THE EFFECTS OF FLUOXETINE AND ENVIRONMENTAL ENRICHMENT ON RECOVERY OF FUNCTION FOLLOWING FOCAL DENTATE GYRUS LESIONS

Michael C. Salling

A Thesis Submitted to the
University of North Carolina Wilmington in Partial Fulfillment
of the Requirements for the Degree of
Master of Arts

Department of Psychology

University of North Carolina Wilmington

2008

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ABSTRACT

New neurons are formed in the dentate gyrus of the mammalian hippocampus throughout adulthood. Rates of adult neurogenesis can be manipulated by pharmacological and environmental factors. Specifically, two factors that lead to increased neurogenesis are the antidepressant fluoxetine which increases the proliferation of neural progenitor cells and environmental enrichment which increases neuronal survival. Although the putative function of adult neurogenesis is unknown, there is accumulating evidence that it plays a role in hippocampal-dependent learning and memory and as a self-repair response following brain insult. The aim of the present study was investigate whether increasing neurogenesis in rats could promote a recovery of spatial function following dentate gyrus damage. Intradentate infusions of colchcine selectively ablated the majority of dorsal dentate gyrus granule cells. Rats where then tested on the Morris water maze and matched to treatment groups. In the treatment paradigm aimed at increasing rates of neurogenesis, rats were given daily saline or fluoxetine injections and either lived in standard housing or a novel enriched environment for 5 weeks with BrdU injections occurring in the middle. Ki67-staining revealed a decrease in cell proliferation associated with the enriched environment. Doublecortin-staining revealed that fluoxetine increased cell survival in the standard housing. BrdU/NeuN-colabeling qualitatively revealed that neurogenesis did occur in the damaged dentate gyrus, but at a low rate. Overall, dentate gyrus lesions significantly decreased the proliferation and survival of new neurons following treatment. We concluded that the colchicine dose used profoundly disrupted the neurogenic niche and

that the enriched environment was inhibiting proliferation because it was more stressful than the standard housing. In the post-treatment behavioral testing, lesion rats had significant spatial memory deficits but did improve and enriched rats improved more than standard housed rats. On the probe test, lesion rats outperformed sham rats and lesion rats in the enriched environment outperformed all other rats on target search but not target crossings which may be interpreted as an increased resistance to extinction.

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INTRODUCTION

A major goal of neuroscience is to develop treatments that ameliorate behavioral and cognitive impairments that result from brain injury and neurodegenerative diseases. One strategy involves injecting stem cells directly into damaged brain regions to promote the regeneration of neural systems. Though promising, the regeneration of neural tissue via stem cell replacement therapy faces complex challenges, including obtaining stem cell sources and managing the invasive surgical procedures used to deliver stem cells to target brain areas. An alternative approach to neural regeneration focuses on triggering the proliferation of endogenous neuronal progenitor cells, termed adult neurogenesis, that are present in several regions of the adult brain. The latter approach may be particularly feasible in situations where brain damage is limited to the hippocampus, a region of the brain that contains large numbers of neural progenitor cells.

The hippocampus is a structure in the brain that is crucial to the formation of specific types of memories. As classically demonstrated in the patient H.M., bilateral damage to human hippocampus causes deficits in declarative memory. For instance, H.M. suffered from anterograde amnesia and could not form memories for facts and autobiographical information (Scoville & Milner, 1957; Marr, 1971). The hippocampus is arranged such that it receives and processes sensory information through bilateral connections to the cortex. It is therefore functionally organized to integrate information about items in the surrounding environment and transmit that information to other areas

of the brain for long-term memory storage (Amaral & Witter, 1995; Eichenbaum, 2001). Behavioral tests have revealed that damage to the rodent hippocampus disrupts the binding together of contextual information leading to deficits in spatial navigation and the ability to acquire configural representations (O'Keefe & Nadel, 1978; O'Reilly & Rudy, 2001). The discovery of adult neurogenesis in the hippocampus has lead to the idea that if these new neurons are functional then they are likely to contribute to hippocampal-dependent forms of learning and memory. Furthermore, it may be possible to recover hippocampal function following damage by replacing lost neurons with new ones.

Rats are ideal animal models for addressing the relationship between hippocampal function and adult neurogenesis. The organization and physiology of the rat hippocampal formation has been well characterized and is considered highly homologous to the human hippocampus in terms of its structure and information processing (Manns & Eichenbaum, 2006). Memory tasks designed for rats like the Morris water maze, a cue-dependent spatial navigation task, are very sensitive to the integrity of the rat hippocampus (Morris, 1981). Elaborations of this procedure using virtual reality paradigms show deficits on spatial navigation tasks extend to the human pathology associated with hippocampal damage (Astur, Taylor, Mamelak, Philpott, & Sutherland, 2002; Spiers et al., 2001). Rats are also advantageous in vivo systems for studying adult neurogenesis. The adult rat forms new hippocampal neurons at a significant rate and a number of techniques have been developed in the rat to visualize and measure adult neurogenesis (Kempermann, Jessberger, Steiner, & Kronenberg, 2004; Ming & Song 2005). In addition, the area of the brain where adult neurogenesis

occurs is a relatively isolated and stable system compared to the developing embryonic brain which allows for a less complicated functional analysis of factors that influence rates of neurogenesis. As a result, there are a number of genetic, physiological, pharmacological and environmental factors that have been identified that modulate mammalian adult neurogenesis (Kempermann et al., 2004; Ming & Song 2005). My thesis aims at utilizing factors that influence adult neurogenesis to increase the number and sustainability of newly formed neurons following hippocampal damage thereby increasing the likelihood of functional recovery in a hippocampal-dependent task.

History of Mammalian Adult Neurogenesis

For the majority of the twentieth century, the scientific community was highly skeptical of the idea that new brain cells are formed during adulthood despite evidence that suggested otherwise (Gross, 2000; Kaplan, 2001). Altman and Das (1965) first described mammalian adult neurogenesis in 1960s after utilizing [^{3}H]-thymidine autoradiography to tag cells that have undergone recent DNA synthesis associated with mitotic division. Within the same tissue, phenotypic identification methods were employed to determine if labeled cells were neurons. They found that adult neurogenesis occurs in an area of the hippocampus called the dentate gyrus (Altman & Das, 1965) and in the olfactory bulb of the adult rat (Altman, 1969). Altman and Das (1967) were the first to suggest that because of its location in the hippocampus, adult neurogenesis likely has a role in learning and memory. These early reports received

little support due to the central dogma in neuroscience cells are not created during adulthood (Gross, 2000; Kaplan, 2000).

Resistance to mammalian adult neurogenesis continued during the next 20 years and the phenomenon remained largely unexplored. However, reports began to emerge that replicated earlier findings and expanded to include characterization of the cellular properties of newly formed adult neurons. Kaplan and colleagues (Kaplan & Hinds, 1977; Kaplan & Bell, 1984) used electron microscopy in conjunction with [^{3}H]-autoradiography to show that new neurons in the adult rodent dentate gyrus and olfactory lobe are capable of surviving and forming synapses with other neurons. Additional findings by Stanfield and Trice (1988) showed that new neurons extend axonal projections into the CA3 region of the hippocampus. This evidence demonstrated that new neurons formed during adulthood are morphologically similar to mature granule cells in the dentate gyrus.

Parallel discoveries occurring in canaries in the 1980s by Fernando Nottebohn and his colleagues supported the notion that adult neurogenesis has a functional role. They reported significant rates of neurogenesis occur in the high vocal area (HVA) of the adult canary and later positively correlated adult neurogenesis in the HVA with song production (Goldman & Nottebohn, 1983; Alvarez-Buylla, Kirn, & Nottebohn, 1990). Electrophysiological evidence obtained via electrodes implanted in HVA indicated that some neurons positively labeled as “new” had generated electrical responses to auditory stimuli (Paton & Nottebohn, 1984), compelling evidence that new neurons have functional properties and may be involved in information processing. Additionally, the high vocal area of the canary is a multimodal association center that shows a high

degree of learning-associated neuroplasticity (Cayre, Malatarre, Scotto-Lomassesse, Strambi, & Strambi, 2002). Therefore, it seems plausible that newly generated neurons could contribute to the complex requirements of the HVA and may play a similar role in homologous and dynamic structures like the mammalian hippocampus.

Despite progress in the avian world, the acceptance of functional, mammalian adult neurogenesis was not as well received. Pasko Rakic (1985) argued that adult neurogenesis in the mammalian brain would be maladaptive. In his view, the complex primate brain needed long lasting stability to serve its memory requirements. New neurons would create strain on brain structure and interfere with existing neuronal circuitry. Using [^{3}H]-thymidine autoradiography, he reported an absence of adult neurogenesis in rhesus monkeys and later suggested that adult neurogenesis was naturally selected out of complex species like primates (Rakic, 1985; Eckenhoff & Rakic, 1988). As a result of the findings and arguments marshaled during this time, the field accepted the existence of adult neurogenesis in birds, reptiles, and fish but maintained that rodents do not have significant levels of neurogenesis and that primates ceased forming neurons following sexual maturity.

The re-emergence of mammalian adult neurogenesis occurred in the 90s, when a surge of new cell labeling techniques and improvements in the visualization of molecules armed researchers with the tools sufficient for ending the controversy surrounding mammalian adult neurogenesis. Using a mitotic marker called bromodeoxyuridine (BrdU) in conjunction with neuron- and glia- specific markers, Elizabeth Gould and her colleagues demonstrated that adult neurogenesis occurs at significant levels in rodents as well as marmosets and macaque monkeys (Gould,

Tanapat, and McEwan, 1998; Gould et al., 2000). In 1998, terminally ill cancer patients received injections of BrdU and their brains were observed postmortem. This landmark study demonstrated that significant adult neurogenesis occurred in the dentate gyrus of humans; evidence that adult neurogenesis has been evolutionarily conserved across species (Eriksson et al., 1998). The discovery of adult neurogenesis in the human implied that it may be clinically relevant to human pathologies. Furthermore, it offered an alternative stem cell source that obviates many of the moral issues surrounding obtaining embryonic stem cells. As a result, the study of mammalian adult neurogenesis has seen rapid expansion over the last 10 years. Currently, maladaptive adult neurogenesis has been linked to a many psychiatric and neurodegenerative diseases including depression, schizophrenia, epilepsy, and dementia (Kempermann, Krebs, & Fabel, 2008; Grote & Hannan, 2007).

Current methodology

Technological advances have driven the rediscovery of adult neurogenesis and continue to set the pace for characterizing the phenomenon. An important advancement has been the development of immunohistochemistry, a technique that uses antibodies to specifically bind to target molecules. The antibodies can then be linked to chromagens or fluorophores that allow the researcher to visualize the localization and relative amount of the target molecule. This technique has enabled researchers to label DNA and proteins that are expressed during specific time points in the neuronal life cycle more efficiently and reliably than traditional autoradiography (Taupin, 2006). As a

result, cellular structures and changes in protein expression can be visualized, illuminating the physical world of the cell.

In the adult mammalian brain, newly formed cells can take the road of gliagenesis, occurring throughout the brain, or neurogenesis, which is only substantial in restricted areas of the mammalian brain like the dentate gyrus. Therefore, it is necessary for a researcher to identify these cells as being recent and having attained a neuronal identity. The advent of BrdU-labeling has enabled neuroscientists to identify new brain cells by labeling cells that have experienced DNA synthesis in the S phase of mitosis (Takahashi, Nowakowski, & Caviness, 1992). BrdU given in vivo will label cells for approximately two hours following an injection, literally identifying the birth date of new cells. It is used concomitantly with neuronal markers like NeuN to positively identify neurons. Equally important, advances in microscopy have been crucial to confirming the existence and advancing our knowledge of adult neurogenesis. In particular, confocal microscopy has given researchers the ability to view cells on an orthogonal plane and reliably identify co-labeled cells. These methods are not perfect, for instance BrdU can label cells undergoing repair and NeuN has been known to stain non-neural cells even under ideal doses and conditions (Cameron & McKay, 2001; Kuhn & Cooper-Kuhn, 2007). The solution has been to use conservative, standardized criterion concomitantly with multiple markers of neurogenesis to validate findings. In addition sampling techniques must be defined a priori and involve blind experimenters when using a direct counting method (West, 1999). That being said, there are a number of neuronal and mitotic identification methods that provide converging evidence and permit the quantification of adult neurogenesis. In addition to BrdU and NeuN, there are multiple

commonly markers of adult neurogenesis including mKi67, a protein expressed during the late G1, S, G2, and M phase of the cell cycle and doublecortin, a microtubule associated protein that is expressed in newly formed neurons from 3 to 6 weeks during periods when the cell is elongating, migrating, and integrating into existing circuits (Kempermann, Jessberger, Steiner, & Kronenberg, 2004). Protein-specific antibodies can be used to identify cells during different stages of neuronal development (Christie & Cameron, 2006; Kemperman, 2004).

Neuronal life cycle

The generation of a neuron in both the embryo and adult begins with a neural stem cell. To be identified as a stem cell, a cell must satisfy two requirements: 1) multipotency, the ability to form multiple cell types and 2) unlimited self-renewal, the ability to divide over unlimited generations (McKay, 1997). A stem cell can divide symmetrically to yield two identical stem cells or asymmetrically, forming a stem cell and a daughter cell called a progenitor cell. Progenitor cells are more limited than stem cells in terms of potency and self–renewal. They serve as an intermediate state prior to differentiation and have limited cell division, however, the rate of division can be much higher than in stem cells leading to the term transiently amplifying progenitor cells (Gage, 2000). Other progenitor cells remain in a quiescent state where they may not divide for several days. A progenitor cell can divide and yield more progenitor cells or form limited potency precursor cells that are committed to a particular cell-fate (Kemperman et al., 2004). In addition to neural stem cells, other cells like radial glia,

which have a supportive function, have been identified as a significant source of neural progenitor cells, especially in the adult dentate gyrus (Seri, Garcia-Verdugo, Collado-Morente, McEwan, & Alvarez-Buylla, 2004).

The progression from a stem cell to a neuron follows a series of distinct developmental stages (Figure 1). The first stage is proliferation where endogenous stem cells or radial glia divide and form neural progenitors, the precursor to a neuron (Figure 1A). Neural progenitors commit to either attaining a glial, oligodendrocyte, or neuronal cell fate depending on intrinsic factors and the extracellular regulatory factors that influence gene expression in the second stage termed differentiation (Figure 1B). Neural precursors then form neuroblasts and migrate either independently by using finger-like leading process called filipodia or attaching to radial glial “guide wires” (Gashghaei, Lai, & Anton, 2005) (Figure 1C). Factors in the extracellular environment influence the rate and direction of migrating cells as well as what cell population will be their final destination. Once a developing neuron has reached its final location, its fate depends on making connections with other neurons. In the fourth stage, integration, the immature neuron extends axons and dendrites to form synapses with other neurons in a process called synaptogenesis (Figure 1D). Neural adhesion molecules on the dendrite and axon attach to receptors and activate intracellular signaling cascades that initiate the cellular machinery that organizes proteins and cytoskeletal structures to create a viable synapse. In addition, it is necessary for the new neuron to make connections with supportive cells like glia and oligodendrocytes so that the neuron can be supported and fire action potentials. Once these events have been coordinated and the new neuron has formed synapses with other neurons, communication and incorporation into that

neural circuit can begin. Neurons that do not integrate lack the necessary trophic support which leads to a separate signaling cascade that initiates and organizes the degradation of cellular structures and the destruction of the neuron. This type of programmed cell death is called apoptosis (Figure 1E).

In the adult brain, two areas have been identified as having neural stem cell sources: the subventricular zone and the subgranular zone. This paper will be primarily concerned with the subgranular zone, the source of new neurons in the hippocampus.

Figure 1. *The neuronal life cycle.* Neural stem cells can divide symmetrically to form two neural stem cells or asymmetrically to form a daughter cell called a neural progenitor cell and another neural stem cell (1A). Radial glia can also divide asymmetrically to form neural progenitor cells (1B). Neural progenitor cells can divide rapidly as transiently amplifying progenitor cells causing large increase in proliferation (1C). Neural progenitor cells make cell fate decisions and differentiate to attain either a neural, glial, or oligodendrocyte phenotype (1D). Neural precursors migrate as neuroblasts by extending filopodia that attach to radial glia and help them move along the glial processes (1F). Immature neurons integrate into neural circuitry by extending dendrites, dendritogenesis, and axons, axonogenesis, and forming synapses with other neurons, synaptogenesis. Integration leads to the establishment of a mature neuron (1E). If they do not occur then intracellular signaling cascades will be activated in the neuron that organize the degradation of neural structures and eventually cell death, or apoptosis.

Adult Neurogenesis in the Dentate Gyrus

Adult neurogenesis occurs in an area of the hippocampus called the dentate gyrus (Figure 2). The dentate gyrus is a densely packed, C-shaped region of relatively small neurons called granule cells. These neurons receive sensory input from entorhinal cortex via the perforant path and project axons to the pyramidal neurons of the CA3 region. Proliferation occurs in the subgranular zone of the dentate gyrus, an area lateral to the granule cell layer. Neural progenitor cells migrate along radial glia a short distance from the subgranular zone to the granule cell layer at which point they differentiate into granule cells (Schinder & Gage, 2002). Although many of these cells die, some integrate into neuronal circuits by extending dendrites and forming synapses on the commissural fibers of the perforant path. The axons from the granule cells project to the mossy fibers of the CA3 region of the hippocampus (Stanfield & Trice, 1988). Studies have shown that new granule cell neurons formed in adulthood have neuronal properties including cell firing and vesicular release (van Praag Christie, Sejnowski, & Gage, 2002). In addition, new neurons have glutamatergic and GABAergic inputs and the majority of cells release glutamate while a small proportion can release GABA (Overstreet-Radiche, Bromberg, Wadiche, & Westbrook, 2005, Walker, Ruiz, & Kullman, 2001) New neurons comprise a significant proportion of the DG, generating 9000 new neurons each day in the dentate gyrus equating to 250,000 per month or 6% of the total granule cell population (Cameron & McKay, 2001).

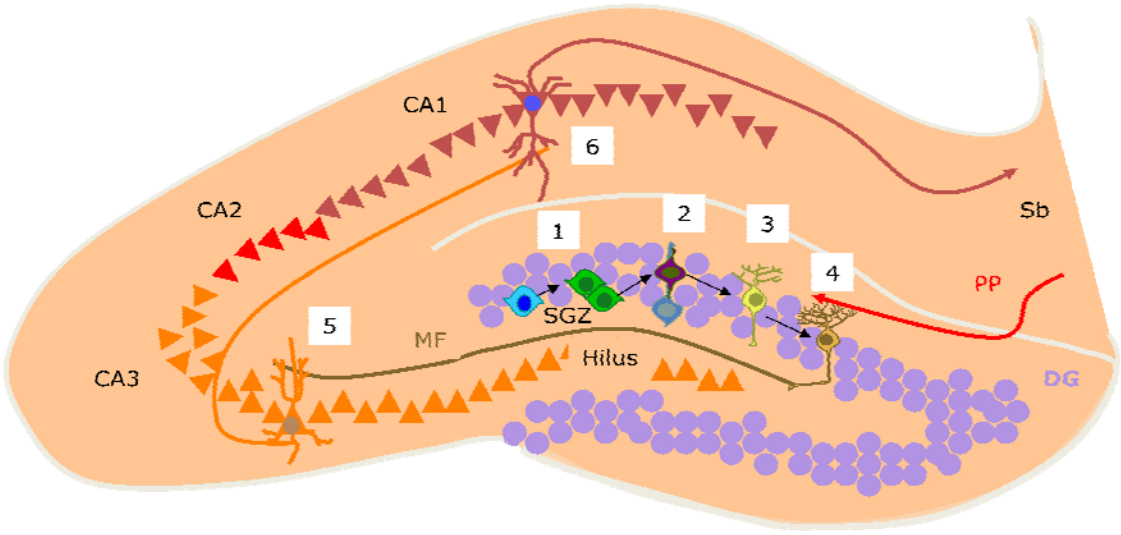


Figure 2. *Adult neurogenesis in the rodent dentate gyrus*. The DG is a cell layer of densely-packed granule cells in the hippocampus (purple cells). (1) Proliferation of neural progenitor cells occurs in the subgranular zone (SGZ) that exists between the hilus and the granule cell layer. Neural progenitors form neuroblasts and migrate (2) a short distance along radial glial in the granule cell layer. (3) Differentiated neurons establish their final position in the DG and begin integration into neural circuitry. (4) New neurons establish postsynaptic connections to projections from the entorhinal cortex called the perforant path (PP). (5) These neurons extend axons called mossy fibers (MF) to neurons called pyramidal cells in the CA1 region of the hippocampus. (6) CA1 neurons project to pyramidal cells the CA3 region and form a trisynaptic circuit. CA3 neurons are the main output of the hippocampus, sending information to the cortex via the subiculum (Sb).

Factors that Influence Adult Neurogenesis

There is an expanding list of factors that influence the rate of adult neurogenesis. Experiments *in vivo* have identified multiple factors that influence adult neurogenesis that may occur naturally, are due to pharmacological manipulation or depend on the organism's interaction with the environment. The majority of known neurogenic regulators can be categorized as having endogenous or exogenous sources and either increase or decrease rates of neurogenesis. Many regulators have been characterized as having specific effects on the particular stages of the neuronal life cycle including proliferation and survival.

Endogenous regulators of neurogenesis include neurotransmitters. Glutamate is the central nervous system's main excitatory neurotransmitter and is a major signaling molecule in the dentate gyrus. Administration of glutamate in the hippocampus suppresses cell proliferation in the dentate gyrus (Cameron, McEwan, & Gould, 1995). Separately, damage to neurons that release glutamate and pharmacological blockage of the ionotropic glutamate receptors that influx calcium (NMDA receptors) result in increased rates of neurogenesis (Cameron et al., 1995). Another major neurotransmitter, serotonin, has been shown to influence cell proliferation. Selective serotonin reuptake inhibiters (SSRis) like the antidepressants fluoxetine increase the amount of serotonin in the synapse and are correlated with increases in the proliferation of neural progenitor cells (Malberg, Estler, Eisch, & Duman, 2000). Following pharmacological inhibition or damage to serotonergic neurons a dramatic decrease of adult progenitor proliferation in the dentate gyrus and subventricular zone is observed

(Brezun & Daszuta, 1999; Rosenbrock, Koros, Bloching, Weiss, & Borsini, 2005). Transplantation of serotonergic neurons into the raphe nucleus restores neurogenesis following raphe nucleus damage (Brezun & Daszuta, 2000).

In addition to neurotransmitters, hormones have also been shown to have a profound effect on adult neurogenesis. Hormones that regulate the stress response are called glucocorticoids and are secreted from the adrenal gland under stressful conditions. Rats that have undergone adrenalectomies, which aim at removing the source of corticosteroids and decreases its levels significantly, increases in neurogenesis (Yehuda, Fairman, & Meyer, 1989). Conversely, rats that are treated with the glucocorticoid corticosterone or glucocorticid receptor agonists show dose-dependent decreases in neurogenesis (Cameron & Gould, 1994) Treating adrenalectomized rats with corticosterone can restore rates of neurogenesis to normal levels (Montaron et al., 1999). Adrenal hormones are thought regulate adult neurogenesis by effecting both the proliferation of neuronal precursors and the survival of immature neurons (Wong & Herbert, 2004). However, this may occur through more than one mechanism. Neuronal precursors express glucocorticoid receptors and binding may influence cell division. In addition, there are high levels of glucocorticoid receptors expressed on mature neurons throughout the hippocampus which can modulate the release of glutamate transmission into the subgranular zone of the dentate gyrus (Dranovsky & Hen, 2006). The negative effect of glucocorticoids on adult neurogenesis is dependent on NMDA receptor activity suggesting that multiple mechanisms are responsible (Cameron et al., 1998).

Other important endogenous regulators of neurogenesis are the extracellular signaling molecules known as neural growth factors (NGFs). Much of the research regarding growth factors originally came from development biology where they were identified as directing the expansion of stem cells and functioning to promote proper development of the organism in a time and location specific manner. Growth factors are able to transduce membrane signals to the nucleus via signaling cascades that activate transcription factors and downstream genes that are required for cell expansion and differentiation into neurons. Accordingly, their important role in nervous system development led to research that asked how they would affect the development of neurons in the adult. One NGF, Epidermal growth factor (EGF), is a powerful mitogen that causes rapid expansion of stem cells in vitro and in vivo (Jin et al., 2002; Jin et al., 2005). Subsequent experiments tested EGF's regenerative potential in the adult and it was found that following site-directed hippocampal ischemia, EGF infused in to the ventricles of the adult brain causes dramatic increases in neural progenitor proliferation and enhances regeneration (Nakatomi et al., 2002). Vascular endothelial growth factor (VEGF) is another growth factor that increases cell proliferation when infused into the dentate gyrus and decreases neurogenesis when its expression is reduced (Cao et al., 2004). It is also required for pharmacological and environmental factors that increase in adult neurogenesis (Warner-Schmidt & Duman, 2007). Brain-derived neurotrophic factor (BDNF) is an additional growth factor that is known to play a role in neural plasticity. Infusions of BDNF into the rat hippocampus have been shown to increase adult neurogenesis (Scharfman et al., 2002).

The diverse array of experiences that an animal encounters in its environment can also modulate rates of neurogenesis. Voluntary exercise can be effectively modeled by allowing access to a running wheel. Rats will run excessively on these wheels which leads increased progenitor proliferation and increased neurogenesis (Van Praag et al., 1999). Simply restricting access to food can lead to robust increases in new neurons by increasing neuronal survival (Lee, 2000). Most laboratory rats live in an impoverished environment compared to their natural habitat. Studies that involve placing adult rats in environments that include more space, toys, tunnels, and social interaction demonstrate that rats living in more "enriched" environments have significantly higher levels of cells identified as new neurons due to increased neuronal survival (Kempermann, Kuhn, & Gage, 1997). Although environmental enrichment is a relative term that is difficult to operationally define, this finding has been validated across many different environments that have a similar array of complex stimuli (Will, Galani, Kelche, Rosenzweig, 2004). Levels of BDNF are increased following exposure to an enriched environment or dietary restriction and have been shown to be required for the increase in neurogenesis due to environmental enrichment using transgenic mice (Lee, 2000; Rossi et al., 2006). Specific types of learning positively correlate with adult neurogenesis. Increased levels of neurogenesis have been observed in rats that are trained on hippocampal-dependent learning tasks like the Morris water maze (Gould, Beylin, Tanapat, Reeves, & Shors, 1999). Conversely, some experiences have negative effects on neurogenesis. Chronic restraint stress has been shown to decrease protein levels and cellular expression of Ki-67, suggesting stress decreases progenitor proliferation (Rosenbrock, Koro, Bloching, Podhorna, & Borsini, 2005). Rats exposed to other forms of chronic stress such as

predator odor and social dominance show decreases in cell proliferation (Tanapat, Hastings, Rydel, Galea, & Gould, 2001, Kozorovitsky & Gould, 2004). As a result, the total volume of the dentate gyrus is reduced in stressed rats, a finding that has been related to cognitive deficits on hippocampus-dependent tasks (Gould, Tanapat, & McEwan, 1998).

Function of Adult Neurogenesis

It is widely accepted that adult hippocampal neurogenesis occurs, yet little is known about whether these new cells contribute to hippocampal function. Two possibilities have been suggested. First, adult neurogenesis may be a vestigal developmental process without any functional role. The other possibility is that new neurons are incorporated into functional neuronal circuits and contribute to information processing (Schinder & Gage, 2004). Support for the latter hypothesis has comes from several sources. First, as mentioned above, the rate of hippocampal neurogenesis and the survival of new neurons is greater in animals that are engaged in behaviors that are dependent on the hippocampus, such as spatial navigation and exercise. Second, when neurogenesis is halted via exposure to antimitotic agents or x-irradiation, the dentate gyrus gradually loses volume and has been associated with behavioral effects like spatial deficits and depression-like symptoms (Gould et al., 1998). Finally, from a theoretical perspective, it is important to consider that creation of a new neuron expends a relatively large amount of resources and energy. If adult neurogenesis did not serve

an important function, it would seem likely that it would not have been favored by natural selection and conserved across species.

The most widely discussed candidate for the function of adult hippocampal neurogenesis is a role in learning and memory. This hypothesis predicts that some forms of learning require or are enhanced by the contribution of new neurons. In their study, learning hippocampal-dependent tasks increased rates of neurogenesis while tasks that do not require the hippocampus did not have an effect (Gould et al., 1999). In addition, the learning hypothesis predicts that decreases in neurogenesis will hinder acquisition and performance on memory tasks. Evidence from a study by Shors and others supports this hypothesis. In her study, rats were administered methylazoxymethanolacetate (MAM) a drug that blocks cell division. MAM treated rats performed poorly on a trace eye-blink conditioning, a hippocampal-dependent task, while their performance on delayed eye-blink conditioning, a hippocampal-independent task was normal (Shors et al., 2001). However, in a later study decreased neurogenesis using MAM did not impair performance on other hippocampal-dependent tasks like the Morris water maze swim task (Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002). Adverse side effects associated with neurogenic blockers like MAM and x-irradiation may serve as potential confounds. The push for more selective neurogenic blockers seeks to alleviate conflicting evidence and definitively identify the role of neurogenesis in learning and memory.

Neuropysiological evidence has also supported its role in learning and memory. Long-term potentiation (LTP) is a form of synaptic plasticity where neurons that fire in close temporal proximity form strengthened synapses. It has long been postulated as

the physiological manifestation of learning and is required for many types of hippocampal dependent-learning. In the dentate gyrus, new neurons demonstrate a lower threshold for the induction of LTP (Van Praag et al., 2002). In addition, experimentally inducing LTP increases rates of adult neurogenesis in the DG (Bruel-Jungerman, Davis, Rampon, & Laroche, 2006). This evidence suggests that new neurons may have specific properties of synaptic plasticity that enable them to contribute to hippocampal-dependent learning.

Functional Recovery

The hippocampus responds to injury by increasing the production of new neurons. This has led to the hypothesis that the newly formed neurons may play a role in self-repair (Kozorovitskiy & Gould, 2003). This hypothesis requires that a population of progenitor cells remains stable or emerges following insult and that new neurons are capable of replacing lost ones. In addition, the microenvironment that these cells exist in must remain conducive to the maturation and survival of a newly formed neuron. In previous experiments, the precise balance needed to support cell growth has been difficult to achieve following insult. Brain injury leads to the production of proinflammatory cytokines like interferon alpha and subsequent activation of stress pathways can inhibit proliferation and neuron survival (Kaneko et al., 2006). Additionally, a sufficient amount of vascularization and supportive glia must be spared so that they can supply the oxygen, trophic factors, and support that are necessary for neuronal survival. Despite these caveats, researchers have observed positive

correlations between recovery of function and rates of neurogenesis. Rats that have received focal ischemic injury to the dentate gyrus have been able to recover some spatial ability following exposure to an enriched environment (Dahlqvist, Ronnback, Bergstrom, Soderstrom, & Olsson, 2004). In addition, pharmacological treatment coupled with enriched environment has been shown to be more effective in recovery of function than environmental enrichment alone (Puruunun, Jolkkonen, Sirvio, Haapalinna, & Sivenius, 2001). These findings serve as compelling evidence that adult neurogenesis may be manipulated by the experimenter or possibly the clinician to achieve an improved outcome following brain damage.

Rationale and Hypothesis of Present Study

The focus of my thesis project was to evaluate the therapeutic potential of adult neurogenesis. To address this, I selectively ablated hippocampal granule cells, the type of neurons that are formed from adult neurogenesis, using colchicine, an alkaloid that binds to tubulin that is particularly toxic to granule cells. Intradentate colchicine infusions caused performance deficits on the Morris water maze, a hippocampal-dependent spatial memory task. I then devised a novel treatment that would theoretically increase progenitor cell proliferation using fluoxetine and enhance the survival of newly formed granule cells by placing rats in an enriched environment. My hypothesis was that if it is possible to recapitulate the loss of a specific population of hippocampal neurons by influencing adult neurogenesis, one would be able be able to observe a recovery of hippocampal function.

METHODS

Subjects

Sixty-seven male, Long Evans, hooded rats were used in the experiment. Rats were between 87-96 days old when they entered the experiment. Eight rats were removed because they sustained injuries that appeared to be a result of fighting or injections during the treatment phase. Prior to entering the experiment, rats were housed in pairs in Plexiglass tubs in the UNCW Psychology Department vivarium. Behavioral testing occurred during the light phase of a 12 hour light/dark cycle. Rats had access to rat chow and water *ad libitum*. All animals were treated in accordance with the International Animal Care and Use Committee (IACUC) regulations.

Surgery Procedure

For each surgery rats were anesthetized with isofluorane (4% with 2 liters per minute of oxygen and 2% after a surgical plane was established). Body temperature was maintained with a heating pad placed under the rat. A midline incision was made in the scalp and periosteum. Eight holes (4 overlying each hemisphere) 0.5 mm in diameter were drilled in the skull overlying the target tissue. The stereotaxic coordinates of the holes, relative to Bregma, were A-P, 3.14, 4.16, 5.2 and 6.0 mm and lateral 1.2, 2.4, 3.1 and 4.4 mm. A 30-gauge injection needle was stereotaxically lowered through each hole to the target depth 3.6, 3.4, 3.4, and 4.4 (mm). Microinjections of colchicine (1.25 mg/ml in 0.1 M PBS) were made at each of three sites in the hippocampus. An

infusion of 0.5 µl of solution was injected at each site at a rate of 0.125 µl/min. The injection needles remained in place for 4 minutes following each injection to allow the colchicine solution to diffuse away from the needle tip. The needles were then removed and the scalp closed with sutures and surgical glue. An intramuscular injection of antibiotic (0.2 ml) was given immediately following the closing of the scalp. Each rat was carefully monitored until it fully recovered from the anesthetic and given a subcutaneas injection of morphine (0.2 ml of 5.0 mg/ml) to alleviate postoperative pain. Rats were returned to their familiar cages for a postoperative recovery period of 7-10 days (Figure 3).

Surgery	Drug	Housing	Group (n)
Sham	Saline	Standard	SSS(8)
		Enriched	SSE(8)
	Fluoxetine	Standard	SFS(7)
		Enriched	SFE(8)
DG Lesion	Saline	Standard	LSS(7)
		Enriched	LSE(7)
	Fluoxetine	Standard	LFS(7)
		Enriched	LFE(7)

Table 1. *Experimental conditions*. Categorization of experimental treatment groups into surgery, drug, and housing treatments. Groups are shown with abbreviations and number of subjects.

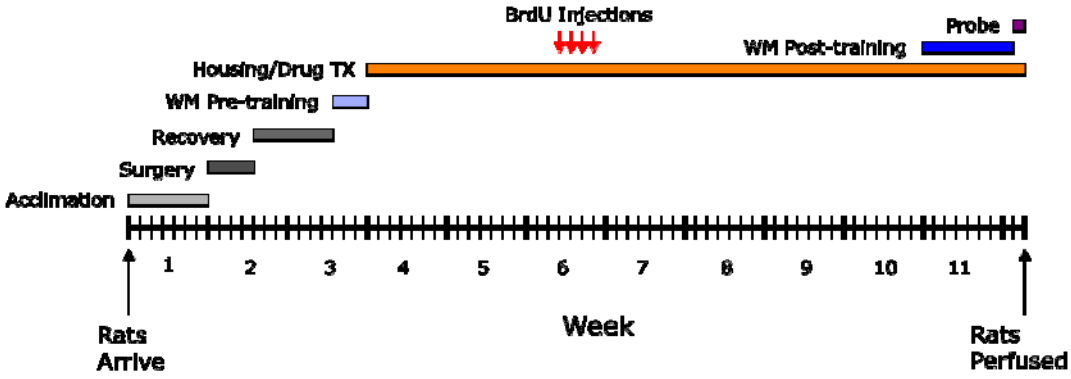


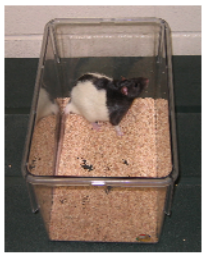
Figure 3. *Experimental timeline*. For each cohort, 16-20 Adult rats arrive and are given a week to acclimate to the colony (light gray bar). They then undergo DG lesion or sham surgery that occurs over 4 days (dark gray bar) and are given 7-10 days to recover (medium gray bar). They all enter the pre-training phase on the Morris water maze at the same time which occurs over 3 days (light blue bar). Following the third day, they are matched to housing and drug treatment conditions. During this time they are weighed daily and either receive saline or fluoxetine (5.0 mg/kg) daily injections and live in either standard laboratory housing or enriched environment throughout the remainder of the experiment (orange bar). On days 16-19 they receive daily injections of BrdU (60 mg/kg) (red arrows). Post-treatment training occurs at approximately week 11 and lasts for 8 days (blue bar). On the 9th day a probe trial session (purple bar) occurs and they are perfused afterwards.

Treatment Conditions

Drug Condition: During the drug treatment phase of the experiment, rats received daily injections of either saline (0.9%) or fluoxetine hydrochloride (1.0 mg/kg of 5.0 mg/ml), (Eli Lilly, Indianapolis, Indiana, USA) for a total of 44 days (Figure 3). Fluoxetine was prepared by dissolving it in distilled water (pH = 7.35). The treatment length was chosen because fluoxetine is known to have effects on proliferation at 2 weeks, and new neurons mature and integrate into circuits beginning 2 weeks following mitosis (Santerelli et al. 2000; Zhao, Deng, & Gage, 2008). Rats were weighed on each day and given intraperitoneal injections (1.0ml/kg) according to standard laboratory procedures.

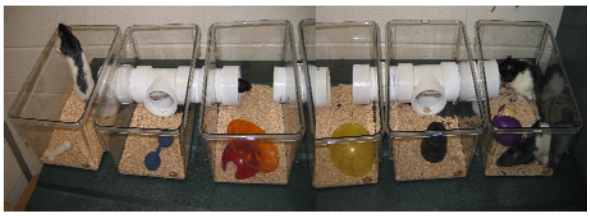
Housing Condition: In the housing condition, rats were either housed in the standard laboratory (Figure 4a) housing previously mentioned or housed in an enriched environment (Figure 4b) during the 44 day treatment period (Figure 3). The enriched environment (EE) was created using 6 standard Plexiglass tubs that were modified to accommodate horizontal connections of PVC piping The EE provided additional space and increased social interaction than standard laboratory housing. In addition, the environment included tunnels, nesting material, and rat toys that were cycled every two days to maintain their novelty.

A



Standard Housing

B



Enriched Environment

Figure 4. *Housing condition.* Rats were placed in either standard housing condition (4A) where they were singly housed or and enriched environment where they were group housed (4B). The enriched environment included toys, tunnels, and nesting material.

BrdU treatment. Bromodeoxyuridine is an analog of the thymidine nucleotide that incorporates into newly synthesized DNA during the S-phase of mitosis with a bioavailability of 2 hours post injection. Single injections of BrdU (60 mg/kg; 10 mg/ml in 0.007N NaOH/0.9% saline) were administered during the 16^{th}, 17^{th}, 18^{th} and 19^{th} days of treatment approximately 24 hours apart (Figure 4). The days were chosen because at this timepoint BrdU should label dividing cells after fluoxetine proliferative effects should begin and with enough time for the cells to differentiate into neurons (Malberg et al 2000; Zhao et al, 2008,).

Histology

Histological Procedures: Following the probe trial, rats were euthanized by injecting sodium pentobarbital (2.0 ml of 5.0%) intraperitoneally. Once the rat became unresponsive it was transcardially perfused on a Perfusion One^{TM} pump system (Coretech Holdings, St. Louis, MO) using the following procedure: 5% cold sucrose for 10 minutes followed by a buffered 4% PFA/picric acid fixative for 15 minutes. The brains were harvested, post-fixed overnight in individual glass containers filled with 4% PFA/picric acid fixative, and then placed in 30% sucrose and stored at 4^{o} C. Fixed brains were sectioned serially on a cryostat through the entire hippocampus at a thickness of 40 µm sections. Every fifth section through the hippocampus was collected on slides for lesion verification. The remaining sections were placed in 0.1 M PBS in 1.5 mL Eppendorf tubes for immunohistochemistry procedures.

Lesion Verification. Sections collected on slides were stained with dehydrated and rehydrated in a series of alcohols before staining with cresyl violet, clearing with citrasolve and coverslipped with permount. Images corresponding to sections 33, 38, and 45 of Paxinos and Watson's (1998) rat brain atlas were taken on an Olympus light microscope with a Motic camera using a 2x objective lens. A tablet PC was used to hand trace and calculate the area of the dentate gyrus using Motic software.

Immunohistochemistry

Ki67, DCX, and BrdU/NeuN Staining: 3 vials containing free-floating sections in PBS were stained for Ki67, Brdu/NeuN, and doublecortin . For Ki67 staining, tissue was washed and incubated overnight in primary antibody solution: 1:500 Ki67 rabbit polyclonal (Novocastra, Newcastle upon Tyne, UK), 0.3% Triton-X, 1% normal goat serum (Jackson ImmunoResearch) in 0.1 M PBS. Tissue was washed and incubated for one hour at room temperature in secondary antibody solution: 1:1000 Anti-rabbit IgG biotinylated antibody (Vector Laboratories) in PBS. Tissue was washed and placed in 1:500 Streptavidin Alexa Fluor 568 conjugate (Molecular Probes) for 45 minutes at room temperature., Tissue was then washed, mounted onto subbed slides (1% gel, 0.2% chromalum) and coverslipped using glycerol mounting medium (1–3% *n*-propyl gallate in 1 part 0.1 M phosphate buffer and 9 parts glycerol). For doublecortin staining, tissue was washed and incubated in 0.3% H_2O_2 in 0.1 M PBS for 25 min at room temperature. Tissue was washed then placed overnight in primary antibody solution: 1:1000 anti-DCX goat polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), 0.5% Triton-X, and 2%

normal rabbit serum (Vectastain Elite ABC Kit Goat IgG, Vector Laboratories, Burlingame, CA), in 0.1 M PBS. Tissue was washed and placed in secondary antibody solution: 1:1000 biotinylated anti-goat IgG (Vectastain Elite ABC Kit Goat IgG, Vector Laboratories), in 0.1 M PBS for 1 h at room temperature. Tissue was washed and incubated in ABC solution (Vectastain Elite ABC Kit Goat IgG, Vector Laboratories) for 45 min at room temperature. Tissue was washed and incubated in DAB with nickel solution (Vector Laboratories) until color changed (2–10 min). Tissue was washed and mounted onto subbed slides (1% gel, 0.2% chromalum). Slides were dehydrated with an ascending series of ethanol, cleared in CitriSolv (Fisher Scientific), and coverslipped using Permount (Fisher Scientific) mounting medium. For BrdU and NeuN staining, the tissue was washed, treated with 50% Formamide/2 SSC buffer for 2 h at 65 °C, washed in 2× SSC, incubated with 2 M HCl at 37 °C for 30 min, and washed over 2 h. Tissue was then placed in primary antibody overnight: 0.3% Triton-X (Sigma, St. Louis, MO), 2% normal goat serum (Jackson ImmunoResearch, West Grove, PA), 1:200 rat anti-BrdU (Accurate Chemical & Scientific Corp., Westbury, NY), and 1:1000 mouse anti-NeuN (Chemicon, Temecula, CA) in 0.1 M PBS. Tissue was washed in 0.1 M PBS and placed overnight in secondary antibody: 1:250 goat anti-mouse IgG Alexa Fluor 488 (Molecular Probes, Eugene, OR), and 1:500 biotinylated goat anti-rat IgG (Chemicon, Temecula, CA) in 0.1 M PBS. Tissue was washed and placed in streptavidin–Alexa 568 (1:500) in 0.1 M PBS for 45 min. Tissue was washed then mounted onto subbed slides (1% gel, 0.2% chromalum) and coverslipped using glycerol mounting medium (1–3% *n*-propyl gallate in 1 part 0.1 M phosphate buffer and 9 parts glycerol).

Stereological Procedures. Sections that corresponded to plates 33, 38, and 44 of Paxinos and Watson's rat brain atlas (1998) were used for quantification using unbiased stereological procedures. Ki67 quantification occurred on an Olympus fluorescence microscope using a 10x objective. Quantification of doublecortin occurred on an Olympus light microscope using a 20x objective. For BrdU/NeuN quantification, unbiased stereological procedures took place on an Olympus confocal laser scanning microscope. Z-stacks were acquired to confirm double-labeling. Experimenters involved in the counting procedure were blind to experimental condition.

Behavioral Testing

Behavioral Apparatus. The Morris swim task (Morris, 1981) was used to test spatial learning and memory. The apparatus is a pool 1.5 m in diameter enclosed by 4 rectangular curtains with salient cues on each curtain (Figure 5a). The pool was filled with approximately 28° C water and evaporated milk was added make the pool water opaque. In the hidden platform paradigm used in the experiment, a platform 10 cm in diameter was submerged just below the surface of the water in the same specified quadrant location for each trial. Location of the platform was changed for each of the four cohorts to rule out quadrant preference. On the first day of each testing phase, the rat was acclimated to the arena by being directed to the platform by the experimenter on the surface of the water and placed on the platform for 10 seconds before any testing began. Each daily session consisted of four trials from four separate drop locations (North, South, East, West) in which the order was chosen randomly. For each trial, the

rats were given 90 seconds to find the platform and were removed after reaching it. When the rat did not find the platform, it was placed on the platform by the experimenter for 5 seconds, returned to the transport cage, and the next trial followed. During each swim, a digital video tracking system (HVS Image®), interfaced with a PC computer, recorded and stored data on swim paths, speed, heading, and escape latency.

Behavioral Testing. In the pre-treatment phase, rats had 3 sessions on 3 consecutive days. Average latency measures and standard deviations from the third day were used to rank and match each rat to the 8 treatment groups (Table 1). Following treatment, rats were given 8 sessions on 8 consecutive days tested. The platform location for each cohort was opposite of their pre-treatment phase. On day 9, the probe trial, the platform was removed and each rat was placed in the same starting location for one probe trial that lasts 60 seconds. Two additional measurements, target crossing and the time spent in each quadrant, were recorded during this phase. Experimenters responsible for the behavioral testing were blind to the group assignment of each rat.

Data Analyses

Cell counts, lesion size, and probe data (target quad percentage, target crossings) were analyzed using a three-way analysis of variance (ANOVA). Pre-treatment measures and post-treatment acquisition measures were analyzed using a mixed model three-way repeated measures ANOVA on measures of latency, distance,

and velocity. Inter-reater reliability was measured using a Pearson's correlation. All statistical analyses were performed using SPSS software and graphs were created using Graphpad Prism. Significance determined at p values < 0.05.

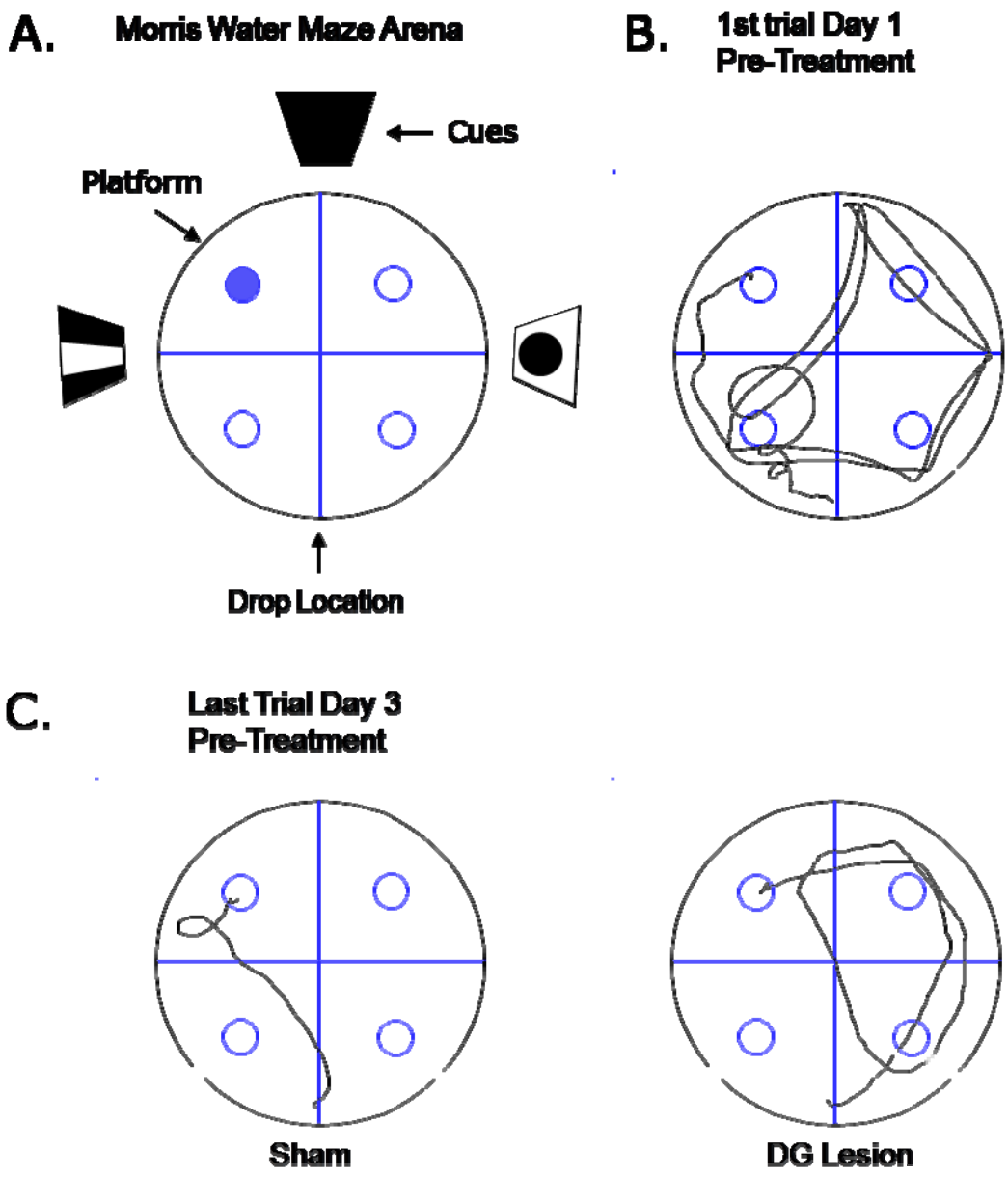


Figure 5. *Morris water maze and representative swim paths*. Diagram (5A) of the water maze with cues placed along the outside of the maze. Drop location and platform are labeled. Swim path of the median latency value for the first trial of the first day (5B). After three days training, median latency value swim paths for a rat in the sham (5C) and the lesion condition (5D) demonstrate deficits seen in the lesion condition.

RESULTS

Histology

Lesion Quantification: Histological analysis using the neuronal marker cresyl violet demonstrated that colchicine (1.25 mg/ml) effectively and reliably ablated dentate granule cells (Figure 6A, red arrow). A three-way ANOVA of granule cell layer area showed a significant main effect for surgery [$F(1, 50) = 380.27$, $p < .001$] where colchicines infusions ablated over 95% of the dorsal DG compared to controls. There were no other effects for DG area. In addition to the loss of DG, a subset of surgery rats showed partial loss of the CA3 and CA1 pyramidal neurons and cells in the subiculum near infusion sites. The loss of cells altered the structural integrity of the hippocampus, causing an observable decrease in the overall size and an increase in the ventricular space surrounding the hippocampus (Figure 6A, white arrow). The loss of these cells was restricted to the dorsal hippocampus and extended along the entire anterior-posterior axis and did not cause significant damage to the ventral hippocampus. Both hemispheres appeared to be equally affected and there did not appear to any injections that missed their targets. Cell loss was not observed in regions other than the hippocampus. The extent of cell loss in the hippocampus was more extensive than expected and that had been seen in pilot experiments at the same injection sites.

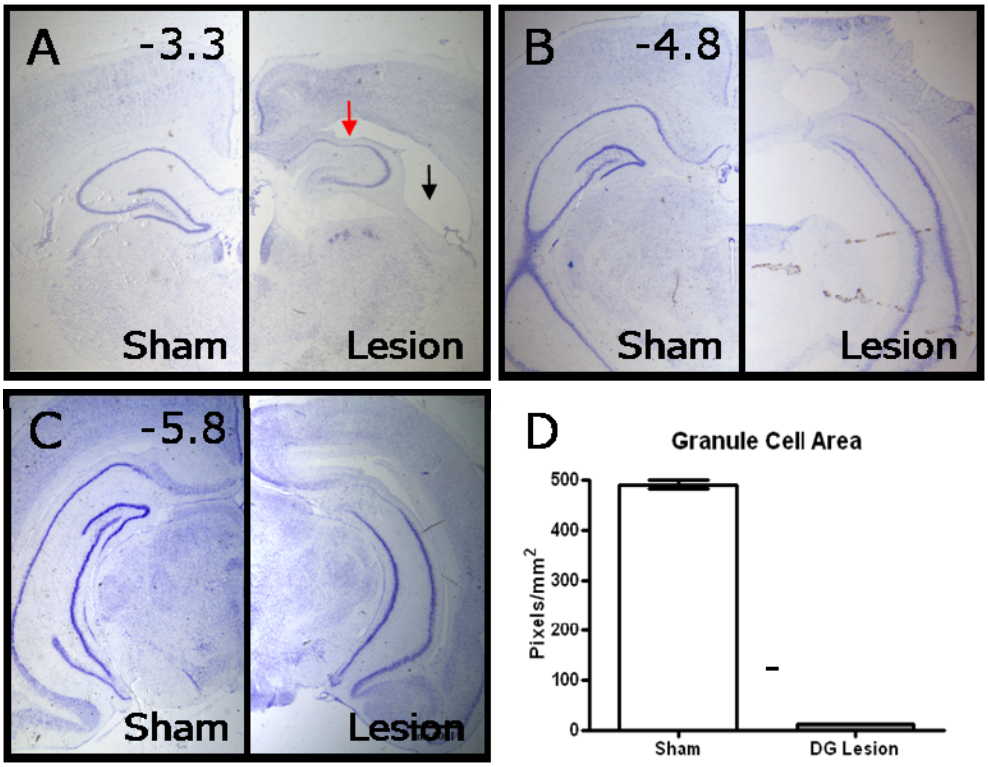


Figure 6. *DG lesion histology.* Micrographs of cresyl violet staining of three sections from sham and DG lesion operated rats (6A-6C). Values (upper left corner) correspond to posterior distance (mm) from bregma. Images depict near complete ablation of the dorsal DG with increases in ventricle size (black arrow) and some loss of CA3 neurons (red arrow) (6A). Results are quantified (6D)

Immunohistochemistry

Ki67: Quantification of Ki67 labeled cells occurred on a wide field fluorescent microscope (Figure 7). Due to autofluorescence observed in the damaged brains, cells identified as positive for Ki67 were required to not fluoresce in the green channel (Figure 7D-E). Cells positively labeled were identified and counted by two blind experimenters and then averaged to get a final measure. Inter-rater reliability was good ($r^2 = 0.65$) A three way ANOVA on cell count averages revealed that there was a main effect for surgery group [$F(1,50)= 5.182$, $p < 0.05$) were sham rats had significantly more Ki67 cells than rats that received dentate gyrus colchicine injections. Additionally, the main effect was found for the housing condition [$F(1,50) = 10.042$, $P < .005$], where rats placed in the enriched environment had significantly less Ki67 labeled cells than the enriched group.

DCX: DCX-positive cells were identified and counted by two blind experimenters over three sections per brain and the counts were averaged to achieve a final measure (Figure 9). A three-way ANOVA on cell count averages revealed that there was a main effect [$F(1,50)= 606.5$, $p< .001$) for surgery where sham rats (Figure 9A) had significantly more DCX-positive cells than rats with lesions (Figure 9B). A significant interaction ($F(1,50)= 4.165$, $p <.05$) of drug by housing occurred where rats in the fluoxetine standard housing group had significantly more DCX-labeled cells than the other groups. Experimenters had a high inter rater reliability rating ($r^2 = 0.98$).

BrdU and NeuN. Analysis of BrdU and NeuN double-labeled cells were performed qualitatively. Confocal microscope images at 20x and 40x demonstrated the presence of three week old neurons in every rat (data not shown) Z-stacks (Figure 9B) were used to confirm the presence of both labels in each cell. There was an observable difference between sham and surgery rats (Figure 9A, C, D).

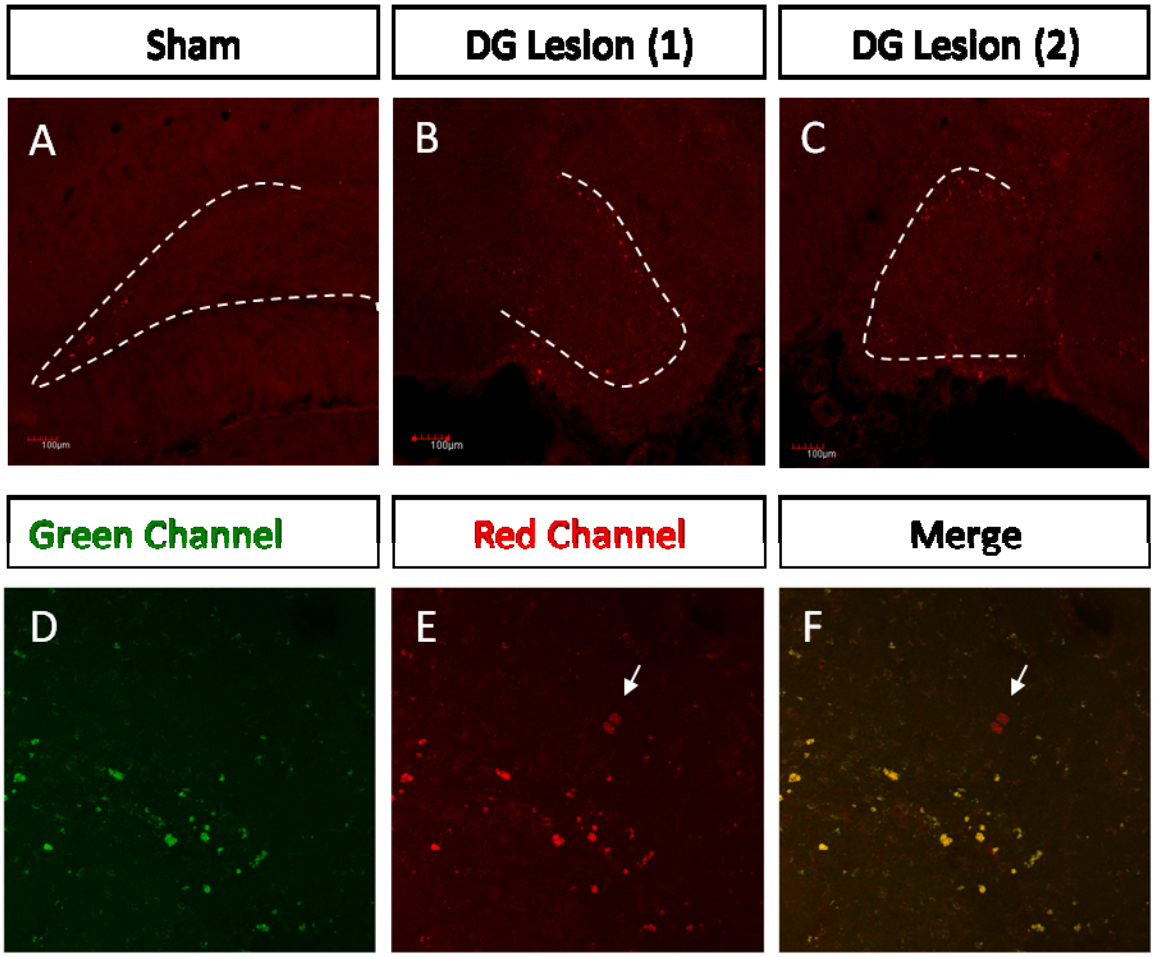


Figure 7. *Representative micrographs of Ki67 staining*. Ki67 staining appears in red in images (20x) taken of the dentate gyrus (DG) (represented by dashed white line) for sham operated (5A) and DG lesion operated rats (5B-C). Staining occurs along the inside of the DG for both surgery conditions and there appears to be an increase of Ki67 stained cells in the DG lesion condition. However, it was observed that autoflourescence occurred frequently in the damaged brains. Therefore, Ki67 cells were identified (white arrow) if they did not fluoresce in the green field (5D) but did in the red field (5E). Merging the images demonstrates this effect and shows two Ki67 cells that are dividing (5F).

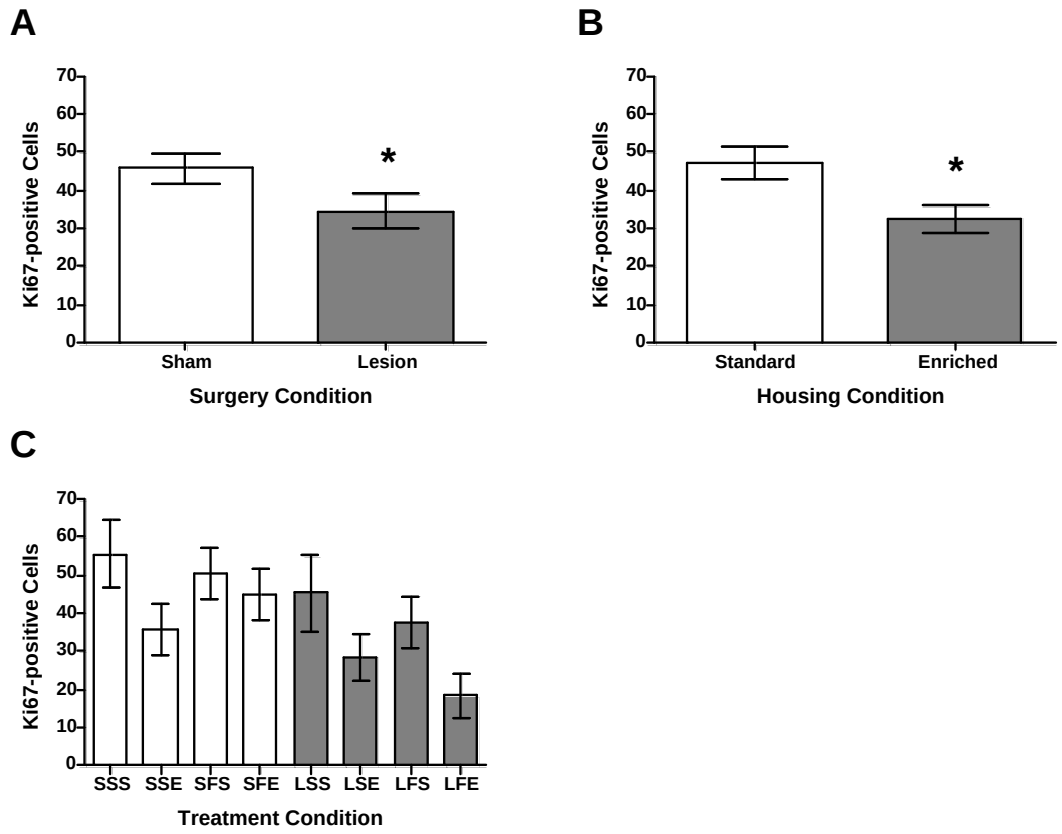


Figure 8. *Quantification of Ki67-positive cells*. Statistical analysis revealed there were significantly more Ki67-positive cells in sham rats compared to rats with a DG lesion (8A) and in standard housed rats compared to environmentally enriched rats (8B) (*, $p < .05$). Comparison of all treatment groups are also shown (8C).

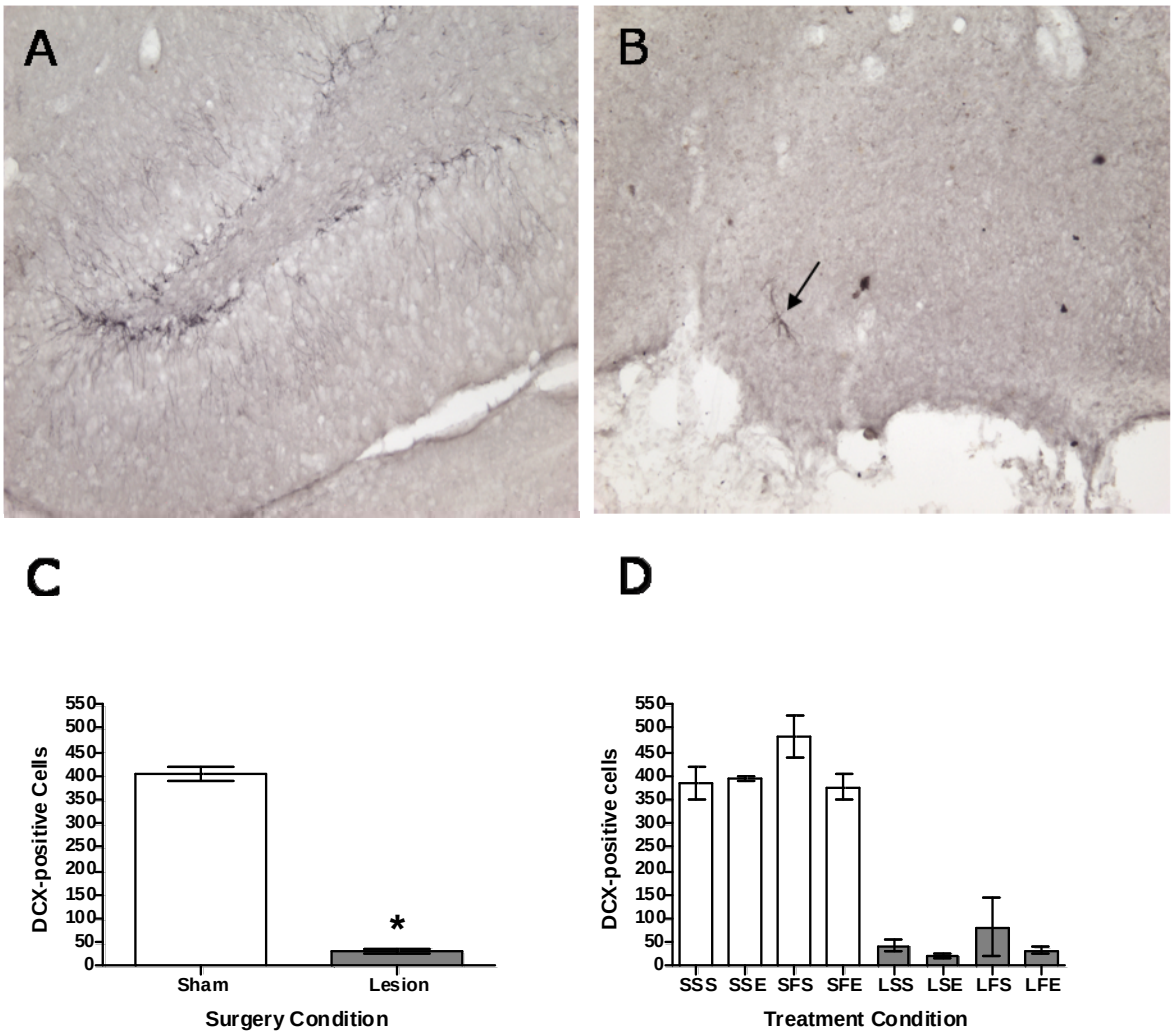


Figure 9. *Representative light micrographs of DCX staining*. DCX staining appears as dark brown on images (20x) taken of the dentate gyrus (DG). In the sham condition (9A), cell body staining appears along the granule cell layer with processes extending outward. In the lesion condition (9B), granule cell layer was mostly absent and there were very few cells observed (black arrow). Analysis revealed there were significantly more DCX-positive cells in the sham rats compared to rats with a DG lesion (9C) (*, p <.001). Comparison of treatment groups are shown (9D).

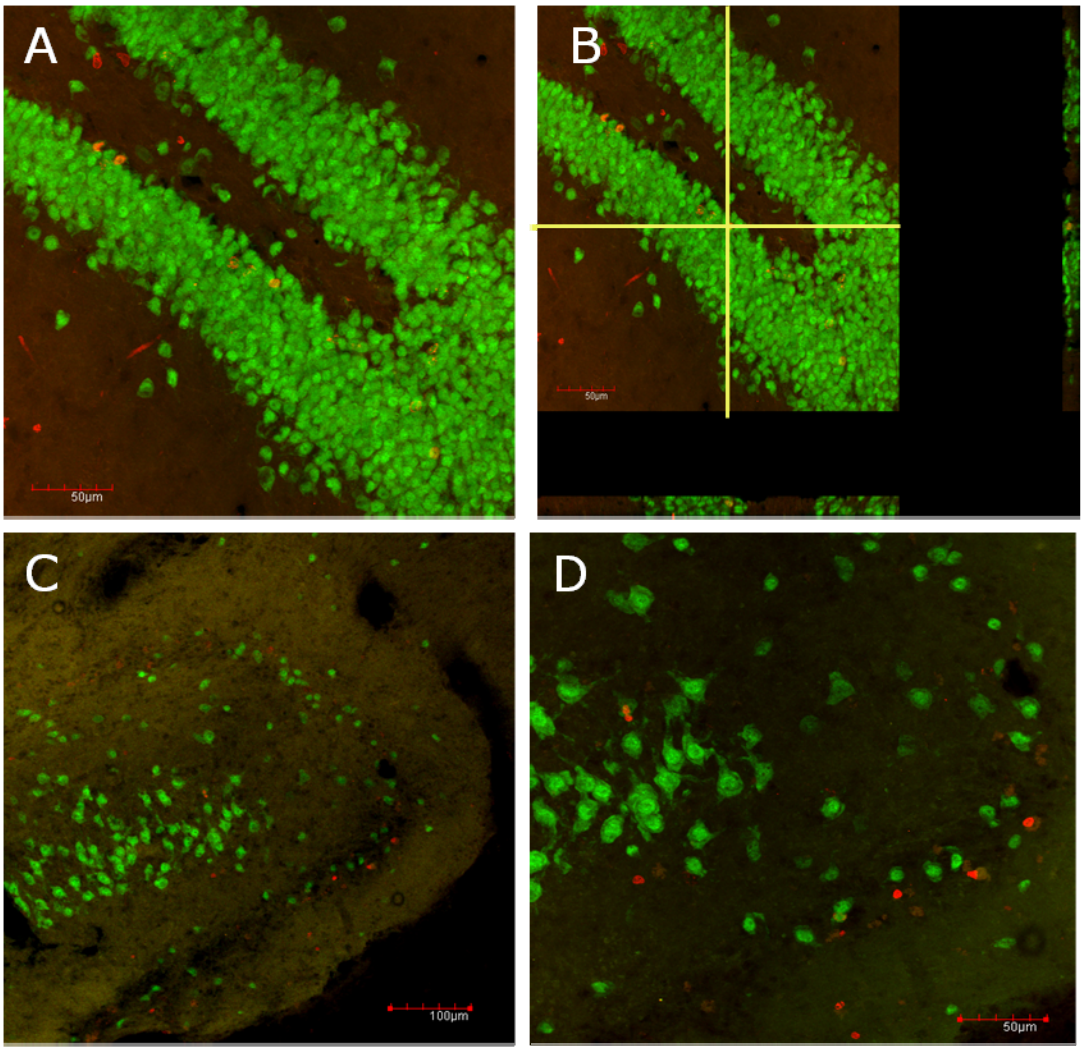


Figure 10. *Representative confocal micrographs of BrdU/NeuN staining*. BrdU labeled cells are appear as red, and NeuN labeled cells appear as green. In the sham condition images (40x) revealed a dense granule cell layer labeled with NeuN with BrdU staining occurring along the inner portion (10A). Z-stacks confirmed the presence of double-labeled cells (yellow lines, 10B). In the lesion condition (10C) images (20x) show there was a near complete loss of the granule cell layer and higher magnification (40x) demonstrates that there were a number of BrdU labeled cells along the remanants of the granule cell layer (10d).

Behavioral Testing

Pre-treatment Behavioral Testing: In the pre-treatment phase of behavioral testing, spatial memory for each rat (n=66) was assessed on the Morris water maze. Pre-testing occurred following a recovery period from surgery and consisted of 4 trials for 3 consecutive days. The latency average of the third day for each rat was used to assign groups by separating lesion and sham groups and matching to drug and housing groups. As a result, each surgery subgroup had similar means and standard deviations for latency as well as velocity and distance (Table 2). As expected, the latency average for the lesion group was much higher during the three testing days, demonstrating that hippocampal colchicine injections caused spatial memory deficits. Lesion groups were not ideally matched due to the removal of one rat during treatment in the fluoxetine-enriched group with a poor pre-treatment performance. Rat spatial memory performance of treatment groups was analyzed using a three-way repeated measures analysis of variance (ANOVA) which revealed that there was no significant main effect of drug treatment [$F(1,51)= 1.619$, $p= .209$] or housing treatments [$F(1,51) = 1.287$, $p= .262$]), only for surgery [$F(1,51)= 58.161$, $p< .001$] and day as shown in Figure 10).

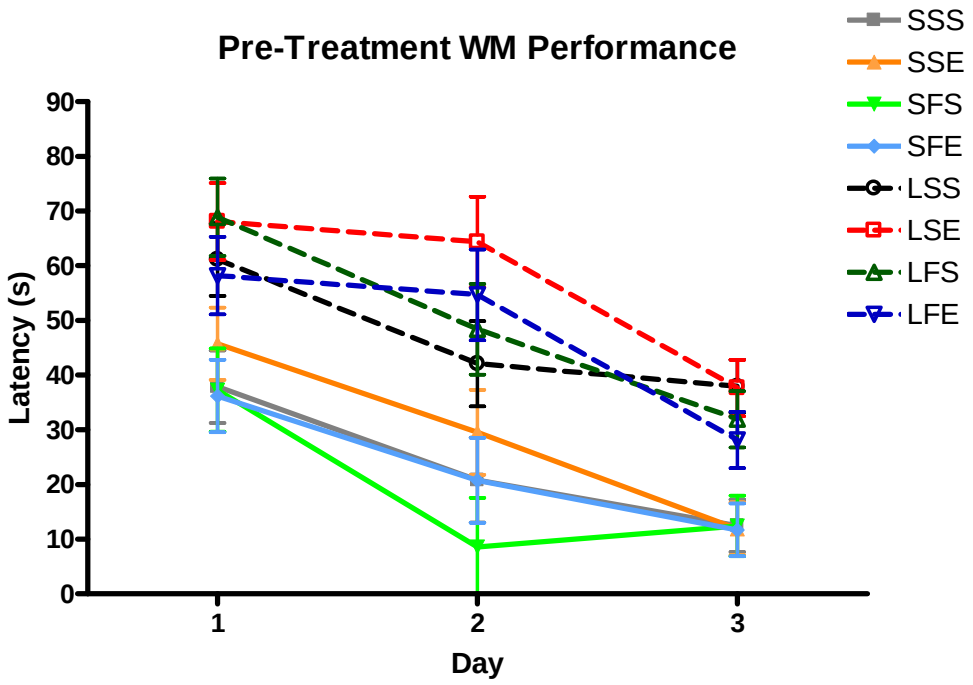


Figure 11. *Pre-treatment performance on Morris water maze*. Graph showing average latency(s) to find platform on each pre-treatment day for each treatment group.

	SSS	SSE	SFS	SFE	LSS	LSE	LFS	LFE
Latency	12.5(3.6)	12.6(4.0)	12.2(2.6)	12.1(2.5)	37.4(5.5)	37.1(6.9)	36.7(7.1)	28.8(4.8)
Distance	105.2(43.4)	107.8(27.1)	98.3(23.5)	105.5(22.0)	351.6(63.2)	332.5(67.2)	348.1(74.3	269.3(53.6)
Velocity	9.5(0.4)	9.7(0.4)	8.4(0.8)	9.0(0.3)	9.4(0.5)	9.0(0.5)	10.0(0.6)	9.7(0.4)

Table 2. *Matching to groups from pre-treatment performance*. Average latencies on last day of pre-treatment were used to match rats to treatment groups and swim distance and velocity measures are included. Values are shown as mean (SEM).

Post-treatment Acquisition. Following drug and housing treatment, spatial memory was retested in the Morris water maze at platform locations opposite the pre-testing phase. Rats were given 4 trials per day for 8 days. A 3-way repeated-measures three-way ANOVA on escape latency revealed that there was a main effect for surgery [$F(1,51)= 56.74$, $p< .001$] where sham rats performed significantly better than lesion rats in escape latency. The analysis revealed a main effect for day [$F(1, 51)= 14.33$, $p< .001$] due to the improvement of rats in all groups across days 1-8. A day x surgery interaction was revealed [$F(1, 51) = 2.70$, $p = .019$), which reflects the continual improvement of the lesion group and the floor effect of the sham group (Figure 12). In days 6-8, in the post-treatment phase, surgery rats approached sham rats suggesting that they learned the task, albeit at a slower rate than intact rats. In addition, a day by housing interaction occurred [$F(1, 51)= 2.28$, $p < .05$] where the enriched group showed a significantly more improvement than did the standard housing group.

Post-treatment Probe Analysis: On the ninth day of post-treatment, rats were given a probe trial where they were placed in the water maze without a platform for 60 seconds while their swim time and location were recorded. Performance on the probe trial was measured by the percentage of time spent in target quadrant and by the number of target crossings. A three-way ANOVA revealed that here was no effect for surgery on either target quadrant percentage and target crossing frequency suggesting that the similar times seen during last days of acquisition carried over to the probe test. A main effect was found for housing [$F(1,50) = 4.194$, $p < .05$] where environmentally enriched rats spent a significantly longer time in the target quadrant than rats in the standard housing group. Additionally there was a significant interaction of surgery by

housing [$F(1,50) = 9.565$, $p < .05$] where, interestingly, rats in the lesion and enriched groups outperformed all other groups as shown in (Figure 13).

An alarming side effect of chronic fluoxetine injections was that in many of the fluoxetine- and not saline-treated rats, there appeared to be significant damage to many of their internal organs like the intestine and stomach near the injections site. These harmful side effects could have altered fluoxetine's positive influence on cell proliferation by causing pain and raising stress levels. In the MWT, fluoxetine did not affect performance swim speed which suggests that the damage was not debilitating, but may have had other nonspecific effects.

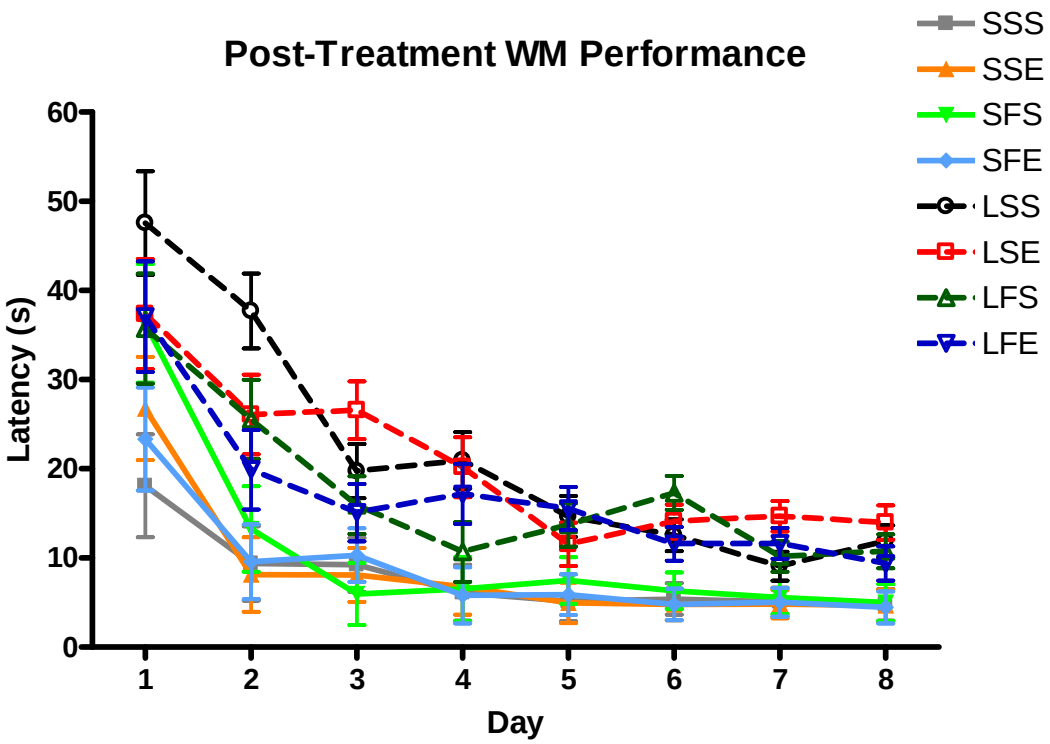


Figure 12. *Post-treatment performance on Morris water maze. Graph* showing average latency(s) to find platform on the 8 post-treatment days for each treatment group.

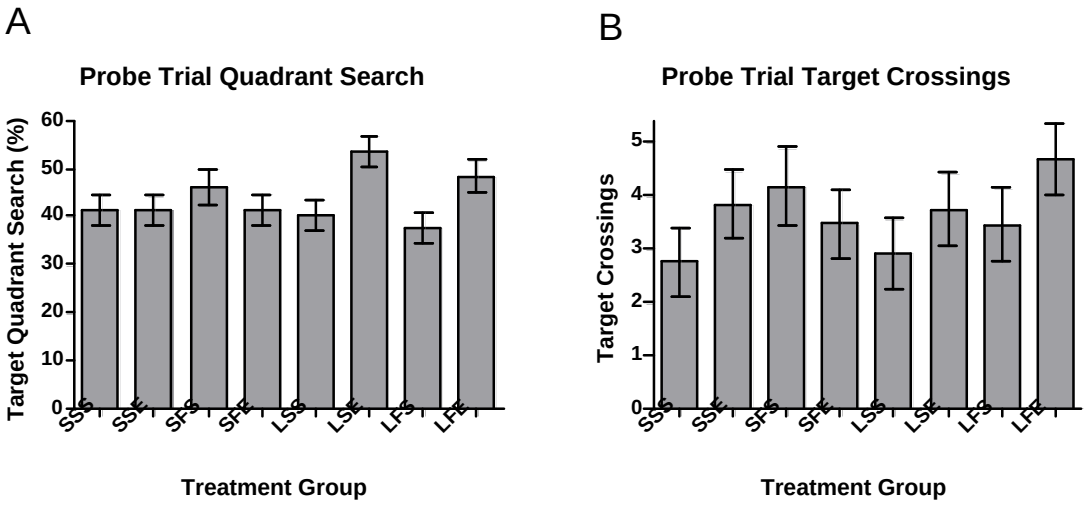


Figure 13. *Probe-trial performance*. Treatment groups are compared for the probe- trial measures quadrant search (13A) and target crossings (13B).

DISCUSSION

In the current investigation, we attempted to address two main hypotheses. The first posited that selective ablation of dentate granule cells would lead to observable behavioral deficits in performance on the Morris water maze. We found that four-site bilateral infusion of colchicine (1.25 mg/ml) into the dorsal dentate gyrus (DG) caused a near complete ablation of the dentate granule cells (97%). Rats with this lesion demonstrated significant performance deficits in the Morris water maze (MWM) on pretreatment testing. Intradentate colchicine infusions lead to a nearly 3-fold increase in latency average on the third day of testing in the absence of swim speed deficits. The second hypothesis was that treatments that increased hippocampal adult neurogenesis would improve functional recovery in the MWM. To address this hypothesis we attempted to influence the regeneration of granule cells by increasing the rate of progenitor cell proliferation through chronic flouxetine injections given systemically and the rate of neuronal survival by placing rats in an enriched environment. Unexpectedly, systemic injections of fluoxetine increased cell survival as demonstrated by an increase in doublecortin (DCX)-expressing neurons compared to saline injections in the standard housing condition. An equally surprising result was that environmental enrichment decreased rates of proliferation as assessed by Ki67 labeled cells compared to standard housing. Furthermore, DG granule cell ablation significantly decreased cell proliferation and the survival of new neurons suggesting the establishment of an unfavorable microenvironment for the regeneration of neurons.

Histological analysis of the hippocampus demonstrated that the colchicine dose chosen (1.25mg/ml) caused a near complete loss of granule cells of the superior and inferior blade of the fascia dentata. The selective loss of granule cells following colchicine infusions into the hippocampus is consistent with what has been previously reported (Goldschmidt and Steward 1982, Walsh 1986, Jeltsch et al. 2002). Some cells were lost in the CA3, CA1 and subiculum, however the loss was generally isolated near the infusion site and did not pervade throughout the rostracaudal axis of the hippocampus. Overall the size of the hippocampal formation was reduced and surrounding ventricles were enlarged compared to sham rats. The extent of DG damage did not vary much across subjects and there was no evidence of misplaced or incomplete infusions. We used a 4-site bilateral 1.25 mg/ml infusion which is smaller dose than the 3-site 1.75 dose that we have reported previously in our lab that is sufficient in causing spatial memory deficits (Keith, Wu, Epp, & Sutherland, 2007). However, we observed increased DG damage from the 4-site infusion. The most likely explanation is that the 4-site dorsal-ventral coordinates targeted the dorsal DG and did not vary as much as in the 3-site injection resulting in a higher concentration of colchicine per brain volume and more complete damage along the rostrocaudal axis.

To address our second hypothesis we used a Ki67 antibody to label proliferating cells and immature neurons in the dentate gyrus following treatment. We expected fluoxetine to cause increases in proliferation and environmental enrichment to be associated with increases in cell survival, however this was not the case. Fluoxetine did not increase numbers of Ki67 cells, evidence that it was not affecting cell proliferation at the 44 day time point. Fluoxetine was however associated with an increase in DCX-

labeled cells in rats living in the standard housing group compared to the enriched environment. As previous experiments have repeatedly associated increased cell proliferation with chronic fluoxetine treatment (Malberg et al., 2000), and not cell survival, this result came as a surprise. In addition, other experiments have associated enriched environments, similar to ours, with increases in both cell proliferation and neuronal survival (Kemperman et al., 1997; Nilson, Perfilieva, Johansson, Orwar, & Eriksson, 1999). However in our case, we saw that environmental enrichment did not affect the number of DCX- positive neurons but did show an overall decrease in Ki67-positive cells.

Although these findings appear to be inconsistent with the literature concerning environmental enrichment, evidence from experiments looking at the role of stress on neurogenesis may explain this result. In our typical lab protocol, paired rats are placed together when they arrive at the lab and remain that way throughout an experiment. In this experiment, rats are initially paired, undergo surgery and recovery, are matched to a group and then placed in an environment with increased overall space, but not space per rat and without acclimation to the other rats. Compounding this problem is that damage to the hippocampus has been associated with deficits in emotional regulation and object recognition which may have extended to other rats (Clark, Zola, & Squire, 2000). In the enriched environment we observed increased fighting and bite wounds compared to what we typically see in paired housing so it is likely that the enriched environment not only was more complex, but more stressful than the standard housing. A number of reports that have found that increases in stress hormones either by pharmacological or environmental factors caused decreases in proliferation and

neuronal survival (Cameron & Gould, 1994; Dranovsky & Hen, 2006) Moreover, a rat's position in the social dominance hierarchy is a typical outcome of enriched environments and has been related to changes in neurogenesis with the subordinate rats having lower levels (Kozorovitskiy & Gould, 2004). In our case, it is quite possible that the stressful properties of the enriched environment are the cause of the decreased cell proliferation compared to standard housing. What is not accounted for, however, is that fluoxetine has been able to reverse the down regulation of DG neurogenesis caused by stress (Malberg & Duman, 2003) which our data does not support. Fluoxetine and saline-treated rats in the enriched environment did not differ in Ki67- and DCX-expressing cells.

Surpisingly, fluoxetine treatment increased neuronal survival in the standard housed rats but had no effect on proliferation which may be due to the time course of treatment. Rats received fluoxetine injections for approximately 7 weeks, with behavioral testing occurring on the last 9 days. Immediately following testing rats were perfused and their brains were harvested. Ki67 is thought to be expressed in cells that are undergoing mitosis immediately preceding sacrifice (Endl & Gerdes, 2000). Fluoxetine has been shown to increase proliferation in as little as 2 weeks and begin to plateau at 6 weeks (Malberg et al., 2000). It may be that when the brains were taken the proliferation effect of fluoxetine had returned to normal levels. The increase in doublecortin positive neurons seen in the fluoxetine group follows the time course of DG adult neurogenesis. Fluoxetine may have increased proliferation through weeks 2-6, and from these cells the peak of doublecortin expression would have occurred during at weeks 4-7 consistent with when they would have differentiated into immature neurons

and began their synaptic integration (Zhao et al., 2008). The fluoxetine effect seen in the standard housing may be predicted by the increased number of proliferating cells caused by fluoxetine were being sustained in an standard housing which may have been more conducive to cell survival. Injections of BrdU at days 16-19 theoretically would be labeling new cells during the window where others see increases in neurogenesis associated with fluoxetine, unfortunately quantification of BrdU-labeled cells did not occur.

DG lesions caused a dramatic deficit in swim task performance. In pre-treatment testing that occurred one week following surgery, rats with DG lesions performed significantly worse than sham rats during the first three training sessions. In addition, sham rats improved more rapidly and demonstrated near mastery by the third day as their swim distance nearly equaled the distance to the platform. Lesions did not affect the ability to swim as measured by swim velocity, as seen previously (Keith et al., 2007) and we conclude that this effect was due to spatial memory deficits. In the post-treatment group, rats were tested on a new platform location and a similar effect was seen during the eight days where DG lesion rats had significantly longer platform latencies without an effect on swim speed. Sham rats appeared to master the task by day two as opposed to DG lesion rats where they had higher latencies, but improved and did reach a stable latency by day 5. Overall, this behavioral data demonstrates that dentate gyrus lesions caused significant deficits in spatial memory and that rats with DG lesions were able to improve significantly over the 8-day training period. Our results are consistent with previous experiments in rats with full and partial hippocampal damage in the Morris water maze (Nanry, Mundy, & Tilson, 1989) and in navigating a complex

environment (Jarrard, 1995; Winocur, Moscovitch, Fogel, Rosenbaum, & Sekeres, 2005) as well as a similar pattern of delayed learning of a new environment by the hippocampal-damaged amnesic patients, H.M. and K.C (Rosenbaum et al., 2000; Corkin, 2002). Taken together this data suggests that the initial spatial memory deficits seen in full hippocampal damage may be mediated specifically by the dentate gyrus.

Evidence suggests that hippocampal damage can disrupt the learning of a new reward specific location (Winocur et al., 2005). In our experiment, the platform location was reversed following relative to its pre-treatment location after five weeks. Therefore, surgery or treatment condition may have had an effect on long-term memory storage that could be seen at the beginning of post-treatment testing. Secondary analysis, on the first trial of the first day revealed that there was not a bias effect to the old platform location suggesting that surgery or treatment group did not affect any location-specific, long-term memory.

On the ninth day post-treatment, a probe test in the absence of a platform was used to determine retention of the platform location. The probe trial analysis assumes that the more time spent in the target quadrant serves as a positive measure of spatial memory. Interestingly, the lesion group outperformed the sham group and the enriched lesion group dwelled in the target quadrant for the largest percentage of the trial. One interpretation is that rats with DG damage adapt by developing compensatory strategies to navigate their environment. For instance, the presubiculum and areas of the sensory cortex are involved in what is termed egocentric spatial memory, where body positioning serves as the navigational cue (Burgess, Maguire, & O'Keefe, 2002). This type of learning would have been spared and in the absence of allocentric cue learning,

proprioceptive sensory information may have been sufficient to locate the general area of the platform, but not the exact location. In the enriched environment, they experience long-term exposure to a demanding learning environment may have enhanced this effect. This is supported by the improvement of the enriched group during the early period of the acquisition phase. Another interpretation of these results may be that the probe analysis does not specifically measure spatial memory retention, but instead or additionally measures resistance to extinction. If this were the case, the sham rats would have rapidly extinguished searching for the platform in the learned location while the lesion rats, having extensive hippocampal damage, may have a deficit in their ability to extinguish their response. The inhibitory model (Gray & McNaughton, 2000) proposes that the hippocampus isn't specifically involved in spatial memory but a more simplistic process of inhibiting responding that have negative consequences. This model was developed following behavioral observations in rats with hippocampal lesions trained on classical conditioning experiments. Rats with lesions simply do not extinguish responses associated with a negative affect (Davidson & Jarrard, 2004). Our data appears to support the inhibitory model, as the negative consequence of not finding the platform location appears to be resistant to extinction in rats with lesions more than those with intact hippocampi.

Extensive loss of the DG resulted in a large decrease in Ki67- and DCX-positive cells. In other experimental paradigms, brain damage has been associated with increases in neurogenesis, however this is likely due to differences in the damage model. In our rats the loss of neurogenesis in rats with lesions is may be due to the overall damage to the DG which likely included supportive cells. Without

oligodendrocytes or glial support, neural precursors would not have been able to be generated, migrate, or survive. In addition, one common cellular response to invasive brain injury is the release of cytokines (Wang & Shuiab, 2002), secreted inflammatory molecules that have been known to prevent neurogenesis and glial scarring, the formation of a glial barrier around damaged areas (Silver & Miller, 2004) These are mechanisms that the brain uses to prevent a spreading of neuronal damage by suppressing neuron action and to isolating the damaged area for a period following injury (Silver & Miller, 2004). The response may have suppressed neurogenesis and prevented new neurons from migrating to the damaged area of the DG. When the brains of DG lesion rats are examined more closely, there appears to be a transition zone from high to low damage in the caudal, more ventral areas the hippocampus and in some instances there were large clusters of DCX-expressing cells suggesting that a smaller dose of colchicine may have resulted in a cellular environment more permissible to neuronal survival. In noninvasive injury models such the use of arterial clamping to model ischemia, there would presumptively be less glial scarring. In addition ischemia is known to promote neurogenesis by increasing in pro-neurogenic growth factors like BDNF and NGF and proteins associated with synapse remodeling like CPG15 (Lindvall, Kokaia, Bengzon, Elmer, Kokaia, 1994; Croll & Weigh, 2001; Han et al., 2007). A growing body of literature has demonstrated successful attempts at obtaining recovery of function using enriched environment and ischemia models of DG damage which supports our original hypothesis (Nilsson et al., 1999; Dahlqvist et al., 2004).

The novel treatment we employed did not appear to be successful in promoting recovery of DG function. There are a number of methodological difficulties that emerged

that may have prevented us answering our experimental question. First, the dose and or locations of colchicine infusions caused more damage then we desired. The neurogenic niche and supportive tissue were likely compromised, as a result there was a lack of supportive cells and extracellular cues necessary to rebuild the DG. In future experiments, lowering the dose or varying the injection sites as well as other methods of DG damage such as ischemia or hypoxia may be more successful in identifying treatments that promote recovery of function using an endogenous neuron replacement strategy. Currently there are multiple labs working on creating transgenic mouse lines that use inducible transgenes to selectively ablate granule cells by activating apoptotic pathways. This may prove to be very useful, not only in looking at functional recovery, but identifying the putative role of the dentate gyrus in learning and memory. Second, the novel enriched environment may not have been beneficial to neurogenesis or functional recovery. Although using a more established environment with more space may have yielded more positive results, it highlights the limitations of enriched environments. It is not known how the enriched environment promotes neurogenesis and increases learning and whether they are directly related. Until aspects of the EE are systematically tested for their contribution both alone and in combination, similar mistakes are likely to occur. Third, solely assessing spatial memory may not be adequate or sensitive enough in demonstrate a recovery of function. There may have been other cognitive tasks for instance trace eyeblink conditioning that may have benefited from increased adult neurogenesis. In the future, employing a battery of behavioral tests will increase the odds of identifying functional recovery.

Finally, there are conceptual issues that need to be addressed and may be improved upon in future experiments. First, the role of adult neurogenesis in learning and memory remains unknown with the evidence for being correlational. Therefore, the idea of triggering adult neurogenesis as a therapy for improving learning and memory is a bit presumptive. Experiments focused on identifying the specific properties of new neurons and then triggering or blocking those properties to change learning and memory may prove to be more successful at functionally demonstrating its role in learning and memory. Secondly, although fluoxetine and environmental enrichment have a reliable and robust positive relationship with adult hippocampal neurogenesis, they have very complex neurobiological actions. Using direct modulation of neurogenesis like administering the trophic factors VEGF and BDNF that are required for the actions of antidepressants and enriched environment on proliferation or neuronal survival may prove to be more effective as well as less mysterious. Lastly, it is probable that many more parameters of the neurogenic niche need to be controlled before a neuron can become functional and not maladaptive. Some of these parameters may include decreasing the activity of microglia that produce inflammatory cytokines, inhibiting the formation of the glial scar, using extracellular cues to direct proper migration and promote integration into the correct circuitry. Coupling a cocktail of techniques that specifically inhibit and promote the expression of these pro-neurogenic factors, for instance blocking expression of a specific inflammatory cytokine with RNA interference and administering a peptide that increases glutamatergic integration directly to the site of injury, along with a environment that encourages a targeted

behavior like spatial learning may prove to be an effective strategy for increasing functional recovery.

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