THE EFFECTS OF SPATIAL LEARNING ON ADULT NEUROGENESIS IN THE DENTATE GYRUS

by

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ABSTRACT

In the hippocampus, unlike most areas of the mammalian brain, new neurons are continuously produced throughout life. Studies have demonstrated that spatial learning, a process dependent on the hippocampus, regulates neurogenesis (i.e. the survival of new neurons) in the hippocampus. Studies have found that spatial learning either causes an increase, no change, or a decrease in neurogenesis. It was the goal of this thesis to determine under what conditions neurogenesis is increased, decreased or unaffected by spatial learning using the Morris water task. Experiment 1 demonstrated that there was a critical period in the developmental of new neurons in adult rats, at 6-10 days old, during which spatial learning can increase neurogenesis. Experiment 2 showed that spatial learning at a later time point (days 11-15) decreased cell survival but this decrease occurred gradually and as a result may only be seen if several days are waited after training prior to examining levels of neurogenesis. Experiment 3 demonstrated that although neurogenesis may be increased if spatial training occurs when the new neurons are 6-10 days old in adult rats, this effect can be eliminated or reversed by increasing the difficulty of the spatial task. Finally, in Experiment 4 I showed that the effect of spatial learning is affected by the strain of rats used, possibly due to differences in the rate of maturation of new neurons. Sixteen day old bromodeoxyuridine-labeled cells were increased in both Sprague-Dawley and Long-Evans rats following spatial learning but doublecortin-labeling (which labels a broader age of new neurons) is increased only in Sprague-Dawley rats after spatial learning. Thus, numerous methodological factors must be considered when examining the effects of spatial learning on neurogenesis. There are likely more factors (i.e., stress, age, sex, etc.) that interact with spatial learning and neurogenesis than described here however these studies have clarified many prior conflicting studies.
TABLE OF CONTENTS

ABSTRACT ............................................................................................................................. ii

TABLE OF CONTENTS ........................................................................................................ iii

LIST OF TABLES .................................................................................................................. vi

LIST OF FIGURES ............................................................................................................... vii

ABBREVIATIONS ................................................................................................................ ix

ACKNOWLEDGEMENTS ..................................................................................................... xi

CO-AUTHORSHIP STATEMENT .......................................................................................... xii

1 INTRODUCTION .............................................................................................................. 1

Hippocampus structure and function .................................................................................. 1

Germative regions in the adult brain .................................................................................... 3

Neurogenesis in the adult hippocampus ............................................................................... 5

The process of adult neurogenesis ..................................................................................... 6

Immunohistochemical markers of adult neurogenesis ......................................................... 8

Function of adult generated neurons .................................................................................. 10

Thesis experimental outline ............................................................................................... 18

References .......................................................................................................................... 21

2 HIPPOCAMPUS-DEPENDENT LEARNING PROMOTES SURVIVAL OF NEW
NEURONS IN THE DENTATE GYRUS AT A SPECIFIC TIME DURING CELL
MATURATION 1 .................................................................................................................... 32

Experimental procedures .................................................................................................. 35

Results ................................................................................................................................... 40

Discussion .......................................................................................................................... 55
3 ACTIVATION AND SURVIVAL OF IMMATURE NEURONS IN THE DENTATE GYRUS WITH SPATIAL MEMORY IS DEPENDENT ON TIME OF EXPOSURE TO SPATIAL LEARNING AND AGE OF CELLS AT EXAMINATION

Experimental procedures ................................................................. 74
Results .................................................................................................. 81
Discussion ........................................................................................... 93
References .......................................................................................... 104

4 TASK DIFFICULTY IN THE MORRIS WATER TASK INFLUENCES THE SURVIVAL OF NEW NEURONS IN THE DENTATE GYRUS

Experimental procedures ................................................................. 111
Results .................................................................................................. 118
Discussion ........................................................................................... 129
References .......................................................................................... 138

5 STRAIN DIFFERENCES IN NEUROGENESIS AND ACTIVATION OF NEW NEURONS IN THE DENTATE GYRUS IN RESPONSE TO SPATIAL LEARNING

Experimental procedures ................................................................. 146
Results .................................................................................................. 154
Discussion ........................................................................................... 167
References .......................................................................................... 176

6 GENERAL DISCUSSION

Interpretations ...................................................................................... 182
Limitations .......................................................................................... 188
Future experiments .............................................................................. 191
Conclusions ......................................................................................... 193
References .................................................................................................................................................. 194

APPENDICES ........................................................................................................................................ 198

Appendix A: zif268 expression in the dentate gyrus .............................................................................. 198

Appendix B: corticosterone concentrations following MWT training .................................................. 199

Appendix C: UBC animal care certificates ............................................................................................ 200
LIST OF TABLES

Table 1.1: Previous studies investigating the impact of spatial learning on cell survival ..........20
Table 2.1: Percentage of BrdU-labeled cells also expressing NeuN ..................................45
Table 2.2: The number of rats classified as ‘good’ or ‘poor’ learners ..................................47
Table 2.3: BrdU labeling in ‘good’ or ‘poor’ learners ..........................................................49
Table 2.4: Volume of the GCL and hilus for each group ......................................................55
Table 3.1: Timeline of training and perfusion ....................................................................75
Table 3.2: Volume of the GCL and hilus from each group ..................................................93
Table 4.1: Cells counted in the hilus for each group .........................................................127
Table 4.2: Percentage of BrdU labeled cells that also express NeuN .................................129
Table 4.3: Volume of the GCL and hilus from each group ................................................129
Table 5.1: Volume of the GCL from each group .................................................................158
Table 5.2: Total number of BrdU-labeled cells counted in the hilus ....................................159
Table 5.3: Density of BrdU-labeled cells in the GCL .........................................................159
Table 5.4: Percentage of BrdU/NeuN double-labeled cells in the GCL ...............................165
Table 6.1. Previous Studies on the effects of spatial learning on cell survival ..................185
LIST OF FIGURES

Figure 1.1: Anatomical representation of the hippocampus (A) and a detailed view of the dentate gyrus (B).............................................................................................................................................................. 2

Figure 1.2: Cartoon depiction of the timeline of the process of adult neurogenesis in the dentate gyrus........................................................................................................................................................................ 7

Figure 2.1: Photomicrographs of BrdU-labeled tissue ................................................................................................................................. 39

Figure 2.2: Latency to reach the hidden platform during acquisition of the MWT for place and cue trained rats and corresponding BrdU labeling in the GCL and hilus ............................................ 43

Figure 2.3: Mean density of BrdU-labeled cells in the GCL of good and poor learning rats..... 50

Figure 2.4: Correlations between BrdU-labeled cells and performance in the MWT for rats trained on the place and cue versions .................................................................................................................. 51

Figure 2.5: Correlations between BrdU-labeled cell survival and performance in the MWT for rats trained on the place and cue versions of the task ........................................................................ 53

Figure 3.1: Representative labeling of BrdU and c-fos ................................................................................................................................. 80

Figure 3.2: Latency to reach the platform for each session ........................................................................................................................... 82

Figure 3.3: Total latency to reach the platform across all trials and sessions .............................................................................................. 84

Figure 3.4: Performance during the probe trial ........................................................................................................................................ 85

Figure 3.5: The total number of BrdU-labeled cells in the GCL and hilus .................................................................................................... 87

Figure 3.6: The percentage of BrdU-labeled cells that express c-fos ........................................................................................................... 89

Figure 3.7: Correlations between BrdU/c-fos double labeling and learning and memory ........ 90

Figure 3.8: The percentage of BrdU-labeled cells that express NeuN ...................................................................................................... 92

Figure 4.1: The layout of the testing room .............................................................................................................................................. 113

Figure 4.2: Photomicrographs of BrdU labeled cells ................................................................................................................................. 117
Figure 4.3A: The mean latency to reach the platform during each session.................................120

Figure 4.3B. The total latency across all training sessions and the total latency across the first
two trials of each session........................................................................................................121

Figure 4.4A: The mean swim distance per session. .................................................................122

Figure 4.4B The Total swim distance during all trials and during the first two trials of each
session ........................................................................................................................................123

Figure 4.5. The total numbers of BrdU-labeled cells in the GCL ............................................126

Figure 4.6: The total numbers of Ki67-labeled cells in the GCL are shown. .........................128

Figure 5.1: Representative photomicrographs of BrdU, DCX and zif268 labeling..................150

Figure 5.2: Doublecortin labeling in the GCL ........................................................................151

Figure 5.3: The distance traveled during each session ............................................................157

Figure 5.4: The time spent in the virtual quadrant of the pool where the hidden platform had
been located ...............................................................................................................................158

Figure 5.5: Total number of BrdU-labeled and DCX-labeled cells in the GCL .......................162

Figure 5.6: The percentage of BrdU-labeled cells and DCX-labeled cells that also express
zif268 ........................................................................................................................................163

Figure 5.7: The percentages of DCX-labeled cells that have no processes, short unbranched
processes or long/branched processes are shown as a factor of strain (panel A) or training
(panel B) .....................................................................................................................................164

Figure 5.8: The percentage of BrdU-labeled cells that express DCX ......................................165

Figure 5.9: Correlations between the percentage of DCX-labeled cells that had long/branched
processes and total distance .......................................................................................................166

Figure A1: Zif268 positive cells in the dorsal GCL ..................................................................198

Figure B1. Corticosterone concentrations following MWT training ....................................199
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Ara-C</td>
<td>Cytosine-β-d-arabinofuranoside</td>
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<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<td>CA</td>
<td>Cornu Ammonis</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
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<td>DCX</td>
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<td>Dentate gyrus</td>
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<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<td>Granule cell layer</td>
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<td>Green fluorescent protein</td>
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<td>I.P.</td>
<td>Intraperitoneal</td>
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<td>IEG</td>
<td>Immediate early gene</td>
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<td>KCC2</td>
<td>Potassium, chloride cotransporter</td>
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<td>LE</td>
<td>Long-Evans</td>
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<tr>
<td>LTP</td>
<td>Long term potentiation</td>
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<tr>
<td>MAM</td>
<td>Methylazoxymethanol</td>
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<td>MWT</td>
<td>Morris water task</td>
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<td>NaCl</td>
<td>Sodium chloride</td>
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<td>NDS</td>
<td>Normal donkey serum</td>
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<td>NeuN</td>
<td>Neuronal nuclei</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NHS</td>
<td>Normal horse serum</td>
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<tr>
<td>NKCC1</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
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<td>Normal rabbit serum</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>PVA-DABCO</td>
<td>Polyvinyl alcohol - 1,4-Diazabicyclo[2.2.2]octane</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague-Dawley</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SGZ</td>
<td>Subgranular zone</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
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<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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1 INTRODUCTION

**Hippocampus structure and function**

The hippocampus is a highly organized structure located within the temporal lobe. The structure and function of the hippocampus appears to be highly conserved across mammalian species, however for the purpose of this thesis I will describe the rodent hippocampus unless otherwise specified. There are a number of principle subdivisions of the hippocampus including the dentate gyrus, CA1, CA2 and CA3 (Figure 1.1). The primary cells of the CA fields are pyramidal cells and the primary cells in the dentate gyrus are granule cells. Although highly simplified, the connectivity of the hippocampus is often described in terms of a tri-synaptic circuit. This circuit begins with inputs arriving in the dentate gyrus from the entorhinal cortex via the perforant path. Then, axons from the dentate gyrus granule cells project onto the pyramidal cells of CA3 via the mossy fiber pathway. The CA3 pyramidal neurons synapse onto the CA1 cells via the Schaffer collaterals (Amaral & Witter, 1989). Although it is beyond the scope of this document it should be noted that there are numerous additional intrinsic and extrinsic connections, including back propagating pathways that operate against the standard unidirectional flow through the tri-synaptic loop (for review see Amaral & Witter, 1989). The dentate gyrus is a unique three-layered sub-region within the hippocampus. The principal cell layer of the dentate gyrus is the granule cell layer, comprised predominately of densely packed granule cells. The granule cell layer is comprised of a suprapyramidal (lying closest to CA1) and infrapyramidal blade. The granule cells are aligned such that their dendrites extend into the superficial molecular layer. Between the suprapyramidal and infrapyramidal blades and bordered by the edge of CA3 is the hilus (polymorphic layer).
Figure 1.1: Anatomical representation of the hippocampus (A) and a detailed view of the dentate gyrus (B).
At the border between the hilus and the granule cell layer is a narrow region (20-50µm) referred to as the subgranular zone (see Figure 1.1). The subgranular zone contains progenitor cells that retain the ability to divide to produce new neurons in the adult brain in most mammalian species. It is the regulation of the process of adult neurogenesis by spatial learning that is the subject of this thesis.

The function of the hippocampus has been extensively studied and it is well accepted that the region plays an important role in numerous types of learning and memory including contextual memory (Rudy et al., 2002; Corcoran et al., 2005), configural representations (Sutherland et al., 1989), trace conditioning (Solomon et al., 1986; Kim et al., 1995), spatial memory (Morris et al., 1982; Sutherland et al., 1983) and is believed to be responsible for the processes of pattern separation and pattern completion (McClelland & Goddard, 1996; Leutgeb et al., 2007; Leutgeb & Leutgeb, 2007). One of the most crucial and widely accepted forms of hippocampus-dependent learning is spatial learning (Martin & Clark, 2007). The hippocampus displays a large amount of plasticity in adulthood including, but not limited to, the production of adult generated neurons and this plasticity is likely related to the ability of the hippocampus to play such an important role in learning and memory. The subgranular zone of the dentate gyrus harbors one of only two germinal regions in the brain that persist into adulthood. This region not only contains the progenitor cells that give rise to adult generated neurons but also acts as a permissive and instructive environment for the proliferation and differentiation of new granule cells.

**Germative regions in the adult brain**

The existence of progenitor cells that give rise to adult generated neurons has only been confirmed in two regions: the subventricular zone (SVZ) and the subgranular zone (SGZ,
The subventricular zone is located along the walls of the lateral ventricles and produces a continuous supply of new neurons that migrate a relatively long distance, by way of the rostral migratory stream, to the olfactory bulbs. The subgranular zone contains progenitor cells that produce new neurons that migrate only a short distance to the adjacent granule cell layer where they incorporate as mature granule cells, the principal cell type of the dentate gyrus. There have been claims of adult generated neurons outside of these two regions for example in the neocortex and striatum (Gould et al., 1999; Cameron and Dayer et al., 2008) although the existence of new neurons in these regions remains hotly debated. The immunohistochemical markers of adult neurogenesis have not gone through the same level of rigorous verification in other regions of the brain and it is possible that cell types other than neurons may express some of these markers. Furthermore, and perhaps more importantly, the identification of an immature neuron somewhere else in the brain does not necessarily indicate that it was produced locally. An immature neuron in the neocortex may have been produced in the subventricular or subgranular zone and migrated to an alternate location (Gould et al., 1999). If new neurons are in fact produced in other regions of the adult brain far fewer of them are produced than in the germative zones of the SVZ and SGZ, a fact that may be due to the specific environment needed to produce large quantities of neurons.

In order for adult neurogenesis to occur, both progenitor cells and a permissive environment are needed. It is believed that the vasculature of the subgranular zone is a critical component of this environment. Cell proliferation occurs in clusters which tend to occur around blood vessels and where endothelial and neuronal progenitor cells reside side by side (Palmer et al., 2000). Furthermore, angiogenesis and neurogenesis appear to be highly correlated in the SGZ (Palmer et al., 2000; Shen et al., 2004) suggesting that the presence of a vascular niche allows for the maintenance and proliferation of neuronal progenitor cells. In addition to this
vascular environment it is likely that adult neurogenesis is dependent upon the presence of
growth factors such as vascular endothelial growth factor (VEGF) that is present in this region
and is also involved in the angiogenic response.

**Neurogenesis in the adult hippocampus**

The intensive study of adult neurogenesis has only begun relatively recently. This is due
to the long held belief that new neurons were not generated throughout life. Instead, it was
thought that neurons were only produced during early development. However, in 1962 Joseph
Altman claimed that he had identified newly generated neurons in the rodent hippocampus.
Several more studies soon demonstrated that these new neurons persisted in three month (Kaplan
and Hinds, 1977) and even nine-month old rats (Kaplan and Bell, 1983). However, these early
reports were not widely accepted because the techniques they were based on, autoradiography
and visual morphological analysis did not provide compelling evidence that the immature cells
actually had a neuronal phenotype. In recent years the existence of adult neurogenesis has
become widely accepted due to technical advances that have allowed for definitive identification
of immature neurons in the dentate gyrus of the hippocampus as well as the subventricular
zone/olfactory bulbs (for review see Alvarez-Buylla et al., 2002). Immunohistochemical
identification of proteins specific to immature neurons as well as co-labeling of S-phase markers
with mature neuronal markers has provided much of the evidence for adult generated neurons.
The use of retrograde tracers and synaptic markers (and more recently GFP expressing mice)
have also provided evidence that the immature neurons incorporate appropriately into the
existing circuitry sending axons along the mossy fiber pathway to area CA3 and projecting
dendrites into the molecular layer. Furthermore, electrophysiological studies and immediate
early gene activation studies have provided evidence that not only do new neurons exist but that
they also mature and make functional contributions to the hippocampus (van Praag et al., 2002; Ambrogini et al., 2004; Schmidt-Heiber et al., 2004 Ramirez-Amaya et al., 2006; Kee et al., 2007; Tashiro et al., 2007; Snyder et al., 2009, 2009b).

**The process of adult neurogenesis**

Adult neurogenesis is a complex process but can be broken down into three main stages; cell proliferation, migration/differentiation and, survival (Figure 1.2). Cell proliferation occurs when a progenitor cell divides either symmetrically or asymmetrically a process that takes approximately 25 h in the rat brain (Cameron & McKay, 2001). Symmetric division can produce either two progenitor cells (expansion of the progenitor cell population) or to two daughter cells (in which case the proliferative capacity is lost). Asymmetric division gives rise to a progenitor cell and an undifferentiated daughter cell. The daughter cell(s) then migrates from the subgranular zone to the inner layers of the granule cell layer. At the same time the daughter cell undergoes a process of fate determination at which point the cell will differentiate into either a neuron or a glial cell (Cameron et al., 1993; Brown et al., 2003). Axon extension occurs within 4-10 days after cell proliferation (Markakis & Gage, 1999; Hasting & Gould, 1999; Zhao et al., 2006). Then, there is a period of continued cell maturation that lasts for weeks to months and finally results in a new mature neuron (van Praag et al., 2002; Esposito et al., 2005). During this developmental period the immature neurons first form dendritic GABAergic connections at approximately 1 week of age followed by the onset of glutamatergic signaling by 18 days of age (Espositio et al., 2005).

It is important to note that changes in neurogenesis within the dentate gyrus may be observed as a result of changes to any of the three stages. Neurogenesis may be increased by
increasing the rate of cell proliferation, increasing the percentage of cells that differentiate into neurons or by increasing the percentage of new neurons that survive to maturity.

**Figure 1.2:** Cartoon depiction of the timeline of the process of adult neurogenesis in the dentate gyrus. Blue circles = progenitor cells; green circles = adult generated neurons; red circles = activated neurons.

For example, both environmental enrichment (Kempermann et al., 2002; Brown et al., 2003; Bruel-Jungerman et al., 2005) and voluntary exercise (van Praag et al., 1999; Holmes et al., 2004) increase adult neurogenesis in the dentate gyrus. However, environmental enrichment appears to exert its effects by increasing cell survival independently of affecting cell proliferation and voluntary exercise increases cell proliferation independently of affecting cell survival (Olson et al., 2006).
Immunohistochemical markers of adult neurogenesis

One of the most widely used tools for studying adult neurogenesis, and the one applied throughout the experiments in this thesis, has been the application of immunohistochemistry to label immature neurons. The basic principle of this technique is to produce antibodies against various proteins (or exogenous compounds) that are expressed specifically by immature or mature neurons. In order to study cell proliferation there are a number of markers that are expressed as cells progress through the cell cycle such as PCNA and pH3. Currently, the most common markers of proliferation are Ki67 and BrdU. Ki67 is an endogenous protein expressed in all phases of the cell cycle except for $G_0$ and early $G_1$. Thus cells expressing Ki67 represent actively cycling cells. Bromodeoxyuridine (BrdU) is an exogenous thymidine analogue that can be injected into an animal to label cells that are in S-phase (synthesizing DNA). BrdU has a bioavailability time of approximately 2 hours (Packard et al., 1973) during which time it will become incorporated into cells that are synthesizing new DNA. Antibodies targeted against BrdU can then be used to visualize new cells later. If examined up until 24 hours later (approximately the length of time for one cell division; Cameron and McKay, 2001) a measure of cell proliferation is obtained. Longer survival periods of days to months or even years will give a measure of cell survival, or in other words, how many of the cells produced at a given time point have survived and matured until the time of sacrifice. Thus BrdU can be used as a marker of cell proliferation or survival, depending on the time of sacrifice relative to injection. It is of importance here to mention that there are some potential problems associated with the use of BrdU (for a comprehensive review see Taupin et al., 2007). For example, many studies utilize multiple injections of BrdU spread out over a period of hours or days (for example, Ambrogini et al., 2000; Dobrossy et al., 2003; Ehninger & Kempermann, 2006). The justification for this methodology is to label a greater number of dividing cells by overcoming the short
bioavailability of BrdU as well as by labeling multiple populations of cells (i.e. those going through S-phase at different times). However, this can lead to difficulties in the interpretation of results. Typically, it is important to know the age of the population of cells being examined as treatments could affect cell proliferation (such as chronic antidepressants Malberg) or survival (such as enriched environments) independently and these effects are masked by multiple injections of BrdU. Using a single injection of BrdU allows for a more accurate determination of the age of the BrdU-labeled cells. The longer the period of BrdU administration is the larger the variability in the age of the BrdU-labeled cells will be.

Another potential issue with the use of BrdU is that the proper dose must be used. High doses of BrdU are toxic in juvenile rats (Kolb et al., 1999) and can potentially disrupt neurogenesis (Qu et al., 2004). However, in adult rats a safe and not disruptive dose of BrdU (approximately 200 mg/kg) exists that also labels the majority of cells that are actively synthesizing DNA (Cameron and Mckay, 2001; Eadie et al., 2005; Hancock et al., 2009)

Finally, as BrdU is incorporated into any dividing cell it does not provide a specific marker of neurogenesis. In order to determine the phenotype of BrdU-labeled cells, co-labeling with a mature or immature neuronal marker (depending on the experimental timeline) is required. NeuN is a transcription factor that is expressed in mature neurons and is often used in neurogenesis research to determine the proportion of new cells that become mature neurons (Mullen et al., 1992). Doublecortin (DCX) is a microtubule-associated protein expressed in immature neurons during the first few weeks after proliferation (Brown et al., 2003). DCX can be used in combination with BrdU to determine the proportion of new cells that are immature neurons or by itself as a general measure of adult neurogenesis (Couillard-Despres et al., 2005). In addition to studying the number of new neurons it is also possible to co-label immature neurons with immediate early gene products (markers of cellular activation) to study the activity
of new neurons (Ramirez-Amaya et al., 2006; Kee et al., 2007; Tashiro et al., 2007; Snyder et al., 2009, 2009b). There are a wide variety of markers that can be used for this purpose but the most commonly used are c-fos, ARC and Zif268.

**Function of adult generated neurons**

Within the adult dentate gyrus there are three subpopulations of granule cells based on their production and maturation that must be considered; developmentally derived mature neurons, adult generated mature neurons and adult generated immature neurons. Adult generated mature neurons become morphologically and electrophysiologically indistinguishable from developmentally derived granule cells by 4 months of age but possibly as early as 7 weeks. (van Praag et al., 2002; Laplagne et al., 2006). Thus, the terminology “mature neuron” will be used to refer to granule cells of both developmental and adult origin that no longer possess the unique characteristics of immature neurons. Adult generated immature neurons do differ from mature neurons in terms of morphological and electrophysiological properties. For example, Immature neurons formed within the adult brain initially lack connectivity with the surrounding cellular network. However, beginning as early as 4-10 days after cell division, these new neurons extend axons into CA3 and dendrites into the molecular layer (Hastings and Gould, 1999; Markakis and Gage, 1999; Zhao et al., 2006). The growth of these projections and the subsequent formation of synapses continue over a period of several weeks culminating with adult generated neurons that have the same soma size as mature granule cell by 4 months. (van Praag et al., 2002; Esposito et al., 2005, Zhao et al., 2006). Initially for a period of 3-4 weeks these immature neurons are highly excitable (Piatti et al., 2006).

The difference in excitability between immature and mature cells in the adult brain recapitulates a phenomenon that occurs during brain development. During early development
GABA, the principle inhibitory neurotransmitter in the central nervous system, does not exert inhibitory control (Wang et al., 2000). Interestingly, the same effect can be seen in immature adult generated neurons. In fact, there is evidence that GABA depolarizes immature neurons (Ben-Ari, 2002; Ge et al., 2006). The reason GABA is able to excite immature neurons is the presence of high levels of the chloride transporter NKCC1 (Ge et al., 2006). The NKCC1 transporter causes cells to have a high internal chloride concentration allowing GABA to depolarize immature neurons. As the cells mature there is a switch in expression from NKCC1 to the chloride exporter KCC2. The KCC2 exporter causes a decrease in internal chloride concentration and the effect of GABA on the cell becomes hyperpolarization. The importance of this difference is that for a period of time immature neurons are highly excitable compared to mature neurons and as a result may confer a degree of excitability to a region that is otherwise not very excitable. A specific type of long-term potentiation (LTP), a putative mechanism of associative learning, can be induced in hippocampal slices in the absence of GABAergic inhibition as a result of the excitability of immature neurons (Snyder et al., 2001). However, either blocking the NR2B subunit of the NMDA receptor (expressed highly during development) or using irradiation to block neurogenesis prevented the expression of this type of LTP (Snyder et al., 2001). Thus, while mature neurons may not respond to weak stimulation, immature neurons are not under the same type of inhibition and are more likely to be excited. There is further evidence that immature neurons may be preferentially recruited for the storage of hippocampus-dependent learning. A study using the immediate early gene products c-fos and ARC has demonstrated that new neurons between 4 and 8 weeks of age are more likely to be activated in response to spatial memory retrieval than the existing granule cell population (Kee et al., 2007). Together, these findings suggest that adult neurogenesis is an important component of hippocampus-dependent learning.
In addition to sending projections to the granule cells of the dentate gyrus the entorhinal cortex also projects directly to area CA3 (Jones, 1993). In fact, the same fibers give rise to both the direct and indirect pathways to CA3 suggesting a duplication of information received in the dentate gyrus and CA3. If the necessary information from the entorhinal cortex reaches CA3 directly then what is the purpose of the dentate gyrus? The most widely held theory is that the dentate gyrus provides a mechanism of pattern separation (McNaughton and Morris, 1987; O’Reilly & McClelland 1994, Treves et al., 2008). Pattern separation is a process thought to be necessary to prevent the overlap between patterns of activity that correspond to similar memories. Although convincing experimental evidence has yet to be provided, there is a large theoretical framework, which implicates the dentate gyrus in the process of generating well separated patterns of cellular activity. Thus a pattern of activity in the entorhinal cortex becomes compressed in the dentate gyrus and it is this compressed pattern of activity that is strengthened within CA3. Dentate granule cells are well suited for reducing interference between different patterns of cell because they are only sparsely activated in comparison to cells in the entorhinal cortex or CA3 (Chawla et al., 2005). The theoretical role of pattern separation in the dentate gyrus does not necessarily require the continuous addition of new neurons but there are some reasons to believe that they may assist in this process (Wiskott et al., 2006; Clelland et al., 2009). As new neurons mature they progress through several developmental stages. They are initially unresponsive, followed by a period of time when they are highly excitable and finally this excitability decreases to the level of normal mature granule cells (Esposito et al., 2005). The addition of new neurons provides a continuum of neurons of different excitabilities, which corresponds to a continuum of probabilities of being activated by a pattern of inputs. Similar events that occur close together in time will activate a similar population of new neurons and different populations of mature neurons. Events occurring farther apart in time will have
different populations of immature and mature neurons activated resulting in patterns of activity that are more distinct than if the events had occurred around the same time. Thus, new neurons may aid in pattern separation by providing a mechanism for separating temporally distinct events (Aimone et al., 2006).

**Correlative evidence for an interaction between learning and neurogenesis**

There is little remaining debate over whether adult generated neurons become mature functional neurons. However, it remains somewhat unclear what specifically immature neurons are required for. There has been a great deal of interest in the possible role of adult neurogenesis in hippocampus dependent learning and memory. Several lines of evidence have provided support for this hypothesis. First, a number of correlations have been shown between levels of neurogenesis and learning and memory abilities. For example, neurogenesis declines significantly in aged animals and low levels of neurogenesis have been shown to predict cognitive decline (Drapeau et al., 2003; Driscoll et al., 2006). Chronic stress, at least in males, decreases adult neurogenesis (Pham et al., 2003) and is also known to have a negative impact on hippocampus-dependent learning (Luine et al., 1994; Conrad et al., 1996). X-irradiation is known to reduce adult neurogenesis in rodents and impairs long-term memory (Synder et al., 2005). The same relationship is seen in humans as similar cranial radiotherapy treatment is often associated with memory impairments (Dietrich et al., 2008). In addition, positive regulators of adult neurogenesis such as voluntary exercise (van Praag et al., 1999; Holmes et al., 2004) and environmental enrichment (Kempermann et al., 2002; Brown et al., 2003; Bruel-Jungerman et al., 2005) are associated with increased cognitive performance on hippocampus-dependent tasks. Although these correlative observations are intriguing, the treatments listed above have a number
of other effects that could explain changes in learning and memory without a specific role for adult neurogenesis. For example, Meshi and colleagues (2006) demonstrated that reducing adult neurogenesis with x-irradiation did not block the cognitive enhancement seen following environmental enrichment. Furthermore, at least one study has shown a correlation between the age-related decline in neurogenesis and cognitive performances such that lower levels of neurogenesis was indicative of better performance (Bizon et al., 2004).

**The effects of reducing neurogenesis on learning and memory**

A second line of evidence for an interaction between adult neurogenesis and hippocampus-dependent learning and memory involves manipulating levels of adult neurogenesis. Several methodologies have been used in an attempt to specifically eliminate adult neurogenesis in order to observe what functions are lost. These techniques fall into three main categories: anti-mitotic agents, irradiation, and genetic ablation. The earliest attempts to reduce adult neurogenesis utilized anti-mitotic agents such as methylazoxymethanol (MAM) or cytosine-β-d-arabinofuranoside (Ara-C) administered either systemically or locally (Shors et al., 2001; Doetsch et al., 1999). Tracy Shors and colleagues (2001, 2002) demonstrated that MAM treatments caused impairments on a type of eye blink conditioning that depends on the hippocampus (trace conditioning) but did not impair a hippocampus-independent version (Delay conditioning). This finding provided some of the first evidence that hippocampus-dependent learning required intact neurogenesis. However, they also demonstrated that not all types of hippocampus-dependent learning were impaired by MAM treatment (Shors et al., 2002). Spatial learning was not impaired by MAM suggesting that there may be a specific aspect of hippocampus-dependent learning that involves adult neurogenesis.
However, it should be noted that the dose of MAM used in these studies was not sufficient to completely eliminate neurogenesis and higher doses needed to significantly reduce neurogenesis are known to have negative effects on the health of the animal (Dupret et al., 2005). It is possible that the lack of impairment of spatial learning may be due to residual levels of adult neurogenesis. It is unknown what exactly new neurons are contributing to hippocampus-dependent learning and therefore it is also unclear how many new neurons might be required for a given type of learning.

X-irradiation is believed to cause a greater reduction in neurogenesis with fewer side effects than anti-mitotic treatment but high doses do still compromise the health of the animal and a small percentage of progenitor cells remain intact and capable of producing a limited number of new neurons (Hellstrom et al., 2009). However, irradiation studies have provided some interesting connections between hippocampus-dependent learning and adult neurogenesis. A common finding is that contextual fear conditioning is impaired by focal hippocampal irradiation (Saxe et al., 2006; Winocur et al., 2006; Wojtowicz et al., 2008). In contrast, spatial learning in the Morris water task is invariably unaffected by irradiation (Madsen et al. 2003; Snyder et al., 2005; Saxe et al., 2006) similar to the pattern of results seen with MAM treatment. Although spatial and contextual memories are the most often tested types of hippocampus-dependent learning, other impairments have been found following irradiation. Non-match to sample training at delays over 120 seconds, in which performance requires the integrity of hippocampus, between sample and choice phase was found to be impaired by irradiation (Winocur et al., 2006). This type of learning is similar to trace eye blink conditioning (which is impaired by MAM treatment) in that it involves the maintenance of information across a temporal delay. Another test of hippocampus function that has been disrupted by irradiation is place recognition (Madsen et al., 2003). Place recognition is a type of spatial learning but unlike
learning in the Morris water task is acquired rapidly (like contextual conditioning). Finally, there is evidence that although spatial learning in the Morris water task may not be impaired by irradiation, long-term retention of this type of learning is impaired (Snyder et al., 2005). Thus, adult neurogenesis in the dentate gyrus may have a variety of roles. New neurons may be important for enhancing learning of tasks that require rapid acquisition or involve the encoding of temporal intervals and may still participate in long-term retention of these and other types of memory.

Several studies have now utilized inducible genetic knockdowns to study the function of adult neurogenesis and there have been some inconsistent results between irradiation and inducible knockdowns. Two studies have found impaired spatial learning following genetic knockdown of neurogenesis unlike what is reported following irradiation (Dupret et al., 2008; Zhang et al., 2008). Contextual fear conditioning on the other hand was found to be unaffected in one study (Zhang et al., 2008) but not another (Saxe et al., 2006). Finally, a study by Clelland and others (2009) comparing irradiation and a genetic ablation strategy found consistent impairments using a spatial discrimination task. It remains unclear why the inconsistent results exist between irradiation and genetic knockdown strategies. It should be noted that these techniques do not cause as great of a reduction in neurogenesis as irradiation does. Furthermore, due to the transgenic nature of these techniques they have only been applied in mice thus far. It is also possible that the genetic manipulations applied in these studies may have some as of yet unknown non-specific effects. Recently it has been demonstrated that compared to rats, adult generated neurons in mice mature more slowly and do not appear to be as important to hippocampal function (Snyder et al., 2009). Therefore, it is also possible that species differences between rats and mice may in part account for the differences observed between genetic ablation studies using mice and irradiation studies that have predominately used rats.
The effects of learning on adult neurogenesis

One of the earliest convincing pieces of evidence that adult neurogenesis is related to hippocampus dependent learning and memory was from a study showing that spatial learning increased the number of new cells that survived to maturity (Gould et al., 1999). In that study, rats were injected with BrdU to label dividing cells and were then trained in the Morris water task one week later. When the rats were perfused following training it was found that rats trained on the place task had higher levels of BrdU-labeling compared to rats trained on the hippocampus-independent cue version, naive cage controls and a swim stress control group. Furthermore, the rats that received cue-training did not differ from the naive controls or the swim controls indicating that the effect was specific to hippocampus-dependent learning and not simply induced by stress, exercise or environmental novelty. Several subsequent studies have provided supporting evidence of this interaction between hippocampus-dependent learning and enhanced cell survival (Ambrogini et al., 2000; Dobrossy et al., 2003; Leuner et al., 2004; Hairston et al., 2005; Olariu et al., 2005; Dalla et al., 2007; Keith et al., 2008; Dalla et al., 2009). In contrast, some studies have shown that hippocampus-dependent learning either had no effect on cell survival (Dobrossy et al., 2003; Olariu et al., 2005; Van der Borght et al., 2005; Ehninger & Kempermann, 2006; Mohapel et al., 2006) or decreased cell survival (Ambrogini et al., 2004; Mohapel et al., 2006). As a result, it remains uncertain whether spatial learning increases cell survival and if so under what conditions this occurs. There are numerous methodological differences between the various studies that have examined the effects of spatial learning on cell survival that may explain the different findings (Table 1.1).

One of the main differences between the studies in Table 1.1 is when the rats (or mice) were administered BrdU relative to when training commenced. BrdU administration labels a
specific age of new cells that will be studied because BrdU is incorporated for a period of approximately two hours after being injected. Different effects of spatial learning on cell survival might be expected based on the age of the new cells at the time of learning because of the different developmental stage of those new cells. For example, a previous study has shown that estradiol increases cell survival if administered on days 6-10 after BrdU administration but does not change the survival of either younger (days 1-5) or older (days 11-15) populations of neurons (Ormerod et al., 2004). As mentioned previously, studies that use multiple injections of BrdU will ultimately label a wider age of cells than if only a single injection is used (Taupin et al., 2007). Thus, if a critical age of cells exists when learning may alter cell survival it will be more difficult to identify with multiple BrdU injections.

**Thesis experimental outline**

Given the inconsistencies that exist in the literature regarding the effects of spatial learning on adult neurogenesis, the principle aim of the experiments presented here was to identify factors that may alter this interaction in order to reconcile the various findings. The experiments presented in the following chapters compare the levels of neurogenesis in rats trained on the hippocampus-dependent place version of the Morris water task to rats trained on the Hippocampus-independent cue version of the task. The place version of the task assesses the rats ability to navigate based on the geometry of the distal cues in the room. However, in the cue version of the task the rats must learn to swim to a visible but moving platform. Rats in the cue condition still have access to the same distal cues available to rats in the place version (ie not curtain is drawn around the pool) but these cues no longer provide any information about the location of the platform. The following experiments are all conducted in male young adult rats.
In chapter two I examined the effects of spatial learning at different time-points (days 1-5, 6-10 or 11-15) after BrdU administration. This experiment was performed to determine whether spatial learning would alter cell survival dependent on the age of the immature neurons being examined (Epp et al., 2007). In chapter three I examined whether spatial learning on days 1-5, 6-10 or 11-15 after BrdU administration would cause a differential activation of immature neurons in response to spatial memory retrieval five days after learning. This study also allowed me to determine whether the variable time after training prior to perfusion utilized in chapter two, had an effect on cell survival (Epp et al., submitted). In chapter four I explored the whether changing task difficulty would change the impact of spatial learning on cell survival (Epp et al., 2009). Finally, in chapter five I compared the effects of spatial learning on neurogenesis and immature cell morphology, and activation in two common strains of laboratory rats (Epp et al., submitted).
Table 1.1: Previous studies investigating the impact of spatial learning on cell survival

<table>
<thead>
<tr>
<th>Study</th>
<th>BrdU injection</th>
<th>Days of training</th>
<th>Day of perfusion</th>
<th>Species/ Strain</th>
<th>Age (days)</th>
<th>Sex</th>
<th>Cell survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gould et al., 1999</td>
<td>1 200mg/kg</td>
<td>Days 7-10 (4)</td>
<td>Day 11 or Day 18</td>
<td>Rat SD</td>
<td>300-350 grams</td>
<td>Male</td>
<td>INC</td>
</tr>
<tr>
<td>Ambrogini et al., 2000</td>
<td>2 x 3 days 50mg/kg</td>
<td>Days 3-7 (5)</td>
<td>Day 18</td>
<td>Rat SD</td>
<td>60 days</td>
<td>Male</td>
<td>INC</td>
</tr>
<tr>
<td>Dobrossy et al., 2003</td>
<td>1 x 4 days 50mg/kg</td>
<td>Days -4 to 3 (8)</td>
<td>Day 4 or day 30</td>
<td>Rat SD</td>
<td>60 days</td>
<td>Male</td>
<td>INC</td>
</tr>
<tr>
<td></td>
<td>1 x 4 days 50mg/kg</td>
<td>0 - 7 (8)</td>
<td>Day 8</td>
<td>Rat SD</td>
<td>60 days</td>
<td>Male</td>
<td>DEC</td>
</tr>
<tr>
<td>Ambrogini et al., 2004</td>
<td>2 x 3 days 50mg/kg</td>
<td>Days 10-14 (5 with 2 session per day)</td>
<td>Day 17</td>
<td>Rat SD</td>
<td>150 days</td>
<td>Male</td>
<td>DEC</td>
</tr>
<tr>
<td>Hairston et al., 2005</td>
<td>2 x 1 day 100 mg/kg</td>
<td>Days 7-10 (4 with 2 sessions per day)</td>
<td>Day 10</td>
<td>Rat ? (albino)</td>
<td>?</td>
<td>Female</td>
<td>INC</td>
</tr>
<tr>
<td>Van der Borght et al., 2005</td>
<td>1 x 3 days 100mg/kg</td>
<td>Days 9-12 (5 with 2 platform locations)</td>
<td>Day 13</td>
<td>Rat SD &amp; Wistar</td>
<td>338±24 grams</td>
<td>Male</td>
<td>N.C.</td>
</tr>
<tr>
<td>Ehninger &amp; Kempermann, 2006</td>
<td>2 x 2 days 50mg/kg</td>
<td>Days 6-9 (4 with new platform location on day 4)</td>
<td>Day 10</td>
<td>Mouse C57BL/6</td>
<td>70 days</td>
<td>Female</td>
<td>N.C.</td>
</tr>
<tr>
<td>Mohapel et al., 2006</td>
<td>4 x 1 day 50mg/kg</td>
<td>Days 7-10 (4)</td>
<td>Day 10</td>
<td>Rat SD</td>
<td>300-350 grams</td>
<td>Male</td>
<td>N.C.</td>
</tr>
<tr>
<td></td>
<td>4 x 1 day 50mg/kg</td>
<td>Days 1-14 (14)</td>
<td>Day 14</td>
<td>Rat SD</td>
<td>300-350 grams</td>
<td>Male</td>
<td>DEC</td>
</tr>
</tbody>
</table>

1. Number of BrdU injections per day. The first day of BrdU injections is set as day 0. Dose given is per injection
2. Time of training relative to BrdU injection (or first BrdU injection in the case of multiple injections). The number in parentheses denotes the number of days of training and the number of sessions per day (if more than one session).
3. Weight is given for cases in which age was not reported
4. Change in cell survival following spatial learning. N.C. = no change, INC = increase, DEC = decrease.
References


Sutherland RJ, McDonald RJ, Hill CR, Rudy JW (1989) Damage to the hippocampal formation in rats selectively impairs the ability to learn cue relationships. Behav Neural Biol 52:331-356.


In the adult mammalian brain, new neurons continue to be added to the dentate gyrus of the hippocampus (Altman and Das, 1965; Kaplan and Hinds, 1977; Cameron et al., 1993; Eriksson et al., 1998). The function of these new neurons is not well understood but it is clear that they develop the morphology of mature granule cells and make appropriate synaptic connections (Cameron et al., 1993; Hastings and Gould, 1999; van Pragg et al., 2002). The major axonal projection from the dentate gyrus terminates in area CA3 of the hippocampus (for review see Knowles, 1992) and adult generated neurons have been shown to send axonal projections to this region (Hastings and Gould, 1999; Markakis and Gage, 1999) suggesting that these cells may in fact form connections with the correct targets. Further evidence has shown that adult generated neurons display electrophysiological properties similar to mature granule cells (Van Praag et al., 2002).

It has been suggested that because the hippocampus plays such a predominant role in many types of learning, such as spatial learning (Morris et al., 1982, Sutherland et al., 1983), adult neurogenesis may play a functional role in the processes underlying hippocampus-dependent learning and memory. Training on hippocampus-dependent tasks has been shown to

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1 A version of this chapter has been published. Epp, J.R., Spritzer, M.D. and Galea, L.A.M. (2007) Hippocampus-dependent learning promotes the survival of new neurons in the dentate gyrus at a specific time during cell maturation. *Neuroscience.* 149: 273-285
increase new neuron survival compared to training on hippocampus-independent tasks (Gould et al., 1999). Gould et al., (1999) demonstrated that rats trained on a spatial version of the Morris water task had a greater number of surviving new neurons than did rats trained on a cued version of the task. This finding indicates that the survival of adult generated neurons may be modulated by local network activity, in this case through acquisition of a hippocampus-dependent task. These findings have been supported by other studies showing that several types of hippocampus-dependent learning enhance the survival of newly proliferated cells (Ambrogini et al., 2000; Leuner et al., 2004; Hairston et al., 2005; Olariu et al., 2005). However, these findings remain contentious based on several reports that show hippocampus-dependent learning has either no effect on cell survival (Dobrossy et al., 2003; Van der Borght et al., 2005; Mohapel et al., 2006) or decreases cell survival compared to hippocampus-independent learning (Ambrogini et al., 2004; Pham et al., 2005, Olariu et al., 2005; Mohapel et al., 2006).

One possible explanation is that learning may influence cell survival differently if the new cells are at different developmental ages. Adult generated neurons in the dentate gyrus advance through several developmental stages after they are born and it is likely that their susceptibility to trophic factors may be altered during these different stages. During the early phase of cellular development, days 1-5 after division, the major events are migration from the subgranular zone to the granule cell layer and differentiation. Doublecortin, a microtubule associated protein required for normal neuronal migration is highly expressed in the subgranular zone during this time and peaks at 4 days after cell division (Brown et al., 2003), suggesting that neuronal phenotype is determined during this early time period. The middle phase of cellular development, days 4-10 after division, involves a rapid extension of axons into area CA3 (Hastings and Gould, 1999) and it is during this period that cells begin to form connections with
target cells and start to become integrated into the existing network. Within 10-15 days after division many of these new cells continue to mature and grow, begin to develop a dendritic arbor (Esposito et al., 2005; Zhao et al., 2006), become responsive to external stimuli (Jessberger and Kempermann, 2003) and begin to express mature neuronal protein markers (Cameron et al., 1993).

In support of the idea that enhancement of new neuron survival may be time sensitive, a previous study demonstrated that the survival-enhancing effect of estradiol on new neurons occurs only if administered 6-10 but not 1-5 or 11-15 days after division (Ormerod Lee and Galea, 2004). This period corresponds approximately to the axon outgrowth phase of cellular development suggesting that survival-enhancing factors are maximally effective during a discrete time frame. At earlier stages of cellular development it may be that the immature neurons are not yet susceptible to changes in network activity because they are not yet connected to the surrounding cells. However, at later stages of development of new neurons, as axonal connections are established, activity received by the new neuron may be more likely to enhance its survival. At even later stages of development connections may be stable enough in the network that their survival is less likely to be altered. Different effects of spatial learning have been found in studies that conducted spatial learning during time periods that correspond roughly to those above. Ambrogini et al. (2000) found that early spatial training (occurring 3-7 days following BrdU administration) enhanced survival of new cells. However, in a subsequent study Ambrogini et al. (2004) observed a decrease in cell survival following training on a spatial task 10-14 days after BrdU administration. Therefore, the maturity of the new cells may be one factor that determines if and how hippocampus dependent learning will affect cell survival. Furthermore, Tashiro et al. (2007) show that environmental enrichment occurring at various times after BrdU administration result in cell survival of different magnitudes with the maximal
effect occurring when exposure began at 11 days after the initial BrdU injection. Although it may not necessarily be the same time window for all neurogenic factors, together these findings suggest that the ability to enhance new cell survival changes as the cell matures.

In this study we examined the effect of spatial learning in the Morris water task on survival of adult generated neurons in the hippocampus. We sought to determine whether the maturity of the cells at the time of learning is an important factor in regulating their rate of survival. Therefore, we injected rats with bromodeoxyuridine (BrdU), a marker of DNA synthesis, and then utilized either a hippocampus-dependent or hippocampus-independent version of the Morris water task. Rats were trained during one of three time points after BrdU administration, early phase (1-5 days), middle phase (6-10 days) or late phase (11-15 days), to determine whether cell survival was enhanced by spatial training during developmentally discrete time periods. We also sought to determine whether ‘good’ or ‘bad’ performance can alter survival of new neurons in the dentate gyrus.

**Experimental procedures**

**Subjects**

Subjects were 48 male Sprague-Dawley rats (Charles River, Saint-Constant Quebec), weighing between 300-350 g at the start of testing. All testing was carried out in accordance with the Canadian Council for Animal Care guidelines and was approved by the animal care committee at the University of British Columbia. Rats were individually housed in standard cages with free access to food and water. Rats were allowed to habituate to their new housing conditions after arrival for seven days. They were then handled for 5 days before behavioral testing began. Half of the rats were trained on a hidden platform (hippocampus-dependent) version of the Morris water task; the remaining 24 rats were trained on a visible platform
(hippocampus-independent) version of the task (Sutherland et al., 1989). Hidden and visible platform groups were both subdivided into three groups that were trained in the Morris water task during days 1-5, 6-10 or 11-15 after BrdU injections.

All rats were given a single intraperitoneal injection of BrdU (a thymidine analogue, which labels all cells in the synthesis phase at the time of injection) on day 0 of the experiment. BrdU (Sigma; Oakville, ON, Canada) was prepared just prior to administration and was dissolved in warm 0.9% saline (NaCl) to a final concentration of 20mg/ml. Each rat received an injection of 200mg/kg of BrdU.

**Apparatus**

A circular pool, 180 cm in diameter, was filled 30 cm deep with water, approximately 21° C. The water was rendered opaque using white non-toxic paint. Large and distinct distal cues were placed on the room walls surrounding all sides of the pool. A camera mounted above the pool was connected to a computer running HVS Water (HVS image; Hampton, UK) and was used to track the movement of the rats in the pool.

**Procedure**

Five daily training sessions were given, each consisting of 4 trials per rat. Each trial began from one of the four cardinal compass points and start locations were never repeated within a session. The order of start locations was randomized for each session but was the same for each group. For rats trained to locate a hidden platform a 10 cm diameter platform was submerged 2 cm below the surface of the water in the center of the northeast quadrant of the pool. The location of the platform remained fixed for the duration of training. For rats trained to swim to a visible platform the position of the visible platform was randomly moved after each
trial to the middle of a new quadrant so that a place-learning strategy could not be used to locate the platform. The surface of the platform extended 2 cm above the surface of the water so that it was visible to the rats.

On day 16 after BrdU administration, rats were given an overdose of sodium pentobarbital and perfused transcardially with 60ml of 0.1M phosphate buffered saline followed by 120ml of 4% paraformaldehyde (Sigma). The brains were extracted and post-fixed in 4% paraformaldehyde for 24 hours and then transferred to 30% sucrose in 0.1M PBS. Brains were then sectioned on a vibratome (Leica; Richmond Hill, ON, Canada) into 40µm thick sections and stored in 0.1M TBS. Series of every 10th section were collected throughout the rostral-caudal extent of the hippocampus.

**BrdU immunohistochemistry**

For BrdU immunohistochemistry, the tissue was incubated in 0.6% H₂O₂ for 30 minutes, rinsed in 0.1M TBS and then transferred to 2N HCL and incubated for 30 minutes at 37°C. Then, the tissue was placed in a borate buffer wash for 10 minutes at room temperature and rinsed in 0.1M TBS. The primary antibody solution contained 1% Triton-X, 3% normal horse serum and 1:200 mouse anti-BrdU (Roche; Mississauga, ON, Canada) in 0.1M TBS. Tissue was incubated in the primary solution for 48 hours at 4°C and then rinsed in 0.1M TBS. The secondary antibody used was horse anti-mouse (Vector; Burlington, ON, Canada) diluted 1:100 in 0.1M TBS. Sections were incubated in the secondary antibody for 4 hours at room temperature and were then washed in 0.1M TBS. An ABC kit (Vector) was used and prepared according to the kit instructions. Labeling was visualized by incubating tissue in 5mg/ml 3,3’-diaminobenzidine (DAB) for 3 minutes. Finally, the tissue was mounted on glass slides, counterstained with cresyl violet and cover slipped with Permount.
BrdU/NeuN double-labeling

Unless stated otherwise, all incubations were carried out at 4°C and were followed by three 10-minute rinses in TBS. Tissue was first incubated for 48 hours in a NeuN primary antibody solution containing a 1:200 dilution of mouse monoclonal anti-NeuN (Chemicon; Temecula CA), 1.5% normal donkey serum (Vector), 0.3% triton-X diluted with 0.1M TBS. Then, the tissue was transferred to a 1:200 solution of Alexa-488 Donkey anti-mouse (Molecular Probes; Eugene OR) in TBS for 24 hours. Tissue was fixed with a 10 minute incubation in 4% PFA at room temperature and then washed and transferred to 2N HCl for 30 minutes at 37°C. Tissue was placed for 48 hours in the BrdU primary solution containing a 1:200 dilution of rat anti-BrdU (Oxford Biotechnology; Raleigh, NC), 1.5% normal donkey serum, 0.3% triton-X and diluted with 0.1M TBS. The tissue was then incubated for 24 hours in a solution containing 1:200 donkey anti-rat conjugated cy-3 (Jackson ImmunoResearch Laboratories Inc.; West Grove, PA) diluted with TBS. Sections were mounted on 2% gelatin coated slides and cover slipped with PVA DABCO, anti-fading mounting medium.

Quantification

BrdU-labeled cells were counted in every 10th section throughout the granule cell layer (including the subgranular zone) and the hilus to obtain an estimate of the total number of labeled cells in each region. Counting was performed using a 100x oil immersion objective on a Nikon E600 Light microscope. Corresponding area measurements were made of the dentate gyrus (granule cell layer and hilus calculated separately) using the software program ImageJ. Volume estimations of the dentate gyrus were then calculated using Cavalieri’s principle (Gundersen and Jensen, 1987) by multiplying the aggregated areas by the distance between
sections (400µm). Total cell counts were calculated by summing the number of BrdU-labeled cells per animal by 10. Densities of BrdU-labeled cells/mm³ in each region (granule cell layer or hilus) were also calculated by dividing the total number of BrdU-labeled cells by the volume of either region (granule cell layer or hilus).

The percentage of BrdU/NeuN double-labeled cells (Figure 2.1) was assessed by examining 25 randomly selected BrdU labeled cells from 4-5 sections per brain using an epifluorescent Nikon microscope with 40x objective.

Figure 2.1: Photomicrographs of BrdU-labeled tissue. (A) A BrdU-labeled cell in the subgranular zone of the dentate gyrus. (B) The granule cell layer showing cells immunoreactive for NeuN (neurons) in green. (C) A merged image showing a BrdU(red)/NeuN(green) double-labeled cell, indicating a new neuron. Arrows in panels A-C indicate the same cell. (D) BrdU labeled cells in the dentate gyrus visualized with DAB and counterstained with cresyl violet. Scale bar in D refers to 50µm in panels A-C.
**Data analyses**

Dependent variables of latency and distance to reach the platform were each analyzed using repeated measures analysis of variance (ANOVA) with day of training (1-5, 6-10, 11-15) and training type (place, cued) as between-subjects factors and session (1,5) as the within-subjects factor. The total number of BrdU-labeled cells and volume were each analyzed with repeated-measures ANOVA with day of training (1-5, 6-10, 11-15) and training type (place, cued) as between-subjects factors and region (granule cell layer, hilus) as the within-subjects factor. Median splits on total distance traveled to reach the platform on all trials and similarly on total latency to reach the platform over all trials were calculated to indicate ‘good’ versus ‘poor’ performers. A repeated-measures ANOVA on density of BrdU-labeled cells with training type (place, cued) and performance (good, poor) as between-subjects factors and region (dentate gyrus (includes subgranular zone), hilus) as the within-subjects factor was also conducted. Post-hoc tests utilized Tukeys procedure. Pearson product-moment correlations were performed on densities of BrdU-labeled cells in the dentate gyrus and hilus (separately) of ‘good’ and ‘poor’ cue and place learners with performance (total latency or total distance). All analyses were performed using the software program Statistica (Statsoft Tulsa, OK).

**Results**

*Cue-trained rats had shorter latencies to reach the platform than did placed-trained rats on days 1 and 2 of training only*

One animal from the place trained (6-10) group was removed from all analyses due to fact that on the last day of training this rat did not find the platform on 50% of the trials and had an average latency nearly three times greater than the group mean during the final session and as
such was deemed an outlier. Due to a lack of BrdU labeling seven additional rats were removed from the study.

For latency to reach the platform there was a significant session by training type interaction effect (F(4, 136)=6.54, p<.00008) but no significant three-way interaction (p≤.30). Post-hoc tests indicated that cue-trained rats had shorter latencies to reach the platform than did place-trained rats on days 1 and 2 of training, regardless of time of training (Figure 2.2 A, D, G). There were no other significant differences between groups. For distance to reach the platform, there was a significant three-way interaction effect of day of training by training type and session (F(8, 136)=3.90, p<.0004). Post-hoc tests revealed significant differences between training type with cue-trained rats swimming shorter distances before reaching the platform than place-trained rats (data not shown) when trained on days 1-5 on sessions 1 and 2 of training, but no other significant differences between group were observed for rats trained on days 6-10 or days 11-15.

Place-trained rats had increased new cell survival compared to cue-trained rats only when training occurred 6-10 days after BrdU administration

There was a significant main effect of region (F(1, 34)=769.314, p<0.00001), with more BrdU-labeled cells in the granule cell layer than in the hilus, but no other significant main or interaction effects. However, a priori we expected that rats trained on the place version would show an increase in number of BrdU-labeled cells compared to rats trained on the cue version of the Morris water maze. A priori tests revealed that rats trained on the place version of the task during the middle time period (6-10 days) had a significantly greater number of BrdU-labeled cells in the dentate gyrus than did the cue-trained rats at the same time point (p≤0.019; Figure 2.2). However, neither the 1-5 day or 11-15 day periods displayed differences between place- and cue-trained groups with respect to number of BrdU-labeled cells (p’s <.84). The same
pattern of results was seen using density of BrdU-labeled cells rather than total BrdU-labeled cells. There was a main effect of region \((F(1,34)=911.099, p<0.00001)\) and an *a priori* test indicated that place learners had a significantly greater density of BrdU-labeled cells than cue learners at the 6-10 day time point \((p<0.03, \text{one-tailed})\).

**Rats trained on days 1-5 had significantly greater percentage of BrdU-labeled cells that co-expressed NeuN**

There was a significant main effect of day of training on the percentage of BrdU-labeled cells that were co-labeled with NeuN \((F(2,31), F=7.14, p<.0028)\), with rats trained 1-5 days after BrdU injection showing a greater percentage of co-labeling compared to training on other days, regardless of training type. Note however, that the mean percentage of co-labeling was 67.3% in days 1-5 versus 60.0 and 55.1 in those rats trained on days 6-10 and 11-15, respectively (see Table 2.1), thus suggesting an average of 7% or 12% greater increase in new neuron survival in the early trained groups. There was no significant difference in the number of double-labeled cells between place- and cue-trained groups at the 1-5 day time point \((p = 0.35)\) 6-10 day time point \((p= 0.55)\) or the 11-15 day time point \((p=0.48)\).
Figure 2.2: (A) Mean (± SEM) latency to reach the hidden platform during acquisition of the Morris water task for place and cue trained rats 1-5 days after BrdU administration. (B) Mean (± SEM) total number of BrdU labeled cells in the granule cell layer and the hilus are shown for rats trained 1-5 days after BrdU administration. (C) Mean (± SEM) density of BrdU labeled cells in the granule cell layer and the hilus are shown for rats trained 1-5 days after BrdU administration. (D) Mean (± SEM) latency to reach the hidden platform during Morris water task acquisition for rats trained 6-10 days after BrdU injection. (E) Mean (± SEM) total number of BrdU labeled cells in the granule cell layer and hilus of rats trained 6-10 days after BrdU administration. Rats receiving place training have significantly more BrdU labeled cells in the GCL than cue-trained rats. (F) Mean (± SEM) density of BrdU labeled cells in the granule cell layer and hilus of rats trained 6-10 days after BrdU administration. Place-trained rats have a significantly greater density of BrdU-labeled cells than cue trained rats. (G) Mean (± SEM) latency to reach the hidden platform during Morris water task acquisition of rats trained 11-15 days after BrdU administration. (H) Mean (± SEM) total number of BrdU labeled cells in the granule cell layer and hilus of rats trained in the Morris water task 11-15 days after BrdU administration. (I) Mean (± SEM) density of BrdU-labeled cells in the granule cell layer and hilus of rats trained in the Morris water task 11-15 days after BrdU administration. Asterisk indicates significantly different (p≤.05).
Table 2.1: Mean (± SEM) percentage of BrdU-labeled cells also expressing the neuronal marker NeuN in all groups. Rats trained during days 1-5 after BrdU administration had significantly more BrdU-labeled that co-expressed NeuN, regardless of training.

<table>
<thead>
<tr>
<th>Condition</th>
<th>1-5 days</th>
<th>6-10 days</th>
<th>11-15 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Place training</td>
<td>64.8±1.5%</td>
<td>58.7±3.4%</td>
<td>53.3±3.0%</td>
</tr>
<tr>
<td>Cue training</td>
<td>68.1±2.4%</td>
<td>61.1±4.3%</td>
<td>56.6±2.5%</td>
</tr>
</tbody>
</table>

*Place learning enhances cell survival compared to cue learning in ‘poor’ learners but not in ‘good’ learners*

We divided the learning groups into ‘poor’ versus ‘good’ learners using a median split based on the total latency or total swim distance to reach the platform across all training trials. The median was calculated separately for cue and place trained rats. The median total latency for place learners was 458s and for cue learners was 282s. The median total distance for place learners was 97m and for cue learners was 52m. Table 2.2 indicates the sample size based on median splits in all the day of training and type of training groups. Due to the low sample size in each day of training groups a repeated-measure ANOVA on density of BrdU-labeled cells with training type (place, cued) regardless of time of training, and performance (good, poor) as between-subjects factors and region (granule cell layer, hilus) as the within-subjects factor was conducted. However, the mean density and total BrdU counts for each training time are shown in Table 2.3. We chose to analyze density of BrdU labeled cells in addition to the total number of BrdU cells as the dependent variable due to the strong trend for differences in dentate gyrus volume between ‘good’ and ‘poor’ learners (see below). There were significant three-way interactions on density of BrdU-labeled cells between good versus poor learners defined by
either latency or distance to reach the platform (latency: \( F(1, 36) = 7.70, p \leq 0.0097 \);
distance: \( F(1, 36) = 5.346, p \leq 0.027 \)). Post-hoc tests revealed that ‘poor’ place learners had a
significantly greater density of BrdU-labeled cells in the granule cell layer than did ‘poor’ cue
learners when defined by either latency (\( p \leq 0.031 \)) or distance (\( p \leq 0.0013 \)), but no significant
difference in the density of BrdU-labeled cells between ‘good’ place learners and ‘good’ cue
learners (\( p \)’s > 0.57; Figure 2.3).

There was a significant main effect of total BrdU labeled cells between ‘good’ and ‘poor’
learners (\( F(1, 36) = 4.213, p \leq 0.047 \)) when performance was defined by total distance. Post-hoc
tests showed that ‘poor’ place learners had significantly more BrdU labeled cells than ‘poor’ cue
learners (\( p \leq 0.046 \), Figure 2.3). There was no significant difference in total BrdU-labeled cells
between ‘good’ place and ‘good’ cue learners (\( p \leq 0.44 \)). The overall ANOVA was not
significant for total BrdU labeled cells between good and poor learners based on latency.
However, an a priori test revealed that there was a trend for ‘poor’ place learners to have more
BrdU labeled cells than ‘poor’ cue learners (\( p \leq 0.068 \)) but there was no significant difference
between ‘good’ place and ‘good’ cue learners (\( p \leq 0.35 \)).
Table 2.2: The number of rats classified as ‘good’ or ‘poor’ learners from each type of learning condition and day of training groups after the median splits for either total distance traveled or total latency to reach the platform. Median splits were done separately for distance and latency for each cue and place-based learning. The overall median split regardless of training time generated the same number of rats for both distance and latency measures and is represented in the table as ‘all’.

<table>
<thead>
<tr>
<th>Performance</th>
<th>Total Distance</th>
<th>Total Latency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-5</td>
<td>6-10</td>
</tr>
<tr>
<td>Training time</td>
<td>All</td>
<td></td>
</tr>
<tr>
<td>Place</td>
<td>Good</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Cue</td>
<td>Good</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>7</td>
</tr>
</tbody>
</table>

We correlated the total number of BrdU labeled cells in the granule cell layer and the hilus with the total latency and total distance to find the platform separately for both place and cue trained rats (Figure 2.4). In place-trained rats we observed a strong trend for a significant positive correlation between total latency and total BrdU labeled cells in the granule cell layer ($r = 0.46, p \leq 0.057$). There was also a trend towards a positive correlation between the total BrdU labeled cells in the hilus and the total latency across all trials to reach the platform in place-trained rats ($r=0.45, p \leq 0.063$). There were no significant correlations between total distance to reach the platform across all trials and total BrdU labeled cells in either the granule cell layer or hilus in rats trained on the place version. In cue-trained rats neither total latency or distance
traveled across all trials was significantly correlated with the total number of BrdU labeled cells in the granule cell layer. However, there were significant positive correlations between total BrdU labeled cells in the hilus and both total latency \((r = 0.48, \ p \leq 0.026)\) and total distance traveled across all trials in cued-trained rats \((r = 0.43, \ p \leq 0.044)\).

Additionally, because there was a strong trend for a difference in dentate volume between ‘good’ and ‘poor’ learners (see below) we correlated the density of BrdU labeled cells with the total latency and total distance to find the platform across all trials separately for both place and cue trained rats (Figure 2.5). In rats trained on the place version of the Morris water task we found a significant positive correlation between density of BrdU labeled cells in the granule cell layer and total latency \((r = 0.66, \ p \leq 0.003)\) and a trend for a positive correlation between density of BrdU labeled cells in the granule cell layer and total distance \((r = 0.42, \ p \leq 0.079)\). In cue-trained rats there was a trend for a positive correlation between density of BrdU labeled cells in the hilus and total latency \((r = 0.40, \ p \leq 0.066)\). There were no significant correlations for cue learners between BrdU labeled cell density in the granule cell layer and total latency or total distance.

**Dentate gyrus volume did not statistically differ between groups**

There was no significant difference in the volume of the granule cell layer across any of the 6 groups \((p \leq 0.21, \text{see Table 2.4})\). However, there was a strong trend for a significant effect of good/poor learners on dentate gyrus volume \((F(1,39), F=1.88, \ p \leq 0.057)\), with poor learners having a larger dentate gyrus volume than good learners based on a median split with distance traveled. However there were no significant differences on dentate gyrus volume when the rats were split based on median latency to reach the platform \((p<0.45)\).
Table 2.3: Mean values (± SEM) of total BrdU cell number and density of BrdU labeled cells in the granule cell layer at each training time in rats that are characterized as ‘good’ or ‘poor’ learners. Performance was defined based on median splits of either total distance or total latency across trials separately for cue and place trained rats.

<table>
<thead>
<tr>
<th>Distance</th>
<th>Density of BrdU labeled cells</th>
<th>Total BrdU labeled cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Good</td>
<td>Poor</td>
</tr>
<tr>
<td>1-5</td>
<td>Cue</td>
<td>Place</td>
</tr>
<tr>
<td>n=0</td>
<td>-</td>
<td>2056±194 n=7</td>
</tr>
<tr>
<td>-</td>
<td>n=0</td>
<td>2196±181 n=6</td>
</tr>
<tr>
<td>6-10</td>
<td>Cue</td>
<td>Place</td>
</tr>
<tr>
<td>n=5</td>
<td>2044±31</td>
<td>1958±419 n=2</td>
</tr>
<tr>
<td>n=4</td>
<td>1987±112</td>
<td>3019±396 n=2</td>
</tr>
<tr>
<td>11-15</td>
<td>Cue</td>
<td>Place</td>
</tr>
<tr>
<td>n=6</td>
<td>2139±95</td>
<td>1869±86 n=2</td>
</tr>
<tr>
<td>n=5</td>
<td>2047±194</td>
<td>2631 n=1</td>
</tr>
<tr>
<td>Latency</td>
<td>Cue</td>
<td>Place</td>
</tr>
<tr>
<td>n=5</td>
<td>2071±277</td>
<td>2020±145 n=2</td>
</tr>
<tr>
<td>n=3</td>
<td>2010±106</td>
<td>2382±344 n=3</td>
</tr>
<tr>
<td>6-10</td>
<td>Cue</td>
<td>Place</td>
</tr>
<tr>
<td>n=3</td>
<td>2092±7</td>
<td>1965±171 n=4</td>
</tr>
<tr>
<td>n=4</td>
<td>1987±112</td>
<td>3019±396 n=2</td>
</tr>
<tr>
<td>11-15</td>
<td>Cue</td>
<td>Place</td>
</tr>
<tr>
<td>n=3</td>
<td>2176±54</td>
<td>2009±98 n=5</td>
</tr>
<tr>
<td>n=2</td>
<td>2168±505</td>
<td>2132±209 n=4</td>
</tr>
</tbody>
</table>
Figure 2.3: Mean (+ SEM) density of BrdU-labeled cells in the granule cell layer of good and poor learning rats using a median split with either (A) total latency to reach the hidden platform or (B) total distance to reach hidden platform across all trials. BrdU-labeled cell survival is enhanced only in poor place learners compared to poor cue learners in both graphs. There is no significant difference in cell survival between good place and good cue learners. Mean total BrdU labeled cells in the granule cell layer of good and poor learning rats using a median split of either (C) total latency to reach the platform or (D) total distance to reach the platform across all trials. Cell survival is enhanced in poor place learners compared to poor cue learners when performance is defined by total distance and there is a trend for poor place learners to have more BrdU labeled cells than poor cue learners when performance is defined based on latency. Asterisk indicates significantly different (p≤0.05).
**Figure 2.4:** Correlations between BrdU-labeled cell survival and performance in the Morris water task for rats trained on the place and cue versions. (A) There is a significant positive correlation between density of BrdU-labeled cells in the granule cell layer and total latency across all trials for place trained rats, $r = 0.66, p \leq 0.003$. (B) There is a trend for a positive correlation between density of BrdU labeled cells in the hilus and total latency across all trials for cue-trained rats. (C) There is a trend for a positive correlation between density of BrdU-labeled cells in the granule cell layer and total distance across all trials for place-trained rats, $r = 0.42, p \leq 0.079$. (D) There are no significant relationships between density of BrdU-labeled cells in the hilus and total distance for either place or cue trained rats. Solid lines represent place-trained rats and dashed lines represent cue-trained rats. Black symbols represent those rats trained in place version, while open symbols represent those rats trained in the cued version of the Morris water task.
Figure 2.5: Correlations between BrdU-labeled cell survival and performance in the Morris water task for rats trained on the place and cue versions. (A) There is a trend towards a significant positive correlation between total number of BrdU-labeled cells in the granule cell layer and total latency across all trials for place trained rats, $r = 0.46$, $p \leq 0.057$. (B) There is a trend for a positive correlation between total number of BrdU labeled cells in the hilus and total latency across all trials for place-trained rats, $r = 0.45$, $p \leq 0.063$, and a significant positive correlation in cue trained rats, $r = 0.47$, $p \leq 0.026$. (C) There were no significant correlations between density of BrdU-labeled cells in the granule cell layer and total distance across all trials for place-trained rats or cue trained rats. (D) There was no significant relationship between total number of BrdU-labeled cells in the hilus and total distance for place-trained rats across all trials. However, there was a significant positive correlation between total distance and total number of BrdU labeled cells in the hilus of cue trained rats, $r = 0.43$, $p \leq 0.044$. Solid lines represent place-trained rats and dashed lines represent cue-trained rats. Black symbols represent those rats trained in place version, while open symbols represent those rats trained in the cued version of the Morris water task.
Table 2.4: Mean (± SEM) volume of the granule cell layer and hilus for each group. There were no significant differences between groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Granule cell layer</th>
<th>Hilus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Place</td>
<td>2.94±0.23 mm³</td>
<td>5.84±0.47 mm³</td>
</tr>
<tr>
<td>Cue</td>
<td>3.32±0.21 mm³</td>
<td>6.39±0.31 mm³</td>
</tr>
<tr>
<td>6-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Place</td>
<td>3.34±0.15 mm³</td>
<td>6.15±0.13 mm³</td>
</tr>
<tr>
<td>Cue</td>
<td>3.09±0.15 mm³</td>
<td>6.41±0.32 mm³</td>
</tr>
<tr>
<td>11-15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Place</td>
<td>2.90±0.11 mm³</td>
<td>5.77±0.16 mm³</td>
</tr>
<tr>
<td>Cue</td>
<td>2.85±0.07 mm³</td>
<td>5.87±0.12 mm³</td>
</tr>
</tbody>
</table>

Discussion

The primary goal of this study was to determine whether spatial learning would differentially affect the survival of newly proliferated neurons in the dentate gyrus of adult rats dependent on the stage of maturation of these new neurons when rats were trained. We demonstrate here that new neurons in the middle phase of development, during which many of these new cells rapidly extend axons, are more susceptible to the survival altering effects of hippocampus-dependent learning than are neurons in earlier or later phases of development. However, spatial learning did not significantly alter new neuron survival if BrdU was administered between days 1-5 or 11-15. Furthermore, we show that, collapsed across time of
training, spatial, but not cue, learning enhanced neurogenesis among the ‘poor’ learners but not among ‘good’ learners.

These findings showing a critical period during the cell maturation cycle when spatial learning can enhance neurogenesis are consistent with the findings of Ormerod, Lee and Galea (2004) and the ideas of Ambrogini et al., (2000, 2004). Ormerod et al (2004) found that only male meadow voles injected with estradiol during days 6-10 (but not 1-5 or 11-15) after BrdU administration exhibited enhanced cell survival compared to controls. Ambrogini et al.(2000, 2004) demonstrated an increase in cell survival with spatial learning when training occurred at an early time point (4 days after BrdU) but a suppression in cell survival when learning occurred later on (11 days after BrdU). Other factors that enhance neurogenesis in the dentate gyrus may also have a critical period but the time window may be slightly different. Tashiro et al. (2007) demonstrated that environmental enrichment maximally enhances cell survival when provided 11 days following the initial BrdU injection. Exposure to an enriched environment one week earlier or later also enhanced cell survival but to a lesser extent, while exposure to an enriched environment 2 weeks later had no significant effect on hippocampal neurogenesis (Tashiro et al., 2007) Taken together with the current study these data are suggestive of a critical period during which various factors may have a greater impact on the survival of new neurons.

Curiously in this study we also found that any type of training during the early part of cell maturation (days 1-5) enhanced the percentage of BrdU-labeled cells co-expressing NeuN, suggesting that early activation can affect cell phenotype. Both cue and place learning result in some activation of the hippocampus albeit to a different extent (Teather et al., 2005). Coupled with our findings this suggests that both types of stimulations can influence neuronal phenotype but that the greater activation with place learning is required to enhance the survival of these new neurons and that there is a critical time window associated with each effect.
Time and maturation-dependent influence of hippocampus-dependent learning on cell survival

One way that hippocampus-dependent learning may enhance new neuron survival is through direct stimulation of the cells via network activation. Thus, it is conceivable that in order for hippocampus-dependent learning to alter survival, new immature neurons must first form connections with the existing network. Axon projection to the CA3 region begins rapidly, within 4 days, with most axons reaching the CA3 region by day 10 or 11 after cell birth (Hastings & Gould, 1999; Zhao et al., 2006). During the early phase of development (days 1-5) the newborn cells are not yet connected to the existing circuitry and therefore, network stimulation via spatial learning may have little impact on these cells. During the middle phase (days 6-10) as the immature neurons extend axons and begin making synaptic contacts it may become possible for network activation to affect the survival of new neurons. In the late phase (days 11-15) cell survival no longer appears dependent on spatial learning perhaps due to the neurons having matured to the point that their survival is no longer uncertain or perhaps a greater activation of the network is needed to alter survival at this time point.

This explanation is compatible with much of the existing literature (Ambrogini et al., 2000; Gould et al., 1999; Hairston et al., 2005). Gould and colleagues (1999) originally demonstrated enhanced cell survival following spatial learning. Training in that study began one week after BrdU administration and closely matches our 6-10 day time period. In another study that found enhanced cell survival with learning (Ambrogini et al., 2000), spatial training commenced 4 days after the first BrdU injection. Although slightly earlier than our 6-10 day time point their spatial training still occurs during the time of axon extension (Hastings and Gould, 1999). Other studies utilizing the spatial Morris water task beginning one week after
BrdU administration have found enhanced cell survival (Hairston et al., 2005; Sisti et al., 2007). The present results also complement findings involving other types of hippocampus-dependent learning. Both trace conditioning and social transmission of food preference occurring one week after BrdU administration are associated with increased hippocampal cell survival (Leuner et al., 2004; Olairu et al., 2005).

Ambrogini and colleagues (2004) showed a suppression in cell survival in the dentate gyrus associated with spatial learning when learning occurred 11 days after the first BrdU injection. This is inconsistent with the findings from the present study as we did not observe any significant change in cell survival at this time point. However, there were some methodological differences that could explain the different findings. First, Ambrogini et al (2004) used 5-month-old rats that were pair housed while in the present study we used 2-month-old rats that were housed individually. Second, Ambrogini used multiple daily injections of BrdU as opposed to a single injection used in the present study and using multiple injections of BrdU can be problematic for a variety of reasons (see Taupin, 2007 and discussion below) and would label a heterogeneous pool of new neurons that could alter or mask an effect of learning on neurogenesis.

It should also be mentioned that differences in number of BrdU-labeled cells between cue- and place-trained rats might have been due to task difficulty, as cue-trained rats had shorter latencies and distances to reach the platform on days 1 and 2 of training. However, the difference in cell survival between place and cue trained rats in the 6-10 day group is not likely a result of a larger difference in performance at this time point compared to all other time points of training as there was no overall difference in performance across the days of training within either the place-trained or cue-trained groups. Regardless of training time, cue and place trained rats only differed in terms of latency during the first two sessions.
BrdU administration protocols are not consistent across studies of learning and new cell survival and therefore, this factor offers another potential explanation for equivocal findings in the literature (Taupin, 2007). We chose to use a single injection of BrdU in order to ascertain the age of labeled cells as closely as possible. Several studies however have used multiple injections either on one day or across several days (Ambrogini et al., 2000; Ambrogini et al., 2004; Van der Borght et al., 2005). It is difficult to assess the full impact the number of BrdU injections might have had on the effects of spatial learning and neurogenesis. However, labeling a more heterogeneous pool of new neurons at different stages of maturation might mask a potential effect of learning if a critical period for survival enhancement does exist as our findings suggest. Another explanation for the contradictory findings regarding learning and neurogenesis has been provided by Ehninger and Kempermann (2006). Stress is known to have a negative impact on hippocampal neurogenesis (Cameron and Gould, 1994; Gould et al., 1997; Thomas et al., 2007) and Ehninger and Kempermann (2006) have suggested that learning-induced effects on neurogenesis may be explained by this factor. Pre-exposure to the testing apparatus has been shown to decrease the stress response associated with behavioral training (Holscher et al., 1999). Ehninger and Kempermann (2006) further demonstrated in mice that pre-exposure to the apparatus eliminated the decrease in neurogenesis they found without pre-exposure. However, Gould and colleagues (1999) demonstrated in rats that naïve controls did not differ from cue-trained rats or rats that were time yoked to place-trained rats. Taken together this suggests that stress may play more of a role in water maze training in mice than rats. Mohapel and colleagues (2006) reported that corticosterone levels increase in rats trained in the Morris water task but do so equally in rats trained either on a cue or place version of the task, suggesting similar stress levels in cue versus place version learners. Furthermore, stress of training is likely at least partially dependent on a number of other factors such as previous handling and temperature of
the water. In the present study rats were handled for 5 minutes daily for 5 days prior to maze training and temperature of water was maintained at 21°C to minimize the stress of training.

**Cell survival is increased with place learning in ‘poor’ but not ‘good’ learners**

The present data show that the place learning can promote the survival of new granule neurons regardless of day of training but only in rats that were categorized as ‘poor’ learners, irrespective of whether ‘poor’ performance was defined by latency or by distance to reach the platform. Our results also show that increased cell survival correlates with longer total latencies to find the hidden platform with 43% of the variance in the density of BrdU-labeled cells of place-trained rats was accounted for by total latency to find the hidden platform, regardless of day of training. This is in agreement with the results of Dobrossy et al (2003) who found that better performance in the Morris water task was correlated with lower levels of cell survival in the hippocampus. In that study, rats were trained on the spatial task and were administered BrdU during the first 4 days of training. Cell survival was assessed the day following completion of training and was then correlated with performance over the first 4 days of training. The BrdU administration and water maze training protocols used by Dobrossy et al (2003) are not comparable to the ones used in our study and thus makes it difficult to compare results directly. However, it is still of interest to note that better performance may be associated with lower cell survival just as we have demonstrated here. Our results also show a trend towards a negative correlation between total latency and cell survival in the hilus of cue-trained rats. That is, there is a trend for better performance in cue-trained rats to be associated with greater cell survival in the hilus. It is unknown what if any function adult generated neurons play in the hilus (Scharfman 2007) and as such it is unclear what the consequences of this finding are. However, training in a cued version of the Morris water task does cause a small activation of the
hippocampus (Teather et al. 2005) that may have been responsible for a small increase in BrdU labeling in the hilus.

Sisti et al (2007) recently showed that spatial learning increased cell survival in good learners compared to poor learners. In our study we did not find a statistical difference between good and poor spatial learners but we did find that that poor place learners had a greater number of BrdU-labeled cells than poor cue learners. It is not immediately clear why our study demonstrated a different result with regards to good versus poor place learners. It is possible that methodological differences may explain the differing results. For example, our rats were sacrificed 16 days following BrdU administration but Sisti et al. (2007) sacrificed rats 11 days following BrdU administration, Sisti et al. (2007) trained rats for 4 consecutive days while in the present study we trained for 5 consecutive days and finally Sisti et al. (2007) defined good and poor performance based on the last 4 trials whereas in the present study we used a median split of total latency or distance to reach the platform across all trials. More research will be needed to clarify the interactions between good and poor spatial learning and hippocampal neurogenesis. Other factors such as the complexity of cues in the testing room may also increase or decrease the importance of the hippocampus for acquiring the task and thus may explain some of the differences between our results and those of Sisti et al. (2007). Furthermore, in the present data the means favored the findings that good place learners had greater cell survival than poor place learners in the groups trained 11–15 days after BrdU administration (when using latency to define good and poor performance) indicating a possible time of training effect.

‘Good’ performance versus ‘poor’ performance on a task may be considered an indicator of the efficiency of the neural substrate that supports it. Thus, it is plausible that increased hippocampal neurogenesis in poor place learners is acting to improve the efficiency of a poorly functioning hippocampus. Neurogenesis has also been shown to play a role in long-term
memory storage in the hippocampus (Snyder et al., 2005). Snyder et al. (2005) found that rats given focal irradiation prior to learning, to reduce neurogenesis, showed normal acquisition of a spatial location in the Morris water task and normal retention 1 week later but were impaired when probe trials were given 2 or 4 weeks after acquisition. Thus, taken together with our findings, increased cell survival may improve long-term memory retention for a spatial task despite poorer initial learning.

Recently Saxe et al. (2007) have proposed that increased GABAergic tone resulting from exposure to a novel environment may explain why certain quickly acquired tasks may enhance neurogenesis while others that take longer to acquire do not. As is the case during development, GABA acts as an excitatory rather than inhibitory neurotransmitter for adult generated young neurons (Wang et al., 2000; Ambrogini et al., 2004b). Therefore in the presence of a novel situation the new neurons play a larger role in synaptic transmission because GABAergic tone is increased in response to novel environments (Bianchi et al., 2003; Davis et al., 2004). This hypothesis may also help explain the results presented here. ‘Poor’ learners may have acquired less about the testing environment and as a result perceive it as being more novel than a ‘good’ learner would. This would likely result in a lengthened increase in GABAergic tone in ‘poor’ learners that could contribute to the survival of new neurons.

Alternatively, it is possible that the difference between ‘good’ and ‘poor’ place learners is a result of task difficulty. ‘Poor’ learners may perceive the task as more difficult than ‘good’ learners and this may somehow cause a change in cell survival. It has been shown that increasing task difficulty can render a hippocampus-independent task dependent on the hippocampus (Beylin et al., 2001). Thus, if the performance of ‘poor’ cue learners is a result of task difficulty then they would likely have had increased activation of the hippocampus and should have had higher levels of cells survival than ‘good’ cue learners. However, this was not
the case. Our finding was that poor place learners had enhanced cell survival compared to poor cue learners, suggesting that hippocampal neurogenesis is more vulnerable to the survival promoting effects after place learning rather than to cue learning likely due to greater activation of the hippocampus by place learning than by cue learning in poor learners.

Blocking adult hippocampal neurogenesis is the only definitive way to determine whether neurogenesis is involved in learning and memory, however, the current methods are not without controversy. Antimitotic agents such as methylazoxymethanol acetate (MAM) have been used to inhibit proliferation but this technique does not provide complete ablation and has a variety of side effects (Dupret et al., 2005). Irradiation has also been used to ablate adult neurogenesis and while the reduction is more complete than with MAM there may still be side effects on brain structure and function including upregulation of microglia which itself has been shown to suppress neurogenesis (Monje et al., 2003). Nevertheless, previous work has shown that limiting hippocampal neurogenesis impairs hippocampus-dependent learning (Shors et al., 2002; Winocur et al., 2006; Saxe et al., 2007) and memory (Snyder et al., 2005), indicating a direct involvement of adult hippocampal neurogenesis on learning and memory. There is controversy in the field as not all forms of hippocampus-dependent learning appear to be affected by reducing neurogenesis via antimitotic agents (Shors et al., 2002). However, recently it has been shown that focal irradiation disrupts many forms of hippocampus learning and memory such as non-match-to-sample learning and contextual fear conditioning (Winocur et al 2006; Saxe et al., 2007). At least two studies have showed that ablation of neurogenesis (via either MAM or focal irradiation) did not impair spatial learning in the Morris water task (Shors et al., 2002; Snyder et al., 2005). Interestingly, as mentioned earlier decreased neurogenesis in the dentate gyrus resulted in long-term memory deficits in the Morris water task (Snyder et al., 2005). Therefore it appears as though new neurons generated in the dentate gyrus are not
required for learning but that learning may enhance survival of some new neurons, those extending axons and making synaptic contacts, and these new cells are required for proper long-term memory.

As more data is generated regarding the interaction between adult neurogenesis and hippocampal-dependent learning, it is becoming evident that the relationship is a complex one. Future studies investigating learning and neurogenesis should acknowledge the importance of the timing of BrdU injections and of subsequent behavioral testing, type of learning and also learning performance.
References


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3 ACTIVATION AND SURVIVAL OF IMMATURE NEURONS IN THE DENTATE GYRUS WITH SPATIAL MEMORY IS DEPENDENT ON TIME OF EXPOSURE TO SPATIAL LEARNING AND AGE OF CELLS AT EXAMINATION

It is now widely accepted that in the adult mammalian brain, neurogenesis occurs throughout life in the dentate gyrus of the hippocampus ((Altman and Das, 1965; Kaplan and Hinds, 1977; Cameron et al., 1993; Eriksson et al., 1998). The function of these new cells remains uncertain and controversial however, mounting evidence points towards a role in hippocampus-dependent learning and memory (for review see Leuner et al., 2006). New neurons that are produced in the subgranular zone of the dentate gyrus migrate into the granule cell layer and extend axons into the CA3 region as they integrate, (Stanfield and Trice, 1989; Hastings and Gould, 1999; Markakis and Gage, 1999), become electrophysiologically active and mature into functional granule cells (van Praag et al., 2002).

Numerous studies have demonstrated that training rats in certain hippocampus-dependent tasks may alter the number of cells that survive to maturity (Gould et al., 1999; Ambrogini et al 2000; Leuner et al., 2004; Hairston et al., 2005; Olariu et al., 2005; Epp et al., 2007; Epp et al., 2009). Gould and colleagues (1999) first showed that rats trained in a spatial navigation version of the Morris water task (MWT) had a greater number of new surviving cells compared to rats trained in a non-spatial (hippocampus-independent) version of the MWT. These results

2 A version of this chapter has been submitted for publication. Epp, J.R., Haack, A.K. and Galea, L.A.M. (2010). Activation and survival of immature neurons in the dentate gyrus with spatial memory is dependent on time of exposure to spatial learning and age of cells at examination.
suggested that memory acquisition was responsible for stabilizing new neurons in the dentate gyrus, presumably in an activity dependent manor. However, several studies have reported that hippocampus-dependent learning does not alter cell survival (Dobrossy et al., 2003; van der Borght et al., 2005; Mohapel et al., 2006) or may even decrease cell survival following spatial learning (Ambrogini et al., 2004; Epp et al., 2009). We and others have shown that the effect of spatial learning on neurogenesis in the hippocampus is dependent on a variety of factors such as timing, quality of learning and task difficulty (Epp et al., 2007; Sisti et al., 2007; Epp et al., 2009).

Recent evidence suggests that timing may be critical in explaining why only some studies have found enhanced cell survival in response to spatial learning. We have recently demonstrated that spatial learning increases cell survival specifically when training occurs 6-10 days following bromodeoxyuridine (BrdU) administration but there was no change in cell survival when training occurred either 1-5 days or 11-15 days after BrdU administration (Epp et al., 2007). This finding provided evidence of a critical period for enhancing cell survival but also reconciled some of the conflicting data regarding cell survival following spatial learning. In several studies that showed increased cell survival following spatial learning the animals received training during a time approximately equivalent to 6-10 days following BrdU (Gould et al., 1999; Ambrogini et al., 2000; Epp et al., 2009). However, one study described a decrease in cell survival following spatial learning that occurred 11-15 days following BrdU administration (Ambrogini et al., 2004). However, despite training at the same time period following BrdU administration (11-15 days) we previously found no change in cell survival (Epp et al., 2007). Thus, although the time of training is an important factor in determining the rate of cell survival in response to spatial learning, there must be other factors involved. In the Ambrogini (2004) study, they perfused the rats three days after training and thus the time after training was longer.
than in our previous study (Epp et al., 2007) and may have allowed for a delayed decrease in cell survival that had not yet occurred one day following training.

In addition to demonstrating that the time window between labeling new cells with BrdU and training is important for detecting changes in cell survival, the first aim of the current study is to determine whether the time between training and perfusion is also important. The second aim of the current study was to examine whether changes in cell survival that result from spatial learning at different times after BrdU administration cause a change in the number of new neurons that are activated by memory retrieval. Several studies have used immediate early gene (IEG) activation as a marker of cellular recruitment in new neurons in response to hippocampus dependent learning and memory (Ramirez-Amaya et al., 2006; Kee et al., 2007; Tashiro et al., 2007; Snyder et al., 2009, 2009b). These studies have been useful in demonstrating that new neurons are recruited by spatial learning and recent evidence indicates that new neurons in the dentate gyrus are preferentially activated during spatial learning compared to mature neurons (Kee et al., 2007). In the current study we sought to determine whether the critical period for enhancing cell survival (6-10 days after BrdU administration) has a corresponding increase in recruitment of these new cells compared to training on days 1-5 or 11-15.

To address these aims, we trained rats in the MWT at one of three time points following BrdU administration (1-5, 6-10 or 11-15 days) and perfused all groups five days following training in order to allow sufficient time for any delayed changes in cell survival to occur after the completion of training. Two hours prior to perfusion all rats were given a single probe trial in the MWT to assess spatial memory and to determine whether memory retrieval differentially actives new neurons dependent on the age of the cells at the time of training.
Experimental procedures

Subjects

Subjects were 96 male Sprague Dawley rats (Charles River; Saint-Constant, Quebec, Canada) 70-75 days old and weighing between 300 and 350 grams at the beginning of testing. Rats were housed individually in standard cages with a polyvinylchloride tube, paper towels, cedar bedding and free access to food and water. Rats were habituated to their housing conditions for one week, and were then handled five minutes per day for five days prior to the start of the experiment. All testing was carried out in accordance with the Canadian Council for Animal Care guidelines and was approved by the animal care committee at the University of British Columbia. All efforts were made to reduce the number of animals used and to minimize their suffering.

Apparatus

A circular pool, 180 cm in diameter was filled with approximately 21°C water to a depth of 30 cm. Non-toxic white tempura paint was added to the water to render it opaque. Numerous large cues were placed around the room on all sides of the pool (see Epp et al., 2009). A camera mounted above the pool was connected to a computer running Anymaze tracking software (Stoelting Co; Wood Dale, IL, USA) and was used to record latency and distance to reach the platform during training as well as the percentage of time spent in each quadrant and in the vicinity of the platform location during the probe trial.

Procedure

All rats were given a single intraperitoneal injection of BrdU (200mg/kg; Sigma, Oakville, ON, Canada) on day 0 of the experiment. Then, rats were divided into three groups to
be tested at three different time points, days 1-5, 6-10 or 11-15 after BrdU administration. See Table 3.1 for a timeline of the training procedure). Half of the rats at each time point were trained on the place (hippocampus-dependent) version of the task and half were trained on the cue (hippocampus-independent) version of the task. In the place version of the task, a hidden platform was located in the center of one quadrant of the pool, 2cm below the surface of the water. In the cue version of the task, the top of the platform extended 2cm above the surface of the water and moved randomly to the center of a new quadrant following each trial so that a spatial strategy could not be used. In either case, all rats were given one daily training session for five days with four trials per session starting from a different one of the four cardinal compass points on each trial.

Table 3.1: Timeline of training and perfusion

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B=BrdU injection, W= Morris water task training, P= probe trial and perfusion

Five days following the final training session all rats were given a single probe trial. During this trial the rats swam for 30 seconds in the same pool without an escape platform to assess retention of the platform location. We compared the amount of time spent in the quadrant of the pool that had previously contained the hidden platform (the target quadrant) as a gross
measure of probe trial performance. We also compared the amount of time spent in a smaller area comprising 10% of the pool area centered over the platforms location (the platform zone).

Two hours following the probe trial the rats were deeply anesthetized with sodium pentobarbital and were then perfused transcardially with 60 ml of 0.9% saline followed by 120 ml of 4% paraformaldehyde (Sigma) in 0.1M phosphate buffered saline (PBS; Sigma). The brains were extracted and placed in the same 4% paraformaldehyde solution for 24 hours and were than transferred into a solution containing 30% sucrose (Sigma) in 0.1M PBS for at least four days. A Leica vibratome (VT1000S; Richmond Hill, ON, Canada) was used to section the brains. Throughout the rostral-caudal extent of the hippocampus 10 series of 40μm sections were collected. The sections were then stored in antifreeze (ethylene glycol/ glycerol; Sigma) at -20°C until processing.

**BrdU immunohistochemistry**

The tissue was rinsed 3 times and left overnight in 0.1M PBS to remove the antifreeze solution. Tissue sections were incubated at room temperature in 0.6% H2O2 (Sigma) for 30 minutes followed by three rinses in 0.1M PBS prior to a 30 minute incubation at 37°C in 2N HCl (Sigma). The tissue was then rinsed in 0.1M borate buffer for 10 minutes (pH 8.5) followed by three more rinses in 0.1M PBS. The tissue sections were then transferred to a primary antibody solution containing 1:200 mouse anti-BrdU (Roche; Mississauga, ON, Canada), 3% normal horse serum (Vector Laboratories; Burlingame, CA, USA) and 1% Triton X (Sigma) in 0.1M PBS for 24 hours at 4°C on a shaker. The tissue was rinsed 3 times in 0.1M PBS and was then transferred to a secondary antibody solution containing a 1:500 dilution of horse anti-mouse (Vector Laboratories) in 0.1M PBS. The tissue was incubated in the secondary solution for 4 hours at room temperature. Following three rinses in 0.1M PBS, an ABC kit (Vector
Laboratories) was used and prepared according to the kit instructions. BrdU labeling was visualized by incubating tissue in nickel enhanced diaminobenzidine (DAB; Sigma) for approximately 3 minutes. Finally, the sections were mounted on glass slides and coverslipped with Permount (Fisher Scientific; Ottawa, ON, Canada).

**BrdU/NeuN double labeling**

Neuronal phenotype was assessed in a subset of randomly selected rats (5 per group). The tissue was rinsed 3 times and left overnight in 0.1M PBS to remove the antifreeze solution. Tissue was incubated at 4°C for 24 hours in a primary antibody solution containing a 1:200 dilution of mouse anti-NeuN (Millipore; Billerica, MA, USA), 3% Normal donkey serum (Vector Laboratories) and 3% triton-x in 0.1M PBS. The sections were then rinsed three times in PBS and were then incubated in a solution containing a 1:250 dilution of donkey anti-mouse Alexa 488 (Jackson ImmunoResearch; West Grove, PA, USA) in 0.1M PBS for 16 hours at 4°C. The tissue was rinsed three times in PBS and was then fixed in 4% paraformaldehyde for 10 minutes and rinsed three times in 0.9% saline. DNA denaturation was accomplished by incubating the tissue for 30 minutes at 37°C in 2N HCl. The tissue was rinsed three times in PBS prior to incubation in a primary antibody solution containing rat anti-BrdU (AbD Serotec; Raleigh, NC, USA), 3% normal donkey serum and 3% Triton-X in 0.1M PBS for 24 hours at 4°C. The tissue was then rinsed three times in PBS and then incubated in a secondary antibody solution containing donkey anti-rat Cy3 (Jackson ImmunoResearch; West Grove, PA, USA) for 16 hours at 4°C. The tissue was rinsed three times and was then mounted on glass slides and coverslipped with PVA-DABCO.
**BrdU/c-fos double labeling**

The tissue was rinsed 3 times and left overnight in 0.1M PBS to remove the antifreeze solution. Then, a 1/10 series of tissue was first incubated in 2N HCl for 30 minutes at 37°C and was rinsed three times in PBS. Then, the tissue was transferred to a primary antibody solution containing 1:500 mouse anti-BrdU (Roche), 1:1000 rabbit anti-c-fos (Santa Cruz Biotechnology; Santa Cruz, CA, USA), 3% normal donkey serum and 3% Triton-X in 0.1M PBS and incubated for 24 hours at 4°C. The tissue was then rinsed three times in PBS and was then transferred to a secondary antibody solution containing 1:1000 dilutions of donkey anti-rabbit Alexa 488 (Jackson ImmunoResearch) and donkey anti-mouse Cy3 (Jackson ImmunoResearch) for 16 hours at 4°C. The tissue was rinsed three times in PBS and was then mounted on glass slides and coverslipped with PVA-DABCO.

**Cell counting**

BrdU-labeled cells (Figure 3.1A) were counted in every 10th section throughout the entire granule cell layer (GCL; includes the 50µm thick subgranular zone) and the hilus to obtain an estimate of the total number of BrdU-labeled cells. The counting of BrdU-labeled cells was performed with a Nikon E600 brightfield microscope and a 100x oil immersion objective lens. To obtain an estimate of the total number of cells we multiplied by the sampling fraction of 10 using the same procedure as in previous studies (Kronenberg et al., 2003; Eadie et al., 2005; Epp et al., 2007; Epp et al., 2009). The percentage of BrdU/NeuN double-labeled cells was obtained by selecting 50 BrdU cells arbitrarily from at least 5 sections per brain and determining whether those cells also expressed NeuN. BrdU/c-fos double labeling was carried out with the same procedure as for BrdU/NeuN labeling except that 100 BrdU-labeled cells were examined in each
brain. Fluorescent imaging was performed on a Nikon E600 epifluorescent microscope under 400x magnification.

**Data analyses**

We analyzed dependent variables of latency to reach the platform, distance to reach the platform and swim speed using repeated-measures analysis of variance (ANOVA). Day of training (1-5, 6-10, 11-15) and type of training (place, cue) were used as between-subjects factors and session (1-5) was the within subject factor. Volume and number of BrdU-labeled cells were each analyzed with repeated measures ANOVAs using day of training and type of training as between-subjects factors and region (GCL, hilus) as a within-subjects factor. The percentage of BrdU/NeuN and BrdU/c-fos double labeling and probe trial performance (percent time in the target quadrant and platform zone) were analyzed using ANOVAs with time of training and type of training as between-subjects factors. Post-hoc tests utilized the LSD procedure. Pearson product moment calculations were performed on the percentage of BrdU/c-fos labeled cells and the percentage of time in the platform zone and target quadrant during the probe trial.
Figure 3.1: A) Representative peroxidase labeling of BrdU labeled cells in the granule cell layer is shown. B) A c-fos labeled cell (green) is shown. C) An example of a fluorescent BrdU-labeled cell. D) BrdU and c-fos double labeling is shown (arrow). Magnification is 1000x for panel A and 400x for panels B C and D.
Results

*Place-trained rats had longer latencies and swim distances in the Morris water task than cue-trained rats*

In terms of latency to reach the escape platform (Figure 3.2 A-C) there was a significant three way interaction between session, time of training and type of training (F(8,204) = 3.58, \( P \leq 0.00066 \)). Post-hoc tests showed that place-trained rats had significantly longer latencies than cue-trained rats during all sessions at all three training time points (\( P \leq 0.037 \)). We further analyzed the total latency across all trials and sessions (Figure 3.3 A). There was a significant main effect of type of training (F(1,51) = 262.70, \( P \leq 0.000001 \)) with cue-trained rats having shorter total latencies. There was no significant main effect of time of training \( (P=0.27) \) or training by time interaction \( (P=0.61) \).

For distance traveled to reach the platform (Figure 3.2 D-F), there was a significant three-way interaction between session, time of training and type of training (F(8,204) = 2.38, \( P \leq 0.018 \)). Post-hoc tests indicated that cue-trained rats swam shorter distances than place-trained rats during all sessions and at all three time points \( (P \leq 0.040) \) except for session five at the 1-5 day time point which was not significantly different \( (P=0.091) \). For total distance to reach the platform across all trials (Figure 3.3 B), cue-trained rats had significantly shorter swim distances than place-trained rats \( (F(1,51) = 260.30, P \leq 0.000001) \). There was no significant main effect of time of training \( (P=0.60) \) and no significant training by time interaction \( (P=0.35) \).
Figure 3.2: Latency to reach the platform for each session is shown for rats trained on days 1-5 (A) 6-10 (B) and 11-15 (C). Cue-trained rats had shorter latencies than place-trained rats during all sessions at all three time points. Distance to reach the platform is shown for rats trained on days 1-5 (D) 6-10 (E) and 11-15 (F). Cue-trained rats swam shorter distances than place-trained rats during all sessions and at all three time points. Data represents mean ± SEM.
Place-trained rats spent more time in the target quadrant and the platform zone than cue-trained rats

In regards to time spent in the target quadrant there was a significant main effect of type of training \((F(1, 51) = 10.18, P \leq 0.0024)\) but not time of training \((P=0.41)\) and there was no significant time by training interaction \((P=0.38)\). Place-trained rats spent a greater percentage of time swimming in the target quadrant than cue-trained rats (Figure 3.4 A). Similarly, in terms of time spent swimming in the platform zone (Figure 3.4 B) there was a significant main effect of type of training with place-trained rats spending more time in the platform zone than cue-trained rats \((F(1, 51) = 17.97, P \leq 0.000094)\). There was no significant main effect of time of training \((P=0.34)\) and no significant time by training interaction \((P = 0.17)\).
**Figure 3.3:** Total latency to reach the platform across all trials and sessions is depicted in Panel A. Cue-trained rats had shorter total latencies than place trained rats but there was no significant effect of time of training. Total Distance to reach the platform across all trials is shown in panel B. Cue-trained rats had significantly shorter total distances than place-trained rats. There was no significant main effect of type of training. Bars represent mean ± SEM.
Figure 3.4: Performance during the probe trial is shown. Panel A shows the percentage of time spent in the quadrant of the pool where the hidden platform had previously been located during place training. Panel B shows the percentage of time spent in a circular zone centered over the platforms previous location that encompasses 10% of the pool area. Place trained rats spent significantly more time in the target quadrant and the platform zone than cue-trained rats. Bars represent mean ± SEM and dashed lines represent chance performance.
Morris water task training increases and decreases cell survival depending on the age of the new cells at the time of exposure to training

For the total number of BrdU-labeled cells in the dentate gyrus there was a significant three-way interaction between region type of training and time of training (F(2,51) = 4.54, $P \leq 0.015$). There were significant main effects of time of training (F(2,51) = 5.85, $P \leq 0.0052$) and region (F(1,51) = 732.359, $P \leq 0.000001$) and significant interactions between time and training (F(2,51) = 4.47, $P \leq 0.016$) and region and time (F(2,51) = 3.27, $P \leq 0.046$). Post-hoc tests indicated that at the 6-10 day training time (Figure 3.5) there were significantly more BrdU-labeled cells in the GCL of place-trained rats compared to cue-trained rats ($P=0.0084$). However, at the 11-15 day time point (Figure 3.5) there were significantly fewer BrdU-labeled cells in the GCL of place-trained rats than cue-trained rats ($P=0.014$). There was no significant difference in BrdU-labeling in the GCL of rats trained on the place or cue task on days 1-5 ($P=0.089$) although there was a trend for lower BrdU-labeled cell counts in place learners (Figure 3.5). There were no differences between place and cue trained rats in terms of BrdU labeling in the hilus at any of the training time points ($P \geq 0.78$).

We next correlated the number of BrdU-labeled cells in the GCL with the total distance traveled during the training sessions as an indicator of overall learning (Figure 3.5). There were no significant correlations for rats trained on the cue task on days 1-5 ($r= -0.15, P=0.75$), 6-10 ($r= 0.49, P=0.22$) or 11-15 ($r= -0.057, P=0.86$). With place training there was a significant negative correlation for rats trained on days 11-15 ($r= -0.75, P=0.013$) but not for rats trained on days 1-5 ($r= -0.55, P=0.20$) or days 6-10 ($r= -0.057, P=0.86$).
**Figure 3.5:** The total number of BrdU-labeled cells in the GCL and Hilus are shown for rats trained on days 1-5 (A) 6-10 (B) and 11-15 (C). There was no significant difference between place and cue training on days 1-5. Place training on days 6-10 increased the number of BrdU-labeled cells compared to cue training. Place training on days 11-15 decreased the number of BrdU-labeled cells compared to cue training. There were no significant differences between place and cue learners for BrdU labeling in the hilus at any of the training times. Bars represent mean ± SEM. Panel D shows the significant negative correlation between BrdU labeling in the GCL and total distance traveled during training for rats trained on the place version of the task on days 11-15.
**Place training increases the activation of BrdU-labeled cells**

We were unable to identify any BrdU/c-fos double-labeled cells in the groups of rats trained on days 1-5 following BrdU administration and perfused on day 10. Thus we statistically analyzed only the 6-10 and 11-15 day time points. There was a significant main effect of time of training ($F(1,39) = 26.57, P \leq 0.000008$) with a greater number of double-labeled cells at the 11-15 day time point compared to the 6-10 day time point. Furthermore, there was a significant main effect of type of training ($F(1,39) = 4.54, P \leq 0.039$) on the percentage of BrdU-labeled cells that co-expressed c-fos, with more BrdU/c-fos labeled cells in place trained rats compared to cue trained rats (Figure 3.6). There was no significant time by training interaction ($P=0.24$). However, *a priori* we predicted that there would be an increase in the number of BrdU-labeled cells activated by place training on days 11-15 (20 day old cells) versus days 6-10 (15 day old cells) based on the reasoning that more of the new older cells will have matured to the point that they are physically capable to being activated. We found that significantly more BrdU-labeled cells expressed c-fos after place training on days 11-15 compared to place training on days 6-10 ($P=0.00003$).

**The number of new activated 20 day old neurons were positively correlated with spatial performance in the MWT**

Next we correlated probe trial performance in the MWT with the percentage of BrdU/c-fos labeled cells. There was a significant positive correlation between the percentage of BrdU/c-fos double-labeled cells and the percentage of time spent in the target quadrant during the probe trial ($r= 0.63, P=0.05$) for rats trained on the place task on days 11-15 (Figure 3.7). No significant correlations were found for the cue-trained rats at days 11-15 ($r= -0.051, P=0.88$) or the 6-10 day place-trained rats ($r= -0.19, P=0.54$) or cue-trained rats at days 6-10 ($r= -0.17,$
between percentage of BrdU/c-fos double-labeled cells and percentage of time spent in the target quadrant. There were no significant correlations between the percentage of time spent in the platform zone and the percentage of BrdU/c-fos double-labeled cells for place- or cue-trained rats on days 6-10 (r= -0.018, P=0.95; r= 0.20, P=0.63 respectively) or place- or cue-trained rats on days 11-15 (r= -0.19, P=0.46; r= -0.14, P=0.67 respectively).

Figure 3.6: The percentage of BrdU-labeled cells that express the immediate early gene product c-fos is shown. A significantly greater percentage of double-labeled cells was found in place-trained rats compared to cue-trained rats. No double-labeled cells were found in the 1-5 day groups. Rats trained on days 11-15 had significantly more double-labeled cells than rats trained on days 6-10. Bars represent mean ± SEM.
Figure 3.7: Panel A shows a significant positive correlation between BrdU/c-fos double labeling and percent time spent in the target quadrant during the probe trial for place trained rats on days 11-15. Panel B shows a significant positive correlation between BrdU/c-fos double labeled cells and total distance traveled during training for rats trained on the place task on days 11-15. Panel C shows a significant negative correlation between BrdU/c-fos double labeled cells and total distance traveled during training for rats trained on the place task on days 6-10. Panel D shows a significant negative correlation between the total number of BrdU labeled cells in the GCL and the percentage of BrdU/c-fos labeled cells for rats trained on the place task on days 11-15.
We also correlated the percentage of BrdU/c-fos double-labeled cells with the total distance traveled during the training sessions. No significant correlations were found for rats trained on the place task on days 6-10 (r = -0.09, P = 0.77), the cue task on days 6-10 (r = -0.13, P = 0.76) or the cue task on days 11-15 (r = -0.29, P = 0.37). However, there was a significant positive correlation for rats trained on the place task on days 11-15 (r = 0.83, P = 0.003).

However, there were two clear outliers present in the number of BrdU/c-fos double-labeled cells for the place 6-10 day group. With these cases removed there was a significant negative correlation for place-trained rats on days 6-10 (r = -0.59, P = 0.05). Finally, we correlated the percentage of BrdU/c-fos double-labeled cells with the total number of BrdU-labeled cells in the GCL and we found a significant negative correlation in the rats trained on the place task on days 11-15 (r = -0.62, P = 0.05). However, there were no significant correlations for rats trained on the place task on days 6-10 (r = -0.25, P = 0.42) or on the cue task on days 6-10 (r = -0.003, P = 0.99) or days 11-15 (r = -0.068, P = 0.083).

**The percentage of NeuN/BrdU labeled cells increases with the age of the BrdU-labeled cells**

For the percentage of BrdU labeled cells co-expressing the neuronal marker NeuN (Figure 3.8) we found a significant main effect of time of training (F(2,24) = 13.98, P ≤ 0.000094). There were significantly fewer BrdU/NeuN double-labeled cells at the 1-5 day training time compared to the 6-10 day group (P = 0.0016) and the 11-15 day group (P = 0.000027). There was no significant difference between the 6-10 and 11-15 day groups (P = 0.12). There was no significant main effect of type of training (P = 0.89) or time by training interaction effect (P = 0.80).
Figure 3.8: The percentage of BrdU-labeled cells that express the mature neuronal marker NeuN is depicted. There was no significant difference between place and cue trained rats but there were significantly fewer double labeled cells in rats trained on days 1-5 compared to days 6-10 and 11-15. Bars represent mean + SEM.

No significant differences in granule cell layer volume between groups

For GCL and hilus volume there were significant interactions between region and training (F(2,51) = 4.29, P ≤ 0.043) and region and time (F(2,51) = 3.35, P ≤ 0.0043) but no significant three way interaction (P=0.59). There was a significant main effect of type of training (F(1,51) = 11.90, P ≤ 0.0011) and a significant main effect of region as expected with the
hilus being larger than the GCL ($F(1,51) = 1059.48, P \leq 0.000001$). All other main or interaction effects were not significant. Despite the significant two-way interactions, post-hoc tests failed to find significant differences in the volumes of the GCL ($P's \geq 0.76$) or hilus ($P's \geq 0.078$) at any of the training time points. There was also no significant difference in GCL volume between place- and cue-trained rats ($P=0.15$). The only significant volume difference was the comparison of hilus volume between place- and cue-trained rats ($P=0.0048$), with cue-trained rats having a significantly greater hilus volume (Table 3.2).

### Table 3.2: Mean (± SEM) volume of the granule cell layer and hilus from each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Granule cell layer (mm$^3$)</th>
<th>Hilus (mm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Place (1-5)</td>
<td>3.24 ± 0.15</td>
<td>5.07 ± 0.21</td>
</tr>
<tr>
<td>Cue (1-5)</td>
<td>3.66 ± 0.087</td>
<td>5.99 ± 0.23</td>
</tr>
<tr>
<td>Place (6-10)</td>
<td>3.34 ± 0.095</td>
<td>5.76 ± 0.18</td>
</tr>
<tr>
<td>Cue (6-10)</td>
<td>3.46 ± 0.096</td>
<td>6.16 ± 0.18</td>
</tr>
<tr>
<td>Place (11-15)</td>
<td>3.26 ± 0.12</td>
<td>5.60 ± 0.18</td>
</tr>
<tr>
<td>Cue (11-15)</td>
<td>3.49 ± 0.15</td>
<td>5.95 ± 0.19</td>
</tr>
</tbody>
</table>

There were no significant differences between groups for GCL volume. Cue-trained rats had significantly larger hilus volumes than place-trained rats.

**Discussion**

The goal of the current study was to determine whether spatial learning alters cell survival dependent on the developmental stage of the new cells relative to the amount of time
following training prior to perfusion and whether the different training time points would
differentially activate new neurons. As we have previously shown (Epp et al., 2007), our results
indicate that spatial training, compared to cue training, in the MWT enhances cell survival but
only if training occurs 6-10 days following BrdU administration. Furthermore, we show that
spatial learning can decrease cell survival compared to cue training if the rats receive training
11-15 days following BrdU administration and are perfused an additional five days later. Early
spatial training (days 1-5) was found to have no effect on cell survival. Furthermore, place
training, in comparison to cue training, increased activation of new neurons that were at least 15
days old.

**Cell survival may respond to spatial learning in a competitive fashion**

The decrease in neurogenesis in the hippocampus of place-trained rats at 11-15 days is in
agreement with the findings of Ambrogini et al., 2004 but differed from our previous results
(Epp et al., 2007. In our original study rats were perfused the day following training (day 16)
whereas in the Ambriogini study rats were perfused several days following training (day 18). In
the present study we waited 5 days to perfuse the rats after training, which more closely matched
the Ambriogini study. Thus, the fact that we observed a decrease in survival of BrdU-labeled
cells in the current study, consistent with Ambriogini’s results, may be due to the fact that we
allowed a period of 5 days instead of one day to elapse following training before perfusing rats.
Tashiro and colleagues (2006) demonstrated using a single-cell gene knockout technique that
survival of new cells is dependent on NMDA-receptor activation. However, death of cells that
do not receive NMDA-receptor activation does not occur until 18 days post infection. Thus, the
time that elapses between spatial training and perfusion may be important in observing changes
in cell survival. It is possible that the additional days following training (1 day (Epp et al, 2007)
versus 5 days in the present study) may allow sufficient time for the removal of degenerating
BrdU-labeled cells. In addition to observing a decrease in BrdU-labeled cells, Ambrogini and
colleagues (2004) also found an increase in apoptosis following spatial training compared to
non-spatial training. The increase in Tunel+ cells suggests that a possible mechanism for
reduced cell survival is apoptosis of newly generated cells. However, the effects of cell death on
the number of BrdU-labeled cells may not be immediately apparent if the unused cells are not
removed immediately from the network. This may explain our current findings that spatial
training on days 11-15 after BrdU injection, with perfusions occurring on day 20, decreases cell
survival. It may take several days following spatial training for the cells that are not
incorporated into the hippocampus to be removed via cell death pathways. Interestingly, a recent
study has demonstrated that spatial learning requires both an increase and a reduction in cell
survival depending on the age of the new cells (Dupret et al., 2007). In that study administration
of an anti-apoptotic agent both blocked cell death and impaired spatial learning. Thus, there
appears to be a functional link between the reduction or enhancement of survival of adult
generated neurons and learning ability. Indeed we found better learning (as assessed by total
distance) was associated with more surviving BrdU-labeled cells but with fewer activated BrdU-
labeled cells when training occurred 11-15 days after BrdU administration.

Although it is not immediately clear why cell survival would have been reduced when
training occurred on days 11-15 and perfusions occurred on day 20 it may be due to an
interaction/competition with other non-labeled populations of cells. Our present results and
previous results (Epp et al., 2007) show that the survival of cells that are 6-10 days old when
training occurs is enhanced. It is important to note that when a single injection of BrdU is given,
a specific age of cell is labeled but other populations of new neurons still exist. Thus, in rats
injected with BrdU 11-15 days before training there is still a population of cells 6-10 days old
that will preferentially be incorporated into the hippocampus as a result of spatial learning. A possible reason for the reduced survival of BrdU-labeled cells in the group trained on the place version on days 11-15 may be that a younger population of cells is preferentially incorporated. As a result, the older population of new cells receives less input and is eliminated in a competitive manor. This interpretation is consistent with findings showing that the selective integration of new neurons is based on a competition for NMDA-receptor activation (Tashiro et al., 2006). Thus the survival of adult generated neurons appears to be subject to at least two types of selection. There is an early phase (identified by training on days 6-10) where network activation promotes the survival of a young population of highly excitable new neurons. Due to the lack of glutamatergic connectivity at this time point the change in cell survival that occurs may be due to GABAergic transmission (which begins by 8 days of age in mice; Esposito et al., 2005). This is followed by a later phase (identified by training on days 11-15) where network activation leads to the pruning of cells that are not utilized for memory formation. This type of selection may provide the pool from which certain young neurons will be engaged in memory formation. In terms of the activation of these new cells during memory retrieval, spatial training on days 6-10 or 11-15 should increase the number of activated cells compared to cue training, which is indeed what we found in the current study. Furthermore, due to the pruning of unused cells, rats trained on days 11-15 may also have a greater percentage of BrdU-labeled cells that are involved in spatial memory storage, consistent with our current findings.

**Immature neurons may participate in the formation of hippocampus-dependent memories**

We also demonstrate here that compared to cue training, rats trained on the place version of the MWT have a greater percentage of new cells that are activated by recall of that memory. The increase in c-fos and BrdU co-labeled cells in place-trained rats compared to cue-trained rats
suggests that these new neurons may be playing an important role in the retention of spatial memories. Regardless of type of training there was also an increase in the number of BrdU-labeled cells that express c-fos at the 11-15 day time point. This is likely due to the fact that the cells examined in the 11-15 day group were more mature (20 days old compared to 15 days old cells in the 6-10 day training group) at the time of learning and retention testing. This reasoning also explains the lack of double-labeled cells in the 1-5 day group that were in fact only 10 days old. In mice, the onset of glutamatergic connectivity occurs once the immature neurons are approximately 18 days old (Esposito et al., 2005). However, this may occur slightly earlier in rats as it has been shown that new neurons develop slightly faster in terms of IEG activation in rats compared to mice (Snyder et al., 2009). Thus, the activated cells seen in the rats trained on days 6-10 and perfused on day 15 may be active in response to the probe trial only. However, the cells activated in the rats trained on days 11-15 and perfused on day 20 may be active in response to retrieval of the previously acquired memory. This would provide a potential explanation for the training effect seen in the 11-15 day group but not in the 6-10 day group.

Only rats trained on the place task on days 11-15 and given a probe trial on day 20 had evidence of a positive correlation between probe trial performance and the percentage of BrdU-labeled cells expressing c-fos. In other words, the number of activated 20 day old cells may be predictive of how well the hidden platform location is remembered. This correlation at the 11-15 day time point is interesting as this corresponds to the group that showed a reduction in cell survival. This might indicate that though there are fewer remaining BrdU-labeled cells those that do survive are more likely to be those that were used in the formation of new memory traces. Because the BrdU/c-fos double labeling at the 6-10 day time point does not correlate significantly with memory retention (on day 15) it is possible that although more of these cells survive they are not as likely as a slightly older population of cells to participate in the formation
or retention of spatial memories. Alternatively, it may simply be the case that fewer of the cells from the 6-10 day training group were activated because they were younger and were possibly too young to be effective at either the time of training or memory recall. This is certainly a possibility and fits with the complete absence of activated cells following training on days 1-5. It is not clear from the current study whether training on days 6-10 and probe trial/perfusion on day 20 would have resulted in an increase in BrdU/c-fos double-labeling similar to what was seen with training on day 11-15. Regardless, our results do show that once the immature neurons reach an adequate stage of maturation they are activated by the retrieval of a previously acquired spatial memory.

In agreement with our current findings, previous studies have demonstrated that newborn neurons are activated perhaps preferentially, in comparison to mature granule cells, as a result of spatial exploration (Ramirez-Amaya et al., 2006) and spatial learning (Kee et al., 2007; Snyder et al., 2009). Young neurons are functionally different from mature neurons in a number of ways that may explain why they may be preferentially recruited by spatial learning. Most importantly, young neurons are more excitable than mature neurons (Wang et al., 2000; Ambrogini et al., 2004; Schmidt-Hieber et al., 2004). The result of this is that less excitation (i.e. through spatial exploration or learning) is required to trigger action potentials in young neurons and therefore they may be more likely to become incorporated into a new circuit when the animal is learning. In fact, long-term potentiation, a putative mechanism of hippocampal learning can only be induced in the presence of GABA inhibition if immature neurons are present in the circuit (Wang et al., 2000). Thus, these new neurons appear to play a substantial role in hippocampus dependent learning and memory.
Impact of quality of learning on cell survival

Several previous studies have examined whether the survival of BrdU-labeled cells is related to the quality of learning (Dobrossy et al., 2003; Sisti et al., 2007; Epp et al., 2007). Although there is some indication that poor learning produces greater cell survival (Dobrossy et al., 2003; Epp et al., 2007) this is not conclusive because another study has shown that cell survival is enhanced in good learners but not poor learners (Sisti et al., 2007). In the current study our results demonstrate that the total swim distance is negatively correlated with the total number of BrdU-labeled cells in the GCL of place-trained rats on days 11-15. This suggests that better performance is associated with greater numbers of BrdU-labeled cells. In our previous study we demonstrated that cell survival was enhanced only in poor place learners but not in good place learners when performance was subdivided based on the median total latency or distance (Epp et al., 2007). The current data lend support to the idea that enhanced cell survival is related to better performance at least when certain ages of cells are examined, as in the current study this negative correlation only held for the days 11-15 place-trained group. The age of the new cells at the time of training may be one factor that interacts with the quality of learning and the number of surviving cells. That is, better learning may decrease or not change (depending on factors such as the difficulty and type of task) the survival of young neurons but may enhance the survival of slightly older new neurons. In addition we also found that more activated new neurons were associated with better memory for the platform (but worse performance during training) when training occurred on days 11-15. However, when training occurred on days 6-10 the opposite relationship between learning ability and activation of BrdU-labeled cells was found. At the earlier time point (days 6-10) better learning was related to more activated cells during the probe trial. This provides further support of a differential effect of quality of learning.
on the incorporation and function of new neurons dependent on the age of the cells at the time of training.

**Factors related to spatial learning that may alter cell survival**

The existing evidence on the effect of hippocampus-dependent learning on adult neurogenesis is mixed (ie. Gould et al., 1999; Ambrogini et al., 2004; Van der Borght et al., 2005). Although much of this literature seems contradictory there are numerous factors that when taken into account help to reconcile the diverse findings. First, the population of new cells being studied must be sufficiently old enough at the time of learning. Cells that are too young may not respond to activation by spatial learning because they lack connectivity with the existing hippocampal network. Likewise, cells that are too old at the time of training may already be stable in the cellular network and thus learning will not affect their survival. However, we and others have shown that there is a critical age at which cell survival will respond positively to spatial learning (Epp et al., 2007) or other factors such as hormone treatment (Ormerod et al., 2004) or spatial exploration (Tashiro et al., 2007). In the case of spatial learning we have shown that the critical age for enhancing cell survival occurs when training takes place 6-10 days following proliferation (present study; Epp et al., 2007). Training outside of this time period may explain why some studies have not found increases in cell survival following spatial training (Dobrossy et al., 2003; Ambrogini et al., 2004; Van der Borght et al., 2005). Spatial learning can also decrease cell survival and there are a number of factors that may explain this response. First, as shown here and in a previous study, training during days 11-15 after BrdU administration decreases cell survival (Ambrogini et al., 2004) but only if several days elapse before perfusion (Epp et al., 2007). Findings of unaltered cell survival with late training may be
explained by a gradual removal of BrdU-labeled cells that may be missed if rats are perfused immediately following training.

Even if training occurs during the critical period (days 6-10) there is some evidence that cell survival may be reduced. One of the factors that appear to eliminate the increase in cell survival is the difficulty of the task (Epp et al., 2009). We showed that increasing task difficulty, by removing most of the distal cues, decreases cell survival compared to visible platform training (Epp et al., 2009). Finally, there is some evidence that stress and physical exercise may interfere with the effects of spatial learning on cell survival (Ehninger and Kempermann, 2006) so these factors should also be considered possible explanations for the variability of results in the literature. Based on the above factors that may alter cell survival it is important to cautiously interpret the current findings. It may be possible that small differences in spatial learning ability or memory retention at the time of the probe trial could affect the survival or activation of new neurons. However, we did not observe any significant differences in learning ability between rats trained on days 1-5, 6-10 or 11-15. Furthermore, although memory retention was not extremely strong (at least in terms of quadrant preference) it was not significantly different between the different training times. Thus, it is unlikely that differences in training or memory are responsible for the differences in cell survival or cell activation at the different training time points.

An interesting set of correlations observed specifically in the place-trained rats at the 11-15 day time point may help to explain the role of adult neurogenesis in spatial learning. First, slow learning (i.e. longer total distance) was predictive of having fewer BrdU-labeled cells in the day 11-15 place group. However, slow learning also predicted greater activation of BrdU-labeled cells during the probe trial in this same group. In addition, with fewer BrdU-labeled cells available in the 11-15 day place group a greater percentage of them were activated during
the probe trial than in the 6-10 day place group. This suggests that the BrdU-labeled cells of this age that do survive are participating in memory retrieval to a greater extent. Furthermore, this idea is supported by the positive correlation between time spent in the target quadrant during the probe trial and the number of activated BrdU-labeled cells in the day 11-15 place rats (but not in the 6-10 day place rats). It is important to emphasize that none of the above correlations were significant in any of the groups except the place trained rats trained on days 11-15. This suggests that although place training on days 6-10 may enhance cell survival, the biggest impact on activation of new neurons in response to spatial learning occurs at a later time point.

**Conclusions**

In summary, our main findings are that spatial learning has different effects on the survival of cells when training occurs at different ages. Training that occurs when cells are very immature (days 1-5) has no effect on cell survival, training at an intermediate age (days 6-10) increases cell survival and late training (days 11-15) decreases cell survival when rats are perfused five days after training. In combination with our previous study we demonstrate here that the decrease in cell survival that occurs with spatial learning on days 11-15 is not immediately apparent if rats are perfused one day later (Epp et al., 2007) but can be seen when perfusion is delayed until day 20 (present study). Finally, the current study provides evidence that despite a decrease in cell survival with late training and perfusion five days after training, cells of this age are recruited for storage/recall of the spatial location of the hidden platform. Thus it appears that the percentage of new cells that are active may be more important than the total number of cells that survive following spatial learning. In conclusion the total number of new cells available can be altered by spatial learning and the number of new neurons appears to
be an important factor in determining the percentage that are actively involved in the storage of a given memory.
References


4 TASK DIFFICULTY IN THE MORRIS WATER TASK INFLUENCES THE
SURVIVAL OF NEW NEURONS IN THE DENTATE GYRUS

The dentate gyrus of the hippocampus contains progenitor cells that continue to produce new neurons throughout life (Altman & Das, 1965; Kaplan & Hinds, 1977; Cameron et al., 1993; Eriksson et al., 1998). Continued adult neurogenesis is rare in the mammalian brain and has been confirmed only within the neurogenic niches of the subventricular zone and the subgranular zone of the dentate gyrus. The persistence of this phenomenon in restricted areas presents the notion that neurogenesis in the adult hippocampus may have a critical function although it remains unclear what that may be. Several lines of evidence have shown that adult generated neurons in the hippocampus develop to become functional neurons with similar projections to mature neurons (Stanfield & Trice, 1988; Markakis & Gage, 1999; Hastings & Gould, 1999; Zhao et al., 2006). As early as 10 days of age they begin to express mature neuronal markers (Brown et al., 2003) and mature further to become electrophysiologically active (van Praag et al., 2002) even as early as 17 days after cell division (Toni et al., 2008).

The hippocampus is critically involved in a variety of types of learning and memory and there are numerous examples of hippocampus-dependent learning modulating both the rate of proliferation (Pham et al., 2005; Epp and Galea, 2009) and subsequent survival of newly generated neurons (Gould et al., 1999; Ambrogini et al., 2000; Ambrogini et al., 2004; Olariu et al., 2005; Mohapel et al., 2006; Epp et al., 2007). For example, spatial learning (one type of

hippocampus-dependent learning) has been shown to enhance the survival of new cells produced approximately one week prior to training (Gould et al., 1999). Although this result has been replicated several times (Ambrogini et al., 2000; Leuner et al., 2004; Hairston et al., 2005; Olariu et al., 2005; Epp et al., 2007; Keith et al., 2008) there are a number of studies that have shown no change in cell survival (Dobrossy et al., 2003; van der Borght et al., 2005; Mohapel et al., 2006; Epp et al., 2007) or even decreased cell survival (Ambrogini et al., 2004; Olariu et al., 2005; Mohapel et al., 2006) in response to spatial learning. One explanation for these differences may in some cases be the timing of the experiment as there is a critical period during which new cells are more vulnerable to the effects of behavioral manipulations (Epp et al., 2007; Tashiro et al., 2007). For example, we found that rats trained on a hippocampus-dependent version of the Morris water task 6-10 days following BrdU administration had increased cell survival compared to rats trained on a hippocampus-independent version of the task. However, there were no differences in cell survival if training occurred either 1-5 or 11-15 days following BrdU administration (Epp et al., 2007).

There are also other methodological differences, specifically in terms of behavioral training that may affect whether a change in cell survival occurs with spatial training. There are numerous variations that can be made to training in the Morris water task that can change how well the task is acquired such as the number of trials, time spent on the platform after each trial or pre-exposure to the platform location (Sutherland & Linggard, 1982; Keith & McVety, 1988). Additionally, there are a number of less controllable differences that may still alter learning or the difficulty of the task such as the ambient light level in the testing room, salience of the pool wall (Hamilton et al., 2008), and the number of or salience of the distal cues (Fenton et al., 1994).
Although, it is known that spatial learning can enhance the survival of adult generated neurons, if BrdU is given 6-10 days prior to training, it is unclear whether the magnitude of the change in cell survival is related to the difficulty of the task. However, it seems likely to be the case as several studies have now identified that the quality of learning or performance efficiency is critical to the level of cell survival following spatial training. Sisti et al., (2007) showed that rats that performed better in the Morris water task had a greater number of surviving new neurons than poor learners. Conversely, a negative correlation between performance and cell survival has also been shown (Dobrossy et al., 2003). We have previously shown that spatial learning in the Morris water task enhances cell survival, but only in poor learners. Good spatial learners on the other hand did not show any increase in cell survival compared to good cue learners (Epp et al., 2007). From these results it is not clear what the relationship between quality of learning (i.e. how well the solution to the task is acquired) and cell survival is but it does appear as though there is an interaction. For example, it may well be that in our previous study only the rats that found the task ‘difficult’ showed the enhancement with spatial learning. In other words, perhaps there is some sort of a threshold for engaging the hippocampus that will lead to enhanced cell survival.

To determine whether increasing the difficulty of a spatial task affects the magnitude of the change in cell survival, we administered BrdU to rats, and one week later trained them in one of four versions of the Morris water task. We utilized four different training methods; a hippocampus independent visible platform version of the task and three hippocampus dependent versions including, a four trial per session hidden platform version, a reduced-cue hidden platform version and a two trial per session hidden platform version. We then compared the number of proliferating and surviving cells in the dentate gyrus. We expected that hippocampus-
dependent learning would increase the survival of newly generated cells in the dentate gyrus and that the difficulty of the task would further influence cell survival.

**Experimental procedures**

**Subjects**

Subjects were 40 male Sprague-Dawley rats obtained from the animal care centre at the University of British Columbia. All rats were 60 days of age at the start of testing. Rats were housed individually in standard cages with cedar bedding, paper towels and a polyvinylchloride tube. Access to food (Purina rat chow) and water was provided *ad libitum*. After arrival the rats were given five days to habituate to their new environment and were then handled for five minutes per day for an additional five days. All testing was carried out in accordance with the Canadian Council for Animal Care guidelines and was approved by the animal care committee at the University of British Columbia. All efforts were made to reduce the number of animals used and to minimize their suffering.

**Apparatus**

The water maze used was a circular pool 180cm in diameter and 60 cm in height. The pool was filled with 21°C water and nontoxic white tempura paint was added to render the water opaque. For visible platform training the pool was filled to a depth of 27cm and for hidden platform training to a depth of 33cm. The platform itself was 30cm tall with a diameter of 10cm. A camera mounted to the ceiling above the pool was used to record the movements of rats during testing. Tracking was performed using *Anymaze* tracking software (Stoelting). Numerous prominent distal cues were placed on the walls of the testing room for all testing conditions except the reduced-cue hidden platform condition (Figure 4.1). There were also a
number of distinct features to the room that were available as cues to all training conditions such as the door, and a divider separating the pool from the computer station and experimenter. The area of the testing room containing the pool was made up of 3 walls and a room divider. The dimensions of this area were 2.75m by 2.75m. The pool was positioned in the centre of the room approximately 48cm from the wall on all sides.

**Procedure**

On day 0, all rats received a single intraperitoneal injection of BrdU (200mg/kg) and were then left in their home cages until the start of training on day six. Training in the Morris water task continued for five days with one training session per day. Rats were trained on one of four conditions (n=10 per condition). The first condition utilized the visible platform version. In this task the rats received four trials per session. The platform extended approximately three cm above the surface of the water and was placed in the center of a different quadrant on each trial. The second condition was a hidden platform version in which the platform was submerged three cm below the surface of the water in a fixed location in the pool for the entire duration of the experiment and each rat was given four trials per session. The third condition, termed the reduced-cue hidden platform version (in comparison to all other testing procedures used in the current study), was the same as the previous hidden platform version except that the majority of the distal cues were removed from the room so that only two prominent cues, the divider and the door remained. Finally, the fourth condition was a hidden platform version with all of the cues present as in conditions one and two but each rat was only given two trials per session rather than four. In all cases an inter trial interval of 8-10 minutes occurred for each rat. Latency, distance and average speed to reach the platform was recorded and analyzed using ANYMAZE (Stoelting). In addition the total distance or latency to reach the platform was recorded across all
sessions including all four trials per session or only the first two trials per session. On the day following the completion of training all rats were given a lethal overdose of sodium pentobarbital and were perfused transcardially with 60ml of 0.1M phosphate buffered saline (PBS) followed by 120ml of 4% paraformaldehyde in PBS. The brains were post-fixed in 4% PFA in PBS for 24 hours and were then transferred to 30% sucrose in PBS until sectioning. A Leica vibratome (VT1000S) was used to section the brains into 10 series of tissue at a thickness of 40µm. Tissue was collected throughout the entire rostral-caudal extent of the hippocampus.

**Figure 4.1:** The layout of the testing room is shown. Panel A shows the configuration of distal cues used for visible platform testing, four-trial hidden platform testing and two-trial hidden platform testing. Panel B shows the reduced cue conditions used for reduced-cue hidden platform testing.
**BrdU immunohistochemistry**

Tissue sections were incubated at room temperature in 0.6% H₂O₂ for 30 minutes followed by three rinses in 0.1M PBS prior to a 30 minute incubation at 37°C in 2N hydrochloric acid. The tissue was then rinsed in 0.1M borate buffer for 10 minutes (pH 8.5) followed by three more rinses in 0.1M PBS. The tissue sections were then transferred to a primary antibody solution containing 1:200 mouse anti-BrdU, 3% normal horse serum and 0.1% Triton X in 0.1M PBS for 24 hours at 4°C on a shaker. The tissue was rinsed 3 times in 0.1M PBS and was then transferred to a secondary antibody solution containing a 1:500 dilution of horse anti-mouse in 0.1M PBS. The tissue was incubated in the secondary solution for 4 hours at room temperature. Following three rinses in 0.1M PBS, an ABC kit (Vector) was used and prepared according to the kit instructions. BrdU labeling was visualized by incubating tissue in diaminobenzidine (DAB) for approximately 3 minutes. Finally, the sections were mounted on glass slides and coverslipped with Permount.

**Ki67 immunohistochemistry**

Tissue was first incubated at room temperature in 0.6% H₂O₂ for 30 minutes and was then rinsed 3 times for 10 minutes each in 0.1M PBS. The sections were then transferred to a primary antibody solution containing a 1:500 Rabbit anti-Ki67 monoclonal antibody (Vector), 3% Normal goat Serum and 0.3% Triton-X in 0.1M PBS. Tissue was incubated in the primary solution for 24 hours at room temperature on a shaker and was then rinsed 3 times for 10 minutes per rinse in 0.1M PBS. The tissue was then incubated for 16 hours at room temperature in a secondary antibody solution containing a 1:500 dilution of goat anti-rabbit (Jackson). After rinsing the tissue 3 times in 0.1M PBS, the tissue was next incubated in an ABC solution (VECTOR LABs) as per the kit instructions for 2 hours. The tissue was rinsed in 0.1M PBS and
was then developed with DAB for approximately 2 minutes. The sections were then mounted on glass slides and were coverslipped with Permount.

**BrdU/NeuN Immunohistochemistry**

Double labeling for BrdU and NeuN was performed to assess mature neuronal phenotype. A series of brain tissue was incubated at 4°C for 24 hours in mouse anti-NeuN diluted 1:250 in 0.1M PBS containing 0.3% triton-X. The tissue was then rinsed three times in 0.1M PBS and was then incubated for 16 hours at 4°C in donkey anti-mouse Alexa 488 diluted 1:500 in 0.1M PBS. The tissue was then rinsed three times in 0.1M PBS and was then fixed in 4% PFA for 10 minutes and was then rinsed in 0.9% NaCl for 10 minutes each. DNA was denatured by heating sections to 37°C for 30 minutes in 2N hydrochloric acid. BrdU labeling was then carried out by incubating the tissue for 24 hours at 4°C in a 1:500 dilution of Rat anti-BrdU (Oxford Biotech) in 0.1M PBS containing 0.3% Triton-x. Following three rinses in 0.1M PBS the tissue was then transferred to a solution containing a 1:500 dilution of Donkey anti-rat Cy3 (Jackson) for 16 hours at 4°C. Finally, the tissue was rinsed three times in 0.1M PBS and the sections were then mounted on glass slides and coverslipped with PVA-DABCO.

**Cell counting**

All counting was conducted by an experimenter blind to training condition. BrdU and Ki67-labeled cells were counted in every 10th section throughout the entire granule cell layer (GCL), including the subgranular zone (SGZ) and the hilus (separately) to obtain an estimate of the total number of labeled cells. The subgranular zone was defined as the 20µm zone between the GCL and hilus. Cell counts were performed in the hilus as a control for any effects that could alter BrdU incorporation such as differences in blood brain barrier permeability. Counts
were performed with a Nikon E600 light microscope and a 100x oil immersion objective lens. The total number of BrdU and Ki67-labeled cells was estimated by multiplying the total number counted by 10 as previously described (Kempermann et al., 2003; Eadie et al., 2005). The percentage of BrdU/NeuN double-labeled cells (Figure 4.2) was obtained by selecting 50 BrdU cells arbitrarily from at least 5 sections per brain and determining what percentage of these cells also expressed NeuN. Fluorescent imaging was performed on a Nikon epifluorescent microscope under 400x magnification. Area measurements of the granule cell layer and hilus were separately calculated using the software program ImageJ (NIH). The volume of each region was estimated using Cavalieri’s principle (Gundersen & Jensen, 1987). Briefly, the sum of the measured areas was multiplied by the distance between measured sections (400µm) to give the volume of the region.

**Data analyses**

Latency and distance to reach the platform and average swim speed were analyzed using a repeated-measures analysis of variance (ANOVA) with session (1-5) as the within-subjects factor and training type (visible, four-trial hidden, two-trial hidden reduced-cue hidden) as the between-subjects factor. The total latency and total distance across all trials were also analyzed using one-way ANOVAs. Total BrdU cell counts, Ki67 cell counts and volumes were analyzed using repeated measures ANOVAs with region (GCL+SGZ and hilus) as the within subjects factor and training type (visible, four-trial hidden, two-trial hidden, reduced-cue hidden) as the between-subjects factor. BrdU/NeuN double labeling was analyzed using a one-way ANOVA with type of training as the between-subjects factor. Post-hoc tests utilized the Newman-Keuls procedure. Pearson product-moment correlations were calculated on total BrdU-labeled cells in the GCL with cell proliferation and with performance (total distance and total latency) for each
training type. All analyses were performed using the software package Statistica (Statsoft, Tulsa, Ok, USA).

**Figure 4.2:** Photomicrographs of BrdU labeled cells. Panel A shows a section of the granule cell layer labeled with the mature neuronal marker NeuN. Panel B shows a BrdU labeled cell in the granule cell layer. Panel C shows co-labeling of BrdU and NeuN indicating a new neuron (magnification is 200X for A, B and C). Panel D shows BrdU-labeled cells in the granule cell layer visualized with DAB (magnification is 1000X).
**Results**

*Rats trained on the more difficult versions of the Morris water task had longer latencies and greater swim distances during training*

For latency to reach the platform (Figure 4.3 A) there was a significant main effect of session \( (F(4,116) = 38.84, P < 0.00001) \) a significant main effect of training type \( (F(3,29) = 26.99, P < 0.00001) \) and a significant session by training type interaction \( (F(12, 116) = 2.59, P < 0.0043) \). Post-hoc tests indicated that rats trained on the two-trial hidden platform version exhibited longer mean latencies than rats trained on the visible platform version during session two \( (P<0.019) \) and had longer mean latencies than the other three groups during session three \( (P’s< 0.0030) \). No other comparisons were significantly different.

In terms of total latency spent locating the platform across all trials (Figure 4.3 B) there was a significant main effect of type of training \( (F(3,29) = 16.74, P < 0.000002) \). Post hoc tests indicated that rats trained on the reduced-cue version of the hidden platform spent more time searching for the platform than rats in all other groups (visible platform :\( P=0.00016 \); four-trial hidden platform: \( P=0.00028 \); two-trial hidden platform: \( P=0.0003 \)). However in order to compare swim latency of rats trained on the two-trial version of the hidden platform with other groups we also analyzed the total latency across session using only the first two trials of each session. There was a significant main effect of training type \( (F(3,29) = 15.85, P < 0.000003) \). Post-hoc tests indicated that rats trained on the reduced-cue hidden platform version spent more time searching for the platform than rats in the visible platform group \( (P=0.0002) \) and the four-trial hidden platform group \( (P=0.002) \) but not the two-trial hidden version \( (P=0.35) \). In addition, rats trained on the two-trial version of the hidden platform had longer latencies than rats trained on the visible platform \( (P=0.00017) \) or the four-trial hidden platform version \( (P=0.00053) \).
For distance to reach the platform (Figure 4.4 A) there were significant main effects of training type ($F(3,29) = 20.70, \ P = 0.00001$) and session ($F(4, 116) = 37.79, \ P = 0.00001$) as well as a significant training type by session interaction ($F(12, 116) = 2.69, \ P = 0.0031$). Post-hoc tests indicated that rats trained on the two-trial hidden platform version had a significantly greater mean swim distance than rats trained on the visible platform on day two ($P=0.022$) and a greater mean swim distance than all other groups on day three ($P's < 0.0054$). No other pairwise comparisons were significantly different.

Analysis of total swim distance across all trials (Fig 4B.) demonstrated a significant main effect of type of training ($F(3,29) = 10.29, \ P < 0.000089$). Post-hoc tests showed that rats trained on the reduced-cue hidden platform version traveled longer distances than rats trained on the four-trial hidden platform version ($P=0.015$), the visible platform ($P=0.0002$) and the two-trial hidden version ($P=0.007$) of the Morris water task. However, in order to directly compare rats trained on the two-trial hidden platform version to other training groups we compared swim distances across sessions but included only the first two trails of each session. There was a significant main effect of training type ($F(3,29) = 13.33, \ P < 0.000012$). Rats trained on the reduced-cue hidden platform version had longer distances to reach the platform than rats trained on the visible platform ($P=0.0022$) and the four-trial hidden platform version ($P= 0.023$) and tended to have shorter distances to reach the platform than rats trained on the two-trial hidden platform version ($P=0.08$). Rats trained on the two-trial hidden platform trained rats travelled longer distances than rats trained on the visible platform ($P=0.0002$) and four-trial hidden platform version ($P=0.00075$).
**Figure 4.3A:** The mean latency to reach the platform during each session is depicted. Rats given two training trials per session have significantly greater latencies during sessions two and three than rats trained on the visible platform (*) and had significantly greater latencies than all other groups during session 3 (#). Data points represent mean latency ± SEM.
Figure 4.3B. The total latency across all training sessions (black bars) and the total latency across the first two trials of each session (white bars) are shown. Rats trained on the reduced-cue hidden platform version and the two-trial hidden platform version had significantly longer latencies than rats trained on the four-trial hidden platform version or the visible platform version. Bars represent mean + SEM. * indicates a significant increase compared to visible platform training for all trials. # indicates a significant increase compared to visible platform training for the first two trials per session.
**Figure 4.4A:** The mean swim distance per session is depicted. Rats given two trails each session swam a greater distance than all other groups during session 3 (#) and swam a greater distance than visible platform trained rats during sessions 2 (*) and 3. Data points represent mean distance + SEM.
Figure 4.4B Total swim distance during all trials (black bars) and during the first two trials of each session (white bars) are shown. Rats trained on the reduced-cue and two-trial hidden platform versions swam greater distances than rats trained on the four-trial hidden platform or the visible platform version of the task. Bars represent mean + SEM. * indicates a significant increase compared to visible platform training for all trials. # indicates a significant increase compared to visible platform training for the first two trials per session.

In terms of average swim speed there were main effects of training type ($F(3,29) = 3.36, P < 0.032$) and session ($F(4, 116) = 22.37, P < 0.00001$) and a significant interaction between
training type and session (F(12, 116) = 3.75, \( P < 0.00008 \)). However, post-hoc tests indicated that there were no significant differences between types of training during any of the sessions.

**Morris water task training increases and decreases cell survival depending on the task**

For the total number of BrdU-labeled cells in the dentate gyrus there was a significant main effect of training type (F(3,29) = 5.96, \( P < 0.0027 \)) and a significant main effect of region (F(1, 29) = 411.82, \( P < 0.00001 \)) with a greater number of BrdU-labeled cells in the GCL+SGZ than in the hilus as expected (Fig 5). Furthermore, there was a significant interaction between training type and region (F(3,29) = 8.88, \( P < 0.00025 \)). Post-hoc tests indicated that rats trained on the four-trial hidden platform version of the task had significantly more BrdU-labeled cells in the GCL+SGZ than rats trained in all other groups (visible platform version (\( P =0.0004 \)) reduced-cue hidden platform version (\( P = 0.00016 \)) two-trial hidden platform version (\( P=0.0004 \)). There was no significant difference between rats trained on the visible platform version and rats trained on the two-trial hidden platform version of the task (\( P = 0.63 \)). Rats trained on the two-trial hidden platform version had significantly greater number of BrdU-labeled cells than rats trained in the reduced-cue hidden platform version of the task (\( P = 0.003 \)). There were no significant differences in the number of BrdU-labeled cells in the hilus as a factor of training type (Table 4.1).

We correlated the total number of BrdU-labeled cells in the granule cell layer with total latency and total distance to locate the platform. There were no significant correlations for any of the training conditions between total latency and total BrdU in the GCL+SGZ (visible \( P=0.58 \); four-trial hidden \( P=0.75 \); reduced-cue hidden \( P = 0.72 \); two-trial hidden \( P=0.70 \)) or between total distance and total BrdU in the GCL+SGZ (visible \( P=0.45 \); four-trial hidden \( P=0.65 \); reduced-cue hidden \( P = 0.94 \); two-trial hidden \( P=0.81 \)). Additionally, we correlated the
total number of BrdU-labeled cells in the GCL with the total number of Ki67 labeled cells in the GCL. There were significant positive correlations for rats trained on the four-trial hidden platform version ($r=0.84$, $P<0.035$) and the two-trial hidden platform version ($r=0.69$, $P<0.028$).

**Training with a reduced number of daily trials decreased cell proliferation**

For the total number of Ki67 labeled cells in the dentate gyrus (Figure 4.6) there was a significant main effect of type of training, $F(3,29) = 3.64$, $P < 0.024$, and a significant main effect of region, $F(1,29) = 453.82$, $P < 0.0001$, with fewer Ki67 labeled cells in the hilus than in the GCL+SGZ. There was also a significant region by training type interaction ($F(3,29) = 2.93$, $P < 0.049$). Post-hoc tests indicated that rats trained on the two-trial hidden platform version of the task had significantly fewer Ki67 labeled cells in the granule cell layer than rat trained on the visible platform ($P=0.0032$), the four-trial hidden platform version ($P=0.025$) and the reduced-cue hidden platform version ($P=0.047$). No other comparisons were significantly different ($P>0.05$) in either the hilus (Table 4.1) or granule cell layer.
Figure 4.5. The total numbers of BrdU labeled cells in the granule cell layer. Compared to rats trained on the visible platform there was a significantly greater number of BrdU labeled cells in the granule cell layer of rats trained on the four-trial hidden platform version of the Morris water task and significantly fewer BrdU labeled cells in the granule cell layer of rats trained on the reduced-cue version of the hidden platform. Bars represent mean cell counts ± SEM. * indicates a significant increase and # indicates a significant decrease compared to visible platform training.
Table 4.1: Mean (± SEM) cells counted in the hilus for each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>BrdU+ cells</th>
<th>Ki67+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visible</td>
<td>1517.5 ± 102.92</td>
<td>752.5 ± 118.9</td>
</tr>
<tr>
<td>Hidden (four trials)</td>
<td>1626.7 ± 230.34</td>
<td>623.3 ± 69.9</td>
</tr>
<tr>
<td>Hidden (cue-deprived)</td>
<td>1584.4 ± 164.58</td>
<td>557.8 ± 58.4</td>
</tr>
<tr>
<td>Hidden (two trials)</td>
<td>1340.0 ± 151.11</td>
<td>336.0 ± 40.4</td>
</tr>
</tbody>
</table>

There were no significant differences between groups.

Morris water task training did not alter the number of new mature neurons

The percentage BrdU-labeled cells that co-expressed the neuronal marker NeuN was 18.6% after visible platform training, 22.0% following four-trial hidden platform training, 27.5% after reduced-cue hidden platform training and 24.0% following two-trial hidden platform training. There was no significant difference in the percentage of BrdU/NeuN double-labeled cells in the granule cell layer as a factor of training type, \( P = 0.59 \) (Table 4.2).

Dentate gyrus volume did not differ statistically between training conditions

There was no significant region by training type interaction in terms of dentate gyrus volume (\( P = 0.46 \)) and no main effect of training type (\( P = 0.18 \)). There was a significant main effect of region (\( F(1,29) = 1298.14, P < 0.00001 \)) with the hilus being significantly larger in volume than the GCL (Table 4.3)
Figure 4.6: The total numbers of Ki67 labeled cells in the granule cell layer are shown. Rats trained on the two-trial hidden platform version had significantly fewer BrdU labeled cells in the granule cell layer than all other training groups. Bars represent mean cell counts ± SEM. * indicates a significant decrease compared to visible platform training.
**Table 4.2:** Mean (± SEM) Percentage of BrdU labeled cells that also express NeuN

<table>
<thead>
<tr>
<th>Group</th>
<th>BrdU/NeuN double-labeled cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visible</td>
<td>18.6 ± 3.28</td>
</tr>
<tr>
<td>Hidden</td>
<td>22.0 ± 3.97</td>
</tr>
<tr>
<td>Hidden (cue-deprived)</td>
<td>27.5 ± 5.10</td>
</tr>
<tr>
<td>Hidden (2 trials)</td>
<td>24.0 ± 4.66</td>
</tr>
</tbody>
</table>

There were no significant differences between groups.

**Table 4.3:** Mean (± SEM) volume of the granule cell layer and hilus from each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Granule cell layer (mm$^3$)</th>
<th>Hilus (mm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visible</td>
<td>3.68 ± 0.16</td>
<td>6.41 ± 0.16</td>
</tr>
<tr>
<td>Hidden</td>
<td>3.13 ± 0.23</td>
<td>5.86 ± 0.42</td>
</tr>
<tr>
<td>Hidden (cue-deprived)</td>
<td>3.25 ± 0.08</td>
<td>6.21 ± 0.18</td>
</tr>
<tr>
<td>Hidden (two trials)</td>
<td>3.02 ± 0.12</td>
<td>6.03 ± 0.25</td>
</tr>
</tbody>
</table>

There were no significant differences between groups.

**Discussion**

The results described here demonstrate that training on a four-trial hidden platform version of the Morris water task increased the survival of new neurons in the dentate gyrus compared to training on a hippocampus-independent version of the task when trained 6 days after BrdU injection and examined 12 days later. This result was expected on the basis of numerous previous findings (Gould et al., 1999; Ambrogini et al., 2000; Epp et al., 2007).
Furthermore we demonstrate here for the first time that decreasing the number of distal cues in the testing environment results in a *decrease* in cell survival compared to rats trained on either a visible platform version of the task or a four-trial hidden platform version with a greater number of distal cues. Surprisingly, we found that training rats using the two-trial hidden platform procedure did not significantly affect cell survival in the dentate gyrus.

Our behavioral data showing longer swim distances and escape latencies in rats trained on the two altered hidden platform conditions, suggest that these versions were more difficult to acquire than the four-trial hidden platform version or in the case of the two-trial hidden platform version were not learned as well due to decreased reinforcement and practice. Thus it appears that changing the demands of the task or training can have implications for the effect of that task on cell survival. Our results also indicated that neither four-trial hidden platform training nor reduced-cue hidden platform training caused a change in cell proliferation in the dentate gyrus compared to visible platform training at the end of training. However, two-trial hidden platform training resulted in decreased cell proliferation compared to all other training procedures. This result may indicate that training with fewer trials, or perhaps in general in conditions where learning cannot be accomplished as quickly is more stressful after 5 days of training because cell proliferation is negatively regulated by stress (Holmes and Galea, 2002; Falconer and Galea, 2003).

*Reducing the number of cues decreased the survival of new neurons*

Rats trained with the reduced-cue hidden platform condition had decreased cell survival compared to both non-spatial training with a visible platform and spatial training with a greater number of cues present. Reducing the number of cues in the testing environment results in fewer items that can be used to form a spatial representation of the environment, and thus, makes it
more difficult to locate a goal region within that representation. Although it remains uncertain what exactly newly generated neurons contribute to hippocampus-dependent learning, this finding suggests that the learning produced by the Morris water task training under reduced-cue hidden platform conditions may be inhibitory to adult neurogenesis. Glutamatergic signaling via NMDA receptors plays a role in spatial learning (Uekita et al., 2006) and has a complex regulatory role in adult neurogenesis (Nacher & McEwen, 2006). Both increases (Joo et al., 2007) and decreases (Cameron et al., 1995) in adult neurogenesis have been reported following modulation of NMDA receptor activity. It is possible that learning-induced changes in NMDA receptor activity in the hippocampus may regulate neurogenesis differently as task demands change. In response to the sparse cue conditions it is also possible that the rats in this condition may have attempted to solve the task a different way than rats trained on the four-trial hidden platform version of the task. For example, these rats may have used more Euclidean cues such as the distance of the platform from the pool wall and geometry of the room to solve the task in the sparse cue condition. Hamilton et al. (2008) demonstrated that rats are capable of solving the Morris water task using a true spatial navigation strategy but preferentially utilize a directional navigation strategy. Furthermore, a recent report from the same group has demonstrated that although rats may use a spatial strategy early on in training they soon switch to a directional strategy (Hamilton et al., 2009). It is possible that the reduction in distal cues may have caused some rats to use a spatial strategy for a different period of time because their learning was progressing at a slower rate and by doing so altered the effect of task training on cell survival. Although this explanation is plausible it has not yet been tested whether directional or spatial learning has different effects on cell survival or whether a reduction in cues precipitates the use of a spatial learning strategy. There is some evidence that these two strategies are dependent on different neurobiological systems or processes (Akers et al., 2009). Thus they may cause
differential activation of the dentate gyrus and a resulting difference in cell survival. It has been demonstrated however that rats can use a spatial learning strategy when only two salient distal cues are present in the environment (Fenton et al., 1994).

Alternatively, rather than a switch in strategy per se, the reduction in distal cues might involve differential (less or more) activation of the dentate gyrus to learn the task. The time window during which cell survival can be enhanced by spatial learning (and when training was carried out in the present study) corresponds to the time when the new neurons are becoming incorporated into the existing network (Hastings & Gould 1999; Zhao et al., 2006). Thus, it is assumed that when hippocampus-dependent learning enhances cell survival this effect is mediated by direct stimulation of the new neurons by activation of the hippocampal network.

Numerous theories suggest that the hippocampus functions to form relational associations among cues (Rudy & Sutherland, 1995; Alvarez et al., 2001; Kesner et al., 2008). Fewer nodes and connections would be required in a network in order to represent an environment containing fewer salient cues. When learning to navigate in an environment containing fewer cues, the dentate gyrus may be encoding less information as it is forming fewer cue associations, which could possibly lead to a decrease in the total number of BrdU-labeled cells. Less activity in the hippocampus would allow for less possible activation of new immature neurons and because the survival of new neurons is regulated by the activation of their NMDA receptors (Tashiro et al., 2006) fewer new cells may survive. Alternatively fewer salient cues may result in more activation in the dentate gyrus as a result of the animal requiring more computation in order to locate the platform. Unfortunately, it is unknown what effect our different training conditions have on activity in the dentate gyrus and thus these interpretations are only speculative. Future studies addressing the differences in activation and encoding of spatial learning under various cue conditions may shed light on our current findings.
**Do altered stress levels account for differences between groups**

Another possible interpretation of the decrease in cell survival seen after reduced-cue hidden platform training seen in the present study is that the harder training condition was more stressful than the four-trial hidden platform procedure. Thus because exposure to stress or elevated levels of the stress hormone, corticosterone, decreases cell survival (Wong & Herbert, 2004; Lee et al., 2006) an increase in stress hormones under harder training conditions may have decreased the survival of new neurons. Although this is a valid explanation, and cannot be completely ruled out based on the current data, this interpretation seems unlikely for a couple of reasons. First, exposure to stress and to corticosterone have well documented inhibitory effects on cell proliferation in male rats (see Mirescu & Gould, 2006). However, we found no significant changes in cell proliferation, as measured by Ki67 labeling, between visible platform, four-trial hidden platform and reduced-cue hidden platform training at the end of training. This suggests that possible differences in stress levels as a result of these different training protocols were not salient enough to significantly alter cell proliferation. Further, there was no significant correlation between the number of Ki67 labeled cells and BrdU labeled cells in the granule cell layer for rats trained in the reduced-cue hidden platform condition. If the resulting decrease in cell survival is to be attributed to stress it would be expected that cell proliferation would correlate positively with cell survival as the rats experiencing the most stress should have the lowest levels of both cell proliferation and survival. However, it is important to note that this argument does not fully discount the impact of stress on cell survival. The BrdU labeling and Ki67 labeling analyzed here represent two distinct populations of cells, those that were dividing at the time of BrdU administration and those proliferating at the end of training. Thus it remains possible that the different training procedures varied in degree of stress activation early on in
training but not by the completion of training. However it is likely that if a procedure is stressful at the end of 5 days of training it was likely stressful at the start of training.

**Two-trial hidden platform training reduced cell proliferation**

In the present study we found that two-trial hidden platform training did result in reduced cell proliferation but these rats did not differ from the visible platform group in terms of cell survival. In the two-trial hidden platform condition we trained a group of rats with the same distal cues as the four trial hidden platform group but instead of receiving four trials per session they were only given two trials. Cell survival in this group of rats was not significantly different than rats trained on the visible platform version of the task. Thus although increasing task difficulty may decrease cell survival it does not appear to be the case that reducing the amount of learning (i.e. the terminal accuracy of the performance) results in a decrease in cell survival.

A possible interpretation of the finding of decreased cell proliferation in the two-trial hidden platform training is that this training protocol was inherently more stressful than visible platform or four-trial hidden platform training. This interpretation is harder to rule out for the two-trial hidden platform condition because the level of cell proliferation was decreased in this group compared to all other training conditions. There was also a significant positive correlation between cell survival and cell proliferation in the two-trial condition. It is possible that cell survival was not enhanced by this type of training simply because the training was more stressful than four-trial hidden platform training rather than the quality or rate of learning being lower. Although it is clear that stress can cause a decrease in cell proliferation, a decrease in cell proliferation accompanying behavioral training is not necessarily limited to this interpretation. Decreases in cell proliferation have been linked to hippocampus-dependent learning previously in situations where stress does not seem to explain the result. For example, Pham et al. (2005)
demonstrated that rats trained on a contextual conditioning task had a reduction in cell proliferation compared to controls despite there being no significant difference in serum corticosterone levels. Furthermore, we have recently shown that rats trained in a cue competition version of the Morris water task that preferentially utilize a hippocampus-dependent strategy during a probe trial have significantly lower cell proliferation than rats that use a hippocampus-independent strategy (Epp et al., 2009). Although, stress levels were not explicitly examined in that study all rats received identical training and differed only in their response during the probe trial so it seems unlikely that stress would have been a factor. Thus, the decrease in cell proliferation seen in the present study may be a result of the more difficult training condition, which may or may not be related to stress per se.

Decreasing the number of trials per session but keeping all other task demands the same likely does not change how the task must be learned but merely slows the rate of acquisition and decreases the final accuracy of performance on the task. Given that the type of learning is likely the same as in the four-trial hidden platform version it is interesting to note that cell survival was not increased. This cannot be attributed to a decrease in exercise as there were no significant differences in total swim distance or total escape latency in rats trained on the four-trial hidden platform version compared to the two-trial hidden platform condition (see figures 4.3 B and 4.4 B). Instead, the critical factor might be the amount or rate of learning that occurs during training. Although there is an overall difference in performance between these training conditions, the level of performance by the end of training does not differ significantly in rats that received four trials versus two trials per session. A number of studies have investigated the effects of spatial learning on adult neurogenesis and several of these studies have noted relationships between cell survival and how well an animal is able to acquire the task (Dobrossy et al., 2003; Epp et al., 2007; Sisti et al., 2007). These findings may help clarify some of the
contradictory studies regarding cell survival and neurogenesis. Dobrossy et al. (2003) observed a correlation such that the rats trained in the Morris water task that exhibited better performance had lower levels of cell survival. This finding suggested that cell survival was enhanced to a greater extent in poor learners. A similar study that directly compared good and poor learners demonstrated that cell survival is enhanced by spatial learning in the subpopulation of rats that exhibit poor learning but not in those that are good learners (Epp et al., 2007). However, an inconsistent finding from Sisti et al., (2007) evaluated the effects of massed versus spaced training in the Morris water task and found no effect of trial distribution but did observe that good learners had increased cell survival compared to poor learners. Although this finding is in opposition to the results of Dobrossy et al. (2003) and Epp et al. (2007) it confirms that the quality of learning plays an important role in determining the number of surviving cells. How well an animal learns a task is clearly influenced by the difficulty of the task. Based on our current findings that task difficulty can influence cell survival it may be the case that the effects of quality of learning are in some way mediated by or related to task difficulty.

As a result of the current findings, the variability of task demands should be considered as a partial explanation for inconsistencies in the literature regarding spatial learning and adult neurogenesis. As an example, an early study that did not find an increase in cell survival following spatial training in the Morris water task used two trials per session for an extended period of time (van Praag et al., 1999). Similarly, the rats trained here with two trials per session did not show increased cell survival. It seems that the number of trials along with other variables, which may seem minor, can have a substantial effect on the interaction between spatial learning and cell survival. Changing a parameter such as pre-exposure to the test apparatus has also been shown to eliminate the effect of spatial learning on cell survival, possibly via reduced stress/anxiety or by decreasing the difficulty of the task (Ehninger and Kempermann, 2006).
Conclusions

The results of the current study demonstrate that there is a complex interaction between Morris water task training, task performance and cell survival. Although the four-trial hidden platform training increases cell survival compared to visible platform training when BrdU is given 6 d prior to the training, decreasing the number of training trials per session eliminates this effect. Additionally, reducing the number of available distal cues causes a reduction in cell survival compared to visible platform training. Regardless of the possible mechanisms it is important to note that altering either the number of trials or the number of cues present in the test environment is sufficient to cause a change in cell survival and cell proliferation in response to spatial learning. Thus seemingly small differences in training procedures, including those that have only minor behavioral consequences, may result in disproportionately large effects on measures of neurogenesis and this may be another possible factor for the disparate findings in the literature. As a result a greater degree of standardized testing procedures may be required to reduce the number of contradictory findings surrounding adult neurogenesis and spatial learning.
References


Neurogenesis occurs throughout life in the dentate gyrus of the mammalian hippocampus (Altman and Das, 1965; Cameron et al., 1993; Eriksson et al., 1998). Several types of hippocampus-dependent learning have been linked to adult neurogenesis (Leuner et al., 2006). Spatial learning can increase (Gould et al., 1999; Ambrogini et al., 2000; Leuner et al., 2004; Hairston et al., 2005; Olariu et al., 2005; Epp et al., 2007; Epp et al., 2009), decrease (Ambrogini et al., 2004; Mohapel et al., 2006; Epp et al., 2009) or have no effect on cell survival (Dobrossy et al., 2003; Van der Borght et al., 2005; Mohapel et al., 2006; Epp et al., 2009). The ability of spatial learning to enhance cell survival depends on numerous factors, including the maturity of cells during spatial learning, task difficulty and quality of learning (Epp et al., 2007; Sisti et al., 2007; Epp et al., 2009).

How well the task is learned influences the impact of learning on cell survival. While two studies showed that cell survival was enhanced in rats that performed relatively poorly, but not in those that performed better, in the Morris Water task (MWT) (Dobrossy et al., 2003; Epp et al., 2007), another study showed the opposite effect (Sisti et al., 2007). These inconsistencies may be related to task difficulty and we found that differences in task difficulty in the MWT alter cell survival (Epp et al., 2009). There is likely a complex relationship between how well animals learn, task difficulty, and the effect of spatial training on cell survival.

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4 A version of this chapter has been submitted for publication. Epp, J.R., Scott, N.A. and Galea, L.A.M. (2010). Strain differences in neurogenesis and activation of new neurons in the dentate gyrus in response to spatial learning.
One factor that could alter the effect of spatial learning on adult hippocampal neurogenesis is strain. Studies have used Wistar rats (Van der Borght et al., 2005), Long-Evans (LE) rats (Keith et al., 2008; Epp and Galea, 2009) or mice (Ehninger and Kempermann, 2006) but the vast majority of studies examining effects of spatial learning on neurogenesis have utilized Sprague-Dawley (SD) rats (Gould et al., 1999; Ambrogini et al., 2000; Dobrossy et al., 2003; Ambrogini et al., 2004; Leuner et al., 2004; Mohapel et al., 2006; Dalla et al., 2007; Dupret et al., 2007; Epp et al., 2007; Sisti et al., 2007; Dalla et al., 2009; Epp et al., 2009). Because task difficulty and quality of learning influence cell survival it might be expected that SD rats could respond differently to spatial training than other strains. SD rats have poor visual acuity (Prusky et al., 2002) and thus task difficulty may be increased in this strain due to decreased cue salience. Furthermore, SD rats do not perform as well on spatial tasks as LE rats (Tonkiss et al., 1992; Harker and Whishaw, 2002). In addition, changes in the number of activated new neurons may occur independently of differences in cell survival and might underlie possible differences in neurogenesis or spatial learning. Several studies have examined the functional incorporation of immature neurons in the hippocampus using immediate early genes (IEG) to mark cellular activation (Ramirez-Amaya et al., 2006; Kee et al., 2007; Tashiro et al., 2007; Snyder et al., 2009, 2009b). These studies have provided supporting evidence for a role of adult generated neurons in spatial learning and memory and have helped to identify a time course for the development of immature neurons in relation to spatial learning and memory. Kee and colleagues (2007) demonstrated that new neurons 4-8 week of age at the time of spatial learning were later activated to a greater extent than the existing granule cell population during memory retrieval. In another recent study using IEG labeling, it was shown that species differences exist in the rate of maturation and activation of new neurons. In that study mice were
found to express IEGs in new neurons at a later time point and had fewer IEG activated new neurons in response to spatial learning than rats (Snyder et al., 2009). Therefore, in the current study we sought to determine whether hippocampal neurogenesis in LE and SD rats responds differently to spatial learning. Furthermore, using IEG labeling we investigated whether there were any differences in the activation of new neurons in response to spatial memory.

**Experimental procedures**

**Subjects**

All experiments were conducted in accordance with the Canadian council on animal care and were approved by the animal welfare committee at the university of British Columbia. A total of 52 male rats (26 LE and 26 SD) were obtained from Charles River (St. Constant, Quebec, Canada). Upon arrival, rats were housed individually in standard cages containing a polyvinyl chloride tube, paper towels and *ad libitum* access to food (Purina rat chow) and water. The rats were allowed to habituate to their new surroundings for five days and were then handled daily for an additional five days prior to training. All rats were between 60 and 65 days of age at the start of behavioral testing.

**Morris water task training**

Each rat received a single 200mg/kg i.p. injection of bromodeoxyuridine (BrdU; Sigma) six days before the commencement of behavioral training. The rats were randomly assigned to one of six groups, place learning (n=10 per strain), cue learning (n=10 per strain) and cage controls (n=6 per strain). Cage control rats were handled upon arrival but otherwise remained in their cages throughout the experiment. MWT training was recorded with an overhead camera.
connected to a computer running the tracking software AnyMaze (Stoelting, Wood Dale, Illinois, USA) in order to analyze the mean distance and latency to reach the platform each session as well as the total distance and latency across all trials. During the probe trial we also measured the time spent in the correct quadrant of the pool. Place and cue-trained rats were given five daily training sessions in the MWT consisting of four trials per session beginning 6 days after BrdU injection. The cue training procedure involved a visible platform that extended 3cm above the surface of the water and was moved to the center of a new quadrant following each trial. The place learning procedure involved training rats to find a submerged platform always located in the same place within the pool. Numerous distal cues were located on the walls of the testing room to aid in spatial navigation under both conditions. Following completion of water task training the rats were left undisturbed for five days. The rats were then returned to the testing room and were given a single 30-second probe trial in which the platform was not in the pool. Ninety minutes later all rats were perfused transcardially with saline followed by 4% formaldehyde. Thus all rats were perfused 16 days after BrdU injection.

**Histology**

The brains were extracted and post-fixed overnight in 4% formaldehyde and then transferred to 30% sucrose for cryoprotection. Ten series of 40 μm sections were collected throughout the rostral-caudal extent of the dentate gyrus using a Leica SM2000R freezing sliding microtome (Richmond Hill, Ontario, Canada).
**BrdU immunohistochemistry**

BrdU labeling (Figure 5.1 A) was performed as described previously (Epp et al., 2009). Briefly, sections were incubated in 0.6% hydrogen peroxide for 30 minutes, rinsed 3 times in PBS and were then incubated in 2N HCl at 37°C for 30 minutes. A primary antibody solution containing 1:200 mouse anti-BrdU (Roche; Toronto, Ontario, Canada), 3% normal horse serum, and 0.1% triton-x was applied for 24 hours at 4°C. Then, the tissue was incubated in 1:500 biotinylated horse anti-mouse (Vector Labs; Burlington Ontario, Canada) for 18 hours at 4°C. An ABC kit (Vector Labs; Burlington Ontario, Canada) was then used according to kit instructions and finally BrdU-labeling was visualized with Nickel enhanced DAB (Sigma; Oakville, Ontario Canada).

**Doublecortin immunohistochemistry**

A second series of tissue was labeled for doublecortin (DCX) expression (Figure 5.2) as described previously (Epp et al., 2009). Briefly, free-floating tissue sections were incubated in a 1:500 dilution of goat anti-DCX (SC-8066, Santa Cruz Biotechnology; Santa Cruz, California, USA) for 24 hours followed by an 18 hour incubation in a secondary antibody solution containing a 1:500 dilution of Alexa-488 conjugated donkey anti-goat (Jackson ImmunoResearch; West Grove, Pennsylvania, USA). Sections were mounted and coversliped.

**Doublecortin and zif268 double labeling**

An additional series of tissue was double-labeled for DCX and the product of the IEG zif268 (Figure 5.1 B). Free-floating sections were incubated for 24 hours in a primary antibody solution containing a 1:500 dilution of goat anti-DCX (SC-8066, Santa Cruz Biotechnology; Santa Cruz, California, USA) and 1:1000 dilution of rabbit anti-zif268 (Egr-1 SC-189, Santa
Cruz Biotechnology; Santa Cruz, California, USA) with 4% normal donkey serum and 0.03 % Triton-X in 0.1M TBS. Following three rinses in TBS the tissue was then transferred to a secondary solution containing 1:500 donkey anti-rabbit Cy3 (Jackson ImmunoResearch; West Grove, Pennsylvania, USA) and 1:250 donkey anti-goat Alexa 488 (Jackson ImmunoResearch; West Grove, Pennsylvania, USA) in 0.1M TBS. Tissue was incubated overnight at 4°C, rinsed 3 times in 0.1M TBS, counterstained with DAPI and mounted and coverslipped with PVA-DABCO.

**BrdU and zif268 double labeling**

BrdU and zif268 double labeling (Figure 5.1 C) was carried out by incubating a series of tissue in a primary antibody solution containing 1:1000 anti-zif268 antibody (Egr-1 SC-189, Santa Cruz Biotechnology; Santa Cruz, California, USA) with 4% normal donkey serum and 0.03% Triton-x for 24 hours at 4°C followed by incubation with 1:500 donkey anti-rabbit Alexa 488 (Jackson ImmunoResearch; West Grove, Pennsylvania, USA) for 18 hours at 4°C. Then, the tissue was fixed in 4% formaldehyde for 10 minutes. The tissue was then incubated in rat anti-BrdU (Oxford) (1:500) for 24 hours at 4°C and then for 18 hours at 4°C in1:500 donkey anti-rat cy3 (Jackson ImmunoResearch; West Grove, PA, USA) for 18 hours. Finally the tissue was counterstained with DAPI, mounted on glass slides and coverslipped with PVA-DABCO.

**BrdU and doublecortin double labeling**

Double labeling for DCX and BrdU (Figure 5.1 D) was performed as above for BrdU and zif268 except that the anti-zif268 antibody was replaced by 1:1000 goat ant-DCX (SC-8066, Santa Cruz Biotechnology; Santa Cruz, California, USA) and was detected using 1:500 donkey anti-goat Alexa 488 (Jackson ImmunoResearch; West Grove, PA USA). Anti-BrdU was
detected using 1:500 donkey anti-rat cy3 (Jackson ImmunoResearch; West Grove, Pennsylvania, USA).

**Figure 5.1:** Panel A shows a BrdU-labeled cell in the granule cell layer visualized with DAB. Panel B shows doublecortin (green) and zif268 (red) labeling in the granule cell layer. The arrow indicated a double-labeled cell. Panel C shows BrdU (red) and zif268 (green) double labeling in the granule cell layer. Panel D shows BrdU (red) and doublecortin (green) double labeling in the granule cell layer. Photomicrographs are magnified 1000x.
Figure 5.2: Panel A shows doublecortin labeling in the granule cell layer. Magnification is 400x. Panel B shows a representative immature doublecortin cell with no process. Panel C shows a representative doublecortin cell with a short unbranched process. Panel D shows a mature doublecortin cell with a long branched process. Magnification of panels B-D is 1000x
**BrdU and NeuN double labeling**

Double labeling for NeuN and BrdU was performed as above for BrdU and zif268 except that the anti-zif268 antibody was replaced by 1:500 mouse anti-NeuN (Millipore; Billerica, Massachusetts, USA) and was visualized with 1:500 donkey anti-goat Alexa 488 (Jackson ImmunoResearch; West Grove, Pennsylvania, USA) and Anti-BrdU was visualized with 1:500 donkey anti-rat cy3 (Jackson ImmunoResearch; West Grove, Pennsylvania, USA).

**Cell counting**

An experimenter blind to treatment conditions performed all quantifications. A Nikon Eclipse E600 microscope with epifluorescent illumination was used for all quantification at a total magnification of 1000X. All BrdU-labeled and DCX-labeled cells in the granule cell layer (GCL) and the subgranular zone (SGZ; the 50 µm border region between the hilus and GCL) were counted on every 10th section throughout the rostral-caudal extent of the dentate gyrus. The number of BrdU-labeled cells in the hilus was also calculated to control for any possible differences in blood brain barrier permeability. Total cell counts were calculated as the sum of the counts from each section multiplied by the sampling fraction of 10. To estimate GCL volume the area of the GCL was traced in each section using the software package ImageJ (NIH). The sum of the areas was then multiplied by the distance between sections (400 µm) to give the estimated total volume of the GCL. Densities were calculated as the total cell counts divided by the volume of the GCL.

The percentages of cells that co-express DCX and BrdU and NeuN and BrdU were determined by examining 50 arbitrarily chosen BrdU-labeled cells located in the GCL and observing whether these cells also expressed DCX or NeuN. Zif268/DCX and zif268/BrdU double labeling was assessed in a similar manner. However, due to the lower frequency of
double-labeled cells 100 arbitrarily chosen DCX-labeled or BrdU-labeled cells were used to help ensure accurate counting. For DCX/zif268 double labeling, only cells with visible processes were used to avoid counting cells too immature to express IEGs. Cells were sampled equally from dorsal and ventral sections of the GCL.

To determine whether any differences exist in the maturational stage of DCX labeled cells we examined the appearance of 100 arbitrarily chosen DCX-labeled cells. Cells were classified as those with no visible processes (Figure 5.2 B), short unbranched processes (Figure 5.2 C) or long and/or branched processes (Figure 5.2 D) as described previously (Plümpe et al., 2006).

**Data analyses**

Data were analyzed using the software program Statistica (Statsoft; Tulsa, OK, USA). The dependent variables latency and distance to reach the platform were each analyzed with a repeated-measures analysis of variance (ANOVA) with session (1-5) as the within-subject variable and strain (SD, LE) and training (cue, place) as the between-subjects variables. BrdU-labeled cell counts were analyzed with a repeated-measures ANOVA using region (GCL+ SGZ, Hilus) as the within-subjects factor and strain (SD, LE) and training (Cage, Cue, Place) as the between subjects variables. The maturation of DCX-labeled cells was analyzed using a repeated-measures ANOVA with stage of maturation (no processes, short unbranched processes, long/branched processes) as the within-subjects factor and strain (SD, LE) and training (Cage, Cue, Place) as the between-subjects variables. We compared the total number of DCX-labeled cells, percentage of BrdU/DCX double-labeled cells, percentage of BrdU/zif268 and DCX/zif268 double-labeled cells in the GCL using a two-way factorial ANOVA with type of training (cage control, cue, place) and strain (SD,LE) as between-subjects factors. Post-hoc tests
utilized the Newman-Keuls procedure. Pearson product-moment correlations were performed on the total distance, total latency and percent time in the target quadrant during the probe trial with the percentage of DCX labeled cells that had no processes, short unbranched processes, long/branched processes as well as the percentage of DCX-labeled cells that expressed zif268 and the total number of DCX-labeled cells.

**Results**

**Cue-trained rats swam shorter distances to reach the platform**

For distance to reach the platform across sessions (Figure 5.3 A) there were significant main effects of type of training ($F(1,34) = 105.03, P \leq 0.000001$) and session ($F(4,136) = 60.44, P \leq 0.000001$). Furthermore, there was a significant session by training interaction effect for distance to reach the platform ($F(4,136) = 13.34, P \leq 0.000001$). However, there was no main effect of strain ($P=0.30$) and no strain by training ($P=0.89$) session by strain ($P=0.98$) or session by strain by training interaction effects ($P=0.80$). Place-trained rats had significantly longer distances than cue-trained rats during sessions one ($P=0.00016$) two ($P=0.00013$), three ($P=0.0013$) but not session four ($P=0.06$) or session five ($P=0.91$). We also analyzed the total distance traveled across all trials and sessions (Figure 5.3 B). As expected there was a main effect of type of training ($F(1,34) = 105.03, P \leq 0.000001$) with cue-trained rats swimming a shorter total distance than place-trained rats. However, there was no main effect of strain ($P \geq 0.30$) or strain by training interaction ($P \geq 0.89$).

**Place-trained rats spent more time in the target quadrant**

We analyzed length of time spent in the target quadrant during the 30 second probe trial (Figure 5.4). There was a significant main effect of type of training ($F(1,34) = 28.39.03, P \leq $
0.000006) with place-trained rats spending a greater amount of time in the target quadrant. However, there was no significant main effect of strain ($P \geq 0.96$) or strain by training interaction effect ($P \geq 0.59$).

**Morris water task training influences GCL volume**

For volume of the GCL there was a significant main effect of training ($F(2,44) = 6.12, P \leq 0.0045$) but no significant main effect of strain ($P=0.12$) nor a significant training by strain interaction ($P=0.55$). Post-hoc tests demonstrated that cage control rats had significantly greater GCL volume than place-trained rats ($P=0.0024$) and cue-trained rats ($P=0.015$). However no other comparisons were found to be significantly different (Table 5.1).

**BrdU-labeling is enhanced by spatial learning regardless of strain**

For the total number of BrdU-labeled cells in the GCL (Figure 5.5 A) there was a significant main effect of type of training ($F(2,25) = 5.74, P \leq 0.0089$) and region ($F(1,25) = 776.80, P \leq 0.000001$) but not strain ($P \leq 0.83$). There was a significant region by training interaction effect ($F(2,25) = 7.48, P \leq 0.0028$) but no significant strain by training ($P=0.96$), region by strain ($P=0.49$) or region by strain by training ($P=0.99$) interaction effects. Post hoc tests demonstrated that place-trained rats had significantly more BrdU-labeled cells in the GCL than cue-trained rats ($P=0.00040$) but not cage control rats ($P=0.11$). Cue-trained rats also had significantly fewer BrdU labeled cells than cage control rats ($P=0.0071$). There were no significant differences in BrdU labeling in the hilus (Table 5.2) as a factor of type of training (all $P$’s $\geq 0.71$). Although the total number of BrdU-labeled cells did not differ between Place-trained and cage-control rats as we had predicted there was a difference in GCL volume between these groups. Thus we also controlled for volume by calculating the density of BrdU-labeled
cells between these groups and in doing so we found that the density of BrdU-labeling was significantly enhanced in place-trained rats compared to cage controls ($P=0.00059$; Table 5.3).

**Spatial learning enhanced doublecortin labeling only in Sprague-Dawley rats**

For the total number of DCX-labeled cells in the GCL (Figure 5.5 B) there was a significant main effect of strain ($F(1,44) = 4.35$, $P \leq 0.043$) but not training ($P=0.37$) and there was a significant strain by training interaction effect ($F(2,44) = 8.24$, $P \leq 0.00092$). Post-hoc tests showed that SD place-trained rats had a significantly greater total number of DCX-labeled cells in the GCL than all other groups ($P \leq 0.020$). No other comparisons were significant.

**Spatial learning increased the activation of BrdU-labeled cells only in Long-Evans rats**

There was a significant strain by training interaction ($F(2,24) = 4.09$, $P \leq 0.030$) for the number of BrdU/zif268 co-labeled cells (Figure 5.6 A). Post-hoc tests showed that the LE place-trained group had significantly more BrdU/zif268 double-labeled cell than the SD place-trained group ($P=0.046$) as well as the LE cue-trained rats ($P=0.013$) and LE cage controls ($P=0.039$). No other comparisons were significantly different (all $P's \geq 0.084$).

**Place learning increased the activation of doublecortin-labeled cells in Sprague-Dawley rats only**

There was a significant strain by training interaction ($F(2,44) = 4.12$, $P \leq 0.023$) for the percentage of DCX labeled cells that express zif268 (Figure 5.6 B). Post-hoc tests showed that the SD place-trained group had a greater number of DCX/zif268 co-labeled cells than all other groups ($P's \leq 0.036$). No other comparisons were significantly different ($P's \geq 0.69$).
Figure 5.3: Panel A shows the distance traveled during each session. Place-trained rats had significantly longer distances on sessions 1-3 but there was no significant effect of strain. Panel B shows the total distances traveled across all trials. There was no significant effect of strain but place-trained rats had significantly longer distances than cue-trained rats. Data represent mean ± SEM.
Figure 5.4: The time spent in the virtual quadrant of the pool where the hidden platform had been located is shown during a 30 second probe trial. Place-trained rats spend significantly more time in the correct quadrant than cue-trained rats. The dashed line represents the level of chance. Bars represent mean ± SEM.

Table 5.1: Mean (± SEM) volume of the granule cell layer from each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Sprague-Dawley (mm$^3$)</th>
<th>Long-Evans (mm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cage control</td>
<td>3.99 ± 0.14</td>
<td>3.65 ± 0.16</td>
</tr>
<tr>
<td>Cue trained</td>
<td>3.51 ± 0.17</td>
<td>3.46 ± 0.078</td>
</tr>
<tr>
<td>Place trained</td>
<td>3.41 ± 0.10</td>
<td>3.29 ± 0.0.094</td>
</tr>
</tbody>
</table>

Cage control rats had significantly greater granule cell layer volumes than cue or place trained rats.
Table 5.2: Mean (± SEM) total number of BrdU-labeled cells counted in the hilus

<table>
<thead>
<tr>
<th>Group</th>
<th>Sprague-Dawley</th>
<th>Long-Evans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cage control</td>
<td>111.21 ± 55.60</td>
<td>113.43 ± 46.31</td>
</tr>
<tr>
<td>Cue trained</td>
<td>126.10 ± 56.39</td>
<td>70.36 ± 28.72</td>
</tr>
<tr>
<td>Place trained</td>
<td>204.51 ± 102.26</td>
<td>100.93 ± 41.20</td>
</tr>
</tbody>
</table>

There were no significant differences between groups.

Table 5.3: Mean (± SEM) density of BrdU-labeled cells in the GCL

<table>
<thead>
<tr>
<th>Group</th>
<th>Sprague-Dawley (cells/mm³)</th>
<th>Long-Evans (cells/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cage control</td>
<td>1028.52 ± 39.53</td>
<td>1115.68 ± 69.09</td>
</tr>
<tr>
<td>Cue trained</td>
<td>912.84 ± 61.31</td>
<td>945.58 ± 46.41</td>
</tr>
<tr>
<td>Place trained</td>
<td>1288.77 ± 157.85</td>
<td>1355.33 ± 101.72</td>
</tr>
</tbody>
</table>

Place-trained rats had significantly greater BrdU-labeling than cue-trained rats or cage-control rats.

Sprague-Dawley rats have more mature doublecortin labeled cells than Long-Evans rats

Because we found DCX labeling only increased in SD and not LE place-trained rats we looked at maturity of DCX cells to determine whether there were more mature DCX-labeled cells in SD than in LE rats. For the maturation of DCX-labeled cells there was no significant main effects of strain ($P=0.37$) or training ($P=0.41$) or strain by training interaction ($P=0.41$). However, there was a significant main effect of the stage of development ($F(2,88) = , P<0.05$).
0.000001) as well as significant stage by strain interaction (Figure 5.7 A; F(2,88) = 25.48, \( P \leq 0.000001 \)) and a stage by training interaction (Figure 5.7 B; F(2,88) = 5.34, \( P \leq 0.00068 \)). The three way stage by strain by training interaction was not significant (\( P = 0.21 \)). Post-hoc tests showed regardless of strain, place-trained rats had significantly fewer cells without visible processes than cage controls (\( P = 0.00012 \)) but cue-trained rats had significantly more DCX cells without processes than cage controls (\( P = 0.00012 \)). Compared to cage controls both place-trained (\( P = 0.00013 \)) and cue-trained (\( P = 0.00012 \)) rats had significantly more DCX cells with short unbranched processes. The proportion of DCX cells with long/branched processes was found to be significantly greater in place-trained rats compared to cage controls (\( P = 0.00012 \)) but cue-trained rats had significantly fewer DCX cells with long/branched processes than cage controls. (\( P = 0.00012 \)). Regardless of type of training SD rats had significantly fewer DCX cells without processes (\( P = 0.00013 \)) and DCX cells with short unbranched processes (\( P = 0.00013 \)) than LE rats but had a significantly greater percentage of long/branched DCX cells (\( P = 0.00012 \)), indicating perhaps differences between strains in the timing of maturation of these cells.

*Spatial learning enhances the percentage of BrdU cells that express doublecortin in Sprague-Dawley rats compared to Long-Evans rats*

There were no significant main effects of either strain (\( P = 0.24 \)) or training (\( P = 0.61 \)) on the percentage of BrdU labeled cells that co-expressed DCX. However, there was a significant strain by training interaction effect (F(2,26) = 3.39, \( P \leq 0.049 \)). Newman-Keuls tests did not reveal any significant comparisons. However, we also performed planned comparisons (LSD using bonferroni corrections, corrected \( \alpha \) value = 0.0167) to determine the effect of strain on BrdU/DCX labeling in each of the three treatment conditions. SD rats trained on the place task
had a significantly greater percentage of BrdU-labeled cells double-labeled with DCX than LE rats \((P=0.013, \text{Figure 5.8})\). There was no significant main effect of strain on cue learners \((P=0.73)\) or cage controls \((P=0.35)\).

**Morris water task training increases the percentage of BrdU-labeled cells that express NeuN**

There was no significant main effect of strain \((P=0.44)\) and no significant strain by training interaction \((P=0.72)\). However, there was a significant main effect of training, \(F(2,26) = 7.12, P \leq 0.0034\), (Table 5.4). Newman-Keuls tests revealed that cage-control rats had significantly fewer BrdU/NeuN double-labeled cells than place-trained rats \((P=0.034)\) or cue-trained rats \((P=0.0022)\). There was no significant difference between place- and cue-trained rats \((P=0.13)\).

**Correlations**

In place-trained rats regardless of strain the percentage of DCX cells that coexpressed zif268 was significantly correlated with the percentage of long/branched DCX cells \((r=0.63, P=0.004)\) and was negatively correlated with the percentage of short unbranched DCX labeled cells \((r=-0.49, P=0.031)\). There was also a significant positive correlation between the percentage of DCX labeled cells with short unbranched processes and total distance \((r=0.58, P=0.009)\). When analyzed by strain there was a significant positive correlation between total distance and percentage of DCX cells with short unbranched processes in SD rats \((r=0.75, P=0.012)\) and a trend for LE rats \((r=0.64, P=0.063)\). Finally, there was a significant negative correlation between the percentage of DCX cells with long branched processes and total distance (Figure 5.9) in SD rats \((r=-0.77, P=0.010)\) but not LE rats \((P=0.71)\) trained on the place version of the task.
**Figure 5.5:** Panel A shows the total number of BrdU-labeled cells in the granule cell layer (including the subgranular zone). Place-trained rats had significantly greater BrdU densities than cue-trained rats. There was no significant effect of strain. Panel B shows the total number of doublecortin-labeled cells in the granule cell layer. Place training significantly increased doublecortin labeling in SD rats compared to all other groups. No other comparisons were significant. Bars represent mean + SEM.
Figure 5.6: Panel A shows the percentage of BrdU-labeled cells that also express zif268. LE place trained rats have significantly more zif268 labeled BrdU cells than SD place-trained rats. Panel B shows the percentage of doublecortin labeled cells that express zif268. SD place-trained rats have significantly more zif268-labeled doublecortin cells than LE place-trained rats. Bars represent mean + SEM.
**Figure 5.7:** The percentages of doublecortin-labeled cells that have no processes, short unbranched processes or long/branched processes are shown as a factor of strain (panel A) or training (panel B). SD rats had significantly fewer cells without processes and short unbranched processes and more cells with long/branched processes than LE rats. Place training increased the number of cells with long/branched and short unbranched processes but decreased the number of doublecortin cells lacking processes compared to cage controls. Cue training however, decreased the number of cells with long/branched processes but increased both the number of cells without processes and those with short unbranched processes. Bars represent mean + SEM.
**Figure 5.8:** The percentage of BrdU-labeled cells that express doublecortin is shown. Place training increased the number of BrdU and doublecortin double-labeled cells in SD rats only. Bars represent mean ± SEM.

**Table 5.4:** Mean (± SEM) percentage of BrdU/NeuN double-labeled cells in the GCL

<table>
<thead>
<tr>
<th>Group</th>
<th>Sprague-Dawley (%)</th>
<th>Long-Evans (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cage control</td>
<td>25.0 ± 1.9</td>
<td>27.3 ± 2.2</td>
</tr>
<tr>
<td>Cue trained</td>
<td>36.0 ± 3.6</td>
<td>40.6 ± 3.3</td>
</tr>
<tr>
<td>Place trained</td>
<td>34.0 ± 4.2</td>
<td>33.3 ± 3.2</td>
</tr>
</tbody>
</table>

Cage control rats had significantly fewer BrdU/NeuN double-labeled cells than place- or cue-trained rats.
Figure 5.9: Correlations between the percentage of doublecortin cells that had long/branched processes and total distance traveled during training is shown for SD place-trained rats (A) and LE place-trained rats (B). The correlation, which was only significant for SD place-trained rats, showed that longer total distances (i.e. poorer learning) was associated with fewer doublecortin cells that had long/branched processes ($r = -0.77$, $P=0.010$).
**Discussion**

The aim of this study was to determine whether strain differences exist in the neurogenic response to spatial learning. Our results show an intriguing pattern of similarities and differences between SD and LE rats. Both strains performed similarly in the MWT task and spatial learning enhanced cell survival (BrdU) to a similar extent in both LE and SD rats but only increased DCX expression in SD rats. Furthermore, we show here for the first time that there are strain differences in the activation patterns produced by memory retrieval. SD rats had a greater percentage of DCX-labeled cells that showed IEG activation compared to LE rats, while LE rats had a greater percentage of BrdU-labeled cells with IEG activation in response to spatial memory. Another novel finding in this study is the difference in DCX morphology observed between the two strains. SD rats had a greater percentage of DCX-labeled cells with long branched processes compared to LE rats and also had a greater percentage of BrdU-labeled cells that expressed DCX, possibly indicating different maturation time lines for the new neurons of each strain. However, there were no strain differences in the percentage of BrdU-labeled cells that also expressed NeuN, indicating that neuronal differentiation was not different between strains. Finally, performance in MWT was correlated with the number of mature DCX cells in SD rats but not LE rats, further suggesting that greater DCX maturation was important for spatial learning performance in SD rats. Furthermore more mature DCX cells were associated with activation of DCX cells suggesting that the more mature DCX cells were the ones that were activated in response to spatial learning.

**Spatial learning enhances cell survival in Long-Evans and Sprague-Dawley rats**

Spatial learning in SD rats increased the number of BrdU-labeled cells in the dentate gyrus consistent with previous work (Gould et al., 1999; Ambrogini et al., 2000; Epp et al.,
2007). Furthermore there was a similar increase in the survival of BrdU-labeled cells following spatial learning in LE rats. This is not unexpected given that both strains performed similarly in the MWT and that cell survival is increased in LE rats following another type of hippocampus-dependent learning (Olariu et al., 2005). While most studies have used SD rats to study the effects of hippocampus-dependent learning on neurogenesis (Gould et al., 1999; Ambrogini et al., 2000; Dobrossy et al., 2003; Ambrogini et al., 2004; Leuner et al., 2004; Pham et al., 2005; Mohapel et al., 2006; Dalla et al., 2007; Epp et al., 2007; Sisti et al., 2007; Dalla et al., 2009; Epp et al., 2009), two studies have utilized LE rats (Olariu et al., 2005; Keith et al., 2008). Both studies found increased neurogenesis associated with training but due to methodological differences neither study can readily be compared to the numerous experiments using SD rats. For example, Keith et al., trained LE rats for eight days in the MWT and then perfused 32 days later with a probe trial two days before perfusion and found an increase in DCX-labeled cells. The two-day period between probe trial and perfusion is sufficiently long to produce many new DCX-labeled cells (Keith et al., 2008). It is possible that the increase in DCX labeling in the Keith et al (2008) study was a result of the probe trial and not the initial training. Here, we perfused our rats 90 minutes after the probe trial to eliminate the effect of the probe trial on neurogenesis (as well as for IEG analysis).

**Long-Evans and Sprague-Dawley rats do not differ significantly in the Morris water task**

Our results did not support the notion that LE rats exhibit superior spatial learning as suggested by previous studies (Tonkiss et al., 1992; Harker and Whishaw, 2002). Here we used a more gradual acquisition procedure (four trials per day over five days) compared to a condensed training procedure (two days of testing with 12 trials per day; Tonkiss et al., 1992) and a longer training procedure (2 trials per day for 10 days; Harker and Whishaw, 2002) in
which strain differences have been found. Although our training procedure may not have been optimal for detecting strain differences in learning abilities, it was chosen because it is the procedure known to produce changes in neurogenesis in SD rats (Gould et al., 1999; Epp et al., 2007). Furthermore, comparable performance between strains allows for a direct comparison of the effects of spatial learning of neurogenesis without the confounding variable of quality of learning.

*Rats trained in the Morris water task had decreased granule cell layer volumes compared to cage controls*

An unexpected difference in GCL volume was found with cage control rats having a larger GCL than place or cue trained rats. Chronic stress is known to produce changes in the volume of the hippocampus in rats (Lee et al., 2009). However, we are unaware of any reports of changes in volume as a result of the mild stress associated with 5 days of Morris water task training. In fact, in one of our previous studies we showed that 10 days of Morris water task training did not change the volume of the GCL compared to cage controls (Epp and Galea, 2009). It may have been the case that stress or some other facet of exposure to the task may have decreased GCL volume but it is also possible that there was a spurious preexisting difference between these groups despite random assignment. It should also be noted that training in the Morris water task increased the percentage of new neurons (as measured by double labeled BrdU/NeuN cells), suggesting that despite possible reduction in dentate gyrus volume, more new neurons are surviving with Morris water task training.
Strain differences in neurogenesis in response to spatial learning may indicate different rates of cell maturation

Although our findings indicate that spatial learning has a similar effect on BrdU-labeled cell survival in SD and LE rats, we also found that spatial learning increased the number of DCX-labeled cells in SD, but not LE, rats. Importantly there was no strain difference in DCX-labeling in cue-trained rats or in untrained rats consistent with a previous study (Epp et al., 2009), indicating that the differential response between strains is due to spatial learning. The increase in DCX-labeling following spatial learning in SD rats could be due to 1) increased cell proliferation, 2) increased cell survival, 3) increased neuronal differentiation or 4) a change in the maturation of the immature neurons in response to spatial learning. An increase in cell proliferation in SD spatial learners is unlikely because our results show that LE, and not SD, rats have a greater percentage of DCX-labeled cells without processes. These cells correspond to the population of DCX cells that are proliferative (Plumpe et al., 2006). Similarly, it is unlikely that differences in cell survival accounted for the differences between SD and LE rats, as the number of BrdU-labeled cells was not significantly different between SD and LE spatial learners. Therefore, it seems more likely that place learning differentially altered either the differentiation or maturation of the new cells in SD rats compared to LE rats. In line with this, SD rats had more long/branched DCX cells than LE rats indicating more mature DCX-labeled cells in SD rats and possibly in place trained rats. Furthermore in possible contrast there were similar proportions of BrdU-labeled cells expressing NeuN.

Our finding that the percentage of BrdU-labeled cells that co-express DCX is higher in SD rats than LE rats that received spatial training suggests that spatial learning altered the differentiation of new cells in SD rats to a greater degree than in LE rats. In most studies the percentage of neurons produced does not change after hippocampus-dependent learning
(Dobrossy et al., 2003; Leuner et al., 2004; Pham et al., 2005; Mohapel et al., 2006; Epp et al., 2007; Keith et al., 2008; Epp et al., 2009; but see Hairston et al., 2005). However, in the current study we did not find a strain difference in the percentage of BrdU-labeled cells that express the mature neuronal marker NeuN. This suggests that differentiation of BrdU-labeled cells into mature neurons did not differ between SD and LE spatial learners. Instead, the increase in DCX/BrdU double labeling in our study could indicate that in SD spatial learners new neurons mature more slowly than in LE spatial learners. DCX is transiently expressed for up to 30 days but expression drops off drastically at seven days with most new cells losing this marker between two and three weeks in mice (Brown et al., 2003). Spatial learning in SD rats may have changed the timeline of DCX expression and possibly the maturation of the BrdU-labeled cells causing them to express DCX for a longer time. This interpretation is partially consistent with our finding that SD rats have a greater percentage of DCX-labeled cells with long/branched processes than LE rats, although this difference was seen regardless of type of training.

Although we did not find strain differences in the percentage of BrdU/NeuN double-labeled cells it is possible that there are still differences in the maturation rates in response to spatial learning as spatial training actively engaged more immature neurons in the SD rats compared to the LE rats. However, the number of DCX-labeled cells did not differ between strains under cage control or cue-training conditions.

*Spatial learning differentially activates new neurons in Sprague-Dawley and Long-Evans rats*

The activation of immature cells was assessed by co-labeling of the IEG zif268 (a marker of cellular activation, Davis et al., 2003) with BrdU and DCX. This technique has been used previously by several groups to demonstrate that immature neurons are in fact activated in response to stimuli such as learning or exploration (Ramirez-Amaya et al., 2006; Tashiro et al.,
One study has even shown the importance of adult generated neurons by demonstrating that immature neurons are preferentially activated relative to the mature granule cell population (Kee et al., 2007). In the current study, memory retrieval increased activation of DCX-labeled cells in SD but not LE rats. Intriguingly, activation of BrdU-labeled cells was increased in LE but not SD rats. One injection of BrdU labels a more homogeneous age of cells than DCX, which is expressed for a longer time. DCX expression begins shortly after cell proliferation with 80% of BrdU labeled cells expressing DCX four days later (Brown et al., 2003). Spatial learning might preferentially recruit new neurons of different ages in SD versus LE rats possibly as a result of the neurons maturing at a different rate and/or the amount of time these immature neurons are in different stages. Immature neurons are highly excitable but lose this characteristic as they age (Wang et al., 2000; Ambrogini et al., 2004; Schmidt-Hieber et al., 2004). The new neurons that seem to mature more slowly in SD rats may remain highly excitable for a longer time and this may be why a greater percentage of the DCX cells that have long/branched processes express zif268 in SD than in LE rats. In spatial learners, zif268/DCX-labeling correlates positively with the percentage of DCX cells with long/branched processes suggesting it is the older DCX cells that are primarily activated by spatial memory retrieval. Furthermore, there is a negative correlation between the percentage of short unbranched DCX cells and their percentage of zif268/DCX labeled cells suggesting that the younger DCX cells are not as active. Although without knowing the exact ages of the activated DCX cells it is difficult to make this conclusion. However, a recent study by Snyder et al. (2009) showed that IEG expression increased linearly in BrdU-labeled cells between 2-4 weeks of age (in SD rats) with almost no co-expression prior to 2 weeks of age. Thus it seems likely that the age of the cells activated by place learning in SD rats is older than 2 weeks of age. Furthermore, because we did not observe an increase in activation of BrdU-labeled cells in SD rats we can
quite confidently predict that the activated cells in that strain were older than 16 days of age (the maximum age of the BrdU-labeled cells). The age of the cells activated by place learning in the LE rats on the other hand appear to be predominantly 16 days of age because activation was enhanced in BrdU-labeled cells but not DCX-labeled cells. Finally, this also suggests that the age of activation was more specific in LE rats because there was no enhancement in activation of DCX-labeled cells.

Another novel aspect of the current study is the correlative data showing the importance of the maturational stage of the DCX labeled cells for learning ability. Regardless of strain, our results show that the greater the percentage of DCX-labeled cells with a relatively immature (short unbranched processes) phenotype the worse the learning was. Thus, immature cells may actually interfere with learning until they reach a certain age. This idea fits well with a couple of recent findings that suggest high rates of cell proliferation are not necessarily beneficial for hippocampus dependent learning (Galea et al., 2001; Epp et al., 2009). Furthermore a number of studies have demonstrated that high levels of cell proliferation in pathological conditions such as traumatic brain injury and seizures are correlated with impaired learning (Gould and Tanapat, 1997; Jessberger et al., 2007).

In SD, but not LE, spatial learners there was a significant negative correlation between distance traveled and the percentage of DCX cells with long/branched processes such that rats with more mature DCX cells demonstrated better learning. This may indicate that immature neurons are less important for spatial memory in LE rats than SD rats or may support the idea that SD and LE rats preferentially incorporate new neurons of different ages for spatial learning. Our finding that the BrdU-labeled cell population is specifically activated by spatial memory recall in LE rats also supports the latter possibility. In other words spatial learning may recruit cells of a wider age range in SD rats but LE rats utilize a more specific age of immature cells.
Therefore, it may be necessary to further dissociate the developmental stages of DCX-labeled cells in order to find a more specific age at which spatial learning ability correlates with the DCX-labeling in LE rats.

Our results appear to provide some evidence that there is a difference in the rate of maturation of new neurons between strains but only in response to spatial learning. However, it is possible that differences in DCX expression might simply reflect a difference in the time course of DCX expression that is not linked to the physical maturity of the cells. This is an unlikely scenario because of the importance of the DCX protein for growth and migration (maturation processes) but must be considered. To definitively determine whether new cells mature at different rates in different strains and whether spatial learning impacts this, the functional maturity of the new cells must be determined. In a recent study the rate of maturation of young neurons of SD rats versus C57BL/6 mice was assessed using a functional rather than morphological assessment. By counting the number of BrdU-labeled cells that expressed zif268 in response to kainic acid induced seizures at one to 10 weeks following BrdU administration they demonstrated that new neurons become functionally mature faster in SD rats than in mice. (Snyder et al., 2009). This method for determining functional maturity may prove to be beneficial in future studies to provide further evidence that new neurons mature at different rates in different strains of rats.

**Conclusions**

Despite similar BrdU-labeled cell survival, SD and LE rats exhibit a number of differences in the relationship between spatial learning and adult neurogenesis. Changes in the rate of maturation, differentiation and ultimately the activation of new cells may represent alternative mechanisms, other than enhancing overall cell survival, by which learning may
optimize the efficacy of the immature neuron population. Future exploration of the differences between strains may help to characterize the mechanisms that underlie the learning dependent changes in neurogenesis.
References


6 GENERAL DISCUSSION

Throughout the preceding studies in Chapters 2-5 I have demonstrated that spatial learning has a variety of effects on adult neurogenesis that depend upon both the specific training conditions and the timing of the experiment. In Chapter 2, I have demonstrated that there is a critical period in the maturational timeline of new cells (6-10 days after cell proliferation) during which new cell survival can be enhanced by spatial learning (Chapter 2; Epp et al., 2007). Before (days 1-5) or after (days 11-15) this time window, spatial learning did not have an effect on cell survival. However, I have also shown that if the post-training time is increased from 1 to 5 days, spatial learning on days 11-15 decreases cell survival (Chapter 3; Epp et al. submitted), suggesting that not only is the time of exposure important but so is the age of cells that are examined and/or the amount of time between learning and sacrifice.

In Chapter 3, the results of the c-fos and BrdU double labeling indicated that more new cells are active following a probe trial if the rats were trained on the place task on days 11-15. Thus, despite a decrease in cell survival following place learning on days 11-15 there is an increase in the percentage of the remaining cells that are activated by memory retrieval. Conversely, activation of new cells was not increased in place learners compared to cue learners when training occurred on days 6-10 despite cell survival being increased by this type of training. Age of cells likely played a role in this finding though as glutamatergic connectivity is not yet established in cells at this age (Esposito et al., 2005)

In Chapter 4 (Epp et al., 2009) I confirmed that spatial learning on days 6-10 after BrdU administration increases cell survival but also found that this effect can be eliminated or even reversed by increasing the difficulty of the task. Increasing task difficulty by decreasing the number of trials per session did not change cell survival relative to training on a visible platform.
task. Reducing the number of distal cues in the environment decreased cell survival compared to visible platform training.

In Chapter 5 I compared the effects of spatial learning on cell survival in two strains of rats, Long-Evans and Sprague-Dawley. I found that spatial learning on days 6-10 increased cell survival equally in both strains. However, spatial learning increased the number of immature neurons (doublecortin) in Sprague-Dawley rats but not Long-Evans rats. There were also differences in the activation of new neurons between strains. In response to a probe trial, Long-Evans, but not Sprague-Dawley, rats trained on the place task showed an increase in the percentage of BrdU-labeled cells that express zif268. However, in Sprague-Dawley, but not Long-Evans rats, training on the place task increased the percentage of doublecortin-labeled cells that expressed zif268 following a probe trial. Regardless of strain, place learning increased the percentage of doublecortin-labeled cells that had a long branched phenotype and decreased the percentage of doublecortin cells that had no or very short processes. However, cue learning had the opposite effect, with the percentage of immature doublecortin-labeled cells increased and the percentage of mature doublecortin cells decreased. Furthermore regardless of type of training, Sprague-Dawley had a greater percentage of doublecortin cells with long branched processes but a smaller percentage with no or short unbranched processes compared to Long-Evans rats.

**Interpretations**

**The effects of spatial learning on cell survival**

The current dissertation was conducted in order to gain a greater understanding of how spatial learning regulates cell survival and to potentially reconcile controversial findings in the literature. Table 6.1 shows a list of studies that have examined the effect of spatial learning on the survival of BrdU-labeled cells, which are divided in their results. The data collected in
Chapter 2 (Epp et al., 2007) provide the most explanatory power for most of the results of the studies in Table 6.1. That is, the critical period for enhancing cell survival that was observed here on days 6-10 corresponds roughly to the time of training utilized by three of the previous studies that also found increased cell survival (Gould et al., 1999; Ambrogini et al., 2000; Hairston et al., 2005).

Another study (Van der Borght et al., 2005) found no change in cell survival when training was conducted on days 9-12 with perfusion on day 13. This time point is approximately in between the 6-10 and 11-15 day time points used here in Chapter 2. However, from the lack of increase in cell survival it appears as though training on days 9-12 may be outside of the critical window for increasing cell survival. If so, this indicates that the critical window exists for a shorter period of time than shown in Chapter 2 (Epp et al., 2007). Another interpretation is congruent with the data collected in Chapter 4 (Epp et al., 2009) as task difficulty likely played a role. Van der borght and colleagues (2005) trained rats on the place task with a fixed platform location for the first three days and were then trained to find a new platform location on the last two days. The switch in platform locations likely resulted in a more difficult task. I have shown in Chapter 4 (Epp et al., 2009) that increased task difficulty by reducing the number of training trials resulted in no change in cell survival compared to cue learning. Regardless of whether training in the Van der borght study (2005) occurred during or after the critical window it is possible that the lack of change in cell survival in that study may have been due to the difficulty of the particular version of the place task that was used. A similar training protocol with two platform locations was used in another study that found no change in cell survival when training occurred 6-9 days after BrdU administration (Ehninger & Kempermann, 2006). Furthermore a study by Mohapel and colleagues (2006) used an even more challenging working memory task in which the platform location changed after every block of trials. Cell survival was
not changed by four days of this type of training. Fourteen days of training on the other hand decreased cell survival suggesting that this type of training, possibly as a result of task difficulty, decreased cell survival, consistent with our findings in Chapter 4.

Taken together, the results of Chapter 2 (Epp et al., 2007) and Chapter 3 demonstrate that place learning on days 11-15 has no effect on cell survival if the rats are perfused on day 16 but decreases cell survival if training occurs on day 20. Thus the age of cells at the time of examination or the amount of time that passes after spatial learning has an effect on whether or not cell survival is decreased. Ambrogini and colleagues (2004) trained rats at a similar time point (days 10-14) and then perfused the rats three days later. With this very similar design these authors also found a decrease in cell survival consistent with our results in Chapter 2.

The final study in Table 6.1 (Dobrossy et al., 2003) cannot be explained as easily by the current studies in this dissertation. In that study, place training on days 0-7 and perfusion on day 8 decreased cell survival in the hippocampus. However, in Chapters 2 and 3 I found no change in cell survival with early training (days 1-5), whether perfusion occurred 5 days or 10 days after training. The difference in results between my studies and Dobrossy’s study may be attributable to the fact that Dobrossy and colleagues (2003) injected BrdU only 30 minutes prior to the start of training. It is possible that the stress of training inhibited cell proliferation and the incorporation of BrdU, as stress is known to decrease cell proliferation in male rats (Holmes and Galea, 2002). In the same Dobrossy study another experiment resulted in an increase in cell survival if BrdU was administered once rats achieved asymptotic performance on the place task and perfusion occurred on either day 4 or 30. The training that occurred after the rats achieved this level of performance and received BrdU could be considered early training based on the current thesis because it occurred immediately after BrdU administration. However, the nature of the training at this time point is much different than what was done in the present studies.
because the rats were already at asymptotic performance. Furthermore, as stress hormone levels are reduced by pre-training (Beiko et al., 2004) it might also be argued that maintenance of a spatial memory is less difficult than initial encoding or at least is not as stressful. These reasons may help explain why an increase in cell survival was observed by Dobrossy and colleagues (2003) following spatial learning that occurred at a time relative to BrdU administration that would not have been expected to be effective based on the timeline for enhancing cell survival established in this dissertation.

**Effects of spatial learning and memory on the integration and activation of adult generated neurons**

Although the primary focus of the experiments in this thesis was to determine the impact of spatial learning on cell survival it is also important to discuss the impact of spatial learning on the related processes of integration and activation of new neurons.

The process of cell survival is highly intertwined with the integration of the new neurons. Previous work has shown that survival of new neurons is dependent on activation of their own NMDA receptors indicating that survival requires functional activation (Tashiro et al., 2006). Furthermore in addition to increasing cell survival, spatial learning increases the dendritic length, branching and spine number of new neurons, further emphasizing the importance of spatial learning for neuronal integration (Ambrogini et al., 2009; Tronel et al., 2010). Similarly, in Chapter 5 (Epp et al., submitted) I analyzed the phenotype of doublecortin-labeled cells based on three morphological characteristics. I found that spatial learning relative to cage controls increased both the percentages of new neurons with no processes and those with long branched processes but decreased the percentage of neurons with an intermediate phenotype. This suggests that there may be a simultaneous increase in the number of new neurons produced as
well as a promotion of the maturation of intermediate aged neurons. Once the new neurons are properly incorporated, their activity in the network in response to spatial learning or memory retrieval can be determined by measuring an immediate early gene product such as zif268 or c-fos. This approach was used in Chapters 3 (Epp et al., submitted) and 5 (Epp et al., submitted).

I was able to demonstrate in Chapter 3 that although cell survival is enhanced when training occurs on days 6-10, this population of cells is not that likely to be active in response to spatial memory retrieval on day 15 after birth. However, despite a decrease in cell survival when training occurs on days 11-15 activation of these new cells in response to spatial memory retrieval is enhanced on day 20 after birth. This result is likely due to difference in the age of the cells (day 15 versus day 20) and the differences in their maturational stage at the time of learning and memory retrieval. The older population of cells is more likely to have formed functional synapses and to have become properly incorporated into the hippocampus. It is also possible that the increase in cell survival when training occurs on days 6-10 may have little to do with encoding or storing the current spatial memory but may be a mechanism to replace the population of unused neurons that is available for a future learning episode. Conversely, the 11-15 day old immature neurons exposed to spatial learning may in fact be more important for spatial learning and memory. Not only does spatial memory increase the percentage of new neurons that are active but also correlatives positively with the percentage of immature neurons that are active in the 11-15 day old immature neurons exposed to spatial training. The same correlation does not exist when training occurred on days 6-10 (Chapter 3, Epp et al., submitted).

Thus, in addition to analyzing the effects of spatial learning on cell survival I and others have demonstrated that is also important to consider the maturation, integration and activity of those new neurons.
Table 6.1. Previous studies on the effects of spatial learning on cell survival

<table>
<thead>
<tr>
<th>Study</th>
<th>BrdU injections</th>
<th>Days of training</th>
<th>Day of perfusion</th>
<th>Species/strain</th>
<th>Cell survival</th>
<th>Relevant thesis chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gould et al., 1999</td>
<td>1 200mg/kg</td>
<td>Days 7-10 (4)</td>
<td>Day 11 or Day 18</td>
<td>Rat SD</td>
<td>INC</td>
<td>Chapter 2, Epp et al., 2007</td>
</tr>
<tr>
<td>Ambrogini et al., 2000</td>
<td>2 x 3 days 50mg/kg</td>
<td>Days 3-7 (5)</td>
<td>Day 18</td>
<td>Rat SD</td>
<td>INC</td>
<td>Chapter 2, Epp et al., 2007</td>
</tr>
<tr>
<td>Dobroissy et al., 2003</td>
<td>1 x 4 days 50mg/kg</td>
<td>Days -4 to 3 (8)</td>
<td>Day 4 or day 30</td>
<td>Rat SD</td>
<td>INC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 x 4 days 50mg/kg</td>
<td>0 - 7 (8)</td>
<td>Day 8</td>
<td>Rat SD</td>
<td>DEC</td>
<td></td>
</tr>
<tr>
<td>Ambrogini et al., 2004</td>
<td>2 x 3 days 50mg/kg</td>
<td>Days 10-14 (5 with 2 session per day)</td>
<td>Day 17</td>
<td>Rat SD</td>
<td>DEC</td>
<td>Chapter 3, Epp et al., Submitted</td>
</tr>
<tr>
<td>Hairston et al., 2005</td>
<td>2 x 1 day 100 mg/kg</td>
<td>Days 7-10 (4 with 2 sessions per day)</td>
<td>Day 10</td>
<td>Rat ? (albino)</td>
<td>INC</td>
<td>Chapter 2, Epp et al., 2007</td>
</tr>
<tr>
<td>Van der Borght et al., 2005</td>
<td>1 x 3 days 100mg/kg</td>
<td>Days 9-12 (5 with 2 platform locations)</td>
<td>Day 13</td>
<td>Rat SD &amp; Wistar</td>
<td>N.C.</td>
<td>Chapter 2 &amp; 4, Epp et al., 2007; Epp et al., 2009</td>
</tr>
<tr>
<td>Ehninger &amp; Kempermann, 2006</td>
<td>2 x 2 days 50mg/kg</td>
<td>Days 6-9 (4 with new platform location on day 4)</td>
<td>Day 10</td>
<td>Mouse C57BL/6</td>
<td>N.C.</td>
<td>Chapter 4, Epp et al., 2009</td>
</tr>
<tr>
<td>Mohapel et al., 2006</td>
<td>4 x 1 day 50mg/kg</td>
<td>Days 7-10 (4)</td>
<td>Day 10</td>
<td>Rat SD</td>
<td>N.C.</td>
<td>Chapter 4, Epp et al., 2009</td>
</tr>
<tr>
<td></td>
<td>4 x 1 day 50mg/kg</td>
<td>Days 1-14 (14)</td>
<td>Day 14</td>
<td>Rat SD</td>
<td>DEC</td>
<td>Chapter 4, Epp et al., 2009</td>
</tr>
</tbody>
</table>

1.Number of BrdU injections per day. The first day of BrdU injections is set as day 0. Dose given is per injection
2.Time of training relative to BrdU injection (or first BrdU injection in the case of multiple injections). The number in parentheses denotes the number of days of training and the number of sessions per day (if more than one session).
3.Change in cell survival following spatial learning. N.C. = no change, INC = increase, DEC = decrease.
4.Relevant thesis chapter that helps explain why the previous study found and increase, decrease or no change in cell survival.
Limitations

Although the factors explored in the preceding experiments are unlikely to be an exhaustive list of factors that regulate the effects of spatial learning on neurogenesis, these results have mitigated much of the confounding results in the literature. However, there are several potential limitations to these studies that should be noted. First, all of the studies utilized the thymidine marker BrdU to label dividing cells and to measure the number of new cells that subsequently survive until the time of perfusion. BrdU is a valuable tool but there are a number of drawbacks associated with its use. First, BrdU is a toxic compound and may be unsuitable in some situations such as in embryonic and early postnatal studies where it is known to have negative effects on brain development (Kolb et al., 1999). The toxicity of BrdU is most extreme during embryonic and early postnatal development. However, by the time the blood brain barrier develops (postnatal day 10 in rats) most of the negative effects of BrdU are no longer apparent. In adults it does not appear as though the doses of BrdU used to study adult neurogenesis have cytotoxic effects (Hancock et al., 2009) but it is possible that there may be long-term effects of BrdU treatment, particularly if high and multiple doses of BrdU are given. However, in the studies reported here I used a single injection of 200 mg/kg, which has been demonstrated to be a safe dose in adult rats (Cameron and McKay, 2001; Cooper-Kuhn and Kuhn, 2002). Secondly, the specificity of BrdU has been questioned by some researchers (See Taupin, 2007 for review) because cells undergoing DNA repair may also incorporate BrdU. While this may be true, the number of cells that incorporate sufficient BrdU to be detectable as a result of cell repair is likely extremely low. For example, focal irradiation of the hippocampus can be used to largely reduce the percentage of dividing cells by damaging progenitor cells (see Wojtowicz, 2006 for review). If cell repair or terminal cell division was responsible for a measurable increase in BrdU-labeling it would be expected that irradiation would cause an
increase in BrdU-labeling and this is clearly not the case as focal irradiation causes a large
decrease in BrdU incorporation (Snyder et al., 2005; McGinn et al., 2008). Further, many
previous studies, including some of the studies in the present thesis have used endogenous
markers of cell proliferation and neurogenesis (such as Ki67 and doublecortin) in conjunction
with BrdU and found similar patterns of results (Rao and Shetty, 2004; Leuner et al., 2009).
This shows that in adults BrdU is not (or at least at a very low rate) labeling cell death or repair
but is a legitimate marker of cell synthesis.

Throughout this thesis, a distinction has been drawn between place and cue learning in
the Morris water task. Cue training has been described as hippocampus-independent while place
learning is described as hippocampus-dependent. However, in reality, training in both tasks
engages the hippocampus to some extent (Teather et al., 2005; also see appendix A). In both
types of training the hippocampus will function to form a spatial map of the testing environment.
Thus, it is not possible to completely dissociate between hippocampus-dependent and -
independent tasks. However, during place learning, the hippocampus is required to navigate to
the location of the hidden platform. During cue learning a spatial map of the environment is
likely created but cannot be used to locate the platform because the platform location moves
within the spatial representation after each trial. Therefore, the hippocampus is required for
place but not cue learning but it is important to note that it is engaged by both types of learning.
Activation studies, including those reported here (Chapter 3 and Chapter 5) have shown that cue
learning activates fewer granule cells than place learning (Teather et al., 2005; also see Figure 1
in Appendix A). In addition to the degree of dependence on the hippocampus, cue and place
learning may differ in other ways that should be considered. For example, cue learning and
place learning likely differ in their degree of difficulty. It is possible that cell survival is
enhanced by place learning because this task is more difficult than cue learning. However, as
observed in Chapter 4, increasing the difficulty of the place task resulted in a decrease in cell survival. Therefore, it seems unlikely that differences in cell survival between cue and place learning could be explained by differences in task difficulty but it would be worth comparing place learning to a more difficult form of cue learning in future studies.

It is also possible that different training paradigms such as cue and place learning as well as the various versions of the place task used in Chapter 4 may be more or less stressful and this rather than learning may explain some of the differences in cell survival that have been observed. There is some evidence that after four days of visible or hidden platform training levels of the stress hormone corticosterone are elevated but do not differ based on type of training (Mohapel et al., 2006). However it is unclear whether there are differences in stress levels over the course of place versus cue training that may influence cell survival. We and others have found that corticosterone, the main glucocorticoid in rats, does increase in response to spatial training in the Morris water task (Appendix B). One study has indeed suggested that if the stress of training is reduced by providing pre-exposure to the task then the increase in cell survival following spatial learning is abolished (Ehninger & Kempermann, 2006). The interpretation provided by the authors in that study is that water maze training acts as a stressor that down regulates neurogenesis, particularly in cue-trained mice and thus the difference in cell survival is not a result of hippocampus-dependent learning. However this interpretation may be flawed on a number of levels. First we, and others, have not found differences in cell proliferation rates following hippocampus-dependent or -independent learning compared to cage controls (Epp et al., 2009; Gould et al., 1999) thus suggesting that cue-trained rats do not have down regulated neurogenesis in relation to cage control rats. Second, Ehninger and Kempermann (2006) used female mice in their study and the relationship between neurogenesis and hippocampus-dependent learning is not as important in mice as it is in rats (Snyder et al., 2009). In fact,
Snyder and colleagues also demonstrated that immature neurons develop less quickly in mice than rats and are also less numerous. As a result, it may be unwise to directly compare rats and mice in terms of the relationship between learning and neurogenesis. As further evidence of this, it has been shown that mice do not perform as well as rats in the Morris water task but are equally able to solve a dry-land spatial task suggesting that the differences in performance in the water maze are due to decreased competency in the water (Whishaw and Tomie, 1996). In addition, the fact that female animals were used may be an important factor to consider. It is currently unclear whether male and female rodents exhibit the same changes in neurogenesis following spatial learning. There is some evidence that this may be the case as female rats learn a different hippocampus-dependent task (trace conditioning) better than males and also exhibit a greater enhancement in cell survival following training than do males (Dalla et al., 2009). Thus, it is unclear if the pre-training effect observed by Ehninger and Kempermann (2006) would be applicable to rats but would be worth investigating.

**Future experiments**

In addition to the factors addressed here that were found to have an impact on cell survival following spatial learning, there are a number of additional ideas that were beyond the scope of this thesis but should be addressed. Based on the IEG activation data in Chapter 3, it appears as though rats trained on days 11-15 had greater activation of BrdU-labeled cells than rats trained on days 6-10. However, it is not clear whether this is due to a difference in the age of the new cells at the time of training (days 6-10 versus days 11-15), or if it is due to the age of the new cells at the time of memory retrieval and perfusion (day 15 versus day 20). A future experiment may answer this question by training rats on days 6-10 or 11-15 and administering the probe trial and perfusing the rats on day 20 regardless of the time of training. This will allow
for a comparison of the time of training on cell activation without the impact of the age of the new cells at the time of memory retrieval. Equal activation of BrdU-labeled cells in rats trained on days 6-10 and 11-15 would suggest that the age of the new cells at the time of memory retrieval was responsible for the differences observed in c-fos labeling in Chapter 3.

It would also be interesting for future studies to expand the results of Chapter 4. In that experiment, task difficulty was increased by either reducing the number of distal cues in the testing environment or by reducing the number of trials the rats received. Both methods reduced cell survival compared to our standard hidden platform training protocol. It is possible that this result was due to an increase in stress associated with the more difficult training methods. It would be informative to compare the effects of these different training protocols on the levels of circulating corticosterone by collecting blood samples at the end of each training session. In addition, it would be interesting to intentionally alter the stressfulness of the training itself to determine whether this changes the effect of spatial learning on cell survival. This could be achieved by training rats in either warm or cold water or by training a group of rats in the presence of a stressful white noise.

Finally, in Chapter 5 I demonstrated that there are strain differences in the maturation of DCX-labeled cells. Sprague-Dawley rats trained on the place task had a greater percentage of DCX-labeled cells that had a mature phenotype than did Long-Evans place learners. Thus, maturity was assessed by examining the morphology of the new neurons. However, it is possible that this does not correlate precisely with the functional maturity of the new neurons. In other words, the age at which the new cells can first be activated may be different between strains. It would be interesting to determine if Sprague-Dawley and Long-Evans rats have different activation time courses. To do so, zif268 co-labeling of BrdU-labeled cells could be carried out in response to a single session of spatial learning on each day after BrdU labeling
from 1-15 days to determine when new neurons first respond to spatial learning in each strain and whether there are strain differences in this time point.

**Conclusions**

The experiments described here demonstrate that a wide variety of factors must be accounted for in order to determine the influence of spatial learning on adult neurogenesis in the hippocampus. Additionally, it is clear that spatial learning has numerous effects on adult generated neurons in the dentate gyrus including enhanced and decreased cell survival depending on the time of training as well as changes in the maturation and activation of new neurons. Ultimately, the experiments described here should help to pinpoint the exact contributions that adult generated neurons make to the function of the hippocampus, especially in regards to learning and memory. The enhanced excitability of the immature neurons suggests a mechanism by which they may participate in hippocampus-dependent learning perhaps even preferentially (Kee et al., 2007). The substantial positive and negative modulations in cell survival that can occur in response to cell survival provide a means to regulate the population of new neurons that will be accessible not necessarily for the current learning episode but more likely for future learning.
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Tronel, S., Fabre, A., Charrier, V., Oliet, S. H., Gage, F. H. & Abrous, D. N. Spatial learning


APPENDICES

Appendix A: zif268 expression in the dentate gyrus

Figure A1: Zif268 positive cells in the dorsal granule cell layer were counted to determine if there was a strain difference in the overall activation of the dentate gyrus. There was no significant main effect of strain or strain by training interaction. However, there was a significant main effect of training. Place-trained rats had greater activation that cue-trained rats and cage controls. Cue-trained rats also had significantly greater activation than cage controls. Sections used for this analysis were from the same rats used in Chapter 5.
Appendix B: corticosterone concentrations following MWT training

Using the same standard training protocols utilized for MWT training throughout this dissertation blood was collected from separate groups of rats after either cue or place training as well as from Naïve cage controls. Blood was collected immediately after the final (fourth) trial on days 1, 3, and 5 of training. Blood from cage controls was collected within 3 minutes of disturbing the cage. Corticosterone concentrations (Figure B1) in the blood sample were determined by performing a radioimmunoassay.

Figure B1. Corticosterone concentrations following Morris water task training. There was a significant main effect of group ($F(2,25) = 129.12, P < 0.00001$). Cage controls were significantly different than cue-trained ($P = 0.00015$) and place-trained rats ($P = 0.00013$). Place and cue trained rats did not differ significantly ($P = 0.13$).
Appendix C: UBC animal care certificates

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0335
Investigator or Course Director: Liisa Galea
Department: Psychology, Department of Animals:

- Rats Sprague-Dawley 26
- Rats Long Evans 250
- Rats Long Evans 168
- Rats Sprague Dawley 152

Start Date: October 15, 2007 Approval Date: May 15, 2009
Funding Sources:

| Funding Agency: | Canadian Institutes of Health Research (CIHR) |
| Funding Title:  | Gonadal hormone effects on adult hippocampal neurogenesis in the male and female rodent |

| Funding Agency: | Natural Sciences and Engineering Research Council of Canada (NSERC) |
| Funding Title:  | Hormones and learning and memory |

Unfunded title: N/A

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

Application Number: A06-0011
Investigator or Course Director: Lisa Cales
Department: Psychology, Department of

Animals:
- Rattus Long Evans 24
- Rattus Sprague-Dawley 78
- Rattus spraguei dawley 280
- Rattus Sprague-Dawley 60
- Rattus Sprague-Dawley 48
- Rattus spraguei dawley 48
- Rattus Sprague-Dawley 30
- Rattus Long Evans 48

Start Date: February 1, 2006
Approval Date: March 26, 2009

Funding Sources:

Funding Agency: National Alliance for Research (US)
Funding Title: Models of post-partum depression: Effects on behavior, stress reactivity and hippocampal neurogenesis in both mother and offspring

Funding Agency: Pacific Alzheimer Research Foundation
Funding Title: Effects of estrogens on neurogenesis in the adult hippocampus in young and aged rodents

Funding Agency: National Alliance for Research (US)
Funding Title: Models of post-partum depression: Effects on behavior, stress reactivity and hippocampal neurogenesis in both mother and offspring

Funding Agency: Pacific Alzheimer Research Foundation
Funding Title: Effects of estrogens on neurogenesis in the adult hippocampus in young and aged rodents

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Estrogen and progesterone effects on neurogenesis in the adult mammalian hippocampus

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Estrogen and progesterone effects on neurogenesis in the adult mammalian hippocampus

Unfunded title: N/A

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