ESTRADIOL-INDUCED CHANGES IN THE PRODUCTION AND SURVIVAL OF GRANULE NEURONS BORN IN THE DENTATE GYRUS OF ADULT RODENTS

by

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ABSTRACT

Controlling neurogenesis constitutive to the hippocampal dentate gyrus of adult mammals could improve strategies geared toward replacing neurons lost in the diseased or injured human CNS. This thesis resolves and expands upon conflicting reports about estradiol’s influence over neurogenesis (progenitor proliferation and daughter cell differentiation and survival) in the adult rodent dentate gyrus. Chapter 2 showed that reproductive status regulates neurogenesis in the dentate gyri of adult laboratory-reared female meadow voles. Specifically, reproductively inactive (low estradiol) females had more dividing cells than reproductively active (high estradiol) females or females exposed to estradiol for 48 h. However, females exposed to estradiol for 4 h had more dividing cells than reproductively inactive females, suggesting that estradiol dynamically regulates cell proliferation. Because the ratio of new cells surviving 5 weeks versus 2 h was higher in the dentate gyri of reproductively active versus inactive females, estradiol appeared to enhance the survival of young cells. Chapters 3 and 4 confirmed that estradiol dynamically regulates dentate cell proliferation robustly across rodent species.

Cell proliferation in the dentate gyri of female rats (Chapter 3) and meadow voles (Chapter 4) increased 4 h after but decreased 48 h after estradiol- versus vehicle-treatment. In part, estradiol suppressed proliferation by stimulating adrenal activity because adrenalectomy eliminated the suppression in adult female rats. Consistent with the effects reported in other species, NMDAr activation decreased and NMDAr inactivation increased proliferation in the dentate gyri of adult female voles but estradiol did not stimulate NMDArs to influence cell proliferation.

Chapter 5 showed that estradiol potently enhances young granule neuron survival and that enhanced survival is related to improved hippocampus-dependent memory (but not learning). Specifically, estradiol doubled the number of 16-day old neurons in the dentate gyri of adult male meadow voles when administered over Days 6-10 after the neurons are born. Estradiol-
treated voles (Days 6-10), exhibited similar hormone-free performance Morris water maze training trials but outperformed vehicle-treated voles on a probe trial. Chapter 6 discusses how estradiol-induced changes in components of neurogenesis may influence normal hippocampus function and discusses how the findings of this thesis may relate to neuronal replacement strategies.
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Estrogen could time-dependently influence cell proliferation in the dentate gyrus of adult rodents through numerous pathways.

Changes in cell proliferation within the dentate gyri of intact females support that estradiol alters cell proliferation dynamically.

New neurons appear functional and influence hippocampus-dependent behavior.

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Estradiol regulates different components of neurogenesis that occurs in adulthood.

New neurons have functional characteristics.

Neurogenesis and hippocampus-dependent behaviour in adulthood.

5.5 IMPLICATIONS

CHAPTER 6

GENERAL DISCUSSION

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<th>Description</th>
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<td>adrenocorticotropic hormone</td>
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CHAPTER 1
GENERAL INTRODUCTION

Over the last decade, evidence has been gathered to demonstrate that new neurons are added daily to the hippocampal dentate gyri and the olfactory bulbs of mammals throughout adulthood (Eriksson et al., 1998; see Gage, 2000, Cameron and McKay, 2001; Gould and Gross, 2002 and Magavi and Mackliss, 2002 for review). Widespread acceptance that neurogenesis occurs within the adult mammalian central nervous system (CNS) has ignited research aimed toward identifying the mechanisms that control the process, largely with the agenda of improving neuronal replacement strategies to restore the neuronal loss associated with neurodegenerative disease or neurotrauma. However, the field is young and the mechanisms regulating the proliferation of progenitor cells and the differentiation and survival of daughter cells either in vivo or in vitro need to be more fully understood before such an approach is attempted. An equally important research avenue being currently undertaken strives to understand the role that neurogenesis through adulthood plays in normal hippocampus and olfactory bulb function. Evidence suggests that new neurons are functionally integrated into the hippocampus and olfactory bulbs and that depleting young neuron number in either area can impair associated behaviours (Gheusi et al., 2000; Shors et al., 2001, 2002; van Praag et al., 2002). These findings tempt speculation that adult-generated neurons could be used to replace neurons lost in the diseased or injured CNS to perhaps restore lost function.

Several groups have proposed that the symptoms of neurodegenerative diseases or neurotrauma with relatively homogenous aetiologies will be those most imminently improved by neuronal replacement strategies (see Shihabuddin et al., 1999; Björklund and Lindvall, 2000; Rossi and Cantaneo, 2002). For example, Parkinson's disease is associated with a progressive loss of dopaminergic neurons in the substantia nigra that culminates in the depleted
striatal dopamine levels thought to underlie the abnormal motor symptoms of bradykinesia, rigidity and tremor (Duvoisin, 1992). Björkland and Lindvall (2000) suggest that neuronal replacement could alleviate the symptomology of a disease, such as Parkinson’s, in two ways. First, cells able to differentiate into the appropriate neuronal phenotype could be grafted into the affected CNS region to replace lost neurons by establishing appropriate efferent and afferent connections. Second, cells genetically engineered to secrete growth factors or neurotransmitters could be grafted into the affected region to promote the survival or regeneration of existing neurons. In fact, fetal mesencephalic tissue grafted into the striatum of Parkinson’s disease patients appears to produce long-lasting (5-10 yr) improvements in symptomology both by becoming integrated into striatal circuitry and by increasing striatal dopamine (Björklund and Lindvall, 2000). Despite the apparent success of fetal-tissue derived grafts in alleviating the symptoms of some Parkinson’s disease patients, using fetus-derived tissue for medical purposes is problematic for several reasons. Acquiring enough fetus-derived tissue is difficult (the tissue from several embryos is required to treat 1 Parkinson’s patient), samples are seldom standardized and the use of fetal tissue for medical purposes is ethically controversial. Clearly, sources of cells that are abundant, standardizable and ethically acceptable that could be grafted into the diseased or damaged CNS could improve neuronal replacement strategies.

Adult CNS progenitor cells are a potentially viable source of cells for grafting either homotopically (into the donor CNS) or heterotopically (into a non-donor CNS) into the diseased or injured CNS. Progenitor cells can be isolated surgically from neurogenic (subventricular and subgranular zones) and non-neurogenic (spinal cord, septum and striatum) adult CNS regions (Morshead et al., 1994; Gage et al., 1995; Palmer et al., 1995,1997; Weiss et al., 1996; Shihabuddin et al., 1997; Kukekov et al., 1999; Roy et al., 2000; Aresenijevic et al., 2001). Recently, progenitor cells have even been isolated from neurogenic and non-neurogenic
regions of the human cadaver CNS (Palmer et al., 2001), further demonstrating their accessibility and potentially bypassing ethical controversy regarding their use. Adult CNS-derived progenitor cells can be expanded long-term in culture to produce many clones and can generate multiple CNS cell types both in vitro and when grafted into neurogenic CNS regions (Gage et al., 1995; Weiss et al., 1996; Palmer et al., 1995, 1997), suggesting that the variety of cell types required to treat different diseases could be generated. In fact, Gage and his colleagues (Sakurada et al., 1999) were able to direct the differentiation of adult rat hippocampus-derived progenitor cells to a dopamine neuron phenotype in vitro. Adult rat hippocampus-derived progenitors grafted into various CNS regions survive for several months (Gage et al., 1995; Suhonen et al., 1996), indicating that trophic support for new cells is abundant throughout the adult CNS. However, these cells only adopt a neuronal fate when transplanted into neurogenic regions (Suhonen et al., 1996). Thus, neuronal replacement in non-neurogenic regions of the adult CNS using this type of approach may require the grafting of cells that have been partially differentiated in vitro or the co-transplantation of cells engineered to secrete factors that direct the differentiation of progenitors.

Alternatively, by understanding the cues that regulate neurogenesis (progenitor cell differentiation, daughter cell differentiation and the survival of young neurons) it may become possible to manipulate endogenous progenitor cells in situ to replace neurons lost in the diseased or injured CNS. In the normal rodent CNS, thousands of neuroblasts are produced daily in the subventricular zone and the hippocampal subgranular zone (Lois and Alvarez-Buylla, 1994; Cameron and McKay, 2001; Seri et al., 2001; Chapters 3, 4, 5). Neuroblasts migrate several mms from the subventricular zone to the olfactory bulbs or several μms from the subgranular zone to the granule cell layer where they differentiate into neurons of the appropriate phenotype (Cameron et al., 1993b; Lois and Alvarez-Buylla, 1994). Therefore, if
the cues that regulate daughter cell differentiation and neuroblast migration in vivo were understood, neuroblasts of the appropriate phenotype could be enticed to migrate into affected CNS areas. As mentioned previously, progenitor cells are situated throughout the CNS. In fact, targeted cell death in the cortex can induce resident progenitors to generate neurons that morphologically resemble cortical pyramidal neurons (Magavi et al., 2000). Therefore, the potential to stimulate neurogenesis could also be present throughout the adult mammalian CNS.

Although the biochemical and behavioural regulators of neurogenesis constitutive to the dentate gyrus are beginning to be understood, studies seldom investigate the cumulative effects of a single factor on the proliferation of progenitor cells, the differentiation of daughter cells and survival of young neurons. Understanding the cumulative effects of a single factor on these components of neurogenesis is important because neuronal replacement strategies strive to increase neuron number in an affected CNS area. A factor that increases progenitor cell proliferation but decreases the survival of young neurons would produce no net change in new neuron number, and would therefore yield limited therapeutic benefit if used in isolation to increase neuron number. The experiments described in the present thesis adopt the approach of investigating the influence of estradiol upon multiple components of neurogenesis in the dentate gyrus of adult rodents. The findings of this thesis are that estradiol first increases (within 4 h) but then stimulates adrenal activity to decrease (within 48) cell proliferation, does not affect the differentiation of daughter cells and enhances the survival of new granule neurons in the dentate gyrus of adult rodent. Increased cell survival is related to improved hormone-free performance on a retention trial, but not on acquisition trials in the hippocampus-dependent Morris water maze, suggesting that memory (but not learning) is improved by an estradiol-induced increase in young neuron number. The findings are discussed in the context of how estradiol-induced changes in dentate neurogenesis could influence normal hippocampal function and neuronal replacement strategies.
1.1 HISTORICAL SYNOPSIS OF ADULT CNS NEUROGENESIS RESEARCH

"...once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centres the nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated. It is for the future to change, if possible, this harsh decree."

Ramón Y Cajal (1913-1914) translated by Raoul May

Forty years ago, Joseph Altman challenged the dogma that the adult mammalian CNS was ‘fixed and immutable’ by using the cell synthesis marker $[^3\text{H}]$-thymidine to demonstrate that new cells, which eventually become morphologically indistinguishable from neighbouring granule neurons, are added daily to the dentate gyrus and olfactory bulbs of adult rats (Altman, 1962; Altman and Das, 1965; Altman, 1969). Few papers describing the topic of adult neurogenesis were published in years following Altman’s very interesting discovery. In their 1984 paper, Kaplan and Bell suggest that researchers resisted the idea that neurogenesis persisted throughout adulthood in mammals because of scepticism about whether adult neural “stem” or “blast” cells could exhibit mitotic potential in vivo and whether the apparently low-level $[^3\text{H}]$-thymidine incorporation observed to occur in the rodent hippocampus represented DNA repair rather than cell division. They attempted to dispel the scepticism by identifying synapses on the soma and processes of $[^3\text{H}]$-thymidine-labelled cells under the electron microscope. Nonetheless, scientists largely resisted the idea that new cells produced in the adult mammalian CNS differentiated into neurons based upon a purely morphological analysis, likely because glia also receive synaptic input (Ventura and Harris, 1999 for example). Nottebohm did manage to stimulate interest in adult neurogenesis by showing that more new cells are added to the higher vocal centre (HVC) of adult male canaries during the fall non-breeding season when new song is learned than during the spring breeding season when song is stable (Goldman and Nottebohm, 1983; Nottebohm et al., 1986; Nottebohm, 1989). However,
Nottebohm's discovery was touted as an interesting avian phenomenon after Rakic (1985a; 1985b) reported that although new cells were produced in the hippocampus of adult rhesus monkeys, he could not verify the phenotype of the new cells using techniques available at that time.

Fortunately, stereological and immunohistochemical methods that could be used to more accurately count and identify the phenotype of new cells in the adult mammalian CNS were developed. Gould and her colleagues first used immunohistochemistry to show that many cells that incorporate the cell synthesis marker $[^3]H$thymidine in the adult rat hippocampus eventually express mature neuronal protein (Cameron et al., 1993b). Coupled with the report made a year earlier by Reynolds and Weiss (1992) that cells derived from the adult rat forebrain generated both primary and secondary neurospheres in vitro, a property indicative of stem cell potential, this evidence prompted some interest in discovering how neurogenesis constitutive to the adult rodent CNS is regulated. In the years following Gould's rediscovery, neurogenesis was identified in the hippocampus of adult tree shrews, marmosets and macaques (Gould et al., 1997a, 1999a,b,c; Kornack and Rakic, 1999). Then Eriksson and his colleagues (1998) found new neurons in the post mortem dentate gyrus and olfactory bulbs of 57 to 72 year-old patients who had received BrdU to monitor tumour growth up to 2 yrs prior to succumbing to cancer. Importantly, post-mortem examinations found no evidence of tumour metastasis to cerebral tissue in that study. This finding demonstrated that neurogenesis is not a phenomenon merely vestigial to the CNS of species lower in phylogenetic rank to humans and that the human brain may in fact retain some regenerative capacity during adulthood. Most important, this finding ignited research in what appears to be the emerging field of 'adult stem cell biology'. In fact, a report created by the National Institutes of Health and Published by The Department of Health and Human Services (2001, June 17) states "...the field of stem cell biology is advancing at an incredible pace with new discoveries being reported in the scientific
literature on a weekly basis". Indeed, the often-cited decree that within the adult mammalian CNS "everything may die and nothing may be regenerated" appears lifted.

1.2 DEFINING STEM CELLS

Because the field of adult neural stem cell biology is relatively new and advancing at such a rapid pace, the terminology used in publications is generally inconsistent and confusing. The Department of Health and Human Services recently published a report (2001) developed by The National Institutes of Health regarding the current state of understanding about stem cells and their potential and pitfalls for therapeutic use. One of the goals of the report was to establish conventions about the terminology used for describing cells with mitotic potential found in neural or other tissue. According to the report, stem cells make copies of themselves (or exhibit clonality) for the host organism’s lifetime and produce all of the cells that compose specialized organs and tissues derived from the three embryonic germ layers (mesoderm, ectoderm and endoderm; or exhibit pluripotentiality). Adult stem cells are derived from specialized tissue (i.e. neural tissue), exhibit clonality for the host organism’s adult life and produce all the cell types unique to the specialized tissue they were derived from (or exhibit multipotentiality; adult neural stem cells produce neurons, astrocytes and oligodendrocytes). Precursor or progenitor cells divide symmetrically (or asymmetrically for a short time) to produce cells of the specialized tissue they were derived from and typically are the mediaries between adult stem and specialized cells. Although the terms precursor and progenitor are often used interchangeably in the literature, Fabel and colleagues (2003) suggest that the term ‘progenitor cell’ is becoming used more often to describe stem-like cells in which either the property of clonality or multipotentiality has not been tested.

The generally accepted view is that new neurons are only generated by cells resident to the hippocampal subgranular zone and the lateral ventricle subventricular zone (Temple and
Alvarez-Buylla, 1999; Gage, 2000; Mao and Wang, 2001; Gould and Gross, 2002; Rakic, 2002). Although reports that neurons are added to the normal feline visual cortex, non-human primate neocortex and amygdala and rodent cortex and amygdala exist (Kaplan, 1981; Gould et al., 1999c, 2001; Bernier et al., 2002; Bedard et al., 2002; Fowler et al, 2002), these results have not generally been replicable (Magavi et al., 2001; Kornack and Rakic, 2001; Koketsu et al., 2003). However, neurogenesis can be induced in the rodent neocortex by targeted cell death (Magavi et al., 2001). In vivo characterization of proliferative cells within the subventricular and subgranular zones is difficult technically, primarily because stem and progenitor cells likely remain quiescent until they divide and at least do not express known proteins that could be used as specific stem cell markers (Gage, 2000; Temple 2001). Therefore, most information about adult neural stem or progenitor cells has been derived using a combination of in vitro and transplant techniques.

One question posed by adult neural stem cell biologists asks whether cells in the proliferative zones of the adult CNS are neural stem cells (i.e. are they clonogenic and multipotential?). Studies have shown that adult human and rodent hippocampus-derived cells cultured on an adherent substrate in media containing fibroblast growth factor (FGF-2) can generate progeny for at least one year, the majority of which express markers found in stem cells, such as nestin, O2-4 and A2B5 (Gage et al., 1995; Palmer et al., 1995,1997; Kukekov et al., 1999; Roy et al., 2000). More convincing evidence that these cells are clonogenic has been shown by studies that genetically tagged cultured adult hippocampus-derived cells to track their lineage and then verified the presence of clones after many divisions using Southern blot analysis (to verify cell genotypes; Palmer et al., 1997). Growth factor withdrawal, high-density cell cycle arrest or the addition of factors such as retinoic acid, cAMP or neurotrophic factors, induces cultured cells to differentiate into multiple CNS cell lineages in vitro (Gage et al., 1995; Palmer et al., 1995,1997; Roy et al., 2000). For example, under these conditions cells
either tend to acquire neuronal- or glial-like processes, to express markers typical of neurons, glia or oligodendrocytes and cells that express neuronal protein can generate sodium and potassium currents typical of neurons. When cultured adult hippocampus-derived cells are grafted heterotopically into either the hippocampus or olfactory bulbs, they generate phenotypically appropriate glia and neurons (Gage et al., 1995; Suhonen et al., 1996), further demonstrating that adult hippocampus-derived cells are capable of producing multiple CNS cell phenotypes.

Cells derived from the adult mammalian subventricular zone also appear able to self-renew and generate multiple CNS cell lineages. Adult mouse and human subventricular zone-derived cells aggregate into spheres of proliferating cells when plated on a non-adherent substrate in media containing either epidermal growth factor (EGF) or FGF-2, and individual cells derived from these spheres can produce secondary spheres, suggesting that these cells are capable of self-renewal. When these sphere-producing cells are plated on an adherent substrate in either EGF or FGF-2 many daughter cells acquire processes and express neuronal or glial markers (Reynolds and Weiss, 1992; Lois and Alvarez-Buylla, 1993; Morshead et al., 1994; Reynolds and Weiss, 1996; Gritti et al., 1996; Chiasson et al., 1999; Kukekov et al., 1999; Arsenjivec et al., 2001), suggesting that these cells are also multipotential. Palmer and his colleagues (1995) have confirmed that adult rat subventricular zone-derived cells, like cells derived from the hippocampus, are clonogenic and multipotential by identifying genetically tagged clones that generate multiple cell lineages after many divisions in culture using Southern blot analysis. Clearly, cells located within the proliferative adult dentate gyrus and subventricular zone exhibit the properties of self-renewal and multipotentiality and, therefore, could be adult neural stem cells.

Because gliogenesis persists throughout the adult mammalian CNS (Ichikawa and Hirata, 1982; Korr et al., 1983), the possibility that stem cells exist in non-proliferative CNS
regions exists. In fact, the first adult CNS-derived cells shown to exhibit stem cell-like properties in culture were isolated from the mouse striatum (Reynolds and Weiss, 1992). Since then, adult neural stem-like cells have been isolated and cultured from the adult rat septum and striatum (Palmer et al., 1995), the rat and human cortex (Palmer et al., 1999; Arsenijevic et al., 2001), the mouse and human rostral migratory stream and olfactory bulbs (Pagano et al., 2000; Gritti et al., 2002) and the mouse and rat spinal cord (Weiss et al., 1996; Shihabuddin et al., 1997). Interestingly, more cells that exhibit stem-cell properties in culture can be isolated from neurogenic versus quiescent CNS regions (Lois and Alvarez-Buylla, 1993; Morshead et al., 1994; Palmer et al., 1995; Seaberg and van der Kooy, 2002). Because stem-like cells can be isolated from normally quiescent regions of the adult CNS, the potential for neurogenesis in these areas must exist. However, the finding that adult hippocampal progenitors only adopt a glial phenotype when grafted into these quiescent CNS regions (Gage et al., 1995; Suhonen et al., 1996) suggests that these areas do not express the cues that induce neuronal differentiation and guide the migration of neuroblasts.

There is debate about whether the stem-like cells in different adult mammalian CNS regions share the same phenotype and about how primitive these cells in each region may be. In fact, at least five different cell classes have been purported to be the source of new olfactory bulb neurons. For example, Johansson and his colleagues (1999) reported that a sub-population of ependymal cells lining the third ventricle generates progenitor cells that then generate neurons and glia, in vitro. However, van der Kooy and his colleagues (Chiasson et al., 1999) argue that subependymal cells (but not ependymal cells) self-renew and generate multiple CNS cell types in their culture system. Doetsch and her colleagues (1997) suggest that undifferentiated cells found in the rostral migratory stream are self-renewing and multipotential while Alvarez-Buylla and his colleagues (García-Verduga et al., 1998) have reported that rostral migratory stream astrocytes exhibit stem cell properties in vitro. Finally, Gritti and her
colleagues (2002) suggest that cells in the rostral extension of the olfactory peduncle and bulb exhibit stem cell-like characteristics in culture. Recently, debate about the phenotype of cells that produce new dentate granule neurons has begun. Although Gage and his colleagues (Palmer et al., 1997) have described adult dentate gyrus-derived cells as clonogenic and multipotential in their culture system, van der Kooy and his colleagues (Seaberg and van der Kooy, 2002) argue the same cells exhibit limited self-renewal in their culture system and that subependymal zone cells are the only true stem cell located in the hippocampus. Seri and her colleagues (2001) have argued that astrocytes within the subgranular zone exhibit self-renewal in culture and generate multiple CNS cell types. Reconciling the findings of these studies is difficult because the host strain and species, dissection methods, culture substrate and media are rarely controlled between studies (see Gage, 2000 for review). In fact, Palmer and his colleagues (1997 but see Palmer et al., 1999) have shown that cultures initiated from hippocampus-derived tissue containing mixed cell types retain a normal diploid karyotype for approximately 35 population doublings when cultured in FGF-2 but then spontaneously transform genetically to become increasingly aneuploid, demonstrating that culture conditions can transform CNS-derived cells. Understanding the behaviour of adult CNS progenitor/stem cells in vitro would benefit from studies that control all of these variables.

Taken together, these findings demonstrate that adult mammalian CNS-derived cells exhibit long-term self-renewal and can generate both neurons and glia in vivo and when transplanted back into the CNS and could be stem cells (Weiss et al., 1996; Fisher et al., 1995; Palmer et al., 1997). In fact, the proliferative cells found within the adult CNS are often referred to as adult neural stem cells (Shihabuddin et al., 1995; Gage 2000 for example). However, the property of clonality has not been demonstrated in vivo and therefore, cells with proliferative capacity in the subventricular and subgranular zones should remain classified as progenitor or precursor cells. In fact, according to Fred Gage (2002) "...no individual neural
stem cell has been identified and isolated adequately to separate it unambiguously from other, more committed [progenitor or precursor] cells in vitro or in vivo. Thus in the experiments described in this thesis, cells that incorporate BrdU upon dividing and subsequently produce daughter cells that express either neuronal or glial protein will be referred to as progenitor cells.

1.3 NEUROGENESIS IN THE ADULT MAMMALIAN BRAIN

Another line of research undertaken by adult stem cell biologists is aimed toward understanding how neurogenesis in the adult mammalian CNS is controlled. To visualize dividing progenitor cells or their progeny in situ, animals are commonly injected with $[^3]H$thymidine or the thymidine analogue bromodeoxyuridine (BrdU). Any cell in the synthesis phase of its cell cycle or undergoing DNA repair will preferentially incorporate either nucleotide into its DNA instead of endogenous thymidine (Packard et al., 1973; Miller and Nowakowski, 1988; Cameron and McKay, 2001). Then, the tissue of interest can be processed autoradiographically or immunohistochemically to reveal progenitor cells that divided in the approximately 2 h that $[^3]H$thymidine or BrdU, respectively, was bioavailable or their progeny depending upon the amount of time that elapses before perfusion (see Figure 1). BrdU and $[^3]H$-thymidine labelling studies have demonstrated that cells located in the subgranular zone divide producing daughter cells that migrate several μms, possibly along radial glia, into the granule cell layer (Altman and Das, 1965; Cameron et al., 1993b; Palmer et al., 2000; Figure 1). These markers have also demonstrated cells located in the subventricular zone divide producing daughter cells that chain migrate several mms through the rostral migratory stream and then along radial glia to the olfactory bulbs (Lois and Alvarez-Buylla, 1993; Rousselot et al., 1995).
Many daughter cells produced in the subgranular zone eventually differentiate into granule neurons upon migrating into the granule cell layer. Morphologically, they become indistinguishable from mature dentate granule neurons (Altman and Das, 1965; Cameron et al., 1993b), acquire synapses on their dendrites and soma (Kaplan and Bell, 1983; Kaplan and Hinds, 1977; Markakis and Gage, 1999) and extend an axon to the CA3 layer of the hippocampus within 4-10 days after birth (Stanfield and Trice, 1987; Hastings and Gould, 1999; Markakis and Gage, 1999). Three-dimensional confocal laser microscopy has been used to confirm the neuronal or glial phenotype of new cells by colocalizing fluorescent probe tagged anti-BrdU with fluorescent probe-tagged antibodies that recognize either neuronal or glial protein in single cells. This technique has revealed that approximately 60-70% of new cells found in the granule cell layer express immature neuronal markers such as doublecortin (DCX) and tubulin-β (Tuj 1β) and then mature neuronal markers such neuron specific enolase (NSE) and calbindin and approximately 15-20% express glial protein such as glial acidic fibrillary protein (GFAP; see Figure 2 for examples). These percentages are reasonable considering that most labelled cells migrate into the granule cell layer and the granule cell layer contains few glia (Kosaka and Hama, 1986; Cameron et al., 1993b; Palmer et al., 2000). The remainder of labelled cells do not express known proteins (Cameron et al., 1993b; Kempermann et al., 1997a; Eriksson et al., 1998; Tanapat et al., 1999; Palmer et al., 2000; Ormerod et al., 2002) and therefore could be daughter cells that retain a parent progenitor cell phenotype.

Today, BrdU is chosen more often than [3H]-thymidine to label dividing cells in adult mammals primarily because immunohistochemical processing is faster than autoradiography, working with radioactivity is labour-intensive and confocal laser microscopy can be used to phenotype BrdU-labelled cells. However, both markers are advantageous for labelling cells in
Inject BrdU

2h

24h

4-10d

2-3wks

BrdU-labelled progenitor cells

neuroblasts, glioblasts and possibly progenitor cell copies; neuroblasts begin expressing immature neuronal protein

Neuroblasts extend axons and express immature neuronal protein

new neurons express mature neuronal protein

Figure 1. Representation of a coronal hippocampal section from the rat with inset depicting neurogenesis and a time course for the maturation of new neurons.

Mature neurons situated in the granule cell layer (GCL) extend dendrites into the molecular layer and an axon through the hilus that synapses with pyramidal cells in the CA3 region of the hippocampus (Amaral and Witter, 1995). Cells located in the subgranular zone (the ≈50μm band between the hilus and GCL) divide producing daughter cells. Many daughter cell migrate into the GCL, and extend and axon into the CA3 region and dendrites into the polymorphic layer. Experimentally, changes in cell proliferation are assessed 2 h after a cell synthesis marker, such as BrdU, is injected as this amount of time is not sufficient for progenitor cells to complete mitosis. Twenty-four h after BrdU is injected, the number of daughter cells can be counted, but very few daughter cells express known proteins by this time making identification of their phenotypes difficult. Presumably neuroblasts migrate deeper into the granule cell layer between 1-4 days after birth and begin to express immature neuronal proteins (Chapter 5). Neuroblasts extend axons between 4 and 10 d after birth (Hastings and Gould, 1999) and 2-3 week old neurons begin to express mature neuronal proteins (Cameron et al., 1993b; Palmer et al., 2000).
different situations. In processed tissue, each $[^3]H$-thymidine substitution stoichiometrically appears as a silver grain, permitting the quantification of labelling degree (heavily labelled cells are progenitor cells or their progeny) and the number of divisions among daughter cells (heavily labelled cells are first generation and lightly labelled cells are subsequent generation; Packard et al., 1973; Nottebohm, 2002). Immunohistochemistry involves a series of steps that amplify the BrdU signal and therefore, is more sensitive but the amplification makes BrdU non-stoichiometric (Nowakowski and Hayes, 2000). Therefore, the hypothesis that BrdU-labelled cells could represent a population of cells that repaired rather than synthesized DNA has been forwarded. However, no evidence of BrdU labelling DNA repair in vivo has ever been published and several lines of evidence dismiss the DNA repair hypothesis. First, labelled cell number approximately doubles between 2 and 24 h after BrdU is injected (Cameron and McKay, 2001). Second, studies using BrdU immunohistochemistry to detect DNA damage in cultured cells deliver insults that induce the replacement of approximately 100 nucleotides at each damaged site whereas in vivo, only 1-2 nucleotides are replaced and current immunohistochemical techniques are not sensitive enough to detect this low-level repair (Schmitz et al., 1999 for review). Third, many BrdU-labelled cells come to exhibit morphology and express proteins similar to mature granule neurons in the granule cell layer (see previous paragraph). Finally, Palmer and his colleagues (2000) demonstrated that the BrdU incorporated by cultured fibroblasts following gamma irradiation, which produces relatively similar damage to what is observed in vivo, could not be detected immunohistochemically. Taken together, the evidence available suggests that BrdU can only be detected in cells that are synthesizing rather than repairing DNA.

Another advantage associated with BrdU is that anti-BrdU can penetrate the relatively thick tissue sections required to maintain the integrity of fixed tissue whereas emulsion only reveals $[^3]H$thymidine in the top 3 μm of a section (Feinendagen, 1971; Miller and Nowakowski, 1988). Therefore, stereological estimates of total labelled cell number can be determined on BrdU-labelled tissue (Gunderson et al., 1988; West et al., 1991; Cameron and McKay, 2001). Stereology was designed to estimate the total number of cells in a structure that
would be observed if the entire structure could be visualized under the microscope (Gunderson et al., 1988; West et al., 1991). For example, if BrdU-labelled cells were counted on every section through the dentate gyrus, then the total number of labelled cells would be underestimated because only those observed in the focal plane of the microscope (i.e. typically the top 0.005 mm of each section using a 100x light objective) can be counted. Stereology, as applied to BrdU-labelled tissue, projects the number of labelled cells counted in the focal plane of the objective through the section thickness and if cells are counted on sections of equal intervals (i.e. every 10th section) through the dentate gyrus then cells are projected through its estimated volume (see Chapter 4 for more detail). The application of stereological techniques to estimate BrdU-labelled cell number through the dentate gyrus of adult mammals appears partially responsible for the relatively recent surge in interest regarding the phenomenon. In fact, Kaplan and Bell (1984) suggested that few researchers were interested in investigating adult neurogenesis partially because the phenomenon appeared to be quite low-level based upon the densities of [3H]thymidine-labelled cells reported (see Altman and Das, 1965; Cameron et al., 1993b). Using stereology, Cameron and McKay (2001) have determined that up to 9,000 new cells are produced daily in the dentate gyri of adult rats (Cameron and McKay, 2001).

Other methods of visualizing dividing cells or their progeny have also been used and can be advantageous in certain situations. Retroviral vectors carrying a reporter gene such as Lac-Z or green fluorescent protein can be used to visualize entire dividing cells and their progeny (see Sanes et al., 1986; van Praag et al., 2002). Because these vectors are expressed in the soma, dendrites and axon of new cells, they can be useful for localizing new neurons in tissue slice preparations used for electrophysiological recording, for example. However, because infection rates can be low-level and retroviral expression can be downregulated once the progeny of dividing cells differentiate (Gage, 2000) their usefulness for stereologically estimating the total number of new cells may be limited. Endogenous markers of mitosis such as Ki67 and PCNA appear as effective as BrdU or [3H]-thymidine for detecting proliferating cells (Nacher et al., 1996; Tanapat et al., 1999; Wojtowicz) and do not require an injection.
However, the transient expression of endogenous mitosis markers makes phenotyping progeny difficult because cells are in s-phase only for approximately 9.5 h (Cameron and McKay, 2001). Clearly, the choice of a proliferation marker depends upon the question being asked in an experiment.

To understand the mechanisms and function of adult neurogenesis, the factors affecting progenitor cell proliferation (mitosis in progenitor cells) must be delineated from the factors that affect the survival (differentiation and maturation) of young neurons. Altering the rate of cell proliferation, the differentiation of daughter cells or the survival of young neurons could increase or decrease net neurogenesis (literally, the creation of neurons) in the dentate gyrus. An experimental manipulation administered just before or during the time that BrdU is bioactive affects cell proliferation whereas an experimental manipulation administered after BrdU is bioactive can affect the survival of labelled cells in the process of differentiating, migrating or maturing. In both cases, the effect is reflected in the number of labelled cells observed hours, days or weeks after BrdU is injected. Alternatively, a manipulation could be administered just prior to or during BrdU uptake and then labelled cell number could be assessed weeks later to verify the number of new neurons surviving. However, in this case BrdU-labelled cells should also be assessed 2-24 h after BrdU is administered (the time required for 1 mitotic division; Cameron and McKay, 2001) to disentangle effects on proliferation versus survival. Although the number of labelled cells has been assessed after multiple daily BrdU injections in some studies to investigate the effect of housing condition, physical activity or genetic strain on neurogenesis (Kempermann et al., 1997a, 1997b; van Praag et al., 1999a, 1999b), interpreting the results of these studies is difficult. In the case that no difference between groups in BrdU-labelled cell number emerges following multiple daily injections, it could be that proliferation was increased and the survival of young neurons decreased or vice versa. Generally, administering a single injection of cell synthesis marker either after or before a treatment facilitates the determination of whether neurogenesis has been affected and whether the manipulation has influenced progenitor cell proliferation or the survival of young neurons, respectively.
1.4 ARE NEURONS PRODUCED IN THE DENTATE GYRUS FUNCTIONAL?

Currently, theories about the functional role of neurogenesis constitutive to the adult CNS are speculative. However, granule neurons are produced in the dentate gyrus of every eutherian mammalian group studied including rodents (Altman, 1962; Kempermann 1997a; Galea and McEwen, 1999; Lavenex et al., 2000; Ormerod et al., 2002), lagomorphs (Gueneau et al., 1982), carnivores (Wyss and Sripanidkulchai, 1985), tupaiids (Gould et al., 1997a) and human/non-human primates (Gould et al., 1998; 1999a; 1999b; Eriksson et al., 1998; Kornack and Rakic 1999) throughout adulthood. The conservation of this phenomenon across species certainly suggests that new granule neurons serve some functional role. In fact, the daily addition of thousands of new neurons to the adult dentate gyrus (Cameron and McKay, 2001) surely impacts the flow of information through the hippocampus.

Information prominently travels through the trisynaptic circuit of the hippocampus (entorhinal cortex to the dentate gyrus to area CA3 and to area CA1) and to a lesser extent through disynaptic (entorhinal cortex to CA3 to CA1) and monosynaptic circuits (entorhinal cortex to CA1; for review see Amaral and Witter, 1995). The neurons that are added to the dentate gyrus appear to be electrophysiologically plastic (Wang et al., 2000; Snyder et al., 2002; van Praag et al., 2002) and could alter how information is transferred through the hippocampus, via the trisynaptic circuit. For example, Wang and colleagues (2000) found that long-term potentiation (LTP) could be induced in presumably young granule neurons located near the subgranular zone in presence of intact GABA_A receptor-induced inhibition whereas LTP can only be induced in mature granule neurons when GABA_A receptor-induced inhibition is antagonized. Recent work using a GFP-tagged retrovirus to birth-date granule neurons produced in the dentate gyrus of mice found electrophysiological responses typical of mature granule neurons 4 weeks after infection and a mature granule neuron morphology (dendritic...
complexity and similar spine counts) 4 months after infection (van Praag et al., 2002). In addition, evidence suggests that if young neurons (a few days to 3 weeks old) are reduced by approximately ½ by gamma irradiation, artificial cerebrospinal fluid-induced LTP that is normally observed cannot be generated, suggesting that young neurons do influence activity within the dentate gyrus (Snyder et al., 2001). Because new dentate granule neurons rapidly extend axons to the CA3 region (Hastings and Gould, 1999), they could rapidly influence hippocampus-dependent function. In addition, because new neurons are extremely plastic, electrophysiologically, they may be primed to participate in learning and/or memory.

In fact, hippocampus-dependent learning enhances the number of young neurons that survive in the dentate gyri adult rats. The acquisition of a trace-conditioned eyeblink response and the Morris water maze (localization of a hidden platform) are dependent upon the integrity of the hippocampus whereas neither the acquisition of delay-conditioned response nor the localization of a visible water maze platform require an intact hippocampus (Morris et al., 1982; Morris et al., 1990; McEchron et al., 1998; Weiss et al., 1999). Gould and her colleagues (1999) found more 12 day-old neurons in the dentate gyri (but not subventricular zone) of rats that were trained on a trace eyeblink conditioning task or in the Morris water maze versus a delay eyeblink conditioning task or a visible platform water maze task, beginning one-week post-BrdU injection. No difference in neuron number was found between groups of rats trained on either trace eyeblink conditioning trials or delay eyeblink conditioning trials prior to BrdU administration (Gould et al., 1999), indicating that cell proliferation is not influenced differentially by these tasks. Although hippocampus-dependent learning can enhance the survival of younger (4 to 7 day-old) neurons, the effect on older (1-2 week-old) neurons is much more robust (Ambrogini et al., 2000 versus Gould et al., 1999). These findings suggest that hippocampus-dependent learning enhances the survival of young granule neurons in the dentate gyrus of adult rodents.
Because thousands of granule neurons are added daily to the dentate gyrus of adult rodents (Cameron and McKay, 2001), preventing the integration of young neurons could produce deficits in performance on hippocampus-dependent tasks. In fact, Shors and her colleagues (2001, 2002) found that rats’ abilities to acquire either a trace-conditioned eyeblink response or a trace-conditioned fear response are impaired if cell proliferation is prevented for 14 d (but not for 6 d) using a cytostatic agent just prior to training. The acquisition of the trace-conditioned fear response has been shown to rely upon the integrity of the hippocampus (McEchron et al., 1998; Weiss et al., 1998). Animals given 21 d to recover from the effects of the cytostatic agent readily acquire the trace-conditioned eyeblink response (Shors et al., 2001). Surprisingly, Shors and colleagues (2002) found that performance across training trials in the Morris water maze was not impaired following treatment with a cytostatic agent over 14 days just prior to training. Taken together, this evidence suggests that 1-2 week old neurons are critical for some, but not all forms of hippocampus-dependent learning.

Therefore, new neurons appear to participate in or are influenced by hippocampus-dependent learning most significantly just after they begin extending their axons (4-10 after birth) and are presumably forming synapses with CA3 region pyramidal neurons because they begin to express the mature neuronal protein NSE by 14 d after birth (Cameron et al., 1993b; Hastings and Gould, 1999). Less is known about the relationship between new neurons and hippocampus-dependent memory. However, relatively specific and complete destruction of the granule cell layer produces severe deficits in performance on retention trials, as well as across training trials in a large Morris water maze (Xavier et al., 1999), suggesting that young granule neurons could influence this aspect of hippocampus-dependent function. Shors and her colleagues (2002) found that rats treated with cytostatic agent for 14 days prior to the onset of Morris water maze training performed as well as control rats on a retention trial in the task, suggesting that young neurons may not influence retention on this hippocampus-dependent
task. However, rats with hippocampal damage are capable of solving the Morris water maze task using non-spatial strategies (Whishaw, 1985; Long and Kesner, 1996; Pouzet et al., 2002), and rats treated with antimitotic agent prior to training could have also learned to solve the Morris water maze task using a non-spatial strategy that would permit good performance on a retention trial. Administering an antiproliferative agent after training trials have been administered (and prior to a retention trial) could provide better insight as to how young granule neurons influence memory for a platform position acquired using spatial information. In addition, increasing young neuron number prior to training trials could reveal their influence over spatial learning and/or memory. Chapter 5 describes the effect of increasing young granule neuron number on hippocampus-dependent learning and memory.

1.5 ESTRADIOL INFLUENCES HIPPOCAMPAL NEUROGENESIS

Estradiol is one factor that has been shown to influence neurogenesis in the dentate gyrus of adult rodents, however the effects reported in rats and voles differ. Galea and McEwen (1999) reported that adult female meadow voles trapped during the non-breeding season (when estradiol levels are low) had more proliferating cells in their dentate gyri (measured 24 h after a single injection of $[^{3}H]$thymidine) than females trapped during the breeding season (when estradiol levels are high) or males trapped during either season. In fact, Galea and McEwen (1999) found that serum estradiol was correlated negatively with labelled cell density in the dentate gyri of adult female meadow voles. Then, Tanapat and her colleagues (1999) found more labelled cells in the dentate gyri of gonadally intact adult female rats than in the dentate gyri of males (measured 2 h after BrdU was injected). The rate of cell proliferation was related to estrous cycle phase because more new neurons were produced during the afternoon of the proestrus phase (when estradiol levels are high) relative to either the estrus or diestrus phase (when estradiol levels are low). In rats, ovariectomy reduces dentate cell proliferation and this
reduction is reversed 2 h after an estradiol injection (Tanapat et al., 1999; Banasr et al., 2001). Taken together evidence suggests that estradiol decreases cell proliferation in the dentate gyri of adult female meadow voles but increases cell proliferation in the dentate gyri of adult female rats.

One reason why the effects of estradiol on cell proliferation in the dentate gyri of adult female meadow voles versus rats could differ is because the meadow voles that Galea and McEwen (1999) trapped during the breeding or nonbreeding season were feral and several factors that have been shown to influence cell proliferation cannot be controlled in a feral sample. For example, all of the females that Galea and McEwen (1999) captured during the breeding season were pregnant and evidence has shown that pregnancy can affect neurogenesis in the dentate gyrus of adult rats (Madonia et al., 2000; Banasr et al., 2001). Cell proliferation declines in the dentate gyri of aged rodents (Kuhn et al., 1996; Seki and Arai, 1995; Montaron et al., 1998; Cameron and McKay, 1999). Because reproductively successful adult female meadow voles establish and defend territories while inhibiting the reproductive status of subadults within their territories (Madison, 1980; Madison et al., 1985), the pregnant females that Galea and McEwen (1999) trapped during the breeding could have been older females. Finally, access to a running wheel increases cell proliferation in the dentate gyrus of adult mice (van Praag 1999a; 1999b). Female meadow voles reduce their space use and territory size during the breeding season (Madison, 1985; Sheridan and Tamarin, 1988), and therefore presumably run less during the breeding season relative to during the non-breeding season and relative to males in either season. Thus, replicating the effect of season on cell proliferation in the dentate gyri of a sample of laboratory-reared female meadow voles would permit control over these potentially confounding variables to shed light upon the relationship between reproductive status, estradiol and cell proliferation in the dentate gyri of adult voles.
Another reason why the effects of estradiol on cell proliferation in the dentate gyri of adult female meadow voles versus rats could differ is that the duration that each species was exposed to high-level circulating estradiol was different. The reproductive physiologies of adult female rats and meadow voles are very different. Whereas female rats experience elevated estradiol levels only on the afternoon of proestrus (Buckingham et al., 1978), female meadow voles experience elevated circulating estradiol levels that persist for up to 45 d only after ovulation is induced by male contact (Lee et al., 1970; Seabloom et al., 1985; Nubbemeyer, 1999). Tanapat and colleagues (1999) injected rats with BrdU at 2 pm, and therefore proestrus females would have likely been exposed to high circulating estradiol for a few hours (see Buckingham et al., 1978). Galea and McEwen (1999) injected females that were trapped in either the breeding or nonbreeding with $[^3]$Hthymidine and, therefore, females in the breeding season would likely have been exposed to high circulating estradiol levels for a longer time period (Lee et al., 1970; Seabloom et al., 1985; Nubbermeyer, 1999). Therefore, following short duration exposure to estradiol cell proliferation could have been elevated in proestrus rats (Tanapat et al., 1999) and following longer exposure to high circulating estradiol cell proliferation could have decreased in the dentate gyri of adult female meadow voles (Galea and McEwen, 2001). High-level estradiol could affect cell proliferation differentially in a duration-dependent manner. This hypothesis is tested in Chapters 3 and 4.

Banasr and her colleagues (2001) demonstrated that estradiol stimulates serotonin synthesis to increase cell proliferation within 2 h of its administration and there is evidence to suggest that estradiol could suppress cell proliferation following longer exposures by stimulating adrenal activity. High-level adrenal steroids (corticosterone injection, stress) suppress and low-level adrenal steroids (low dose corticosterone replacement in adrenalectomized rats) enhance cell proliferation in the dentate gyri of adult male rats (Cameron and Gould, 1994; Cameron et al., 1995; Cameron and McKay, 1999; Tanapat et al.,
2001). Dividing progenitor cells express neither Type I nor Type II glucocorticoid receptors (Cameron et al., 1993a), suggesting that adrenal steroids regulate cell proliferation indirectly. In fact, Cameron and her colleagues (1998) demonstrated that NMDA receptor activation works downstream of adrenal steroids to suppress cell proliferation in the dentate gyri of adult rats. NMDA receptor activation increases and NMDA receptor blockade decreases cell proliferation in the dentate gyri of adult rats and tree shrews (Cameron et al., 1994; Cameron et al., 1995; Gould et al., 1997; Bernabau and Sharp, 2000; Nacher et al., 2001; Nacher et al., 2003 but see Bernabau and Sharp, 2000 and Arvidsson et al., 2001) and NMDA receptor activation can prevent an adrenalectomy-induced increase and NMDA receptor blockade can block an adrenal steroid-induced decrease in cell proliferation in the dentate gyri of adult male rats (Cameron et al., 1998). Interestingly, estradiol stimulates the rat and meadow vole hypothalamic-pituitary-adrenal axis (Coyne and Kitay, 1969; Christian, 1969; Burgess and Handa, 1992; Handa et al., 1994) and has been shown to increase both the sensitivity and number of NMDA receptors in the rat hippocampus (Weiland et al., 1992; Gazzaley et al., 1996). Therefore, estradiol could eventually suppress cell proliferation by stimulating adrenal activity and/or by stimulating NMDA receptor activity. Chapter 3 describes an experiment that tests whether estradiol stimulates adrenal activity and Chapter 4 describes experiments that test whether estradiol interacts with NMDA receptors to suppress cell proliferation.

1.6 ESTRADIOL ENHANCES CELL SURVIVAL IN DIFFERENT SYSTEMS

Although thousands of new neurons are added daily to the dentate gyri of adult rodents, many of the neurons appear to die between 2 and 4 weeks after birth (Gould et al., 1999; Cameron and McKay, 2001). Studies using [3H]thymidine or relatively high doses of BrdU (200-600 mg/kg) have demonstrated that the number of labelled cells approximately doubles between 24 h and 1 week post-label, but that only about ½ of the labelled cells observed at 24 h
survive longer than 2-4 weeks (Cameron et al., 1993b; Gould et al., 1999; Cameron and McKay, 2001). Although some labelled cells appear to continue dividing to the point that BrdU and [³H]thymidine are too diluted to be detected immunohistochemically or autoradiographically (Cameron et al., 1993b; Nowakowski and Hayes, 2002), the average number of silver grains in [³H]thymidine-labelled cells does not diminish until 3 weeks post-label suggesting many cells die between 2 and 3 weeks (Cameron and McKay, 2001). Therefore, by increasing the number of new granule neurons that survive 2 weeks or more, net neurogenesis could be increased.

Estradiol enhances the survival of numerous cell types (see Garcia-Segura et al. 2001 for review) including hippocampal neurons in various models of injury both in vivo and in vitro. Estradiol increases the viability, differentiation and survival of cultured hippocampal neurons (Sudo et al., 1998). In addition, estradiol rescues cultured hippocampal neurons from excitotoxicity-, oxidative injury- and β-amyloid toxicity-induced death (Goodman et al., 1996; Weaver et al., 1997). In vivo, estradiol reduces the death of CA1 region hippocampal neurons and decreases infarct size following experimental ischemia in gerbils (Chen et al., 1998). Estradiol significantly reduces the number of dentate gyrus hilar interneurons that die following the administration of convulsive doses of kainic acid in ovariectomized female rats (Azcoitia et al., 1998, 1999). In addition, pretreatment with estradiol can prevent quinolinic acid-induced cell death in the hippocampus of adult male rats (Favata et al., 1998; Kuroki et al., 2001).

The most compelling evidence to suggest that estradiol could enhance the survival of granule neurons produced in the adult rodent dentate gyrus is that estradiol has also already been shown to enhance the survival of new neurons in the adult avian song circuit. More labelled cells were observed in the song circuits of female zebra finches surgically implanted with estradiol-filled versus empty silastic capsules 24 h after [³H]thymidine was injected
(assessed 18 days after implant; Burek et al., 1995), demonstrating that estradiol enhances the survival of new neurons. Hidalgo and colleagues (1995) found more labelled cells in the song circuit of canaries implanted with an estradiol- versus cholesterol-filled silastic capsule 32 days after the onset of 8 daily \(^{3}\text{H}\)thymidine injections. Because Hidalgo et al. (1995) only found estrogen receptors (ERs) on cells in the migratory pathway of new neurons but not on the new neurons themselves, they concluded that estradiol indirectly influenced the survival of new migrating neurons. Recent work by Loissant and his colleagues (2001) has demonstrated that estradiol enhances the survival of cultured avian song circuit neurons by inducing BDNF expression in endothelial cells. Concurrent changes in angiogenesis and neurogenesis have been reported in the adult rodent dentate gyrus (Palmer et al., 2000) suggesting that estradiol could enhance the survival of new granule neurons in the dentate gyrus of adult rodents and songbirds through a similar mechanism. Both estrogen receptor subtypes ER\(_{\alpha}\) and ER\(_{\beta}\) are expressed in the dentate gyrus (Shughrue et al., 1997; Milner et al., 2001). In fact, electron microscopy has revealed that not only are interneurons in the dentate gyrus ER\(_{\alpha}\)-immunoreactive but the axons and dendrites of granule neurons are also ER\(_{\alpha}\)-immunoreactive (Milner et al., 2001). Whether young neurons express either ER is unknown but the presence of ERs in the dentate gyrus provides a means by which estradiol could enhance the survival of young neurons, at least indirectly. Chapter 5 describes the effect of estradiol on the survival of young granule neurons.

1.7 OVERVIEW AND OBJECTIVES

The experiments described in this thesis investigate the effect of estradiol on components of neurogenesis in the adult rodent dentate gyrus and to determine whether changes in young neuron number influence hippocampus-dependent behaviour. The reported
effects of estradiol on progenitor cell proliferation in the dentate gyri of adult female rats and meadow voles are different. Elevated circulating estradiol levels are associated with increased proliferation in female rats (Tanapat et al., 1999) but decreased proliferation female meadow voles (Galea and McEwen, 1999). Several factors could account for the differences reported in the relationship between estradiol and cell proliferation in the dentate gyri of rats and voles. For example, Galea and McEwen (1999) used feral voles as subjects in their study and many factors that alter adult neurogenesis cannot be controlled in a feral sample. Moreover, rat and meadow vole reproductive physiologies differ such that each species would have experienced high circulating estradiol for different durations prior to the administration of cell synthesis marker and estradiol could influence cell proliferation in a time-dependent manner. Currently the effect of estradiol on the survival of young neurons produced in the adult rodent dentate gyrus unknown. However, estradiol enhances the survival of new neurons produced in the rodent CNS developmentally and in the avian song circuit during adulthood and, therefore, could enhance the survival of young neurons produced in the dentate gyrus. If estradiol enhances the survival of young granule neurons, then hippocampus-dependent behaviour could be altered. Indeed, reports that hippocampus-dependent behaviour enhances the survival of young neurons (Gould et al., 1999) and that some forms of hippocampus-dependent behaviour are impaired when young granule neurons are depleted (Shors et al., 2001, 2002) have been published. Therefore, the objectives of the present thesis are as follows:

1. To determine whether reproductive status influences the number of dividing and new cells in the dentate gyri of adult laboratory-reared female meadow voles (Chapter 2). An attempt to replicate the finding that cell proliferation is suppressed in the dentate gyri of adult female meadow voles trapped during the breeding season when they are reproductively active compared to the non-breeding season when they are reproductively inactive will be made using sample of laboratory-reared voles to control
potentially confounding variables. Females will be acclimated to a short- or long-photoperiod to simulate the winter non-breeding or summer breeding season, respectively, and a male or female cage partner will be introduced to manipulate reproductive status, which will be verified by ovary mass and serum estradiol level. BrdU or $[^3]$H-thymidine will be injected to assess the number of dividing cells (2 h after BrdU) and new cells (5 weeks after $[^3]$H-thymidine), respectively. Because cell proliferation is elevated in nonbreeding versus breeding feral adult female meadow voles, I expect to observe more labelled cells in the dentate gyri of reproductively inactive (with low circulating estradiol levels) versus active (with high circulating estradiol levels) laboratory-reared females, 2 h after a BrdU injection. Furthermore, because estradiol has been shown to promote the survival of neurons in other systems, I expect to observe more labelled cells in the dentate gyri of reproductively active versus inactive females, 5 weeks after a $[^3]$H-thymidine injection.

2. To determine whether the effect of reproductive status on cell proliferation in the dentate gyrus of adult female meadow voles can be mimicked by estradiol and whether estradiol influences cell proliferation time-dependently (Chapter 2). Reproductively inactive females will be injected with estradiol four h (the time reported to increase cell proliferation in rats) or 48 h (the time that female meadow voles are paired with a cage partner to manipulate reproductive status) before a BrdU injection. Voles will be perfused 2 h later to assess the density of proliferating cells in their dentate gyri. These data will address some of the controversy in the literature by determining whether the effect of estradiol on cell proliferation in the dentate gyrus of adult female meadow voles is time-dependent. I hypothesize that female voles exposed to estradiol for 4 h will have more labelled cells and females exposed to estradiol for 48 h will have fewer labelled cells in their dentate gyri than reproductively active females.
3. To test the robustness of estradiol's time-dependent effects on cell proliferation across species (Chapters 3 and 4). To test the robustness of the time-dependent effect of estradiol on cell proliferation, ovariectomized adult female rats and voles will be given and injection of estradiol or vehicle 4 h or 48 h prior to an injection of BrdU. The animals will be perfused 2 h, 24 h or 4 d later to determine the number of dividing progenitors (2 h post-BrdU in voles), new cells (24 h post-BrdU in rats) and new neurons (4 days post-BrdU in rats). I expect that the number of labelled cells will increase in the dentate gyri of rats and voles treated with estradiol versus vehicle for 4 h but decrease in the dentate gyri of rats and voles treated with estradiol versus vehicle for 48 h prior to a BrdU injection. Because previous work has shown that estradiol does not alter the percentage of BrdU-labelled cells that acquire a neuronal phenotype, I also expect to observe a similar percentage of BrdU-labelled cells that express glial or neuronal protein in estradiol- versus vehicle-treated rats (assessed 4 days post-BrdU).

4. To determine how estradiol suppresses cell proliferation in the adult rodent dentate gyrus (Chapters 3 and 4). Previous studies have shown that adrenal steroids and NMDA receptor activation suppress cell proliferation in the dentate gyrus of adult rats, and estradiol stimulates adrenal activity and increases both the number and sensitivity of NMDA receptors. Therefore, estradiol could stimulate adrenal activity or NMDA receptor activity to suppress cell proliferation within 48 h. To test whether estradiol stimulates adrenal activity to suppress cell proliferation, ovariectomized/adrenalectomized rats will be exposed to estradiol or vehicle for 48 h and then injected with BrdU and perfused 24 h later to assess the density of labelled cells. I predict that removing estradiol's influence over adrenal activity will either eliminate (no difference between BrdU-labelled cell number will be observed between groups) or reverse (more BrdU-labelled cells will be observed in the estradiol- versus vehicle-treated group) the
estradiol-induced suppression in cell proliferation in the dentate gyri of adult female rats. To test whether estradiol influences NMDA receptor activity to suppress cell proliferation, ovariectomized female meadow voles will be injected with estradiol or vehicle and then NMDA or saline 3 h later or MK-801 or saline 47 h later. One h after the injection of NMDA, MK-801 or saline, BrdU will be injected and the voles will be perfused 1 h later. I predict that MK-801 will eliminate or reverse the estradiol-induced suppression in cell proliferation.

5. **To determine whether estradiol influences the survival of young granule neurons produced in the dentate gyrus of adult rodents (Chapter 5).** The effect of estradiol on the survival of young granule neurons in the adult mammalian dentate gyrus has not been tested specifically. To specifically test the effect of estradiol on the survival of new neurons independent of its effects on cell proliferation, castrated male meadow voles will injected twice with BrdU and then no treatment will be administered for at least 24 h, as this is enough time for cells proliferating in the adult rodent dentate gyrus to complete one mitotic division. This design enables the effect of estradiol on the survival of young neurons independent of its effects on cell proliferation, to be tested. Then males will be injected once per day with estradiol or vehicle either over Days 1-5, Days 6-10 or Days 11-15 post BrdU and then perfused on Day 16 to assess the total number of labelled cells (and their phenotypes). The time-periods were chosen to roughly correspond with differentiation and migration, axon extension and maturation, respectively. Because estradiol has been shown to promote the survival of immature neurons during development and migrating neuroblasts in the avian forebrain I hypothesize that more labelled neurons will survive in the dentate gyrus of estradiol-versus vehicle-treated males, possibly when estradiol is administered when cells are migrating (Days 1-5) similar to what has been observed in the avian songcircuit.
6. To determine whether increasing the number of young granule neurons influences hippocampus-dependent behaviour (Chapter 5). Previous work has demonstrated that hippocampus-dependent learning enhances the survival of young neurons and that depleting the number of young neurons with a cytostatic agent impairs some forms of hippocampus-dependent behaviour. Therefore, if estradiol increases the survival of young neurons, then the increase may influence hippocampus-dependent behaviour.

Castrated males will be given two injections of BrdU and then estradiol over Days 6-10 post BrdU (the time frame shown to enhance the survival of new neurons in Experiment 7; Chapter 5). Over Days 16-19, voles will be trained in the same spatial Morris water maze task shown previously to enhance the survival of new neurons. On Day 20, a probe trial will be administered to test the voles' retention of the platform location over training trials and then reversal trials will be administered to test the voles' tendencies to perseverate. Because performance across training trials is unaffected when young neurons in the dentate gyri of adult rats are depleted, I predict that both groups may perform similarly across training trials but that estradiol-treated voles may outperform vehicle-treated voles on the probe trial.

Chapters 2-5 will describe experimental data collected to address the objectives described. All experimental data has been published or submitted for publication in manuscript form. The details of those findings will not be reiterated in the General Discussion, rather the findings will be discussed generally and then in terms of how estradiol-induced changes in neurogenesis may impact normal hippocampus function and may influence neuronal replacement strategies.
CHAPTER 2

REPRODUCTIVE STATUS INFLUENCES CELL PROLIFERATION AND CELL SURVIVAL IN THE DENTATE GYRUS OF ADULT FEMALE MEADOW VOLES: A POSSIBLE REGULATORY ROLE FOR ESTRADIOL

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2.1 INTRODUCTION

Although most neurons are integrated into the central nervous system during discrete developmental periods, recent studies have shown that the hippocampus of adult mammals retains the ability to produce and incorporate new granule neurons (Kaplan and Hinds, 1977; Kaplan and Bell, 1984; Cameron et al., 1993b). New cells are produced when progenitor cells within the subgranular zone of adult laboratory mammals (Altman and Das, 1967; Angevine, 1965; Gueneau et al., 1982; Wyss and Sripanidkulchai, 1985; Cameron et al., 1993b; Gould et al., 1997a; 1997b; Galea and McEwen, 1999; Gould et al., 1999a; 1999b; Kornack and Rakic, 1999) and humans (Eriksson et al., 1998) divide. Evidence suggests that the majority of the progeny differentiate into neurons. New cells migrate into the granule cell layer, extend an axon into the CA3 region by 10 days after birth and exhibit granule neuron morphology (Cameron et al., 1993b; Hastings et al., 1999). These new cells express immature neuronal markers, such as TOAD-64 (turned on after division-64kD), within 24 hours after division (Minturn et al., 1995; Tanapat et al., 1999) and then mature neuron-specific proteins, such as neuron-specific enolase, 2 to 3 weeks later (Cameron et al., 1993b; Gould et al., 1997a; Eriksson et al., 1998). New granule neurons are likely functional as they receive synaptic input (Markakis and Gage, 1997), show paired-pulse facilitation comparable to mature granule neurons and enhanced electroplasticity (via lower threshold for the induction of long-term potentiation in absence of suppressed inhibition; Wang et al., 2000). In adult rodents, the rate of cell proliferation is elevated by serotonin (Jacobs et al., 1998; Brezun and Daszuta, 1999; 2000).
and physical activity (van Praag et al., 1999a; 1999b) but diminished by dopamine agonists (Teucherdt-Noodt et al., 1997), N-methyl-D-aspartate-receptor (NMDAr) activation (Cameron et al., 1995; Cameron et al., 1998) corticosterone (CORT; Gould et al., 1992; Cameron and Gould, 1994; Cameron et al., 1995; Cameron and McKay, 1999; Cameron et al., 1998) and ageing (Seki and Arai, 1995; Kuhn et al., 1996; Montaron et al., 1998; Cameron and McKay, 1999).

Although neurogenesis has been extensively studied in laboratory animals, only a few studies have investigated the phenomenon in natural populations (Barnea and Nottebohm, 1994; 1996; Galea and McEwen, 1999; Tramontin and Brenowitz, 1999; Lavenex et al., 2000). In the wild adult female meadow vole, Galea and McEwen (1999) found that the rate of cell proliferation within the dentate gyrus fluctuates with season of capture. Specifically, rates of cell proliferation were significantly lower in female meadow voles trapped during the breeding season than in females trapped during the non-breeding season. Galea and McEwen (1999) also found that the rate of cell proliferation was negatively correlated with serum estradiol level in these animals. Female meadow voles are reflex ovulators and thus, serum estradiol is rapidly elevated with the induction of behavioural estrous and remains elevated throughout the breeding season as the majority of female meadow voles are either in behavioural estrous or are pregnant (Clulow and Mallory, 1970; Lee et al., 1970; Prentice and Shepherd, 1978; Boonstra and Boag, 1992). Therefore, the suppression in cell proliferation observed by Galea and McEwen (1999) was likely mediated by estradiol.

However, Tanapat et al. (1999) reported that estradiol transiently increased the level of neurogenesis in the dentate gyrus of adult female laboratory rats. Specifically, cell proliferation was increased during proestrous (associated with high serum estradiol levels) relative to either estrous or diestrous (associated with low or medium serum estradiol levels) in normally cycling rats (Buckingham et al., 1978; Butcher et al., 1974; Tanapat et al., 1999). In addition, Tanapat
et al. (1999) found that an ovariectomy-induced diminution in granule neuron proliferation could be reversed in the 2 hours following an injection of estradiol benzoate. Therefore, while high levels of endogenous estradiol in wild female meadow voles are associated with suppressed rates of granule cell proliferation (Galea and McEwen, 1999), high levels of endogenous or exogenous estradiol in the laboratory-reared female rat are associated with increased rates of granule cell proliferation (Tanapat et al., 1999).

There are several possible explanations for the discrepant results found between the two studies, other than potential species' differences. The Galea and McEwen (1999) study utilised wild animals and therefore, many variables could have suppressed cell proliferation in female meadow voles captured during the breeding season. For example, in Galea and McEwen's (1999) study, all of the females captured during the breeding season were pregnant and pregnancy affects hippocampal morphology and could influence cell proliferation Galea et al., 2000). Ageing is associated with a decline in the rate of cell proliferation in rodents (Kuhn et al., 1996; Seki and Arai, 1995; Montaron et al., 1998; Cameron and McKay, 1999). Because reproductively successful adult female meadow voles establish and defend territories while inhibiting the reproductive status of subadults within their territories (Madison, 1980; Madison et al., 1985), the pregnant females that Galea and McEwen (1999) trapped could have been older females. Alternatively, experience is associated with an increase in cell proliferation and cell survival (Scott et al., 1998; Gould et al., 1999). Female meadow voles reduce their space use and territory size during the breeding season (Madison, 1985; Sheridan and Tamarin, 1988), which presumably changes experience within their environment relative to the non-breeding season and relative to males in either season. Thus, while investigating cell proliferation in natural populations is advantageous many uncontrolled variables in a wild sample could potentially influence the rate of cell proliferation observed. Therefore, the present study was conducted to determine the effect of reproductive status on cell proliferation and
survival in a sample of laboratory-reared meadow voles. By using laboratory-reared meadow voles, the potentially confounding variables of pregnancy, age, and experience could be controlled.

2.2 METHODS

All animals were treated in strict accordance with the guidelines set forth by the Canadian Council on Animal Care and The University of British Columbia regarding the ethical treatment of animals used for the purposes of research. Every effort was made to minimise the number of animals used per group and their suffering.

Animals

Adult male (>35 g and 60 days old) and female (>25 g and 60 days old) voles from our breeding colony at The University of British Columbia were used for all experiments. All voles were reared in a 16 hr light/8 hr dark colony room (lights on at 0700h) and were given access to Lab Diet #5012 (Jamieson) and tap water, ad libitum. Twenty-one day old animals were weaned and housed in a bedding-lined (Care Fresh; Absorption Corporation) polyurethane cage with a same-sex littermate until 60 days of age. At 60 days of age, the voles were housed individually and kept in either the long-photoperiod (16 hr light/8 hr dark) or moved to a short-photoperiod (10 hr light/14 hr dark) to simulate breeding or non-breeding season day-length, respectively.

Procedure

After a 4-week acclimation period (to photoperiod), voles were paired with either a male or female cage partner for 48 hours to manipulate reproductive status. Previous studies
have shown that estradiol levels in female meadow voles are elevated 12-18 hours after exposure to male pheromone only during the long-photoperiod (Lee et al., 1970) and remain elevated for at least 22 days (Seabloom, 1985). Similarly, exposure to a male vole induces a rapid increase in the serum estradiol level (about 200%; Cohen-Parsons and Carter, 1987) that persists for at least 3 weeks (Dluzen and Carter, 1979). Therefore, introducing a male cage partner for 48 hours was sufficient to shift female reproductive status and thereby elevate the associated hormones, particularly estradiol. Female-paired female meadow voles housed in either the long- or short-photoperiod were considered reproductively inactive (RI) and male-paired female meadow voles housed in the long-photoperiod were considered reproductively active (RA). Serum estradiol levels further verified reproductive status.

Experiment 1 was conducted to determine whether reproductive status influences the density of proliferating cells within the dentate gyrus of adult female meadow voles. In addition, separate groups of RI females were given a single injection of estradiol benzoate (EB) to determine whether estradiol mimicked the effect of reproductive status because estradiol level is rapidly and dramatically increased with the onset of behavioural estrous in meadow voles (Prentice and Shepherd, 1978). Thus, female meadow voles were given a single injection of estradiol benzoate (EB) 4 hours (EB4) or 48 hours (EB48) prior to bromodeoxyuridine (BrdU) labelling. These time points for EB administration were chosen to match the possible onset of estradiol increase in RA females (4 hours) and the maximum duration that RA females would be exposed to elevated estradiol prior to sacrifice (48 hours). RA females, RI females, EB4 and EB48 (n=5 per group) were given a single intraperitoneal (i.p.) injection of the thymidine analogue, BrdU (50 mg/kg; Sigma Aldrich Chemicals), between 1230 and 1300h). The voles were anaesthetised deeply with sodium pentobarbital (0.1 ml) and then perfused transcardially with 4.0% paraformaldehyde in 0.1-M phosphate buffered saline (PBS), 2 h after BrdU was injected. All brains were extracted and post-fixed overnight in perfusate. Unfixed adrenal
glands and ovaries were removed and weighed. The following day, the brains were sectioned and the sections were processed immunohistochemically for BrdU. Prior to perfusing the animals, blood samples were taken from the right ventricle and were stored at 4° Celsius. Twenty-four hours later, the blood samples were centrifuged at 4g for 10 minutes and then the serum was drawn and frozen at -70° Celsius until radioimmunoassays were performed.

Experiment 2 was conducted to determine whether the initial difference in cell proliferation observed in RI versus RA females persisted for 5 weeks. Long-photoperiod and short-photoperiod female meadow voles were housed with a cage partner for 48 hours and composed three experimental groups: 1) long-photoperiod male-paired females (n=7, reproductively active [RA]), 2) long-photoperiod female-paired females (n=5, reproductively inactive [RI]) and 3) short-photoperiod female-paired females (n=7, RI). Forty-eight hours after being paired, the voles were given a single i.p. injection of $[^3]$Hthymidine (5 μCi/g; between 1230 and 1300h). Five weeks after $[^3]$Hthymidine injection, the voles were deeply anaesthetised with sodium pentobarbital (0.1 ml) and then perfused transcardially with 4.0% paraformaldehyde in 0.1-M phosphate buffered saline (PBS). Brains were extracted and post-fixed overnight in perfusate. The following day, the brains were sectioned and the sections processed autoradiographically for $[^3]$Hthymidine. BrdU was used in Experiment 1, $[^3]$Hthymidine was used in Experiment 2 to label dividing cells. However, BrdU and $[^3]$Hthymidine label proliferating cells at very similar relative rates in proliferation (the present study and see Galea and McEwen, 1999) and survival studies (Cameron and Gould, 1994 versus Cameron and McKay, 1999).

Drug preparation
17β-Estradiol benzoate (EB; Sigma Aldrich Chemicals) was prepared by dissolving EB in sesame oil (Sigma Aldrich Chemicals) to a concentration of 10μg of EB/0.1 ml sesame oil. The solution was then stored in a light insensitive container. All voles were given a 0.10 ml injection (subcutaneous) of the solution (containing 10μg of EB). This dose was chosen because Carter and colleagues (1987) reported that lordosis behaviour could be induced in female prairie voles 48 hours after a single 10μg injection of EB. BrdU was prepared just prior to administration by dissolving BrdU in freshly prepared isotonic saline containing 0.7% 2 N NaOH to a concentration of 10 mg BrdU/ml saline. BrdU was injected i.p. in a volume of 0.5ml/100g.

Histology

All brains were sliced into 40 μm thick sections through the entire dentate gyrus with an oscillating tissue slicer (OTS 3000, Electron Microscopy Sciences) using a bath of 0.1-M phosphate buffer (PB). Slices prepared for peroxidase immunohistochemistry were pre-treated in a solution of PB with 0.2% H2O2 for 20 min and then rinsed before being mounted on slides treated with 3% 3-aminopropyltriethoxy-silane in acetone. Slices prepared for autoradiography were mounted on 2% gelatin-coated slides and left to dry overnight

Peroxidase immunohistochemistry

Tissue was processed for BrdU-immunoreactivity by applying solutions directly to the slide-mounted sections. Unless otherwise specified, phosphate buffered saline (0.1 M sodium phosphate heptahydrate in 0.9% saline; pH 7.4) was used for all rinses and slides were rinsed repeatedly between each step. 1) Cells in the sections were permeabilized with 0.05% Trypsin (Sigma Aldrich Chemicals) in Tris-HCl buffer (pH 7.5) containing 0.1% CaCl2 for 10 min. 2)
DNA was denatured by applying 2N HCl for 30 min and then the sections were repeatedly rinsed (pH 6.0). 3) Sections were blocked in 5.0% normal horse serum for 30 min and then incubated overnight in mouse monoclonal antibody against BrdU (1:100 + 3% NHS + 0.5% Tween 20; Boehringer Mannheim) at room temperature. 4) Sections were incubated in mouse secondary antisera (1:29 + 3.0% normal horse serum; Vector Laboratories) for 4 hrs. 5) Sections were incubated in avidin-biotin horseradish peroxidase (AB; 1:50; Vector Laboratories) for 60 min. 6) Sections were reacted for 10 min in 0.02% diaminobenzidine (DAB; Sigma Aldrich Chemicals) with 0.003% H$_2$O$_2$ and then counterstained with cresyl violet acetate (Baker), dehydrated and coverslipped with Permount (Fisher Scientific).

For $[^{3}H]$thymidine autoradiography, slides were dipped in autoradiographic emulsion (NTB2, Kodak) and stored at 4EC for 4 weeks. Then the slides were developed in Dektol (Kodak), fixed in Ektaflo (Kodak), counterstained with cresyl violet acetate, dehydrated in ethanol, preserved with xylene and coverslipped with Permount.

**Hormone assays**

Serum corticosterone (CORT) levels were analysed in the Department of Anatomy using a radioimmunoassay protocol described in detail by Weinberg and Nezio (1987). Briefly, antiserum was obtained from Immunocorp (Montreal, Canada) and tracer was obtained from Mandel Scientific (Guelph, Canada). Dextran-coated charcoal was used to adsorb and precipitate free steroids after incubation.

Serum estradiol levels were analysed using a Coat-a-Count kit (Diagnostic Products Corporation, Los Angeles, CA) modified for low expected levels of estradiol. The sensitivity of the estradiol assay was 5 pg/ml. Values below the detection threshold of the assay were arbitrarily given a value of 0 pg/ml (n=3; RI females).
Data analyses

Slides were coded prior to the analysis to blind the experimenter to the treatment conditions. Six sections of the middle portion of the dentate gyrus (where the dentate gyrus is positioned horizontally beneath the corpus callosum and the suprapyramidal and infrapyramidal blades are joined at the crest; between A -3.3 and A -4.8 in rats) were analysed, per subject (see Gould et al., 1992; Cameron et al., 1993b; Gould et al., 1997a; Cameron et al., 1998; Gould et al., 1999b), using a Nikon Eclipse (C 600) Light microscope (100X objective). For the BrdU analyses, cells were considered immunoreactive if they were intensely stained and exhibited appropriate morphology (medium-sized round or oval cell bodies; Cameron et al., 1993b; Figure 2A). For the $[^{3}]$H thymidine analysis, granule cells were considered labelled if the number of silver grains was at least 20x the background level and the cell exhibited mature granule cell morphology (medium-sized round or oval cell bodies; Cameron et al., 1993b; see Figure 2B). Pyknotic cells were counted for all sections using the criterion set forth by Gould and colleagues (1991). Briefly, pyknotic cells lacked a nuclear membrane, had pale or absent cytoplasm and darkly stained spherical chromatin (Figure 2C). The total number of $[^{3}]$H thymidine-labelled or BrdU-immunoreactive and pyknotic cells found in the granule cell layer or hilus was counted and divided by the total respective area. The area of the granule cell layer and hilus was determined using the digitising software Analytical Imaging Station (Imaging Research Inc, Brock University, Ontario, Canada). The data are expressed as densities (the number of labelled cells per mm$^2$).

Statistical analyses

The dependent variables from Experiment 1 (density of BrdU-labelled cells and density of pyknotic cells) were each analysed using a one-way analysis of variance (ANOVA) with
condition (RA, RI, EB 4, and EB 48) as the independent variable. Pearson product-moment correlation tests were conducted on the density of labelled and pyknotic cells and hormone levels. Data were derived from 19 females, as some sections from the brain of one RI female were lost during processing. The data from Experiment 2 (density of $[^3]$Hthymidine-labelled cells and density of pyknotic cells) were each analysed using an independent t-test with status (RI and RA) as the independent variable. Data were derived from the brains of 14 animals as some sections from two RA females and two RI females were damaged during processing. In addition, one RI female died prior to completing the experiment. Unless otherwise specified, Post hoc tests utilised the Newman-Keuls procedure. All statistical procedures set $\alpha = 0.05$.

2.3 RESULTS

Experiment 1. Reproductive status, possibly via estradiol, influences cell proliferation in the dentate gyrus of adult female meadow voles.

Density of BrdU-labelled cells and pyknotic cells in the granule cell layer and hilus

Figure 2A shows a BrdU-labelled cell found in the subgranular zone of an adult female meadow vole. BrdU-labelled cells were observed to occur primarily in clumps located in the subgranular zone. The density of BrdU-labelled cells in the granule cell layer was significantly greater in RI and EB4 females compared to RA females ($p < 0.0007$) and EB48 females ($p < 0.0013$, $F_{(3,15)} = 21.016$, $p < 0.0001$; see Table 1). Similarly, the density of BrdU-labelled cells in the hilus of RI females was greater compared to all other groups; RA ($p < 0.016$), EB4 ($p < 0.027$) and EB48 females ($p < 0.014$; $F_{(3,15)} = 5.267$, $p < 0.011$; see Table 1). No significant difference was found between groups in the area of the GCL ($p < 0.09$) or the hilus ($p < 0.57$).
Figure 2. Microphotographs of a bromodeoxyuridine (BrdU)-labeled cell, a $[^3]$H-thymidine-labeled cell and a pyknotic cell in Experiments 1 and 2.

A) Depicts a BrdU-labelled cell in the subgranular zone between the granule cell layer and hilus of the dentate gyrus of an adult female meadow vole. The majority of BrdU-labelled cells were observed to occur in clumps in the subgranular zone of the dentate gyrus. B) Depicts a $[^3]$H-thymidine-labelled cell located within the granule cell layer of an adult female meadow vole. Most $[^3]$H-thymidine-labelled cells were located in the granule cell layer. C) Depicts a pyknotic cell located in the hilus of an adult female meadow vole. GCL = granule cell layer and H = hilus. Scale bar (1c) = 20 µm.
Table 1. Mean (+SEM) density of BrdU-labelled and pyknotic cells in the granule cell layer and hilus of adult female meadow voles measured 2h after BrdU was injected in Experiment 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Labelled cells (Granule Cell Layer)</th>
<th>Pyknotic cells (Granule Cell Layer)</th>
<th>Labelled cells (Hilus)</th>
<th>Pyknotic Cells (Hilus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RI (n=5)</td>
<td>88.61±18.81</td>
<td>19.31±11.27</td>
<td>17.31±6.29</td>
<td>0.92±1.30</td>
</tr>
<tr>
<td>RA (n=4)</td>
<td>5.93±1.99**</td>
<td>13.09±3.46</td>
<td>2.09±0.50*</td>
<td>1.61±0.76</td>
</tr>
<tr>
<td>EB4 (n=5)</td>
<td>126.22±16.99†</td>
<td>23.65±1.01</td>
<td>5.59±1.01*</td>
<td>0.53±0.93</td>
</tr>
<tr>
<td>EB48 (n=5)</td>
<td>3.66±0.93**</td>
<td>21.38±0.50</td>
<td>0.35±0.16*</td>
<td>1.84±1.60</td>
</tr>
</tbody>
</table>

* indicates a significant difference (p < 0.050) relative to the RI group
**indicates a significant difference (p < 0.002) relative to the RI group
† indicates a tendency toward a significant difference (p ≤ 0.067) relative to the RI group

A pyknotic cell is shown in Figure 2C. There was no main effect of condition on the density of pyknotic cells in the granule cell layer (p ≤ 0.49) or the hilus (p ≤ 0.51) of female meadow voles (see Table 1). The density of labelled cells was not correlated with the density of pyknotic cells in either group (Tables 1 and 2; granule cell layer \( r_{(19)} = 0.145; p < 0.62 \), hilus \( r_{(19)} = -0.287; p < 0.32 \)).

Table 2. Pearson product-moment correlations between dependent variables measured in adult female meadow voles in Experiment 1.

<table>
<thead>
<tr>
<th>Serum EB‡</th>
<th>BrdU-ir Cells (GCL)</th>
<th>BrdU-ir Cells (hilus)</th>
<th>Pyknotic Cells (GCL)</th>
<th>Pyknotic Cells (hilus)</th>
<th>Adrenal Mass (mg)</th>
<th>Ovary Mass (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>†</td>
<td>( r = -0.657* )</td>
<td>( R = -0.530 )</td>
<td>( r = -0.177 )</td>
<td>( r = -0.074 )</td>
<td>( r = 0.733* )</td>
<td>( r = 0.798** )</td>
</tr>
<tr>
<td>Serum CORT</td>
<td>( r = 0.362 )</td>
<td>( R = -0.447 )</td>
<td>( r = -0.756 )</td>
<td>( r = -0.732 )</td>
<td>( r = 0.188 )</td>
<td>( r = 0.314 )</td>
</tr>
<tr>
<td>Adrenal Mass</td>
<td>( r = -0.692 )</td>
<td>( R = -0.496 )</td>
<td>( r = -0.400 )</td>
<td>( r = -0.175 )</td>
<td>( r = 0.672* )</td>
<td></td>
</tr>
<tr>
<td>Ovary Mass</td>
<td>( r = -0.841** )</td>
<td>( R = -0.672* )</td>
<td>( r = -0.461 )</td>
<td>( r = 0.484 )</td>
<td>( r = 0.720* )</td>
<td></td>
</tr>
</tbody>
</table>

Degrees of freedom for all r values = 9
* indicates a significant difference (p < 0.050)
**indicates a significant difference (p < 0.005)
‡ P-values are one-tailed

Hormone levels and ovary and adrenal masses

There was a significant effect of condition on adrenal mass \( F_{(3,14)} = 4.171, p \leq 0.026 \); Table 3). Post-hoc analyses revealed that RA females had significantly heavier adrenal masses than did RI females \( p \leq 0.029 \) and EB4 females \( p \leq 0.013 \). Similarly, there was a significant effect of condition on ovary mass \( F_{(3,15)} = 28.87, p < 0.0001 \); Table 3). Post-hoc analyses
revealed that both RA females and EB48 females had significantly heavier ovary masses than did RI females (p ≤ 0.0002 and p ≤ 0.02, respectively) and EB4 females (p ≤ 0.0002 and p ≤ 0.005, respectively and see Table 2). Due to heterogeneity of variance between groups on serum estradiol levels ($\chi^2 (3) = 38.42, p < 0.001$) a non-parametric Kruskal-Wallis test was used and revealed a significant difference between groups ($\chi^2 (3) = 12.842, p < 0.005$). Post-hoc analyses revealed that all high estradiol groups (RA, EB4 and EB48) had significantly greater serum EB levels than did the RI group (p < 0.05; Table 3). In addition, post-hoc analyses confirmed that RA females had significantly higher serum EB levels than did RI females (p ≤ 0.05; Table 3) and that EB4 females had significantly higher serum estradiol levels than did EB48 females (p ≤ 0.05; Table 3).

### Table 3. Mean (±SEM) adrenal mass, gonad mass, serum CORT level and serum estradiol level measured in adult female meadow voles in Experiment 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Adrenal Mass (mg)</th>
<th>Ovary Mass (mg)</th>
<th>Serum estradiol (pg/ml)</th>
<th>Serum CORT (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RI (n=5)</td>
<td>17.6±1.8</td>
<td>12.6±1.2</td>
<td>4.69±3.26</td>
<td>634.32±87.04</td>
</tr>
<tr>
<td>RA (n=4)</td>
<td>28.5±5.8*</td>
<td>31.8±1.1**</td>
<td>22.00±5.84*</td>
<td>509.15±92.63</td>
</tr>
<tr>
<td>EB4 (n=5)</td>
<td>12.3±4.1</td>
<td>9.8±2.9</td>
<td>250.52±50.08**</td>
<td>719.70±54.80</td>
</tr>
<tr>
<td>EB48 (n=5)</td>
<td>21.0±5.2</td>
<td>19.0±5.7**</td>
<td>44.56±4.07*</td>
<td>609.02±39.04</td>
</tr>
</tbody>
</table>

* indicates a significant difference (p < 0.050) relative to the RI group
**indicates a significant difference (p < 0.001) relative to the RI group

Table 2 shows the correlations between hormone levels, adrenal and ovary masses, the density of BrdU-immunoreactive (ir) cells and the density of pyknotic cells in the RI and RA female groups. The density of BrdU-labelled cells in the granule cell layer was negatively correlated with ovary mass and serum estradiol level (also see Tables 1 and 3). Serum estradiol was positively correlated with ovary and adrenal mass (also see Table 3). In addition, adrenal mass was positively correlated with ovary mass (also see Table 3).
Experiment 2. The elevated labelled cell density observed in RI adult female meadow voles persists for 5 weeks.

Density of [3H]thymidine-labelled and pyknotic cells in the granule cell layer and hilus

The majority of [3H]thymidine-labelled cells were found within the granule cell layer, consistent with the data reported by Cameron and colleagues (1993; see Figure 2B). There was no statistical difference between the RI female groups (female-paired females housed in the long-photoperiod versus the short-photoperiod) in the density of [3H]thymidine-labelled cells (granule cell layer, p ≤ 0.46; hilus, p ≤ 0.67). Therefore, these groups were combined and referred to as RI females in subsequent analyses. RI females had a significantly greater density of [3H]thymidine-labelled cells in the granule cell layer than did RA females (t(11) = 2.944, p < 0.013, see Table 4).

<table>
<thead>
<tr>
<th>Group</th>
<th>Labelled cells</th>
<th>Pyknotic cells</th>
<th>Labelled cells</th>
<th>Pyknotic Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>RI (n=9)</td>
<td>5.94±0.85</td>
<td>17.24±3.00</td>
<td>1.44±0.28</td>
<td>1.45±0.70</td>
</tr>
<tr>
<td>RA (n=5)</td>
<td>2.93±1.99*</td>
<td>22.26±2.98</td>
<td>1.72±0.60</td>
<td>0.80±0.70</td>
</tr>
</tbody>
</table>

* indicates a significant difference (p < 0.050) relative to the RI group

However, densities of [3H]thymidine-labelled cells in the hilus (p ≤ 0.44) and pyknotic cells in the granule cell layer (p ≤ 0.367) and the hilus (p ≤ 0.109) did not statistically differ between groups (see Tables 3 and 4). The density of pyknotic cells was not correlated with the number of labelled cells in either the granule cell layer (r(14) = 0.154, p ≤ 0.29]) or the hilus (r(14) = -0.458, p ≤ 0.116; Table 4). The number of pyknotic cells was ≈ 285% and ≈ 650% greater than the number of [3H]thymidine-labelled cells in RI and RA voles, respectively (compare densities in Table 4). There was 1 pyknotic cell labelled with [3H]thymidine in the granule cell layer of
an RA female. No significant differences were found between the RI and RA groups in the area of either the GCL (p ≤ 0.99) or the hilus (p ≤ 0.28).

2.4 DISCUSSION

The density of proliferating cells in the dentate gyrus was elevated in reproductively inactive (RI) relative to reproductively active (RA) females and was negatively correlated with serum estradiol level (Tables 1 and 2). These findings indicate that reproductive status regulates cell proliferation in female meadow voles. Exposing female meadow voles to EB for 48 hours, but not 4 hours could reproduce the degree of suppression in cell proliferation observed in RA versus RI females. In fact, exposure to EB for 4 hours tended to elevate the number of proliferating cells in the granule cell layer compared to RI females (Table 1). This finding demonstrates that the effect of reproductive status can be mimicked by estradiol in a duration-dependent manner. Similar patterns were observed with cell survival as the RI female meadow voles had greater densities of labelled cells than did RA females, 5 weeks after the injection of [3H]thymidine (Table 4). No significant difference in pyknotic cell density was found in either the granule cell layer or the hilus between RI and RA female meadow voles, in either experiment (Tables 1 and 4).

Reproductive status rapidly influences cell proliferation in the dentate gyrus of adult female meadow voles

Reproductive status influences cell birth in the dentate gyrus of laboratory-reared female meadow voles. The density of proliferating cells in the granule cell layer and hilus of RI females was approximately 9-times higher than in RA females. These results replicate Galea and McEwen's (1999) finding that non-breeding female meadow voles had significantly greater
densities of \(^{3}\text{H}\)thymidine-labelled cells (approximately 10 times) in the granule cell layer compared to breeding female meadow voles. The density of labelled cells in the hilus of the RI group was larger than in the RA group (about 8x). This finding is also consistent with that of Galea and McEwen (1999) that the density of labelled cells in the hilus was larger in non-breeding versus breeding voles, although the magnitude of their difference was lower (about 3x). Typically, cells found in the hilus express proteins consistent with a glial phenotype (Cameron et al., 1993b) and, as observed in the present study, fewer new cells are found in the hilus relative to the GCL. Interestingly, EB4 (high estradiol) females in this study tended to have more labelled cells in the GCL but fewer labelled cells in the in the hilus relative to RI females. Based upon the findings of the current and previous studies, it is tempting to speculate that different mechanisms may regulate the genesis of cells found in the GCL (mostly neuronal) versus the hilus (mostly non-neuronal).

The density of proliferating cells in the dentate gyrus of female laboratory-reared meadow voles was inversely related to serum estradiol level, which also complements Galea and McEwen’s (1999) finding in wild female meadow voles. In fact, the amount of variance in proliferating cells accounted for by serum estradiol alone is 43%, and for ovary mass alone is 70%. The present study demonstrated that cell proliferation in the dentate gyrus of laboratory-reared female meadow voles was initially increased but subsequently decreased by estradiol exposure. Specifically, a single high dose of estradiol tended to increase cell proliferation 4 hours after exposure but significantly suppressed cell proliferation 48 hours after exposure relative to RI females. Females exposed to a high level of estradiol for 4 hours exhibited rates of cell proliferation similar to RI females, while females exposed to the same dose of estradiol for 48 hours had rates of cell proliferation similar to RA females. Therefore, the duration of exposure to estradiol (endogenous or injected) appeared to produce the differences in the rate of cell proliferation observed at four versus 48 hours. The duration-dependent effect of estradiol
on cell proliferation may explain the apparent discrepancies regarding the effects of estradiol on neurogenesis in the dentate gyrus of rodents reported in the literature (Galea and McEwen, 1999; Tanapat et al., 1999). Tanapat and colleagues (1999) found that rats in proestrus had significantly higher rates of cell proliferation than rats in estrous or diestrous. According to their protocol rats in proestrus would have been exposed to high levels of estradiol for approximately 2-7 hours before BrdU was injected while rats in estrous would have experienced the proestrous estradiol surge approximately 30 hours before BrdU was injected (see Butcher et al., 1974). Thus, the meadow voles in the present study were exposed to high a level of EB for roughly the same durations that rats in the Tanapat et al (1999) study were exposed to proestrus level estradiol (E4 mimics proestrus and E48 mimics estrous).

The duration-dependent effect of estradiol on cell proliferation likely reflects an interaction between estradiol and other hormones or neurotransmitters. For example, breeding season-dependent increases in estradiol enhance the secretion of adrenocorticotropic hormone (ACTH) and corticoid binding thereby producing compensatory adrenal growth and secretion in female meadow voles after a lag time (Coyne and Kitay, 1969; Christian, 1975). This lag time must be between 4 and 48 hours, as changes in adrenal mass were only observed, in the present study, after 48 hours of exposure to estradiol (either injected or induced). Estradiol may regulate CORT level similarly in rats as circadian fluctuations of serum ACTH and CORT are enhanced during proestrus (Buckingham et al., 1978) and estradiol upregulates glucocorticoid density in the hippocampus (Ferrini and DeNicola, 1991). High levels of circulating adrenal steroids suppress cell proliferation (Gould et al., 1992; Cameron and Gould, 1994; Cameron et al., 1995; Cameron et al., 1999; Cameron et al., 1998). Therefore, estradiol could initially increase and subsequently decrease cell proliferation in the dentate gyrus of adult female rodents by upregulating CORT secretion from the adrenal glands (Christian, 1975) and CORT receptor mRNA in the brain (Ferrini and DeNicola, 1991). In the present study, adrenal masses
were significantly larger in RA females compared to RI females which is in agreement with the observations of Christian (1975) and were positively correlated with ovary mass. Our method of blood removal (prior to perfusion) may have prevented accurate assessments of pre-sacrifice differences in serum CORT level between groups by producing a ceiling effect. Of course, estradiol could influence cell proliferation by any number of interactions within the dentate gyrus. Estradiol has been shown to increase the number of NMDA receptor binding sites on CA1 pyramidal neurons (Weiland, 1992) and upregulate expression of the obligatory NMDA receptor subunit NR1 (Gazzaley et al., 1991). NMDA receptor activation also suppresses cell proliferation in the dentate gyrus of adult rodents (Cameron et al., 1995; Cameron et al., 1998) and shrews (Gould et al., 1997a).

*Estrogen receptor localization and effects within the hippocampus*

Generally, estradiol both downregulates estrogen receptor_α_ expression in the hippocampus (Weiland et al., 1997) and can differentially regulate estrogen receptor_β_ mRNA expression (McEwen and Alves, 1999) which is more abundantly expressed in the hippocampus (Shughrue et al., 1997). Any estrogen receptor_α_-mediated effect on cell proliferation is likely indirect as this receptor is expressed in GABAergic interneurons within the hippocampal formation of rats (Weiland et al., 1997). Less is known about the phenotype of cells that express estrogen receptor_β_ but this receptor subtype is more abundant within the hippocampus of rats and is expressed at low levels within the subgranular zone where the progenitors of new granule neurons reside (Shughrue et al., 1997). Of course, definitive statements about the localization and regulation of estrogen receptor subtypes cannot currently be made as studies utilising putatively specific antibodies, ligands and probes are quite recent (Shughrue et al., 1997; McEwen and Alves, 1999). Thus, the effect of EB on cell proliferation
in this study could have been mediated by either the \( \alpha \)- or \( \beta \)-receptor as both demonstrate high affinity for EB (Shughrue et al., 1999).

Estrogen receptor subtypes activate different second messenger pathways (MAPK and cAMP) and activate gene transcription via different response elements (CRE, SRE and AP-1; for review see McEwen and Alves, 1999) and therefore, it would not be surprising to find that cell proliferation could be differentially influenced by estradiol along very different time lines. The female meadow vole is an advantageous model for investigating the mechanism(s) by which estradiol influences cell proliferation as estradiol in this animal is maintained at either a high or low level (Seabloom, 1985) and estrogen receptor mRNA is distributed similarly across rodent species (Koch and Ehret, 1989; Simerly et al., 1989; Hnatchuk et al., 1994).

**Neurogenesis in natural and laboratory populations**

While the labelling efficacy of BrdU versus \(^{3}\text{H}\)-thymidine has not been directly assessed, the densities of labelled cells in the granule cell layer of both RA and RI laboratory-reared female meadow voles in the present study were very comparable to those found in wild voles by Galea and McEwen (1999). This finding also suggests that 10\(^{th}\) to 12\(^{th}\) generation wild female meadow voles that have been reared and housed in relatively impoverished laboratory conditions experience the same degree of cell proliferation during the non-breeding and breeding season as meadow voles reared in the wild (Galea and McEwen, 1999). Indeed, Kempermann and colleagues (1997) have shown that mice housed in an enriched environment have more surviving new neurons but not more proliferating granule cells than mice housed in impoverished conditions. Although they did not assess cell proliferation, Barnea and Nottebohm (1994) found that seasonal increases in the density of surviving neurons (labelled 6 or more weeks after \(^{3}\text{H}\)thymidine injection) were enhanced in free-ranging relative to captive
black-capped chickadees, demonstrating that birds' natural environments enhanced cell survival beyond that observed in an enriched laboratory environment (a large outside aviary). Another study that directly assessed cell proliferation in wild grey squirrels reported seasonal fluctuations in brain volume but no change in cell proliferation in the dentate gyrus (Lavenex et al., 2000). However, the longevity of wild grey squirrels (up to 6 years; Gurnell, 1987) increases the probability that senescent squirrels were included in the sample analysed and cell proliferation is diminished in senescent rats (Seki and Arai, 1995; Kuhn et al., 1996; Montaron et al., 1998; Cameron and McKay, 1999). In summary, while cell proliferation in adult wild animals may be seasonally regulated, the survival of new neurons may be enhanced by experience.

Reproductive status influences granule cell survival in the dentate gyrus

In the present study, females were only paired with a cage partner for 48 hours, but cell survival was likely affected by reproductive status. Behavioural estrous, and therefore elevated estradiol levels, persist for at least 3 weeks following exposure to a male (Seabloom, 1985; Cohen-Parsons and Carter, 1987). In fact, RI females had more proliferating cells (Experiment 1) and more surviving new cells (Experiment 2) compared to RA females. However, the percentage of cells labelled at 5 weeks (Experiment 2) versus 2 hours (Experiment 1) was lower in the RI female group (granule cell layer ≈ 7%, hilus ≈ 24%) compared to the RA female groups (see Tables 1 and 4; granule cell layer ≈ 49%, hilus ≈ 82%). This finding suggests that although RI females had more proliferating cells, RA females had a greater rate of cell survival (density of labelled cells persisting 5 weeks). Studies have shown that survival can be regulated independent of cell proliferation. For example, enriched housing (Kempermann et al., 1997) and hippocampus-dependent learning (Gould et al, 1999a) enhance the survival of
new granule neurons but have no effect on cell proliferation. Interestingly, in the avian higher vocal centre, the survival of new neurons is modulated by older estrogen-ir neurons that lie within the migratory pathway (Goldman, 1999).

There was no effect of photoperiod on cell survival in female meadow voles (the number of new cells surviving 5 weeks) as RI females housed in either the long- or short-photoperiod had comparable densities of both labelled and pyknotic cells. This finding coupled with previous work in males suggests that there is a possible sex difference in the effect of photoperiod on cell survival. For example, photoperiod has been shown to regulate the number of new neurons surviving 7 weeks after BrdU injection in adult male golden hamsters (Huang et al., 1998). Male golden hamsters housed in a short-photoperiod had more seven week-old neurons than male golden hamsters housed in a long-photoperiod. Observing the effect of reproductive status on cell proliferation in male meadow voles would be interesting because while reproductive status in females is dependent upon the environment (exposure to male pheromone or a male), reproductive status in male meadow voles is photoperiod-dependent (Seabloom, 1985).

Reproductive status does not influence the density of pyknotic cells in the dentate gyrus of adult female meadow voles

While reproductive status influenced cell proliferation in the dentate gyrus of female meadow voles, no effect on pyknotic cell density was found in Experiment 1. Furthermore, the density of labelled cells (proliferating or mature) within the dentate gyrus of female meadow voles was not related to pyknotic cell density at either 2 hours or 5 weeks. Studies utilising experimentally induced pathologies, such as kindling (Bengzon et al., 1997; Parent et al., 1998; Scott et al., 1998), ischemia (Liu et al., 1995) and lesions (Gould and Tanapat, 1997) have found evidence that dying cells may induce the proliferation of progenitor cells. Interestingly,
Galea and McEwen (1999) found that during the non-breeding season (when estradiol levels are low) the density of pyknotic cells, along with the density of proliferating cells was elevated. The RA females (present study) had the same density of pyknotic cells as wild female meadow voles trapped during the non-breeding season by Galea and McEwen (1999). Interestingly, while pyknotic cell densities drop significantly during the breeding season in wild voles (Galea and McEwen, 1999) they remain elevated in RA females (present study). As mentioned, serum CORT level is inversely related to pyknotic cell density in the dentate gyrus of adult rodents (Sloviter et al., 1989; Gould et al., 1990; Gould et al., 1991) and serum CORT levels measured in the present study were much lower than those reported by Galea and McEwen (1999). Therefore, pyknotic cell densities may have been artificially high in the present study due to chronically low CORT level.

Reproductive status-related changes in cell proliferation and/or survival in the female meadow voles may be related to maternal behaviour in natural settings

The observation that reproductive status, possibly through estradiol, regulates the density of proliferating cells within the dentate gyrus of adult female meadow voles is intriguing, as these animals are very successful breeders. Behavioural estrous and subsequent ovulation can be induced in female meadow voles on the day of parturition (Lee et al., 1970). Because meadow voles are promiscuous, females are either in behavioural estrous or pregnant throughout much of the breeding season (Anderson et al., 1976). The observation that the majority of new granule neurons express mature neuronal markers (neuron-specific enolase, for example; Cameron et al., 1992) at approximately 21 days after their birth is compelling as gestation is 21 days in meadow voles (and other rodents). Therefore, reproductive status-dependent shifts in cell proliferation might profoundly impact hippocampus-dependent behaviour at the time a mother meadow vole gives birth to her litter. If the number of new
granule neurons that form mature synapses affects hippocampal function, then hippocampus-dependent behaviour should be affected about 21 days after any shift in the rate of proliferating cells within the dentate gyrus; i.e. during parturition in the adult female meadow vole. Adult female meadow voles contract their home range size at parturition and expand their home range size during weaning (Madison, 1978). In addition, studies have shown that female meadow voles decrease their home range size during the breeding season (when cell proliferation rates are low) relative to the non-breeding season (Madison, 1985; Sheridan and Tamarin, 1988). Perhaps analogously, low estradiol female meadow voles learn a spatial version of the water maze better than high-estradiol female meadow voles (Galea et al., 1995). Thus, the functional consequence of reduced cell proliferation may be reduced spatial ability, which could be advantageous near parturition for female meadow voles. By remaining close to her nest, a mother meadow vole likely reduces her probability of predation and is better able to protect her pups. In fact, Sheridan and Tamarin (1988) found that smaller home range size is related to better reproductive success in female voles.

In addition to influencing cell proliferation, estradiol produces morphological and electrophysiological changes within the hippocampus. For example, CA1 pyramidal cell spine and synapse density is increased by estradiol (Woolley et al., 1990; Woolley and McEwen, 1992; Woolley and McEwen, 1993). Woolley (1998) has hypothesised that estradiol-induced changes in hippocampal morphology may be related to maternal behaviour in female rodents. Early studies have demonstrated that aspects of maternal behaviour are hippocampus-dependent. Indeed, fimbria lesions impair pup retrieval, nest building and nursing (Kimble et al., 1967; Steele et al., 1978). Certainly, estradiol-induced morphological changes within the hippocampus could prime maternal behaviour and estradiol-induced decreases in the density of proliferating cells could impair spatial ability.
2.5 IMPLICATIONS

In conclusion, reproductive status, possibly through estradiol, regulates cell proliferation and survival in the dentate gyrus of adult female meadow voles. Initially, estradiol tends to increase cell proliferation in the dentate gyrus of rodents but subsequently induces a mechanism (perhaps CORT mediated) that suppresses cell proliferation. Although the density of labelled cells remains elevated in RI females relative to RA females, 5 weeks later, the rate of survival of newly formed granule cells in the granule cell layer is enhanced in RA females, indicating that estradiol may enhance cell survival. Because this change is reproductively linked, the long-term functional consequence of a changed rate of cell proliferation could be that maternal behaviour is enhanced and spatial ability is decreased, both of which could potentially enhance the reproductive success of female meadow voles.
CHAPTER 3

ESTRADIOL INITIALLY ENHANCES BUT SUBSEQUENTLY SUPPRESSES (VIA ADRENAL STEROIDS) PROGENITOR CELL PROLIFERATION WITHIN THE DENTATE GYRUS OF ADULT FEMALE RATS


3.1 INTRODUCTION

The dentate gyrus of the hippocampus is a structure that exhibits neurogenesis throughout adulthood (for review see Kempermann and Gage, 1999; Fuchs and Gould, 2000; Ormerod and Galea, 2000). Estrogen is one factor that can regulate neurogenesis within the adult rodent dentate gyrus (Galea and McEwen, 1999; Tanapat et al., 1999; Ormerod and Galea, 2001). High levels of estradiol increase progenitor cell proliferation in intact female rats relative to vehicle controls (Tanapat et al., 1999). In contrast, the density of new cells is correlated negatively with plasma estradiol levels in wild adult female meadow voles (Galea and McEwen, 1999). This differential nature of estradiol's regulation of cell production within the rat versus vole dentate gyrus could be time-dependent. We have found that estradiol exposure for 4 h enhances whereas estradiol exposure for 48 h suppresses cell proliferation in the dentate gyrus of adult laboratory-reared female meadow voles (Ormerod and Galea, 2001). Understanding how estradiol dynamically regulates granule cell production in the rodent hippocampus will facilitate our ability to understand and potentially control neurogenesis.

The differential time-dependent nature by which estradiol regulates the production of new cells may be mediated by its stimulatory effects on the hypothalamic-pituitary-adrenal (HPA) axis. One possibility may be that estradiol-induced high levels of circulating adrenal steroids suppress the number of new cells produced in the female rodent dentate gyrus. Indeed, high levels of adrenal steroids suppress cell proliferation in the dentate gyrus of adult male rats (Gould et al., 1992; Cameron and Gould, 1994; Cameron et al., 1995; Cameron and McKay,
In addition to stimulating corticosterone (CORT) secretion in adult male and female rodents, estradiol elevates adrenocorticotropic hormone (ACTH) levels, which gradually produce adrenal enlargement (Coyne and Kitay, 1969; Christian, 1975; Burgess and Handa, 1992; Handa et al., 1994). Consistent with these findings, we (2001) found larger adrenal masses in adult female meadow voles exposed to estradiol for 48 versus 4 h and adrenal mass size is indicative of HPA activity (Akana et al, 1983; Lemaire et al, 1997; Lemaire et al, 2000). Because CORT suppresses cell proliferation estradiol could, in turn, suppress cell proliferation indirectly by stimulating adrenal activity.

The present study investigated whether estradiol influences the production of new cells time-dependently in the dentate gyrus of adult female rats and whether the estradiol-induced suppression in cell proliferation depends on estradiol-stimulated adrenal activity. We hypothesized that the number of new cells observed in the dentate gyrus of adult female rats would increase following a 4 h but decrease following a 48 h exposure to estradiol. Because estradiol stimulates adrenal function, we hypothesized that the suppression of new cells could be eliminated or reversed by adrenalectomizing animals to prevent estradiol-induced stimulation of adrenal activity. Understanding the interaction between estradiol and adrenal steroids on neuron production may provide insight into how different estrogen replacement therapies can either benefit or exacerbate the symptoms of Alzheimer Disease (Hogervorst et al., 2000). Indeed the cognitive deficits and hippocampus atrophy associated with Alzheimer disease may be associated with abnormal levels of cortisol (Lupien et al., 1998,1999,2002).

3.2 METHODS

All animals were treated in strict accordance with the guidelines set forth by the Canadian Council on Animal Care and The University of British Columbia regarding the ethical
treatment of animals used for the purposes of research. Every effort was made to minimise the number of animals used per group and their suffering.

Animals

Sixty-six female Sprague-Dawley rats (225-250 g) were obtained from Charles River Canada. Upon arrival, the rats were housed in groups of four in hanging metal cages in a temperature controlled colony room (21±1°C) with a 12:12 h light:dark cycle (lights on at 0700h). Beginning the day after arrival, rats were handled for 5 min daily. One week after arrival, rats were ovariectomized (Experiment 3) or ovariectomized and adrenalectomized (Experiment 4) and then given another week to recover from surgery before testing began. During recovery, the rats were housed singly in bedding-lined (Care Fresh; Absorption Corporation) polyurethane cages. Food (Lab Diet #5012; Jamieson) and tap water were available, ad libitum throughout the experiments. To maintain salt balance, adrenalectomized rats were given 0.9% NaCl in their drinking water.

Procedure

Experiment 3 was conducted to determine whether exposure to estradiol for 4 h increases and exposure to estradiol for 48 h decreases the number of new (post-mitotic) cells in the dentate gyrus of adult female rats. Previously, Tanapat et al (1999) reported that rats in the proestrus (a high estradiol phase) had more dividing (assessed 2 h post-BrdU) and more new (assessed 2-14 d post BrdU) cells in their dentate gyri than rats in the estrus (low estradiol) phase. In addition, they found that estradiol reversed an ovariectomy-induced reduction in the number of new neurons. These results suggest that estradiol increases neurogenesis. Ormerod and Galea (2001) then demonstrated that female meadow voles exposed to estradiol for 4 h before BrdU
was injected had more but females exposed to estradiol for 48 h had significantly fewer dividing (assessed 2 h post-BrdU) cells than females that were reproductively inactive (with undetectable circulating estradiol levels). These results suggest that estradiol enhances then suppresses neurogenesis by increasing and then decreasing the number of progenitor cells that leave the cell cycle to complete mitosis. Interestingly, the observation made by Tanapat and colleagues (1999) that estrous versus proestrous rats had fewer dividing and new cells in their dentate gyri supports the hypothesis that estradiol influences cell proliferation time-dependently because rats in estrus would have experienced their proestrus surge 24-48 h before receiving BrdU. Therefore, we hypothesized that females exposed to estradiol for 4 h prior to an injection of BrdU would have more new or labelled cells and that females exposed to estradiol for 48 h before an injection of BrdU would have fewer new cells than females exposed to vehicle. To test whether estradiol time-dependently influences the number of new cells produced, OVXd female rats were given a subcutaneous injection of either estradiol benzoate (EB; 10 µg in 0.10 ml of sesame oil) or vehicle (V; 0.10 ml sesame oil) either 4 h (EB4 group, n=7 and V4 group, n=6) or 48 h (EB48 group and V48 group, n=6 per group) before a single injection of the cell synthesis marker, bromodeoxyuridine (BrdU; 200 mg/kg, i.p.). We were interested in determining whether estradiol influenced the number of new rather than dividing cells because Nowakowski and Hayes (2001) argue that treatment-induced changes in cell proliferation could actually reflect changes s-phase length that would artificially suggest the number of dividing cells has changed. Because progenitor cell division is complete within ≈ 24 h of an injection of BrdU (Cameron and McKay, 2001) we perfused rats 24 h after BrdU was injected to determine the number of newly divided cells after one mitotic division. To investigate whether the expression of immature neuronal protein (TUC-4 and doublecortin; DCX) increases in BrdU-labelled cells with a longer survival time, a subset of EB4 and V4 rats were sacrificed 4 days
(or 96 h) after BrdU was injected (n=4 per group). In addition, we sacrificed separate groups of ovariectomized animals either 4 or 48 h after an injection of EB or vehicle (n=4 per group) so that we could assess the relationship between serum estradiol and CORT at the time that animals in the other groups were injected with BrdU.

**Experiment 4** was conducted to determine whether adrenal steroids mediate the suppressive effect of estradiol on the production of new cells. Previous work has shown that estradiol increases cell proliferation through a serotonin-mediated mechanism (Banasr et al., 2001). Therefore, we investigated whether we could eliminate (no difference in BrdU-labelled cell number would be observed between estradiol- and vehicle-treated groups) or reverse (the estradiol-induced increase in BrdU-labelled cell number would persist) the estradiol-induced suppression in the number of new cells by removing estradiol’s influence on circulating adrenal steroid levels (via adrenalectomy). Exposure to high-level estradiol for 48 h induces adrenal enlargement in meadow voles (Coyne and Kitay, 1969; Ormerod and Galea, 2001) and increases CORT secretion in rats (Burgess and Handa, 1992) and in dispersed adrenocortical cells (Nowak et al., 1995). In addition, circadian fluctuations in serum ACTH and CORT are potentiated during proestrus in the rat (Buckingham, 1978). High levels of the adrenal steroid CORT have been found to suppress cell proliferation within the dentate gyrus of adult male rats (Gould et al., 1992; Cameron and Gould, 1994; Cameron and Gould, 1996; Cameron et al., 1999). Thus, a plausible hypothesis is that estradiol suppresses cell proliferation 48 h after exposure by increasing circulating adrenal steroids. OVXd +ADXd female rats received an injection of either estradiol (ADX+E48 group; 10 μg in 0.10 ml of sesame oil, s.c.; n=9) or vehicle (ADX+V48 group; 0.10 ml sesame oil, s.c.; n=8) 48 h prior to receiving a single injection of BrdU (200 mg/kg, i.p.). A low oral dose of CORT (25μg per ml 0.9% saline) was added to the rats’ drinking water over 4 days beginning the day prior to the estradiol injection.
This dose has been shown to produce low circulating physiological levels of CORT (Gould et al., 1990; Cameron and Gould, 1994). Low-level CORT was administered orally to maintain basal levels because we wanted to prevent a possible ceiling effect with an enhancement of cell proliferation by both ADX and high-level estradiol that would mask a potential reversal of the suppression. In addition, low level CORT replacement eliminates the enhanced granule cell death response to ADX in the dentate gyrus of adult rats (Gould et al., 1990; Cameron and Gould, 1998) and cell death stimulates the division of neuronal progenitors (Gould and Tanapat, 1997). Rats were perfused 24 h after receiving BrdU to analyse the number of new cells produced by a 48 h exposure to estradiol.

**Histology**

Twenty-four h or 4 d after BrdU was injected, rats were anaesthetized with sodium pentobarbital and then perfused with 4% paraformaldehyde. A 24 h survival time after BrdU injection was chosen to allow for one mitotic division. The phenotype of BrdU-ir cells was verified in both the 24 h and the four-day survival group by assessing anti-TUC-4 (Turned on After Division-64kD/ULIP/CRMP; TUC-4 is a protein expressed by postmitotic granule neurons; Quinn et al., 1999; Tanapat et al., 1999; Shors et al., 2001), anti-doublecortin (a protein expressed by migrating and differentiating granule neurons; Jin et al, 2001; Gleeson et al, 1999; Francis et al, 1999; used only in 4 d survival group) and anti-glial fibrillary acidic protein (GFAP; an astroglia marker; Cameron et al., 1993b) immunoreactivity. Before perfusing the rats, blood samples were taken transcardially to verify serum estradiol and CORT levels by radioimmunoassay. Blood samples were taken at the time of perfusion to minimize any effect of stress that may occur by taking blood at the time of BrdU injection. Intact adrenal glands were removed and immediately weighed. Adrenal mass is expressed as mg/100g body
mass. Following perfusion, brains were extracted and stored overnight in perfusate at 4°C. The following day, the brains were sliced into 40 μm thick sections through the entire dentate gyrus (8-9 sections per rat) an oscillating tissue slicer (Leica VT1000S) in a bath of 0.1-M phosphate buffer (PB). Slices prepared for peroxidase immunohistochemistry were pre-treated in a solution of 0.2% H₂O₂ in PB for 20 min and then rinsed in PB before being mounted on slides treated with 3% 3-aminopropyltriethoxy-silane in acetone (Sigma Chemicals). BrdU-labelled cells were counted on peroxidase treated tissue and the phenotype of new cells was determined on fluorescent probe-treated tissue.

Peroxidase immunohistochemistry

Tissue was processed for BrdU-immunoreactivity by applying solutions directly to the slide-mounted sections. Unless otherwise specified, phosphate-buffered saline (0.1 M sodium phosphate heptahydrate in 0.9% saline; pH 7.4) was used for all rinses and slides were rinsed repeatedly between each step. 1) Cells in the sections were permeabilized with 0.05% Trypsin (Sigma Aldrich Chemicals) in Tris-HCl buffer (pH 7.5) containing 0.1% CaCl₂ for 10 min. 2) DNA was denatured by applying 2N HCl for 30 min and then the sections were repeatedly rinsed (pH 6.0). 3) Sections were blocked in 5.0% normal horse serum for 30 min and then incubated overnight in mouse monoclonal antibody against BrdU (1:100 + 3% NHS + 0.5% Tween 20; Boehringer Mannheim) at room temperature. 4) Sections were incubated in mouse secondary antisera (1:29 + 3.0% normal horse serum; Vector Laboratories) for 4 hrs. 5) Sections were incubated in avidin-biotin horseradish peroxidase (ABC Elite Kit; 1:50; Vector Laboratories) for 60 min. 6) Sections were reacted for about 10 min in 0.02% diaminobenzidine (DAB; Sigma Aldrich Chemicals) with 0.003% H₂O₂ and then counterstained with cresyl violet acetate (Baker), dehydrated and coverslipped with Permount (Fisher Scientific).
Fluorescence immunohistochemistry

Separate sets of slide-mounted sections were triple-stained with fluorescent probes to assess BrdU-, TUC-4- and GFAP-immunoreactivity (ir) or double-stained with fluorescent probes to assess BrdU- and doublecortin-ir. Unless stated otherwise all sections were rinsed several times with tris-buffered saline (TBS; pH 7.5) between steps. 1) Endogenous peroxidase was quenched for 10 min with 2% H$_2$O$_2$ in TBS. 2) Sections were incubated for 2 h in a solution of deionized formamide in 2XSSC at 65°C. 3) DNA was denatured by applying 2N HCl for 30 min at 37°C. 3) Sections were incubated in 0.1 M borate buffer for 10 min. 4) Sections were blocked in 5.0% normal donkey serum (Jackson Immunoresearch) for 30 min and then incubated overnight in a cocktail containing rat anti-BrdU (ascites 1:100; Oxford Biochemicals Incorporated), rabbit monoclonal anti-TUC-4 (1:500; Chemicon) and mouse monoclonal anti-GFAP (1:2000; Novacastra) or were incubated in a cocktail of rat anti-BrdU and goat anti-doublecortin (1:2000; Santa Cruz) at 4°C. 5) 5% normal donkey serum (Jackson Immunoresearch) was applied to slides. 6) Sections were incubated in a cocktail of donkey anti-rat fluorescein (FITC; to visualize anti-BrdU) and donkey anti-rabbit Cy5 (to visualize TUC-4) and donkey anti-mouse Cy3 (to visualize GFAP; all diluted 6μl/ml; Jackson Immunoresearch) or in a solution of donkey anti-rat FITC (to visualize BrdU) and donkey anti-goat Cy3 (to visualize doublecortin) for 4 hrs. 5) Sections were then rinsed and coverslipped with the anti-fading agent diazobicyclooctane (DABCO; 2.5% DABCO, 10% polyvinyl alcohol and 20% glycerol in TBS; Sigma).

Hormone assays

Serum estradiol and CORT assays were performed as described in Ormerod and Galea (2001). Briefly, blood samples were stored overnight at 4°C and then were centrifuged at 10g
for 10 min. Serum estradiol was assayed using a Coat-A-Count kit (Diagnostic Products
Corporation, Los Angeles, CA) modified for low expected levels of estradiol. The intra-assay
coefficient of variation was 1.35 %.

Serum corticosterone levels were analysed using a radioimmunoassay protocol
described by Weinberg and Nezio (1987). Briefly, antiserum was obtained from Immunocorp
(Montreal, Canada) and tracer was obtained from Mandel Scientific (Guelph, Canada).
Dextran-coated charcoal was used to adsorb and precipitate free steroids after incubation. The
intra-assay coefficient of variation was 1.55 %.

Data analyses

Prior to analysis, slides were coded so that the experimenter was blind to the treatment
conditions. Total BrdU-ir and pyknotic cells through the granule cell layer and subgranular
zone (defined as approximately the 50 μm band between the granule cell layer and the hilus;
Palmer et al., 2000) were stereologically estimated using peroxidase-treated tissue. On
peroxidase-treated tissue, BrdU-ir and pyknotic cells were counted on every 10th section
through the dentate gyrus per rat. Cells were considered BrdU-labelled if they were intensely
stained and exhibited medium round or oval cell body morphology (see Figure 3A and
Cameron et al., 1993b; Ormerod and Galea, 2001). Cells were considered pyknotic if they
lacked a nuclear membrane, had pale or absent cytoplasm and darkly stained spherical
chromatin (see Figure 3B and Gould et al., 1991; Ormerod and Galea, 2001). Pyknotic and
BrdU-ir cells were counted with a 100X objective on a Nikon Eclipse (e600) light microscope
and the total number of cells was estimated using a modified version of the optical fractionator
method (West et al., 1991) for an estimate of total cell counts:

\[
N = \sum \text{Cells}_{\text{BrdU}} \left( \frac{t}{h} \ast \frac{1}{\text{asf}} \ast \frac{1}{\text{ssf}} \right)
\]
Figure 3. Photomicrographs of BrdU-labeled and pyknotic cells and confocal images of neurons or glia in the dentate gyri of adult female rats in Experiments 3 and 4.
A) Photomicrograph of a BrdU-labelled cell clump in the subgranular zone (SGZ) of an OVX'd female exposed to estradiol for 4h (100x objective). B) Photomicrograph of a pyknotic (dying) cell in the SGZ (100x objective). C) Confocal image (63X objective) of neurons in the granule cell layer of a female rat exposed to estradiol for 48h. The white arrows point to cells expressing the neuronal protein doublecortin (indicated by the red label). The yellow arrow points to BrdU-ir cell (green label; a new cell) expressing the neuronal protein doublecortin. The cell is therefore a new neuron. D) Confocal image of cells expressing the glial protein GFAP (red label). The white arrows point to glia and the yellow arrow shows a new cell (that is not a new glia). Scale bar represents 10 μm.
where, $\sum_{Cells_{BrdU}}$ is the total number of BrdU-labelled cells counted on all sections, $t =$ section thickness (0.04mm), $h =$ height of the counting frame (plane of focus, 0.005mm), $asf =$ area sampling fraction (in our case the section area of dentate gyrus), and $ssf =$ sections sampled fraction (1/10). Areas were measured using the digitizing software Analytical Software Imaging Station (Imaging Research, Brock University, Ontario, Canada) and dentate gyrus volume estimates were made using Cavalieri’s principle (Gunderson et al., 1988). Because we have previously reported BrdU-ir cell densities (Ormerod and Galea, 2001; Galea and McEwen, 1999) we also calculated BrdU-ir cell densities (# of cells/area) on 6 anatomically matched sections per rat (where the dentate gyrus lies just beneath the corpus callosum and the infrapyramidal and suprapyramidal blades are joined at the crest; between A -3.3 and A -4.8 in rats) in order to compare the density of BrdU-ir cells with stereological estimates of total BrdU-ir cells in the dentate gyrus.

BrdU-ir cell phenotypes were analyzed on fluorescent probe-treated tissue. Twenty-five BrdU-labelled cells on four sections per brain (400 μm apart; $n = 3$ per group) taken where the infrapyramidal and suprapyramidal blades join at the crest were identified on a Zeiss fluorescence microscope and their phenotype verified using a confocal laser scanhead (BioRad 2000) and a 63X objective. The percentage of BrdU-ir cells that expressed a neuronal (TUC-4-ir or DCX-ir; Figure 3C) or glial phenotype (GFAP-ir; Figure 3D) was determined. Z-sections at 0.4 μm intervals were taken and optical stacks of 10 images were created with NIH Image for PC (http://www.scioncorp.com/pages/menu.htm) and imported into Adobe Photoshop for channel merging. Digital manipulations were restricted to contrast enhancements and colour level adjustments.

Statistical analyses
In Experiment 3, the dependent variables (total BrdU-ir cells, BrdU-ir cell density, total pyknotic cells, percentage BrdU/GFAP-ir cells, percentage BrdU/TUC-4-ir cells or BrdU/DCX-ir cells; serum hormone levels) were analysed using an analysis of variance (ANOVA) with group (V4, EB4, V48 and EB48, respectively) as the independent variable. Newman-Keuls procedure were used in post-hoc analyses unless the groups violated the assumption of homogeneity of variance and in this case, the Welch’s $t$ test was used. In Experiment 4, dependent variables were analyzed with Student’s independent t-tests with group (ADX+V48 versus ADX+E48) as the independent variables. In Experiment 3, Spearman Rank correlations were conducted between the dependent variables (V4 vs. E4 and V48 vs. E48) because comparisons included hormone assay values that fell below the threshold of detection and were arbitrarily given a value of zero. In Experiment 4, Pearson product-moment correlations were run between dependent variables with the exception of analyses that included serum hormone levels. All statistical procedures set $\alpha = 0.05$.

**3.3 RESULTS**

*Experiment 3. Relative to vehicle, the number of BrdU-labelled cells observed in the dentate gyrus of adult female rats increases following exposure to EB for 4 h but decreases following exposure to EB for 48 h.*

The number of BrdU-labelled cells significantly differed between groups ($F_{(3,21)} = 4.49$, $p \leq 0.014$). EB4 females had significantly more labelled cells ($p \leq 0.04$) and EB48 females had significantly fewer BrdU-labelled cells ($p \leq 0.006$) relative to the V4 and V48 groups, respectively (see Figure 4). We found similar results when comparing the density of BrdU-labelled cells between groups and found a significant positive correlation between total labelled cell estimates and labelled cell densities ($r_{s(38)} = 0.95; p \leq 0.001$). Neither total number of
pyknotic cells ($p \geq 0.23$; see Figure 4) nor pyknotic cell density ($p \geq 0.97$) significantly differed between groups. No significant linear relationship between BrdU-labelled cell number and pyknotic cell number was observed between groups ($p \leq 0.57$). Total volume of the granule cell layer and subgranular zone did not statistically differ between groups ($F_{(3,21)} = 0.63$, $p \leq 0.63$; $V_{4}=1.360 \pm 0.05$ mm$^3$; $EB_{4}=1.47 \pm 0.05$ mm$^3$; $V_{48}=1.40 \pm 0.08$ mm$^3$; $EB_{48}=1.42 \pm 0.08$ mm$^3$). No significant difference was observed between groups in the percentage of BrdU-

**Figure 4.** Mean ($\pm$SEM) number of new cells or pyknotic cells in the dentate gyrus of adult female rats given BrdU either 4 h or 48 h after estradiol or vehicle and sacrificed 24 h later in Experiment 3.

White bars represent the data of females given sesame oil vehicle either 4 or 48 h before BrdU ($n=6$ per group) and grey and black bars represent data of females given estradiol either 4 h ($n=7$) or 48 h ($n=6$) before BrdU, respectively. A) Mean number new (BrdU-ir) cells observed in the dentate gyrus of adult female rats when BrdU was injected 4 h or 48 h after an injection of estradiol or vehicle. Estradiol significantly increased the number of BrdU-ir cells (approximately 316%) found within the dentate gyrus of adult female rats within 4 h ($p=0.01$) but significantly decreased the number of cells (approximately 60%) within 48 h ($p=0.006$). B) Mean number of pyknotic cells in the dentate gyrus of adult female rats given estradiol or vehicle. Estradiol exposure for either 4 or 48 h did not influence the number of pyknotic cells relative to vehicle.

**denotes $p \leq 0.01$  *denotes $p \leq 0.05$  *denotes $0.10 > p \leq 0.05$
ir cells expressing TUC-4 or GFAP in animals perfused 24 h (TUC-4 \(\sim 22\%\), \(= 0.21\) and GFAP \(\sim 17\%\), \(= 0.48\); see Table 5) after BrdU was injected.

### Table 5. Mean (±SEM) % BrdU-ir cells expressing a neuronal (TUC-4-ir) or glial (GFAP-ir) phenotype measured 24 h after BrdU was injected did not significantly differ in adult female rats exposed to estradiol or vehicle for either 4 or 48 h in Experiment 3.

<table>
<thead>
<tr>
<th>Group</th>
<th>TUC-4 ir</th>
<th>GFAP-ir</th>
</tr>
</thead>
<tbody>
<tr>
<td>V4 (n=6)</td>
<td>14.7±4.9</td>
<td>17.0±2.9</td>
</tr>
<tr>
<td>V48 (n=6)</td>
<td>18.7±3.5</td>
<td>18.7±2.7</td>
</tr>
<tr>
<td>EB4 (n=7)</td>
<td>19.3±4.7</td>
<td>14.7±3.5</td>
</tr>
<tr>
<td>EB48 (n=6)</td>
<td>25.3±3.5</td>
<td>18.7±2.4</td>
</tr>
</tbody>
</table>

As expected, EB4 (\(p \leq 0.0003\)) and EB48 (\(p \leq 0.04\)) females had higher serum estradiol levels than V4 and V48 females, respectively (\(F(3,21) = 29.09, p \leq 0.001\); see Table 6). Planned comparison revealed that adrenal mass was significantly heavier in the E48 versus V48 females (\(p \leq 0.02\)) but was similar in E4 and V4 females (\(p \leq 0.54; F(3,21) = 2.26, p \leq 0.11\); see Table 6). Serum CORT levels tended to differ between groups (\(F(3,21) = 2.66, p \leq 0.07\)) and interestingly, planned comparisons showed that levels did not differ between E48 and V48 females (\(p = 0.38\)) but were higher in the E4 versus V4 females (\(p \leq 0.02\); see Table 6).

### Table 6. Mean (±SEM) serum hormone levels and adrenal masses in adult female rats injected with BrdU either 4 or 48 h after estradiol administration in Experiments 3 and 4.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Serum Estradiol (pg/ml)</th>
<th>Serum CORT (ng/ml)</th>
<th>Adrenal Mass (mg/100g bw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V4 (n=6)</td>
<td>N/A(^w)</td>
<td>377.9±7.99</td>
<td>27.21±0.96</td>
</tr>
<tr>
<td>EB4 (n=7)</td>
<td>28.97±3.91(^**)</td>
<td>595.9±2.93(^*)</td>
<td>25.46±2.19</td>
</tr>
<tr>
<td>V48 (n=6)</td>
<td>N/A(^w)</td>
<td>558.7±3.33</td>
<td>23.93±2.64</td>
</tr>
<tr>
<td>EB48 (n=6)</td>
<td>7.13±1.62(^*)</td>
<td>482.3±8.16</td>
<td>31.01±1.68(^*)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADXV48 (n=9)</td>
<td>N/A(^w)</td>
<td>96.4±2.74</td>
<td>155.6±3.72</td>
</tr>
<tr>
<td>ADXE48(n=8)</td>
<td>8.84±0.94(^*)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^*\) indicates a significant difference (\(p < 0.050\)) relative to the control group  
\(^**\) indicates a significant difference (\(p < 0.001\)) relative to the control group  
\(^w\) N/A indicates that serum hormone levels were below the detection threshold of the radioimmunoassay (5 pg/ml)

Because blood samples were taken 28 and 72 h after EB was injected and serum CORT assay values are likely not reflective of the values that would be observed at the time that BrdU
was injected (4 or 48 h after EB was administered). Thus, we assessed the relationship between serum estradiol and serum CORT level in animals that were sacrificed, rather than injected with BrdU, either 4 or 48 h after EB was injected. In animals that were sacrificed 4 h after EB was injected, serum CORT assay values were 100% of control values whereas in animals sacrificed 48 h after EB was injected, serum CORT assay values were 175% of control values (Table 7).

Table 7. Mean (±SEM) serum hormone levels and adrenal masses in samples taken from animals either 4 or 48 h after an estradiol injection in Experiment 3 (no BrdU injected).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Serum Estradiol (pg/ml)</th>
<th>Serum CORT (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle - 4 h</td>
<td>7.14±2.41</td>
<td>247.1±68.28</td>
</tr>
<tr>
<td>Estradiol - 4 h</td>
<td>339.08±66.29**</td>
<td>248.9±91.94</td>
</tr>
<tr>
<td>Vehicle - 48 h</td>
<td>N/A</td>
<td>68.4±16.62</td>
</tr>
<tr>
<td>Estradiol - 48 h</td>
<td>20.51±4.52*</td>
<td>120.3±43.56*</td>
</tr>
</tbody>
</table>

* indicates a significant difference (p < 0.050) relative to the control group
** indicates a significant difference (p < 0.001) relative to the control group
N/A indicates that serum hormone levels were below the detection threshold of the radioimmunoassay (5 pg/ml)

In EB4 and V4 females, serum estradiol was significantly correlated positively with total BrdU-ir cell count ($R_{(13)}=0.61; p \leq 0.02$) with higher levels of estradiol being associated with more BrdU-ir cells (Figure 4 and Table 6). In EB48 and V48 females, serum estradiol was significantly correlated negatively with total BrdU-ir cell count ($R_{(12)}=-0.63; p \leq 0.03$), with more BrdU-ir cells associated with lower levels of serum estradiol (Figure 4 and Table 6).

There were no other significant correlations observed between any of the dependent variables.

In animals perfused 4 days after BrdU was injected, EB4 females had significantly more labelled cells than V4 females ($p \leq 0.02$; vehicle = 888.2±333.4 and EB4 = 4052±1322.23). No difference was observed between vehicle- and EB-treated rats in the percentage of BrdU-labelled cells expressing the neuronal protein TUC-4 (~27%, $p = 0.56$) or in the percentage of BrdU-labelled cells expressing GFAP (~23%, $p = 0.86$; see Table 8). A greater percentage of BrdU-labelled cells expressed the neuronal protein DCX (~65%) relative to TUC-4 or GFAP
Table 8. Mean (±SEM) % BrdU-ir cells expressing a neuronal (TUC-4-ir or doublecortin-ir) or glial (GFAP-ir) phenotype measured 4 days after BrdU was injected did not significantly differ in adult female rats exposed to estradiol for 4 h in Experiment 3.

<table>
<thead>
<tr>
<th>Group</th>
<th>TUC-4 ir</th>
<th>Doublecortin-ir</th>
<th>GFAP-ir</th>
</tr>
</thead>
<tbody>
<tr>
<td>V4 (n=4)</td>
<td>28.0±2.3</td>
<td>64.0±4.6</td>
<td>22.7±5.3</td>
</tr>
<tr>
<td>EB4 (n=4)</td>
<td>26.7±1.3</td>
<td>66.0±2.0</td>
<td>24.0±4.6</td>
</tr>
</tbody>
</table>

Experiment 4. The Estradiol-induced Suppression in Cell Proliferation Within the Dentate Gyrus of Adult Female Rats is Reversed by Adrenalectomy.

Total BrdU-ir and pyknotic cells did not significantly differ between groups (p = 0.43 and p = 0.54, respectively; Figure 5) and the density of BrdU-labelled cells was similar between groups (p ≤ 0.82; data not shown). Non-significant differences were observed between groups in the percentage of cells expressing TUC-4 (~ 19%, p = 0.56) or GFAP (~ 18%, p = 0.72; Table 9).

Table 9. Mean (±SEM) % BrdU-ir cells expressing a neuronal (TUC-4-ir) or glial (GFAP-ir) phenotype in adrenalectomized females following a 24 h survival time after BrdU injection in Experiment 4 did not significantly differ between groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>TUC-4-ir</th>
<th>GFAP-ir</th>
</tr>
</thead>
<tbody>
<tr>
<td>V48 (n=9)</td>
<td>17.3±3.5</td>
<td>18.7±2.7</td>
</tr>
<tr>
<td>EB (n=8)</td>
<td>20.0±2.3</td>
<td>20.0±2.4</td>
</tr>
</tbody>
</table>

Serum estradiol (measured 24 h after BrdU) was elevated in estradiol-treated rats versus controls (p ≤ 0.015; Table 6). However, no significant differences between groups in serum CORT (measured 24 hr after BrdU) were observed. Table 6 shows that the dose of CORT replaced in the drinking water of rats did produce circulating levels that were lower than the endogenous levels observed in Experiment 3. In addition, there were no significant correlations
found between the dependent variables. Total volume of the granule cell layer and subgranular zone did not differ between adrenalectomized rats exposed to vehicle 48 h before BrdU (ADXV48: $1.580 \pm 0.103 \, \text{mm}^3$) and adrenalectomized rats exposed to EB 48 h before BrdU (ADXEB48: $1.586 \pm 0.104 \, \text{mm}^3$) groups ($p \leq 0.97$).

![Figure 5. Mean (±SEM) number of new cells or pyknotic cells in the dentate gyrus of adrenalectomized adult female rats when BrdU was administered 48 h after estradiol or vehicle in Experiment 4.](image)

White bars represent the data of females given sesame oil vehicle (n=8) and black bars represent data of females given estradiol (n=9). A) Mean number new (BrdU-ir) cells in the dentate gyrus of adult female rats 4 h after an injection of estradiol. No difference was observed between groups given estradiol versus vehicle in the number of BrdU-ir cells found within the dentate gyrus of adult female rats following adrenalectomy ($p= 0.43$). B) Mean number of pyknotic cells in the dentate gyrus of adult female rats given estradiol or vehicle. Estradiol did not influence the number of pyknotic cells ($p=0.54$). Note, however that more pyknotic cells were observed in adrenalectomized animals (compare with Figure 4).

3.4 DISCUSSION

In adult female rats, we found that estradiol dramatically alters the number of new (BrdU-labeled cells) in a biphasic manner. Relative to vehicle, exposure to a high dose of estradiol for 4 h increases whereas exposure for 48 h decreases the both the number and density of BrdU-labeled cells. The dual effect of estradiol on the number of new cells could be either duration or dose-dependent, however several lines of evidence suggest the effect is dose-dependent. First, in the present study, estradiol levels remained elevated in the group that received estradiol 48 h before BrdU (Experiment 3; Table 6), relative to the group that received
vehicle, but the number of labeled cells was significantly lower. This result is similar to what we have observed previously in adult female meadow vole. We found that although females exposed to estradiol for 48 h had serum levels intermediate to females exposed to estradiol for 4 h and females with low circulating estradiol levels, they had significantly fewer labeled cells in their dentate gyri (Ormerod and Galea, 2001). In fact, the number of labeled cells was suppressed to the level observed in the dentate gyri of females with chronically high circulating serum estradiol levels (Ormerod and Galea, 2001). Second, Tanapat and colleagues (1999) found that the rate of cell proliferation was similar during the diestrus and estrus phase although estradiol levels differ during these phases. Finally, we have preliminary data to suggest that prolonged exposure to estradiol suppresses the production of new cells within the dentate gyrus of adult female rats (E.M. Falconer and L.A.M. Galea, personal communication).

Estradiol suppresses the number of new cells, at least in part, by stimulating adrenal activity because adrenalectomy eliminated the estradiol-induced decrease in the number of labeled cells observed (Experiment 4). Previous work has shown that estradiol stimulates adrenal activity (Coyne and Kitay, 1969; Buckingham, 1978; Burgess and Handa, 1992; Nowak et al., 1995; Ormerod and Galea, 2001) and that adrenal steroids suppress cell proliferation in the dentate gyrus of adult male rats (Gould et al., 1992; Cameron and Gould, 1994; Cameron and Gould, 1996; Cameron et al., 1999). Here we show adrenalectomized females exposed to estradiol have similar numbers of BrdU-labeled cells in their dentate gyri as females exposed to vehicle. To fully understand how adult neurogenesis is regulated, it is important to determine how a factor, such as estradiol, dynamically regulates components of the process. This study shows that estradiol first enhances and then suppresses the production of new cells by stimulating adrenal activity. This finding is important because the manipulation of endogenous progenitor cells could lead to advances in therapy geared toward alleviating neurodegenerative disease.
Stress, working through an elevation in adrenal steroids, has been shown to suppress cell proliferation in the dentate gyrus of adult male rats (Tanapat et al., 2001). Thus, the possibility that subcutaneous injection is stressful enough to elevate CORT and diminish the production of new cells exists. In fact, a previous study has shown that the number of new cells produced in the dentate gyrus of adult rats may be reduced by a subcutaneous injection (Cameron and Gould, 1994). That finding is consistent with our study, in which we noted that serum CORT levels were higher 4 h (V4 and E4) versus 48 h (V48 and E48) after a subcutaneous injection of estradiol (Table 6). In addition, the number of BrdU-labeled cells tended to be suppressed in the V4 group relative to the V48 group (p ≤ 0.06; see Figure 4). However, relative to their controls, E48 rats had higher levels of CORT than E4 rats, suggesting again that estradiol has a stimulatory effect on adrenal function 48 h after exposure. In addition, the enhanced level of CORT in the EB4 group did not block the estradiol-induced increase in cell proliferation.

Interestingly, we did not find a significant relationship between cell birth and cell death in our experiments, however more pyknotic cells were observed following adrenalectomy, which is consistent with previous reports (Gould et al., 1992; Cameron and Gould, 1994; Cameron et al., 1995; Cameron and McKay, 1999). Across experiments estradiol did not alter the phenotype of new cells.

**Estradiol interacts with adrenal steroids to suppress cell proliferation**

In the present study adrenal steroids mediated the estradiol-induced suppression in the number of new cells observed. High levels of adrenal steroids suppress cell proliferation in the dentate gyrus of adult male rats (Gould et al., 1992; Cameron and Gould, 1994). Previous work has shown that estradiol increases serum CORT levels in female rodents (Christian, 1975;
Coyne and Kitay, 1969; Buckingham, 1978; Handa and Burgess, 1994) and increases adrenal mass in adult female meadow voles 48 h but not 4 h after estradiol is administered (Ormerod and Galea, 2001). Consistent with this literature, we found heavier adrenals in E48 but not E4 females, relative to controls. Relative to vehicle, rats sacrificed 4 h after estradiol was injected (when V4 and E4 rats would have been injected with BrdU) had similar serum CORT values whereas rats sacrificed 48 h after estradiol was injected (when V48 and E48 rats would have been injected with BrdU) had serum CORT values that were 175% of their controls' values.

The estradiol-induced suppression in cell proliferation in the dentate gyrus of adult female rats was eliminated but not reversed by adrenalectomy (Experiment 4). This observation suggests that the estradiol-induced suppression in cell proliferation may be mediated by another factor. Estradiol upregulates Type I glucocorticoid receptor expression in the hippocampus (Ferrini and De Nicola, 1991). Thus the low level replacement of CORT used in this study could have masked a possible adrenalectomy-induced reversal of the estradiol-mediated suppression in cell proliferation. In addition, Cameron et al (1995) found that N-methyl-D-aspartate receptor (NMDAr) activation suppresses cell proliferation in the dentate gyrus of adult male rats downstream of CORT. Estradiol increases NMDAr binding sites in the hippocampus (Weiland, 1992) and upregulates the NMDAr NR1 subunit (Gazzaley et al., 1996). Therefore in addition to increasing serum CORT levels, estradiol could mediate the synergistic effect of CORT and NMDAr activation by increasing NMDAr and/or Type I glucocorticoid receptor expression to suppress cell birth at 48 h after its administration.

**Estradiol could act time-dependently through numerous pathways**

The current study found that exposure to estradiol for 4 h increases and for 48 h decreases the number of new cells (assessed 24 h post-BrdU) complementing previous work (Ormerod and Galea, 2001; Tanapat et al., 1999). Thus, estradiol appears to influence
neurogenesis by altering cell proliferation or the number of progenitor cells that exit the cell cycle to produce new cells as an estradiol-induced change in the length of s-phase in progenitor cells already dividing would not presumably change the number of daughter cells produced (see Nowakowski and Hayes, 2001). A previous study has shown that estradiol initially enhances cell proliferation by influencing serotonin activity (Daszuta et al., 2001) and in the present study we have evidence that estradiol subsequently reduces cell proliferation by influencing adrenal steroids.

Because adrenalectomy eliminated but did not completely reverse the estradiol-induced suppression we observed in the number of new cells produced, estradiol may also work through other mechanisms to suppress the production of new cells. There are two estrogen receptor isoforms (ERα and ERβ) expressed within the hippocampus (Shughrue et al., 1997; Milner et al., 2001). Although progenitor cell ER expression has not been investigated, weak ERβ receptor mRNA expression and extranuclear ERα expression is present in the dentate gyrus including the subgranular zone (Shughrue et al., 1997; Weiland et al., 1997; Milner et al., 2001). FURTHERMORE, ERβ has been localized on astrocytes in the subgranular zone (Garcia-Segura et al., 1999), and astrocytes have been putatively identified as neural stem cell precursors (Seri et al., 2001). Based upon ER localization, the estradiol-induced increase in cell proliferation could be directly mediated by activation of the ERβ isoform or indirectly by the ERα isoform. Estradiol could regulate cell proliferation time-dependently by changing ER expression (Shughrue et al., 1992) or by activating second messenger pathways and gene transcription as estradiol activates cAMP (Gu and Moss, 1996), calcium (Improta-Brears et al., 1999), inositol-3 phosphate kinase, diaglycerol, src (Arnold et al., 1995), CREB, ERK-1 and ERK-2 (Sing et al., 1999) cascades. Moreover, both ligand bound ER isoforms can stimulate gene transcription of an estrogen-response element (ERE)-driven reporter gene and can
modulate the activity of other transcription factors (NFκB, AP-1 and SFRE; Shyamala and Guiot, 1992; Webb et al., 1999; McDonnell and Norris, 2002). A plausible hypothesis is that estradiol enhances cell proliferation via second messengers and eventually suppresses cell proliferation by interacting with transcription factors or that ERαs and ERβs differentially influences adult neurogenesis. Clearly, the time-dependent effect of estradiol on cell proliferation could be mediated by several factors.

_Estradiol time-dependently influences many forms of plasticity within the hippocampus_

In addition to neurogenesis, estradiol mediates several forms of plasticity within the adult rodent hippocampus. High-level estradiol enhances both long-term potentiation (LTP) and long-term depression in hippocampal CA1 pyramidal neurons (Cordoba Montoya and Carrer, 1997; Desmond, Zhang, & Levy, 2000; Good, Day, & Muir, 1999; Warren et al., 1995). However, high levels of estradiol reduce LTP in the dentate gyrus (Gupta et al., 2001). High estradiol levels increase dendritic spine and synapse density on hippocampal CA1 pyramidal neurons but not in the dentate gyri of young female rats (Woolley et al., 1990). Interestingly 4-10 d old granule neurons extend axons to CA3 pyramidal neurons (Hastings and Gould, 1999), which in turn synapse with CA1 pyramidal neurons (Amaral and Witter, 1995). Increases in CA1 pyramidal dendritic spine density are maximal from 48 h to 96 h after estradiol administration before gradually declining (Woolley and McEwen, 1993). Thus, increases in CA1 dendritic synapse density coincide with the time that estradiol-induced new granule neurons begin to extend axons into the CA3 region. Similarly, CA1 dendritic spine density recedes at the time when estradiol-induced decreased numbers of new neurons would begin to extend their axons into the CA3 region. Thus estradiol may regulate plasticity of hippocampal circuitry time-dependently.
3.5 IMPLICATIONS

Estradiol increases but then decreases cell proliferation in the dentate gyrus of adult female rats and voles (Ormerod and Galea, 2001; current study) suggesting that this time-dependent effect is robust across rodent species. Neurogenesis occurs in the dentate gyrus during adulthood in many species (Cameron et al., 1993b; Kempermann et al., 1997; Gould et al., 1997, 1998, 1999a, 1999b; Kornack and Rakic, 1999; Ormerod and Galea, 2001) including humans (Eriksson et al., 1998). Putative younger neurons are more electrophysiologically plastic than older granule neurons (Wang et al., 2000). In fact recent electrophysiological work has shown that new neurons born in adulthood mature into functional granule neurons in the mouse dentate gyrus (van Praag et al, 2002). Recent work has shown that hippocampus-dependent learning requires and enhances the survival of 1-2 week old neurons (Gould et al., 1999; Shors et al., 2001). Therefore, estradiol-induced changes in the number of new cells produced could profoundly influence hippocampus-dependent behavior. Indeed, hippocampus-dependent learning is impaired by a high dose but enhanced by a low dose of estradiol in female rats (Galea et al., 2001; Holmes et al., in press). These findings suggest that there may be an optimal level of neuron production and survival for optimal performance on hippocampus-dependent tasks. Of course, the effect of estradiol on cell proliferation would have to be dissociated from its effect on learning before definitive statements about the functional role of estradiol-induced changes in cell proliferation can be made.

Intriguingly, high levels of estradiol for 48 h decreases cell proliferation in the dentate gyrus of adult female meadow voles (Ormerod and Galea, 2001) and increases cell proliferation in the subventricular zone (but not the dentate gyrus) of adult female prairie voles (Fowler et al., 2001; Smith et al., 2001), indicating that the functional role of estradiol-induced changes in neurogenesis may differ among strains of voles with very different social patterns (meadow
voles are promiscuous and prairie voles are monogamous). Both the subventricular zone and dentate gyrus are likely to mediate different subsets of behaviours and while $\text{ER}_\beta$ is expressed in both the rat dentate gyrus and the olfactory area, $\text{ER}_\alpha$ is expressed at very low levels if at all in the olfactory area (Shughrue et al., 1997). Future work could take advantage of this dissociation in the effect of estradiol on cell proliferation to investigate the functional role of these new neurons.
CHAPTER 4
NMDA RECEPTOR ACTIVITY AND ESTRADIOL: INDEPENDENT REGULATION OF CELL PROLIFERATION IN THE DENTATE GYRUS OF ADULT FEMALE MEADOW VOLES
(In press, Journal of Endocrinology)

4.1 INTRODUCTION

New granule neurons are added to the dentate gyri of all mammalian species that have been studied, including humans throughout adulthood (Altman and Das, 1965; Cameron et al., 1993; Gould et al., 1997, 1998, 1999; Eriksson et al, 1998; Kornack and Rakic, 1999). The number of granule neurons added to the mammalian dentate gyrus appears substantial. In rats, approximately 9,000 new cells are produced daily and many of these new cells differentiate into granule neurons (Cameron and McKay, 2001). Altering progenitor cell proliferation, the fate of daughter cells or the survival of new granule neurons could increase or decrease neurogenesis in the dentate gyrus. Understanding how different components of adulthood neurogenesis are influenced by a single factor is important because a factor that both increases cell proliferation and decreases the survival of young neurons could produce no net change in new neuron number. Estradiol dynamically influences neurogenesis within the adult rodent dentate gyrus by first increasing and then decreasing cell proliferation (Ormerod and Galea, 2001; Ormerod et al., 2003) as well as by enhancing the survival of young neurons (Ormerod et al., 2002). This study was designed to better understand how estradiol dynamically influences cell proliferation in the adult rodent dentate gyrus.

Several studies have shown that estradiol influences cell proliferation in the dentate gyri of adult rodents. For example, short-term exposure to estradiol (2-4 h) stimulates cell proliferation in the dentate gyri of adult ovariectomized (OVXd) female rats (Tanapat et al., 1999; Banasr et al., 2001; Ormerod et al, 2003). Interestingly, we have previously shown that estradiol initially enhances cell proliferation (within 4 h) but subsequently suppresses cell
proliferation (within 48 h) in the dentate gyrus of OVXd adult female rats, suggesting that estradiol time-dependently influences cell proliferation (Ormerod et al., 2003). In fact, estradiol appears to dynamically regulate cell proliferation across rodent species. Ormerod and Galea (2001) found that a 4 h exposure to estradiol tended to increase whereas a 48 h exposure significantly decreased the density of proliferating cells in the dentate gyrus of reproductively inactive female meadow voles (with low circulating estradiol levels). However, in that study all females had intact ovaries and the possibility that other ovarian steroids could also have influenced cell proliferation exists. Therefore, one objective of this study was to investigate whether the dynamic changes in cell proliferation observed in the dentate gyri of OVXd adult female rats and intact female meadow voles following estradiol administration also occur in dentate gyri of adult OVXd female meadow voles. This finding would verify that the dynamic effects of estradiol are robust across rodent species with diverse reproductive strategies and physiologies.

The mechanisms by which estradiol differentially influences cell proliferation in the adult rodent dentate gyrus are beginning to be explored. Banasr and colleagues (2001) have shown that the estradiol-induced increase in cell proliferation is mediated by serotonin. Specifically, the estradiol-induced increase in cell proliferation in the dentate gyrus of adult OVXd female rats is abolished by the administration of the serotonin synthesis inhibitor PCPA (Banasr et al., 2001). We found that the estradiol-induced suppression in cell proliferation is partially mediated by adrenal steroids because the suppression is eliminated (but not reversed) in adult female rats that are adrenalectomized (Ormerod et al., 2003). Therefore, estradiol must influence another factor, in addition to stimulating adrenal activity, to suppress cell proliferation with longer exposure.

Estradiol exposure for 48 h increases the number and sensitivity of N-methyl-D-aspartate (NMDA) receptors in the hippocampus of adult rats (Weiland, 1992; Gazzaley et al.,
Furthermore, NMDA receptor activation (via NMDA) decreases and NMDA receptor inactivation (via MK-801 or CGP43487) increases cell proliferation in the dentate gyri of adult male rats, tree shrews and aged female rats (Cameron et al, 1994, 1995; Gould et al., 1997; Bernabau and Sharp, 2000; Nacher et al., 2001; Nacher et al., 2003 but see Bernabau and Sharp, 2000 and Arvidsson et al., 2001). Interestingly, Cameron and colleagues (1998) demonstrated that NMDA receptor activation works downstream of adrenal steroids to suppress cell proliferation as the effects of low-level or high-level corticosterone can be blocked by NMDA receptor activation or inactivation, respectively. Therefore, another objective of the current study was to investigate whether longer exposure to estradiol influences NMDA receptor activity to suppress cell proliferation.

We investigated whether estradiol influences neurogenesis in the dentate gyrus of adult OVXd female meadow voles by first increasing (within 4 h) and then decreasing (within 48 h) cell proliferation and whether estradiol stimulates NMDA receptor activity to suppress cell proliferation. We also examined whether NMDA receptor activation and inactivation influenced cell proliferation as it does in adult male and aged female laboratory rats and tree shrews. Based upon our previous work using adult OVXd rats and intact adult female meadow voles, we hypothesized that estradiol would increase cell proliferation within 4 h but decrease cell proliferation within 48 h in the dentate gyri of OVXd adult female meadow voles. We also hypothesized that because estradiol influences NMDA receptor number and sensitivity and NMDA receptor activation influences cell proliferation, administration of the NMDA receptor antagonist MK-801 could reverse the estradiol-induced suppression in cell proliferation.

Discovering how estradiol mediates its diverse effects on neurogenesis in the adult dentate gyrus could promote the development of strategies to control the process in order to replace neurons lost in disease or trauma. Moreover, fully characterizing the time-dependent effects of estradiol on neurogenesis may be important for patients on long-term estrogen replacement
therapies, given that neurogenesis has been linked to hippocampus-dependent behavior (Gould et al, 1999; Shors et al., 2001, 2002). For example, estrogen replacement therapy has been purported to reduce the risk and severity of Alzheimer's disease as well as the associated cognitive impairment (Henderson et al., 1994; Sherwin, 1997; Kawas et al., 1997).

4.2 METHODS

All animals were treated in strict accordance with the guidelines set forth by the Canadian Council on Animal Care and The University of British Columbia regarding the ethical treatment of animals used for the purposes of research. Every effort was made to minimise the number of animals used per group and their suffering.

Animals

Forty-one adult female meadow voles (at least >25 g and 60 d old) reared in our breeding colony at The University of British Columbia were used as subjects. The voles were bred and reared in a colony room that was temperature controlled (21±1°C) with a 16:8 h (lights on at 0700h) light:dark cycle. All animals were housed in polyurethane paper bedding-lined (Carefresh; Absorption Corporation) cages that contained enrichment supplies (plastic and/or cardboard containers). At 21 days of age, the voles were weaned and housed either with same sex siblings or individually (if the vole was the only female sibling of her litter) until 60 days of age when all voles were housed individually. The voles had free access to tap water and Jamieson Lab Diet #5012 for the duration of the experiment and were given weekly sunflower seed, alfalfa pellet, carrot, and apple food supplements. All voles were ovariectomized (OVXd) using sterile surgical techniques under Halothane anaesthesia delivered at 3% (reduced as
required to maintain stable respiration) and given one week to recover from surgery before participating in an experiment.

Procedure

*Experiment 5* was conducted to determine whether 1) short-term (4 h) exposure to estradiol increases cell proliferation, 2) NMDA receptor activation decreases cell proliferation, and 3) estradiol influences NMDA receptor activity within 4 h to alter cell proliferation in the dentate gyri of adult OVXd female meadow voles. OVXd female meadow voles were injected either with estradiol benzoate (EB4; 10 µg) or sesame oil (OIL; 0.05 ml) and then either NMDA (30 mg/kg) or vehicle (VEH; 0.05 ml saline) 3 h later. Four hours after the injection of estradiol or oil, the voles were given a single injection of the cell synthesis marker, bromodeoxyuridine (BrdU; 50 mg/kg) and were perfused 1 h later to assess cell proliferation (see Figure 6 for experiment timeline).

![Figure 6. Time line of Experiment 5.](image)

Voles were injected with oil (0.05 ml) or estradiol (10 µg) and then either with vehicle or NMDA (30 mg/kg) 3 h later and BrdU (50 mg/kg) 4 h later.

Therefore, effects on cell proliferation were tested in four groups in Experiment 1: OIL4+VEH (n=5), EB4+VEH (n=6), OIL4+NMDA (n=5) or EB4+NMDA (n=5). If estradiol influenced NMDA receptor activity within 4 h to alter cell proliferation, then we would expect to observe no difference or perhaps a suppressed number of labelled cells in the EB4+NMDA-treated versus the OIL4+NMDA-treated voles.
Experiment 6 was conducted to investigate whether 1) estradiol exposure for 48 h suppresses cell proliferation in the dentate gyrus of adult OVXd female meadow voles, 2) MK-801 stimulates cell proliferation, and 3) estradiol stimulates NMDA activity to suppress cell proliferation in the dentate gyrus of adult OVXd female meadow voles. To test whether estradiol exposure for 48 h decreases cell proliferation, OVXd voles were injected (s.c.) with either estradiol benzoate (EB48; 10 µg) or sesame oil (OIL; 0.05 ml) and then either MK-801 (1 mg/kg) or vehicle (VEH; 0.05 ml saline) 47 h later. Forty-eight hours after the injection of estradiol or sesame oil vehicle, the voles were given a single injection of the cell synthesis marker, bromodeoxyuridine (BrdU; 50 mg/kg, i.p.) and were perfused 1 h later to assess cell proliferation (see Figure 7).

Figure 7. Time line of Experiment 6.
Voles were injected with oil (0.05 ml) or estradiol (10 µg) and then either with vehicle or MK-801 (1 mg/kg) 47 h later and BrdU 48 h later. In both experiments, voles were perfused 1 h after BrdU was injected.

Therefore, effects on cell proliferation were tested in four groups in Experiment 2:
OIL48+VEH, EB48+VEH, OIL48+MK-801 or EB48+MK-801 (n=5 per group). If estradiol stimulates NMDA receptors to suppress cell proliferation in the dentate gyrus of adult OVXd female meadow voles, then we would expect to find that antagonizing NMDA receptor activity with MK-801 would eliminate or reverse the estradiol-induced suppression in cell proliferation.

Drug preparation

Estradiol benzoate (EB; Sigma Aldrich Chemicals) solution was prepared by dissolving EB in sesame oil (Sigma Aldrich Chemicals) to a concentration of 10 µg of EB/0.05 ml sesame oil.
The solution was then stored in a light insensitive container and used for all experiments. All voles were subcutaneously injected with 0.05 ml of the solution (10 μg EB per vole). Although estradiol-induced changes in blood-brain permeability could, in theory, account for differences in BrdU-labeling between groups, estradiol only alters rat blood brain barrier permeability after at least 3 weeks of exposure (Ziylan et al., 1990). In addition, previous work has shown that the number BrdU-labeled cells is elevated in the rostral migratory stream (but not dentate gyrus) of adult female prairie voles with high- versus- low estradiol levels (Smith et al., 2001; Fowler et al., 2001). N-methyl-D-aspartate (NMDA; Tocris) was prepared just prior to its use in Experiment 1. NMDA was dissolved in isotonic saline to a concentration of 30 mg/ml and was injected intraperitoneally (i.p.) in a volume of 0.1ml/100g body weight making the dose of NMDA 30 mg/kg. The noncompetitive NMDA receptor antagonist MK-801 (Tocris) was prepared just prior to its use Experiment 2. MK-801 was dissolved in isotonic saline to a concentration of 1 mg/ml and was injected i.p. in a volume of 0.1ml/100g body weight making the dose of MK-801 1 mg/kg. The doses and durations of exposure to NMDA or MK-801 were chosen because they have been shown previously to influence cell proliferation in the dentate gyri of adult rats (Cameron et al., 1995, 1998). Bromodeoxyuridine (BrdU; Sigma Aldrich Chemicals) was prepared just prior to use by dissolving BrdU in freshly prepared saline buffered with 0.7% 2 N NaOH to a concentration of 10 mg BrdU/ml saline. Voles were injected intraperitoneally with 0.5ml/100g of the solution (50 mg/kg). This dose has been used to label cells dividing in the dentate gyrus of mice (Kempermann et al., 1997) and voles (Ormerod and Galea, 2001; Smith et al., 2001; Fowler et al., 2002).
Figure 8. Photomicrographs of a BrdU-labelled cells and a pyknotic cell.
A) Microphotograph of BrdU-labelled cells located in the subgranular zone of an OVXd female exposed to estradiol for 4 h before BrdU was injected. These cells are representative of those counted in the dentate gyrus of all groups. B) Microphotograph of a representative pyknotic or dying cell in the subgranular zone (SGZ) between the GCL and hilus. Scale bar represents 10 μm.
Histology

At the end of each experiment, voles were anaesthetized with sodium pentobarbital and then perfused with 4% paraformaldehyde, 1 h after BrdU was injected. Following perfusion, brains were extracted and refrigerated overnight in perfusate at 4°C. The following day, the brains were sectioned 40 μm thick sections through the entire dentate gyrus using an oscillating tissue slicer (Leica VT1000S) in a bath of 0.1-M phosphate buffer (PB). Sections were pre-treated in a solution of 0.2% H₂O₂ in PB for 20 min and then rinsed in PB before being mounted on slides treated with 3% 3-aminopropyltriethoxy-silane in acetone (Sigma Chemicals) to enhance slide adherence.

Peroxidase immunohistochemistry

Tissue was processed to reveal BrdU labelling by applying solutions directly to the slide-mounted sections as described previously (Cameron et al., 1993b; Gould et al., 1999; Tanapat et al., 1999; Ormerod and Galea, 2001; Ormerod et al., 2003). The sections were rinsed repeatedly between steps in phosphate-buffered saline (0.1 M sodium phosphate heptahydrate in 0.9% saline; pH 7.4) unless stated otherwise. Sections were incubated in 0.05% Trypsin (Sigma Aldrich Chemicals) in 0.1% CaCl₂ Tris-HCl buffer (pH 7.5) for 10 min to permeabilize cells. DNA was then denatured by applying 2N HCl for 30 min and then the sections were repeatedly rinsed in PBS (pH 6.0). Sections were blocked with 5.0% normal horse serum for 30 min and then incubated overnight in mouse monoclonal antibody against BrdU (1:100 + 3% NHS + 0.5% Tween 20; Boehringer Mannheim) at room temperature. The following day, sections were incubated in mouse secondary antisera (1:29 + 3.0% normal horse serum; Vector Laboratories) for 4 hrs and then in avidin-biotin horseradish peroxidase complex
(ABC Elite Kit; 1:50; Vector Laboratories) for 60 min. Sections were reacted for about 10 min in 0.02% diaminobenzidine (DAB; Sigma Aldrich Chemicals) and 0.003% \( \text{H}_2\text{O}_2 \) in Tris-buffered saline and then counterstained with cresyl violet acetate (Baker), dehydrated and coverslipped with Permount (Fisher Scientific) so that pyknotic cells could also be counted.

Data Analyses

Prior to analysis, slides were coded to blind the experimenter to the treatment conditions. Total BrdU-ir (intensely stained medium round or oval cells; Fig 8A and Cameron et al., 1993b; Ormerod and Galea, 2001; Ormerod et al., 2003) and pyknotic cells (with pale or absent cytoplasm, dark spherical chromatin and no nuclear membrane; Fig 8B and Gould et al., 1991; Ormerod and Galea, 2001) through the granule cell layer and subgranular zone (the \( \approx 50 \mu \text{m} \) border between the hilus and granule cell layer; Palmer et al., 2000) of the dentate gyrus were stereologically estimated. To stereologically estimate cell numbers, total BrdU-ir and pyknotic cells were counted on every 10\(^{th}\) section (8 sections per vole; \( p = 1.00 \)) through the rostral-caudal extent of the dentate gyrus per rat using a 100X objective under a Nikon Eclipse (\( \geq 600 \)) light microscope. The counts were then applied to a modified version of the optical fractionator formula (West et al., 1991; described in Ormerod, Lee and Galea, in press) to project what was counted on every 10\(^{th}\) section to what would be counted on the entire dentate gyrus. Areas were measured using the digitizing software Analytical Software Imaging Station (Imaging Research, Brock University, Ontario, Canada) and dentate gyrus volume was estimated using Cavalieri’s principle (Gunderson et al., 1988). Because we have previously reported BrdU-ir cell densities (Ormerod and Galea, 2001; Galea and McEwen, 1999) we also calculated BrdU-ir cell densities (\# of cells/area) on 5 anatomically matched sections per rat (where the dentate gyrus lies just beneath the corpus callosum and the infrapyramidal and
suprapyramidal blades are joined at the crest; between A -3.3 and A -4.8 in rats) in order to compare the density of BrdU-ir cells with stereological estimates of total BrdU-ir cells in the dentate gyrus. Our relative densities and stereologically estimated total BrdU-labelled cell numbers were similar to those reported by Cameron and McKay (2001).

**Statistical analyses**

In Experiment 5, the dependent variables (total BrdU-ir cells, BrdU-ir cell density, and total pyknotic cells) were analysed using an analysis of variance (ANOVA) with hormone (EB4, OIL4) and drug (NMDA, VEH) as the independent variables. In Experiment 6, the dependent variables (total BrdU-ir cells, BrdU-ir cell density, and total pyknotic cells) were analyzed using an ANOVA with hormone (EB48, OIL48) and drug (MK801, VEH) as the independent variables. For both experiments, Pearson product-moment correlations were run between dependent variables and the Newman-Keuls procedure was used as the post-hoc analysis. All statistical procedures set $\alpha = 0.05$.

**4.3 RESULTS**

Experiment 5. Relative to vehicle, estradiol increased and NMDA decreased cell proliferation and a 4h estradiol exposure did not appear to stimulate NMDA receptor activity to influence cell proliferation.

Estradiol significantly increased (main effect of hormone: $F_{(1,17)} = 14.27, p < 0.001$) and NMDA significantly decreased (main effect of drug: $F_{(1,17)} = 9.62, p < 0.006$) the total number of BrdU-labelled cells in the dentate gyri of adult female meadow voles (Figure 9A). Estradiol did not stimulate NMDA receptors within 4 h to alter cell proliferation because hormone treatment did not interact with drug treatment to influence labelled cell number ($p < 0.77$;
Figure 9. Mean (±SEM) number of new cells or pyknotic cells in the dentate gyrus of adult female voles injected with BrdU 4 h after estradiol or oil and 1 h after NMDA or vehicle in Experiment 5.

The white bar represents the data of OIL4+VEH females (n=5), the light gray bar represents the data of EB4+VEH females (n=6), the dark gray bar represents the data of OIL4+NMDA (n=5) females and the black bar represents the data of EB4+NMDA females (n=5). A) Mean number new (BrdU-ir) cells observed in the dentate gyrus of adult female voles. Relative to oil, estradiol increased (p_< 0.001) and NMDA decreased (p_< 0.006) BrdU-labelled cell number in the adult female vole dentate gyrus. Estradiol increased the number of BrdU-labelled cells both in the absence (OIL4+VEH versus E4+VEH; p_< 0.01) and in the presence (OIL4+NMDA versus E4+NMDA; p_< 0.03) of NMDA. B) Mean number of pyknotic cells in the dentate gyrus of adult female voles. The number of pyknotic cells did not differ between groups.

* Newman-Keuls result p<0.001  ** Newman-Keuls result p<0.05
Figure 9A). In fact, Figure 9A shows that estradiol enhanced cell proliferation regardless of
drug condition. We found similar results when comparing BrdU-labelled cell density
(OIL4+VEH=1.20 ± 0.18 cells; EB4+VEH= 2.28 ± 0.34 cells; OIL4+NMDA=0.32 ± 0.40
cells; EB4+NMDA=1.47 ± 0.20 cells) between groups; estradiol increased (F_{1,17} = 21.17, p <
0.0003) and NMDA decreased (F_{1,17} = 11.96, p < 0.003) the density of labelled cells but no
interaction effect was observed (p ≤ 0.95). In fact, BrdU-labelled cell density was strongly
correlated positively with total BrdU-labelled cell number (r_{231} = 0.93; p ≤ 0.001). Neither the
total number of pyknotic cells (p ≥ 0.68; see Figure 9B) nor pyknotic cell density (p ≥ 0.79)
significantly differed between groups. The total area on which BrdU-labelled cells were
counted (granule cell layer + subgranular zone) was similar between groups (F_{1,17} = 0.70, p ≤
0.54; OIL4+VEH=2.51 ± 0.17 mm³; EB4+VEH= 2.63 ± 0.10 mm³; OIL4+NMDA=2.38 ± 0.09
mm³; EB4+NMDA=2.41 ± 0.18 mm³), verifying that differences observed between groups in
BrdU-labelled cell number were not volumetric.

Experiment 6. Relative to vehicle, estradiol significantly decreased and MK-801 significantly
increased cell proliferation but estradiol did not interact with NMDA receptors to alter cell
proliferation within 48 h.

Estradiol significantly decreased (main effect of hormone: F_{1,16} = 10.13, p ≤ 0.006) and
MK-801 significantly increased (main effect of drug: F_{1,16} = 36.37, p ≤ 0.0001) the number of
BrdU-labelled cells. Similar to Experiment 5, estradiol did not appear to stimulate NMDA
receptors within 48 h to influence cell proliferation because the hormone x drug interaction
effect was non-significant (p ≤ 0.80; Figure 10A). In fact, Figure 10A shows that estradiol
suppressed cell proliferation, both in the absence and presence of MK-801. BrdU-labelled cell
Figure 10. Mean (±SEM) number of new cells or pyknotic cells in the dentate gyrus of adult female voles injected with BrdU was administered 48 h after estradiol or oil and 1 h after MK-801 or vehicle in Experiment 6.

The white bar represents the data of OIL48+VEH females, the light gray bar represents the data of E48+VEH females, the dark gray bar represents the data of OIL48+MK-801 females and the black bar represents the data of E48+MK-801 females (n=5 per group). A) Mean number new (BrdU-ir) cells in the dentate gyrus of adult female voles. Relative to oil, estradiol significantly decreased ($p < 0.006$) and MK-801 significantly increased ($p < 0.0001$) the number of labelled cells in the dentate gyrus of adult female voles. Relative to oil, coadministration of estradiol and MK-801 tended to increase ($p < 0.06$) the number of labelled cells. B) Mean number of pyknotic cells in the dentate gyrus of adult female voles. The treatments did not influence pyknotic cell number.

** Newman-Keuls result $p<0.001$  * Newman-Keuls result $p<0.05$  a  Newman-Keuls result $p<0.06$
density differed similarly to BrdU-labelled cell number between groups (OIL48+VEH=1.56 ± 0.30 cells; EB48+VEH= 0.57 ± 0.09 cells; OIL48+MK801=3.89 ± 0.58 cells; EB48+MK801=2.86 ± 0.37 cells); estradiol decreased ($E(1,16) = 7.19$, $p \leq 0.016$) and MK-801 increased ($E(1,16) = 37.64$, $p \leq 0.0001$) the density of labelled cells but no significant interaction effect was observed ($p \leq 0.95$). In fact, BrdU-labelled cell density was strongly correlated positively with total BrdU-labelled cell number ($r_{(20)} = 0.94$; $p \leq 0.001$). Neither the total number of pyknotic cells ($p \geq 0.91$; see Figure 10B) nor pyknotic cell density ($p \geq 0.89$) significantly differed between groups. The total area that BrdU-labelled cells were counted on did not differ between groups ($E(1,16) = 1.17$, $p \leq 0.30$; OIL48+VEH=2.76 ± 0.16 mm$^3$; EB48+VEH= 2.45 ± 0.15 mm$^3$; OIL48+MK801=2.54 ± 0.17 mm$^3$; EB48+MK801=2.54 ± 0.06 mm$^3$), verifying that differences observed between groups in BrdU-labelled cell number were not related to volumetric differences.

4.4 DISCUSSION

These data demonstrate that estradiol dynamically influences cell proliferation in the dentate gyrus of adult female meadow voles but does not interact with NMDA receptors to mediate its effects on cell proliferation. Estradiol increased cell proliferation within 4 h but decreased cell proliferation within 48 h in the dentate gyri of OVXd adult female meadow voles, consistent with what we have reported previously in the dentate gyrus of adult female OVXd rats and intact meadow voles (Ormerod and Galea, 2001; Ormerod et al., 2003). In addition, NMDA receptor activation (via NMDA) suppressed and NMDA receptor blockade (via MK801) enhanced cell proliferation which extends previous findings showing that NMDA receptor activity regulates cell proliferation in the dentate gyri of adult rats and tree shrews (Cameron et al., 1994; Cameron et al., 1995; Gould et al., 1997; Bernabau and Sharp, 2000; Nacher et
Estradiol did not appear to influence NMDA receptors to alter cell proliferation at either the 4 h or 48 h time point because estradiol increased proliferation in the presence or absence of NMDA (within 4 h) and decreased proliferation in the absence or presence of MK-801 (within 48 h; see Figures 8A and 9A). Pyknotic cell number did not differ between groups and total pyknotic cell number was similar to what we have reported previously (Ormerod and Galea, 2001; Ormerod et al., 2003).

We believe that the differential effects of estradiol on cell proliferation observed within 4 versus 48 h of its administration are time- rather than dose-dependent. Serum estradiol levels are high 4 h after an estradiol injection, intermediate 48 h after an estradiol injection and low or undetectable following a vehicle injection but the number of labeled cells is elevated 4 h after an estradiol injection and suppressed 48 h after an estradiol injection relative to a vehicle injection (Ormerod and Galea, 2001; Ormerod et al., 2003). In addition, cell proliferation is suppressed 4 h after an estradiol injection (same dose used as the present study) in the dentate gyri of adult female rats exposed to low dose-estradiol (via silastic implant) for 1 week (Falconer and Galea, personal communication). Future work could verify whether the differential effect of estradiol on cell proliferation is time-dependent by keeping dose constant over 48 h.

*Estradiol suppresses cell proliferation by stimulating adrenal activity but not NMDA receptor activity*

Previous work has shown that estradiol increases cell proliferation in the dentate gyrus of adult rats by stimulating serotonin synthesis (Banasr et al., 2001) and suppresses cell proliferation partially by stimulating adrenal steroids in the adult rodent dentate gyrus (Ormerod et al., 2003). However, removing estradiol’s stimulatory effect on the HPA axis via
adrenalectomy eliminates but does not reverse the suppression in cell proliferation observed to occur in the dentate gyrus of adult female rats 48 h after an estradiol injection (Ormerod et al., 2003), suggesting that estradiol stimulates a factor, perhaps in addition to adrenal steroids, to suppress cell proliferation. Similar to effects shown previously in the dentate gyri of adult male rats (Cameron et al., 1994; Cameron et al., 1995; Gould et al., 1997; Bernabau and Sharp, 2000; Nacher et al., 2001), we found that NMDA receptor activation decreased and NMDA receptor blockade increased cell proliferation in adult female meadow voles. One other study, demonstrated that NMDA receptor blockade enhances cell proliferation in the dentate gyri of gonadally intact adult female rats (Nacher et al., 2003). Our results demonstrated similar effects in adult OVXd female meadow voles. However, NMDA receptor-mediated effects on cell proliferation observed in the current study occurred independently of estradiol’s effects on cell proliferation. This result was somewhat surprising given that estradiol administration for 48 h has also been shown to increase the sensitivity and number of NMDA receptors in the hippocampus of adult female rats (Weiland 1992; Gazzaley 1996) and NMDA receptors work downstream of corticosterone to suppress cell proliferation (Cameron et al., 1998). In fact, Cameron and colleagues (1998) postulated that another factor likely operates the suppressive corticosterone/NMDA receptor pathway because dividing progenitor cells do not appear to express glucocorticoid receptors (Cameron et al., 1993a). Because estradiol stimulates both the HPA axis and NMDA receptor activity, in theory, estradiol could have modulated cell proliferation through its effects of NMDA receptor activity.

Of course, we cannot completely dismiss that estradiol interacts with NMDA receptors. Perhaps the use of CGP43487, a longer-lasting NMDA receptor antagonist than MK-801 (Schmutz et al., 1990; Cameron et al., 1995) would have yielded different results. However, our finding that similar relative estradiol-induced increases (within 4 h) in cell proliferation in the presence or absence of NMDA and similar relative estradiol-induced decreases (within 48 h) in
presence or absence of MK-801 exist suggests that the effects we observed are straightforward. Within our experimental parameters estradiol did not work through NMDA receptors to influence cell proliferation. Although we found that estradiol and NMDA receptors influenced proliferation independently, the effects of co-administering estradiol+NMDA or estradiol+MK-801 on cell proliferation were additive. Remembering that the effects of stress (Gould et al., 1997, 1998; Tanapat et al., 2001; Holmes and Galea, 2002), some forms of learning (Gould et al., 1999), exercise (van Praag et al., 1999a,b) and hormones (Cameron and Gould, 1994; Cameron et al., 1998; Cameron and McKay, 1999; Tanapat et al., 1999; Banasr et al., 2001; Ormerod et al., 2003) can all influence cell proliferation, perhaps independently, is important as most non-laboratory-reared mammals likely experience all these phenomena daily. The effects could be additive or cancel one another out.

Estrogen could time-dependently influence cell proliferation in the dentate gyrus of adult rodents through numerous pathways.

Both known estrogen receptors (ER) subtypes ERα and ERβ are expressed in the hippocampus, including the subgranular zone (Shughrue et al., 1997; Weiland et al., 1997; Milner et al., 2001) and can activate numerous second messenger pathways as well as stimulate gene expression (Kawata, 1995; Beyer, 1999 for review). A recent study has shown that progenitor cells derived from the adult rat ventricular lining express ERβ and to a lesser extent ERα (Brännvall et al., 2002). In these progenitors, estradiol reversed an epidermal growth factor-stimulated increase in proliferation (Brännvall et al., 2002) suggesting that estradiol mediates differential effects upon adult-derived progenitor cells in vitro depending upon the presence of other factors. Of course, cells derived from the ventricular subependyma appear to
exhibit stem cell properties but the subgranular zone derived cells appear to exhibit the properties of more restricted progenitors (Seaberg and van der Kooy, 2002).

Interestingly, ER\(_{\alpha}\) is expressed at very low levels in the olfactory area of rats (Shughrue et al., 1997) and cell proliferation is elevated in subventricular zone and the rostral migratory stream (but not dentate gyrus) of adult female prairie voles 60 h after the onset of daily estradiol versus vehicle treatments (Smith et al., 2001). In addition, estradiol does not influence the proliferation of progenitors in the avian song circuit (Burek et al., 1995; Hidalgo et al., 1995; Loissant et al., 2002). Relative differences in the distribution of ER\(_{\alpha}\) and ER\(_{\beta}\) may produce differences in the effect of estradiol on cell proliferation in these systems. In addition, the interesting dichotomy between the effects of estradiol on cell proliferation in the dentate gyri of adult prairie versus meadow voles may reflect the well known differences in mating and affiliative systems between these two species (Dewsbury, 1987). Changes in cell proliferation within the dentate gyri of intact females support that estradiol alters cell proliferation dynamically.

Natural fluctuations in cell proliferation within the dentate gyri of intact adult female rodents also suggest that estradiol regulates the process time-dependently. For example, cell proliferation increases in the rat dentate gyrus during proestrus relative to diestrus or estrus but increases in the dentate gyrus of adult female meadow voles that are reproductively inactive versus active (Galea and McEwen, 1999; Tanapat et al., 1999; Ormerod and Galea, 2001). Whereas female rats experience elevated estradiol levels only on the afternoon of proestrus (Buckingham et al., 1978), female meadow voles experience elevated circulating estradiol levels that persist for up to 45 d only after ovulation is induced by male contact (Lee et al., 1970; Seabloom et al., 1985; Nubbemeyer, 1999). Tanapat and colleagues (1999) injected rats with BrdU at 2 pm, and therefore proestrus females would have likely been exposed to high
circulating estradiol for a few hours (see Buckingham et al., 1978). Ormerod and Galea (2001) injected females with BrdU 48 h after introducing a male or female cage partner and, therefore, reproductively active females would likely have been exposed to high circulating estradiol levels for approximately 30-36 h as estradiol levels rise between 12-16 h upon male contact (Lee et al., 1970; Seabloom et al., 1985; Nubbermeyer, 1999). Therefore, the duration of exposure that intact rats and meadow voles would be exposed to elevated circulating estradiol levels to increase (Tanapat et al., 1999) and then decrease (Ormerod and Galea, 2001) cell proliferation are similar to the durations found in this study to increase and then decrease cell proliferation in the dentate gyrus of adult OVXd female meadow voles.

In addition to influencing cell proliferation in the adult rodent dentate gyrus (Ormerod and Galea, 2001; Ormerod et al., 2003; current study), estradiol also has been shown to enhance the survival of young neurons. Estradiol enhances the survival of neurons migrating in the adult avian songbird forebrain (Burek et al., 1995; Hidalgo et al., 1995) by upregulating the production of brain-derived growth factor in endothelial cells (Loissant et al., 2002). We have found that when estradiol is administered 6-10 days, but not 1-5 or 11-15 days post BrdU, twice as many new neurons survive in the dentate gyrus of adult male meadow voles (Ormerod et al., 2002 and submitted for publication). Clearly, the observations that estradiol time-dependently influences cell proliferation as well as enhances the survival of young neurons could complicate the interpretation of results from studies utilizing intact female animals to investigate the effect of estradiol, or other factors, on neurogenesis.

Changes in cell proliferation within the dentate gyri of intact females support that estradiol alters cell proliferation dynamically

Approximately 270,000 new cells are produced monthly within the dentate gyri of adult rats and many of these cells differentiate into neurons (Cameron and McKay, 2001). These new
granule neurons extend axons to the CA3 region within 4-10 days after birth (Hastings and Gould, 1999) and resemble mature granule neurons electrophysiologically by 4 weeks after birth (van Praag et al., 2002). Therefore, young granule neurons could contribute rapidly to the influence that the dentate gyrus has over hippocampus activity and therefore, hippocampus-dependent behaviour. Because a large number of new granule neurons are added to the dentate gyrus monthly, the possibility that new granule neurons influence hippocampus-dependent behaviour seems plausible. In fact, Gould and colleagues (1999) found that more new neurons survive in the dentate gyrus of rats that engage in hippocampus-dependent (trace eyeblink conditioning and spatial Morris water maze acquisition trials) versus non-hippocampus-dependent (delay eyeblink conditioning and cued water maze trials) behaviour. Then, Shors and colleagues (2001, 2002) found that animals with depleted young neuron number following the administration of a cytostatic agent exhibited impaired performance on training trials in some hippocampus-dependent tasks (trace eyeblink conditioning and context conditioning) but not others (spatial Morris water maze training trials). We recently discovered that young granule neuron number in the dentate gyrus of adult male meadow voles is not related to performance on spatial Morris water maze training trials but increased young neuron number is related to improved performance on a probe trial (Ormerod et al., 2002 and submitted for publication). Because estradiol does not appear to alter the differentiation of new daughter cells produced in the dentate gyrus of adult rodents (60-70% neuronal differentiation; Tanapat et al., 1999; Ormerod et al., 2003; Ormerod et al., 2002), the estradiol-induced increase and then decrease in cell proliferation likely results in an increased and then decreased number of new granule neurons that are integrated into existing hippocampal circuitry and could influence hippocampus-dependent behaviour.

*New neurons appear functional and influence hippocampus-dependent behaviour*
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4.5 IMPLICATIONS

Estradiol diversely influences neurogenesis in the adult rodent dentate gyrus by first increasing and then decreasing progenitor cell proliferation as well as by enhancing the survival of young granule neurons. Estradiol can increase cell proliferation by stimulating serotonin activity and suppress cell proliferation by stimulating adrenal steroids (but not via NMDA receptors). Discovering the mechanisms by which estradiol mediates its diverse effects over the production and survival of new neurons in the dentate gyrus may facilitate our understanding of how to control the process to perhaps restore the neuronal loss associated with disease or trauma in the hippocampus and other areas of the adult central nervous system. Hippocampus-dependent behavior enhances the survival of young granule neurons and young granule neurons appear necessary for the successful performance of some hippocampus-dependent tasks. Estradiol-induced increases in neuron number are associated with improved retention in the Morris water maze in meadow voles. Clearly understanding estradiol’s role over adult neurogenesis could improve neuronal replacement strategies and assist the development of strategies that manipulate new neurons in situ. This study provides insight into the mechanism by which estradiol suppresses cell proliferation in the adult rodent dentate gyrus and demonstrates that estradiol robustly influences cell proliferation in the dentate gyri of rodents with diverse cycles (reflex ovulating voles and cycling rats) in a similar fashion.
CHAPTER 5

ESTRADIOL ENHANCES NEUROGENESIS BY INCREASING THE SURVIVAL OF YOUNG NEURONS AND THE INCREASE IS RELATED TO BETTER SPATIAL MEMORY IN ADULT MALE RODENTS

(submitted to Hippocampus)

5.1 INTRODUCTION

The discoveries that cells derived from the adult mammalian central nervous system (CNS) exhibit stem cell properties in culture (Reynolds and Weiss, 1992) and that many cells born within the neurogenic adult rat dentate gyrus express neuronal protein (Cameron et al., 1993) verified Altman’s (Altman and Das; 1965) early observation that new neurons are produced in the adult mammalian CNS. Eriksson and colleagues’ (1998) discovery that new granule neurons are produced in the adult human dentate gyrus fuelled extensive research into discovering how the production and survival of neurons is regulated in the adult mammalian CNS (see Kuhn et al., 2001 for review). Clearly, the ability to regulate and manipulate neurogenesis in neurogenic regions or to induce neurogenesis in non-neurogenic regions of the adult rodent central nervous system could lead to strategies for replacing neurons lost in diseases such as Alzheimers (West et al., 1994; Guela, 1998) or Parkinson’s (Uhl et al., 1985) to potentially promote restoration of function (for review see Kempermann and Gage, 1999).

Among the many factors that influence neurogenesis in the adult rodent hippocampus (Kuhn et al., 2001), estradiol has been shown to regulate neurogenesis dynamically by affecting both the production and possibly the survival of new granule neurons. For example, estradiol dramatically alters the rate of cell proliferation in the dentate gyrus of adult female rats. In the dentate gyrus of adult female rats and voles, the number of dividing progenitor cells increases following short-term (4 h) but decreases following longer-term (48 h) estradiol exposure (Ormerod and Galea, 2001; Ormerod et al., 2003). In the song circuit of adult canaries and
zebra finches, estradiol promotes the survival of new neurons as they differentiate and migrate to their final destinations (Hidalgo et al., 1995; Burek et al., 1995; 1997). Previous evidence from our laboratory suggests that estradiol also promotes the survival of new neurons born in the dentate gyrus of adult rodents. We found a higher proportion of new cells surviving 5 weeks versus 2 h in the dentate gyrus of adult female meadow voles with high versus low circulating estradiol (Ormerod and Galea, 2001). However, in that study, we did not directly manipulate cell survival as estradiol levels were different between the groups both before and after the injection of cell synthesis markers, and therefore cell proliferation as well as cell survival was affected. Treatments can influence cell proliferation (the division of progenitor cells) and/or the survival of young neurons and increasing either cell proliferation or survival could produce a net increase net neurogenesis (the number of neurons produced). In the present study, we directly tested whether estradiol could directly influence the survival of new neurons independent of its effects on cell proliferation.

Therefore, the first objective of this study was to determine whether estradiol specifically enhances the survival of new granule neurons in the dentate gyrus of adult male meadow voles. To this end, we injected adult male meadow voles with bromodeoxyuridine (BrdU) and waited 24 h before administering estradiol or vehicle as most progenitor cells complete mitosis within 24 h (Cameron and McKay, 2001). Because previous work with avian species (Hidalgo et al., 1995; Burek et al., 1997) has shown that estradiol enhances the survival of new migrating neurons, we tested whether new neurons born in the dentate gyrus of adult meadow voles were more vulnerable to the trophic effect of estradiol at various time points roughly associated with different stages of their maturation. Therefore, we injected voles with estradiol over three different 5 day periods after BrdU was administrated; either Days 1-5 (which coincide with the time that proteins associated with differentiation and migration begin to be expressed; Tanapat et al., 1999; Quinn et al., 1999; Cameron and McKay, 2001; Jin et al.,
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Days 6-10 (which coincide with axon extension; Hastings and Gould, 1999) and Days 11-15 (which coincide with the onset of the expression of mature neuronal protein; Cameron et al., 1993b; Kempermann et al., 1997; Tanapat et al., 1999). Based upon the work done by Hidalgo and colleagues (1995) and Burek and colleagues (1995; 1997) showing that more new migrating neurons survived in the avian forebrain of estradiol-treated songbirds, we expected that estradiol may increase the number of young neurons in the dentate gyrus of adult meadow voles treated with estradiol just after the administration of BrdU.

The functional consequences of changes in neuron number are just beginning to be explored. Recent work suggests that hippocampus-dependent behaviour influences the number and relies upon the presence of relatively young dentate gyrus granule neurons. For example, animals that engage in hippocampus-dependent Morris water maze place training or trace-eyeblink conditioning have more new granule neurons surviving in their dentate gyri than animals that engage in non-hippocampus-dependent visible platform training or delay-eyeblink conditioning (Gould et al., 1999; Ambrogini et al., 2000). Moreover, Shors and colleagues (2001) found that when progenitor cell proliferation is reduced 80%, by the administration of a cytostatic agent over 14 days but not over 6 days just prior to training, trace- but not delay-eyeblink conditioning is impaired. However, recent work by Shors and colleagues (2002) showed that performance on training trials in the Morris water maze is not affected when progenitor cell proliferation is reduced by the same treatment, suggesting that young granule neurons influence learning in some but not all hippocampus-dependent tasks. These studies suggest that hippocampus-dependent behaviour increases the number of young granule neurons and that hippocampus-dependent behaviour is impaired by a significant reduction in the number of 1-2 week old neurons. In rats, estradiol has been shown to influence both neurogenesis in the dentate gyrus (Galea and McEwen, 1999; Tanapat et al., 1999; Ormerod and Galea, 2001; Ormerod et al., 2003) and learning and memory (Galea et al., 2001; Holmes
et al., 2002). Therefore, estradiol may produce some of its effects on hippocampus-dependent behaviour by influencing neurogenesis. Our second objective, therefore, was to determine whether any estradiol-induced change in the number of young neurons influences hippocampus-dependent behaviour. To test whether estradiol-induced changes in neuron number influence hippocampus-dependent behaviour, we observed the performance of animals that had been treated with either estradiol or vehicle over Days 6-10 post BrdU (with hormone treatment ending 6 days prior to training) in the standard Morris water maze place task.

We found that estradiol increased the number of young neurons surviving in the dentate gyrus of adult male meadow voles, but only when administered over Days 6-10 after BrdU when the young neurons are 6-10 days old (presumably in the process of extending axons; Hastings and Gould, 1999). Furthermore during a probe trial (on Day 20 after BrdU administration), voles treated with estradiol over Days 6-10, spent significantly more time in the quadrant of the Morris water maze that had previously housed the platform than vehicle-treated males. The results suggest that spatial memory is improved by an increased number of young neurons.

5.2 METHODS

All animals were treated in strict accordance with the guidelines set forth by the Canadian Council on Animal Care and The University of British Columbia regarding the ethical treatment of animals used for the purposes of research. Every effort was made to minimise the number of animals used per group and their suffering.

Animals
We used adult male meadow voles (n=45; >35 g and 60 d old) reared in our breeding colony at The University of British Columbia as subjects. The voles were bred and reared in polyurethane bedding-lined (Carefresh; Absorption Corporation) cages that contained plastic and cardboard containers. At 21 days of age, the voles were weaned and housed either with same sex siblings or individually (if no sibling of the same sex existed) until 60 days of age when they were all housed individually. The colony room was temperature controlled (21±1°C) colony room and the light:dark cycle was set at 16:8 h (lights on at 0700h). The voles had free access to tap water and Lab Diet #5012 (Jamieson) for the duration of the experiment and were given sunflower seed/alfalfa pellet supplements once per week.

**Surgery**

At 70-80 d old, male meadow voles (5-6 per group) were castrated, using sterile surgical techniques. First animals were put into a chamber to which halothane was delivered at a flow rate of 5% (flow rate of O₂ was 2%) until anaesthesia was visibly induced. Then animals were transferred to a nose cup to which halothane was delivered initially at a flow rate of 3% but was reduced to as low as 1% to maintain a stable respiratory rate. A small incision was made through each scrotal sac to extrude the testis. Each vas deferens was then tied off using surgical silk and the testis was surgically removed before the scrotal sac was sutured. Animals recovered for one week prior to the onset of each experiment.

**Drug preparation**

The cell synthesis marker bromodeoxyuridine (BrdU; Sigma Aldrich Chemicals), a marker of dividing cells (Nowakowski et al., 1989), was prepared just prior to injection. BrdU was dissolved to a concentration of 10 mg/ml freshly prepared 0.9% saline (buffered with 7μl
2N NaOH/ml saline) and injected intraperitoneally in a volume of 0.5ml/100 g body weight (50 mg/kg). This dose is typically used to investigate neurogenesis in mice (Kempermann et al., 1997; Kempermann et al., 1998; Kempermann and Gage, 2002) and meadow voles (Ormerod and Galea, 2001; Smith et al., 2001; Fowler et al., 2002). Estradiol (17-β estradiol benzoate; Sigma Aldrich Chemicals) was dissolved over low heat in sesame oil (Sigma Aldrich Chemicals) to a concentration of 10μg estradiol/0.5μl sesame oil. Once dissolved, the estradiol solution was stored in a light insensitive container. Subcutaneous injections of the estradiol solution or sesame oil vehicle were given in a volume of 0.5μl.

Procedure

Experiment 7 was conducted to investigate whether estradiol influences the survival of new neurons. Because previous work in canaries has shown that estradiol improves the survival of new neurons within the song circuit as they migrate (Hidalgo et al., 1995; Burek et al., 1997), we were interested in determining whether young neurons in adult male meadow vole dentate gyrus were more vulnerable to the trophic effect of estradiol at different stages of their maturation. To test the effect of estradiol on the survival of new neurons, voles were injected twice with BrdU on Day 0, once at 0800 h and then again at 1000 h. We then waited 24 h after the second injection as that amount of time allows progenitor cells that have incorporated BrdU to complete one mitotic division (Cameron and McKay, 2001). To test whether new (BrdU containing) cells were more vulnerable to the trophic effect of estradiol at different stages of their maturation, voles were injected with either vehicle or estradiol for 5 consecutive days either over Days 1-5 (V1-5 and E1-5; n=5 and n=6, respectively), Days 6-10 (V6-10 and E6-10; n=6 and n=5, respectively), or Days 11-15 (V11-15 and E11-15; n=5 and n=6, respectively). These time periods were chosen to roughly correspond with the onset of migration and differentiation.
(Days 1-5; Tanapat et al., 1999; Quinn et al., 1999; Cameron and McKay, 2001; Jin et al., 2001), to roughly correspond with the time that new cells extend axons (Days 6-10; Hastings and Gould, 1999) or to roughly correspond with maturation (Days 11-15; Cameron et al., 1993b; Kempermann et al., 1997; Tanapat et al., 1999) of new granule neurons. All animals were perfused on Day 16 post-BrdU administration. All animals were perfused on Day 16 so that differences in new neuron number between groups could be compared independent of differences that would likely occur due to time-dependent naturally occurring cell death observed to occur over time (Cameron et al., 1993b; Cameron and McKay, 2001). The time line of the experiment is depicted in Figure 11.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 0 (BrdU)</th>
<th>Days 1-5 (estradiol or vehicle injections)</th>
<th>Days 6-10 (estradiol or vehicle injections)</th>
<th>Days 11-15 (estradiol or vehicle injections)</th>
<th>Day 16 (Perfuse)</th>
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<tbody>
<tr>
<td>V1-5</td>
<td></td>
<td>✓ ✓</td>
<td>✓ ✓ ✓ ✓</td>
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<td>✓</td>
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<td>E1-5</td>
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<tr>
<td>V6-10</td>
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<td>✓ ✓</td>
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<td>E6-10</td>
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<td>E11-15</td>
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</table>

Figure 11. Timeline of Experiment 7.
Voles were injected with either vehicle or estradiol either over Days 1-5 (V1-5 and E1-5; n=5 and n=6, respectively), Days 6-10 (V6-10 and E6-10; n=6 and n=5, respectively), or Days 11-15 (V11-15 and E11-15; n=5 and n=6, respectively) to test whether new (BrdU containing) cells were more vulnerable to the trophic effect of estradiol at different stages of their maturation. All voles were perfused on Day 16.

Experiment 8 was conducted to investigate whether an estradiol-induced increase in the number of new granule neurons influences hippocampus-dependent behaviour. Previous work has shown that adult male rats that engage in water maze place training (standard Morris water maze training) have more new neurons than males that engage in a cued version of the task (Gould et al., 1999) when training occurs 7-10 days after BrdU is administered (i.e. when the new neurons are 7-10 days old). Because the place version of the task relies upon the presence
of an intact hippocampus whereas the cued version does not (Morris et al., 1982), the results of that study suggest that the survival of young neurons is influenced by hippocampus-dependent behaviour. Furthermore, Shors and colleagues (2001) found that rats treated with the cytostatic agent methylafoxymethanol (MAM) for 14 days but not 6 days prior to training exhibited impaired trace-eyeblink and trace fear conditioning but not impaired Morris water maze acquisition or contextual fear conditioning. Interestingly, in Experiment 7, we found that estradiol increases the number of young neurons in the dentate gyrus of adult male meadow voles when administered Days 6-10 after their birth (after BrdU was injected). Thus, we used the same injection paradigm to investigate whether the estradiol-induced increased number of new neurons influences learning and/or retention in the standard Morris water maze task. We injected voles with BrdU twice on Day 0, once at 0800 h and again at 1000h and then with either estradiol (E6-10; n=6) or vehicle (V6-10; n=6) over Days 6-10 post BrdU. Voles then began Morris water maze training hormone free on Day 16-post BrdU (6 days after the last estradiol or vehicle injection). All animals were perfused on Day 24 after the completion of Morris water maze training. The time line of this experiment is depicted in Figure 12.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 0 (BrdU)</th>
<th>Days 1-5 (Estradiol or Veh)</th>
<th>Days 6-10 (Estradiol or Veh)</th>
<th>Days 11-15 (Estradiol or Veh)</th>
<th>Day 16 (MWM)</th>
</tr>
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<tbody>
<tr>
<td>V6-10</td>
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<td>E6-10</td>
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**Figure 12. Timeline of Experiment 8.**

Voles were injected twice with BrdU on Day 0, once at 0800 h and again at 1000h and then with either estradiol (E6-10; n=6) or vehicle (V6-10; n=6) over Days 6-10 post BrdU. This was done to test the effect of changes in young neuron number on hippocampus-dependent behaviour. Voles then began Morris water maze training drug free on Day 16-post BrdU (6 days after the last EB or vehicle injection).

**Morris water maze training**

All animals in Experiment 8 were trained in the standard Morris water maze task (Morris et al., 1982; Morris, 1984). The maze (90cm diameter x 45cm height) was filled with
water (20±1°C) that was rendered opaque with Tempera nontoxic white paint (Demco) to a height of (30cm). The maze was divided into quadrants and four equally spaced release points were designated N, E, S and W. A platform (7 cm diameter) was hidden 1 cm beneath the water in the centre of the northwest quadrant. Cues (camera, coloured cardboard symbols, television, door, etc) were visible throughout the room. The first day of training consisted of a probe trial, in which swim speed was assessed in absence of the platform. Twenty-four hours after the first probe trial, the voles were given four training trials per day over 4 days, in which they were given 60 s to swim to the platform hidden in the northwest quadrant. If the voles did not find the platform in the allotted time, they were placed on the platform for 15 s (ITI 60s). Over training trials, latency and path-length to reach the platform and average swim speed were assessed. On the fifth day of behavioral testing, a probe session was administered in which the platform was removed from the maze and percent time spent in each quadrant was assessed. Then, the following day the platform was moved to the southwest (opposite) quadrant and the voles were given four reversal-learning trials. Behaviour in the water maze was videotaped and then analyzed with an HVS tracking system (HVS Image, Hampton, UK). Blocks were the average of four trials.

**Histological procedures**

On Day 16 (Experiment 7), voles were anaesthetized with sodium pentobarbital and then perfused with 4% paraformaldehyde. Their brains were extracted and stored overnight in perfusate at 4°C. The following day, 40μm sections were sliced through the entire dentate gyrus with an oscillating tissue slicer (Leica OTS1000) in 0.1M phosphate buffer (PB). Sections were incubated in a 0.2% H₂O₂ PB solution for 20 min and then rinsed before being
mounted on slides treated with a solution of 3% 3-aminopropyl-triethoxysilane (Sigma Aldrich Chemicals) in acetone. Sections were dried overnight then processed immunohistochemically.

Peroxidase immunohistochemistry

Peroxidase immunohistochemistry was performed as described previously to stereologically estimate total BrdU-labelled cell number on slide-mounted tissue (Cameron et al., 1993b; Gould et al., 1999; Tanapat et al., 1999; Ormerod and Galea, 2001; Ormerod et al., 2003). Sections were rinsed repeatedly in phosphate-buffered saline (PBS; pH 7.4) between steps. Cells were permeabilized using a solution of 0.05% trypsin (Sigma Aldrich Chemicals) in TRIS-buffered saline for 10 min and DNA was denatured by incubating tissue in 2N HCl for 30 min at 37°C. Nonspecific antigens were then blocked by incubating the tissue in a solution of 5% normal horse serum (NHS; Vector Laboratories) in PBS (PBS+) for 20 min and then the tissue was incubated overnight in mouse monoclonal anti-BrdU (Boehringer Mannheim; 1:400). The next day, the tissue was rinsed repeatedly and then incubated in mouse secondary antisera (Vector Laboratories; 1:129) in PBS+ for 4 h and then in avidin-biotin horseradish peroxidase complex (ABC Elite; Vector Laboratories) for 1 h. Finally, tissue was reacted for 10 min in 0.2% 3,3'-diaminobenzidine (Sigma Aldrich Chemicals) TRIS-buffered saline and counterstained with cresyl violet (Baker) to assess the number of pyknotic cells before being coverslipped with permount.

Immunofluorescent labelling

Immunofluorescent labelling was done on slide-mounted tissue to verify the phenotype of BrdU-labelled cells as described previously (Gould et al., 1999; van Praag et al., 1999a; van Praag et al., 2002; Ormerod et al., 2003). Sections were rinsed in TRIS-buffered saline (TBS) between steps. Sections were incubated in deionized formamide for 2 h at 65°C and DNA was
Figure 13. Microphotographs of cells analyzed in Experiment 7.
A) A clump of BrdU-labelled cells in the dentate gyrus of a male meadow vole injected with estradiol over Days 6-10. B) A pyknotic cell in the dentate gyrus of an animal treated with vehicle. C) Neurons (immunoreactive for Cy3 conjugated anti-NeuN) are shown in red and a new cell (immunoreactive for FITC conjugated anti-BrdU) is shown in green. The arrow points to a new neuron (immunoreactive for both anti-NeuN and anti-BrdU). No significant difference in the number of new cells expressing NeuN was observed between groups (p>0.80). D) Glia (immunoreactive for Cy3 conjugated anti-GFAP) are shown in red and a new cell (immunoreactive for anti-BrdU conjugated with FITC) is shown in green. The arrow points to a new glia (immunoreactive for both anti-GFAP and anti-BrdU). No significant difference was observed between groups in the number of new cells that expressed GFAP (p>0.38).
denatured in 2N HCl at 37° for 30 min. Sections were blocked in 5% normal donkey serum (NDS; Jackson immunoresearch) in TBS (TBS⁺) for 30 min and then overnight in a cocktail of rat anti-BrdU (ascites 1:100; Oxford Biochemical Incorporated), rabbit monoclonal neuron specific enolase (NSE; 1:2000; Santa Cruz) and mouse monoclonal anti-glia fibrillary acidic protein (GFAP; 1:2000; Novacastra) in TBS⁺ or rat anti-BrdU and mouse anti-GFAP or rat anti-BrdU and mouse anti-neuronal nuclei (NeuN; Chemicon). NSE and NeuN label mature neurons (Cameron et al., 1993b; van Praag et al., 1999; Palmer et al., 2000) and GFAP labels glial cells (Debus et al., 1983; Gould et al., 1999; Smith et al., 2001). The following day, the sections were rinsed and blocked in 5% NDS and then for 4 h in a cocktail of donkey anti-rat fluorescein (FITC; to visualize BrdU), donkey anti-rabbit Cy5 (to visualize NSE) and donkey anti-mouse Cy3 (to visualize GFAP or NeuN; all 6 μl/ml) in TBS⁺. Slides were rinsed and then coverslipped with the anti-fading agent diazobicyclooctane.

Data analyses

All slides were coded to blind the experimenter from the treatment conditions. On peroxidase-treated tissue, BrdU-ir and pyknotic cells were counted on every 10th section through the subgranular zone (∼ the 50 μm band between the granule cell layer and the hilus; Palmer et al., 2000) and granule cell layer using a 100x objective on a Nikon Eclipse E600 light microscope. Cells were considered BrdU-labelled if they were intensely stained and exhibited medium round or oval cell body morphology and pyknotic if they lacked a nuclear membrane and had condensed chromatin (Cameron et al., 1993b; Ormerod and Galea, 2001; Figure 13A and 13B). Areas counted were measured using the digitizing software Analytical Software Imaging Station (Imaging Research, Brock University) so that the total number of BrdU-ir and pyknotic cells that would be present throughout the complete dentate gyrus could be estimated.
using a modified version of the optical fractionator method (West et al., 1991). We estimated the total number of BrdU-labelled cells using a modified version of the optical fractionator method as outlined in Ormerod, Lee and Galea (2003). Areas were obtained using the digitizing software Analytical Software Imaging Station (Imaging Research, Brock University, Ontario, Canada) and dentate gyrus volume estimates were made using Cavalieri’s principle (Gunderson et al., 1988). Because we have previously reported BrdU-ir cell densities (Galea and McEwen, 1999; Ormerod and Galea, 2001; Ormerod et al., 2003) we also calculated BrdU-ir cell densities (# of cells/area) on 6 anatomically matched sections per rat (where the dentate gyrus lies just beneath the corpus callosum and the infrapyramidal and suprapyramidal blades are joined at the crest; between A -3.3 and A -4.8 in rats) in order to compare the density of BrdU-ir cells with stereological estimates of total BrdU-ir cells in the dentate gyrus. BrdU-ir cell phenotypes were analyzed on fluorescent probe-treated tissue. Twenty-five BrdU-labelled cells on 4-6 sections per animal (n=3 per group) were identified using a Zeiss fluorescent microscope and their phenotype analyzed using a confocal laser scanning head (BioRad 2000) with UV (red diode), green HeNe and argon lasers. Z-sections at 0.4 μm were taken and optical stacks of 10 images were created with NIH image for PC so that cells could be rotated in orthogonal planes to verify double labelling. Neurons were double-labelled with BrdU+NSE or BrdU+NeuN (Figure 13C) and glia were double-labelled with BrdU+GFAP (Figure 13D). NIH images were imported into Adobe Photoshop for channel merging and digital manipulations were restricted to contrast enhancements and color level adjustments.

Statistical analyses

In Experiment 7, the dependent variables (number of BrdU-labelled cells, pyknotic cells, density of both BrdU-labelled and pyknotic cells, and percentage of BrdU-
labelled/GFAP-ir, BrdU-labelled/NSE-ir or BrdU-labelled/NeuN-ir labelled cells) were each analyzed using an analysis of variance (ANOVA) with condition (vehicle, E1.5, E6-10 and E11-15) as the between-subjects factor. Pearson product-moment correlations were conducted to assess the relationship between BrdU-labelled cell number and density. In Experiment 8, the dependent variables (latency, path-length and average swim-speed to reach the platform) were each analyzed using repeated-measures ANOVAs with condition (vehicle, E6-10) as the between-subjects factor and session (1, 2, 3, 4) or quadrant (training, adjacent 1, adjacent 2, opposite) as the within-subjects factor. Post-hoc analyses utilized the Newman-Keul’s procedure.

5.3 RESULTS

Experiment 7. Estradiol promotes the survival of new neurons

The number of labelled 16-day old BrdU-labelled cells did not differ between the V1-5, V6-10 and V11-15 groups (p > 0.86) and therefore the data for these groups were collapsed into one group (Vehicle) for subsequent analyses. Relative to vehicle, estradiol increased the total number of 16 d-old BrdU-labelled cells (F(3,29) = 4.55; p < 0.01) when administered over Days 6-10 (p ≤ 0.009) but not over either Days 1-5 (p > 0.41) or Days 11-15 (p > 0.70; see Figure 14A). Similarly, estradiol increased the density of BrdU-labelled cells but only when administered over Days 6-10 (4.82 ± 1.58; p > 0.01) and not Days 1-5 (2.46 ± 0.34; p > 0.72) or Days 11-15 (1.45 ± 0.32; p > 0.41) relative to vehicle (2.16 ± 0.23; F(3,29) = 4.96; p ≤ 0.007).

Our measures of total number and density of BrdU-labelled cells were highly positively correlated (r(33) = 0.91; p ≤ 0.001).
Figure 14. Stereological estimates of BrdU-labelled and pyknotic cells in the dentate gyrus of adult male meadow voles following estradiol or vehicle treatment in Experiment 7.

The white bars depict the data of voles treated with vehicle over Days 1-5, 6-10 or 11-15 after BrdU (data were collapsed as the number of BrdU-labelled cells did not statistically differ between groups). The grey bars depict the data of voles treated with estradiol over Days 1-5, 6-10 or 11-15 after BrdU was injected. A) Stereological estimate of total BrdU-labelled cell number in the granule cell layer of male voles. Relative to vehicle, estradiol nearly doubled the number of BrdU-labelled cells in the dentate gyrus of adult male meadow voles but only when administered over Days 6-10 after BrdU was injected (p<0.009). B) Stereological estimate of total pyknotic cell number in the granule cell layer of adult male meadow voles. More pyknotic cells were found in the granule cell layer of voles treated with estradiol over Days 1-5 (p<0.06) or Days 6-10 (p<0.03) than vehicle-treated voles. **denotes p<0.01 *denotes p<0.05 †denotes 0.10>p<0.05

There were no group differences in dentate gyrus volume indicating that the difference in labelled cell number between estradiol- and vehicle-treated voles was not related to a volumetric difference ($F_{(3,29)} = 0.56; p's \geq 0.60$; see Table 10).

Table 10. Mean ($\pm$SEM) dentate gyrus volume in vehicle- and estradiol-treated adult male meadow voles in Experiment 7.

<table>
<thead>
<tr>
<th>Group</th>
<th>Volume (mm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (n=16)</td>
<td>2.34±0.95</td>
</tr>
<tr>
<td>E$_{1-5}$ (n=6)</td>
<td>2.44±0.14</td>
</tr>
<tr>
<td>E$_{6-10}$ (n=5)</td>
<td>2.37±0.10</td>
</tr>
<tr>
<td>E$_{11-15}$ (n=6)</td>
<td>2.53±0.75</td>
</tr>
</tbody>
</table>

Dentate gyrus volume did not significantly differ between groups.
The majority of 16 day-old BrdU-labelled cells expressed the mature neuronal protein, NSE (≈ 60%) or NeuN (≈ 66%) and this percentage was consistent across groups ($F_{(3,8)} = 0.034; p > 0.99$ and $F_{(3,8)} = 0.033; p > 0.80$, respectively; see Table 11). Fewer BrdU-labelled cells expressed the glial marker GFAP (≈ 16%) and this percentage was similar between groups ($F_{(3,8)} = 1.17; p > 0.38$; see Table 11).

### Table 11. Mean (±SEM) % BrdU-ir cells expressing a neuronal (NSE- or NeuN-ir) or glial (GFAP-ir) phenotype measured 16 d after BrdU was injected did not significantly differ in adult male meadow voles treated with estradiol or vehicle in Experiment 7.

<table>
<thead>
<tr>
<th>Group</th>
<th>NSE-ir ±SEM</th>
<th>NeuN-ir ±SEM</th>
<th>GFAP-ir ±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (n=16)</td>
<td>59.2±3.2</td>
<td>66.2±2.5</td>
<td>16.0±2.3</td>
</tr>
<tr>
<td>E$_{1-5}$ (n=6)</td>
<td>60.0±2.3</td>
<td>63.3±4.1</td>
<td>18.7±2.7</td>
</tr>
<tr>
<td>E$_{6-10}$ (n=5)</td>
<td>60.3±3.2</td>
<td>67.3±1.8</td>
<td>12.1±1.3</td>
</tr>
<tr>
<td>E$_{11-15}$ (n=6)</td>
<td>58.7±1.3</td>
<td>59.2±1.3</td>
<td>17.3±1.3</td>
</tr>
</tbody>
</table>

The total number of pyknotic cells tended to differ between vehicle- and estradiol-treated groups ($F_{(3,29)} = 2.45; p > 0.08$; see Figure 14B). Because previous work has shown that high estradiol levels are associated with lower number of pyknotic cells (Tanapat et al., 1999) and because we observed differences in the number of new (16 d old) neurons in the dentate gyri of estradiol-treated animals (see above), we planned a priori to compare the number of pyknotic cells between the vehicle-treated group and each of the estradiol-treated groups. Relative to vehicle, estradiol reduced the number of pyknotic cells when administered either over Days 1-5 (p < 0.06) or over Days 6-10 (p < 0.03) but not when administered over Days 11-15 (p > 0.17; see Figure 14B).

**Experiment 8. Estradiol-induced changes in young neuron number did not influence performance on water maze acquisition trials**

Both groups exhibited similar swim speeds ($t_{(10)} = 1.68; p ≥ 0.12$) and pathlengths ($t_{(10)} = 0.98; p ≥ 0.35$) on a baseline trial (Day 16) in which the platform was absent from the pool (data not
shown). Over training trials (Days 16-19), we observed that average latency for each session was significantly positively correlated with the respective pathlength for each session ($r$'s $(11) \geq 0.80; p$'s $\leq 0.002$). We found that latency to reach the platform significantly decreased across sessions (main effect of session: $F(3,27) = 8.86; p \leq 0.00003$) in both groups (main effect of condition: $p \geq 0.55$) and that performance did not differ between groups across session (session by group interaction $p \geq 0.90$; see Figure 15A). Newman Keuls post hoc tests revealed that both groups exhibited significantly shorter average latencies during training sessions 2, 3 and 4 relative to session 1 ($p$'s $\leq 0.002$). Similarly, the distance traveled to reach the platform (pathlength) decreased across training sessions (main effect of session: $F(3,27) = 15.04; p \geq 0.00002$) in both groups (main effect of condition: $p \geq 0.82$) and that pathlength did not differ between groups across sessions (condition by session interaction: $p \geq 0.72$; see Fig. 15B). No difference in swim speed between groups was observed across training sessions ($p \geq 0.96$).

**Estradiol-induced changes in neuron number did influence performance on a water maze retention trial**

On probe trial performance (Day 20), a ANOVA on percent time spent each quadrant revealed a significant main effect of quadrant ($F(1,10) = 112.75; p \leq 0.001$) and a significant interaction effect of condition and quadrant (interaction $F(1,10) = 8.16; p \leq 0.03$). Newman-Keuls comparisons revealed estradiol-treated voles ($E_6$-$10$) spent significantly more time there than did vehicle-treated voles ($p \leq 0.015$) in the quadrant that had previously held the platform (Figure 16). Neither total pathlength ($p \geq 0.96$) nor average swim speed ($p \geq 0.92$) varied between groups on probe trial performance. To ensure that the observation that estradiol-treated males more time spent in the training quadrant than vehicle-treated males was better explained by improved memory versus perseveration, we administered reversal trials in which the
Figure 15. Performance of vehicle- versus estradiol-treated meadow voles in training trials of the Morris water maze in Experiment 8.
In both graphs, white circles depict the drug-free performance of voles treated with vehicle over Days 6-10 and black circles depict the data of voles treated with estradiol over the same period. A) Latency did not differ between groups over training trials. Note that both groups acquired the task at the same rate and to the same degree. B) Pathlength was highly correlated with latency (p<0.002) and demonstrates that both groups acquired the task at the same rate and to the same degree.
platform was hidden in the opposite quadrant to that of the training trials one day after the probe trial. Neither a significant main effect of condition ($p > 0.45$; data not shown) nor a condition by trial interaction ($p > 0.29$) was observed, suggesting that both groups learned the new location of the hidden platform at the same rate.

Figure 16. Performance of vehicle- versus estradiol-treated meadow voles on a probe trial in the Morris water maze in Experiment 8.

White bars depict the drug-free performance of voles treated with vehicle over Days 6-10 and black bars depict the data of voles treated with estradiol over the same period. Estradiol-treated voles spent significantly more time in the training quadrant than did vehicle-treated voles ($p<0.015$).

5.4 DISCUSSION

We designed our study to specifically test whether estradiol influences the survival of new neurons, independent of any effect on the proliferation of progenitor cells by administering our
treatment at a time when progenitor cells would have completed mitosis. We found that relative to vehicle, estradiol approximately doubled the number of 16 d old neurons in the dentate gyrus of adult male meadow voles but only when administered over Days 6-10 after the new neurons are born. Estradiol did not influence the differentiation of new daughter cells (approximately 60% differentiated into neurons across groups) but enhanced neurogenesis by increasing the number of young neurons that survived 16 d in the dentate gyrus of adult meadow voles. Furthermore, estradiol-treated voles outperformed vehicle-treated voles on a probe trial in the Morris water maze despite both groups acquiring the task and the post-probe trial reversal training trials at the same rate. Because the voles were trained and tested hormone free in the Morris water the improved retention trial performance observed could be related to the estradiol-induced increase in young neuron number. Our results show that estradiol does indeed promote the survival of young neurons born in the dentate gyrus of adult meadow voles and that this change in neuron number did not influence learning, but was related to improved hippocampus-dependent memory.

Estradiol influences the survival of neurons in various systems

This study shows that estradiol influences the survival of new granule neurons. Relative to vehicle, estradiol approximately doubles the number of new granule neurons surviving 16 days in the dentate gyrus of adult male meadow voles. This is consistent with our previous work showing that the ratio of new cells surviving 5 weeks relative to 2 h was higher in the dentate gyrus of adult female meadow voles with chronically high versus low endogenous estradiol levels (Ormerod and Galea, 2001). However in the previous study, estradiol level was related to rates of cell proliferation as more dividing progenitors were observed in the dentate gyri of the low estradiol females compared to high estradiol females 2 h after BrdU was injected. Because females with chronically high estradiol had more cells surviving 5 weeks,
despite having fewer new cells produced initially, estradiol likely enhanced the survival of young cells in that study. In the current study all animals presumably had the same number of proliferating progenitor cells at the time of BrdU injection (they were castrated and housed in standard conditions) and no treatment was administered until at least 24 h after BrdU was injected. Because progenitor cells complete division in approximately 24 h (Cameron and McKay, 2001), we can conclude that in this study, estradiol enhanced neurogenesis by increasing the survival of new granule neurons independent of an increase in the number of progenitor cells undergoing mitosis.

Estradiol enhances the survival but not production of new neurons in the higher vocal centre (HVC) of the adult songbird forebrain (Hidalgo et al., 1995; Burek et al., 1995; Burek et al., 1997). Recent work has shown elegantly that estradiol induces angiogenesis in the adult songbird HVC and by upregulating the expression of vascular endothelial growth factor (VEGF), its receptor VEGF-R2/Quek1 and induces brain-derived neurotrophic factor (BDNF) expression in endothelial cells which promotes the survival of migrating neurons (Loissant et al., 2002). Estradiol could influence the survival of new granule neurons produced in the dentate gyrus of adult voles via a similar mechanism. In the hippocampus of adult rats and voles, BDNF and its receptor TrkB is widely expressed (Barbacid, 1994; Lindsay et al., 1994; McAllister et al., 1999; Liu et al., 2001) and estradiol significantly increases BDNF mRNA expression most prominently in the dentate gyrus (Gibbs, 1998; Liu et al., 2001). Palmer and colleagues (2000) have shown that angiogenesis and neurogenesis occur concurrently in the dentate gyrus of adult female rats substantiating that estradiol could enhance the survival of new neurons produced there in the same manner observed in the avian HVC. Future work could investigate the link between angiogenesis/neurogenesis to determine whether estradiol upregulates BDNF in endothelial cells to influence the survival of new cells in neurogenic regions of the adult mammalian central nervous system.
Interestingly, the survival-promoting effect of estradiol in male meadow voles is temporally discrete because the number of new granule neurons is increased when estradiol is administered 6-10 days but not 1-5 days or 11-15 days after the neurons are produced. Hastings and Gould (1999) have shown that young granule neurons extend their axons between 4 and 10 days after birth. Thus, the time when new granule neurons are extending their axons is coincident with the time that these new neurons appear receptive to estradiol’s survival-promoting effect. Developmentally, the estrogen receptor subtype $\alpha$ is expressed transiently in various brain areas including the hippocampus thereby promoting neuronal survival and growth as well as neurite extension (Kawata, 1995; Beyer, 1999). Moreover, during development estradiol enhances the activity of survival promoting neurotrophins and most prominently, BDNF and its receptor TrkB mRNA in the dentate gyrus (McAllister et al., 1999; Gibbs, 1998; Davies, 1994). Both BDNF and TrkB are expressed developmentally at times associated with maximal neuronal growth, differentiation and synaptogenesis (Davies et al., 1994). Thus, estradiol could enhance the survival of new granule neurons by providing trophic support, possibly by either increasing growth factor production in the microenvironment of new granule neurons or by inducing the expression of growth factor receptors in new neurons.

In addition to increasing neuronal survival via growth factors, estradiol has also been shown to prevent the death of hippocampal neurons both in vitro and in vivo. Estradiol rescues cultured hippocampal neurons from oxidative stress-, excitotoxicity- and $\beta$-amyloid toxicity-induced death (Goodman et al., 1996; Green et al., 1996) and decreases cell death in the hippocampus of rats that undergo experimentally induced ischemia and excitotoxicity (Hall et al., 1991; Simpkins et al., 1997; Dubal et al., 1998; Azcoitia et al., 1999). Thus, estradiol could have increased the number of 16-day old neurons in the present study by decreasing the expression of proteins associated with cell death. For example, estradiol has been shown to
increase the expression of negative cell death regulators such as Bcl-2 in adult neurons \textit{in vivo} (Garcia-Segura et al., 1998) and Bcl-XL in cultured hippocampal neurons (Pike, 1999). In fact, the bcl-2 promoter contains several putative estrogen response elements with which estradiol could directly interact to influence transcription (Teixeira et al., 1995). Our results do suggest that estradiol influenced the death of young neurons. First, we found that the number of pyknotic cells was significantly lower in voles treated with estradiol on either Days 1-5 or Days 6-10 days after BrdU injection (but not Days 11-15) suggesting that prolonged (5 d) exposure to estradiol reduces the number of dying cells and that the effects may be somewhat delayed and temporally discrete. Second, although we did not note any BrdU-labelled pyknotic cells, we did note that many of the pyknotic cells were located near or in the subgranular zone that harbours presumably young neurons (Wang et al., 1999; van Praag et al., 2002).

\textit{Estradiol regulates different components of neurogenesis that occurs in adulthood}

In addition to influencing the survival of new granule neurons, estradiol dynamically regulates the production of new neurons in the adult rodent dentate gyrus (Tanapat et al., 1999; Galea and McEwen, 1999; Ormerod and Galea, 2001; Ormerod et al., 2003). Specifically, more new neurons are produced in the dentate gyrus of adult female rats on the day of proestrus (when estradiol levels are highest) than on the days of estrus or diestrus and an ovariectomy-induced decrease in the production of new neurons can be reversed by estradiol administration (Tanapat et al., 1999). We found previously that while short-term (4 hour) exposure to estradiol increases the number of new neurons produced longer exposure (48 hour) decreases the number of new neurons produced in the dentate gyrus of both adult female meadow voles and rats and that the decrease is due, at least in part, to estradiol-stimulated adrenal activity (Ormerod and Galea, 2001; Ormerod et al., in press). More new cells are observed in the dentate gyrus of reproductively inactive (low estradiol) versus reproductively active (high estradiol) adult
female meadow voles (Galea and McEwen, 1999; Ormerod and Galea, 2001). Interestingly, the number of BrdU-labelled cells observed is elevated in the rostral migratory stream (but not dentate gyrus) of adult female prairie voles with high versus low estrogen levels (Smith et al., 2001; Fowler et al., 2002). Prairie voles and meadow voles distinctly differ in their affiliative behaviour, spatial behaviour including space use in their native environments and hippocampal volume, indicating that species-specific changes in neurogenesis may support species-specific behaviors. Therefore, estradiol dynamically influences neurogenesis in the dentate gyrus of adult rodents by first increasing and then decreasing (via adrenal steroids) the production of new granule neurons and by increasing their survival. Intriguingly, estradiol influences the survival of new neurons in the avian song circuit and the rodent hippocampus and influences the production of new neurons in the rodent hippocampus but not the avian song circuit (Hidalgo et al., 1995, Burek et al, 1995; 1997; Tanapat et al., 1999; Ormerod et al., 2003; Loissant et al., 2002). Comparative studies investigating differences in the localization of estradiol-induced mitogenic factors between the species may provide insight into the mechanisms that control the proliferation of adult neural stem cells in general.

New neurons have functional characteristics

New neurons are produced in the dentate gyrus throughout adulthood in all mammalian species studied (see Ormerod and Galea, 2001 for review) including humans (Eriksson et al., 1998). In fact, several thousand new cells are produced daily in the dentate gyrus of adult rats (Cameron and McKay, 2001). Many of the new cells produced within the dentate gyrus extend axons through the mossy fibre pathway within 4-10 days to the CA3 region (Stanford and Trice, 1988; Markakis and Gage, 1999; Hastings and Gould, 1999), express immature and then mature neuronal markers (Cameron et al., 1993b; Palmer et al., 2002; Tanapat et al., 1999; for example) and acquire synapses on their dendrites and soma (Kaplan and Hinds, 1977; Kaplan
and Bell, 1984). Electrophysiological studies in rats have shown that presumably young
granule neurons with cell bodies located near the neurogenic subgranular zone exhibit greater
plasticity than presumably older neurons located deeper in the granule cell layer (Wang et al.,
2000). Recent work using a GFP-tagged retrovirus to birth-date granule neurons produced in
the dentate gyrus of mice found electrophysiological responses typical of mature granule
neurons 4 weeks after infection and a mature granule neuron morphology (dendritic complexity
and similar spine counts) 4 months after infection (van Praag et al., 2002). Taken together, this
evidence suggests that new granule neurons could rapidly influence and be influenced by
activity within the hippocampus.

Most attention has been focused upon the role of the hippocampus in spatial processing,
although evidence suggests a role for the hippocampus in declarative memory (Cohen and
Eichenbaum, 1993; Bunsey and Eichenbaum, 1996), working memory (Olton, 1983), stimulus-
stimulus learning (McDonald and White, 1993), and data-based memory (Kesner, 1998).
Destruction of the hippocampal formation impairs rats' performances in the spatial (platform
hidden) but not the nonspatial version (platform visible) of the Morris water maze (Kelsey and
Landry, 1988; Morris et al., 1982; Morris et al., 1990; Schenk and Morris, 1985; Taube et al.,
1992) and in spatial versions of the radial arm maze (Olton and Samuelson, 1976; Jarrard,
1983; Jarrard et al., 1984). Indeed, the performance of rats with fairly specific granule neuron
depletion is impaired on hippocampus-dependent tasks (Sutherland et al., 1983; Whishaw et al.,
1987; McLamb et al., 1988; McNaughton et al., 1989; Nanry et al., 1989; Armstrong et al.,
1993; Conrad and Roy, 1993; Schuster et al., 1997). Recently, Xavier and colleagues (1999)
reported that fairly complete and specific colchicine-induced granule neuron destruction
severely impaired performance across acquisition trials and on a probe trial in the standard
Morris water maze task.
Neurogenesis and hippocampus-dependent behaviour in adulthood

A few studies have shown hippocampus-dependent behaviour can increase young neuron number and that a change in young neuron number can alter the ability of rats to learn some hippocampus-dependent tasks. Gould and colleagues (1999) found that rats trained in trace eye-blink conditioning trials or standard Morris water maze training trials had more new (7-14 day old) dentate gyrus granule neurons (but not subventricular zone-produced neurons) relative to rats trained in delay eye-blink conditioning or cued water maze training trials. Both standard Morris water maze training and trace eyeblink-conditioning requires an intact hippocampus whereas cued water maze training and delay eyeblink-conditioning do not (Morris et al., 1982; Solomon et al., 1986; Moyer et al., 1990; Clark and Squire, 1998). Another study found some evidence to suggest that rats exhibiting good performance in the Morris water maze have more young neurons surviving than rats exhibiting poorer performance, although some rats trained on the cued platform version of the task had as many new cells as the good learners (Ambrogini et al., 2000). Further evidence that new neurons participate in successful hippocampus-dependent performance has been derived from studies utilizing the cytostatic agent methylazoxymethanol (MAM) to halt the proliferation of progenitor (and other) cells. MAM treatment for 14 days (but not 6 days) prior to training impairs trace eyeblink conditioning but delay eyeblink conditioning (Shors et al., 2001). Recent work has shown that MAM administration impairs performance on another associative task, trace fear conditioning, but does not influence performance on Morris water maze acquisition trials, contextual fear conditioning trials or on elevated plus maze exploration (Shors et al., 2002). Taken together, this evidence suggests that more young neurons are found in the dentate gyri of animals that engage in hippocampus-dependent learning and that young neurons participate in
hippocampus-dependent associative tasks but not necessarily acquisition of spatial information used for navigation.

Interspecies comparisons have also shown that baseline neurogenesis and performance in hippocampus-dependent tasks are related. Strains of mice with high levels of baseline neurogenesis (C57BL/6) outperform strains with low levels (129/SvJ and DBA/2, for example) on standard Morris water maze training trials (Kempermann et al., 1997; Kempermann and Gage, 2002). Interestingly, seasonal changes in baseline neurogenesis may relate to seasonal changes in spatial ability. Adult female meadow voles have higher rates of cell proliferation, establish greater territory sizes in their natural habitats and exhibit better performance across Morris water maze training sessions during the non-breeding compared to the breeding season (Sheridan and Tamarin, 1988; Galea et al., 1995; Galea and McEwen, 1999; Ormerod and Galea, 2001). Combined, this evidence suggests high-level baseline neurogenesis is associated with good performance across training trials in the Morris water maze and low-level baseline neurogenesis is associated with poorer performance in the task.

Here, we show that an increase in young neuron number does not influence performance over acquisition trials but does improve performance in the probe trial in the Morris water maze. Voles injected with estradiol over Days 6-10 post-BrdU (but were trained and tested hormone free) exhibit better memory for the maze location of the maze that previously housed the platform during probe trials than vehicle-treated males, despite similar rates of learning on training trials. Importantly, both groups of animals exhibited asymptotic performance across training trials indicating that they had learned the task to the same degree, before the probe test was administered validating the effect on retention that we observed. Many interpretations of probe trial performance have been proposed. For example, the probe test has been proposed to measure either perseverance (or behavioural inflexibility; Eichenbaum et al., 1990; 1992) or strength of spatial learning (or retention; Whishaw, 1985).
Because we observed no difference between groups on reversal training trials, the idea that the
estradiol-induced increased number of young granule neurons is associated with improved
spatial memory is supported. Our observation that performance between estradiol- and vehicle-
treated meadow voles across training trials complements the work of Shors and colleagues
(2002) showing unimpaired performance across training trials in the Morris water maze in rats
when the number of young neurons is drastically reduced. Both lines of evidence suggest that
new granule neurons are not necessary for successful performance across acquisition trials of
the Morris water maze. However, our data demonstrate that an increased number of young
neurons can improve performance on water maze probe trials. These data are interesting given
that granule neurons appear necessary for precise spatial search strategy in the Morris water
maze (Xavier et al., 1999) and young granule neurons appear to be especially plastic (Wang et
al, 1999). In summary, we show that relative to vehicle, estradiol increases the survival of new
neurons and the estradiol-induced increase in new neuron number are related to improved
memory in the Morris water maze.

5.5 IMPLICATIONS

We, and others, have shown that estradiol regulates the production (by increasing and
then decreasing progenitor cell proliferation) and survival of new granule neurons in the rodent
dentate gyrus during adulthood. Our understanding of how to control neurogenesis may be
facilitated by discovering the mechanisms by which estradiol dynamically regulates
neurogenesis within the adult rodent dentate gyrus. Furthermore, previous work has shown that
some forms of hippocampus-dependent learning requires the presence of young granule
neurons (Shors et al., 2001, 2002) and increases the survival of young granule neurons (Gould
et al., 1999; Ambrogini et al., 2000). This study shows that an increased number of new
neurons improves a facet of hippocampus-dependent behaviour; memory for a platform
position in the standard Morris water maze task. The present study adds to our understanding of how estradiol influences components of neurogenesis in the adult mammalian hippocampus and how changes in neuron number influence features of hippocampus-dependent behaviour. By discovering the mechanisms by which estradiol mediates its influence upon different facets of adult mammalian neurogenesis, insight about how to control the process may be achieved.
CHAPTER 6
GENERAL DISCUSSION

Perhaps because the field of adult stem cell biology is young, the effects of a single factor upon different components of neurogenesis have been relatively unexplored. However, to argue that a factor influences neurogenesis, its cumulative effects upon progenitor cell proliferation, daughter cell differentiation and survival of young neurons should be determined to ascertain if that factor actually does alter net neurogenesis. Combined, the experiments in this thesis take the approach of investigating the effects of estradiol on cell proliferation, the differentiation of daughter cells and the survival of young granule neurons in the dentate gyrus of adult rodents. Estradiol first increased (within 4 h) and then decreased (within 48 h), partially by stimulating adrenal activity, cell proliferation in the dentate gyri of adult female rats and meadow voles. Estradiol did not alter the differentiation of daughter cells. Estradiol enhanced the survival of young neurons in the dentate gyri of adult male meadow voles and the estradiol-induced increase in young neuron number was related to improved retention (but not learning) in the spatial Morris water maze task. A general summary of the experimental results is presented first and then their implications for understanding the role of neurogenesis in normal hippocampus-dependent function as well as their potential for neuronal replacement strategies are discussed.

Reproductive status and estradiol influence cell proliferation

Reproductive status influenced cell proliferation in the dentate gyri of adult laboratory-reared female meadow voles. Chapter 2 revealed that reproductively inactive females (with low estradiol levels) had significantly more proliferating cells in their dentate gyri than reproductively active females (with high estradiol levels; Tables 1 and 3). This finding
replicated Galea and McEwen's (1999) work showing that cell proliferation is elevated in the dentate gyri of non-breeding wild adult female meadow voles (with low estradiol levels) relative to breeding wild females (with high estradiol levels). Importantly, the potentially confounding variables of age and activity level (Kuhn et al., 1996; Seki and Arai, 1995; Montaron et al., 1998; Cameron and McKay, 1999; van Praag 1999a; 1999b) were controlled in the laboratory-reared sample to confirm that the seasonal variable influencing cell proliferation in the dentate gyri of adult female meadow voles is reproductive status. In both studies, the finding that serum estradiol level was correlated negatively with labelled cell density suggests that estradiol level mediated the effect of reproductive status on cell proliferation in both samples (Chapter 2 and Galea and McEwen, 1999). In fact, the effect of shifting reproductive status from inactive to active could be mimicked by exposing reproductively inactive females to estradiol for 48 h (Table 1).

In fact, Chapter 2 suggested a novel dynamic effect of estradiol on cell proliferation in the adult rodent dentate gyrus. Although the density of proliferating (BrdU-labelled) cells in the dentate gyri of adult gonadally intact adult female meadow voles decreased within 48 h, it increased within 4 h of a single injection of estradiol, relative to the density of proliferating cells observed in the dentate gyri of reproductively inactive females. The data of Chapters 3 and 4 verified that estradiol differentially influences cell proliferation in the dentate gyri of adult female rodents. The total number of proliferating (BrdU-labelled cells) cells in the dentate gyri of ovariectomized adult female rats (Chapter 3; Figure 4) and meadow voles (Chapter 4; Figure 9) increases within 4 h but decreases within 48 h of a single injection of estradiol versus vehicle. Chapter 4 demonstrated that estradiol-induced increases in other ovarian hormones such as progesterone (see Nubbemeyer, 1999) did not contribute to the changes in labelled cell number observed after 4 versus 48 h of exposure to estradiol in intact voles, because the voles in Chapter 4 were ovariectomized. Importantly, Chapters 2, 3 and 4 combined demonstrate that
the differential effects of estradiol on dentate cell proliferation is robust across species (voles and rats) despite different methods used to analyse cell proliferation (labelled cell density versus stereological estimate of total labelled cell number).

The differential effects of estradiol on cell proliferation in the adult rodent dentate gyrus appeared to be duration- rather than dose-dependent. Serum estradiol levels in rats exposed to estradiol for 48 h were intermediate to those of rats exposed to estradiol for 4 h and to vehicle (Chapter 2; Table 6). In addition, intact female voles exposed to estradiol for 48 h had serum estradiol levels intermediate to females exposed to estradiol for 4 h and reproductively inactive females (Chapter 2; Table 3). If the differential effect of estradiol were dose-dependent, then I would expect that the number/density of proliferating cells in the dentate gyri of female rats and voles exposed to estradiol for 48 h to be intermediate between those found in the dentate gyri of females exposed to estradiol for 4 h and to vehicle/no injection. Of course, this hypothesis could be directly tested in an experiment designed to keep estradiol dose constant while varying duration. For example, if BrdU-labelled cell number increased following one but then decreased following two daily injections of estradiol versus vehicle, then effect could clearly be interpreted as duration-dependent. In fact, a recent study in our laboratory demonstrated that prolonged exposure to estradiol suppresses cell proliferation in the dentate gyri of adult female rats.

Regardless, the differential effects of estradiol on cell proliferation reported in this thesis are consistent with the findings of other studies. Cell proliferation increases in the dentate gyri of adult female rats and voles exposed to high-level estradiol for short durations (2-4 h) before BrdU is injected (Tanapat et al., 1999; Banasr et al., 2001; Chapters 2, 3 and 4). Similarly, estradiol levels rise on the afternoon of proestrus in rats (Buckingham et al., 1978) and cell proliferation increases in the dentate gyri of rats that are injected with BrdU on the afternoon of their proestrous phase (Tanapat et al., 1999). Cell proliferation decreases in the dentate gyri of
adult female rats and voles exposed to high-level estradiol for longer durations (48 h) before BrdU is injected (Chapters 2, 3 and 4). Most adult female meadow voles trapped during the breeding season exhibit signs of behavioural estrus (perforated vagina and pregnancy; Galea and McEwen, 1999) indicating that their estradiol levels must have risen at least 12-16 h before capture (Lee et al., 1970). These voles have reduced rates of cell proliferation in their dentate gyri relative to females trapped during the breeding season (Galea and McEwen, 1999). Taken together, published studies investigating the relationship between estradiol and neurogenesis support the findings of this thesis that short-term exposure to estradiol increases and longer-term exposure to estradiol decreases cell proliferation in the dentate gyri of adult rodents.

_Estradiol partially suppresses cell proliferation by stimulating adrenal activity_

Estradiol increases cell proliferation in the dentate gyri of adult female rats by increasing serotonin synthesis (Banasr et al., 2001) and Chapter 3 demonstrated that estradiol stimulates adrenal activity to suppress cell proliferation in the dentate gyri of adult female rats, because adrenalectomy eliminated the suppression in cell proliferation observed 48 h after estradiol was administered (Figure 5). Adrenalectomy would also likely eliminate or reverse the estradiol-induced suppression in cell proliferation in the dentate gyri of adult female meadow voles because estradiol treatment for 48 h (but not 4 h) increases their adrenal masses (Table 2) and suppresses cell proliferation (Table 1), and future work could examine this possibility. Because the estradiol-induced suppression in cell proliferation in the dentate gyri of adult rats was eliminated but not reversed by adrenalectomy, I hypothesized that estradiol may work through a factor in addition to adrenal steroids to suppress cell proliferation within 48 h.

The hypothesis that estradiol may stimulate NMDA receptors to influence cell proliferation within either 4 or 48 h was formulated from evidence showing that NMDA receptors work downstream from adrenal steroids to suppress cell proliferation (Cameron et al.,
1998) and that estradiol can increase both the sensitivity and number of NMDA receptors (Weiland, 1992; Gazzaley et al., 1996). In essence, estradiol should potentiate the effect of an NMDA receptor agonist-induced decrease and antagonist-induced increase on cell proliferation in the rodent dentate gyrus, if this hypothesis was correct. To expand upon the findings of previous work showing that NMDA receptor activation suppresses and NMDA receptor blockade enhances cell proliferation in the dentate gyri of adult rats and tree shrews (Cameron et al, 1994; Cameron et al., 1995; Gould et al., 1997; Bernabau and Sharp, 2000; Nacher et al., 2001; Nacher et al., 2003), I tested this hypothesis using adult ovariectomized female meadow voles as subjects. As expected, estradiol enhanced cell proliferation within 4 h and suppressed cell proliferation within 48 h in the dentate gyri of adult ovariectomized voles. Consistent with the effects reported in other species, NMDA receptor activation suppressed and NMDA receptor blockade enhanced cell proliferation in the dentate gyri of adult female meadow voles. However, estradiol did not appear to interact with NMDA receptors either following 4 or 48 h of exposure to influence cell proliferation because estradiol enhanced cell proliferation and then suppressed cell proliferation despite the presence of NMDA or MK-801, respectively.

This result was surprising because the results of Experiment 4 (Chapter 3) had shown that estradiol stimulated adrenal activity to suppress cell proliferation in the adult rat dentate gyrus and previous work had shown that NMDA receptor activation works downstream of adrenal steroids to suppress cell proliferation in the dentate gyri of adult rats (Cameron et al., 1998). In the Cameron et al. (1998) study the effects of low-level (via adrenalectomy) or high-level adrenal steroids (corticosterone injection) were blocked by NMDA receptor activation or inactivation, respectively (Cameron et al., 1998). To my knowledge, the distributions of NMDA receptors and NMDA receptor subunit sequences have not been investigated in the meadow vole and the genes encoding NMDA receptor subunits are known to vary between species (Eriksson et al., 2002; Andersson et al., 2001 for example). The expression patterns of
arginine vasopressin and oxytocin receptors are known to vary drastically vole species (Insel and Shapiro, 1992; Insel, Wang & Ferris, 1994) and NMDA receptor distribution patterns could differ between rats and voles. Nonetheless, the finding that NMDA and MK-801 decreased and increased cell proliferation in the dentate gyri of adult meadow voles similarly to what has been reported in adult rats, suggests that NMDA receptor distribution and phenotype is similar between the two species and that estradiol simply does not stimulate NMDA receptor activity to suppress cell proliferation within 48 h. Interestingly, in the paraventricular nucleus of the hypothalamus, estradiol-induced downregulation of ERα expression returns to control levels 48 h after estradiol is injected (Paul Shughrue, personal communication). We are currently investigating whether estradiol mediates effects on cell proliferation in the adult rodent dentate gyrus via ERs.

Estradiol did not influence the differentiation of daughter cells

Estradiol did not influence the differentiation of daughter cells when administered prior to pulse of BrdU. In Experiment 3 (Chapter 3), regardless of whether ovariectomized female rats were exposed to estradiol or vehicle, 60-70% of the BrdU-labelled cells expressed the neuronal marker DCX, 25-30% expressed the neuronal marker TUC-4 and 20-25% expressed the glial marker GFAP by 4 d BrdU injection (Table 8). TUC-4-ir or GFAP-ir was observed in more 4-day old versus 24-h old BrdU-labelled cells but the percentages did not vary with treatment (either estradiol or vehicle exposure for 4 or 48 h; Table 5). These data are consistent with those of Tanapat and her colleagues (1999) who found that 60-70% of the labelled cells in the dentate gyri of adult rats injected with BrdU in either proestrus or estrus expressed TUC-4 or calbindin and approximately 15-20% expressed GFAP between 4 d and 3 weeks after birth.
There are differences in TUC-4-ir BrdU-labelled cells reported in Chapter 3 and by Tanapat et al. (1999) and these differences likely reflect the use of a commercially available (Chemicon) versus homemade (Hockfield laboratory) antibody raised against TUC-4 in the respective studies. The commercially available antibody appears lower in affinity for either TUC-4 or the fluorescent probe-conjugated secondary antibody used to detect it under confocal microscopy than the homemade antibody (Brandi Ormerod, unpublished data; Clive Svendsson personal communication; Martin Wojtowicz, personal communication). The important point is that the percentage of BrdU-labelled cells either expressing a neuronal (DBX and TUC-4) or glial marker (GFAP) was unaffected by treatment in both studies (hormone treatment, duration of hormone treatment, estrus cycle stage). Estradiol also did not influence the differentiation of labelled cells when administered over either Days 1-5, 6-10 or 11-15 after a pulse of BrdU. Chapter 5 demonstrated that 55-60% of BrdU-labelled cells expressed the neuronal marker NSE, 60-70% expressed the neuronal marker NeuN and 12-20% expressed GFAP (Table 11) in the dentate gyri of adult males meadow voles treated with estradiol or vehicle.

The finding that estradiol did not influence the differentiation of daughter cells in the adult rodent hippocampus is interesting because estradiol influences differentiation during development, particularly of cells located in sexually dimorphic nucleii (see Kawata, 1995; Toran-Allerand, 1996; Beyer, 1999 for review). In line with this observation, in vitro evidence suggests that estradiol may influence the differentiation of cells derived from the developing versus adult rodent nervous system differently. Brännvall and her colleagues (2002) found that the proportion of adult neural progenitor derived BrdU-labelled cells that expressed either the neuronal marker β-tubulin versus GFAP did not differ in media containing estradiol versus vehicle but the proportion of embryonic neural progenitor derived BrdU-labelled cells expressed β-tubulin versus GFAP increased upon exposure to estadiol. In their culture system,
estradiol promoted a neuronal fate among daughter cells ‘developmentally’ but not during ‘adulthood’. The potential and phenotype of stem cells is known to change developmentally, likely because of intrinsic changes in the responsiveness of stem cells to growth factors (Spradling et al. 2001, Weissman et al., 2001) and adult stem/progenitor cells could become insensitive to estradiol as a differentiative cue in this manner.

Interestingly, adrenal steroids did not appear to influence the differentiation of daughter cells (Chapter 3). The percentage of BrdU-labelled cells that expressed TUC-4 and GFAP did not differ in the dentate gyrus of adrenal intact (Table 5) and adrenalectomized (Table 9) adult female rats despite differences in circulating corticosterone levels (Table 6). This result is incongruent with the findings of Cameron and McKay (1999) who reported that a significantly higher percentage of BrdU-labelled cells in the dentate gyri of aged adrenalectomized male rats expressed TUC-4 than in the dentate gyri of aged adrenal intact male rats. The same low dose of corticosterone was added to the drinking water of adrenalectomized rats in both studies to prevent cell death and the rats were adrenalectomized one week prior to BrdU injection in both studies, suggesting that the age or sex of the rats used may account for the differences reported. Basal corticosterone levels do increase in aged rats (Sapolsky, 1992) and ligand-bound glucocorticoid and mineralocorticoid receptors are known transcription factors (see Meijer, 2002 for review). Determining the changes in potential and phenotype of adult neural progenitor cells not only between development and adulthood, but across adulthood would be interesting given that adrenal steroids appear to influence the differentiation of cells in the aged but not young adult rodent dentate gyrus. In theory, prolonged exposure to elevated glucocorticoid levels could alter transcription of factors that influence the intrinsic responsiveness of progenitor cells to extrinsic cues guiding differentiation in aged or even between male and female rats.
**Estradiol enhances the survival of young granule neurons**

The experiments described in Chapter 2 suggested that estradiol enhances the number of new cells that survive in the dentate gyri of adult female meadow voles. The proportion of $[^3]$H]thymidine-labelled cells that survived 5 weeks relative to the number of BrdU-labelled cells observed at 2 h was higher in the dentate gyri of reproductively active versus inactive females (Table 4 versus Table 1). Because reproductive status and therefore serum estradiol level differed between the groups prior to and after the administration of cell synthesis marker in each experiment and serum estradiol remains elevated for at least 22 days in reproductively active voles (Seabloom, 1985), both cell proliferation and the survival of new cells were likely affected in Chapter 2. Therefore, Experiment 7 (Chapter 5) was designed to specifically investigate the effect of estradiol on the survival of young granule neurons by injecting BrdU at least 24 h (the time required for adult progenitor cells to complete 1 mitotic division; Cameron and McKay, 1999) before treating the animals with estradiol or vehicle. This design revealed that estradiol approximately doubles the number of 16 day-old neurons in the dentate gyri of adult male meadow voles but only when administered over Days 6-10 (but not Days 1-5 or Days 11-15) post BrdU label. I have since replicated this finding in adult female meadow voles, although the effect appears more robust and not as temporally discrete in females versus males (Figure 17). The more robust and temporally less discrete estradiol-induced enhancement of survival among young granule neurons in the dentate gyri of adult female versus male meadow voles could reflect dimorphisms in the expression of estrogen or other receptors in the adult male and female rodent central nervous system. The number of ER$\alpha$-ir cells is similar in the hippocampus of gonadectomized adult male and female rats (Weiland et al., 1997). However, sex differences in ER$\beta$ expression that have not yet been investigated in the adult rodent hippocampus could account for the differential effect of estradiol on granule neuron survival.
Figure 17. Stereological estimates of BrdU-labelled cells in the dentate gyrus of adult female meadow voles following the administration of estradiol or vehicle.
The white bar depicts the data of female voles treated with vehicle over Days 1-5, 6-10 or 11-15 after BrdU (data were collapsed as the number of BrdU-labelled cells in vehicle groups did not statistically differ). The gray bars depict the data of voles treated with estradiol either over either Days 1-5, 6-10 or 11-15 after BrdU was injected. Relative to vehicle, estradiol increased the number of BrdU-labelled cells that expressed a neuronal protein (NSE or NeuN) in the dentate gyrus of adult female meadow voles when administered over Days 11-15 post BrdU and tended to increase neuron number when administered over Days 1-5 and Days 6-10. ** denotes p<0.05 observed in the dentate gyri of adult male versus female meadow voles. Estrogen increases the survival of migrating neurons in the adult avian songcircuit and in vitro work suggests that estradiol mediates this effect by increasing BDNF expression in endothelial cells (Hidalgo et al., 1995; Loissant et al., 2002). Interestingly, neurogenesis in the adult rodent dentate gyrus is associated with angiogenesis (Palmer et al., 2000). Whether angiogenesis is specifically related to increased proliferation or the survival of young neurons in the adult rat dentate gyrus has not been investigated but estradiol could promote cell survival similarly in the adult rat and avian CNS. Estradiol enhances BDNF expression in the hippocampus of adult female rats, most prominently in the dentate gyrus and in the olfactory bulbs of adult female prairie voles and (Gibbs, 1998; Smith et al., 2001). BDNF increases the survival of adult subventricular zone-derived cells both in vivo and in vitro (Kirschenbaum and Goldman, 1995; Zigova et al., 1996).
Future work could investigate whether estradiol enhances the expression of BDNF to promote the survival of young granule neurons and whether differences in either ERβ, BDNF and/or the BDNF receptor trkA in the dentate gyri of female versus male rodents is related to the robustness and temporal discreteness of this effect.

Estradiol could increase the number of 16-day old neurons in the dentate gyri of adult meadow voles by either providing trophic support or by inducing the expression of antiapoptotic factors to reduce cell death. Potential mechanisms by which estradiol could mediate either effect are described in detail in Chapter 5.

Net neurogenesis and behaviour in adult voles

Chapter 2 showed that although a greater proportion of new cells survived 5 weeks versus 2 h in the dentate gyri of adult reproductively active (with high estradiol levels) versus inactive (with low estradiol levels) meadow voles, the number cells at both time frames was higher in the dentate gyri of inactive versus active females. This finding suggests that net neurogenesis is higher in the dentate gyri of reproductively inactive versus active females. Kempermann and his colleagues (Kempermann et al., 1997; Kempermann and Gage, 2002) have shown that strains of mice with high net neurogenesis (C57BL/6) outperform strains with low net neurogenesis (129/SvJ and DBA/2, for example) on standard Morris water maze trials. Interestingly, Galea and her colleagues (1995) have shown that reproductively inactive female meadow voles (with high net neurogenesis; Chapter 2) outperform reproductively active females (with low net neurogenesis; Chapter 2) on standard Morris water maze training trials. Seasonal changes in net neurogenesis could influence performance on this hippocampus-dependent task.
Conclusive evidence that changes in net neurogenesis influence the performance of adult female meadow voles would require dissociating estradiol's effects on neurogenesis from its effects on learning (Galea et al., 2001; Holmes et al., 2002). Nevertheless, because the reproductive status of adult voles is seasonally regulated and their behaviour in the field has been studied relatively thoroughly, speculation about the influence of new neurons in the dentate gyri of adult meadow voles on normal hippocampus function is tempting. Evidence from previous studies suggests reproductive status-induced changes in net neurogenesis could alter spatial performance in adult female meadow voles despite reproductive status-induced changes in estradiol. Male exposure increases serum estradiol and induces ovulation in another vole species, the prairie vole (Cohen-Parsons and Carter, 1987; Smith et al., 2001).

Interestingly two studies have shown that, when trapped during the breeding season, adult male meadow voles outperform females (with high-level estradiol) on a spatial land maze task but adult male and female (with high-level estradiol) prairie voles exhibit similar performance on the same task (Gaulin and Fitzgerald, 1989; Gaulin et al., 1990). Interestingly, net neurogenesis is diminished in the dentate gyri of reproductively active adult female meadow voles (Chapter 2) but Fowler and her colleagues (2002) have shown that cell proliferation and the survival of new cells is similar in the dentate gyri of reproductively active and inactive adult female prairie voles. Combined, these studies suggest that net neurogenesis in the dentate gyri of adult female voles could influence spatial performance independent of changing estradiol levels.

Chapter 5 demonstrated that estradiol-induced increases in young dentate granule neuron number in the dentate gyri of adult male meadow voles are related to improved retention (but not learning) in the Morris water maze. In these voles, cell proliferation was similar in estradiol- and vehicle-treated voles because BrdU was administered prior to the onset of treatment. Thus, net neurogenesis was enhanced in estradiol- versus vehicle-treated voles because estradiol enhanced the survival of young neurons. These results are consistent with
previous work showing that net neurogenesis is higher in the dentate gyri of adult reproductively active versus inactive male meadow voles and the effect is due to enhanced survival (Ormerod and Galea, in press). Male meadow voles increase their home range sizes during the breeding season to encompass many female ranges within their own (Madison, 1980, 1985; Gaulin and Fitzgerald, 1989). Whether reproductively active males have better retention in the field has not been tested, but a seemingly good strategy for a polygamous breeder like the meadow vole would be to remember where he’s been so that he finds a receptive rather than pregnant female. In addition, male meadow voles compete aggressively for females during the breeding season and remembering where another dominant male’s territory is could prevent wounding that would diminish reproductive fitness (Clarke, 1956; Turner and Iverson, 1978). Future work could verify that the seasonal changes in net neurogenesis that occur in the dentate gyri of male and female meadow voles are related to seasonal changes in spatial ability measured in the laboratory and space use in the field.

Of course, theories about the functional role of neurogenesis constitutive to the adult CNS are currently speculative. On one hand, Alvarado and Newmark (1998) believe the phenomenon to be a “remnant of evolution from more primitive organisms like planaria or fish in which organ renewal affords an advantage in adverse environments”. On the other hand, Gage (2000) has argued that specific CNS regions retained the capacity to produce neurons because new neurons assist normal functioning, like learning and memory in the case of the hippocampus. Structural similarities among the CNS areas in which neurogenesis occurs postnatally could be taken as evidence that adult neurogenesis is a vestigial phenomenon. For example, new neurons are only added in significant numbers postnatally to the granule cell layers the olfactory bulbs, dentate gyrus and for a short time the cerebellum (2-3 weeks; Lois and Alvarez-Buylla, 1993; Cameron et al., 1993; Cameron and McKay, 2001; Altman and Bayer, 1997). These areas are archicortical, or the evolutionarily oldest mammalian cortex
(Reiner, 1993). Nonetheless, new granule neurons produced in the dentate gyri of adult rodent electrophysiologically resemble mature granule neurons (Wang et al., 2000; Snyder et al., 2002; van Praag et al., 2002), are influenced by hippocampus-dependent learning (Gould et al., 1999) and participate in some forms of hippocampus-dependent learning (Shors et al., 2001, 2002; Chapter 5), suggesting that they assist normal hippocampus function. In fact, the theories described by Alvarado and Newmark and by Gage are not mutually exclusive. A trait can evolve and persist after speciation because it is still adaptive in the descendent species. For example, penguins are birds that cannot fly but that swim exceedingly well because of their wings.

_Estradiol-induced changes in components of neurogenesis and their implications for neuronal replacement_

Neurogenesis occurs constitutively in the dentate gyri of both humans and rodents (Eriksson et al., 1998; Cameron et al., 1993; Experiments 1-7). Therefore, discovering the mechanisms by which neurogenesis is controlled in the adult rodent dentate gyrus may profoundly and imminently influence neuronal replacement strategies for human neurodegenerative disease or neurotrauma. Björkland and Lindvall (2000) have proposed that the symptoms of neurodegenerative disease or neurotrauma could be alleviated by replacing lost neurons with new neurons that establish the appropriate efferent and afferent connections and/or by introducing cells genetically engineered to secrete growth factors or neurotransmitters to promote the survival or regeneration of existing neurons. The experimental data of this thesis suggest that estradiol treatment could improve the success of both approaches.

Estradiol treatment could increase net neurogenesis by enhancing both the proliferation of progenitor cells and the survival of young neurons if the suppressive effect of estradiol on
cell proliferation is antagonized. Excessive glucocorticoid levels in Cushing’s disease patients have been successfully decreased with adrenolytic compounds such as metyrapone and ketoconazole (see Morris and Grossman, 2002 for review). Treatment that combines estradiol with metyrapone or ketoconazole could therefore sustain elevated rates of progenitor cell proliferation in the dentate gyrus and enhance the survival of new granule neurons. This approach would most imminently improve the outcome of neuronal replacement strategies that seek to manipulate neuron production in the dentate gyrus.

Combined estradiol/adrenolytic treatment could be used to increase net neurogenesis in extradentate CNS areas as well. Cells produced in the subventricular zone are known to migrate several mm to the olfactory bulbs (Lois and Alvarez-Buylla, 1993; Rousselot et al., 1995), demonstrating that the potential for directed long-distance migration in the adult mammalian CNS exists. If the cues directing the migration of neuroblasts were discovered, then estradiol/andrenolytic-induced increased numbers of neuroblasts produced in the dentate gyrus or other areas could potentially be directed to migrate into affected CNS areas and their rate of survival could be improved. Interestingly, recent evidence suggests that following ischemic injury, cells that differentiate into neurons migrate from the hippocampal periventricular zone into damaged the CA1 region and dentate gyrus (Nakatomi et al., 2002) and estradiol/adrenolytic treatment could enhance this process.

Targeted cell death induces neurogenesis in nonneurogenic regions of the CNS (Magavi et al., 2001) and progenitor cells are located in non-neurogenic regions of the adult rodent and human CNS (Palmer et al., 1995,1997; Shihabuddin et al., 1997; Kukekov et al., 1999; Roy et al., 2000; Aresenijevic et al., 2001), suggesting that neurogenesis could be induced throughout adult mammalian CNS. Targeted cell death is not a reasonable method of increasing neuron number in affected CNS areas, but when those mechanisms mediating the effect of targeted cell death on neurogenesis are discovered, combined estradiol/adrenolytic treatment could
potentially enhance both the proliferation of progenitor cells in normally quiescent CNS regions and promote the survival of their progeny.

Combined estradiol/adrenolytic treatment could also improve the success of transplant technology. For example, increasing the proliferation cultured progenitor cells that are transplanted in the adult CNS as well as enhancing the survival of their progeny would presumably increase the net number of neurons produced. When cultured adult hippocampal progenitor cells are transplanted into various brain regions, a greater percentage of the progeny die than survive (Suhonen et al., 1996). Estradiol/adrenolytic treatment could increase net neurogenesis by increasing the proliferation of these cells *in situ* and by enhancing the number of their progeny that survive. Because the progeny of cultured progenitor cells only differentiate into neurons when transplanted into neurogenic regions (Gage et al., 1995; Suhonen et al., 1996), this treatment would need to be combined with a factor that targets the differentiation of progeny into a neuronal phenotype. Alternatively, estradiol could be administered to enhance the survival of more differentiated cells that are transplanted into non-neurogenic CNS regions. Of course, the effects of estradiol on the proliferation of progenitor cells derived from different CNS areas and on the survival of different neuronal phenotypes would need to be determined to ascertain whether any of these approaches would be feasible.

*Summary and overall implications*

The purpose of this chapter was to integrate the experimental data of this thesis with previous findings to discuss the influence of estradiol over components of neurogenesis in the adult rodent dentate gyrus, discuss the potential consequences of estradiol-induced changes in neurogenesis for normal hippocampus function and to discuss the implications of the experimental findings for potential neuronal replacement strategies. To this end, the overall conclusions of the discussion are as follows.
1) Estradiol influences some but not all components of neurogenesis constitutive to the adult rodent dentate gyrus. Estradiol first increases and then decreases progenitor cell proliferation in the dentate gyri of adult female rats and meadow voles, demonstrating that the effect is robust across species with different reproductive strategies. Estradiol does not influence the differentiation of daughter cell in the dentate gyr of young adult female rats. However, the potential and phenotype of neuronal progenitors may change across lifespan because estradiol influences the differentiation of cells in the developing CNS. Further evidence to support this notion is that adrenal steroids appear to influence the differentiation of daughter cells in the dentate gyri of aged rats but do not influence the differentiation of daughter cells in young adult rats. In part, estradiol stimulates adrenal activity to suppress cell proliferation because the suppression is reversed in the dentate gyr of adult female rats. Although estradiol increases the affinity and upregulates the expression of NMDA receptors and NMDA receptor activation decreases cell proliferation, estradiol does not stimulate NMDA receptors to influence cell proliferation in the dentate gyri of adult female meadow voles. However, NMDA receptor activation and NMDA receptor blockade respectively increases and decreases cell proliferation in the dentate gyri of adult female meadow voles, similar to the effects reported in the dentate gyr of adult rats and tree shrews. Estradiol enhances the survival of young granule neurons but the temporal specificity and robustness of the effect appear different in the dentate gyri of adult female versus male meadow voles.

2) Seasonally regulated changes in net neurogenesis, spatial ability and space use may be related in adult meadow voles. Net neurogenesis decreases (because cell proliferation decreases) in the dentate gyri of laboratory-reared reproductively active versus inactive adult female meadow voles but is similar in the dentate gyri of laboratory-reared reproductively active and inactive adult female prairie voles. Serum estradiol level is similarly elevated in
reproductively active adult female meadow and prairie voles. Whereas a sex difference in spatial ability occurs between reproductively active male and female meadow voles, reproductively active male and female prairie voles exhibit similar spatial ability, suggesting that diminished neurogenesis in the dentate gyri of adult breeding female meadow voles is related to their diminished spatial ability. In contrast, net neurogenesis increases (because cell survival increases) in the dentate gyri of laboratory-reared reproductively active versus inactive adult male meadow voles. Wild adult male meadow voles increase their territory size during the breeding season to encompass the territories of females, suggesting that increased net neurogenesis could be related to increased space use. Perhaps analogously, adult male meadow voles with more surviving young granule neurons in their dentate gyri (and therefore higher net neurogenesis) outperform males with lower young neuron number on a retention trial in the Morris water maze. At least in the meadow vole, seasonal changes in net neurogenesis could be related to seasonal changes in spatial ability and space use.

3) By eliminating estradiol’s influence over adrenal activity, estradiol’s stimulatory effect on progenitor cell proliferation could be prolonged. Cumulatively, estradiol-induced increases in progenitor cell proliferation and the survival of young neurons could increase net neurogenesis in a number of different approaches for replacing neurons lost in the diseased or damaged adult CNS. Estradiol/adrenolytic treatment treatment could improve neuronal replacement strategies by a) enticing neuroblasts to migrate from the dentate gyrus into affected CNS regions and increasing their survival over time, b) enhancing cell proliferation induced by another factor (perhaps associated with targeted cell death) and the survival of progeny and/or c) increasing the survival of relatively differentiated neuroblasts that have been transplanted into non-neurogenic regions of the adult CNS.
Overall, the experiments described in this thesis explored the role of estradiol on multiple components of neurogenesis in the adult rodent dentate gyrus and have provided general insight about the effect of estradiol on net neurogenesis. Furthermore, this thesis establishes a model with which to test the effects of any single factor on multiple components of neurogenesis, not only in the dentate gyrus of adult mammals, but within the adult mammalian CNS.
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