone in adult female but not adult male rats, as measured by cells expressing (A) a pyknotic morphology, (B) active caspase-3, or (C) degenerating neurons (cells immunoreactive with Fluoro-Jade B), as indicated by the density of cells per unit volume of the granule cell layer. Bars show group mean + standard error of the mean. * statistically significantly different (p < 0.05) from oil-injected controls.



There was a higher density of ActC3+ cells in the granule cell layer than in the hilus (main effect of area: $F_{1,16} = 151$; p < 0.001; see Table 2.4 for counts of cells in the hilus), but no other main or interaction effects on the density of ActC3+ cells in the granule cell layer (p ≥ 0.14). Although the ANOVA revealed no significant sex x treatment interaction effect ($F_{1,16} = 1.68$; p = 0.21), *a priori* tests revealed that estradiol treatment decreased the density of cells containing activated caspase-3 in the granule cell layer of female (p = 0.02) but not male rats (p = 0.64; Fig. 2.5b). An ANOVA on the total number of ActC3+ cells in the dentate gyrus found similar results as those found with density; there were more ActC3+ cells in the granule cell layer than in the hilus (main effect of area: $F_{1,16} = 120$; p < 0.001), and no other significant main or interaction effects (p > 0.25). Within each sex, *a priori* tests revealed that estradiol decreased the number of ActC3+ cells in the granule cell layer of female cell has the number of ActC3+ cells in the granule cell layer that estradiol decreased the number of ActC3+ cells in the granule cell layer of females (p = 0.01) but not that of males (p = 0.83; Table 2.3).

There was a trend for a sex x treatment interaction effect on the density of FJ+ cells in the granule cell layer ($F_{1.15} = 3.85$; p = 0.068), but no main effect of sex or treatment (main effect of sex: $F_{1.15} = 0.03$; p = 0.87; main effect of treatment: $F_{1.15} = 2.94$; p = 0.11). *A priori* tests revealed that estradiol treatment decreased the density of FJ+ cells in the granule cell layer of female (p = 0.02) but not male rats (p = 0.87; Fig. 2.5c). An ANOVA on total numbers of FJ+ cells found similar results as those found with density; there was a trend both for a main effect of treatment and for a sex x treatment interaction effect, but no significant main effect of sex on the total number of FJ+ cells (main effect of treatment: $F_{1.15} = 3.77$; p = 0.07; sex x

treatment interaction effect: $F_{1.15} = 3.33$; p = 0.09; main effect of sex: $F_{1.15} = 1.25$; p = 0.28). Within each sex, *a priori* tests revealed that estradiol decreased the number of FJ+ cells in the granule cell layer of females (p = 0.02) but not that of males (p = 0.94; Table 2.3).

Phenotype of surviving BrdU+ cells

The percentage of cells co-expressing BrdU and NeuN (BrdU+/NeuN+) was approximately 81% in the granule cell layer in both males and females. There were no significant differences among groups in the proportion of BrdU+ cells in the granule cell layer that co-expressed NeuN (0.56 < p < 0.95; Table 2.5). When we multiplied the proportion of BrdU+/NeuN+ cells by the total number of BrdU+ cells to estimate the total number of new neurons produced, we found that overall estradiol decreased both the density of new neurons produced (Fig. 2.4b; main effect of treatment: $F_{1.5} = 7.8$; p = 0.04) and the total number of new neurons produced (Table 2.2; $F_{1.5} = 10.6$; p = 0.02). Although there was no main effect of sex on new neuron density, and no other significant main or interaction effect on total new neuron numbers (all p's > 0.19), there was a significant sex x treatment interaction effect on new neuron density ($F_{1,5} = 6.46$; p = 0.05). Post hoc tests revealed that the effect of treatment on the density of new neurons was significant in females (p = 0.046) but not in males (p = 0.87). In addition, a priori tests revealed that the effect of treatment on the total number of new neurons produced was significant in females (p = 0.02) but not in males (p = 0.29).

Table 2.5: Neither sex nor chronic estradiol treatment affected the percentage ofBrdU+ cells that were co-labelled with NeuN 16 days after BrdU injection (p > 0.05).Data are shown as group mean ± standard error of the mean.

<u>Sex</u>	<u>Treatment</u>	Percentage of BrdU+ cells
		co-labelled with NeuN
Female	Oil	87 ± 12
	Estradiol benzoate	76 ± 10
Male	Oil	83 ± 12
	Estradiol benzoate	79 ± 12

Serum estradiol levels

Estradiol levels in all oil-injected rats were below the detection threshold of the ELISA kit (8 pg/mL); the mean estradiol level of estradiol-injected females (60.1 \pm 10.3 pg/mL), did not significantly differ from that of estradiol-injected males (73.7 \pm 9.5 pg/mL; p = 0.19).

Correlations

In keeping with the above results showing no significant effect of estradiol on neurogenesis and cell death in the male hippocampus, there were no significant correlations between any of these measures and estradiol level in males (all p's > 0.2). However, there were significant correlations between estradiol level and

neurogenesis/cell death measures in female rats (Fig. 2.6). The density of BrdU+ cells in the granule cell layer varied negatively with estradiol level in female rats (r = -0.78, p = 0.007), indicating that as estradiol level increased, fewer new cells survived; estradiol level accounted for approximately 61% of the variation in the density of BrdU+ cells in females. There was also a trend for a significant negative relationship between the total number of BrdU+ cells in the granule cell layer and serum estradiol level in female rats (r = -0.60, p = 0.066), suggesting that as estradiol levels increased there was a subsequent decrease in the number of BrdU+ cells surviving.

Similarly, estradiol level was negatively related in females with the number of pyknotic cells in the granule cell layer (r = -0.63, p = 0.049) and the hilus (r = -0.79, p = 0.006), as well as the density of pyknotic cells in the granule cell layer (r = -0.64, p = 0.046) and the hilus (r = -0.85, p = 0.002), indicating that as estradiol level increased, the number and density of pyknotic cells decreased; estradiol level thus accounted for over 40% of the variation in the number and density of pyknotic cells in females in the granule cell layer, and over 60% of the variation in the number and density of pyknotic cells in females in the granule cell layer, and over 60% of the variation in the number and density of FJ+ cells (r = -0.66, p = 0.0003), as well as the density of FJ+ cells in the granule cell layer (r = -0.65, p = 0.0005), indicating that as estradiol level increased, the number and density of FJ+ cells decreased; estradiol level thus accounted for over 40% of the variation in the number and set the density of FJ+ cells in the granule cell layer (r = -0.65, p = 0.0005), indicating that as estradiol level increased, the number and density of FJ+ cells decreased; estradiol level thus accounted for over 40% of the variation in the number and density of FJ+ cells in the granule cell layer. The other measures of neurogenesis and cell death examined did

not vary significantly with serum estradiol level (all $p \ge 0.33$), nor were any of the correlations between measures of neurogenesis or cell death statistically significant in males (all $p \ge 0.1$).

Figure 2.6: Serum estradiol level in female (•), but not male (\Diamond), rats receiving oil or estradiol treatment varied with the density in the granule cell layer (per unit volume of the granule cell layer) of (A) cells labelled with BrdU, (B) the density of pyknotic cells in the granule cell layer of the dentate gyrus, and (C) the density of cells labelled with Fluoro-Jade, but not with (D) the density of cells expressing Ki-67, (E) the density of cells expressing DCX, or (F) the density of cells expressing activated caspase-3.



Discussion

The results from the present study demonstrate that estradiol regulates cell death and neurogenesis in the hippocampus of female, but not male rats. Repeated (15-day) administration of estradiol resulted in a decrease in cell death and new neuron survival, with a slight increase in cell proliferation by the end of treatment, in the dentate gyrus of adult female, but not male, rats. The finding that estradiol decreases overall cell death is consistent with previous reports demonstrating that estradiol can protect neurons against a variety of insults (e.g. Goodman et al., 1996; Garcia-Segura et al., 2001; Lee and McEwen, 2001; Galanopoulou et al., 2003). In light of this, the decrease in BrdU+ cell survival we found may seem paradoxical (decreasing BrdU+ cell survival while decreasing overall cell death), but it is important to note that the dying (pyknotic, activated caspase-3+, and FJ+) cells likely belong to a different population of cells than those that had previously been labelled with BrdU. The BrdU+ cells are likely older than the dying cells observed, because the majority of cells that die in the dentate gyrus do so at early maturation stages, rather than at later stages (Dayer et al., 2003).

Consistent with previous reports, although males had larger dentate gyrus volumes than females, repeated estradiol administration had no significant effect on dentate gyrus volume in either sex (Roof and Havens, 1992; Galea et al., 1999). However, it should be noted that early exposure to testosterone increases granule cell layer volume (Roof and Havens, 1992), and chronic high estradiol levels are

correlated with larger total hippocampus volume in the female meadow vole (Galea et al., 1999). Long-term estradiol administration had no significant effect on cell proliferation, survival, or death in adult gonadectomized male rats. This is consistent with findings that there is no fluctuation in cell proliferation across reproductive status (seasons) in the dentate gyrus of male meadow voles (Galea and McEwen, 1999), no difference in cell proliferation in the dentate gyrus of castrated adult male rats (Spritzer and Galea, 2007) nor changes in cell survival with estradiol in adult male rats (Spritzer and Galea, 2007). The finding that estradiol did not significantly affect new neuron survival in male rats is somewhat inconsistent with Ormerod et al. (2004), who found that 5 days of estradiol treatment enhanced new neuron survival in males, but only when administered during the period in which the new cells were likely extending their axons. Thus the length of exposure to estradiol, and when during the cell maturation cycle estradiol exposure occurs, may determine whether estradiol can affect hippocampal neurogenesis in male rodents.

Repeated estradiol slightly increased the density of proliferating cells after repeated exposure in female but not male rats

After 15 daily injections of estradiol administration, cell proliferation, as measured by the density (but not total number) of cells expressing Ki-67, was increased in the GCL of female, but not male rats. Acute administration of estradiol increases cell proliferation in the GCL after 4h, but suppresses cell proliferation 48h later in adult female rats (Ormerod et al., 2003a). Other studies have found that estradiol exposure for 3 days results in no significant effect on hippocampal cell

proliferation (3 days; Perez-Martin et al., 2003; Fowler et al., 2005). In contrast to the present findings, repeated administration of estradiol to ovariectomized rats via implanted pellets for 3 weeks does not appear to affect cell proliferation in the dentate gyrus of adult female rats (Tanapat et al., 2005). However, there are several differences between that study and our own. First, in the present study we administered estradiol via daily s.c. injections, rather than via pellets as used in the Tanapat et al. study; pulsatile exposure to estradiol via daily injections may have different effects than continuous exposure to a constant high serum concentration of estradiol. Although Tanapat et al. did include a treatment group receiving pulsatile estradiol injections and did not find an effect on cell proliferation, their estradiol injections were 4 days apart, compared to our 24-hr spacing of injections, which may contribute to the differences between their results and those obtained in the current study. Second, we used 17β -estradiol benzoate whereas Tanapat et al. used unconjugated 17 β -estradiol, which has a shorter biological half-life and it is possible that the clearance rate of the particular type of estradiol used may affect the cellular response to estradiol. Third, we administered estradiol benzoate for 15 d whereas Tanapat et al. used implanted pellets for 21 days and over longer time periods the effects of estradiol may be attenuated as the system adjusts to the administration schedule. Fourth, we examined Ki-67, an endogenous protein that labels all actively cycling cells, instead of BrdU which was used by Tanapat et al. (2005) which only labels cells that are in the synthesis phase of the cell cycle within a 2 h period (Packard et al., 1973). BrdU may be insensitive to changes in proliferation if estradiol affects the length of stages of the cell cycle other than the S-phase. Furthermore,

estradiol can alter blood-brain barrier permeability after long-term treatment (Ziylan et al., 1990; Bake and Sohrabji, 2004), thus it is entirely possible the results found by Tanapat et al., (2005), in terms of BrdU-labeled cells, were confounded by effects of estradiol on blood-brain barrier permeability. Because we used an endogenous marker of proliferation (Ki-67) our results would not be influenced by blood-brain barrier permeability changes as a result of repeated estradiol. However, this explanation does not account for the fact that Tanapat et al. (2005) found the same result, namely no significant difference in cell proliferation after 21 days of estradiol treatment using the endogenous marker p-histone H3. Interestingly, in this part of their study they found that the number of p-histone H3-labeled cells did not correlate with the number of BrdU-labeled cells. Thus the difference between our findings with Ki-67 and their findings with p-histone H3 may be due to the fact that Ki-67 is present in cycling cells for a longer period of time than p-histone H3. Finally, we analyzed both total cell numbers and cell densities, and found a statistically significant difference only in the latter measure, whereas Tanapat et al. only analyzed total cell numbers.

Repeated estradiol suppresses new cell and new neuron survival in female but not male rats

Repeated exposure to estradiol *reduced* the number of surviving BrdU+ cells in female, but not male, rats. BrdU was administered prior to estradiol exposure, so our results demonstrate the effect of estradiol on survival of these labelled cells, rather than having any effect on their initial proliferation or BrdU incorporation rate.

Short-term estradiol has been shown to increase cell survival in adult male meadow voles (Ormerod et al., 2004), and acute estradiol tends to protect neurons against a variety of insults (e.g. Garcia-Segura et al., 2001; Lee and McEwen, 2001; Galanopoulou et al., 2003), but few studies have investigated the effects of *repeated* estradiol on neuroprotection. Similarly, the reduction in the number of BrdU+ cells surviving in our study is consistent with findings in female meadow voles that high levels of estradiol for 5 weeks results in lower numbers of ³H-thymidine-labelled cells surviving compared to voles with low levels of estradiol (Ormerod and Galea, 2001). In addition, neither the sex of the animal nor estradiol treatment affected the percentage of BrdU+ cells that co-expressed NeuN, a protein found in mature neurons, consistent with previous reports that repeated estradiol administration does not necessarily regulate the differentiation of newly generated cells in the adult dentate gyrus (e.g. Tanapat et al., 1999; Perez-Martin et al., 2003; Ormerod et al., 2004). Thus, overall we found that repeated estradiol treatment decreased the survival of new neurons in female but not male rats.

Repeated estradiol suppressed cell death in female, but not male, rats

Repeated estradiol administration to female rats in the present study reduced the density of pyknotic, activated caspase-3+, and FJ+ cells, suggesting that cell death is suppressed in female rats with repeated exposure to estradiol. Ovariectomy itself appears to have increased the densities of activated caspase-3+ and FJ+ cells, which were reduced by estradiol administration in females. However, this same pattern was not seen when examining cells with a pyknotic morphology,

as estradiol-injected females showed a reduction in cell death below that of all other groups, and indeed females overall had fewer pyknotic cells than males, in terms of both total numbers and density. A similar effect has been found in intact rats, where female rats in proestrus have fewer pyknotic cells in the dentate gyrus than males (Tanapat et al., 1999). Although counts of pyknotic cells and cells expressing activated caspase-3 can both be used to quantify cell death, these two markers target different time points in cell death, and perhaps slightly different populations of cells. The precise nature of the degenerating neurons stained with Fluoro-Jade dyes is as of yet undetermined, so it is unknown whether it preferentially stains neurons at a particular stage of the degeneration process. However, it has been shown to overlap substantially with activated caspase-3 after neurotoxic insult (Scallet et al., 2004). Caspase-3 is activated early in the apoptotic cascade, whereas the pyknotic morphology presents later in the process of cell death (necrotic and apoptotic) and persists for up to 72 h before the cell is dismantled completely and is no longer visible (Hu et al., 1997). Thus it is possible that any effects of prolonged ovariectomy (as rats would have been deprived of ovarian hormones for 22 days at the time of perfusion) may be specific to the cell maturation stage(s) during which cells are deprived of ovarian hormones.

Repeated estradiol suppresses new cell survival and cell death: a paradox?

There are at several plausible explanations for the apparent discrepancy between the decreased in the number of dying cells (pyknotic, active caspase-3+, and FJ+) and the decrease in the number of BrdU+ surviving cells. First, the 'dying'

cells observed in the present study may have been younger than the BrdU+ cells. New immature neurons are more likely to undergo cell death within the first week after birth (Cameron et al., 1993; Gould et al., 1999) and so the dying cells may have been exposed to estradiol for a shorter period of time than the BrdU+ cells that had been exposed to estradiol for a full 15 days. This is supported somewhat by the pattern of DCX+ cell densities, which roughly tracked the BrdU+ cell densities but do not reach statistical significance; most DCX+ cells are typically younger than 16 days (Brown et al., 2003), and as these cells mature in the presence of estradiol their survival may decrease. Duration of estradiol exposure may strongly modulate estradiol's effects, as treatment of ovariectomized female rats with estradiol valerate for 12 days up-regulates the expression of estrogen receptor (ER)- β , but not ER α (Jin et al., 2005). Interestingly, progenitor cells in the hippocampus show higher expression of ER β than ER α in female rats (Mazzucco et al., 2006). Together these findings suggest that repeated exposure to estradiol may alter the responsiveness of cells to estradiol itself, potentially reversing the survival-enhancing effects of acute estradiol exposure.

Secondly, the dying cells observed in the present study may have been from an *older* population than the BrdU+ cells, and may have been rescued from death by estradiol administration. The preservation of older cells may have reduced the survival of younger cells as production of new neurons in the olfactory epithelium is inhibited by GDF11, a growth and differentiation factor that is produced by mature olfactory receptor neurons and/or their progenitors (Wu et al., 2003). Thus it is

possible that a similar system operates in the granule cell layer of the hippocampus, whereby the presence of older neurons inhibits the production and/or maturation of additional, younger neurons. Alternatively, as repeated estradiol treatment enhances the excitatory effects of γ-aminobutyric acid (GABA) on young neurons (Nuñez et al., 2005), the addition of estradiol may cause GABA transmission to have excitotoxic effects on these cells (Nuñez and McCarthy, 2003). The BrdU+ cells we observed were up to 16 days old, within the 2- to 3-week-long early cell development stage during which GABA acts to depolarize immature neurons (Karten et al., 2006). Thus it is possible that estradiol enhances GABA-induced activation of immature cells, independently of its effects on both progenitor cells that do not yet received synaptic inputs and older neurons in which the effect of GABA is inhibitory. Estradiol may specifically render young neurons susceptible to excitotoxic effects of GABA, thus decreasing the survival of BrdU+ cells in the present study, while having different (potentially neuroprotective) effects on both progenitor (Ki-67+) cells and mature neurons.

Finally, it is possible that the increased cell proliferation found in the GCL with repeated estradiol exposure caused a reduction of the BrdU concentration in surviving cells to below detectable limits. Thus, rather than indicating decreased survival, the apparent reduction in the number of BrdU+ cells in female rats may be due to increased proliferation diluting the BrdU signal. Our current data examining young neuron production unfortunately neither conclusively supports nor necessarily refutes this conclusion. Staining for the endogenous protein DCX revealed fewer

DCX+ cells in females given estradiol, but the effect was not statistically significant, and further investigation into this possibility is warranted.

Sex differences in response to estradiol

Although repeated administration of estradiol had multiple effects on the cells in the dentate gyrus of female rats, we found no significant effects of repeated administration of estradiol in male rats. Although testosterone can be metabolized to estradiol, it has been shown to have neuroprotective properties independent of estradiol pathways (Freeman et al., 1996; Frye and McCormick, 2000). Chronic testosterone exposure promotes hippocampal neurogenesis via cell survival in males and this effect is mediated by dihydrotestosterone but not estradiol (Spritzer and Galea, 2007). Our findings are also consistent with studies in the nigrostriatal dopaminergic systems as estradiol protects the nigrostriatal dopaminergic system against methamphetamine neurotoxicity in female mice, but not in male mice (Anderson et al., 2005). Coupled with our findings, these results suggest the female brain is more responsive to the effects of estradiol than the male brain, whereas the male brain may be more reliant on testosterone.

Interestingly, the normal amount of estradiol present in the brain may not differ much between the sexes; while precise concentrations are not yet known, male rats normally have a substantial amount of estradiol available in the brain, produced locally by the aromatization of testosterone (Naftolin et al., 1975); as aromatase activity is higher in male than female rats, it is conceivable that the local

estradiol concentrations are similar (Wagner and Morrell, 1997). Thus it is unlikely that the sex differences in response to exogenous estradiol are the result of the same dose providing supra-physiological concentrations in one sex but not the other. There is no sex difference in the levels or regional localization of estrogen receptor (ER) subtypes α and β in the hippocampus of adult rats (Weiland et al., 1997; Kalita et al., 2005), so it is unlikely that any sex difference in response to estradiol is a result of differences in the regional distribution of estrogen receptors within the hippocampus. However, there may be differences in the types of cells on which estrogen receptors are located. For example, in intact female rats, young neurons in the dentate gyrus contain ER β (Herrick et al., 2006), and in ovariectomized female rats, cells expressing Ki-67 in the dentate gyrus contain mRNA for both ER α and ER β , though more Ki-67+ cells contain ER β than contain ER α , and only a small fraction (3-10%) of Ki-67+ cells co-localize with either estrogen receptor subtype (Mazzucco et al., 2006). In intact male rats, on the other hand, the majority of Ki-67+ cells (75-80%) contain ER α or β , and the two receptor subtypes do not appear to differ in the extent to which they co-localize with Ki-67 (Isgor and Watson, 2005). These findings may seem paradoxical in light of our results demonstrating that estradiol affects cell proliferation in female but not male rats, as this is opposite to what would be expected if the effects of estradiol on proliferating cells depend on those cells expressing ERs. However, whereas Mazzucco et al. (2006) examined ovariectomized females, and the rats used in the current study were all gonadectomized, Isgor and Watson (2005) used intact males. It is possible that gonadectomy affects the cellular localization of ERs; ovariectomy

in mice decreases overall ER levels in the brain (Ehret and Buckenmaier, 1994), so the removal of gonadal hormones may selectively reduce the level of ER expression in progenitor cells. This reduction in ER level may be specific to ER α , as the number of cells containing ER β is not changed 2 weeks after ovariectomy in adult female rats (Blurton-Jones and Tuszynski, 2002).

Possible mechanisms of estradiol-induced changes in hippocampal neurogenesis and cell death

The presence or absence of ER α or ER β on progenitor or daughter cells does not preclude cells from responding to estradiol via the putative membrane ER (Toran-Allerand et al., 2002) or through another mechanism (independent of or dependent on ERs), such as neurotrophins (Lee and McEwen, 2001; Scharfman and Maclusky, 2005). Intriguingly, previous research in our laboratory has found that agonists of neither ER α nor ER β enhance cell proliferation to the same extent as estradiol alone (Mazzucco et al., 2006). Furthermore, the ER antagonist ICI 182. 780, did not completely eliminate the estradiol-induced enhancement of cell proliferation (Nagy et al., 2006), and administration of ICI 182,170 alone slightly enhanced cell proliferation. This ER antagonist has been suggested to act as a ligand for the putative membrane-bound ER (Zhao et al., 2006). Thus these lines of evidence suggest that estradiol may be working through another mechanism independent of its two known ERs to upregulate cell proliferation. in addition to the membrane-bound ER, another such mechanism could be the neurotrophin, brainderived neurotrophic factor (BDNF) (Scharfman and Maclusky, 2005).

Estradiol can upregulate BDNF through pathways dependent or independent of its ER (Scharfman and Maclusky, 2005). BDNF and its receptor TrkB are expressed at high levels in the hippocampus, and estradiol upregulates BDNF mRNA and protein in the hippocampus (Gibbs, 1999; Zhou et al., 2005) and increases BDNF release from neurons in the dentate gyrus (Sato et al., 2007). Estrogen response elements have been located on the genes for growth factors, including BDNF, indicating that estrogens can activate BDNF gene transcription (see Scharfman and Maclusky, 2005 for review). Many neurons and astrocytes in a variety of brain regions coexpress both TrkB receptors and ER (McCarthy et al., 2002). Furthermore, many structural and electrophysiological changes due to estradiol are mediated through estradiol's interactions with BDNF (Murphy et al., 1998; Scharfman and Maclusky, 2005).

Multiple lines of evidence indicate BDNF promotes hippocampal neurogenesis: dietary restriction and enriched environments have been shown to increase both hippocampal neurogenesis and BDNF (Lee et al., 2000; Lee et al., 2002); BDNF+/- mice, with reduced BDNF levels, have reduced hippocampal neurogenesis (Sairanen et al., 2005); and BDNF infusions upregulate hippocampal neurogenesis (Scharfman et al., 2005). Furthermore, BDNF is required for adult hippocampal progenitors to differentiate into neurons in vitro (Bull and Bartlett, 2005) and BDNF levels also modulate apoptosis (Kalb, 2005). Therefore it is likely that

there is a connection between BDNF, neurogenesis, apoptosis and estradiol, and studies are currently underway to elucidate this link.

Conclusion

We have reported here the first evidence of a sex difference *in vivo* in the effects of repeated administration of estradiol on hippocampal neurogenesis and cell death. Although repeated estradiol exposure increased cell proliferation and decreased cell death in female rats, it had no such effect in male rats. As estradiol has been presented as a potential therapeutic or preventative agent for neurodegenerative diseases, it is important to note that while both men and women suffer from such diseases, the efficacy of any potential treatment or preventative agent cannot be verified in one sex alone. Additionally, although long-term estradiol decreased overall cell death in the female hippocampus, it also reduced the survival of cells that had been labelled with BrdU prior to estradiol treatment. As preventative or therapeutic medical treatments that include estrogens can be expected to consist of repeated or chronic administration, it is important to understand the effect of extended treatment on the brain. The effects of long-term estradiol administration differ from, and may be more complex than, those of acute administration, so caution should be used when evaluating the results of acute or short-term studies in terms of the potential utility of estrogens in preventing or treating neurodegenerative diseases.

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3 SEX AND REGIONAL DIFFERENCES IN ESTRADIOL CONTENT IN THE PREFRONTAL CORTEX, AMYGDALA AND HIPPOCAMPUS OF ADULT MALE AND FEMALE RATS²

In various species, sex differences have been frequently reported on a number of learning and memory tasks (e.g. Gibbs and Johnson, 2008), and any time a sex difference is observed this generally suggests that sex hormones, such as estradiol, play a role in mediating these differences. For example, males usually outperform females on hippocampus-dependent tasks (for reviews, see Galea et al., 1996; Luine, 2008) and greater sex differences are observed in spatial learning when circulating gonadal hormone levels are high in adulthood (Galea et al., 1994; Galea et al., 1995). Generally, high levels of estradiol impair memory while low levels of estradiol facilitate spatial working and reference memory in adult female rats (Holmes et al., 2002). Some studies have found that estradiol can alter spatial performance in adult males (Luine and Rodriguez, 1994; Moradpour et al., 2006 These findings suggest that hippocampus-dependent learning is modulated by estradiol in both adult males and females.

Adult levels of estradiol differentially affect the structure of the hippocampus between the sexes (Leranth et al., 2003; Lee et al., 2004; Galea et al., 2006; Barker and Galea, 2008; McLaughlin et al., 2008). For example, repeated estradiol

² A version of this chapter has been published. Barker, J.M. and Galea, L.A.M. (2009) Sex and regional differences in estradiol content in the prefrontal cortex, amygdala and hippocampus of adult male and female rats. Gen. Comp. Endocrinol. 164:77-84.
administration affects adult neurogenesis and cell death in the dentate gyrus of adult female, but not male, rats (Spritzer and Galea, 2007; Barker and Galea, 2008) and short-term estradiol increases apical dendritic spine density in the CA1 region of the hippocampus in female (Woolley and McEwen, 1993) but not male rats (Leranth et al., 2003). Thus estradiol differentially affects hippocampus structure in adult female, but not male, rats.

Sex differences have also been reported in amygdala-dependent behaviours and structure, which are in part be mediated by estradiol levels in adulthood. Estradiol disrupts conditioned fear performance in female, but not male, rodents (Toufexis et al., 2007; Gupta et al., 2001; Morgan and Pfaff, 2001). There are also sex differences favoring males in size, morphology and responsiveness to sex steroids in various nuclei within the amygdala (Mizukami et al., 1983; Hines et al., 1992; Vinader-Caerols et al., 1998; Cooke et al., 2003; Carrillo et al., 2007). Thus activational effects of estradiol influence amygdala-based structure and function, and may do so differently in males versus females.

Males and females also differ prefrontal cortex-dependent cognition and morphology (Müller et al., 2007; Harness et al., 2008) that is in part dependent on levels of estradiol, (Alejandre-Gomez et al., 2007; Wide et al., 2004). In adult female rats, low levels of estradiol facilitate, and high levels of estradiol impair, performance on a non-spatial working memory task (Wide et al., 2004). In adult male rats, decreasing estradiol improves working memory on a delayed matching to

sample task (Alejandre-Gomez et al., 2007. Further, estradiol increases dendritic spine density in the prefrontal cortex of both male and female rodents (Hao et al., 2006; Wallace et al., 2006; Hajszan et al., 2007). Thus estradiol influences prefrontal cortex function and morphology in both male and female rodents.

Collectively, these studies suggest that estradiol differentially affects the structure and function of the hippocampus, amygdala and prefrontal cortex in male and female rats. However, the amount of estrogen that can be bound in various brain regions - including the cortex, hippocampus, and amygdala - is very similar in male and female rats (Ogren et al., 1976; Barley et al., 1977; Lieberburg et al., 1980). Estrogen receptors (ER)- α and - β are found in the hippocampus and amygdala, and ER β is found in the prefrontal cortex (Shughrue et al., 1997) at similar levels between males and females in these regions (hippocampus ER α : Weiland et al., 1997; amygdala (ER α and ER β : Kalita et al., 2005; Simerly et al., 1990; prefrontal cortex (ER α and ER β : Kritzer, 2002; Simerly et al., 1990). Thus sex differences in the influence of estradiol on the structure or function of these areas may be due to differences in estradiol content after administration in various brain regions. To date the concentration of estradiol in the hippocampus, prefrontal cortex or amygdala has yet to be compared in adult male and female rats. We sought to determine the concentration of estradiol in the amygdala, hippocampus, and prefrontal cortex of intact, gonadectomized, and estradiol-replaced rats of both sexes. We administered either oil or estradiol benzoate over 15 days to gonadectomized rats, a regimen that results in profound sex differences in

neurogenesis and cell death in the hippocampus (Barker and Galea, 2008). We also sought to determine whether the levels of estradiol in the brain reflect those in serum, or are actively regulated independently of serum concentrations. We expect that estradiol levels in the brain tissue of rats treated with estradiol should be higher than those in oil-treated rats, and that sex and regional differences may exist.

Experimental procedures

All experiments were conducted in accordance with the Canadian Council on Animal Care guidelines regarding appropriate treatment of animals and were approved by the University of British Columbia. Every effort was made to minimize the number of animals used per group and to minimize the suffering of animals used throughout all experimental procedures.

Experimental subjects

Twenty-four male and twenty-seven female adult (80-90 days old) Sprague-Dawley rats (Charles River Canada, Québec, Canada) were kept on a 12-hour lightdark cycle (lights on at 0700), housed in same-sex groups of 2-3 rats in opaque polyurethane bins (48 x 27 x 20 cm) with aspen chip bedding and Purina rat chow and tap water ad libitum.

Procedure

Eleven male (n = 5-6 per group) and twelve female rats (n = 6 per group) were gonadectomized under isoflurane anaesthesia one week after their arrival in the laboratory. Briefly, all rats were anaesthetized with 1-chloro-2, 2,2-trifluoroethyl difluoromethyl ether (isoflurane) using an initial flow rate of 4% and a maintenance flow rate of 2% during surgery. All females were bilaterally ovariectomized through bilateral flank incisions, and all males were bilaterally castrated through an incision in the scrotal sac. Beginning 7 days after surgery (Day 1), rats received subcutaneous injections of estradiol benzoate (EB; dissolved in 0.1 mL sesame oil) or sesame oil (vehicle, 0.1 mL) each day, between 1000 h and 1230 h, for 15 consecutive days (Fig. 3.1). This regimen was used as it has been previously shown to result in sex differences in hippocampal neurogenesis and cell death (Barker and Galea, 2008). A dose of 33 μ g/kg EB was used to match previous studies investigating the effects of estradiol on hippocampal neurogenesis and spine density (e.g. Woolley and McEwen, 1993; Tanapat et al., 1999; Leranth et al., 2003; Rössler et al., 2006; Spritzer and Galea, 2007; Barker and Galea, 2008). Beginning 6 days after surgery (Day -1), up to 200 µL of blood was collected from gonadectomized rats via a tail nick every 2-3 days (Fig. 3.1), one hour after injection (Woolley and McEwen, 1993).

The remaining 6 males and 8 females were left intact and untreated, except for daily vaginal lavage of the females each day for 21 days to ensure that females were all in proestrus (and therefore maximal normal serum estradiol concentration)

at the time of tissue collection. On Day 16, all rats (gonadectomized and intact) were heavily anaesthetized with isoflurane and rapidly decapitated. Trunk blood was collected from all rats, and the brain removed and dissected within 10 minutes. The hippocampus, amygdala, and prefrontal cortex were dissected out and stored at -80°C until processing.

Figure 3.1: Experimental treatment timeline. All male and female rats treated with oil or estradiol benzoate were gonadectomized on day -7, and allowed to recover for 6 days. On day -1, prior to the start of injections, blood was collected from the tail. Daily injections were given for 5 days (estradiol benzoate) or 15 days (estradiol benzoate or oil). Trunk blood and brain tissue was collected from all rats on day 16, including intact males and females.



Hormone assays

Following each blood collection, whole blood was allowed to clot at 4°C for 24 hours, then spun in a rotating centrifuge (8000 rpm for 8 minutes). The serum was collected and stored at -20°C until processing. The estradiol concentration of each sample was measured in duplicate using a commercial radioimmunoassay kit (MP Biomedicals). Briefly, 25 µL of each serum sample was added to each of two borosilicate glass tubes, along with a known amount of radioactive estradiol and anti-estradiol antibody. After incubation at 37°C for 90 minutes, a precipitant solution was added to remove the antibody (and bound estradiol) from solution. Tubes were spun in a rotating centrifuge (4° C, 1000 g for 20 minutes), the liquid portion discarded and the radioactivity of the remaining pellets measured using a gamma counter. A standard curve created by adding known amounts of estradiol to tubes was used to calculate the amount of estradiol present in each experimental sample. The detection limit of this assay was 7.2 pg/mL, and the intra- and interassay coefficients of variation were 5.9% and 11.5%, respectively. Cross-reactivity of this kit with estrone is 20%, and below 1.6% with other steroid hormones.

Frozen brain tissue was weighed to obtain the wet weight of each brain region of interest. Tissue was then homogenized with 400 μ L of phosphate-buffered saline (ph 7.4) using a Teflon manual homogenizer, and 25 μ L of this homogenate (each sample in duplicate) was added to borosilicate glass test tubes. Two millilitres of diethyl ether was added to each tube to extract out steroid hormones, and the tubes were centrifuged (4°C, 1000 *g* for 5 minutes), then snap-frozen in a slurry of dry ice

and 70% methanol. The ether fraction was decanted, and dried down under nitrogen at 37°C. The resulting crystalline hormones were re-dissolved in 25 μ L of stripped (estradiol-free) human serum and analyzed using the radioimmunoassay kit as described above. Estradiol concentration in brain tissue was expressed in pg/g wet weight of tissue assayed.

To verify that the method used to measure estradiol was appropriate, additional samples of brain tissue were homogenized as described above. From each of these samples, $25 \,\mu$ L of the homogenate was treated and re-dissolved as described above. In separate tubes, $2.5 \,\mu$ L of 1000 pg/mL estradiol standard from the radioimmunoassay kit was added to an additional 22.5 μ L of the homogenate from each sample. The concentration of estradiol measured in the original samples was used to calculate expected estradiol concentrations in the samples to which standard had been added. These expected values were compared to those obtained by direct measurement by radioimmunoassay. Estradiol recovery was determined by comparing spiked samples with unspiked samples from the same homogenate (n = 35 pairs).

Data analyses

Concentrations of estradiol obtained from tail blood were analyzed using repeated-measures ANOVA, with sex (male, female) and treatment (oil, 15d estradiol) as between-subjects factors, and day (0 through +15) as the within-subjects factor. Estradiol concentrations in trunk blood and brain tissue on day 16

were analyzed using ANOVA, with sex and treatment (oil, 15d estradiol, intact) as between-subjects factors. Post-hoc tests utilized the Newman-Keuls procedure.

Results

Four blood samples were missing and these values were replaced with the mean value of the group in which these animals were assigned, for the day in question (missing values were from one each: oil-treated male and EB-treated female on day +1, oil-treated male on day 7, and EB-treated male on day 15). The overall ANOVA of serum levels during days 0-15 revealed a significant main effect of group (F(1,19) = 182; p < 0.0001) and of day (F(6,114) = 8.47; p < 0.0001), but not of sex (F(1,19) = 0.45; p = 0.51). There was also a significant group x day interaction effect (F(6,114) = 9.22; p < 0.0001). Post-hoc tests revealed that rats treated with estradiol for 15 days had higher serum levels of estradiol than oil-treated rats of the same sex from day 1 onwards (all comparisons p < 0.009; Fig. 3.2A).

An ANOVA of the concentration of estradiol in serum from estradiol-injected, oil-injected, and intact rats at the time of perfusion revealed a significant main effect of group (F(2,31) = 57.0, p < 0.0001) but no significant main effect of sex (F(1,31) = 0.05, p = 0.82) or sex x group interaction effect (F(2, 31) = 2.18, p = 0.13). Post-hoc tests revealed that rats of either sex treated with estradiol for 15 days had higher serum levels of estradiol than rats treated with oil (both comparisons p < 0.0001). The concentration of estradiol in the serum of female rats treated with estradiol for

15 days was also higher than that of intact females in proestrus (p < 0.0002; Fig. 3.2B). In male rats, serum estradiol levels of rats treated with estradiol for 15 days were higher than those in intact males (p < 0.0002).

Figure 3.2: Estradiol concentrations in the serum of gonadectomized rats over time. (A) Blood was collected via tail nick one hour after estradiol or oil injection. Both female (\bigcirc) and male rats (\square) treated with oil for 15 days have low but detectable serum concentrations of estradiol. Female (\bullet) and male rats (\blacksquare) treated with estradiol benzoate for 15 days show an initial increase in serum estradiol concentration, and subsequently maintain high concentrations. Estradiol-treated rats had significantly higher serum estradiol concentrations at all time points examined from day 1 through day 15. (B) On the day of perfusion, 24 hours after the final round of injections, serum estradiol remained higher in the estradiol-treated (females: FEB, males: MEB) than the oil-treated gonadectomized rats (females: FO, males: MO), and was also higher than in intact rats of the same sex (females: FI, males: MI). Data is shown as group mean \pm SEM (standard error of the mean). *significantly different groups (p < 0.05).



To verify the estradiol assay with brain tissue, the concentrations of estradiol measured in brain tissue to which estradiol had been added were highly correlated with those calculated for the same samples that were not spiked with known estradiol (r = 0.93, p < 0.0001, Fig. 3.3). Recovery of estradiol was 104 \pm 7.5% in brain tissue. The experimental brain tissue was therefore also processed using ether extraction, resuspension, and radioimmunoassay.

Figure 3.3: The extraction and assay methods used were adequate for comparing relative concentrations of estradiol in rat brain samples. Brain tissue from adult rats was homogenized and extracted with ether, with or without the addition of a known amount of estradiol to the sample measured. The measured concentration of estradiol in samples to which estradiol had been added was plotted against the expected value. There was a high correlation between the measured and expected values ($r^2 = 0.86$, p < 0.05).



Separate ANOVAs on the concentration of estradiol in the amygdala, hippocampus, or prefrontal cortex revealed a significant main effect of group in the amygdala (F(2,31) = 11.4, p = 0.0002; Fig. 3.4A), hippocampus (F(2,31) = 16.6; p = 0.00001; Fig. 3.4B), and prefrontal cortex (F(2,31) = 11.3, p = 0.0002; Fig. 3.4C). There were no other significant main or interaction effects for any region (all p's > 0.19;). In the amygdala and prefrontal cortex, estradiol treatment increased the concentration of estradiol above that of both oil-treated and intact animals (both p < 0.0004; oil vs. intact p = 0.85). Furthermore, gonadectomy did not significantly reduce the amount of estradiol in the amygdala or prefrontal cortex compared to intact rats (p < 0.69, p < 0.88, respectively). In contrast gonadectomy decreased the amount of estradiol in the hippocampus (p < 0.002). Furthermore, the estradiol treatment resulted in estradiol concentrations above both oil-treated and intact animals p < 0.0001, p < 0.02, respectively). A priori we were interested in sex differences in differential uptake of estradiol, and a priori tests revealed that estradiol treatment raised the estradiol concentration in the hippocampus above that found in intact animals in males (p < 0.001), but not females (p = 0.45).

Figure 3.4: Estradiol concentration in brain tissue. (A) Rats treated with estradiol benzoate for 15d (females: FEB, males: MEB) had higher concentrations of estradiol in the amygdala than gonadectomized oil-treated rats (females: FO, males: MO). However, intact rats of both sexes (females in proestrus: FI, intact males: MI) were similar to oil-treated rats. (B) In the hippocampus, oil-treated gonadectomized rats had lower concentrations of estradiol than either estradiol-treated or intact rats. Estradiol treatment raised levels to above those found in intact rats in male rats only. (C) In the prefrontal cortex, rats treated with estradiol benzoate for 15d had higher concentrations of estradiol than gonadectomized oil-treated rats. However, intact rats of both sexes were similar to oil-treated rats, further male rats had undetectable levels of estradiol in both intact and oil-treated controls. Numbers immediately adjacent to the x-axis indicate the percentage of detectable samples. Bars represent group means + SEM (standard error of the mean). * significantly different groups (p < 0.05).



Discussion

The effects of gonadectomy on estradiol concentration differed with the specific tissue examined. As expected, in serum, gonadectomized rats had lower concentrations of estradiol than their intact counterparts. Long-term gonadectomy (3) weeks) reduced estradiol concentration in the male and female hippocampus (Fig. 3.4B), but not in the male or female amygdala or in the female prefrontal cortex (Figs. 3.4B and 3.4C). This suggests greater local production of estradiol in the male and female amygdala and female prefrontal cortex, but perhaps not in the hippocampus or prefrontal cortex of the adult male rat. Furthermore, exogenous treatment with estradiol increased estradiol content to levels above intact animals in the amygdala, prefrontal cortex and the male hippocampus. These results further suggest that the female rat hippocampus may respond differently to exogenous estradiol than males, and that the female prefrontal cortex differs from that of males in endogenous estradiol content. In addition, our results indicate that changes in serum estradiol concentration alone do not necessarily reflect changes in estradiol concentration in brain tissue. Taken together, our results point to evidence that different brain regions differ in the uptake, storage, or metabolism of estradiol arriving from the periphery.

In intact rats, we did not find evidence for a sex difference in estradiol content in the prefrontal cortex, hippocampus, or amygdala. This is consistent with findings in perinatal rats showing that although there sex differences at birth in estradiol

content in the cortex, favouring males, these sex differences were no longer evident 32 h after birth (Amateau et al., 2004). In contrast to our results from the adult hippocampus, in rat pups there are higher levels of estradiol in the female hippocampus compared to the male hippocampus 32 h after birth (Amateau et al., 2004). Despite differences in serum levels of estradiol seen in the present study, and in neural and behavioural responses to exogenous estradiol administration (e.g. Barker and Galea, 2008; Gibbs and Johnson, 2008), intact rats of both sexes are remarkably similar in the amount of estradiol present in various brain regions.

Evidence for local production of estradiol in the amygdala and prefrontal cortex but limited evidence in the hippocampus of adult female rats

Although serum estradiol was higher in intact rats than in gonadectomized rats of the same sex, this difference was not necessarily reflected in brain tissue. In the male and female amygdala and female prefrontal cortex, the concentration of estradiol was similar in gonadectomized and intact rats, perhaps suggesting local production of estradiol in these two regions of the brain (Baulieu, 1998; Tsutsui et al., 2000). Interestingly, only females demonstrated a reliable supply of estradiol to the prefrontal cortex in the absence of gonadal production, as estradiol levels were undetectable in the prefrontal cortex of gonadectomized or intact males. These findings suggest the absence of local production of estradiol in the male prefrontal cortex. However, it should be noted that the apparent absence of estradiol may be due to the detection limits of the assay we used, and that there could be estradiol present at a very low concentration in the male PFC and amygdala. To our

knowledge, this is the first demonstration of estradiol content in the amygdala and prefrontal cortex of adult rats of both sexes, and the first evidence of local steroid production in the adult amygdala and female prefrontal cortex.

In the hippocampus, estradiol concentration was significantly lower in longterm gonadectomized rats than their intact counterparts, suggesting that the hippocampus did not locally synthesize substantial quantities of estradiol under these conditions in either sex. Male rats had no detectable estradiol in the hippocampus after gonadectomy, suggesting limited local production of estradiol, and estradiol was detected in only a single female rat. Thus it is unlikely that three weeks after gonadectomy adult rats of either sex produce substantial amounts of estradiol in the hippocampus itself under these conditions. In contrast to our findings, local endogenous production of estradiol has been reported in embryonic, perinatal and postnatal tissue of intact rats (Amateau et al., 2004; Rune et al., 2006; Prange-Kiel et al., 2008). However, the sex of the embryos used in these studies was not determined, so the possibility of sex differences in local estradiol production rates in vitro could not be assessed. Consistent with our results, Hojo et al. (2004) found evidence for estradiol in the hippocampus of intact adult male rats, and McCarthy's group showed that *in vivo* inhibition of aromatase in perinatal male rat pups does not affect estradiol content in the hippocampus (Amateau et al., 2004). Although the failure to detect estradiol in the hippocampus of adult male rats may have been due to the detection limits of the assay, it is clear that the level of estradiol in the hippocampus is lower in gonadectomized compared to intact rats.

This suggests that local production of estradiol is limited in the hippocampus in longterm gonadectomized rats. Hippocampal slices from intact adult male rats produce estradiol after incubation with NMDA (Hojo et al., 2004) and hippocampal slices of intact postnatal rats produce estradiol after application of gonadotropin-releasing hormone (GnRH) (Prange-Kiel et al., 2008) suggesting that under different stimulating circumstances local production of estradiol may be seen in the intact adult male and female hippocampus. It is therefore possible that long-term gonadectomy alters the environment of the hippocampus, limiting or eliminating local production of estradiol.

In the present study, we used adult rats whereas prior studies used *in vitro* systems, embryonic, perinatal and/or postnatal tissue, underscoring the possible differences between these different developmental stages and using a dissociated system. Overall, the concentrations we detected in all brain regions are considerably lower than those reported overall in embryonic brain tissue (e.g. less than 25% of the lowest mean reported by Pei et al., 2006) but are on the same order of magnitude as those reported in young postnatal female rats (Bixo et al., 1986) and female human frontal cortex (Bixo et al., 1995). Taken together, these studies suggest that estradiol content is much lower in adult versus embryonic brain tissue.

With the exception of the present study, there have been no studies examining the potential local production of estradiol after long-term gonadectomy in the adult male and female hippocampus, suggesting that the capacity for local

estradiol production in the brain decreases with time after gonadectomy. Certainly there are a number of other changes that occur after long-term gonadectomy that could potentially alter the brain's ability to locally produce estradiol or respond to estradiol: for example, in adult females 3 or 4 wks after ovariectomy estradiol no longer upregulates cell proliferation in the dentate gyrus of the hippocampus (Tanapat et al., 2005) and 3 months after ovariectomy there are decreases in estrogen receptor (ER) β density in the brain (Rose'Meyer et al., 2003). We also cannot exclude the possibility that the hippocampus was producing estradiol and metabolizing it (for example, to catechol estrogens or estrone) or clearing it away at a higher rate than the other areas examined.

Differential metabolism or sequestering of estradiol by different regions of the brain

Systemic administration of estradiol to both males and females increased the estradiol concentration in the amygdala and prefrontal cortex to levels above those found in intact animals of the same sex, suggesting either sequestration or a relatively slow breakdown of estradiol within these brain regions. However, there was a sex difference in estradiol content in the hippocampus. Estradiol administration to gonadectomized male rats increased hippocampal estradiol levels above those found in intact males. In females, however, estradiol administration did not significantly increase hippocampal estradiol above that found in intact females. This may suggest the rapid use of estradiol, or the presence of a mechanism to

restrict estradiol concentration within the female hippocampus, such as blood flow changes, metabolism differences, or lack of sequestering in female rats.

Estradiol access to the hippocampus may be controlled by modulation of blood flow to particular brain regions, as estradiol administration rapidly increases blood flow to most brain regions, most notably to the frontal cortex and hippocampus (Goldman et al., 1976). This effect on blood flow is greater in females than in males, suggesting a potential for differential delivery of peripheral estradiol. Although increased blood flow to these regions may result in increased availability of estradiol, this could be counteracted by a concurrent increase in blood flow *from* these regions, rapidly clearing estradiol or its metabolites and maintaining normal, physiological levels of estradiol despite supra-physiological peripheral levels.

Another possible explanation for our results is differential metabolism of estradiol in different brain regions. For example, estradiol within the hippocampus may be efficiently converted to a variety of metabolites (e.g. by oxidation or conjugation; Martucci and Fishman, 1993; Raftogianis et al., 2000, as well as conversion to estrone Wu et al., 1993; Akinola et al., 1996; Miettinen et al., 1996) that would not necessarily be detectable, but could be related to behavioural or neural effects of estradiol administration (Zhu and Conney, 1998; Barha et al., *in press*). In the amygdala and prefrontal cortex, on the other hand, the metabolism of estradiol may not proceed as rapidly or as efficiently, allowing estradiol arriving from the periphery to accumulate in these regions. Similar effects have been reported

from the intake of dietary phytoestrogens, after which various phytoestrogens (daidzein, genistein, and equol) are increased in the frontal cortex and amygdala, but not in the hippocampus, of intact male rats (Lund et al., 2001). There is evidence for sex differences in the metabolism of estrogens within the brain; for example, the enzyme CYP1B1 (which metabolizes estradiol to catecholestrogens) is widely distributed in the female rhesus monkey brain (including the frontal cortex, hippocampus, and amygdala), but in males is primarily restricted to the hippocampus (Scallet et al., 2005). Thus it is possible that metabolism of estrogens within the brain also differs in males and females.

The differences in estradiol concentrations between brain regions may be due to specific sequestration and storage of estradiol in particular regions. For example, the hypothalamus accumulates more estradiol from the periphery than does the cortex in female rats (Feder et al., 1974), and in male and female guinea pigs (Eaton et al., 1975; Sholl and Goy, 1981). Within the amygdala of both male and female rats, a substantial population of cells selectively take up peripheral estradiol into their nuclei, whereas in the hippocampus a much smaller subset of cells take up peripheral estradiol (Stumpf and Sar, 1971; Pfaff and Keiner, 1973; Parvizi et al., 1985). This sequestration may in part be due to differences in the number of ERs present in the different brain regions, as the presence of more ERs would be expected to increase the amount of estradiol that could be bound and sequestered within a cell. Overall levels of ER β and its mRNA are similar in the hippocampus and amygdala in both sexes (Kalita et al., 2005), but the density of cells containing

ER α mRNA is higher in the amygdala than in the hippocampus, in both male and female rats (Simerly et al., 1990). The amygdala therefore has the potential to hold more estradiol than the hippocampus, and we found estradiol levels in the amygdala approximately twice those in the hippocampus.

Estradiol concentrations do not clearly explain sex differences in the behavioural or neural effects of estradiol treatment

Perhaps surprisingly, the present study did not show dramatic sex differences in estradiol concentration in any brain area examined, despite the fact that estradiol can have disparate effects in males versus females, particularly in the hippocampus and amygdala. The lack of dramatic differences in estradiol concentration in the hippocampus of adult rats was somewhat surprising, given the sex differences in the effects of estradiol on the hippocampus (Leranth et al., 2003; Barker and Galea, 2008). Although the absolute tissue concentration of estradiol was similar in males and females, the relationship between serum and tissue concentrations of estradiol differed between the sexes. For example, intact males had lower serum estradiol concentrations than intact females, but the estradiol concentration in the hippocampus of intact rats was similar in both sexes. Furthermore, exogenous estradiol raised serum estradiol concentrations to similar levels in both sexes, but raised the concentration of estradiol in the hippocampus above that of intact rats only in males. In addition, although serum estradiol levels in gonadectomized rats were similar, ovariectomized females had detectable levels of estradiol in all three brain regions examined, whereas none of the oil-treated males had detectable levels

of estradiol in the hippocampus or prefrontal cortex. Such results could potentially be explained by sex differences in ER levels, however, overall levels of estrogen binding and ER α and ER β levels in the cortex, hippocampus, and amygdala are very similar in male and female rats (Ogren et al., 1976; Barley et al., 1977; Lieberburg et al., 1980; Weiland et al., 1997; Kritzer, 2002; Simerly et al., 1990). However, it remains possible that subtle sex differences in localization of ERs within particular brain regions or their inputs determine the extent to which estradiol can affect those regions and the behaviours they mediate, in males and females(Isgor and Watson, 2005; Mazzucco et al., 2006).

Conclusions

We have demonstrated that intact male and female rats, though they differ in serum concentration of estradiol, have similar estradiol content in the amygdala and hippocampus. Gonadectomy substantially reduces the serum concentration of estradiol in both sexes, but does not reduce estradiol in the female amygdala or prefrontal cortex, consistent with reports that regions of the adult brain are capable of local synthesis of steroid hormones (Naftolin, 1994; Baulieu, 1998; Tsutsui et al., 2000). However, long-term gonadectomy in males reduces estradiol content of the hippocampus to undetectable levels, and both intact and gonadectomized males had no detectable levels of estradiol in the prefrontal cortex, providing little evidence for local production of estradiol in the male hippocampus or prefrontal cortex. It is also possible that levels of estradiol were present in low amounts that were beyond the detection limit of our assay and future studies should examine this possibility.

However this does not negate the fact that levels of estradiol were lower in the male and female hippocampus and the male amygdala after approximately one month after gonadectomy, suggesting limited local production of estradiol under these conditions. Administration of estradiol to gonadectomized rats increases estradiol concentration in the amygdala and prefrontal cortex to roughly the same levels in both sexes. Thus in the current study we provide evidence for sex differences in the concentration of estradiol in the amygdala, PFC and hippocampus under different hormonal conditions.

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4 MALES SHOW STRONGER CONTEXTUAL FEAR CONDITIONING THAN FEMALES AFTER CONTEXT PRE-EXPOSURE³

Sex of subject and gonadal hormones have been shown to affect performance on a variety of learning and memory tasks (for reviews, see Galea et al., 1996; Shors et al., 2000). In general, males tend to outperform females on tasks that are considered hippocampus-dependent (for reviews, see Galea et al., 1996; Luine, 2008) or amygdala-dependent (Fernandes et al., 1999; Aguilar et al., 2003; Lopez-Aumatell et al., 2008). Contextual fear conditioning is dependent on the integrity of the hippocampus and amygdala (Vazdarjanova and McGaugh, 1999; Huff and Rudy, 2004) and performance is influenced by sex and estradiol levels. For example, intact male rats show more rapid conditioning and greater retention of contextual fear (i.e. greater time spent freezing) than intact females (Maren et al., 1994). This sex difference is related to ovarian hormone levels, as females in proestrus spend less time freezing than females in estrus (Markus and Zecevic, 1997), and ovariectomized rats freeze more than estradiol-treated females, but to the same extent as intact males (Gupta et al., 2001). Estradiol modulates the inhibition of fear (Toufexis et al., 2007) and extinction of contextual fear in females (Telegdy and Stark, 1973; Chang et al., 2009). It also improves retention of passive avoidance in male rats (Vazquez-Pereyra et al., 1995), but inhibits passive avoidance in females (Mora et al., 1996). Thus estradiol modulates performance on a variety of fear conditioning tasks, and can affect performance in both sexes.

³ A version of this chapter has published. Barker, J.M. and Galea, L.A.M. (2009) Males show stronger contextual fear conditioning than females after context preexposure. Physiol. Behav. doi:10.1016/j.physbeh.2009.10.014.
The ability of estradiol to modulate fear conditioning may be related to estradiol-induced neuroplasticity in the hippocampus and/or amygdala. In the adult rat, estradiol increases synaptogenesis in the hippocampus of females (Woolley and McEwen, 1993), but not males (Leranth et al., 2003; Lee et al., 2004). Repeated estradiol treatment also decreases the survival of new neurons generated in the dentate gyrus of the female, but not male, hippocampus (Barker and Galea, 2008). In the amygdala, there are sex differences in the size, morphology and responsiveness to sex steroids within various nuclei. For example, the volume of subnuclei of the medial amygdala is larger in males than females (Mizukami et al., 1983; Hines et al., 1992; Cooke et al., 2003). Dendritic spine density in the posteromedial cortical amygdala decreases in males, but not in females after gonadectomy (deCastilhos et al., 2008). Within the basolateral nucleus of the amygdala, which is involved in fear conditioning, the volume and total number of neurons are similar in males and females (Rubinow and Juraska, 2009). However, males have a higher spine density of principle neurons (Rubinow et al., 2009) and a lower number of GABA-immunoreactive neurons than females in this region (Stefanova, 1998). Adult male and female rats have similar distributions of estrogen receptor (ER) alpha in the hippocampus and amygdala (Simerly et al., 1990; Weiland et al., 1997; Kalita et al., 2005), suggesting a similar capacity to respond to estradiol in both sexes. Estradiol is normally present in these regions in both sexes (Henderson et al., 1979; Bixo et al., 1986; Hojo et al., 2004), in similar concentrations in both males and females (Barker and Galea, 2009). However,

estradiol given to gonadectomized rats has region- and sex-specific effects on tissue estradiol concentration (Barker and Galea, 2009). In the amygdala, repeated administration of estradiol increases levels in both sexes to levels above those found in intact animals, whereas estradiol levels in the hippocampus are increased more in males than females (Barker and Galea, 2009). Thus estradiol may play a role in mediating sex differences in cognitive tasks involving these regions.

We have previously demonstrated that repeated administration of estradiol to gonadectomized female, but not male, rats results in decreased numbers of young neurons in the hippocampus (Barker and Galea, 2008). With this in mind, in the present study, we sought to determine whether the sex differences previously seen in the cellular effects of estradiol were coincident with differences in hippocampusand/or amygdala-dependent learning. We used the contextual pre-exposure protocol which is a contextual fear conditioning task that cannot be performed if the hippocampus is compromised at any one or multiple stages of training and testing, unlike other contextual fear conditioning tasks (Rudy et al., 2004). To our knowledge this is the first use of this particular task to investigate sex differences in contextual fear. We administered repeated estradiol injections to gonadectomized rats of both sexes and tested them using a contextual pre-exposure fear conditioning paradigm (Matus-Amat et al., 2004; Huff et al., 2005). Because there is evidence that the young neurons produced in adulthood may be relevant to longer-term memory rather than initial learning of a task (Snyder et al., 2005) and may be related to reactions to novelty (Lemaire et al., 1999), we included additional post-training

test phases and a novel context phase. We further added a cued-conditioning training phase, to determine whether any differences in freezing during the contextual conditioning/testing phases could be explained by effects on amygdalabased learning. If young neurons promote contextual fear conditioning, then female rats given estradiol should demonstrate weaker contextual fear conditioning than oil-treated females. If, however, the young neurons are related to the recognition of novel environments (or their distinction from familiar stimuli), the behaviour of estradiol-treated females should differ from that of oil-treated females in the post-training exposure to a novel context. As repeated estradiol treatment seems to have little effect on the production or survival of cells and young neurons in the male hippocampus (Barker and Galea, 2008), we expect estradiol to have little or no effect on the freezing behaviour of males. Finally, in concordance with past experiments (Maren et al., 1994; Gupta et al., 2001; Barker and Galea, 2008) we expected overall sex differences, favouring males, in contextual fear conditioning.

Experimental procedures

All experiments were conducted in accordance with the Canadian Council on Animal Care guidelines regarding appropriate treatment of animals and were approved by the University of British Columbia. Every effort was made to minimize the number of animals used per group and to minimize the suffering of animals used throughout all experimental procedures.

Experimental subjects

Sixteen male and sixteen female adult (80-90 days old) Sprague-Dawley rats (Charles River Canada, Quebec, Canada) were kept on a 12h:12h light/dark cycle (lights on at 0700h), housed in same-sex pairs in opaque polyurethane bins (48 x 27 x 20 cm) with aspen chip bedding and Purina rat chow and tap water ad libitum.

Procedure

All subjects (males: n = 8 per group, females: n = 16 per group) were gonadectomized under isoflurane anaesthesia one week after their arrival in the colony. Briefly, all rats were anaesthetized with 1-chloro-2, 2,2-trifluoroethyl difluoromethyl ether (isoflurane) using an initial flow rate of 4% and a maintenance flow rate of 2% during surgery. All females were bilaterally ovariectomized through bilateral flank incisions, and all males were bilaterally castrated through an incision in the scrotal sac.

Eight days after surgery (Day 1), rats received s.c. injections of either estradiol benzoate (EB; 33 µg/kg, dissolved in 0.1 mL sesame oil) or sesame oil (vehicle, 0.1 mL) each day for 15 consecutive days. All animals were injected between 1000 h and 1230 h each day. This regimen has been previously shown to affect hippocampal neurogenesis in the female rat (Barker and Galea, 2008) and to increase estradiol levels in the serum and the brain to similar levels in male and female rats (Barker and Galea, 2009). The remainder of the experimental timeline is described below, and shown in Figure 4.1.

Figure 4.1: Experimental timeline. Rats were gonadectomized, allowed to recover, and then given daily injections of estradiol or vehicle (oil) for 15 days. The day following the last injection, rats were tested for thermal pain sensitivity, and began training in the conditioning chambers. The chambers were modified at each stage, with different features present in the chamber for the novel context exposure, cued training, and cued testing, as described in the text.

<u>Day</u>	Treatment	<u>Chamber</u> exposure
Day -7	GDX	n/a
Days 1-15	E2 / oil injections	n/a
Day 16	Pain sensitivity test	n/a
	Pre-exposure	
Day 17	Imm. Shock	
Day 18	Context Test 1	
Day 19	Novel Context	
	Context Test 2	
	Cued Training 📣	
Day 20	Cued Test	

Apparatus

All training and testing occurred in two identical operant conditioning training chambers (30.5 cm x 24.1 cm x 21.0 cm) enclosed in sound-attenuating boxes (Med Associates Inc., St. Albans VT, USA). The chambers were constructed of aluminum (two side walls) and Plexiglas (rear wall, ceiling, hinged front door). Each chamber was illuminated by a single 100-mA house light located in the top center of one wall. Auditory stimuli were delivered via a speaker connected to a programmable audio generator (ANL-926, Med-Associates) located in the top-left corner. Inside each chamber was a stainless steel grid floor (19 4.8 mm rods spaced 1.5 cm apart) wired to an electrical source for the delivery of footshock. The chambers were situated on a load-cell platform that recorded chamber displacement in response to the rats' motor activity as output digitized every 200 ms using the Threshold Activity software (Med-Associates). Locomotor activity was guantified by the raw load cell values (range = 0-100), and freezing behaviour quantified by calculating the number of load cell values below a threshold determined by comparing load cell output with an observer's ratings of freezing behaviour, i.e. the cessation of all movement with the exception of respiration-related movement. In the current study, this threshold was a load cell output of 5. To exclude momentary bouts (i.e. < 1 s) of inactivity, freezing was only scored after five or more contiguous observations below the freezing threshold (for further detail, see also Maren, 1998; Rabinak and Maren, 2008). The time spent freezing displayed by rats was scored on subsequent days in (1) the training context, (2) a novel context in which rats had never been shocked, and (3) the training context a second time. During all conditioning and testing sessions, the

rats' activity was monitored continuously using live video feed, via a small surveillance camera mounted above each chamber, and the data acquisition system described above. We confirmed the accuracy of threshold output of freezing behavior by manually scoring videotaped sessions, during which freezing was defined as the cessation of all movement with the exception of respiration-related movement.

The percentage of time spent actively exploring the chamber was quantified by calculating the number of load cell values above an 'activity threshold' determined by comparing load cell output with an observer's ratings of exploratory behaviour. "Exploratory behaviour" included moving around the chamber and rearing, and excluded freezing, standing still, and grooming. In the current study, this threshold was defined as a load cell output of 10.

Procedures

Pain sensitivity

Acute estradiol administration can affect nociception (e.g. Stoffel et al., 2002; Fischer et al., 2008), and this could act to affect performance on a fear conditioning task by changing the saliency of the shock administered. Therefore, three hours prior to training in the operant chambers (Fig. 4.1), each rat was subjected to a thermal pain sensitivity test (Ratka and Simkins, 1990; Cook and Moore, 2006; Sanoja and Cervero, 2008). Briefly, all male rats and half of the female rats were

placed individually on a hotplate at 50°C, boxed in on all four sides by a cardboard wall 7.6 cm high. The length of time taken for each rat to lick one of its paws was recorded, and the rat immediately removed from the hotplate back to its home cage. Any rat remaining on the hotplate after 120 seconds was removed manually and its score recorded as 120 seconds (one oil-treated male and one estradiol-treated male). This was done to determine whether any treatment effects or sex differences found in performance on the fear conditioning tasks could be accounted for by differences in nociception.

Contextual fear conditioning

To examine the effects of estradiol on contextual conditioning we used a procedure used by Rudy et al. (2004). Briefly, rats were transported in clear cages to a holding room prior to coming into the testing room. Rats were exposed to conditioning chambers with two distinct odour cues (almond and orange, or cinnamon and rum extract), each presented on the end of a cotton swab affixed to opposite corners of each chamber. One house light was presented on either the left or the right side of the box, and either the back wall or the front door to the box was covered with brown wax paper to further distinguish the training and novel contexts.

In the afternoon of the day following the last injection of estradiol or oil (day 16, beginning at 1400h), rats were transported from the holding room to the conditioning chamber. Each rat was lifted from the transport bin to the conditioning chamber, allowed to explore the chamber for 5 minutes, placed back into the

transport bin and returned to its home cage. Seven minutes after the end of its first exposure to the conditioning chamber, each rat was again returned to the chamber to explore for an additional 5 minutes. This second pre-exposure was added to ensure good retention of the association between transport cues and the training context, so that rats could form an association between a shock given immediately in the training context and that context (Rudy and O'Reilly, 2001). After two exposures to the chamber, each rat was returned to its home cage and transported back to the colony room.

The next day (day 17), 24h after initial exposure to the conditioning chamber, each rat was placed in the chamber and given an immediate footshock (2s, 1.80 mA) through the chamber floor. Each rat was returned to its home cage immediately upon conclusion of the footshock. Rats were tested for their response to the conditioning context 24h after they received a footshock (day 18). Each rat was placed in the chamber for 2 minutes, and the percentage of time spent freezing recorded as an indication of fearfulness in response to the context (conditioning chamber). Performance on this task depends on the integrity of the hippocampus (Rudy et al., 2004; Huff et al., 2005).

Novel context and re-exposure to context chamber

In the morning of day 19 (beginning at 0730h), each rat was transported in an empty opaque polyurethane bin (48 x 27 x 20 cm) to a different conditioning chamber, with different odour distribution and types, and lighting and wall coverings

distinct from the context in which they had been shocked. Each rat was permitted to explore the novel chamber for 2 minutes, and its freezing behaviour recorded as described above. Immediately following the session in the novel chamber, each rat was again transported in clear Plexiglas bins to the chamber in which it had been previously shocked for 1 minute, and its freezing behaviour scored. Rats were returned to the colony room until 1400h, when cued fear conditioning training began.

Cued fear conditioning

The afternoon of day 19 (starting at 1400h), rats (all males and half of the females) were transported to a conditioning chamber distinct from any to which they had been previously exposed. This chamber had no added odour cues, the outer box doors were shut (so the testing room was not visible to the rat), and the inner chamber was lit by one of the 'stimulus' lights on one wall of the chamber. Over the course of a 7-minute session, each rat received 3 pairings of a 10s tone (2 kHz, 80 dB) and a 2 s, 1.10 mA footshock delivered during the final 2s of the tone. The following day (day 20), each rat was placed in a conditioning chamber made distinct by variations in lighting and textures within the box. The freezing behaviour of each rat in this chamber was recorded over the course of 7 minutes, including three 15 second long tones (15 sec, 2 kHz; 80 dB) that matched the tones previously paired with footshock. Performance on this task is dependent on the integrity of the amygdala (Phillips and LeDoux, 1992; Rudy et al., 2004).

Behavioural recordings

For all procedures we used the Threshold Activity Software version 3.10 (SOF-806, Med Associates Inc.) for behavioural data recording (i.e. percentage of time spent freezing) during testing sessions. The subjects were tested and scored for the first two minutes of each training and testing session, and the time spent freezing converted to percentage of total time analyzed. Rats were excluded from analyses if they froze for more than 10% of the time during pre-exposure sessions (one oil-treated female, one estradiol-treated female, and one oil-treated male).

Data analyses

For the pre-exposure condition dependent variables were analyzed using a repeated-measures ANOVA with sex (male, female) and treatment (oil, EB) as between-subjects factors, and session (first and second exposure) and time (first and second minute) as within-subjects factors. Percentage of time spent freezing and overall activity were each analyzed using a repeated-measures ANOVA with sex (male, female) and treatment (oil, EB) as between-subjects factors, and time (first and second minute of each test) as the within-subjects factor for the contextual (test 1), novel and cued conditions. For the second exposure to context a repeated-measures ANOVA with sex (male, female) and treatment (oil, EB) and treatment (oil, EB) as between-subjects factors was conducted on the percentage of time spent freezing. Statistica 8.0 (Statsoft, Tulsa, OK, USA) was used for all analyses, and a significance level of $\alpha = 0.05$ was used. Post-hoc comparisons utilized the Newman-Keuls procedure. Based on our findings in a previous study on hippocampal neurogenesis ((Barker

and Galea, 2008)) we expected to find treatment effects on female behaviour in the current study. We therefore ran additional *a priori* comparisons of estradiol- and oil-treated females within each phase of training and testing. The significance level for the a priori tests was set using a Bonferroni correction for each analysis, as specified below.

Results

Pain sensitivity

To determine whether there were group differences in pain sensitivity that might affect our results, we first subjected rats to a thermal pain sensitivity test. Males took longer than females to escape the hotplate (F(1,26) = 5.04, $p \le 0.03$). There was no significant main effect of treatment ($p \le 0.71$) and no significant sex x treatment interaction effect ($p \le 0.69$) (Table 4.1). Because of the sex effect on pain sensitivity we used pain sensitivity as a covariate in all subsequent analyses involving freezing. **Table 4.1**: Latency of rats to escape a painful thermal stimulus (50° hotplate) after repeated estradiol injections. Data are presented as mean \pm SEM.

<u>Sex</u>	<u>Treatment</u>	Time to escape (s)
Female	Oil	23.0 ± 5.3
	Estradiol	23.3 ± 9.4
Male	Oil	55.7 ± 17.0
	Estradiol	45.9 ± 14.1

Pre-exposure freezing behaviour

Repeated-measures ANOVA on the percentage of time spent freezing during pre-exposure sessions revealed that rats exhibited more freezing behaviour in the second session than in the first (main effect of session: F(1,41) = 6.49, $p \le 0.002$) (Table 4.2). Rats also froze more during the second minute of sessions than during the first (main effect of time: F(1,41) = 4.05, $p \le 0.05$). There were no other significant main effects of sex ($p \le 0.71$) or treatment ($p \le 0.55$). Several interaction effects also approached significance, including sex x session x treatment (F(1,41) = 3.10, $p \le 0.09$), sex x time (F(1,41) = 3.67, $p \le 0.06$), time x treatment (F(1,41) = 2.96, $p \le 0.09$), and sex x time x treatment (F(1,41) = 3.40, $p \le 0.07$); all other interaction effects were not significant (p's > 0.13). After including the time to escape the hotplate, repeated measures ANOVA showed no significant main or interaction effect: F(1,25) = 3.03, $p \le 0.10$; session x sex x treatment interaction effect: F(1,25) = 3.03, $p \le 0.10$; session x sex x treatment interaction effect: F(1,25) = 3.03, $p \le 0.10$; session x sex x treatment interaction effect: F(1,25) = 3.03, $p \le 0.10$; session x sex x treatment interaction effect: F(1,25) = 3.03, $p \le 0.08$; all other main and interaction effects p > 0.14).

Table 4.2: Percentage of time spent freezing during pre-exposure tests. Data are

 presented as mean ± SEM.

		First pre-exposure		Second pre-exposure	
<u>Sex</u>	<u>Treatment</u>	First minute	Second minute	First minute	Second minute
Female	Oil	0.02 ± 0.02	0.00 ± 0.00	2.34 ± 1.41	2.19 ± 1.29
	Estradiol	0.15 ± 0.09	0.47 ± 0.34	1.24 ± 0.65	1.23 ± 0.64
Male	Oil	0.24 ± 0.24	0.39 ± 0.25	0.00 ± 0.00	0.42 ± 0.42
	Estradiol	0.00 ± 0.00	0.29 ± 0.19	0.16 ± 0.16	4.57 ± 2.17

Repeated-measures ANOVA on the percentage of time spent actively exploring the chamber during pre-exposure sessions revealed a significant main effects of session (F(1,41) = 57.2, p ≤ 0.0001) and a significant sex x session x time interaction effect (F(1,41) = 10.2, p ≤ 0.003) (Table 4.3). There was no significant main effect of sex (p ≤ 0.14) or treatment (p ≤ 0.44), and no other significant interaction effects (all p's > 0.15). Post-hoc testing revealed that in general rats were more active during the first pre-exposure session than the second, but this was true for females only in the second minute and for males only in the first minute (females: first minute: p ≤ 0.055, second minute: p ≤ 0.0002; males: first minute: p ≤ 0.0001, second minute: p ≤ 0.17). **Table 4.3**: Percentage of time spent actively exploring chamber during pre-exposuretests. Data are presented as mean ± SEM.

		First pre-exposure		Second pre-exposure	
<u>Sex</u>	<u>Treatment</u>	First minute	Second minute	First minute	Second minute
Female	Oil	93.7 ± 1.5	91.2 ± 1.7	83.2 ± 3.2	74.2 ± 3.7
	Estradiol	91.1 ± 2.8	91.9 ± 2.1	86.0 ± 3.7	79.1 ± 3.7
Male	Oil	98.2 ± 1.4	91.8 ± 2.7	82.8 ± 3.2	81.9 ± 9.0
	Estradiol	99.4 ± 0.3	94.1 ± 3.2	78.5 ± 3.7	91.0 ± 3.6

Contextual fear conditioning

During the first exposure to the training context after footshock, males froze more than females (main effect of sex: F(1,25) = 8.92, $p \le 0.006$), and rats froze more during the second minute of the contextual test than during the first (main effect of time: F(1,25) = 14.9, $p \le 0.0007$) (Fig. 4.2A). There were no other significant main or interaction effects (all p's > 0.27). A priori comparisons within females revealed no significant effect of treatment on freezing behaviour during the first context test (first minute: $p \le 0.99$, second minute: $p \le 0.99$).

Repeated-measures ANOVA on the percentage of time spent actively exploring the chamber during the first contextual testing session revealed that exploration was decreased in the second minute relative to the first (F(1,41) = 21.1, $p \le 0.0001$), but there were no other significant main or interaction effects (all p's \le 0.11) (Fig. 4.2B).

During the second exposure to the training context after footshock, males froze more than females (main effect of sex: F(1,41) = 20.9, $p \le 0.0001$) (Fig. 4.2C). This effect was not significant after including time to escape the hotplate as a covariate (covariate effect: F(1,25) = 0.13, $p \le 0.73$; main effect of sex: F(1,25) =3.33, $p \le 0.08$). There was no significant effect of treatment ($p \le 0.26$), and no sex x treatment interaction effect on the percentage of time spent freezing ($p \le 0.99$). A priori comparisons within females revealed no significant effect of treatment on freezing behaviour during this second context test ($p \le 0.30$).

During the second contextual testing session, males were less active than females (F(1,41) = 5.12, $p \le 0.03$), but there were no other significant main or interaction effects (all p's > 0.18) (Fig. 4.2D).

Figure 4.2: Freezing and exploratory behaviour of rats in a conditioning chamber after contextual fear conditioning of gonadectomized male (M) and female (F) rats previously given repeated injections of oil (O) or estradiol benzoate (E). Overall, gonadectomized male rats froze more than female rats during exposure to the training context, (A) 24h and (C) 36 h after a footshock was received in the same context. There was no significant sex difference in exploratory behaviour levels during (B) the first post-training exposure to the training context, but (D) males spent less time than females exploring during the second post-training exposure. Bars represent group means + standard error of the mean; lines above bars indicate statistically significant comparisons (*p < 0.05).



Novel exposure

When exposed to the novel chamber, males froze more than females (main effect of sex: F(1,41) = 16.4, $p \le 0.0002$) and this effect remained significant after

including time to escape the hotplate as a covariate (covariate effect: F(1,25) = 0.37, $p \le 0.55$; main effect of sex: F(1,25) = 4.53, $p \le 0.04$) (Fig. 4.3A). There was also a main effect of time on the percentage of time spent freezing (F(1,41) = 4.48, $p \le 0.04$), and a significant sex x time interaction effect (F(1,41) = 5.02, $p \le 0.03$), but these effects were not significant after including time to escape the hotplate as a covariate (both p's > 0.14). There were no other significant interaction effects on freezing behaviour (all p's > 0.37). Post hoc analysis revealed that males froze more than females (first minute: $p \le 0.0002$, second minute: $p \le 0.0002$), and that males froze more during the second minute than the first ($p \le 0.004$) but females did not change their behaviour over time ($p \le 0.93$) (Fig. 4.3A). A priori comparisons within females revealed that estradiol-treated females spent less time freezing than their oil-treated counterparts during the second minute of testing ($p \le 0.01$), but not the first ($p \le 0.99$).

Repeated-measures ANOVA on the percentage of time spent actively exploring the chamber during exposure to a novel context revealed significant effects of sex (F(1,41) = 5.15, p ≤ 0.03) and time (F(1,41) = 10.8, p ≤ 0.002), but not treatment (p ≤ 0.67) (Fig. 4.3B). There was a significant sex x time interaction effect (F(1,41) = 5.26, p ≤ 0.03), but no other interaction effects (all p's ≤ 0.49). Post hoc analysis revealed that males during the second minute of this test were less active than females at any time (both p's ≤ 0.01), and less active than males during the first minute (p ≤ 0.0004; all other p's > 0.48). **Figure 4.3**: Freezing behaviour and activity levels of conditioned rats in a novel context. Gonadectomized male (M) and female (F) rats previously given repeated injections of oil (O) or estradiol benzoate (E) were placed in a novel chamber after exposure to the familiar training chamber. (A) Males froze more than females, regardless of treatment or time. When exposed to the novel chamber, female rats previously treated with estradiol froze less than their oil-treated counterparts. (B) Males explored the chamber less than females during the second minute of this test. Bars represent group means + standard error of the mean; lines above bars indicate statistically significant comparisons (*p < 0.05).



Cued fear conditioning

To distinguish between effects of estradiol specific to the hippocampus and any effects on the amygdala, we also tested animals on a cued fear conditioning task. There were no significant main or interaction effects of sex or treatment on the percentage of time spent freezing during testing with the cue (all p's > 0.29) (Fig. 4.4A). These findings were not altered after including pain sensitivity as a covariate (covariate effect: F(1,25) = 0.13, $p \le 0.73$). A priori comparisons within females revealed no significant effect of treatment on the percentage of time spent freezing in response to the auditory cue ($p \le 0.54$).

ANOVA on the percentage of time spent active during exposure to the auditory cue revealed that males were more active than females during this test $(F(1,26) = 7.26, p \le 0.01)$ (Fig. 4.4B). There was no significant effect of treatment (p ≤ 0.98), and no interaction effect on the activity level of the rats (p ≤ 0.92).

Figure 4.4: Freezing behaviour and activity levels of conditioned rats in response to an auditory cue that previously predicted footshock of gonadectomized male (M) and female (F) rats previously given repeated injections of oil (O) or estradiol benzoate (E). (A) When presented with the cue, all rats showed robust freezing, but there were no significant sex or treatment effects on this behaviour. (B) Males spent more time exploring the chamber than females during presentation of the auditory cue, but there was no significant effect of treatment on activity level. Bars represent group means + standard error of the mean; lines above bars indicate statistically significant comparisons (*p < 0.05).



Discussion

Gonadectomized male rats froze more than females during exposure to both training and novel contexts after pre-exposure contextual fear conditioning, regardless of treatment (Figs. 4.2A-B, 4.3A). This suggests that overall males may have formed a stronger association of the training context with an aversive footshock. Prior exposure to estradiol resulted in significant behavioural effects in female rats only, with estradiol-treated females showing less freezing behaviour than oil-treated females during post-training exposure to a novel context (Fig. 4.3A), but

not during any other phase of training or testing. This suggests that repeated estradiol may enhance the ability of female rats to distinguish a novel context from a familiar one with which they have associated an aversive stimulus (e.g. footshock). These findings were not explained by differences between groups in pain sensitivity (Table 4.1) or exploratory behaviour (Figs. 4.2C-D, 4.3B, 4.4B).

Sex differences in freezing behaviour using a contextual pre-exposure training paradigm

After training in a contextual fear conditioning task relying on pre-exposure to the training context, male rats froze more than female rats, regardless of treatment group (gonadectomized or gonadectomy + estradiol). This is similar to the differences seen in intact rats, as intact male rats outperform intact females in contextual fear conditioning task without context pre-exposure (Maren et al., 1994). In our study we found that long-term gonadectomized rats demonstrated a persistent sex difference favouring males. Ovariectomy enhances contextual fear conditioning relative to intact females (10 days after surgery), improving the performance of females to match that of intact males (Gupta et al., 2001). On the other hand, castration 4-6 weeks prior to testing impairs contextual fear conditioning in males relative to intact males (Edinger et al., 2004). Taken together, these previous data suggest that ovariectomized female rats may outperform gonadectomized males in a conditioned fear task. However, in the present study we found that castrated males outperformed ovariectomized females, suggesting that longer-term ovariectomy results in worsened performance of female rats. The varying results between studies

may be related to the specifics of the training protocols used or to the strain of rat involved. First, as suggested above, our rats were gonadectomized prior to training for considerably longer than the rats used by Gupta et al. (24 days in the present study compared to 10 days in the previous study) (Gupta et al., 2001). This extended period after gonadectomy may have permitted activation of compensatory responses to the absence of gonadal hormones, or stabilization of the systems and responses affected by gonadectomy, for which 10 days may be insufficient. For example, circulating estrogen concentrations in female rats, though low, gradually increase over time after ovariectomy (Zhao et al., 2005) and the effects of ovariectomy, and estrogen replacement, to influence the hippocampus vary with time since after ovariectomy (Woolley and McEwen, 1993; Tanapat et al., 2005; McAsey et al., 2006; Suzuki et al., 2007). Second, other studies (Gupta et al., 2001; Edinger et al., 2004) have used Long Evans rats whereas we used Sprague-Dawley rats. As albino rats often have poor eyesight relative to their pigmented counterparts (Prusky et al., 2002), we included olfactory cues in the training and testing contexts to ensure the rats were capable of performing the task presented. Very few studies make use of odour as contextual information, so it is possible that the inclusion of olfactory cues affects the nature or demands of the task. For example, repeated exposure to low levels of formaldehyde fumes enhances fear conditioning to a different odour (orange oil) in male rats, but not females (Sorg et al., 2004). Thus it is possible that sex differences in the ability to use, and reliance on, odours may affect performance on a contextual fear conditioning task that includes olfactory cues. Third, the 'contextual pre-exposure' protocol we used is somewhat unique

among contextual fear conditioning tasks, in that the task cannot be learned if the hippocampus is compromised at any one or multiple stages of training and testing (Rudy et al., 2004). The more common protocol used by Gupta et al. makes it possible to solve the task even if the hippocampus is taken off-line (Rudy and O'Reilly, 2001). Although the amygdala is also involved in the contextual task we used, we found no significant difference between the sexes and no significant effect of estradiol treatment on performance in a cued fear conditioning task in which performance requires the integrity of the amygdala but not the hippocampus. This suggests that the sex differences we found in performance on the contextual fear task we used are a result of difference in hippocampal function.

Estradiol administered prior to training and testing has minimal effects on fear conditioning

Within each sex in the present study, treatment with estradiol was found to produce little difference in performance on the tests used. As estradiol seems to affect the male hippocampus only minimally (Woolley and McEwen, 1993; Leranth et al., 2003; Lee et al., 2004; Spritzer and Galea, 2007; Barker and Galea, 2008), this outcome was expected in the male rats. However, long-term estradiol administration has a variety of effects on the female hippocampus, including increasing cell proliferation while decreasing both overall cell death and young neuron survival (Barker and Galea, 2008). In the current study, previous repeated exposure to estradiol did not significantly affect contextual fear conditioning using pre-exposure. This finding varies from the results of Gupta et al., who found that acute high levels

of estradiol decreased the amount of time spent freezing in ovariectomized rats (Gupta et al., 2001). However, it should be noted that Gupta et al. administered estradiol to their rats 48 h and 4 h prior to conditioning, whereas we administered estradiol daily for much a longer period of time (15 d) and ceased administration 24 h prior to initial context exposure. The effects of estradiol and/or ovariectomy on a variety of neural parameters have been shown to vary with time (Woolley and McEwen, 1993; Tanapat et al., 2005; Suzuki et al., 2007). Thus it is not surprising that different timeframes of estradiol administration produce different behavioural effects. Furthermore we have recently shown that there are sex differences in the concentration of estradiol in the hippocampus relative to intact rats after this same regimen of estradiol, with levels of estradiol higher in male rats than intact male rats, but similar to intact rats in females (Barker and Galea, 2009). It is therefore possible that the sex difference in the effects of estradiol is a result of a difference in estradiol uptake into the hippocampus, or of a difference in metabolism of estradiol within the hippocampus.

Prior estradiol administration affects reaction of females to a novel context after fear conditioning

In the present experiment, when exposed to a novel context after training estradiol-treated rats displayed spent less time freezing than oil-treated females. The 'novel' context we used shared some of the individual features of the familiar training context (e.g. general size and shape). Therefore, post-training freezing in a novel context may indicate differences in generalization of the learned context-shock

association, or in the ability to discriminate the training and novel contexts. We have previously shown that long-term estradiol administration to female rats increases cell proliferation in the dentate gyrus while decreasing the production of young neurons and overall cell death (Barker and Galea, 2008), suggesting that estradiol treatment increases cell turnover. This, coupled with our present results, suggests a role of cell turnover in the detection and encoding of subtle changes in the environment or in the recognition of novel stimuli. Indeed, it has been shown that the dentate gyrus of the hippocampus is particularly important for detecting both novelty and subtle changes in the environment (Lemaire et al., 1999; Hunsaker et al., 2008). For example, normally rats will re-explore a familiar object if its position is changed, or if it is moved into a different context (e.g. round enclosure instead of square) (Hunsaker et al., 2008). Rats with lesions to the dentate gyrus fail to re-explore objects after either modification is made (Hunsaker et al., 2008). New cells produced in the dentate gyrus may play a key role in the ability to detect such changes, as rats demonstrating a higher degree of exploration of a novel environment also have high rates of cell proliferation (Lemaire et al., 1999). Consistent with our results, the *survival* of these cells may be inversely related to exploratory behaviour, as rats that perform more exploratory behaviour in a novel environment have fewer cells overall in the granule cell layer of the dentate gyrus (Lemaire et al., 1999). Network models of adult neurogenesis suggest that for optimal learning, both young neuron proliferation and neuron death – i.e. neuronal turnover – should be increased in proportion to the 'novelty' of the new information (Chambers and Conroy, 2007). In the current study, female rats given long-term

estradiol treatment, which increases cell turnover (Barker and Galea, 2008), seemed better able to recognize a novel environment, as demonstrated post-training by reduced freezing in a novel chamber. In addition, these rats froze to the same extent as their oil-treated counterparts when tested a second time in the training chamber, suggesting that the difference in the 'novel' chamber was not due to differences in forgetting or extinction of the learned context-shock association.

Conclusions

In the present study, we found that gonadectomized male rats outperformed females in a contextual fear conditioning task that is dependent on the hippocampus, regardless of treatment. In addition, we have demonstrated that previous exposure to estradiol affects post-training performance of female rats, but not males, in response to a novel context by reducing the amount of freezing in the novel context. Along with previous data suggesting increased cellular turnover in response to estradiol treatment (Barker and Galea, 2008), this supports the hypothesis that adult neurogenesis in the hippocampus is related to the recognition of and/or learning about novel contextual information.

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5 GENERAL DISCUSSION

General findings

I have demonstrated that repeated estradiol administration to gonadectomized adult male and female rats results in:

1) increased cell proliferation, decreased survival of young neurons, and decreased overall cell death in the dentate gyrus of the hippocampus of adult female, but not male, rats (Chapter 2, Barker and Galea, 2008),

2) increased estradiol concentration in the hippocampus, amygdala and prefrontal cortex in both male and female rats (Chapter 3, Barker and Galea, 2009), and

3) subtle changes in the behaviour of female, but not male, rats in response to a novel context following contextual fear conditioning (Chapter 4).

In addition, I found evidence for local production of estradiol in the amygdala and prefrontal cortex of the female rat brain (Chapter 3, Barker and Galea, 2009). I also found regional differences in the effects of gonadectomy and estradiol administration, and that serum concentrations of estradiol are not necessarily reflective of brain tissue concentrations. In Chapter 4 I demonstrated that gonadectomized male rats outperform gonadectomized female rats on contextual conditioning.

Curiously, male rats were unaffected by repeated estradiol administration in terms of hippocampus structure (Chapter 2, Barker and Galea, 2008) and function (Chapter 4). The lack of effect of estradiol in males to modulate behaviour, neurogenesis or cell death (Chapter 2, Barker and Galea, 2008) was not due to a difference in gross estradiol concentration, as estradiol concentrations in the hippocampus following repeated estradiol injections were similar in both male and female rats (Chapter 3, Barker and Galea, 2009). Furthermore the overall distribution of estrogen receptors (ERs) in the hippocampus is similar (Weiland et al., 1997) and I found that in intact rats, estradiol concentrations within the hippocampus were remarkably similar between the sexes (Chapter 3, Barker and Galea, 2009). Collectively, these data suggest that even in the presence of similar concentrations of estradiol in brain and periphery, and similar ER density and distribution, males do not respond to repeated administration of estradiol in the neuronal (Chapter 2, Barker and Galea, 2008) or behavioural (Chapter 4) parameters examined in this thesis. A number of possible explanations for these findings exist. For example, there may be differences between the sexes in the cellular and subcellular localization of ER α and ER β . The effects of activation of either or both ER subtypes would be expected to differ depending on the particular type of neuron (or glia) on which the receptors were located. It is possible that within a cell ERs are differentially available in males and females, for example localization of ERs within the membrane, cytoplasm or nucleus, or different concentrations of cofactors required for ER function may be different between the sexes. Indeed, the ER coactivator SRC-1 is present at higher levels in the hippocampus of male rats than in

female rats (Bousios et al., 2001). Alternatively, the downstream effects in the metabolic pathways affected by the activation of either ER may differ in males and females. For example, administration of estradiol to hippocampal neurons cultured from female rat pups modulates nuclear CREB phosphorylation; importantly, this modulation by estradiol is not observed in neurons cultured from male tissue (Boulware et al., 2005). Further, estradiol *in vivo* also affects pCREB levels in adult female rodents, but not in male rodents (Abrahám and Herbison, 2005; Zhou et al., 2005; Raval et al., 2009). These findings suggest that estradiol differentially affects downstream mechanisms in the hippocampus of males and females. Any or all of these potential explanations may be involved in the sex differences I found in response to estradiol.

Limitations

Administering estradiol to gonadectomized male and female rats has provided valuable information about its potential role in the brain and its effects on behaviour. However, gonadectomy in both males and females has other effects that should be noted. It is possible, for example, that the effects of estradiol may differ in the presence or absence of other hormones affected by gonadectomy, including estrone, progesterone, testosterone, and their metabolites. Gonadectomy initially increases levels of luteinizing hormone and follicle-stimulating hormone, as the negative feedback normally exerted by gonadal hormones on the hypothalamus and pituitary is disrupted (e.g. Wise and Ratner, 1980; Luderer and Schwartz, 1994).

Gonadal hormones also interact with the hypothalamic-pituitary-adrenal axis; for example, testosterone exerts a tonic inhibitory action on the HPA axis in males, inhibiting CRH expression and secretion, whereas estrogen can increase CRH production in males and females (Patchev et al., 1995; Lund et al., 2004). Thus it is important to note in discussing the effects of any one hormone that the results may be dependent on the overall hormonal milieu in which treatment is occurring.

Further, as discussed in Chapter 2 (Barker and Galea, 2008) and Chapter 4, the effects of estradiol administration can vary depending on the dose administered, the length of time post-gonadectomy, the administration schedule and duration of estradiol treatment (e.g. Ormerod et al., 2003; Leuner et al., 2004; Wide et al., 2004; Tanapat et al., 2005; Sinopoli et al., 2006). I opted to use the particular estradiol administration paradigm described based on studies in meadow voles in which gonadectomized males and females were injected with estradiol benzoate (EB) daily for 5 days, either 1-5, 6-10, or 11-15 days after a single BrdU injection, and examined on the 16th day after BrdU administration. Males that were injected with EB on days 6-10 demonstrated an increase in the number of BrdU-labelled cells present in the dentate gyrus on day 16 (Ormerod et al., 2004). In females, estradiol results in an upregulation in cell survival regardless of time of administration (Ormerod & Galea, unpublished data). In an effort to explore the effects of longerterm administration, I therefore mimicked the procedures used in this study but continued estradiol treatment for the entire 15 days. Daily injections provide daily peaks and troughs in peripheral estradiol levels similar to the normal increase (in

intact females) from relatively low estradiol levels in diestrus to a peak during the day of proestrus followed by a decline back to low levels 24 hrs later (Butcher et al., 1974). Daily injections do not, however, include the normal 4-5 day delay that would occur between proestrous peaks of estradiol in intact females. Interestingly, estradiol given to female rats either continuously (via silastic pellets) or on an injection schedule designed to mimic the normal estrous cycle (with or without progesterone) for 21 days did not significantly affect cell proliferation in the dentate gyrus (Tanapat et al., 2005). However, in Chapter 2, I found an increase in cell proliferation (Ki-67+ cells) after 15 daily injections of estradiol benzoate, as well as other changes in cell death and neuron production in the dentate gyrus. Thus estradiol appears to influence neurogenesis in the adult female rat in the short-term (up to 15 d) but this ability may be diminished after longer-term exposure to estradiol (21 d). Further, the ability of estradiol to influence neurogenesis may be dependent on the type of estrogen (17 β -estradiol or estradiol benzoate) or the administration technique (injections versus pellets; compare Barker and Galea, 2008 with Tanapat et al., 2005).

The dose of estradiol benzoate I opted to use in female rats (10 µg) produces supra-physiological levels of estradiol in the peripheral circulation for 1-2 hours after injection, after which levels fall to within the high end of the physiological range for normally cycling female rats (60-90 pg/mL). This dose of systemic estradiol has been used frequently in the literature (e.g. Woolley and McEwen, 1993; Galea et al., 2001; Mazzucco et al., 2008). Acute administration of this dose increases cell

proliferation in the dentate gyrus of ovariectomized female rats (Tanapat et al., 2005; Barha et al., 2009; Ormerod et al., 2003). Interesting, although repeated administration of this dose of estradiol benzoate produces serum concentrations well above those found in intact animals, the change in estradiol concentration is much less pronounced within the hippocampus itself (Chapter 3, Barker and Galea, 2009). A dose-response curve has yet to be produced regarding either brain concentrations or effects on neurogenesis after long-term estradiol administration, but could prove to be an important addition to the current literature.

Within neurogenesis research as a field, thymidine analogues and endogenous proteins are widely used as indicators of a particular cell's age and/or phenotype. As noted in Chapter 1, both applied and endogenous markers have a variety of limitations (e.g. Nowakowski and Hayes, 2000; Rakic, 2002). However, the importance of these concerns can be reduced by combining techniques and markers, such as I have done in Chapter 2 (Barker and Galea, 2008). Whether different measures agree with each other or differ, the combination of a variety of measures provides a powerful tool for elucidating the effects of any treatment. For example, the use of BrdU alone to label new cells would suggest that cell survival is decreased by estradiol treatment (Chapter 2, Barker and Galea, 2008). However, I also found that estradiol decreased cell death using several immunohistochemical markers. This suggests that estradiol may be acting differently on different subpopulations of cells in the hippocampus. This is further supported by my finding that the number of young neurons, as measured by DCX labelling, does not seem

affected by estradiol treatment in females (Chapter 2, Barker and Galea, 2008). BrdU is permanently incorporated into cells that were synthesizing DNA at or about (within 2 h of) the time of BrdU administration (Packard et al., 1973; Cameron and McKay, 2001). Markers of cell death are generally only present in cells during their final days to hours (FluoroJade: Schmued and Hopkins, 2000; activated caspase-3: Porter and Jänicke, 1999; pyknosis: Hu et al., 1997), and thus also provide an indication of events within a relatively narrow time window. Doublecortin on the other hand is present in neuroblasts, and in young neurons as they extend processes approximately 24h – 14 d after birth (Brown et al., 2003; Kempermann et al., 2003; Rao and Shetty, 2004; Seri et al., 2004), providing a wider overview of neurogenesis and the neurogenic capacity of the hippocampus. Combining markers spanning multiple developmental stages of cells (e.g. Chapter 2, Barker and Galea, 2008), can thus provide an indication of the age or type of cell being affected by treatment. Finally, an important caveat to studies of sex differences in neurogenesis is that few studies have been completed to determine whether cell cycle kinetics or the cell maturation timeline of new neurons is similar in male and female rats, and this may prove to be important information. For example, adult female rats in diestrus have fewer late-phase young neurons (expressing DCX and having long processes) than do males, but have more transient amplifying neuroblasts (co-expressing Ki-67 and DCX) (Mandyam et al., 2008). This suggests that exit from the cell cycle may be delayed in new neurons generated by the female hippocampus, relative to those in males. Alternatively, survival of neuroblasts to the young neuron stage may be lower in females than in males. Further investigation

into the kinetics of the cell cycle and maturation of these new cells is needed to elucidate the underlying mechanisms, which may then help to explain the sex differences in reactivity to treatments such as estradiol.

In these studies I found that repeated estradiol affects performance on a hippocampus-dependent task which is coincident with its effects on adult hippocampal neurogenesis, but I have not yet demonstrated a causal relationship. Such experiments are difficult to arrange, as behaviour must be measured in vivo whereas the state of individual cells deep within the brain is possible only upon removal of the brain and post-mortem tissue processing. The use of conditional genetic knockdowns to reduce the expression of ERs within new cells or young neurons specifically could be used to determine whether estradiol exerts its effects on behaviour through direct effects on these cells. Similarly, reducing the number of new cells by a conditional knockdown of proteins required for the production or maturation of young neurons would allow for examination of the effects of estradiol in the absence of hippocampal neurogenesis. While such techniques, of course, come with their own caveats, the combination of different lines of research would help to describe more fully the mechanisms underlying sex differences within the brain.

Finally, rats used in research are bred in captivity and for a particular set of characteristics. Strain differences in baseline neurogenesis have been described (e.g. Perfilieva et al., 2001; Kronenberg et al., 2007; Epp et al., 2009), as have

differences in response to treatments such as fluoxetine (AlAhmed and Herbert, 2008), social isolation (Bjørnebekk et al., 2007), repeated restraint stress (Uchida et al., 2008), and running (Bjørnebekk et al., 2005). Thus while the results I have described above may represent generalizable phenomena, it is important to consider the possibility that different strains and/or species may not respond in the same way. Such differences can be useful, however, as they can lead to further research describing the underlying genetic mechanisms mediating phenomena including the interaction of gonadal hormones with, and effects on, adult neurogenesis and behaviour.

Future studies

Although estradiol affects contextual fear conditioning in female rats (Maren et al., 1994; Markus and Zecevic, 1997; Gupta et al., 2001; Jasnow et al., 2006), the behavioural effect I found in response to estradiol between the sexes was rather subtle. Female rats given long-term estradiol treatment seem better able to recognize a novel environment as distinct from the training environment, as demonstrated post-training by reduced freezing in the novel chamber. Estradiol-treated rats froze to the same extent as their oil-treated counterparts when tested a second time in the training chamber, suggesting that the difference in the novel chamber was not due to forgetting or extinction of the learned context-shock association. It is also important to note that estradiol administration had ceased prior to training and behavioural testing, so the difference found was not due to the

presence of estradiol at the time of testing. I found that repeated exposure to estradiol influences both neurogenesis in the hippocampus (Chapter 2, Barker and Galea, 2009) and hippocampus-dependent context discrimination (Chapter 4) in female rats. Thus it is possible that the two effects are related, as discussed in Chapter 4. The characteristics of the training and testing paradigm used, allow for a variety of additional experiments to further explore the effects of estradiol on behaviour, and the relationship of adult neurogenesis and sex to learning and memory. For example, the effects of continuing long-term estradiol treatment throughout training and/or testing on performance in the fear conditioning task described have yet to be determined. This information would also help elucidate the mechanisms underlying sex differences in performance on fear conditioning, novel context or object recognition, and other tasks that require involvement of the hippocampus. Furthermore, if repeated estradiol treatment affects both neurogenesis and the ability to process new information or distinguish between similar environments, this could provide additional clues as to the function of the new neurons. The hippocampus is important for contextual pattern separation and discrimination (e.g. Leutgeb and Leutgeb, 2007; Bakker et al., 2008; Goodrich-Hunsaker et al., 2008; McTighe et al., 2009), and recent evidence suggests that hippocampal neurogenesis is important for pattern separation (Clelland et al., 2009). Together this provides further evidence to suggest that the subtle effect of novel context discrimination may have been due to the modulation of adult hippocampal neurogenesis by estradiol.

One can address the question of whether the effects of estradiol on neurogenesis cause the effects of estradiol on behaviour by using manipulations that block or reduce the production of new neurons in the hippocampus. Methylazoxymethanol (MAM, e.g. Ciaroni et al., 2002; Shors et al., 2002), focal irradiation of the hippocampus (e.g. Saxe et al., 2006; Wojtowicz, 2006) and genetic manipulations (e.g. Saxe et al., 2006; Dupret et al., 2008) have all been used previously to reduce neurogenesis in the adult hippocampus. If the effects of estradiol on behaviour are still observed, this would suggest that it affects other features of cells in the hippocampus such as dendritic branching or synapse formation. To further this investigation, and that into the structural or biochemical differences in the response to estradiol between males and females, it would also be beneficial to determine the location and quantity (or density) of ER α and ER β at the cellular level. It is possible that the localization of ER subtypes across subpopulations of cells (e.g. cell types) and within individual cells (e.g. at synapses, or near the nucleus) differs between the sexes. Such cellular and subcellular differences could account for the sex differences observed in the behavioural response to estradiol.

It is important to note that estradiol was manipulated systemically in Chapters 2-4, by gonadectomy and peripheral injections, so effects of estradiol on brain regions other than those studied here cannot be ruled out. Local infusion of estradiol directly into the hippocampus, while not without its own important caveats, could help in determining what effects of estradiol are specific to the hippocampus.

It would also be useful to locally infuse estradiol into the hippocampus of gonadally intact animals, to determine the effects on neurogenesis and behaviour of either stabilizing or increasing the amount of estradiol present in this region.

Conclusions

I have demonstrated here a variety of sex differences in the effects of estradiol on cells in the hippocampus and on a hippocampus-dependent learning task. Although the precise mechanisms underlying these differences remain to be determined, there are several possibilities worth examining. These include estrogen receptor subtype localization, the availability of cofactors modulating the binding of activated ERs to the genome, and interactions with other signaling pathways. I have also reported sex differences in estradiol concentrations in the prefrontal cortex and hippocampus, and in performance on a fear conditioning task. I have shown sex differences both between gonadectomized animals and in their responses to estradiol. Such characteristics unique to each sex further our understanding of the function of estradiol within the brain, and of the characteristics of the brain that may help explain sex differences in behaviour and cognition.

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Application Number: A05-1889						
Investigator or Course Director: Liisa Galea						
Department: Psyc	hology, Department of					
Animals:						
R	ats Long Evans 160					
Start Date:	February 28, 2006 Approval Date: May 14, 2009					
Funding Sources:						
Funding Agency: National Alliance for Research (US) Funding Title: Models of post-partum depression: Effects on behavior, stress reactivity and hippocampal neurogenesis in both mother and offspring						
Funding Agency: Funding Title:	Natural Sciences and Engineering Research Council of Canada (NSERC) Hormones and learning and memory					
Funding Agency: Funding Title:	British Columbia Ministry of Children and Family Development Effects of early androgens and corticosterone on stress reactivity and cognition in adulthood					
Unfunded title:	N/A					

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.



Application Nu	mber: A06-0	011				
Investigator or	Course Dire	ctor: Liisa Gak	1			
Department: Ps	ychology, D	epartment of				
Animals:						
	Rats Long I	Svans 24				
	Rats Spragu	e-Dawley 78				
Pate oprague-Dawley 780		e dawley 280				
	Rats Spragu	e-Dawley 60				
	Rats Spragu	e Dawley 48				
Rats sprague dawley 48		e dawley 48				
Rats Sprague Dawley 30		e Dawley 30				
Rats Long Evans 40		Svans 48				
Start Date:	Febr	uary 1, 2006		Approval Date:	March 26, 2009	
Funding Source	51					
Funding Agency	y: 1	National Alliana	e for Research (US)			
Funding Title:		Models of post-partum depression: Effects on behavior, stress reactivity and hippocampal neurogenesis in both mother and offspring				
Funding Agency: P Funding Title: E		Pacific Alzheimer Research Foundation Effects of estrogens on neurogenesis in the adult hippocampus in young and aged rodents				
Noticed (Biose & Description)						
Funding Agency	y	Models of post-	ance for Research (US) st-partum depression: Effects on behavior, stress reactivity and hippocampal neurogenesis in both mother and			
Funding Title: offspring		offspring			,, ,, ,, ,, ,, ,, ,, ,, ,, ,, ,, ,, ,, ,, ,, ,,	
Funding Agency: Pacifi		acific Alzheimer Research Foundation				
Funding Title: Effects of		Effects of estrog	ts of estrogens on neurogenesis in the adult hippocampus in young and aged rodents			
Funding Agenc	v: (Canadian Institu	es of Health Research (CIHE	n		
Funding Title: Parity effects of		Parity effects on	brain morphology and functi	on		
Funding Agency: Canadian Ins		Canadian Institu	es of Health Research (CIHF	0		
Funding Title: Estrogen		Estrogen and pr	gen and progesterone effects on neurogenesis in the adult mammalian hippocampus			
Unfunded title:	1	N/A				

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

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A copy of this certificate must be displayed in your animal facility.



Application Number: A07-0123						
Investigator or Course Director: Liisa Galea						
Department: Psychology, Department of						
Animals:						
	Rats Sprague-Dawley 44					
Rats Sprague Dawley 192						
	Rats sprague dawley 276					
Start Date:	April 1, 2007	Approval Date: June 23, 2009				
Funding Source	e.	••				
Fullding Bources.						
Funding Agency: Pacific Alzheimer Res Funding Title: Effects of estrogens of		arch Foundation neurogenesis in the adult hippocampus in young and aged rodents				
	0					
Unfunded title:	N/A					

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

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Application Number: A07-0335						
Investigator or Course Director: Liisa Galea						
Department: Psychology, Department of						
Animals:						
	Rats Long Evans 168					
	Rats Long Evans 250					
	Rats Sprague Dawley 152					
Start Date:	October 15, 2007	Approval Date:	May 15, 2009			
Funding Sources:						
Funding Agency Funding Title:	 Natural Sciences and E Hormones and learning 	ingineering Research Council of Can 3 and memory	ada (NSERC)			
Unfunded title:	N/A					

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.