ADULT HIPPOCAMPAL NEUROGENESIS
and MEMORY ENHANCEMENT

by

Scellig S. D. Stone

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Institute of Medical Science
University of Toronto

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Adult Hippocampal Neurogenesis and Memory Enhancement

Scellig S. D. Stone
Doctor of Philosophy
Institute of Medical Science
University of Toronto
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Abstract

Hippocampal neurogenesis continues throughout life in mammals. These adult-generated dentate granule cells (DGCs) are generally believed to contribute to hippocampal memory processing and are generated at varying rates in response to neuronal network activity. Deep brain stimulation (DBS) allows clinicians to influence brain activity for therapeutic purposes and raises the possibility of targeted modulation of adult hippocampal neurogenesis. It has recently been shown that DBS may ameliorate cognitive decline associated with Alzheimer’s disease (AD), and while underlying mechanisms are unknown, one possibility is activity-dependent regulation of hippocampal neurogenesis. To this end, whether or not adult-generated DGCs can assume functional roles of developmentally-generated neurons, and stimulation-induced enhanced neurogenesis can benefit memory function in the normal and diseased brain, warrant study. First, we examined separate cohorts of developmentally- and adult-generated DGCs in intact mice and demonstrated similar rates of activation during hippocampus-dependent spatial memory processing, suggesting functional equivalence. Second, we examined the neurogenic and cognitive effects of targeted entorhinal cortex (EC) stimulation in mice using parameters analogous to clinical high frequency DBS. Stimulation increased the generation of DGCs. Moreover, stimulation-induced neurons were functionally recruited by hippocampal spatial memory processing in a cell age-dependent fashion that is consistent with DGC maturation.
Importantly, stimulation facilitated spatial memory in the same maturation-dependent manner, and not when stimulation-induced promotion of adult neurogenesis was blocked, suggesting a causal relationship. Finally, we are in the process of testing whether similar stimulation facilitates spatial memory in a transgenic (Tg) disease model of AD that exhibits amyloid neuropathology and cognitive impairment. Preliminary results suggest stimulation promotes neurogenesis and rescues impaired spatial memory in Tg animals. When considered in the context of promising clinical results, this body of work suggests stimulation-induced neurogenesis could provide a novel therapeutic modality in settings where functional hippocampal regenerative therapy is desirable.
Acknowledgments

I owe my sincerest gratitude to Dr. Andres Lozano for this opportunity and for continued guidance, support, and mentorship. Of the many pieces of wisdom you have provided over the years, one has left the greatest impression upon me: “We can get more of everything except time. Time is your most precious resource. Your challenge is to decide how best to spend that resource”.

I consider forming a working relationship with Dr. Paul Frankland, without which this work would not exist, to be my greatest benefit from this research. I am forever grateful for the countless hours you spent teaching me how to plan, perform, and communicate science, while always treating me as nothing short of a colleague and friend.

I also wish to thank Dr. Martin Wojtowicz for his invaluable contributions as a member of my thesis committee. Most notably, your keen interest and attention to detail was instrumental in raising the scientific quality of this work.

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Curiosity, enthusiasm, work ethic, organization, and compassion are the pillars upon which I am striving to build a meaningful clinician-scientist career. For instilling these in me, and so much more, I thank my parents David and Therese, Glen and Fran, and my brother Daithi.

I owe my deepest gratitude to my wife, Angela, who is most singularly responsible for making the completion of this thesis possible. Thank you for your tireless encouragement, immeasurable sacrifice, and unyielding love.

I dedicate this thesis to our two precious children, William and Adelaide.

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Contributions

Scellig Stone (author) solely prepared this thesis. All aspects of this body of work, including the planning, execution, analysis, and writing of all original research and publications was performed in whole or in part by the author. The following contributions by other individuals are formally and inclusively acknowledged:

Dr. Andres Lozano (Primary Supervisor and Thesis Committee Member) – mentorship; laboratory resources; guidance and assistance in planning, execution, and analysis of experiments as well as manuscript/thesis preparation

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<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>CA</td>
<td>Cornu Ammonis</td>
</tr>
<tr>
<td>CldU</td>
<td>5-Chloro-2'-deoxyuridine</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBS</td>
<td>Deep Brain Stimulation</td>
</tr>
<tr>
<td>DCX</td>
<td>Doublecortin</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate Gyrus</td>
</tr>
<tr>
<td>DGC</td>
<td>Dentate Granule Cell</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic Day</td>
</tr>
<tr>
<td>EC</td>
<td>Entorhinal Cortex</td>
</tr>
<tr>
<td>ECS</td>
<td>Electroconvulsive Shock</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric Acid</td>
</tr>
<tr>
<td>GADD45B</td>
<td>Growth Arrest and DNA-Damage-Inducible Beta</td>
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<tr>
<td>GCL</td>
<td>Granule Cell Layer</td>
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<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HC</td>
<td>Home Cage</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HSV-TK</td>
<td>Herpes Simplex Virus-Thymidine Kinase</td>
</tr>
<tr>
<td>IdU</td>
<td>5-Iodo-2'-deoxyuridine</td>
</tr>
<tr>
<td>IEG</td>
<td>Immediate Early Gene</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>LTP</td>
<td>Long Term Potentiation</td>
</tr>
<tr>
<td>MAM</td>
<td>Methazoxymethanol</td>
</tr>
<tr>
<td>ML</td>
<td>Molecular Layer</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartic Acid Receptor</td>
</tr>
<tr>
<td>NS</td>
<td>Non-Stimulated (or Electrode Insertion Only)</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal Day</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PTZ</td>
<td>Pentylenetetrazole</td>
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<tr>
<td>S</td>
<td>Stimulation</td>
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<tr>
<td>SC</td>
<td>Subcutaneous</td>
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<tr>
<td>SGZ</td>
<td>Subgranular Zone</td>
</tr>
<tr>
<td>SOX2</td>
<td>Sex-Determining Region Y-Box 2 Transcription Factor</td>
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<td>SVZ</td>
<td>Subventricular Zone</td>
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<td>TBS</td>
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<td>TMZ</td>
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Chapter 1  General Introduction
1.1 Preamble

The continued generation of neurons within the adult central nervous system (CNS) was identified over forty years ago in rodents (Altman and Das, 1965) and subsequently confirmed in other mammals including humans (Eriksson et al., 1998). Adult neurogenesis is consistently reported in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG), where adult-generated dentate granule cells (DGCs) eventually integrate into the local neural network (Deng et al., 2010).

Given the widely accepted importance of the hippocampus in forming new memories (Moscovitch et al., 2005; Scoville and Milner, 1957; Squire and Zola-Morgan, 1991), the possibility that hippocampal neurogenesis represents a form of structural plasticity in memory processes has garnered considerable attention (Deng et al., 2010; Shors, 2008). Although mounting evidence over the past several years suggests adult-generated DGCs contribute to the formation of hippocampus-dependent memory, precisely what contribution(s) this entails remain(s) unclear. One key question concerning how these adult-generated DGCs contribute to memory function is whether they are functionally distinct from DGCs generated during development.

The production and survival of adult-generated DGCs varies in response to a number of influences, including local network activity (Zhao et al., 2008). Deep brain stimulation (DBS) uses surgically implanted electrodes to deliver electrical stimulation to precisely targeted areas in the brain and is used clinically as a therapeutic means of modulating brain activity (Awan et al., 2009; Perlmutter and Mink, 2006). Recent human evidence suggests DBS might arrest or slow cognitive decline associated with dementia (Laxton et al., 2010), although the mechanism(s) underlying these effects is(are) unclear. Stimulation of hippocampal afferents (Bruel-Jungerman et al., 2006; Chun et al., 2006; Encinas et al., 2011; Kitamura et al., 2010; Toda et al., 2008) and mossy fibers (Derrick et al., 2000) can reliably increase the production of new neurons in rodents, pointing to one possible mechanism through which DBS might influence cognitive function. However, whether stimulation-induced increases in neurogenesis impact hippocampal function and memory performance is not known.
1.2 Thesis Organization

This thesis is organized in a “multiple paper format” rather than a traditional “continuous design”, using mainly unaltered peer-reviewed content. This structure best reflects the sequential nature of the past, present and future of this continuing project. It is my hope that this will give the reader a sense for how events unfolded, what answers were generated along the way, and how they have spawned multiple routes of ongoing and future investigations. Chapter 2 primarily serves to review adult hippocampal neurogenesis in the context of learning and memory, and is largely derived from a peer reviewed book chapter on the topic (Stone and Frankland, in press). The final section of this chapter outlines the research aims and hypotheses governing the thesis. Chapters 3, 4, and 5 present original research addressing those objectives, each as self-contained manuscripts with similar formatting for consistency of style. Chapter 3 is a reformatted version of a paper published in the journal “Hippocampus” (Stone et al., 2010) along with its supplemental section containing additional supporting experiments. Chapter 4 is a reformatted version of a paper recently published in the “Journal of Neuroscience” (Stone et al., 2011). Chapter 5 introduces aims, preliminary experiments, and data in a disease model and also constitutes an ongoing and future direction for the overall project. The discussion sections within these last 3 chapters are complemented by Chapter 6, which briefly summarizes the key findings and general conclusions of the thesis. In addition, ongoing and specific future directions are outlined in the discussion sections of Chapters 4 and 5 and are complemented by related general thoughts in Chapter 6.
Chapter 2  Adult Hippocampal Neurogenesis and Memory

This chapter is modified from the following:

2.1 Introduction

This review chapter is divided into 3 main sections. The first 2 (2.2 and 2.3) discuss key biological aspects of adult hippocampal neurogenesis followed by its links with and learning and memory. Section 2.3 concludes by placing adult hippocampal neurogenesis within a clinical and therapeutic context. It should be noted that a considerable body of literature implicates adult hippocampal neurogenesis in the pathophysiology of depression (Sahay and Hen, 2007), but such disorders of mood are not studied here and are thus deliberately not discussed. Finally, the third section (2.4) outlines aims and hypotheses of the original research in this thesis.

2.2 Biology of Adult Hippocampal Neurogenesis

2.2.1 Introduction

This section summarizes key biological aspects of neurogenesis, including where it occurs within hippocampal circuits, what characterizes adult-generated dentate granule cells (DGCs), and how this process is regulated.

2.2.2 Anatomy of the Dentate Gyrus

The hippocampal formation includes several distinct cytoarchitectural regions (Figure 2-1): the entorhinal cortex (EC), dentate gyrus (DG), hippocampus proper (including the Cornu Ammonis (CA) subfields 1-3 and sometimes 4 if considered separate from the DG, where it is otherwise referred to as the hilus), and subicular complex (Amaral and Lavenex, 2007; Amaral and Witter, 1989). Table 2-1, listing approximate numbers of neurons in major regions of the rat and human hippocampus, indicates that the DG contains a relatively large pool of hippocampal neurons, of
which the majority are DGCs (Mulders et al., 1997; West and Gundersen, 1990; West and Slomianka, 1998; West et al., 1991). Although these regional totals are less precisely defined in mice, strains commonly used in neurogenesis studies harbor roughly 250,000-500,000 granule cells per DG (Abusaad et al., 1999; Kempermann et al., 1997a). DGCs are the principal excitatory neurons of the DG, receive the majority of their inputs from layer II neurons of the medial and lateral EC via the medial and lateral perforant paths (named in reference to their course which “perforates” the subiculum) respectively, and send mossy fiber terminals to CA3 pyramidal cells (Amaral and Lavenex, 2007). The fact that a small number of EC neurons project to a relatively larger number of DGCs (Table 2-1) has been argued to allow the DG to separate multiple EC inputs (O’Reilly and McClelland, 1994). This coding property will be discussed in greater detail later. These DG connections, along with Schaffer collateral fibers from CA3 pyramidal neurons to CA1 neurons, participate within the classically described trisynaptic circuit of the hippocampus (Andersen et al., 1969). The hippocampal formation communicates extensively with the rest of the brain, mainly through the EC and via the fimbria-fornix pathway. The most numerous hippocampal-cortical connections comprise a loop of EC-mediated neocortical interactions with unimodal and polymodal association areas in the frontal, temporal, and parietal lobes (Lavenex and Amaral, 2000). Many subcortical regions, including diencephalic and limbic structures, also reciprocate connections with the hippocampi. Damage to either the hippocampi or any of these associated regions can produce anterograde amnesia, indicating their importance in memory (Aggleton and Saunders, 1997).
**Figure 2-1. The Hippocampus and Trisynaptic Circuit.** Left inset depicts temporal position and orientation of horizontal section (right). Major signal pathways within the hippocampus (HC) can be represented simplistically as a circuit beginning in the entorhinal cortex (EC), which acts as the main conduit for signal transmission between the HC and neocortical areas. The EC predominantly projects to DGCs via layer II neuron efferents along the perforant path (red). DGCs send mossy fibers (purple) to CA3 pyramidal neurons. Schaffer collateral fibers (orange) extend from CA3 to CA1, which in turn projects back (blue) to the EC and Subiculum (S). EC projections to CA1 are predominantly via the perforant path at more temporal levels (as shown here), and alvear path (salmon) primarily from layer III EC neurons at more septal levels (depicted here for illustrative purposes).

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<thead>
<tr>
<th>Major Hippocampal Region</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entorhinal Cortex Layer 2</td>
<td>110,000</td>
<td>1,100,000 ^b</td>
</tr>
<tr>
<td>Dentate Granule Cell Layer</td>
<td>1,200,000 ^c</td>
<td>15,000,000 ^d</td>
</tr>
<tr>
<td>CA2/3 Subfields</td>
<td>250,000 ^c</td>
<td>2,700,000 ^d</td>
</tr>
<tr>
<td>CA1 Subfield</td>
<td>382,000 ^c</td>
<td>16,000,000 ^d</td>
</tr>
<tr>
<td>Subiculum</td>
<td>300,000 ^a</td>
<td>4,500,000 ^d</td>
</tr>
</tbody>
</table>

^a^ (Mulders et al., 1997); ^b^ (West and Slomianka, 1998); ^c^ (West et al., 1991); ^d^ (West and Gundersen, 1990)

The DG contains three main layers (Figure 2-2): an inner-located hilus (also referred to as either the polymorphic layer or CA4 if considered a hippocampal subfield rather than a portion of the DG (Amaral, 1978)), middle-located granule cell layer (GCL), and outer-located molecular layer (ML) (Amaral and Lavenex, 2007). The hilus primarily contains inhibitory interneurons, glutamatergic mossy cells and DGC axons. Perforant path fibers synapse upon apical dendrites...
of DGCs in the outer region of the ML, with lateral EC originating fibers arriving outermost and medial EC fibers incoming just below (van Groen et al., 2002). Some commissural fibers from the contralateral DG have also been demonstrated medially in the ML (Blackstad, 1956). DGC bodies populate the densely packed GCL, and it is at its interface with the hilus, termed the subgranular zone (SGZ), where adult hippocampal neurogenesis occurs.

**Figure 2-2. Hippocampal Neurogenesis.** Adult-generated DGCs arise from self-renewing progenitor cells within the SGZ of the hippocampus (HC), located between the hilus and granule cell layer (GCL). Maturing DGCs extend axons to CA3 and dendritic trees into the molecular layer (ML). Dendrites receive glutamatergic inputs from entorhinal cortex (EC) originating perforant path fibers (red). Inset depicts position and orientation of coronal section.

### 2.2.3 Identification and Quantification of Neurogenesis

Cellular proliferation within the SGZ of the DG has been characterized using a variety of methods, including the detection of exogenously delivered and endogenously expressed markers of cell division. Altman and Das first demonstrated adult hippocampal neurogenesis in rodents by detecting exogenously administered tritiated thymidine (Altman and Das, 1965). Nucleotide derivatives such as these can be given *in vivo*, for example to rats, are available (for up to 2 h in DG cells) for permanent incorporation within the 7 h S phase (deoxyribonucleic acid (DNA) replication) of the 25 h cell cycle, and can be subsequently detected at later time-points.
Using a short interval between analogue delivery and animal sacrifice generates a labeled cell count that estimates proliferation. A long delay, during which some labeled cells may die, provides an opportunity to estimate cell survival.

Electron microscopy of autoradiographically labeled new DGCs in adult rodents demonstrated neuronal morphology, confirming neurogenesis (Kaplan and Bell, 1984; Kaplan and Hinds, 1977). Combining this technique with immunohistochemical detection of the relatively selective neuron marker neuron-specific enolase (Deloulme et al., 1996; Schmechel et al., 1980) and tracer injection further revealed that the majority of adult-generated cells were likely DGCs extending mossy fibers to CA3 (Cameron et al., 1993; Stanfield and Trice, 1988). While studying neurogenesis in the higher vocal center of adult songbirds, Barnea and Nottenbohm separately confirmed new neuron production in the avian equivalent of the hippocampal formation and noted an increase in neurogenesis with high environmental novelty and spatial memory demand (Barnea and Nottenbohm, 1994, 1996).

Despite its successes, tritiated thymidine labeling use was inherently limited by its radioactivity, requirement for very thin tissue sections and difficulty with multi-labeling, and soon gave way to the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) (Miller and Nowakowski, 1988). BrdU is detectable immunohistochemically in thick sections required for stereology (Figure 2-3), readily amenable to multi-labeling, and capable of signal amplification (West et al., 1991). Accordingly, BrdU became the primary agent for identifying adult-generated cells in the 1990s (Kempermann et al., 1997b; Kuhn et al., 1996). Compelling evidence for neurogenesis was obtained by co-staining tissue for BrdU and one of many endogenous neuron-specific markers. These included NeuN, a nuclear-specific protein (Mullen et al., 1992), and calbindin, a vitamin-D dependent calcium-binding protein (Jande et al., 1981). Using BrdU incorporation to evaluate cell cycle kinetics in the DG, investigators determined that young adult mice and rats gain roughly 3000 and 9000 new DGCs per day respectively, or roughly 0.4% of their total granule cell populations (Cameron and McKay, 2001; Hayes and Nowakowski, 2002). Rodents then lose half or more of these new cells by 1 month, after which most survive for at least several months (Dayer et al., 2003; Kempermann et al., 2003). This rate of production declines precipitously with age, but does not cease (Drapeau et al., 2003; Kempermann et al., 1998b; Kuhn et al., 1996). BrdU labeling also confirmed adult hippocampal neurogenesis in numerous other
mammals including primates and humans (Eriksson et al., 1998; Gould et al., 1997; Gould et al., 1999b; Gould et al., 1998; Kornack and Rakic, 1999). In Eriksson et al.’s seminal study, newly generated DGCs were detected in 5 cancer patients that received BrdU for diagnostic purposes; however, their rate of production in humans remains unknown. The striking degree of evolutionary conservation suggests, at least in general terms, that this process carries some degree of biological importance.

Figure 2-3. Sample BrdU Labeling of Adult-Generated Dentate Granule Cells. Adult-generated cells in the SGZ and inner GCL of the DG are visualized through immunohistochemical staining for exogenously administered and incorporated BrdU. 4',6-diamidino-2-phenylindole (DAPI) counterstain (scale bar = 50 µm).

A collective disadvantage of exogenously delivered cellular proliferation markers is the inherent requirement to deliver a foreign compound, potentially influencing the proliferation, maturation, and survival of new neurons. For instance, stress that animals may experience while receiving injections can alter normal cellular processes including neurogenesis (Gould et al., 1991; Heine et al., 2004). Thymidine analogues can also, in principle, incorporate into any cell undergoing DNA repair and thus falsely identify non-mitotic cells (Kuan et al., 2004). However, mitosis involves the synthesis of the entire genome and typically utilizes several orders of magnitude more nucleotides than DNA repair (Kee et al., 2007a). Consistent with this, the risk of false-positive labeling associated with DNA repair is negligible even at high analogue doses and under conditions that stimulate DNA repair such as inflammation and brain irradiation (Cameron and McKay, 2001; Palmer et al., 2000).

Certain methodological considerations remain important caveats to thymidine analogue use. These include the effect of administration timing and dosage on the number of labeled cells (Cameron and McKay, 2001; Mandyam et al., 2007) and impact of relatively harsh epitope
retrieval steps on multi-labeling success (Wojtowicz and Kee, 2006). Although temporally limited by their natural expression patterns, endogenous markers can obviate these potential confounds and some provide useful cell-cycle kinetic information (Eisch and Mandyam, 2007). Ki67 expression is a common example used to verify thymidine analogue-detected proliferation rates (Dayer et al., 2003; Eisch and Mandyam, 2007; Kee et al., 2002). This nuclear protein has a short half-life and is expressed in dividing cells primarily during the S, G2 and mitosis phases (Brown and Gatter, 2002). Doublecortin (DCX) is a microtubule-associated protein expressed in the cytoplasm for roughly 2 weeks and thus can be used as an endogenous marker of new immature neurons (Brown et al., 2003; Nacher et al., 2001; Sisti et al., 2007).

2.2.4 Adult-Generated Dentate Granule Cell Development

2.2.4.1 Technical Considerations

Three types of approaches have been used to investigate the origins and progressive development of adult-generated DGCs. Initial studies characterized overlapping phases of proliferation, differentiation, and maturation by mapping the temporal expression of cell markers in BrdU-labeled cells (Kempermann et al., 2004; Steiner et al., 2004). More recent work has applied genetic lineage tracking techniques to facilitate the focused study of newly generated cells across their entire lifespan. Specifically, in vivo delivery of reporter transgenes using retroviruses to selectively infect dividing cells has since become a popular means of labeling and tracking newly generated cells (Tashiro et al., 2006b) (Figure 2-4). The signal(s) can be targeted to either the nucleus or cytoplasm, facilitating multi-labeling and morphological analyses. Despite its utility, concerns have arisen over the possibility for reporter protein transfer to post-mitotic neurons (Ackman et al., 2006), although this issue can generally be overcome by detecting additional endogenous markers for cell identity. The temporal specificity of retroviral labeling has also been questioned due to potentially continued slow and fast proliferation of some infected stem and progenitor cells (Breunig et al., 2008). Zhao et al. immunohistochemically examined the frequency with which retrovirally-labeled DGCs co-expressed Ki67, a marker of proliferating cells, and determined that while this fraction is initially nearly 20% at 3 days post-infection, it
declines rapidly to near 0% by 7 days post-infection (Zhao et al., 2006). This suggests, especially at relatively longer time-points post-infection, that the time of virus infection reasonably approximates the birth-date of labeled DGCs. Another method of identifying and tracking adult-generated DGCs is to use Tg reporter animals to genetically label the adult-generated progeny of neural precursors. For example, mice that express markers under Nestin gene regulatory control elements have been used to quantify neural progenitor and stem cells with extremely high labeling efficiency (Imayoshi et al., 2008; Lagace et al., 2007; Ninkovic et al., 2007). Reporter gene expression can also be temporally activated in animals expressing Cre-recombinase bound to a receptor, such as the ligand-binding domain of the estrogen receptor (Hayashi and McMahon, 2002). In this case, Cre-mediated recombination and hence marker expression only occurs when in the presence tamoxifen. Accordingly, new neuron labeling can be initiated at a desired adult time-point.

Figure 2-4. Sample Retroviral-Green Fluorescent Protein Labeling of Adult-Generated Dentate Granule Cells. Adult-generated cells in the SGZ and inner GCL of the DG are visualized through immunohistochemical staining for GFP 6 weeks following DG infection with GFP-encoding retrovirus. This can be combined with stains for endogenous markers, such as the neuronal stain NeuN (red) used here (scale bar = 50 µm).

2.2.4.2 Stem and Progenitor Origins

Adult neural stem cells should be capable of self-renewal and differentiation into all types of CNS cells, including neurons, astrocytes, and oligodendrocytes (Gage, 2000). While at present no definitive marker for neural stem cells has been identified, candidate multipotent neurogenic radial glia-like cells within the SGZ (termed type 1 cells) have been uniquely identified as Glial fibrillary acidic protein (GFAP), Nestin, and Sex-determining region Y-box 2 transcription factor (SOX2) positive (Figure 2-5) (Fukuda et al., 2003; Garcia et al., 2004; Suh et al., 2007). They extend a radial process through the GCL that arborizes in the ML along with a smaller horizontal
process within the SGZ, and possess some astrocytic features (Filippov et al., 2003; Fukuda et al., 2003; Seri et al., 2001). It is possible that these type 1 cells are relatively quiescent neural stem cells, able to generate self-renewing progenitor cells (Mathews et al., 2010; Mu et al., 2010). Highly proliferative type 2a and 2b cells are distinguished by initially remaining Nestin+ but lacking GFAP staining and having only short processes (type 2a), then becoming DCX+ (type 2b). This early detection of DCX importantly indicates lineage commitment prior to cell cycle exit. Whether or not type 2 cells arise from type 1 cells is uncertain, but type 2 Nestin+/SOX2+ cells can self-renew and generate both neurons and astrocytes, suggesting stem cell properties and possibly a reciprocal relationship with type 1 cells (Suh et al., 2007). Type 2 cells give rise to DCX+/Nestin- type 3 cells or neuroblasts, which continue to cycle. Cells become post-mitotic immature neurons in as little as 3 days, transiently express Calretinin, and can already express NeuN. Ultimately DCX expression ceases and Calbindin replaces Calretinin as a mature DGC phenotype is assumed.

2.2.4.3 Morphological Development

Post-mitotic adult-generated DGCs progressively attain mature granule cell morphology, implying functional capacity (Figure 2-2). During this process, primarily elucidated in rodents using Green fluorescent protein (GFP) retroviral labeling (Figure 2-4), most structural modification occurs over roughly 2 months (Esposito et al., 2005; Zhao et al., 2006). Within days some cells begin migrating into the GCL, although most still remain near the hilar border even months later (Esposito et al., 2005; Zhao et al., 2006). Axonal projections reach CA3 by 4 to 11 days (Hastings and Gould, 1999; Zhao et al., 2006) and continue to extend distally thereafter.
Apical dendrites grow and arborize into the ML over the first 3 to 4 weeks (Esposito et al., 2005; Zhao et al., 2006). Dendritic spines are apparent by 2 weeks and display synaptic terminals, with subsequent modification extending beyond this period (van Praag et al., 2002; Zhao et al., 2006). Structurally mature axosomatic, axodentic, and axospinous synaptic inputs have been visualized by 30 days, with the suggestion that some arise from the EC, along with similar output synapses on appropriate CA3 and hilar target neurons (Toni et al., 2008; Toni et al., 2007; van Praag et al., 2002). These findings suggest that adult-generated DGCs possess the requisite morphology to assume some degree of circuit function within a few weeks of birth.

2.2.4.4 Electrophysiologic Development

Developing adult-generated DGCs begin to exhibit functional Gamma-amino butyric acid (GABA) and Glutamate afferents by roughly 1 and 2 weeks of age respectively (Figure 2-2) (Ambrogini et al., 2010; Esposito et al., 2005; Ge et al., 2006). Relative to mature granule cells, immature cells have distinct electrophysiological properties at around 2 to 6 weeks of age, prompting some to propose this represents a period of enhanced plasticity and distinct function (Ge et al., 2007). Features identified include: lower activation thresholds, higher resting potentials, and a greater propensity for long-term potentiation (LTP) (Ambrogini et al., 2004a; Ge et al., 2007; Schmidt-Hieber et al., 2004; Wang et al., 2000). Indeed, eliminating adult-DGC production leads to a significant reduction in perforant path-induced DG LTP in vitro, underscoring the important contribution of adult neurogenesis to DG synaptic plasticity (Snyder et al., 2001; Zhao et al., 2007). These electrophysiologic parameters approach those of mature granule cells by approximately 6 to 8 weeks in rodents (Ge et al., 2007; Laplagne et al., 2006; Laplagne et al., 2007; van Praag et al., 2002). Overall, the developmental sequence of electrophysiologic maturation in adult-generated cells follows closely that of cells in the developing brain (Ambrogini et al., 2004a; Esposito et al., 2005).

Mature new adult hippocampal neurons have similar electrophysiological properties to developmentally-generated DGCs (Esposito et al., 2005; Laplagne et al., 2006; van Praag et al., 2002). Hippocampal slice recordings find mature cell-like membrane potentials, action potentials, and spontaneous post-synaptic currents. Moreover, these cells show post-synaptic responses to perforant path stimulation with similar properties to cells generated during
development (Laplagne et al., 2006; van Praag et al., 2002), and essentially identical responses to numerous GABAergic and Glutamatergic input parameters (Laplagne et al., 2006; Laplagne et al., 2007). This compelling evidence implies that new granule cells become functional members of the hippocampal circuit akin to their developmentally-generated counterparts.

2.2.4.5 Contribution to the Adult Dentate Granule Cell Population

Long-term fate mapping studies using Tg reporter animals demonstrate that adult-generated DGCs primarily reside in the innermost GCLs and constitute 10-15% of the entire DGC population (Imayoshi et al., 2008; Ninkovic et al., 2007). This proportion initially increases over several months, as does the density of DGCs (Imayoshi et al., 2008), reflecting a net addition of granule cells over time. The proportion of DGCs that are generated during adulthood eventually reaches a plateau, likely reflecting declining neurogenesis with age; however, whether a certain degree of dynamic equilibrium between new neuron addition and death is partly responsible for the plateau remains a matter of debate (Ninkovic et al., 2007).

2.2.5 Regulation of Neurogenesis

The microenvironment of the SGZ, including its neural, glial and vascular components, grants a wide variety of influences access to neural stem/progenitor cells (Ma et al., 2008; Morrison and Spradling, 2008). Neurotransmitters and neural peptides, growth factors and neurotrophins, morphogens, cytokines, hormones (including steroids) and drugs (including antidepressants) have all been shown to modulate aspects of adult hippocampal neurogenesis. These elements influence the proliferation, differentiation, survival, and integration of adult-generated DGCs both positively and negatively through a large repertoire of mechanisms, including cell surface receptor-mediated signaling pathways, transcription factor modulation, and epigenetic regulation (Zhao et al., 2008). Collectively, these numerous influences and their mechanisms of action permit the system to respond to complex internal and external stimuli and experiences, such as environmental enrichment, aging, stress, and physical activity (Drapeau et al., 2003;
Given its direct relevance to this thesis, a detailed discussion of activity-dependent regulation follows.

### 2.2.5.1 Activity-Dependent Regulation

Since adult-generated DGCs join hippocampal networks, as suggested by their phenotypic, morphologic, and electrophysiologic development, it is fitting that their genesis responds to activity within those networks. Supraphysiologic generalized increases in neuronal activity, such as that induced by seizures and electroconvulsive shock (ECS), increase DGC generation, although resultant cells can exhibit altered morphology and may arise ectopically in the hilus and inner ML (Jessberger et al., 2007b; Ma et al., 2009; Madsen et al., 2000; Overstreet-Wadiche et al., 2006; Parent et al., 1997). In addition, focal electrical stimulation of limbic targets/the perforant path (Bruel-Jungerman et al., 2006; Chun et al., 2006; Encinas et al., 2011; Kitamura et al., 2010; Toda et al., 2008) and mossy fibers (Derrick et al., 2000) promotes both the proliferation and survival of adult-generated granule cells in vivo. These neuronal activity-induced effects reflect influences on multiple cellular constituents within the neurogenesis pathway, including neural progenitor cells, and to a lesser extent immature DGCs.

Neuronal activity likely increases neurogenesis by influencing neural progenitor cells. In vivo stimulation of brain regions with DG connectivity primarily promotes neurogenesis by increasing DG proliferation (Bruel-Jungerman et al., 2006; Chun et al., 2006; Derrick et al., 2000; Encinas et al., 2011; Kitamura et al., 2010; Toda et al., 2008) without altering baseline rates of neuronal apoptosis (Toda et al., 2008), suggesting a key effect occurs at the progenitor stage. Encinas et al. demonstrated that stimulation of the anterior thalamic nucleus, a limbic structure, promotes hippocampal neurogenesis by specifically affecting type 2 progenitor cells (Encinas et al., 2011). Stimulation in reporter mice increased the number and mitotic activity of these cells, culminating in more adult-generated DGCs. Indeed, other pro-neurogenic stimuli resulting in increased network activity, such as seizures and ECS, primarily increase neurogenesis through upregulated progenitor cell proliferation suggesting similar underlying mechanisms (Parent and Murphy, 2008; Zhao et al., 2008).
Activity-induced release of paracrine neurogenic factors from local mature DGCs may couple neuronal activity to the progenitor cell neurogenic response. Several such factors have been identified to have neurogenesis-inducing properties, and many stimulate neurogenesis through increased progenitor cell proliferation (Zhao et al., 2008). For instance, recent work in this area suggests ECS incites growth and DNA-damage-inducible beta (GADD45B) expression, in-turn causing expression of pro-proliferation and survival factors, such as Fibroblast growth factor 1 and Brain-derived neurotrophic factor, via epigenetic region-specific DNA demethylation (Ma et al., 2009). This could also explain the reported link between stimulation-induced increased neurogenesis and LTP induction (Bruel-Jungerman et al., 2006; Kitamura et al., 2010), since LTP induces the expression of GADD45B (Hevroni et al., 1998) and various growth factors (Patterson et al., 1992). Other known pro-neurogenic growth factors may mediate the promotion of neurogenesis associated with activity, including Insulin-like growth factor 1 (Lichtenwalner et al., 2001) and Vascular endothelial growth factor (Cao et al., 2004). For example, Insulin-like growth factor 1 is exocytosed by neurons in an activity-dependent fashion through signaling by calcium sensing proteins called Synaptotagmins (proteins that act as calcium sensors for synaptic and neuroendocrine exocytosis) (Cao et al., 2011). In addition to growth factors, other diffusible signaling proteins that promote neurogenesis, such as Wnt (Lie et al., 2005) and Sonic hedgehog (Ahn and Joyner, 2005; Banerjee et al., 2005; Lai et al., 2003), may couple activity to neurogenesis. For example, DG Wnt3a is released following DG stimulation and modulates DG LTP (Chen et al., 2006).

Neural progenitor cells also respond to activity-induced release of neurotransmitters, particularly GABA and Glutamate. Although not directly innervated, progenitor cells exist within the densely packed SGZ and thus could undergo nonsynaptic depolarization due to local neuronal activity-related increased extracellular potassium (Gardner-Medwin and Nicholson, 1983), voltage change-induced ephaptic responses (Jefferys, 1995), and ambient Glutamate and/or GABA activating neurotransmitter receptors (Jang et al., 2008). Evidence that Glutamate and GABA modulate progenitor cell proliferation is scant and contradictory (Jang et al., 2008; Tozuka et al., 2005); however, studies do show that progenitor cell activation through Glutamate and GABA induces neuronal differentiation of progeny (Deisseroth et al., 2004; Tozuka et al., 2005).

Neurotransmitter-mediated regulation of hippocampal neurogenesis also occurs at later stages in the neurogenesis pathway. For instance, GABA facilitates the eventual Glutamatergic
innervation of newborn DGCs through its ambient release from interneurons, potentially
directing the integration of new DGCs into hippocampal circuits in an activity-dependent fashion
(Ge et al., 2006). Moreover, retrovirus-mediated gene knockout of N-methyl-D-aspartic acid
receptor (NMDAR) expression in proliferating DG cells impairs their survival during the 3rd
week of development, possibly denoting input-specific selection of developing new neurons
(Tashiro et al., 2006a). A pro-survival effect of Glutamate on immature DGCs may also explain
the increased survival of immature DGCs following perforant path stimulation in vivo (Bruel-
Jungerman et al., 2006; Kitamura et al., 2010). Collectively, these linkages with activity suggest
mechanisms through which hippocampal neurogenesis could respond to network activity arising
from a variety of stimuli, including hippocampus-dependent cognitive processes.
2.3 Evidence Linking Hippocampal Neurogenesis to Learning and Memory

2.3.1 Introduction

The preceding section suggests that DGCs are produced in adulthood and join the hippocampal circuit, and that this process is regulated by various intrinsic and extrinsic variables. Considerable evidence also suggests that learning impacts neurogenesis, and adult-generated cells functionally integrate into memory networks and contribute to learning and memory. However, precisely how adult-generated DGCs contribute to these cognitive processes is less clear. While alterations in hippocampal neurogenesis have been associated with changes in behavioral measures of learning and memory, inconsistent and conflicting data has frequently emerged.

This section discusses evidence for functional integration of adult-generated DGCs into learning and memory processes at the cellular and behavioral level, including evidence for learning- and memory-specific activation of newborn neurons and the impact of manipulating the rate of adult hippocampal neurogenesis on behavior. Also discussed are unresolved issues that impede our progress towards a clearer understanding, and potential technical and interpretative reasons underlying conflicting results.

2.3.2 Learning Impacts Neurogenesis

Learning promotes the survival of adult-generated DGCs. Studies suggest this is characteristic of hippocampus-dependent tasks, correlates with performance, biases precursors towards neuronal differentiation, and is temporally specific. For example, survival of roughly 1 week-old DGCs is enhanced by Morris water maze training and trace eyeblink conditioning in young adult rodents (Anderson et al., 2011; Epp et al., 2010, 2011a; Epp et al., 2007; Gould et al., 1999a; Leuner et
This effect is most prominent in the outer blade of the DG (Ambrogini et al., 2000) and does not extend to the SVZ (Gould et al., 1999a). Interestingly, DG LTP induced by in vivo stimulation of the perforant path has comparable effects (Bruel-Jungerman et al., 2006; Kitamura et al., 2010). The extent of survival also correlates with performance in some reports (Ambrogini et al., 2000; Drapeau et al., 2003; Epp et al., 2011a; Leuner et al., 2004; Sisti et al., 2007; Waddell and Shors, 2008). Since the rate of hippocampal neurogenesis is activity-dependent, it seems logical that multiple forms of learning that engage, but are not necessarily dependent on, the hippocampus could promote survival. Consistent with this reasoning, some propose that increased survival occurs with more robust forms of learning regardless of whether they are hippocampus-dependent (Dalla et al., 2007; Epp et al., 2010).

Adult DGC proliferation may also be stimulated by learning and correlate with performance. For instance, BrdU incorporation studies report increases in proliferation associated with water maze training in rats of varying ages (Drapeau et al., 2003; Lemaire et al., 2000). Greater increases correlated with faster maze learning and better recall. Since BrdU incorporation is permanent, increased cell numbers after even short delays between labeling and detection can partially represent enhanced survival rather than solely proliferation (Prickaerts et al., 2004). Dupret et al. mitigated this potential confound by detecting endogenous expression of Ki67 and confirmed elevated DG proliferation after 5 days of water maze training (Dupret et al., 2007).

Despite evidence for a pro-survival and pro-proliferative influence of learning on adult hippocampal neurogenesis, many studies have failed to detect these effects. In some cases, discrepant results arise when similar procedures are applied to different animal strains (Olariu et al., 2005; Snyder et al., 2005) or ages (Drapeau et al., 2003). Inconsistent findings could also be explained by the complex interplay of cognitive load and stress levels associated with training protocols. For example, one study demonstrated that water maze task difficulty was inversely proportional to survival, and suggested this might reflect animals adopting non-survival-promoting learning strategies when given less spatial cues (Epp et al., 2010). However, it is difficult to separate the potential anti-survival influence of greater task difficulty from that of higher levels of psychological stress that may be associated with more challenging tasks (Thomas et al., 2007). Stress is a pivotal factor in determining whether or not learning modulates neurogenesis, as evidenced by a lack of benefit of water maze training on the survival of 1 week-
old DGCs with protocols that significantly elevate circulating corticosterone levels (Mohapel et al., 2006). Moreover, some experimental procedures require pre-exposing mice to the water maze days before training in order to demonstrate learning-induced increased cell survival (Ehninger and Kempermann, 2006), possibly because pre-exposure to the maze environment lowers stress levels during training (Beiko et al., 2004).

Studies examining several time points in and around behavioral tasks propose a learning-induced selective process of adult-generated neuron retention and removal. For instance, “snapshots” of neurogenesis taken during different periods of learning demonstrate opposing phases of enhanced survival and cell death in adult born DGC populations of different ages (Ambrogini et al., 2004b; Anderson et al., 2011; Epp et al., 2007; Olariu et al., 2005). A homeostatic regulation of adult-generated DGC production and survival that is modulated by learning has been proposed by studies labeling newborn DGCs at multiple time points during extended water maze training regimens. During the early learning phase, corresponding to rapid performance improvement, neurogenesis was reduced and DG apoptosis rates were elevated relative to controls (Dobrossy et al., 2003; Drapeau et al., 2007; Dupret et al., 2007). Interestingly, this reduced neurogenesis also correlated with better performance (Dobrossy et al., 2003; Drapeau et al., 2007). Cells born during or soon after the second phase, when asymptotic performance levels were reached, were more numerous than controls (Dobrossy et al., 2003; Drapeau et al., 2007). Blocking apoptosis during this second phase of learning, by intraventricular injection of a pan-caspase inhibitor, prevented this spike in neurogenesis and impaired memory during learning and testing (Dupret et al., 2007). Collectively, these results suggest that certain forms of learning promote the survival of selected new granule cells, while removing others; however, the precise utility of this process, and why it would occur in such immature cell populations, remain to be determined.

Beyond influencing their number, learning can also potentiate the differentiation and morphological development of new neurons. Water maze training was associated with increases in neuronal differentiation (Epp et al., 2007), as well as dendrite length and branching, and spine density and maturation (Tronel et al., 2010), of DGCs generated either 1 week prior to or during the early phase of water maze training. These morphological effects were not seen in primarily developmentally-generated DGCs, identified using a reporter viral vector that infects all neurons, suggesting adult born DGCs were specifically influenced. Structural changes were also abolished by blocking either apoptosis, pointing to a relationship between the learning-induced homeostatic
regulation of cell survival and that of morphology, or NMDARs, implicating activity-dependent regulation. Alterations were enhanced by a more challenging delayed matching-to-place task (Tronel et al., 2010), and were not seen in a separate study when the water maze training regimen resulted in rapid acquisition (Ambrogini et al., 2010), suggesting greater task difficulty promotes learning-induced changes in morphology.

2.3.3 Adult-Generated Neurons Functionally Integrate and Contribute to Learning and Memory

2.3.3.1 Functional Activation

While the aforementioned electrophysiologic studies have demonstrated synaptic integration of adult-generated DGCs, functional recruitment into hippocampal memory circuits, within the context of hippocampus-dependent memory tasks, has been demonstrated in intact animals using immunohistochemical approaches. In order to identify learning and memory-induced activation of adult born DGCs, immediate early gene (IEG) products are co-detected with new neuron markers, such as BrdU, in a temporally specific fashion (Figure 2-6) (Guzowski et al., 2005; Kee et al., 2007a). Rapid and temporary up-regulation of IEGs (such as Fos, Arc, Zif268) is induced by DGC activation (Farivar et al., 2004), for example following seizures (Dragunow and Robertson, 1987; Jessberger and Kempermann, 2003) and LTP induction (Cole et al., 1989). Accordingly, animals can be given BrdU to label adult-generated DGCs, subsequently learn a hippocampus-dependent task (such as the hidden platform version of the water maze), and be sacrificed when learning- or testing-induced IEG expression reaches its peak (Kee et al., 2007a). The rate of IEG expression within labeled new neurons reflects both task-specific and nonspecific activity (for instance, resulting from swimming activity, stress, or arousal). Consequently, all experimental groups must undergo exactly the same experiences prior to sacrifice so that adult-generated granule cell activation induced by memory processes can be determined by group differences in, rather than raw, activation rates.
Spatial exploration (Alme et al., 2010; Chawla et al., 2005; Marrone et al., 2011; Ramirez-Amaya et al., 2006), and water maze learning and recall (Jessberger and Kempermann, 2003; Kee et al., 2007b; Snyder et al., 2009a; Snyder et al., 2009b; Snyder et al., 2011; Tashiro et al., 2007; Trouche et al., 2009), increase IEG expression rates in DGCs, suggesting functional activation. This expression occurs in a relatively small proportion of DGCs, consistent with the concept of sparse encoding by the DG (Jung and McNaughton, 1993). More importantly, hippocampus-dependent learning and recall increases activation rates in adult-generated DGCs only once they are at least several weeks old at the time of learning, suggesting age-dependent functional recruitment of adult-generated granule cells by hippocampus-dependent memory processing in the DG (Kee et al., 2007b; Snyder et al., 2009a). The timeline of this cell age-dependent recruitment tracks the morphological and physiological maturation of adult-generated neurons.
2.3.4 Changes in Neurogenic Rate Influence Learning and Memory

Associations between neurogenesis levels and mnemonic performance are frequently observed. Many correlative examples have been demonstrated by behavioral studies of animals with differing baseline levels of neurogenesis and following manipulations that increase or decrease the adult-generated DGC population.

Animals with a relatively high rate of endogenous neurogenesis generally outperform similar animals with lower neurogenic rates on hippocampus-dependent learning tasks. For instance, neurogenesis levels in several recombinant strains of mice positively correlate with acquisition in the Morris water maze (Kempermann and Gage, 2002a). Furthermore, declining hippocampal neurogenesis with advancing age has been linked to the emergence of a variety of memory deficits (Aizawa et al., 2009; Kim et al., 2010; Montaron et al., 2006; van Praag et al., 2005). However, the age-related decline in neurogenesis significantly precedes the onset of cognitive impairment, arguing against a straightforward relationship between aging, memory impairment, and lowered neurogenesis.

Experimentally augmenting adult hippocampal neurogenesis correlates with improved hippocampus-dependent learning in many instances. For example, subjecting unaltered young and aged animals to neurogenesis enhancing manipulations including environmental enrichment, physical activity, or proneurogenic drug treatment, and subsequently allowing sufficient time for these adult-generated DGCs to mature, enhances spatial learning in the Morris water maze (Clark et al., 2008; Kempermann et al., 1998a; Kempermann et al., 2002; Kempermann et al., 1997b, 1998b; Kobilo et al., 2011; Koo et al., 2003; Li et al., 2011; Nilsson et al., 1999; Pieper et al., 2010; van Praag et al., 1999a), Y-maze (Van der Borght et al., 2007), and radial arm maze (Diederich et al., 2009). One report also demonstrates increased contextual fear memory after running-enhanced neurogenesis (Clark et al., 2008). Recent studies using genetic knockout means of increasing neurogenesis have revealed similar findings (Li et al., 2011; Sahay et al., 2011a). For example, a conditional knockout mouse in which the pro-apoptotic gene Bax is ablated by a tamoxifen-inducible system, specifically in cells carrying the Nestin promoter, has been used to selectively increase the survival of newly-generated DGCs in adulthood (Sahay et
al., 2011a). Knockout animals performed better in a hippocampus-dependent contextual fear-discrimination task (McHugh et al., 2007) where animals had to discriminate between 2 highly similar shock and non-shock paired contexts. However, these studies cannot exclude the possibility that other consequences of experimental manipulations lead to behavioral improvements rather than increased neurogenesis. For instance, blocking the environmental enrichment-induced increase in neurogenesis did not prevent improved water maze performance following enrichment (Meshi et al., 2006). A stronger link between neurogenesis and learning was demonstrated for a non-spatial novel object recognition task (Bruel-Jungerman et al., 2005). Here, blocking the environmental enrichment-induced increase in neurogenesis, while maintaining baseline levels, prevented the improvement in novel object recognition.

Many studies have sought a causal link by examining the effects of knocking down adult hippocampal neurogenesis (Table 2-2A). A variety of non-genetic means of neurogenesis suppression lead to hippocampus-dependent memory impairments, which in some cases can be rescued by neurogenesis-promoting manipulations. Treatments that down-regulate neurogenesis, including the use of stress, social isolation, perinatal inflammation, constant light exposure, brain irradiation, forebrain cholinergic lesioning, vitamin A deficiency, and toxins (including lead, isoflurane, NMDAR antagonists, temozolamide (TMZ), methotrexate, and methylazoxymethanol (MAM)), have impaired spatial learning in behavioral paradigms that include the Morris water maze, Barnes maze, radial arm maze, Y-maze, and place recognition tasks (Bonnet et al., 2008; Fan et al., 2007; Fujioka et al., 2011; Garthe et al., 2009; Goodman et al., 2010; Hu et al., 2009; Ibi et al., 2008; Lemaire et al., 2000; Lyons et al., 2010; Madsen et al., 2003; Mohapel et al., 2005; Raber et al., 2004; Rola et al., 2004; Snyder et al., 2005; Veena et al., 2009; Wojtowicz et al., 2008). Similar results are reported for other forms of hippocampus-dependent learning, including contextual fear conditioning (Hernandez-Rabaza et al., 2009; Jaako-Movits and Zharkovsky, 2005; Jaako-Movits et al., 2005; Ko et al., 2009; Saxe et al., 2006; Snyder et al., 2009a; Warner-Schmidt et al., 2008; Winocur et al., 2006; Wojtowicz et al., 2008; Yun et al., 2010), novel object recognition (Graciarena et al., 2010), delayed nonmatching-to-sample and place (Clelland et al., 2009; Winocur et al., 2006), touchscreen 2-point discrimination (Clelland et al., 2009), visual discrimination (Winocur et al., 2011), and trace conditioning (Achanta et al., 2009; Shors et al., 2001; Shors et al., 2002). In some cases, these behavioral deficits have been subsequently ameliorated by restoring neurogenesis rates through exercise, environmental
enrichment, ECS, adrenalectomy, selective serotonin reuptake inhibitors, or reversal of the neurogenesis-suppressing treatment (Bonnet et al., 2008; Fan et al., 2007; Graciarena et al., 2010; Ibi et al., 2008; Kim et al., 2010; Lyons et al., 2010; Montaron et al., 2006; van Praag et al., 2005; Veena et al., 2009; Warner-Schmidt et al., 2008; Winocur et al., 2011).
Table 2-2. Studies Ablating Neurogenesis and Examining Learning and Memory.
(A) Studies showing effect.

<table>
<thead>
<tr>
<th>Behavioral tasks</th>
<th>Species</th>
<th>Strain</th>
<th>Sex</th>
<th>Ablation method</th>
<th>Animal age at ablation</th>
<th>Timing of behavioral test</th>
<th>Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morris Water Maze</td>
<td>mouse</td>
<td>Nestin-HSV-TK</td>
<td>B</td>
<td>genetic, GCV induced</td>
<td>n/a, adult</td>
<td>&gt; 4 w post GCV</td>
<td>no effects on acquisition, deficit in retention</td>
<td>Blaiss et al., 2011</td>
</tr>
<tr>
<td>hidden pf 4 trials/d x 11 d</td>
<td>rat</td>
<td>Wistar</td>
<td>M</td>
<td>vitamin A-free diet x 13 w</td>
<td>3 w</td>
<td>during last w of diet</td>
<td>deficits in acquisition &amp; retention</td>
<td>Bonnet et al., 2008</td>
</tr>
<tr>
<td>hidden pf 4 trials/d x 7 d</td>
<td>mouse</td>
<td>Nestin-tk</td>
<td>B</td>
<td>genetic, GCV induced</td>
<td>8 w</td>
<td>1, 3, 5, or 9 w(s) post GCV</td>
<td>no effects on acquisition &amp; short term retention, deficits in long-term retention &amp; extinction for 1 w only</td>
<td>Deng et al., 2009</td>
</tr>
<tr>
<td>visible then hidden pf 3 trials/d x 9 d</td>
<td>mouse</td>
<td>Nestin-rTA; TRE-Bax</td>
<td>M</td>
<td>genetic, Dox induced</td>
<td>2 m</td>
<td>6 or &gt; w post Dox</td>
<td>deficits in acquisition &amp; memory retention</td>
<td>Dupret et al., 2008</td>
</tr>
<tr>
<td>visible then hidden pf 6 trials/d x 5 d</td>
<td>gerbil</td>
<td>Mongolian</td>
<td>M</td>
<td>irradiation</td>
<td>2 m</td>
<td>13 w post irradiation</td>
<td>deficits in acquisition &amp; retention</td>
<td>Fan et al., 2007</td>
</tr>
<tr>
<td>hidden pf 6 trials/d x 3 d, then 2 reversals 6 trials/d x 2 d each</td>
<td>mouse</td>
<td>Nestin-rTA; TRE-PC3</td>
<td>B</td>
<td>genetic, Dox induced</td>
<td>30 or 138 d</td>
<td>65 or 22 d post Dox</td>
<td>deficits in acquisition, reversal learning &amp; retention</td>
<td>Farioli-Vacchioli et al., 2008</td>
</tr>
<tr>
<td>hidden pf 3 trials/d x 5 d</td>
<td>mouse</td>
<td>C57BL/6/J</td>
<td>M</td>
<td>constant light exposure x 3 w</td>
<td>8-10 w</td>
<td>1 d post light</td>
<td>deficits in acquisition</td>
<td>Fujikawa et al., 2011</td>
</tr>
<tr>
<td>hidden pf 6 trials/d x 3 d, then reversal 6 trials/d x 2 d</td>
<td>mouse</td>
<td>C57BL/6</td>
<td>F</td>
<td>TMZ treatment x 4 w</td>
<td>6-8 w</td>
<td>4 w post TM</td>
<td>deficits in acquisition, learning strategy choice during acquisition &amp; reversal, &amp; retention of reversal</td>
<td>Garthe et al., 2009</td>
</tr>
<tr>
<td>visible then hidden pf 12 trials x 1 d</td>
<td>mouse</td>
<td>C57BL/6/J</td>
<td>M</td>
<td>MAM treatment x 14 d</td>
<td>9 w</td>
<td>1 d post MAM</td>
<td>no effect on acquisition, deficits in short- &amp; long-term retention</td>
<td>Goodman et al., 2010</td>
</tr>
<tr>
<td>visible &amp; hidden pf, details n/a</td>
<td>mouse</td>
<td>C57BL/6/J</td>
<td>M</td>
<td>NVP-AAM077 treatment x 2 d</td>
<td>6-7 w</td>
<td>1 &amp; 29 d post NVP-AAM077</td>
<td>no effects on acquisition initially, deficits in delayed re-acquisition &amp; retention</td>
<td>Hu et al., 2009</td>
</tr>
<tr>
<td>hidden pf trials n/a x 6 d</td>
<td>mouse</td>
<td>ICR</td>
<td>M</td>
<td>social isolation x 5 w</td>
<td>3 w</td>
<td>during last w of social isolation</td>
<td>deficits in acquisition &amp; memory retention</td>
<td>Ibi et al., 2008</td>
</tr>
<tr>
<td>hidden pf 4 trials/d x 7 d, then reversal</td>
<td>rat</td>
<td>Sprague-Dawley</td>
<td>B</td>
<td>lentivirus-dnWnt</td>
<td>7-8 w</td>
<td>8-9 w post virus injection</td>
<td>no effects on acquisition, short term retention or reversal learning, deficit in long-term retention</td>
<td>Jessberger et al., 2009</td>
</tr>
<tr>
<td>hidden pf 4 trials/d x 5 d</td>
<td>rat</td>
<td>Sprague-Dawley</td>
<td>B</td>
<td>prenatal stress in last w of gestation</td>
<td>sometime post E15</td>
<td>4 m post stress</td>
<td>deficits in acquisition</td>
<td>Lemaire et al., 2000</td>
</tr>
<tr>
<td>hidden pf 4 trials/d x 7 d</td>
<td>rat</td>
<td>Sprague-Dawley</td>
<td>F</td>
<td>saporin lesion of basal forebrain</td>
<td>12-14 w</td>
<td>3-4 w post lesion</td>
<td>deficits in acquisition &amp; memory retention</td>
<td>Mohapel et al., 2005</td>
</tr>
<tr>
<td>hidden pf 6 trials/d x 3 d</td>
<td>mouse</td>
<td>C57BL/6/J</td>
<td>M</td>
<td>irradiation</td>
<td>21 d</td>
<td>3 m post irradiation</td>
<td>no effects on acquisition, deficit in short-term retention</td>
<td>Rola et al., 2004</td>
</tr>
<tr>
<td>hidden pf 2 trials/d x 7 d</td>
<td>mouse</td>
<td>Nestin-Cre NT3</td>
<td>M</td>
<td>genetic knockout</td>
<td>constitutive</td>
<td>2-4 m of age</td>
<td>deficits in acquisition &amp; memory retention</td>
<td>Shimizu et al., 2006</td>
</tr>
<tr>
<td>hidden pf 8 trials/d x 6 d</td>
<td>rat</td>
<td>Long Evans</td>
<td>M</td>
<td>irradiation</td>
<td>40 d</td>
<td>2 d before, 3-4 d after, or 4 w post irradiation</td>
<td>no effects on acquisition or short-term retention, deficit in retention 4 w later only</td>
<td>Synder et al., 2005</td>
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<tr>
<td>hidden pf 4 trials/d x 6 d</td>
<td>mouse</td>
<td>Liver IGF-1</td>
<td>M</td>
<td>genetic knockout</td>
<td>constitutive</td>
<td>3-4 m of age</td>
<td>deficits in acquisition &amp; memory retention</td>
<td>Trejo et al., 2008</td>
</tr>
<tr>
<td>hidden pf 4 trials/d x 5 d</td>
<td>rat</td>
<td>Long Evans</td>
<td>M</td>
<td>irradiation</td>
<td>4.5 m</td>
<td>3 m post irradiation</td>
<td>no effects on acquisition or short-term retention, deficits in reversal learning</td>
<td>Wojtowicz et al., 2008</td>
</tr>
<tr>
<td>hidden pf 4 trials/d x 7 d, then reversal</td>
<td>mouse</td>
<td>Tlxfl/fl; CMV-CreER</td>
<td>B</td>
<td>genetic, TM induced</td>
<td>8 w</td>
<td>4 w post TM</td>
<td>deficits in acquisition, reversal learning &amp; retention</td>
<td>Zhang et al., 2008</td>
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<td>hidden pf 4 trials/d x 9 d</td>
<td>mouse</td>
<td>MBD1</td>
<td>M</td>
<td>genetic knockout</td>
<td>constitutive</td>
<td>2-5 m of age</td>
<td>deficits in acquisition &amp; memory retention</td>
<td>Zhao et al., 2003</td>
</tr>
<tr>
<td>Behavioral tasks</td>
<td>Species</td>
<td>Strain</td>
<td>Sex</td>
<td>Ablation method</td>
<td>Animal age at ablation</td>
<td>Timing of behavioral test</td>
<td>Phenotype</td>
<td>References</td>
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<tr>
<td>Morris Water Maze CONT'D</td>
<td>hidden pf 8 trials/d x 4 d</td>
<td>mouse</td>
<td>Nestin-Cre FGFRI1</td>
<td>B</td>
<td>genetic knockout</td>
<td>constitutive</td>
<td>6-8 w of age</td>
<td>deficits in acquisition with moving pf, not stationary</td>
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<tr>
<td>Barnes Maze</td>
<td>spatial version</td>
<td>mouse</td>
<td>Nes-CreER, NSE-DTA</td>
<td>M</td>
<td>genetic, TM induced</td>
<td>8 w</td>
<td>41 ds post TM</td>
<td>deficits in acquisition &amp; long-term retention, no effect on short-term retention</td>
</tr>
<tr>
<td></td>
<td>spatial then visual version</td>
<td>mouse</td>
<td>C57BL/6J</td>
<td>M</td>
<td>irradiation</td>
<td>2 m</td>
<td>3 m post irradiation</td>
<td>deficits on spatial version &amp; searching strategies, but not in visual version</td>
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<td>Contextual Fear Conditioning</td>
<td>1 shock</td>
<td>mouse</td>
<td>Nestin-tk</td>
<td>B</td>
<td>genetic, GCV induced</td>
<td>8 w</td>
<td>1 or 5 w post GCV</td>
<td>no effect on context conditioning, deficits in extinction x 5 w only</td>
</tr>
<tr>
<td></td>
<td>1 tone-shock pair</td>
<td>mouse</td>
<td>Nestin-tTA; TRE-PC3</td>
<td>B</td>
<td>genetic, Dox induced</td>
<td>30 or 138 d</td>
<td>65 or 22 d post Dox</td>
<td>deficits in freezing</td>
</tr>
<tr>
<td></td>
<td>1 unsignalled shock</td>
<td>mouse</td>
<td>PC3/Tis21</td>
<td>M</td>
<td>genetic knockout</td>
<td>constitutive</td>
<td>2 m of age</td>
<td>deficits in freezing</td>
</tr>
<tr>
<td></td>
<td>1 unsignalled shock</td>
<td>rat</td>
<td>Long Evans</td>
<td>M</td>
<td>irradiation</td>
<td>9-11 w</td>
<td>9 w post irradiation</td>
<td>deficits in freezing</td>
</tr>
<tr>
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<td>3 tone-shock pairs</td>
<td>mouse</td>
<td>Nes-CreER, NSE-DTA</td>
<td>M</td>
<td>genetic, TM induced</td>
<td>8 w</td>
<td>59 d post TM</td>
<td>deficits in freezing</td>
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<td>Wistar</td>
<td>M</td>
<td>olfactory bulbectomy</td>
<td>2 m</td>
<td>6 w post surgery</td>
<td>deficits in freezing</td>
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<td>3 unsignaled shocks</td>
<td>rat</td>
<td>Wistar</td>
<td>M</td>
<td>lead treatment x 30 d</td>
<td>1 d</td>
<td>50 d post lead treatment</td>
<td>deficits in freezing</td>
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<tr>
<td></td>
<td>3 tone-shock pairs</td>
<td>mouse</td>
<td>C57BL/6</td>
<td>M</td>
<td>irradiation</td>
<td>5 or 8 w</td>
<td>5 w or 11 d post irradiation</td>
<td>no effect on context conditioning, but prolonged hippocampus-dependency of memory</td>
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<tr>
<td></td>
<td>1 unsignalled shock</td>
<td>mouse</td>
<td>C57BL/6</td>
<td>M</td>
<td>irradiation</td>
<td>7-10 w</td>
<td>3 m post irradiation</td>
<td>deficits in contextual conditioning, no effects on extinction</td>
</tr>
<tr>
<td></td>
<td>3 tone-shock pairs</td>
<td>mouse</td>
<td>129/SvEv</td>
<td>M</td>
<td>irradiation</td>
<td>12-25 w</td>
<td>3 m post irradiation</td>
<td>deficits in freezing</td>
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<td>GFAP-tk</td>
<td>M</td>
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<td>12-20 w</td>
<td>after 6-w GCV treatment</td>
<td>deficits in freezing</td>
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<td>mouse</td>
<td>FoxG1</td>
<td>B</td>
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<td>constitutive</td>
<td>12-14 w of age</td>
<td>deficits in freezing</td>
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<tr>
<td></td>
<td>7 tone-shock pairs</td>
<td>rat</td>
<td>Sprague-Dawley</td>
<td>M</td>
<td>irradiation</td>
<td>8-9 w</td>
<td>3, 4, or 8 w post irradiation</td>
<td>deficits in freezing x 4 &amp; 8 w only</td>
</tr>
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<td></td>
<td>3 tone-shock pairs</td>
<td>rat</td>
<td>Sprague-Dawley</td>
<td>M</td>
<td>irradiation</td>
<td>n/a, adult</td>
<td>6 w post irradiation</td>
<td>deficits in freezing</td>
</tr>
<tr>
<td></td>
<td>10 tone-shock pairs</td>
<td>rat</td>
<td>Long Evans</td>
<td>M</td>
<td>irradiation</td>
<td>4 m</td>
<td>4 w post irradiation</td>
<td>deficits in freezing</td>
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<tr>
<td></td>
<td>7 tone-shock pairs</td>
<td>rat</td>
<td>Long Evans</td>
<td>M</td>
<td>irradiation</td>
<td>4.5 m</td>
<td>5 w post irradiation</td>
<td>deficits in freezing</td>
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<tr>
<td></td>
<td>1 tone-shock pair</td>
<td>mouse</td>
<td>ICR</td>
<td>M</td>
<td>restraint stress x 4 w</td>
<td>3 w</td>
<td>1 d post stress</td>
<td>deficits in freezing</td>
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<tr>
<td>Behavioral tasks</td>
<td>Species</td>
<td>Strain</td>
<td>Sex</td>
<td>Ablation method</td>
<td>Timing of behavioral test</td>
<td>Animal age</td>
<td>Phenotype</td>
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<tr>
<td>Trace Fear Conditioning</td>
<td>rat</td>
<td>Sprague-Dawley</td>
<td>M</td>
<td>irradiation</td>
<td>90 d post irradiation</td>
<td>3/3</td>
<td>deficits in freezing</td>
<td>Achanta et al., 2009</td>
</tr>
<tr>
<td>Trace Fear Conditioning</td>
<td>rat</td>
<td>Sprague-Dawley</td>
<td>M</td>
<td>MAM treatment x 14 d</td>
<td>1 d post MAM</td>
<td>n/a, adult</td>
<td>deficits in mobility</td>
<td>Shors et al., 2001</td>
</tr>
<tr>
<td>Eye Blinking Conditioning</td>
<td>rat</td>
<td>Sprague-Dawley</td>
<td>M</td>
<td>MAM treatment x 6 or 14 d</td>
<td>2 d post 6 d, or 2 or 21 d</td>
<td>n/a, adult</td>
<td>deficits in acquisition of conditioned response</td>
<td>Shors et al., 2002</td>
</tr>
<tr>
<td>Working Memory</td>
<td>mouse</td>
<td>C57BL/6J</td>
<td>F</td>
<td>genetic, Dox induced</td>
<td>3-4 m of age</td>
<td>30-36 d post birth</td>
<td>deficits in working memory</td>
<td>Clelland et al., 2009</td>
</tr>
<tr>
<td>Working Memory</td>
<td>mouse</td>
<td>C57BL/6J</td>
<td>F</td>
<td>lentivirus-dnWnt</td>
<td>3-4 m of age</td>
<td>8 w post virus injection</td>
<td>deficits in working memory</td>
<td>Clelland et al., 2009</td>
</tr>
<tr>
<td>Working Memory</td>
<td>rat</td>
<td>Wistar</td>
<td>M</td>
<td>irradiation</td>
<td>2-2.5 m</td>
<td>4 w post irradiation</td>
<td>deficits in working memory</td>
<td>Clelland et al., 2009</td>
</tr>
<tr>
<td>Object Recognition Memory</td>
<td>rat</td>
<td>Sprague-Dawley</td>
<td>M</td>
<td>MAM treatment x 14 d</td>
<td>1 d post MAM</td>
<td>n/a, adult</td>
<td>deficits in acquisition &amp; reference errors</td>
<td>Shors et al., 2002</td>
</tr>
<tr>
<td>Place Recognition Memory</td>
<td>rat</td>
<td>Wistar</td>
<td>M</td>
<td>irradiation</td>
<td>2-2.5 m</td>
<td>21 d post irradiation</td>
<td>deficits in exploring novel arm</td>
<td>Madsen et al., 2003</td>
</tr>
<tr>
<td>Spatial Discrimination</td>
<td>mouse</td>
<td>C57BL/6</td>
<td>F</td>
<td>irradiation</td>
<td>8 w</td>
<td>8 w post irradiation</td>
<td>deficits in proximal but not distal spatial discrimination</td>
<td>Celland et al., 2009</td>
</tr>
<tr>
<td>Spatial Discrimination</td>
<td>mouse</td>
<td>C57BL/6</td>
<td>F</td>
<td>lentivirus-dnWnt</td>
<td>8 w</td>
<td>8 w post viral injection</td>
<td>deficits in proximal but not distal spatial discrimination</td>
<td>Celland et al., 2009</td>
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</table>
(B) Studies showing no effect.

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**Abbreviations:**

- B: Both Male and Female
- D2: Cyclin D2
- DMP: Delayed Matching to Place
- DNMP: Delayed Non-Matching to Place
- DNMS: Delayed Non-Match to Sample
- Dox: Doxycycline
- DTA: Diphtheria Toxin fragment A
- E: Embryonic Day
- FGFR1: Fibroblast growth factor receptor protein 1
- GCV: Ganciclovir
- GFAP: Glial Fibrillary Acidic Protein
- LPS: Lipopolysaccharide
- M: Male
- MAM: Methylazoxymethanol acetate
- MBD1: methyl-CpG binding protein 1
- MTP: Matching to Place
- MTX: Methotrexate
- MWM: Morris Water Maze
- n/a: Not Available
- NMTP: Non-Matching to Place
- NVP-AAM077: NR2A antagonist
- NSE: Neuronal Specific Enolase 2
- P: Postnatal Day
- pf: Platform
- TM: Tamoxifen
- TMZ: Temozolomide
- TRE: Tetracycline Responsive Elements
Genetic methods of disrupting adult hippocampal neurogenesis generate similar behavioral impairments to those induced by non-genetic techniques (Table 2-2A). In theory, genetic manipulations can lead to a more selective effect on neurogenesis, thereby lessening the possibility for confounding links between neurogenic rates and memory function. For example, stereotactic DG infections with dominant-negative Wnt expressing lentivirus disrupts the Wnt signaling pathway specifically in the DG, reduces neurogenesis, and impairs water maze, delayed nonmatching-to-place, and object recognition learning (Clelland et al., 2009; Jessberger et al., 2009). Several Tg knockout mouse lines with constitutive genomic alterations in transcription factors, epigenetic regulators, and growth factors exhibit diminished adult hippocampal neurogenesis and deficits in spatial (Denis-Donini et al., 2008; Trejo et al., 2008; Zhao et al., 2003) and contextual fear (Farioli-Vecchioli et al., 2009; Shen et al., 2006) memory. Using genetic recombination under the control of the Nestin promoter to specifically target genes involved in brain development, FGF receptor 1 (Fgfr1) or Neurotrophin 3 (Ntf3) knockout impedes adult hippocampal neurogenesis, partially alters DG LTP and impairs spatial memory (Shimazu et al., 2006; Zhao et al., 2007).

Inducible, and in some cases reversible, genetic techniques of specific neurogenesis suppression are the latest tools available to tease out functional contributions for adult-generated DGCs in learning and memory (Table 2-2). In principle, these approaches retain many of the benefits of other techniques, but additionally offer 3 advantages: 1) animals develop undisturbed until a desired age, attaining normal adult neural anatomy and function without potentially generating compensatory mechanisms during development, 2) DG neurogenesis can be suppressed with cell-type specificity at a desired age and/or time point relative to memory acquisition, retention, or recall, and 3) in some systems, this effect is reversible such that the potential for rescue of function can be examined.

A variety of Tg mice that enable inducible neurogenesis ablation have been examined, including those that exploit the expression of Herpes simplex virus-thymidine kinase (HSV-TK), Bax protein, Pheochromocytoma cell 3 transgene (Pc3), Diphtheria toxin, and which knockout a key mediator of neural stem cell maintenance. Mice that express HSV-TK under the control of cell-type specific promoters can be treated with the antiviral ganciclovir, eliminating dividing TK+ cells (Garcia et al., 2004). Saxe et al. utilized HSV-TK under GFAP promoter control such that GFAP+ progenitor cells were ablated in adulthood upon drug administration (Saxe et al., 2006).
Following treatment, freezing was significantly reduced in contextual fear conditioning. In another Tg mouse line, HSV-TK is expressed under Nestin promoter control in the brain only (Deng et al., 2009). Here, ablation of dividing TK+ cells ceases upon drug withdrawal, restoring adult hippocampal neurogenesis. Using this method, a selective partial removal of adult-generated DGCs that would have been 1-4 weeks old during water maze training impaired memory retention during subsequent testing (replicated more recently using analogous Nestin-HSV-TK mice (Blaiss et al., 2011)). This memory deficit could not be explained by enhanced extinction. Furthermore, the fact that the memory impairment withstood reversal of the neurogenesis suppression specifically points to the importance of newly-generated DGCs during spatial learning.

Bax and TgPC3 mice over-express the pro-apoptotic Bax protein (Dupret et al., 2008) or pro-neuronal differentiation PC3 protein (Farioli-Veccioi et al., 2008) respectively, using a transcription factor that is activated by exogenous tetracycline analogues. The transcription factor itself is expressed under Nestin promoter control, thus enabling relatively specific adult hippocampal neurogenesis ablation (Bax expression) or altered maturation (Pc3 expression) with drug exposure. Upon induction in adulthood, these animals exhibit deficits in hippocampus-dependent water maze learning (Dupret et al., 2008; Farioli-Veccioi et al., 2008), as well as radial arm maze and contextual fear learning (Farioli-Veccioi et al., 2008).

Tg mice that express diphtheria toxin in newly generated neurons following tamoxifen exposure can be generated by crossing Nestin promoter-controlled inducible Cre-recombinase mice with those carrying a reversibly silenced diphtheria toxin gene coupled to a neuron-specific promoter (Imayoshi et al., 2008). In these animals, neurogenesis ablation resulted in impairments of both Barnes maze performance and contextual fear conditioning. The tamoxifen inducible Cre-recombinase system was also utilized in another study to knockout expression of the orphan nuclear receptor TLX from the adult neural stem cell pool (Zhang et al., 2008). TLX is known to maintain the proliferative capacity of neural stem cells and is critical for normal neurologic development (Shi et al., 2004). Defective adult neurogenesis in these animals impaired spatial learning in the Morris water maze.
2.3.4.1 Inconsistencies in the Literature

Despite the considerable mass of studies supporting an important role for adult-generated DGCs in hippocampus-dependent learning and memory, there are many conflicting data (Table 2-2A,B). While these discrepancies signal the need for cautious interpretation of data and remind us to avoid the trappings of dogma, careful examination of these studies underscores several important technical considerations.

The false attribution of behavioral phenotypes to neurogenesis suppression can arise due to several confounding factors. The specificity of neurogenesis knockdown as well as possible developmental consequences of treatment in early life are potentially important alternative factors in determining behavioral phenotypes. For instance, irradiation interferes with cell cycle proteins and instigates DNA damage, preferentially targeting proliferating cells and thus depleting new neurons in a dose-dependent fashion (Wojtowicz, 2006). The effects of radiation, however, are not neurogenesis-specific (Leuner et al., 2006a), and include an inflammatory response days-weeks (Noonan et al., 2010; Rola et al., 2004; Tan et al., 2011) and months (Monje et al., 2002; Monje et al., 2003; Rola et al., 2004; Rosi et al., 2008) following treatment which might impact memory performance. Some evidence also suggests that contextual fear memory deficits are only seen when a sufficiently high dose of radiation is used (Ko et al., 2009). This could indicate the necessity for a profound decline in neurogenesis to generate a behavioral effect; however, it could also reflect increased nonspecific toxicity associated with higher radiation exposure. Analogous side effect and dose-response concerns have been raised for other anti-neurogenic treatments, such as MAM (Dupret et al., 2005), as well as for viral vector-mediated genetic manipulations (Jessberger et al., 2009; Thomas et al., 2003). Lastly, Tg animals inherently carry a risk for developmental abnormalities, including subtle differences in brain maturation, leading to unique susceptibilities to neurologic impairments (Breunig et al., 2008).

While each individual method of suppressing neurogenesis has its own set of drawbacks, the fact that suppressing neurogenesis using such a wide array of methods produces similar phenotypic impairments provides considerable support for an important role for adult-generated DGCs in hippocampus-dependent memories (Table 2-2A). In spite of this, many studies utilizing similar techniques do not demonstrate a critical role for adult hippocampal neurogenesis in some
hippocampus-dependent learning paradigms (Table 2-2B; (Achanta et al., 2009; Blaiss et al., 2011; Bruel-Jungerman et al., 2005; Clark et al., 2008; Clelland et al., 2009; Denis-Donini et al., 2008; Dupret et al., 2008; Farioli-Vecchioli et al., 2009; Goodman et al., 2010; Hernandez-Rabaza et al., 2009; Jaholkowski et al., 2009; Ko et al., 2009; Madsen et al., 2003; Meshi et al., 2006; Raber et al., 2004; Rola et al., 2004; Saxe et al., 2006; Saxe et al., 2007; Shors et al., 2002; Snyder et al., 2009a; Zhang et al., 2008)). These studies could, of course, show that neurogenesis is not critical to these tasks; however, the volume of work indicating its importance suggests otherwise. Several factors could diminish the likelihood of detecting a phenotypic effect of suppressing neurogenesis, including differences between species and strains, compensatory mechanisms following neurogenesis suppression, variations in behavioral testing protocols, and the age of new neurons at training/testing.

First, it is possible that in the minority of cases, discordant results could be obtained between studies using separate species and strains. These different animals can exhibit distinct baseline rates of adult-generated DGC production and maturation, behavioral phenotypes in hippocampus-dependent learning and memory paradigms, and fundamental differences in the patterns and extent of adult born DGC integration into memory networks (Cameron et al., 1993; Epp et al., 2011b; Hayes and Nowakowski, 2002; Kempermann and Gage, 2002b; Schauwecker, 2006; Snyder et al., 2009a).

Second, a more significant reason that some studies suppressing neurogenesis do not reveal behavioral effects relates to compensatory mechanisms. Virtually all studies knocking down neurogenesis do so prior to training (Table 2-2A,B), creating a potential opportunity for remaining adult- or developmentally-generated DGCs to compensate for this loss. Residual adult-generated DGCs following incomplete knockdown techniques might adequately fulfill functional requirements (Dupret et al., 2005; Jessberger et al., 2009; Ko et al., 2009). This could explain why the profound age-related decline in neurogenesis precedes the appearance of cognitive deficits. In addition, mature adult-generated DGCs exhibit similar neuronal morphology, electrophysiological properties, and afferent and efferent connectivity as developmentally-generated DGCs (Ge et al., 2007; Laplagne et al., 2006; Laplagne et al., 2007; Toni et al., 2008; Toni et al., 2007; Zhao et al., 2006); accordingly, this phenotypic convergence may enable developmentally-generated DGCs to assume the learning function of their missing adult-generated counterparts. One strategy to test whether compensation occurs when adult-
generated DGCs are removed is to lower their functional, rather than physical, presence. For instance, Farioli-Vechioli et al. accelerated the maturational process of immature adult-generated DGCs by conditional expression of a pro-neuronal-differentiation gene in progenitor cells (Farioli-Vechioli et al., 2008). These animals had selectively reduced numbers of immature neurons, and normal numbers but altered morphologies of mature adult-generated neurons. These changes were accompanied by spatial and contextual fear memory deficits for both new and previously learned information, reduced DG LTP, and less testing-induced activation of adult-generated DGCs. One interpretation of these results is that the continued presence of functionally-impaired adult-generated DGCs prevented the initiation of compensatory processes by other DGCs and thus led to the observed phenotype. A more definitive test of whether adult-generated DGCs contribute to learning and memory would be to have animals learn during a period of normal neurogenesis, subsequently eliminate those newly born cells that should have been recruited into hippocampal memory networks, and then look for impaired recall.

Third, protocol variations for hippocampus-dependent memory tasks between studies could significantly influence results by potentially altering underlying neural mechanisms and the performance demands placed on animals. For instance, task difficulty can influence hippocampal dependency, as demonstrated by classical conditioning paradigms becoming hippocampus-dependent only once rendered sufficiently difficult by an extended interstimulus interval (Beylin et al., 2001). It is also possible that hippocampus-dependent tasks must be sufficiently difficult in order to detect subtle differences in performance. Given the relatively small proportional contribution of adult-generated DGCs to hippocampus-dependent memory networks (Kee et al., 2007b; Snyder et al., 2009a; Tashiro et al., 2007; Trouche et al., 2009), it is reasonable to expect that suppressing neurogenesis might only translate into detectable phenotypes when animals are subjected to tasks with sufficiently high hippocampal performance demands. For instance, suppressing neurogenesis may reduce contextual fear conditioning-induced freezing only when brief training is provided (Drew et al., 2010). This is also supported by the fact that Morris water maze acquisition deteriorated with reduced neurogenesis when a moving, rather than fixed, hidden platform location was used in otherwise identical protocols (Zhao et al., 2007), and improved following exercise-induced enhanced neurogenesis only when animals received a small number of daily trials (van Praag et al., 1999a), possibly relating to
greater task difficulty. Clearly, relationships and interactions between neurogenesis, task
difficulty, and hippocampal dependency require further clarification (Shors et al., 2002).

Fourth, the age of removed adult-generated DGCs during behavioral testing could impact the
likelihood for detecting, and the nature of, phenotypic consequences. Given the aforementioned
developmental timeline of adult-generated DGCs, it is unlikely that cells merely days old could
functionally participate in local networks supporting memory. In contrast, as several week-old
adult-generated DGCs approach full maturity, participation resembling that of mature
developmentally-generated DGCs is relatively likely. Inconsistent results between these time-
points (Table 2-2A,B) could, in part, reflect variability in the age of deleted new neurons at the
time of behavioral training/testing (Deng et al., 2010). Indeed, it is likely that painstaking highly-
resolved and carefully controlled experiments to delineate an evolving effect of knocking out
differentially-aged adult-generated cells are needed to better understand this issue.

2.3.5 Cognitive Role in Learning and Memory

Despite evidence indicating adult-generated DGCs are involved in learning and memory,
precisely how they contribute to cognitive processes remains elusive. This is due, in part, to
several issues. First, our evolving biological understanding of adult neurogenesis creates a
moving target for theorists. For instance, recent evidence indicates that adult hippocampal
neurogenesis primarily leads to a net increase in the DGC population over time (Imayoshi et al.,
2008); however, older studies pointed to a greater role in DGC replacement and thus promoted
theories emphasizing neural turnover (Dayer et al., 2003; Lledo et al., 2006). Second, different
theories are not necessarily mutually exclusive, and it is possible that adult-generated neurons, or
subsets of them, contribute to multiple, possibly even opposing, cognitive processes during
different periods of their development (Leuner et al., 2006a). Finally, our incomplete
understanding of hippocampal function also hampers attempts to propose functional roles for
new neurons (Kempermann, 2002). General consensus posits that the hippocampal formation
initially encodes episodic memories regarding context-specific information, and spatial
memories involving environmental relations and orientations. However, the roles of mesial
temporal lobe structures in memory, and their interaction with other brain regions, continue to be debated (Frankland and Bontempi, 2005; Moscovitch et al., 2006; Squire and Zola-Morgan, 1991; Winocur et al., 2010).

Proposed roles, based variably on psychological principles and computational models, include the following: increasing the capacity for information processing, providing memory storage, clearing memory traces, associating memories, and discriminating between similar patterns (for an in depth discussion, including relevant references, please see Appendix A1).

2.3.5.1 Functional Significance of Adult- vs. Developmentally-Generated Dentate Granule Cells

Understanding whether adult- and developmentally-generated DGCs represent functionally distinct or convergent populations of granule cells is important to establishing how adult-generated neurons might contribute to learning and memory at a cognitive level. Indeed, this is a pivotal issue for some theories suggesting adult-generated DGCs are uniquely capable of performing certain cognitive tasks, such as encoding temporal associations in memory (Aimone et al., 2006). As previously discussed, this issue is also fundamental to understanding the functional implications of neurogenesis depletion and potential ensuing compensatory mechanisms. This is also relevant to examining the possibility that harnessing the regenerative capacity of the DGC population might lead to a replacement of diseased DGCs with phenotypically-convergent neurons capable of assuming their lost functions.

Previous attempts to address this question have done so by comparing the functional activation of thymidine-analogue labeled adult-generated DGCs to that of the NeuN+ DGC population as a whole (Kee et al., 2007b; Ramirez-Amaya et al., 2006; Tashiro et al., 2007). Using this indirect comparison, these studies have concluded that adult-generated DGCs are preferentially recruited to participate in hippocampal processing of spatial exploration and learning. However, a direct comparison between adult and developmental cell cohorts labeled with equivalent techniques, with extension to non-spatial hippocampus-dependent memory testing, is required to truly answer this question in a generalizable way. A portion of this thesis is devoted to such a comparison.
2.3.6 Adult Hippocampal Neurogenesis and Dementing Illnesses

Motivations for understanding the relationships between adult hippocampal neurogenesis and memory function partly stem from the growing unmet clinical need for cognitive-restoring therapies for neurodegenerative diseases. Limited and heterogeneous human studies suggest Alzheimer’s disease (AD) patients have increased hippocampal proliferation, variable immature neuron generation, but overall reduced neuronal maturation culminating in less neurogenesis (Crews et al., 2010; Jin et al., 2004b; Li et al., 2008). Interestingly, neurogenesis may remain intact at presenile stages (Boekhoorn et al., 2006; Crews et al., 2010). In AD model mice, similarly heterogeneous results range from increased (Ermini et al., 2008; Gan et al., 2008; Jin et al., 2004a; Lopez-Toledano and Shelanski, 2007), to normal (Choi et al., 2008; Donovan et al., 2006; Ermini et al., 2008; Feng et al., 2001; Gan et al., 2008; Lopez-Toledano and Shelanski, 2007), to reduced (Donovan et al., 2006; Ermini et al., 2008; Fiorentini et al., 2010; Haughey et al., 2002; Rodriguez et al., 2008; Valero et al., 2011; Verret et al., 2007; Wang et al., 2010; Wen et al., 2004) levels of baseline DG neurogenesis. In cases of deficient neurogenesis, studies also inconsistently implicate alterations in proliferation, neuronal differentiation, and/or survival. Aside from methodological reasons for these discrepant results, it is likely that different strain and Tg genetic backgrounds influence neurogenesis rates in differing and poorly understood ways (Winner et al., 2011). Neurogenic rates may also vary during different stages of disease progression, with many examples of baseline neurogenesis roughly matching that of wild type animals before symptom onset, followed by altered neurogenesis once disease pathology is evident (Donovan et al., 2006; Ermini et al., 2008; Gan et al., 2008; Rodriguez et al., 2008). For instance, as suggested in human studies, DG proliferation in mice overexpressing mutated amyloid precursor protein (APP) (containing the human familial AD Indiana mutation) is intact at 2 months of age, prior to symptom onset, but markedly reduced at 12 months of age, once symptoms are established (Donovan et al., 2006).

In addition to examining baseline neurogenesis in AD models, several studies also describe memory benefits following neurogenesis-enhancing treatments in these animals (Fiorentini et al., 2010; Valero et al., 2011; Wang et al., 2010; Wolf et al., 2006). Although, these reports suggest
Deficient neurogenesis is a key determinant of cognitive deficits, others suggest that the benefits of such interventions as environmental enrichment occur despite the absence of increased neurogenesis in certain models (Catlow et al., 2009). Once again, factors including disease status at the time of intervention/testing may critically influence results. For instance, a model of relatively severe disease with rapid deterioration failed to demonstrate enhanced neurogenesis or improved cognition following physical activity/environmental enrichment (Cotel et al., 2010), suggesting earlier disease states harbor the greatest chance for benefit from pro-neurogenic manipulations.

Deficient neurogenesis might also contribute to dementia associated other neurodegenerative diseases, such as late stage Parkinson’s disease (PD) and Huntington’s Disease (HD). One human study demonstrated reduced numbers of proliferating neural progenitor cells isolated from the SGZ of PD patients post-mortem, though the reduction did not correlate with the presence or absence of dementia (Hoglinger et al., 2004). In the laboratory, depleting dopaminergic input to the SGZ has been shown to reduce neurogenesis in rodents (Hoglinger et al., 2004). In addition, α-synuclein, a protein whose accumulation in brain is a key pathologic feature of PD (Lang and Lozano, 1998), impairs neurogenesis in vitro and in animal models (Crews et al., 2008). Several HD models also exhibit reduced neurogenesis (Gil et al., 2005; Lazic et al., 2004; Simpson et al., 2011), although verification in human patients is still lacking. Should emerging non-invasive techniques for visualizing neurogenesis in vivo (Manganas et al., 2007; Pereira et al., 2007) prove robust, they should greatly facilitate additional human studies examining hippocampal neurogenesis in diseases of cognitive impairment. Along with determining whether or not perturbations in neurogenesis underlie these diseases, it is important for future research efforts to examine whether this intrinsic neurogenic capability could lead to new concepts in restoring brain function. The fact that the adult brain can generate and integrate new neurons autonomously at least raises the possibility of exploiting this biology for hippocampal, or even brain, regenerative therapies.

2.3.6.1 Potential Therapeutic Implications: Deep Brain Stimulation

DBS, by delivering electrical current to targeted brain regions through surgically implanted electrodes, has provided remarkable therapeutic benefits for patients with several brain disorders
(Ponce and Lozano, 2010). Much of this success stems from established efficacy in the treatment of movement disorders, including PD (Davis et al., 1997; Deuschl et al., 2006), dystonia (Vidalhlet et al., 2005) and essential tremor (Koller et al., 1997); however, recent studies suggest this technique may also benefit certain patients with psychiatric diseases (Mallet et al., 2008; Mayberg et al., 2005; Shahed et al., 2007). In a new landmark trial of AD patients, limbic targeted DBS increased neurophysiological activity in several brain regions including entorhinal and hippocampal areas (Laxton et al., 2010). More importantly, preliminary outcome data suggests clinical benefits in cognitive function. While the mechanism(s) of action underlying these effects is(are) undetermined, an enticing possibility is that clinical DBS is capable of driving activity-dependent increased neurogenesis, analogous to that observed in rodent models, and that these new neurons functionally contribute to improved cognition. A portion of this thesis explores this possibility.
2.4 Thesis Aims and Hypotheses

The primary aim of this thesis is to examine the nature of focal brain stimulation-induced increased neurogenesis and to evaluate the functional significance of this both at the hippocampal network and behavioral levels in wild type and AD model mice. A secondary aim is to clarify the functional significance of adult-generated DGCs, shedding light on their capacity to assume roles of their developmentally-generated counterparts in memory processes. Broadly speaking, the content of this research has implications for the potential development, and improvement, of therapeutic strategies aimed at enhancing adult-generated DGC production for cognitive benefit.

The specific hypotheses tested are the following:

1) Similar proportions of developmentally- and adult-generated DGCs are activated following hippocampus-dependent memory recall in intact mice (see Chapter 3).

2) EC stimulation increases the production of adult-generated DGCs, and these stimulation-induced DGCs mature normally, demonstrate activation following hippocampus-dependent spatial memory recall, and facilitate spatial memory formation in intact mice (see Chapter 4).

3) EC stimulation increases DG cell proliferation in a mouse model of AD, and facilitates spatial memory formation (see Chapter 5).
Chapter 3  Functional Convergence of Developmentally- and Adult-Generated Granule Cells in Dentate Gyrus Circuits Supporting Hippocampus-Dependent Memory

This chapter is modified from the following:

3.1 Abstract

In the hippocampus, the production of dentate granule cells (DGCs) persists into adulthood. As adult-generated neurons are thought to contribute to hippocampal memory processing, promoting adult neurogenesis therefore offers the potential for restoring mnemonic function in the aged or diseased brain. Within this regenerative context, one key issue is whether developmentally- and adult-generated DGCs represent functionally-equivalent or distinct neuronal populations. To address this, we labeled separate cohorts of developmentally- and adult-generated DGCs and used immunohistochemical approaches to compare their activation by circuits supporting hippocampus-dependent memory in intact mice. First, in the water maze task, recruitment rates of adult-generated DGCs were regulated by maturation, with maximal participation not occurring until DGCs were 5 or more weeks in age. Second, these rates of activation were equivalent for embryonically-, postnatally- and adult-generated DGCs. Third, these findings generalized to another hippocampus-dependent task, contextual fear conditioning. Together, these experiments indicate that developmentally- and adult-generated DGCs are involved in hippocampal memory processing at similar rates, and suggest a functional equivalence between DGCs generated at different developmental stages.
3.2 Introduction

During adulthood, new neurons are continuously added to the dentate gyrus (DG), a subregion of the hippocampus that plays an essential role in memory formation (Ming and Song, 2005; Zhao et al., 2008). Similar to developmentally-generated DGCs, these adult-generated DGCs are thought to eventually contribute to the formation of hippocampus-dependent memory (Deng et al., 2010; Shors, 2008). One key question for understanding how these adult-generated DGCs contribute to memory function is whether they are functionally distinct from DGCs generated during development. While adult-generated DGCs may eventually develop similar cellular phenotypes to developmentally-generated DGCs (Laplagne et al., 2006; Laplagne et al., 2007), adult-generated DGCs differ transiently from their developmentally-generated neighbors. For example, between 2-4 weeks of age the threshold for long term potentiation (LTP) is reduced (Ge et al., 2007; Schmidt-Hieber et al., 2004), and from 4-6 weeks LTP magnitude is increased (Ge et al., 2007). In particular, these transient changes in the threshold and magnitude of LTP may promote integration of newborn neurons into hippocampal memory networks by allowing them to either temporarily or permanently out-compete their developmentally-generated neighbors. Such a competitive mechanism has been proposed to allow the DG to temporally associate event memories that occur close together in time (Aimone et al., 2006, 2009). Alternatively, developmentally- and adult-generated DGCs may be functionally equivalent. According to this view, developmentally- and adult-generated DGCs would be integrated into hippocampal memory circuits at equivalent rates.

Previously this question has been addressed using immunohistochemical approaches to estimate integration rates of DGCs into hippocampal memory networks in intact mice (Kee et al., 2007a, b; Ramirez-Amaya et al., 2006; Tashiro et al., 2007). In these studies, proliferation markers such as BrdU were used to label adult-generated DGCs. Typically, mice were then trained in a hippocampus-dependent task (such as the hidden platform version of the water maze), and expression of immediate early genes (IEGs) (such as Fos, Arc, Zif268) quantified following memory testing. Because IEG expression is regulated by neuronal activity (Guzowski et al., 2005), this approach makes it possible to estimate the proportion of adult-generated DGCs that are functionally recruited by hippocampal memory networks by calculating the likelihood of IEG
expression in BrdU+ cells. Integration rates of developmentally-generated DGCs can then be estimated by calculating the likelihood of IEG expression in cells expressing the neuronal marker NeuN. However, NeuN is expressed in cells as young as 3 days-old (Brandt et al., 2003) and adult-generated DGCs < 2 weeks-old do not express Fos (Jessberger and Kempermann, 2003). Therefore, these analyses likely underestimate recruitment rates for developmentally-generated cells, and potentially lead to the impression that adult-generated DGCs are integrated at preferential rates. To circumvent this issue, in the current study we chose to directly label separate cohorts of embryonically-, postnatally- and adult-generated DGCs by injecting different thymidine analogs (BrdU, IdU, CldU) at different developmental stages, and compare their rates of activation by quantifying IEG expression.
3.3 Materials and Methods

3.3.1 Mice

Male offspring from a cross between C57Bl/6NTacfrBr [C57B6] and 129Svev [129] mice (Taconic, Germantown, NY) were used in these experiments. All mice were bred in our colony at The Hospital for Sick Children, and maintained on a 12 h light/dark cycle with free access to food and water. Behavioral procedures were conducted during the light phase of the cycle, blind to the treatment condition of the mouse and according to protocols approved by the Animal Care Committee at The Hospital for Sick Children.

3.3.2 Water Maze Apparatus and Procedures

The apparatus and behavioral procedures have been previously described (Teixeira et al., 2006). Behavioral testing was conducted in a circular water maze tank (120 cm in diameter, 50 cm deep), located in a dimly-lit room. The pool was filled to a depth of 40 cm with water made opaque by adding white, non-toxic paint. Water temperature was maintained at 28 ± 1 °C by a heating pad beneath the pool. A circular escape platform (10 cm diameter) was submerged 0.5 cm below the water surface, in a fixed position in 1 quadrant. The pool was surrounded by curtains, at least 1 m from the perimeter of the pool. The curtains were white with distinct cues painted on them.

Prior to commencing training, mice were individually handled for 2 min each day over 7 consecutive days. Mice were trained over 5 days. On each training day, mice received 6 training trials (presented in 2 blocks of 3 trials; inter-block interval was ~1 h, inter-trial interval was ~15 s). On each trial they were placed into the pool, facing the wall, in 1 of 4 start locations. The order of these start locations was pseudo-randomly varied throughout training. The trial was complete once the mouse found the platform or 60 s had elapsed. If the mouse failed to find the platform on a given trial, the experimenter guided the mouse onto the platform. Following the completion of training, spatial memory was assessed in a series of 3 probe tests with an inter-test
interval of approximately 3 min. In this test the platform was removed from the pool, and the mouse was allowed 60 s to search for it.

Behavioral data from training and the probe tests were acquired and analyzed using an automated tracking system (Actimetrics). In probe tests we quantified performance in two ways. First, we measured the amount of time mice searched the target zone (20 cm radius, centered on the location of the platform during training) vs. the average of 3 other equivalent zones (O) in other areas of the pool. These zones each represent approximately 11% of the total pool surface. Second, we represented probe test performance as a heat map (or density plot), with hot colors corresponding to areas of the pool that were more frequently visited.

### 3.3.3 Contextual Fear Conditioning Apparatus and Procedures

The apparatus and behavioral procedures have been previously described (Wang et al., 2009). Contextual fear conditioning experiments were conducted in a windowless room containing 4 conditioning chambers. Each conditioning context consisted of a stainless steel conditioning chamber (31 cm × 24 cm × 21 cm; Med Associates), containing a stainless steel shock-grid floor. Shock grid bars (diameter 3.2 mm) were spaced 7.9 mm apart. The grid floor was positioned over a stainless-steel drop-pan, which was lightly cleaned with 70% ethyl alcohol to provide a background odor. The front, top, and back of the chamber were made of clear acrylic and the two sides made of modular aluminum. Mouse freezing behavior was monitored via 4 overhead cameras. Freezing was assessed using an automated scoring system (Actimetrics), which digitized the video signal at 4 Hz and compared movement frame by frame to determine the amount of freezing.

During training, mice were placed in the context for 5 min and were presented with 3 unsignaled footshocks (0.5 mA, 2 s, 1 min apart) starting at 2 min. Following the last footshock mice remained in the context for an additional 1 min, and then were returned to their home cage (HC). On the test day, mice were placed back into the same context for a total of 5 min and freezing was monitored.
3.3.4 Pentylentetrazole Treatment

Mice were initially injected intraperitoneally (IP) with 30 mg/kg of the chemical convulsant Pentylentetrazole (PTZ; Sigma) followed by additional 10 mg/kg injections every 15 min as needed. This procedure was repeated until a seizure occurred, typically requiring a total dose of 60 mg/kg.

3.3.5 Entorhinal Cortex Electrical Stimulation

Mice were pre-treated with atropine sulfate (0.1 mg/kg, IP), anesthetized with chloral hydrate (400 mg/kg, IP) and placed in a stereotaxic frame. The scalp was incised and a hole drilled in the skull above the lateral EC [anteroposterior = -4.0 mm, mediolateral = 3.3 mm relative to bregma] (Paxinos and Franklin, 2000). A concentric bipolar stimulating electrode (FHC Inc.) was inserted to 5.0 mm below bregma into the lateral EC (Paxinos and Franklin, 2000), and high-frequency electrical stimulation (130 Hz, 90 µs pw, 50 µA) was delivered using a Medtronic 3628 screener (Medtronic Inc.) for 1 h. At the completion of surgery, animals were kept anesthetized until sacrifice.

3.3.6 BrdU, CldU, and IdU Administration

BrdU (Sigma) was dissolved in 0.1 M phosphate-buffered saline (PBS) and heated to 50–60 °C, at a concentration of 10 mg/ml. In experiments 2, 3, 6 and 7, animals received 50 mg/kg of BrdU per injection either IP on postnatal day 7 (P7) or 60 (P60), or subcutaneously (SC) around gestational day 18 (E18). Exposure to even higher doses of BrdU at these embryonic and postnatal stages of development does not impact number, development or behavior of progeny (Bick-Sander et al., 2006; Kolb et al., 1999; Vega and Peterson, 2005).

5-Iodo-2’-deoxyuridine (IdU; MP Biomedicals) or 5-Chloro-2’-deoxyuridine (CldU) (IdU and CldU are collectively referred to as XdU) were dissolved in 0.1 M PBS and heated to 50–60 °C, at a concentration of 10 mg/ml. In experiments 4 and 5, animals received the same molar concentration of XdU as 50 mg/kg of BrdU per injection either IP on P7 or P60, or SC around
E18. Actual equimolar doses used were 57.5 mg/kg of IdU, and 42.5 mg/kg of ClU per injection.

3.3.7 Experimental Methods

Experiment 1 (Figure 3-1) evaluated the activity-dependent regulation of Fos, and co-expression of Fos and Arc, in DGCs. Fos expression was assessed in the HC condition (n = 3), following memory recall (n = 3) and after PTZ-induced seizures (n = 3) in 8 week-old mice. Mice analyzed following memory recall underwent water maze training followed by a probe test 1 day later. Age-matched HC animals were sacrificed simultaneously. Fos expression was also examined in mice receiving high frequency EC electrical stimulation (n = 6), electrode-implanted but non-stimulated mice (n = 8) and age-matched HC animals (n = 4). Finally, Fos and Arc expression in DGCs was examined in adult mice (n = 4) that were trained and then tested 1 day later.

Experiment 2 (Figure 3-2) determined the age at which adult-generated DGCs are functionally recruited by spatial memory networks. To label dividing cells, 8 week-old mice were injected with BrdU (2 injections/day, ~12 h apart, for 5 days). Separate groups were then trained in the water maze either 1 week (n = 8), 5 weeks (n = 18), 7.5 weeks (n = 18) or 10 weeks (n = 18) later. Spatial memory was assessed 1 day following the completion of training. To control for cell age, an additional group of mice (n = 10) was trained 1 week following BrdU treatment and then tested 4 weeks later. Further groups were treated with PTZ. Injections occurred either 1 week (n = 2), 5 weeks (n = 3), 7.5 weeks (n = 3) or 10 weeks (n = 3) after BrdU treatment. Age-matched HC groups were sacrificed at the same time as the 1 week (n = 3), 5 weeks (n = 3), 7.5 weeks (n = 3), and 10 weeks (n = 3) post BrdU treatment groups.

Experiment 3 (Figure 3-3) compared recruitment rates of embryonically-, postnatally- and adult-generated DGCs by spatial networks in the DG, controlling for cell age. BrdU was administered to pregnant mothers once around E18 (n = 6), mouse pups once at P7 (n = 6) or adult mice at P60 (2 injections/day, ~12 h apart, for 5 days) (n = 18). All groups were then trained 7.5 weeks later and spatial memory was tested 1 day following the completion of training. Additional groups were first treated with equivalent doses of BrdU at E18 (n = 3), P7 (n = 3) or P60 (n = 3), and
then treated with PTZ. PTZ injections occurred 7.5 weeks following BrdU treatment, matching the age at which water maze animals underwent training and testing.

Experiment 4 (Figure 3-4) tested the sensitivity and specificity of CldU and IdU cell-labeling. One group of mice was treated with CldU once at P7, and then IdU at P60 (2 injections/day, ~12 h apart, for 5 days) (n = 3). A second group received IdU once at P7, followed by CldU (2 injections/day, ~12 h apart, for 5 days) (n = 4). A third group of mice received both CldU and IdU simultaneously for 2 days (n = 3) at P60. All groups were sacrificed 1 day following the completion of injections.

Experiment 5 (Figure 3-5) compared recruitment rates of embryonically-, postnatally- and adult-generated DGCs by spatial memory networks in the same mouse. In the first group, pregnant mothers were treated with CldU once at E18, and then progeny (n = 12), were treated with IdU at P60 (2 injections/day, ~12 h apart, for 5 days). In the second group (n = 14), mice were treated once with CldU at P7, and then with IdU at P60 (2 injections/day, ~12 h apart, for 5 days). Both groups were trained in the water maze 7.5 weeks after IdU treatment, and spatial memory was assessed 1 day following the completion of training.

Experiment 6 (Figure 3-6) evaluated the influence of cell age on activation of adult-generated DGCs by hippocampal networks supporting contextual fear memory. Mice were initially injected with BrdU (2 injections/day, ~12 h apart, for 5 days), and separate groups were trained in the contextual fear conditioning task either 1 week (n = 10) or 6 weeks (n = 18) later. Contextual fear memory was assessed in all mice 4 weeks following training. In addition, Fos expression in BrdU+ cells was examined in HC control mice and mice treated with PTZ either 5 (HC, n = 3; PTZ, n = 2) or 10 (HC, n = 6; PTZ, n = 3) weeks following BrdU treatment.

Experiment 7 (Figure 3-7) compared recruitment rates of developmentally- and adult-generated DGCs by contextual fear memory networks in the DG. One group of mice (n = 6) was treated with BrdU once at P7 and then trained at P100. A second group (n = 27) was treated with BrdU (2 injections/day, ~12 h apart, for 5 days) at P60, and then trained at P100. Contextual fear memory was assessed in both groups 4 weeks after training.
3.3.8 Tissue Handling and Preparation for Stereology

Ninety min following the completion of behavioral testing, PTZ-induced seizure activity or EC stimulation, mice were anesthetized and perfused transcardially with PBS and then 4\% paraformaldehyde (PFA). Brains were removed, fixed overnight in PFA and then transferred to 30\% sucrose solution (PBS for Fos/Arc co-labeling, experiment 1) and stored at 4 °C. Fifty μm coronal cryostat sections (vibratome sections for Fos/Arc co-labeling, experiment 1) were cut beginning from a random starting point and continuing along the entire anterior-posterior extent of the DG. Sections were kept in sequential order and maintained free-floating in PBS. A 1/4 section sampling fraction was used to create 4 sets (each containing sections at 200 μm intervals) for use in immunohistochemical staining. Accordingly, each set comprised a systematic random sample representative of the entire DG for use in quantification analyses. Additional sets were stored in a PBS solution containing 0.02\% sodium azide for later processing.

3.3.9 Immunohistochemistry

For Fos/Arc double labeling, sections were treated with 1\% hydrogen peroxide (Sigma-Aldrich) and ice-cold acetone (Sigma-Aldrich). A rabbit on rabbit staining technique was employed, beginning by incubating sections with 70 ng/ml (1:15000) of rabbit anti-Arc polyclonal antibody (Synaptic Systems). Arc protein was visualized using biotin conjugated donkey anti-rabbit antibody (1:500; Jackson Immunoresearch), avidin-biotin-peroxidase complex (Vector laboratories), tyramide signal amplification (made by mixing biotin succcinimidyl ester (Sigma-Aldrich) and tyramine hydrochloride (Fluka)) and Alexa-568 conjugated streptavidin (1:500; Invitrogen). Next, residual rabbit IgG epitopes were blocked with donkey anti-rabbit IgG monovalent antibody (1:500; Jackson Immunoresearch). Fos protein was visualized using rabbit anti-cFos polyclonal antibody (1:1500; Calbiochem) and Alexa-488 conjugated anti-rabbit antibody (1:500; Invitrogen). To rule out cross-reactivity between the Alexa-488 conjugated anti-rabbit and rabbit anti-Arc antibodies, we carried out staining without anti-cFos antibody and confirmed the absence of a Fos signal.

For BrdU/Fos staining, the BrdU antigen was exposed by incubating the sections in 1 N hydrochloric acid (HCl) at 45°C for 30 min. Incubation for 48 h at 4°C was performed using
primary antibodies against Fos (rabbit anti-Fos polyclonal antibody; 1:1000; Calbiochem) and BrdU (rat anti-BrdU monoclonal antibody; 1:500; Accurate Chemicals). Secondary antibody staining with Alexa-488 goat anti-rat and Alexa-568 goat anti-rabbit (1:500; Molecular Probes) was carried out for 2 h at room temperature. Antibodies were diluted in blocking solution containing 2% goat serum, 1% bovine serum albumin, and 0.2% Triton X-100 dissolved in PBS. Sections were mounted on slides (VWR) with Permafluor anti-fade medium (Lipshaw Immunon).

For BrdU/neuronal-specific nuclear protein (NeuN) double labeling, identical procedures were performed while exchanging anti-Fos primary and anti-rabbit secondary antibodies with mouse anti-NeuN (1:1000; Chemicon) and Alexa-568 goat anti-mouse (1:500; Molecular Probes), respectively.

For BrdU/Fos/NeuN triple labeling, identical procedures were performed using primary rabbit anti-Fos, rat anti-BrdU and mouse anti-NeuN antibodies. As secondary antibodies we used Alexa-488 goat anti-rat, Alexa-568 goat anti-mouse and Biotin-SP-conjugated anti-rabbit (1:500; Jackson ImmunoResearch). Biotin-SP-conjugated anti-rabbit primary antibody was detected by subsequent incubation with cy5 conjugated streptavidin (Zymed).

For XdU/Fos staining, sections were washed with tris-buffered saline (TBS) followed by DNA denaturation in 1N HCl at 45°C for 30 min. Following additional washes with TBS, sections were incubated in primary antibodies: mouse anti-BrdU monoclonal antibody (BD Biosciences) at 1:1000 for IdU, rat anti-BrdU monoclonal antibody (Accurate Chemicals) at 1:500 for CldU, and rabbit anti-Fos polyclonal antibody (Calbiochem) at 1:1000 for 48 h at 4°C. After three washes in 3x TBS-tween 20, sections were incubated for 2 h at room temperature in secondary antibodies: Rhodamine Red-X-conjugated anti-mouse, FITC- conjugated anti-rat and Biotin-SP-conjugated anti-rabbit (Jackson ImmunoResearch). Subsequent to three washes with 3x PBS, biotinylated secondary antibody was detected with cy5 conjugated streptavidin (1:50; Zymed).

For XdU/NeuN staining, identical procedures were performed except sections were first stained with XdU primary and secondary antibodies alone, replacing FITC- conjugated anti-rat secondary with biotinylated goat anti-rat (Vector Laboratories) followed by cy5 conjugated streptavidin (Zymed). At the completion of XdU staining, sections were incubated for 2 h at room temperature with Alexa-488 mouse anti-NeuN (1:1,000, Chemicon).
3.3.10 Imaging

Data and images were acquired using either a Nikon Eclipse 80i or Olympus BX61 epifluorescent microscope, or an Olympus IX81 with DSU or Zeiss LSM710 confocal microscope. Analysis for cell counting used Image J software (National Institute of Health), and for spatial measurements and confocal examination used Image-Pro 6.2 software (Media Cybernetics Inc.) or ZEN 2009 software (Zeiss).

3.3.11 Stereologic Quantification of Cells

Total cell counts obtained from a 1⁄4 systematic random section sampling fraction covering the entire anterior-posterior extent of the DG were divided by the total number of DG sections analyzed. Thus, normalized values for the number of cells per DG section (indicated as “[cells] per section” throughout the figures) were representative of the entire DG and appropriate for group comparisons. Unless otherwise specified, direct counting of labeled cells, identified by unique point criteria, in the entire DG was performed.

Arc and Fos colocalization was quantified initially using an epifluorescent microscope with a 40X objective. These results were compared to subsets of 50-100 cells per animal analyzed with a 40X/1.3 objective on a confocal microscope. Confocal 1 µm Z-stack optical sections were obtained at 15 µm-spaced intervals to prevent duplicate counts of the same cell. In all animals the confocal data matched the epifluorescent results.

The density of Fos+ cells was relatively low (<1.5% of DGCs expressed Fos following behavioral testing) and therefore it was possible to quantify the number of Fos+ cells from images acquired using a 10X or 20X objective.

The total number of NeuN+ cells in the DG was estimated using a method adapted from Barnes and colleagues (Chawla et al., 2005). Two confocal Z-stacks of 1 µm optical sections per DG (one each from the upper and lower blades) were collected from 4 mice with a 40X/1.3 objective. The total number of NeuN+ cells in each 50 µm stack was counted manually and the total area of the middle plane was measured. From this we calculated the density of NeuN+ cells in the DG.
From our analysis of Fos+ cells, we then determined the proportion of NeuN+ cells expressing Fos following behavioral testing and PTZ treatment (see also (Kee et al., 2007a)).

Adult labeled BrdU+ and Fos+/BrdU+ cells were first determined using an epifluorescent microscope with a 40X objective. A subset of Fos+/BrdU+ cells was verified using a 40X/1.3 objective on a confocal microscope.

The numerous developmentally labeled BrdU+, XdU+, Fos+/BrdU+ and Fos+/XdU+ cells were determined from 4 µm-spaced, 1 µm thick, Z-stack optical sections of the entire DG using a 20X objective. Cells were counted by 2 experimenters (unaware of the treatment conditions). A subset of Fos+/XdU+ cells were confirmed with a 40X/1.3 objective on a confocal microscope. Average counts obtained by the 2 observers were used in statistical analyses. These Z-stack images were also used to measure the distance of XdU+ cells from the SGZ.

To estimate the proportions of BrdU+ and XdU+ cells that were NeuN+, and to assess the proportions of XdU+ cells that were mono- or co-labeled for IdU and/or CldU, randomly chosen DG regions of interest were analyzed using confocal Z-stacks of 1 µm optical sections collected from 3 mice per group with a 40X/1.3 objective. Approximately 100 BrdU+ or XdU+ cells were counted per animal and assessed for co-labeling.

3.3.12 Statistical Analyses

Behavioral and Cell counting data were evaluated using parametric ANOVAs or t-tests, where appropriate. Post-hoc Newman-Keuls tests were used to examine significant main effects or interactions. Statistics were performed using STATISTICA 8.0 software (StatSoft Inc.).
3.4 Results

3.4.1 Fos Protein in Dentate Granule Cells is Regulated by Memory Recall

The IEG *Fos* is induced by neural activity, and has been used as an activity marker in a variety of different experimental situations and brain regions (Dragunow and Robertson, 1987; Guzowski et al., 2005; Hunt et al., 1987; Morgan et al., 1987; Saffen et al., 1988). Our current experiments are focused on the DG, and, accordingly, we initially characterized the regulation of Fos in DGCs in a range of conditions. First, recall of a spatial memory induced Fos in approximately 1% of DGCs, consistent with previous IEG (Chawla et al., 2005) and electrophysiological (Jung and McNaughton, 1993) studies showing that a similarly small proportion of granule cells are activated during spatial exploration. These levels are higher than those in HC control mice, and much lower than in mice treated with the chemical convulsant, PTZ ($F_{2,6} = 43.3, P < 0.001$) (Figure 3-1A). Second, high-frequency electrical stimulation of the major afferent input into the DG leads to an upregulation of Fos in a similarly small subpopulation of DGCs, relative to HC or non-stimulated controls ($F_{2,15} = 5.51, P < 0.05$) (Figure 3-1B). Third, Fos expression is limited to neurons following either behavioral testing or PTZ treatment, as almost all Fos+ cells also expressed the neuronal marker, NeuN (Figure 3-1C). Fourth, other IEGs including *Arc* (also known as *Arg3.1*) have been used as activity markers. Following behavioral testing we found similar numbers of Fos+ and Arc+ DGCs, and, these signals were almost completely co-localized. This suggests that *Fos* and *Arc* are regulated in a highly similar manner in DGCs (Figure 3-1D). Together, these data support the idea that Fos can be used as a neuronal activity marker in the DG, and that *Fos* is regulated in a similar manner to other IEGs such as *Arc*. 
3.4.2 Functional Activation of Adult-Generated Dentate Granule Cells is Regulated by Maturation

As adult-generated DGCs mature, they exhibit distinct plastic properties compared to their developmentally-generated neighbors (Ge et al., 2007). We therefore next evaluated whether such transient changes in plasticity coincide with transient increases in rates at which adult-generated DGCs are recruited by hippocampal memory circuits. To address this question, mice were injected with the proliferation marker BrdU and separate groups were trained in the water maze either 1, 5, 7.5 or 10 weeks later. One day following the completion of training, spatial memory was assessed in a series of probe tests (Figure 3-2A). Ninety min following testing, mice were perfused and Fos and BrdU expression in DG tissue was quantified using immunohistochemical approaches (Kee et al., 2007a). As Fos expression is regulated by

Figure 3-1. Activity-Dependent Regulation of Fos in Dentate Granule Cells. (A) Induction of Fos in DGCs following spatial memory recall (R) and PTZ treatment compared to home cage (HC) mice. These and all subsequent graphs show mean number of cells per DG section ± SEM. (B) Induction of Fos in DG cells following stimulation (S) of the lateral EC compared to non-stimulated (NS) and HC control mice. (C) Left, the proportion of Fos+ DG cells that were NeuN+ (a neuronal specific marker) following memory recall (R). Right, the proportion of Fos+ DG cells that were NeuN+ following PTZ treatment (PTZ). (D) Representative confocal images of Fos+, Arc+ and Fos+/Arc+ (merge) cells in the DG following behavioral testing (scale bar = 10 µm). Venn diagram of Fos+ (green) and Arc+ (red) DGCs, indicating that Fos and Arc were almost always co-localized (yellow) in the same DGCs.
neuronal activity (see Figure 3-1), the degree of overlap between BrdU-labeled and Fos-labeled neurons provides an indication of whether adult-generated DGCs have been functionally incorporated into hippocampal memory circuits.

Figure 3-2. Maturation Regulates Adult-Generated Dentate Granule Cell Recruitment by Hippocampal Circuits Supporting Water Maze Memory. (A) Experimental design. (B) Mice searched selectively in each of the three probe tests. In this and subsequent figures, only data from the first probe test are shown. Left, density plots for grouped data showing where mice concentrated their searches. The color scale represents the number of visits per animal per 5 cm × 5 cm area. Right, all groups of mice spent more time searching the target zone (T) compared to other (O) zones. (C) Representative confocal images of BrdU+, Fos+ and Fos+/BrdU+ (arrow) cells in the DG following water maze testing in the 1 week and 7.5 week groups (scale bar = 20 µm). (D) Numbers of BrdU+ cells in the DG. (E) Fos+ cells in the DG following probe tests. (F) Fos expression in BrdU+ cells (orange) or NeuN+ cells (clear). Fos in BrdU+ cells increased with longer delays between BrdU treatment and water maze training. Fos expression in matched BrdU-treated mice is shown following PTZ treatment (red) or in a home cage (HC) condition (light blue). (G) Proportion of BrdU+ cells that are NeuN+ in the GCL 1, 5 or 10 weeks after BrdU treatment.

Over the course of training mice required progressively less time to locate the platform, and escape latencies did not differ between groups (Figure 3-S1A). In the probe test following
training, all groups of mice spent significantly more time in the area of the pool that formerly contained the platform (Figure 3-2B) (paired t-tests; all $P_s < 0.001$), indicating that this training produced robust spatial memory. Following the probe test we identified many Fos+ and BrdU+ cells in the DG (Figure 3-2C). As expected, the number of BrdU+ cells generally declined as the BrdU-training delay increased ($F_{3,58} = 60.6, P < 0.001$) (Figure 3-2D), reflecting reduced survival at longer delays. Fos was also expressed in a relatively small proportion (< 1.5%) of DGCs, consistent with previous studies (Chawla et al., 2005; Kee et al., 2007b) and the idea that spatial information is sparsely encoded in the DG (Jung and McNaughton, 1993). There were differences in Fos expression between groups (Figure 3-2E) ($F_{3,58} = 6.33, P < 0.001$) though, with levels in the 1 week group lower than all other groups ($P_s < 0.05$). Most notably, overlap between Fos+ and BrdU+ cells depended on the delay between BrdU treatment and training (Figure 3-2F). As this delay lengthened, the probability of Fos expression in BrdU+ cells increased ($F_{3,58} = 3.39, P < 0.05$), supporting the idea that integration of newborn DGCs into spatial memory networks is regulated by maturation (Kee et al., 2007b). The most marked difference in Fos expression in BrdU+ cells was between the 1 and 5 week groups. At 5 weeks of age, newborn DGCs exhibit a transient increase in plasticity (i.e., lower induction threshold and increased amplitude LTP (Ge et al., 2007)), and therefore these changes in plasticity might promote integration. Fos expression in BrdU+ cells did not differ in the 5-10 week groups. This suggests that beyond 5 weeks of age, recruitment rates stabilized and enhanced plasticity in 5 week-old DGCs does not result in a transient increase in rates of functional incorporation.

The ability of newborn cells to express IEGs such as Fos may vary as a function of cell age and/or phenotype (Jessberger and Kempermann, 2003). With regard to cell age, in our experiment the BrdU-labeled cells were approximately 1, 5, 7.5 and 10 week(s)-old at the time of testing. To evaluate whether changes in Fos expression reflect cell-age dependent differences in ability to express Fos, we additionally examined Fos expression in a HC condition or following treatment with a chemical convulsant, PTZ, in BrdU-treated matched controls. In the HC condition, Fos expression was uniformly low in all groups (Figure 3-2F). In contrast, PTZ treatment induced robust Fos expression in BrdU+ cells, but these levels were appreciably higher in the 5-10 week groups compared to the 1 week group ($F_{3,7} = 13.9, P < 0.005$) (Figure 3-2F). Therefore, these non-physiological conditions, where activity levels are driven toward ceiling, reveal cell age-dependent differences in Fos regulation (Jessberger and Kempermann, 2003).
Accordingly, they suggest that the low levels of Fos expression in BrdU+ cells in the mice trained 1 week following BrdU treatment likely reflect an inability of 1 week-old DGCs to express Fos, rather than an inability of these newborn cells to integrate.

To address this confound, an additional group of mice was trained 1 week after BrdU treatment, and tested 4 weeks, rather than 1 day, later (Figure 3-2A). This ensures that BrdU-labeled cells are the same age at the time of testing as those in the 5 week group. In the probe test, mice searched selectively (paired t-test, \( P < 0.001 \); Figure 3-2B). Following the probe test numerous Fos+ and BrdU+ cells were identified in the DG (Figure 3-2D-E), and these levels were equivalent to those in the 5 week group (\( Ps > 0.05 \)). Most importantly, there was little overlap between these two populations of cells (Figure 3-2F): The likelihood of Fos expression in BrdU-labeled cells was significantly lower than in the 5 week group (\( P < 0.05 \)) and no different to those in the 1 week (\( P = 0.39 \)) or HC (\( P = 0.61 \)) groups. Therefore, controlling for cell-age confounds, this experiment indicates that 1 week-old adult-generated DGCs are insufficiently mature to be incorporated into spatial networks in the DG, consistent with previous reports from our lab (Kee et al., 2007b). The direct comparison of these 2 groups allows us to rule out three alternative interpretations of the data. First, the fixed delay between BrdU treatment and testing ensures that all labeled cells are the same age at the time of testing. Therefore, any group differences in Fos expression in BrdU+ cells are unlikely to be due to differences in maturation. Second, differential levels of Fos expression between groups indicate that 5 week-old adult-generated neurons do not simply have a lower threshold for activation during memory retrieval. Third, mice in each group underwent exactly the same experience immediately prior to sacrifice, and therefore group differences in numbers of Fos+/BrdU+ cells are specifically related to spatial memory processing and not related to nonspecific aspects of the testing procedure (e.g., handling, activity, mild stress or arousal).

After behavioral testing, induction of Fos is almost exclusively neuronal in the GCL of the DG (Figure 3-1A,B; see also (Kee et al., 2007a, b)). Therefore, a second potential confound is that differences in Fos expression in BrdU+ cells reflect group differences in the proportion of BrdU-labeled cells that differentiated into neurons. To address this issue, we counterstained for the neuronal marker, NeuN, in the groups of mice trained in the water maze 1, 5 and 10 weeks following BrdU treatment. Consistent with previous studies (Brandt et al., 2003), we found that BrdU+ cells were equivalently likely to be NeuN+ across groups (Figure 3-2G) (\( F_{2,6} = 0.13, P = \))
0.89), indicating that group differences in BrdU+ cell phenotype cannot account for differences in Fos expression in BrdU+ cells.

Finally, it is important to note that in the 5-10 week groups, rates of Fos expression in BrdU+ cells were greater than those in NeuN+ cells (Figure 3-2F), as in our previous study (Kee et al., 2007b) (see also Figure 3-S2). While this may suggest that adult-generated DGCs are recruited preferentially, Fos expression in NeuN+ cells provides only an indirect estimate of activation rates for developmentally-generated DGCs. As NeuN is expressed in both immature (as young as 3 days-old: (Brandt et al., 2003)) as well mature neurons, these analyses might underestimate recruitment rates of developmentally-generated DGCs. To address this we will label developmentally-generated DGCs directly in the following experiments.

3.4.3 Recruitment of Equivalently Aged E18, P7 and P60 Dentate Granule Cells

In the developing brain, two major waves of neurogenesis lead to the formation of the DG (Altman and Bayer, 1990; Piatti et al., 2006; Schlessinger et al., 1975). First, late in embryonic development (E15-P1), neural progenitor cells in the secondary dentate matrix generate DGCs that form the outer shell of the upper, and subsequently, lower blade of the DG. A second dentate migration after birth (> P1) leads to the formation of the tertiary dentate matrix, and neural progenitor cells from here migrate radially and generate DGCs that populate the inner part of the GCL. These neural progenitor cells subsequently accumulate in the SGZ and continue to generate DGCs throughout adulthood (albeit at exponentially declining levels). In the above experiment we found that adult-generated DGCs are recruited by spatial memory circuits in a maturation-dependent manner, and once new neurons are 5 weeks or more in age activation rates remained relatively stable. One key question is whether these stable rates of functional activation of mature adult-generated DGCs differ from those of developmentally-generated DGCs.

To address this question, three groups of mice were treated with BrdU at E18, P7 or as adults (P60). To ensure that BrdU-labeled cells were equivalently aged at the time of training and testing, all groups were trained 7.5 weeks after BrdU treatment (Figs. 3A, S1B). In the probe test conducted 1 day following the completion of training, all groups of mice searched selectively in
the region of the pool that formerly contained the platform (Figure 3-3B) (paired t-tests, $P < 0.01$). Following the probe test, immunohistochemical processing revealed many Fos+ and BrdU+ cells in the DG. Decreasing numbers of BrdU+ cells were found following treatment at E18, P7 and P60 respectively (Figure 3-3C) ($F_{2,27} = 181.0$, $P < 0.001$), reflecting age-dependent decline in rates of proliferation. In contrast, the numbers of Fos+ cells were similar across groups (Figure 3-3D) ($F_{2,27} = 0.49$, $P = 0.61$). Most importantly, we found that the likelihood of Fos expression in BrdU+ cells did not differ between groups following the probe test (Figure 3-3E) ($F_{2,27} = 0.20$, $P = 0.82$), suggesting that integration rates of equivalently-aged DGCs are the same, regardless of whether they were born during development (E18, P7) or in adulthood (P60).

We found the same pattern of results in an additional experiment where mice were treated with PTZ to strongly activate DGCs. Similar to the water maze experiment, we found that Fos expression in BrdU+ cells did not differ between E18, P7 and P60 groups (Figure 3-3F) ($F_{2,6} = 3.17$, $P = 0.12$). Together, these experiments suggest that equivalently aged DGCs respond similarly to both physiological and non-physiological stimulation, regardless of whether they were born during development (E18, P7) or in adulthood (P60).

Figure 3-3. Functional Activation of Equivalently Aged E18, P7 and P60 Dentate Granule Cells. (A) Experimental design. Separate groups of mice were treated with BrdU at E18, P7 and P60 and then trained in the water maze 7.5 weeks later. Spatial memory was tested in a series of three probe tests 1 day following the completion of training. (B) Mice searched selectively in the probe tests. Left, density plots for grouped data showing where mice concentrated their searches. Right, all groups of mice spent more time searching the target zone (T) compared to other (O) zones. (C) Number of BrdU+ cells was highest in the E18 group, followed by P7, and then P60, reflecting higher rates of developmental neurogenesis. (D) Equivalent Fos expression in the DG across groups following probe tests. (E) The likelihood of Fos expression in BrdU+ cells following probe tests was similar in all groups, suggesting that developmentally- and adult-generated cells are integrated into DG circuits supporting water maze memory at similar rates. (F) Fos expression in matched BrdU-treated mice is shown following PTZ treatment.
3.4.4 Recruitment of Adult- vs. Developmentally-Generated Dentate Granule Cells (Within Animal Comparison)

The previous experiment indicated that equivalently aged DGCs are functionally recruited by circuits supporting spatial memory at similar rates, regardless of whether they were born during development (E18, P7) or in adulthood (P60). A remaining possibility is that, in the adult mouse, more recently adult-generated DGCs out-compete relatively older DGCs that were born during embryonic or early postnatal development. To address this question we next labeled embryonically- or postnatally-generated DGCs and directly compared their activation rates with adult-generated DGCs in the same animal. To achieve this within-animal comparison mice were treated with two equimolar, chemically-related thymidine analogs, CldU and IdU (Burns and Kuan, 2005; Dupret et al., 2007; Thomas et al., 2007; Tronel et al., 2010; Vega and Peterson, 2005). CldU and IdU are recognized by different antibodies and so separate cohorts of cells may be labeled in the same mice. We first verified that our staining procedures detected CldU and IdU specifically within mice exposed to both compounds (see (Leuner et al., 2009)). To do this, mice were first injected with CldU at P7 and then IdU at P60 (or vice-versa). Tissue from all animals was subjected to identical staining (i.e., incubated with primary and secondary antibodies for both compounds), and many DGCs were identified (Figure 3-4A). Importantly, virtually no DGCs were labeled for both IdU and CldU (Figure 3-4B), indicating that staining was specific for each compound. Furthermore, there were similar numbers of CldU- and IdU-labeled DGCs at both time points and in both groups (Figure 3-4C) ($F_{1,5} = 0.35, P = 0.58$), indicating that these compounds label equivalent numbers of DGCs. Similar experiments performed in adult mice in which animals were injected with either one of the compounds at P60, confirmed the near absence of cross-detection (Figure 3-S3). Importantly, when adult mice received CldU and IdU simultaneously, virtually all cells were co-labeled as expected (Figure 3-4D). Together these results indicate that, using our methodology, CldU and IdU can be used in the same animal to specifically label separate cohorts of DGCs with equivalent sensitivity.
To label embryonically- and adult-generated DGCs in the same mice, pregnant mice were injected with CldU at E18 and then their progeny were subsequently treated with IdU once they reached adulthood (P60). To label postnatally- and adult-generated DGCs in the same mice, mouse pups were treated with CldU at P7, and then with IdU after they reached adulthood (P60). Both groups of mice were then trained in the water maze 7.5 weeks following IdU treatment (Figure 3-S1C) and spatial memory was assessed 1 day following the completion of training (Figure 3-5A). In the probe test, both groups of mice concentrated their search in the region of the pool that formerly contained the platform (Figure 3-5B) (paired t-tests, P < 0.01). Following the probe test, many CldU+, IdU+ and Fos+ cells were identified in the DG. Importantly, the distribution and number (but not phenotype) of CldU+ and IdU+ cells depended on the timing of the treatment. First, while embryonically-labeled DGCs were distributed throughout the GCL, postnatally-generated DGCs were predominantly located in the inner two thirds of the GCL and adult-generated DGCs were largely restricted to the innermost third of the GCL or SGZ (Figure 3-5C). These distinct distributions reflect the sequential waves of neurogenesis that lead to the formation of the DG during development (Piatti et al., 2006). Second, as treatment age increased the number of labeled cells declined (Figure 3-5E,F) (paired t-test [E18/P60]: t11 = 9.16, P < 0.001; paired t-test [P7/P60]: t13 = 17.9, P < 0.001), reflecting an age-dependent decline in proliferation. Third, embryonically-, postnatally- and adult-generated cells were equally likely to differentiate into neurons (Figure 3-5D) (F2,6 = 0.79, P = 0.50). Following the probe test, similar numbers of Fos+ cells were identified in the DG in both groups (Figure 3-5E,G) (t24 = 0.04, P =
Most strikingly, developmentally- and adult-generated DGCs appeared to be recruited at equivalent rates by DG circuits supporting spatial memory: The likelihood of Fos expression in embryonically- or postnatally-generated cells did not differ to that in adult-generated cells (Figure 3-5E,H) (paired t-test [E18/P60]: $t_{11} = 0.39, P = 0.70$; paired t-test [P7/P60]: $t_{13} = 1.03, P = 0.32$). Counter to the idea that adult-generated neurons are recruited preferentially into DG memory circuits, these data suggest that they are recruited at equivalent rates to those of existing (or developmentally-generated) DGCs.

**Figure 3-5. Functional Activation of E18, P7 and P60 Dentate Granule Cells (Within Animal Design).** (A) Experimental design. In group 1, pregnant mice were treated with CldU at E18, and their progeny with IdU at P60. In group 2, mouse pups were treated with CldU at P7 and then IdU at P60. Both groups were trained 7.5 weeks after IdU treatment, and spatial memory was assessed 1 day following the completion of training. (B) Both groups searched selectively in the probe test. Left, density plots for grouped data showing where mice concentrated their searches. Right, both groups spent more time searching the target zone (T) compared to other (O) zones. (C) Representative images of DGCs labeled by CldU injection at E18 (top) or P7 (middle) or by IdU injections at P60 (bottom) (DAPI counterstain, scale bar = 20 µm). The lower graph quantifies the relative distributions of embryonically-, postnatally- and adult-labeled DGCs. (D) Proportion of CldU- or IdU-labeled cells that were NeuN+ following treatment at E18 (top), P7 (middle) or by IdU injections at P60 (bottom). (E) Representative confocal images of CldU+, IdU+, Fos+ and XdU+/Fos+ (merge) cells in the DG following water maze testing in group 1 (E18/P60) (scale bar = 5 µm). Dotted lines outline the GCL and SGZ. (F) XdU- (CldU- or IdU-) labeled cells in the DG for group 1 (upper) and group 2 (lower). These were highest in the E18 or P7 group relative to P60, reflecting higher rates of developmental neurogenesis. (G) Fos expression in the DG following probe tests was similar for both groups. (H) Likelihood of Fos expression in XdU+ cells for both groups following probe tests was similar in corresponding developmentally- and adult-generated DGCs, suggesting similar integration rates into DG circuits supporting water maze memory.
3.4.5 Contextual Fear Conditioning

The above experiments suggest that developmentally- and adult-generated DGCs are recruited at similar rates by hippocampal circuits supporting water maze memory. Since the hippocampus is engaged in a wide range of learning situations, we finally wanted to determine whether this pattern of results would generalize to another form of hippocampus-dependent learning such as contextual fear conditioning (Kim and Fanselow, 1992). In our first experiment we evaluated whether the functional activation of adult-generated DGCs by hippocampal networks supporting contextual fear memory depends on cell age, as is the case in the water maze (Kee et al., 2007b). In this experiment mice were treated with BrdU, and then trained in contextual fear conditioning either 1 week or 6 weeks later (Figure 3-6A). During the retention test 4 weeks later, all mice exhibited similar levels of conditioned freezing ($t_{26} = 0.45, P = 0.66$) (Figure 3-6B). Furthermore, we identified similar numbers of Fos+ cells ($t_{26} = 1.38, P = 0.18$) (Figure 3-6D). Notably, numbers of BrdU+ cells were decreased in mice trained 1 week following BrdU treatment ($t_{26} = 5.17, P < 0.001$) (Figure 3-6C). This might suggest that mild stress associated with training episode was sufficient to reduce survival of relatively immature (i.e., 1 week-old but not 6 week-old) adult-born cells, an observation that is consistent with the idea that stress regulates the survival of newborn cells (Gould et al., 1991). Most importantly, after normalizing for the number of BrdU+ cells, we found that the likelihood of Fos expression in BrdU+ cells was higher in the group of mice treated with BrdU 6 weeks before training ($t_{26} = 2.12, P < 0.05$) (Figure 3-6E). These differences in overlap were not due to either cell age-dependent differences in ability to express Fos between groups, since PTZ-induced Fos expression was similar in age-matched controls (Figure 3-6E) ($t_{3} = 0.18, P = 0.87$), or the age of the memory at the time of testing (Figure 3-S4A-E). Furthermore, this maturation-dependent pattern of activation was limited to the DG and did not extend to the olfactory bulb (Figure 3-S4F-H), another neurogenic region in the adult brain that would not be expected to play a major role in contextual fear conditioning (for similar results in water maze see: (Kee et al., 2007b)). Consistent with the water maze, these data support the idea that adult-generated DGCs are integrated into hippocampal networks supporting contextual fear conditioning memories (see also: (Deng et al., 2009; Hernandez-Rabaza et al., 2009; Ko et al., 2009; Saxe et al., 2006; Warner-Schmidt et al., 2008; Winocur et al., 2006)), and establish that functional activation depends on their maturational state.
Using a between subjects design, we next tested whether developmentally- and adult-generated DGCs are recruited at similar rates by hippocampal networks supporting contextual fear memory. Mice were treated with BrdU either at P7 or during adulthood (P60) (Figure 3-7A). Both groups were then trained at P100 and tested 1 month later. In this test, conditioned freezing levels were similar in both groups of mice ($t_{31} = 0.37, P = 0.71$) (Figure 3-7B), indicating that training produced robust contextual fear memory. We identified significantly more BrdU+ cells in mice treated with BrdU at P7 compared to adulthood ($t_{31} = 27.6, P < 0.001$) (Figure 3-7C,D), reflecting higher rates of neurogenesis during the early postnatal period. Contextual fear conditioning testing induced Fos in the DGCs, and expression levels were similar between groups ($t_{31} = 0.44, P = 0.67$) (Figure 3-7C,E). Most notably, the likelihood of Fos expression in BrdU+ cells did not differ between groups ($t_{31} = 0.55, P = 0.58$) (Figure 3-7F), suggesting that DGCs generated during the postnatal period and adulthood are equally likely to become integrated into DG circuits supporting contextual fear memory. We further replicated these findings in a separate experiment using a within subject design (Figure 3-S5). Therefore, using a task with quite different stimulus properties and performance demands (but nonetheless dependent upon the hippocampus) these data provide convergent evidence that developmentally-generated and adult-generated DGCs are functionally recruited at similar rates by hippocampal memory networks.
Figure 3-7. Postnatally- and Adult-Generated Dentate Granule Cells are Recruited at Similar Rates by Hippocampal Circuits Supporting Contextual Fear Memory. (A) Mice were treated with BrdU either at P7 or as adults (P60). At P100 both groups were trained, and contextual fear memory tested 1 month later. (B) Levels of conditioned freezing were similar in both groups. (C) Low magnification examples of NeuN, Fos, and BrdU immunofluorescence in the DG following contextual fear testing in the postnatal- (P7) and adult- (P60) treated groups (Scale bar = 100 µm). (D) Number of BrdU+ cells was highest in the P7 group, reflecting higher rates of developmental neurogenesis. (E) Following contextual fear memory testing, Fos expression in the DG was similar in both groups. (F) Fos expression in BrdU+ cells was similar in both groups, suggesting that postnatally- and adult-generated cells are integrated at similar rates into DG circuits supporting contextual fear memory.


3.5 Discussion

The persistence of neurogenesis in the hippocampus beyond development means that the adult DG is composed of a heterogeneous pool of developmentally- and adult-generated granule cells. While both developmentally- and adult-generated DGCs are believed to contribute to hippocampal memory processing, whether they represent functionally-equivalent or distinct pools of neurons is not known. Here we used immunohistochemical procedures to study the activation of developmentally- and adult-generated DGCs following memory recall. Four main lines of evidence suggest that developmentally- and adult-generated DGCs were equally likely to contribute to hippocampal memory formation. First, using a water maze task, we found that after adult-generated DGCs reached 5 weeks of age, functional activation rates stabilized. There was no evidence for a transient peak in integration rates that might correspond with known changes in plasticity in maturing, adult-generated DGCs (Ge et al., 2007). Second, 7.5 week-old DGCs are recruited by circuits supporting water maze memory at similar rates, regardless of whether they were born during embryonic or postnatal development or during adulthood. Third, while the adult DG is composed of neurons generated during development and during adulthood, we found corresponding activation rates for embryonically-, postnatally- and adult-generated DGCs in our within-animal comparison. Finally, we found similar rates of recruitment for developmentally- and adult-generated DGCs in another hippocampus-dependent task—contextual fear conditioning. Collectively, these results provide convergent evidence that developmentally- and adult-generated DGCs are recruited at similar rates by hippocampal memory networks, and suggest a functional equivalence between DGCs generated at different developmental stages.

In these studies we quantified the expression of the IEG, Fos, in order to identify newborn DGCs exhibiting memory-related activation. Fos expression is regulated by neural activity and therefore has been used to map neural activation following, for example, seizures (Dragunow and Robertson, 1987; Morgan et al., 1987; Saffen et al., 1988), induction of NMDA-dependent LTP (Cole et al., 1989; Dragunow et al., 1989; Worley et al., 1993), noxious stimulation (Hunt et al., 1987) as well as learning and/or memory recall (Guzowski et al., 2005). Here we showed that both memory recall and stimulation of DG afferents leads to the specific upregulation of Fos in DGCs, and that another IEG product, Arc, is similarly upregulated in the same subpopulation.
While several studies have established that newborn DGCs express IEGs in various experimental situations (Farioli-Vecciochi et al., 2008; Jessberger and Kempermann, 2003; Kee et al., 2007b; Ramirez-Amaya et al., 2006; Takahashi et al., 2009; Tashiro et al., 2007), the major challenge in memory studies is to develop experimental designs that can differentiate between gene expression associated with memory processing from that associated with non-specific aspects of the testing procedure. Our experiments typically included three sequential components—treatment of mice with thymidine analogs (BrdU, IdU, CldU), training and memory testing—the relative timing of which was manipulated across experiments. The critical feature common to all experiments is that all groups underwent exactly the same experience prior to sacrifice. That is, all mice were removed from their HC, transported to the testing room and expressed a spatial or fear memory. Therefore, this ensures that observed between-group differences in Fos expression in thymidine analog-labeled cells (see Figs. 2F and 6E) cannot be attributed to these general features of the testing experience. Accordingly, here we confirmed that activation of newborn DGCs by hippocampal circuits supporting water maze memory is regulated by cell age, with newborn DGCs not maximally contributing until they are 5 weeks or older (Kee et al., 2007b). This delayed time course for functional recruitment follows the establishment and gradual maturation of excitatory connections of newborn DGCs (Piatti et al., 2006; Zhao et al., 2008).

In this study, spatial memory recall induced Fos expression in only a small proportion of mature adult-generated DGCs, consistent with the idea that spatial information is sparsely encoded in the DG (Chawla et al., 2005; Jung and McNaughton, 1993). Subsequent experiments established that these levels were not different to those in developmentally-generated DGCs. This was observed across a range of experimental conditions: When the age of developmentally- and adult-generated DGCs was matched (experiment 3), in a within-mouse comparison (where animal age was fixed, but cell age varied; experiment 5) and using a contextual fear conditioning paradigm (experiment 7). Not in one experiment was the null hypothesis—that developmentally- and adult-generated DGCs are integrated into hippocampal memory circuits at equivalent rates—rejected, and therefore, collectively, these experiments provide no support that the alternative—that developmentally- and adult-generated DGCs are integrated into hippocampal memory circuits at different rates—is true. The lack of evidence for preferential recruitment of adult-generated DGCs is consistent with previous studies establishing that, once they reach maturity, adult-generated DGCs exhibit similar neuronal phenotypes as developmentally-generated DGCs.
(Laplagne et al., 2006; Laplagne et al., 2007; Zhao et al., 2006). For example, developmentally-
and adult-generated DGCs eventually establish equivalent afferent and efferent connectivity,
exhibit similar morphology and electrophysiological properties (excitability and short- and long-
term plasticity) (Ge et al., 2007; Laplagne et al., 2006; Laplagne et al., 2007; Toni et al., 2008;
Toni et al., 2007; Zhao et al., 2006). Therefore, in addition to this phenotypic convergence at the
anatomical, morphological, and electrophysiological levels, the current series of experiments
provide evidence for similar convergence at the behavioral level. They suggest that
developmentally- and adult-generated DGCs are interchangeable, as long as they are sufficiently
mature. Such phenotypic convergence is typical in other organ systems, including skin, blood,
muscle, liver etc and would be advantageous for potential cell replacement therapies: That is,
that stimulation of adult neurogenesis would lead to the repopulation of the DG with
functionally-equivalent cell-types (or replacing like with like).

Our experiments do not support the idea that adult-generated DGCs are recruited preferentially
relative to developmentally-generated cells, and stands in contrast to the conclusions of some
previous studies (Ramirez-Amaya et al., 2006; Tashiro et al., 2007) including our own (Kee et
al., 2007b). In these previous studies, BrdU was injected into adult mice to label adult-generated
DGCs (as in the current experiments). However, unlike the current experiments,
developmentally-generated DGCs were not labeled directly. Rather, IEG expression was
examined in BrdU-labeled (adult-generated) cells vs. NeuN-labeled cells in the same animals.
NeuN may be expressed in cells as young as 3 days-old (Brandt et al., 2003). Therefore, as the
DG is composed of a heterogeneous pool of immature and mature NeuN+ cells (and adult-
generated DGCs < 2 weeks-old do not express Fos (Jessberger and Kempermann, 2003)), these
analyses likely underestimate recruitment rates for developmentally-generated cells. In the
current experiments, we confirmed that the likelihood of Fos expression in BrdU+ cells was
significantly higher than in NeuN+ cells (Figs. 2F and S2).

Our finding that developmentally- and adult-generated DGCs are recruited at similar rates is not
incompatible with the idea that adult neurogenesis plays a critical role in hippocampal memory.
Indeed, suppression of adult neurogenesis disrupts multiple forms of hippocampus-dependent
learning, including the water maze and contextual fear conditioning (Deng et al., 2010; Shors,
2008). This suggests that (at least in some conditions) normal levels of neurogenesis are essential
for hippocampal memory function. Instead, it might be more appropriate to consider that the
critical contribution of adult neurogenesis to hippocampal memory function is at the network, rather than cellular, level. For example, the addition of new DGCs into hippocampal circuitry may degrade existing memories (Meltzer et al., 2005), and such degradation may promote the transformation of memories from hippocampus-dependent to hippocampus-independent forms in the cortex (Feng et al., 2001; Kitamura et al., 2009).

Comparison of activation rates of developmentally- and adult-generated DGCs makes it possible to estimate the overall contribution of adult-generated DGCs to hippocampal memory networks. Given our finding that developmentally- and adult-generated cells were recruited at similar rates, the contribution of adult-generated DGCs to hippocampal memory processing should be directly proportional to their number. In young adult mice, adult-generated cells constitute as much as 15% of the DG (Imayoshi et al., 2008; Ninkovic et al., 2007), and so 15% of DGCs contributing to memory-related activity in the DG would be predicted to have been generated during adulthood. As levels of neurogenesis are modulated across the lifespan (Kuhn et al., 1996) and by environmental factors, such as enrichment and/or exercise (Kempermann et al., 1997b; van Praag et al., 1999b), this model would then predict that under conditions where levels of adult neurogenesis decline (e.g., with age (Kuhn et al., 1996)), the contribution of adult-born neurons to hippocampal memory function declines proportionally.

Finally, in order to label separate cohorts of new neurons within the same animal, we used the thymidine analogs CldU and IdU along with immunohistochemical procedures designed to recognize one or the other exclusively (Burns and Kuan, 2005; Dupret et al., 2007; Thomas et al., 2007; Tronel et al., 2010; Vega and Peterson, 2005). Although techniques vary amongst these reports and can lead to cross-detection (Leuner et al., 2009), we established that our injection and immunohistochemistry methods did not result in nonspecific labeling of one compound by both primary antibodies. Consistent with (Leuner et al., 2009), we also found no difference in the sensitivity of cell-labeling between the two compounds (although sensitivity levels may be below that of BrdU).
3.6 Supplemental | Supporting Experiments

3.6.1 Materials and Methods

3.6.1.1 Sensitivity and Specificity of IdU and CldU

In experiment 4 (Figure 3-4), we verified the sensitivity and specificity of CldU- and IdU-labeling and detection within animals that received one or the other compound at P7, and the converse at P60, as well as within animals that received both drugs simultaneously at P60. In order to test the remaining possible drug treatment combinations in adult animals, four groups of adult mice received either 2 days of CldU injections (n = 3), 2 days of IdU injections (n = 3), 2 days of CldU injections followed 2 weeks later by 2 days of IdU injections (n = 3) or 2 days of IdU injections followed 2 weeks later by 2 days of CldU injections (n = 3) (Figure 3-S3A,B). For illustrative purposes in Figure 3-S3, data from adult animals given 2 days of simultaneous CldU and IdU injections (n = 3) are shown. Animals were sacrificed 1 day following the completion of injections, and tissue handling matched that of the other experiments. Staining was carried out on all groups using primary and secondary antibodies for both CldU and IdU regardless of which compound(s) was(were) injected. 50-100 cells were examined per animal with a 40X/1.3 objective on a confocal microscope.

3.6.1.2 Stability of New Neuron Recruitment

We tested whether adult-generated DGCs are stably recruited by DG circuits supporting contextual fear memory (Figure 3-S4A-E). Mice were trained in contextual fear conditioning 6 weeks after BrdU treatment, and then separate groups were tested either 1 day (n = 9) or 1 month (n = 18) later.
3.6.1.3 **Anatomical Specificity of New Neuron Recruitment**

We tested the specificity of our observed age-dependent functional activation of new neurons by circuits supporting contextual fear memories in the DG by examining another brain region where new neurons are added during adulthood—the olfactory bulb (Figure 3-S4F-H). Mice received BrdU injections, underwent contextual fear conditioning 1 (n = 6) or 6 (n = 8) week(s) later, were tested 10 weeks following BrdU treatment and sacrificed 90 min following testing. Two 50 µm sections containing the granule cell layer of the olfactory bulb were selected from each animal. A confocal microscope was used to quantify Fos+, BrdU+ and Fos+/BrdU+ cells using the optical dissector, stereological method. Cells were counted within two counting frames per section (area of grid squares and counting frames were 100 × 100 µm).

3.6.1.4 **New Neuron Recruitment During Contextual Fear Conditioning (Within Animal Comparison)**

We compared recruitment rates of developmentally- and adult-generated DGCs by contextual fear memory networks in the DG (Figure 3-S5). Mice were injected with CldU and IdU at P7 and P60 respectively (n = 10). Mice were trained in the contextual fear conditioning task 7.5 weeks later, and fear memory was assessed 1 day following training.

3.6.2 **Results**

3.6.2.1 **Water Maze Training**

In the three water maze experiments (Figures 3-2,3,5), learning was evident in all groups (Figure 3-S1A,B,C) through a progressive reduction in escape latencies over the course of training ($F_{4,268} = 223.6, P < 0.001; F_{4,108} = 60.8, P < 0.001; F_{4,96} = 52.2, P < 0.001$ respectively). Furthermore, escape latencies did not differ between experimental groups, suggesting equivalent training performance ($F_{4,67} = 0.87, P = 0.49; F_{2,27} = 0.99, P = 0.38; F_{1,24} = 2.02, P = 0.17$ respectively).
Figure 3-S1. Escape Latencies During Water Maze Training. Learning was evident in all corresponding groups from experiments 2 (A, Figure 3-2), 3 (B, Figure 3-3) and 5 (C, Figure 3-5) through a progressive reduction in escape latencies over the course of training. Furthermore, escape latencies did not differ between experimental groups, suggesting equivalent training performance.

3.6.2.2 Fos Expression in Directly and Indirectly-Labeled Developmentally-Generated Dentate Granule Cells

Some previous studies, including our own, suggested that adult-generated DGCs are recruited preferentially relative to developmentally-generated cells (Kee et al., 2007b; Ramirez-Amaya et al., 2006; Tashiro et al., 2007). In these previous studies, BrdU was injected into adult mice to label adult-generated DGCs (as in the current experiments). However, unlike the current experiments, developmentally-generated DGCs were indirectly identified as all NeuN+ cells. NeuN may be expressed in cells as young as 3 days-old (Brandt et al., 2003). Therefore, as the DG is composed of a heterogeneous pool of immature and mature NeuN+ cells (and adult-generated DGCs < 2 weeks-old do not express Fos (Jessberger and Kempermann, 2003)), these analyses likely underestimate recruitment rates for developmentally-generated cells. In a supplemental analysis, we used pooled data from all compatible experiments to test the likelihood of Fos expression in BrdU- and XdU-labeled adult-generated DGCs relative to NeuN+ DGCs. Animals from all water maze and contextual fear conditioning groups that underwent training 6-7.5 weeks following BrdU- or XdU-labeling of adult-generated DGCs were included (n = 161). Fos expression in BrdU- or XdU-labeled adult-generated DGCs was greater than that in NeuN+ cells (Figure 3-S2) (paired t-test = 3.10, P < 0.005). Together with our findings that directly-labeled adult- and developmentally-generated DGCs are recruited similarly by DG
memory circuits, this large sample pooled analysis suggests indirect methods of labeling developmentally-generated DGCs can underestimate their recruitment rates.

Figure 3-S2. Fos Expression in Adult-Generated and NeuN+ Dentate Granule Cells. Pooled data from water maze and contextual fear conditioning experimental animals, who received BrdU or XdU injections 6-7.5 weeks prior to training, demonstrates greater Fos expression in BrdU- or XdU-labeled adult-generated DGCs relative to NeuN+ cells.

3.6.2.3 CldU- and IdU-Labeling of Dentate Granule Cells is Equally Sensitive and Highly Specific

In order to label separate cohorts of new neurons within the same animal, several studies have used the thymidine analogs CldU and IdU along with immunohistochemical procedures designed to recognize one or the other exclusively (Burns and Kuan, 2005; Dupret et al., 2007; Thomas et al., 2007; Tronel et al., 2010; Vega and Peterson, 2005). Since techniques vary amongst these reports and can lead to cross-detection (Leuner et al., 2009), it was necessary to confirm that our CldU and IdU injection and immunohistochemical staining procedures and protocol result in equal sensitivity for the detection of new DGCs and a high degree of selectivity for each marker. While Figure 3-4A-C explored these issues in animals treated at P7 and P60, similar methods were also applied here to mice treated with both compounds at P60. Tissue from animals who received one or the other thymidine analogue, or both separated in time by 2 weeks in either sequence, subjected to primary and secondary antibodies for both, demonstrated virtually 100% specific staining for each marker (Figure 3-S3A,B). Comparable proportions of cells were either CldU+ or IdU+ in tissue from animals receiving both compounds separated in time by 2 weeks, confirming equivalent sensitivity for these markers (Leuner et al., 2009). Finally, tissue stained from animals that received both CldU and IdU simultaneously revealed virtually 100% co-labeling. Together these results indicate that, using our methodology, CldU and IdU can be utilized in the same animal to specifically label cell cohorts with equivalent sensitivity.
Figure 3-S3. Sensitivity and Specificity of CldU and IdU Cell Labeling and Staining.

(A) Representative low magnification images of IdU+, CldU+, and IdU+/CldU+ (merge) DG cells from mice treated with CldU (top row), IdU (second row), CldU followed 2 weeks later by IdU (third row), IdU followed 2 weeks later by CldU (fourth row) and CldU simultaneously with IdU (bottom row) (scale bar = 50 µm). Dotted white lines outline the GCL and SGZ. Lower-right confocal images illustrate a cluster of CldU+/IdU+ cells (scale bar = 20 µm).

(B) Virtually all labeled cells in mice treated solely with CldU were only CldU+ and IdU were only IdU+. When CldU and IdU injections were separated by 2 weeks, virtually all labeled cells were either only CldU+ or IdU+ regardless of the order in which they were given. Simultaneous injection with CldU and IdU resulted in virtually all double-positive cells. Comparable proportions of cells were either CldU+ or IdU+ in animals that received one followed 2 weeks later by the other, suggesting equivalent sensitivity.
3.6.2.4 Stable Recruitment of New Neurons by Circuits Supporting Contextual Fear Conditioning Memory

It is possible that adult-generated DGCs play either a transient or a sustained role in the expression of contextual fear conditioning memory. To distinguish between these two possibilities, two groups of mice were trained 6 weeks after BrdU treatment and then tested 1 day or 1 month later (Figure 3-S4A). In the retention tests mice showed similar levels of freezing at the recent and remote delays ($t_{25} = 0.61, P = 0.55$) (Figure 3-S4B), indicating no change in memory expression over time. As in our previous studies, we identified many BrdU+ cells, and numbers did not differ between groups ($t_{25} = 2.01, P = 0.06$) (Figure 3-S4C). Following contextual fear conditioning testing we identified many Fos+ cells in the DG, and, notably, Fos expression was similar regardless of the delay between training and testing ($t_{25} = 0.00, P = 0.99$) (Figure 3-S4D). This suggests that, while the hippocampus may not play an essential role in the expression of contextual fear memory at remote time points (Kim and Fanselow, 1992), if it is present it is nonetheless engaged (Frankland et al., 2004; Gusev et al., 2005; Teixeira et al., 2006). Most importantly, the likelihood of Fos expression in BrdU+ cells did not differ between groups ($t_{25} = 0.13, P = 0.89$) (Figure 3-S4E), suggesting that once recruited, new neurons are stably maintained in circuits supporting contextual fear memories as a function of time.
3.6.2.5 *Age-Dependent Recruitment of New Neurons by Circuits Supporting Contextual Fear Memories Does Not Occur in the Olfactory Bulb*

Neural precursors in the SVZ generate neurons that ultimately populate the olfactory bulb (Ming and Song, 2005; Zhao et al., 2008). In order to evaluate the anatomical specificity of our effects, we also examined olfactory bulb sections from animals following contextual fear memory testing. Adult mice received BrdU injections, contextual fear conditioning 1 or 6 week(s) later, and testing 10 weeks following BrdU treatment. Analyses of BrdU and Fos expression (Figure 3-S4F-H) revealed similar numbers of BrdU+ ($t_{12} = 1.14, P = 0.28$), Fos+ ($t_{12} = 1.58, P = 0.14$) and Fos+/BrdU+ ($t_{12} = 0.36, P = 0.73$) cells following the contextual fear memory testing in the 1...
week and 6 week groups. This suggests that the cell age-dependent pattern of recruitment is limited to the DG, and does not extend to other brain regions that are not thought to play a primary role in the storage of contextual fear memories (as demonstrated previously for spatial memories (Kee et al., 2007b)).

### 3.6.2.6 Contextual Fear Conditioning (Within Animal Design)

In a similar fashion to the within animal confirmation for water maze memory, we tested whether developmentally- and adult-generated DGCs are recruited at similar rates by hippocampal circuits supporting contextual fear memory within the same animal. Mice were initially treated with CldU at P7, IdU once they reached adulthood (P60) and trained in contextual fear conditioning 7.5 weeks later (Figure 3-S5A). In the contextual fear memory test 1 day later, mice exhibited robust levels of conditioned freezing (75±6%). Following the test, we identified large numbers of CldU+, IdU+ and Fos+ cells. As in our previous experiments, there were considerably more CldU+ cells than IdU+ cells (Figure 3-S5B) (paired t-test: $t_9 = 6.52$, $P < 0.001$), reflecting greater levels of proliferation during the postnatal period. Again, Fos was robustly expressed in DGCs (Figure 3-S5C). Most notably, the likelihood of Fos expression in BrdU+ cells did not differ between the P7 and P60 groups (Figure 3-S5D) (paired t-test: $t_9 = 0.32$, $P = 0.76$). Therefore, using a task with quite different stimulus properties and performance demands (but nonetheless dependent upon the hippocampus) these data provide convergent evidence that developmentally- and adult-generated DGCs are functionally recruited at similar rates by hippocampal memory networks.
Figure 3-S5. Recruitment of P7 and P60 Dentate Granule Cells by Hippocampal Circuits Supporting Contextual Fear Memory (Within Animals). (A) Experimental design. Mouse pups were treated with CldU at P7 and then with IdU at P60. They were trained in contextual fear conditioning 7.5 weeks later, and fear memory was tested 1 day following training. (B) Number of XdU+ cells was higher in the P7 than P60 group, reflecting higher rates of developmental neurogenesis. (C) Following fear memory testing, expression of Fos in DGCs. (D) Likelihood of Fos expression in XdU+ cells following fear testing was similar in both groups, suggesting that developmentally- and adult-generated DGCs are integrated into DG circuits supporting contextual fear memory at similar rates.
Chapter 4  Stimulation of Entorhinal Cortex Promotes Adult Neurogenesis and Facilitates Spatial Memory

This chapter is modified from the following:

4.1 Abstract

Deep brain stimulation (DBS) is an established therapeutic modality for the treatment of movement disorders, and an emerging therapeutic approach for the treatment of disorders of mood and cognition. For example, recently we have shown DBS of the fornix may ameliorate cognitive decline associated with dementia. However, like other applications of DBS, the mechanisms mediating these clinical effects are unknown. As DBS modulates neurophysiological activity in targeted brain regions, DBS might influence cognitive function via activity-dependent regulation of hippocampal neurogenesis. Using stimulation parameters analogous to clinical high frequency DBS, here we addressed this question in mice. We found that acute stimulation of the entorhinal cortex (EC) transiently promoted proliferation in the dentate gyrus (DG). Cells generated as a consequence of stimulation differentiated into neurons, survived for at least several weeks and acquired normal dentate granule cell (DGC) morphology. Importantly, stimulation-induced promotion of neurogenesis was limited to the DG, and not associated with changes in apoptotic cell death. Using immunohistochemical approaches, we found that, once sufficiently mature, these stimulation-induced neurons were functionally recruited by hippocampal spatial memory processing. Finally, formation of water maze memory was facilitated 6 weeks (but not 1 week) following bilateral stimulation of the EC. The delay-dependent nature of these effects matches the maturation-dependent recruitment of adult-generated neurons by dentate circuits supporting water maze memory. Furthermore, as the beneficial effects of EC stimulation were prevented by blocking neurogenesis, this suggests a causal relationship between stimulation-induced promotion of adult neurogenesis and enhanced spatial memory.
### 4.2 Introduction

DBS uses surgically implanted electrodes to deliver electrical stimulation to precisely targeted areas in the brain. To date more than 60,000 patients have been implanted with deep brain electrodes (Ponce and Lozano, 2010), and its predominant application has been in the treatment of movement disorders, most commonly Parkinson’s disease (PD) (Deuschl et al., 2006; Koller et al., 1997; Vidailhet et al., 2005). However, studies have also begun to explore its potential application to a widening array of neurologic and psychiatric conditions, including disorders of mood and thought. For example, DBS of limbic circuits has shown promise in treating refractory depression (Mayberg et al., 2005), while DBS of basal ganglia circuitry has been used in the treatment of obsessive compulsive disorder (Mallet et al., 2008) and Tourette syndrome (Shahed et al., 2007). Moreover, we recently reported a phase 1 trial investigating DBS of the limbic system for the treatment of dementia/cognitive impairment (Laxton et al., 2010). In this trial, DBS of the fornix was associated with arrest or slowing of cognitive decline in certain patients, measured by two different scales of cognitive functioning. While the mechanism(s) of action underlying these clinical effects, and DBS in general, remain(s) poorly understood (Kringelbach et al., 2007), it is clear that DBS modulates the activity of targeted brain circuits (Davis et al., 1997; Mayberg et al., 2005). For instance, in our trial, fornix stimulation strongly activated the hippocampus and parahippocampal gyrus including the EC (Laxton et al., 2010).

One potential mechanism through which DBS might influence cognitive function is via activity-dependent regulation of hippocampal neurogenesis. New neurons are continuously added to the adult mammalian DG, a sub region of the hippocampus that plays an essential role in memory formation (Ming and Song, 2005; Zhao et al., 2008). Once sufficiently mature, these adult-generated DGCs eventually develop similar cellular phenotypes to developmentally-generated DGCs (Laplagne et al., 2006; Laplagne et al., 2007), and are thought to contribute to the formation of hippocampus-dependent memory (Deng et al., 2010; Shors, 2008). Consistent with the idea that activity regulates neuron production in the adult DG, stimulation of limbic targets in rodents (e.g., the anterior thalamic nucleus (Encinas et al., 2011; Toda et al., 2008), EC/perforant path (Bruel-Jungerman et al., 2006; Chun et al., 2006; Kitamura et al., 2010), or mossy fibers (Derrick et al., 2000) promotes the proliferation and/or survival of adult-generated DGCs in vivo.
While these studies demonstrate that stimulation of hippocampal afferents can reliably increase the production of new neurons, whether these stimulation-induced increases in neurogenesis impact hippocampal function is not known. Our experiments indicate that new granule cells, born as a consequence of EC stimulation, mature normally and assume functional roles in hippocampal circuits supporting spatial memory. Furthermore, stimulation-induced promotion of hippocampal neurogenesis facilitates spatial memory formation, suggesting that enhanced neurogenesis is one mechanism via which DBS may have pro-cognitive effects.
4.3 Materials and Methods

4.3.1 Mice

Male offspring from a cross between C57Bl/6NTacfBr [C57B6] and 129Svev [129] mice (Taconic) were used in these experiments. All mice were bred in the colony at The Hospital for Sick Children, housed in groups of 3-5 mice per cage, maintained on a 12 h light/dark cycle with free access to food and water, and were 8 weeks old at the start of experimentation. Behavioral procedures were conducted during the light phase of the cycle, blind to the treatment condition of the mouse and according to protocols approved by the Animal Care Committee at The Hospital for Sick Children.

4.3.2 Stereotactic Surgery

Mice were pre-treated with IP atropine sulfate (0.1 mg/kg), anesthetized with chloral hydrate (400 mg/kg, IP) and placed in a stereotaxic frame. The scalp was incised and skull hole(s) drilled. Targets with coordinates (mm) relative to bregma in the anteroposterior, mediolateral, and dorsoventral planes were as follows: EC bordering on the lateral and medial entorhinal areas, specifically near the junction of the ventral intermediate, medial, and caudal entorhinal fields (van Groen, 2001) [-4.0, 3.0, 5.1]; DG [-2.0, 1.6, 2.0]; 4th and 5th cerebellar lobules [-6.2, 1.0, 2.0] (Paxinos and Franklin, 2000).

4.3.3 Fluorescent Anterograde Tracer Labeling

1.5 µl of anterograde tracer (0.82 mg/ml fluorescent-labeled 10 KDa dextran [fluoro-ruby D-1817; Invitrogen]) was injected at 0.15 µl/min unilaterally into the EC using a glass micropipette (outer diameter approximately 50 µm) connected to an infusion pump-driven (SP100i; WPI) Hamilton (Co.) syringe. The micropipettes were left in place an additional 10 min to ensure diffusion.
4.3.4 Brain Electrical Stimulation

Electrical stimulation was delivered via concentric bipolar electrodes (CBASC75; FHC), detailed electrical properties of which have been described previously (Gimsa et al., 2005; Gimsa et al., 2006). Electrode integrity was verified by impedance measurement. Stacked contacts include a 100 μm long x 125 μm diameter stainless steel outer pole, 100 μm separation, and 100 μm long x 25 μm diameter platinum/iridium inner pole. Targeting accuracy was refined to ±0.5 mm in the anteroposterior and mediolateral planes and +/- 0.25 mm in the dorsoventral plane during pilot experiments. Stimulation was applied with a clinical screener (model 3628; Medtronic) using frequency (130 Hz) and pulse width (90 μs, square wave) settings approximating high frequency DBS used in clinical practice (Volkmann et al., 2006). Current (0-500 μA), duration (30-120 min), and laterality (uni- or bilateral) of stimulation varied by experiment. Charge density, calculated using electrode geometry (Gimsa et al., 2005; Gimsa et al., 2006; McCreery et al., 1990), was kept below 30 μC/cm²/phase to minimize the risk of stimulation-induced neural injury (McCreery et al., 1990).

4.3.5 Retroviral Labeling of New Neurons

New neurons were labeled by CAG promoter-driven GFP expression following infection with a replication-deficient retroviral vector (based on the Moloney murine leukemia virus) (Tashiro et al., 2006b). Viral vector was prepared by transfecting Plat-gp cells with two plasmids containing an amphotropic envelope (vsvg) and the transgene (pCAG-GFP), followed by collection through ultra-speed centrifugation. Plat-E cells were then infected to generate a stable virus-producing cell line and concentrated virus solution was obtained by ultra-speed centrifugation (average 3.5 x 10⁹ iu/ml). In experimental mice, 1.5 μl/DG of virus solution was infused at 0.12 μl/min via a glass micropipette (outer diameter approximately 50 μm) connected to an infusion pump-driven (SP100i; WPI) Hamilton (Co.) syringe. Micropipettes were left in place an additional 10 min to ensure diffusion.
4.3.6 BrdU, CldU, and IdU Administration

The thymidine analogues BrdU (Sigma), IdU (MP Biomedicals) or CldU (Sigma) were dissolved in 0.1 M PBS and heated to 50–60 °C, at a concentration of 10 mg/ml. BrdU IP injection dosages and dosing intervals are described in the “experimental procedures” section below. IdU and CldU were given IP at equimolar concentrations to 50 mg/kg of BrdU (corresponding to 57.5 mg/kg of IdU and 42.5 mg/kg of CldU) 3 times/day (8 h apart) for 3 consecutive days as indicated in the “experimental procedures” section below.

4.3.7 Temozolomide (TMZ) Administration

TMZ (Sigma) was dissolved in dimethyl sulfoxide (DMSO), diluted in PBS to a concentration of 2.5 mg/ml (10% DMSO), and administered IP at a dose of 25 mg/kg once daily for 3 consecutive days according to a previous protocol (Garthe et al., 2009). Vehicle solution (V) was the identical DMSO/PBS solution but without TMZ, and was administered in volumes consistent with TMZ dosing.

4.3.8 Water Maze Apparatus and Procedures

The apparatus and behavioral procedures have been previously described (Chapter 3; (Teixeira et al., 2006)). In brief, a circular water maze tank (120 cm diameter, 50 cm deep), located in a dimly-lit room, was filled to a depth of 40 cm with water (maintained at 28 ± 1 °C and made opaque by adding white, non-toxic paint). A circular escape platform (10 cm diameter) was submerged 0.5 cm below the water surface, in a fixed position in 1 quadrant. White curtains with distinct cues painted on them surrounded the pool, each ≥ 1 m from the pool perimeter.

Prior to training, mice were individually handled for 2 min each day over 7 consecutive days. Mice were trained with one of two protocols: standard training over 5 days with 6 training trials/day (presented in 2 blocks of 3 trials; inter-block interval ~ 1 h, inter-trial interval ~15 s) or undertraining over 3 days with 3 trials/day (inter-trial interval ~15 s). On each trial mice were placed into the pool, facing the wall, in 1 of 4 start locations (the order of which pseudo-
randomly varied throughout training). The trial was complete once the mouse found the platform or 60 s had elapsed. If the mouse failed to find the platform on a given trial, the experimenter guided the mouse onto the platform. Following the completion of training, spatial memory was assessed in a series of 3 probe tests with an inter-test interval of approximately 3 min. In this test the platform was removed from the pool, and the mouse was allowed 60 s to search for it.

Behavioral data from training trials and probe tests were acquired and analyzed using an automated tracking system (Actimetrics). General training measures included latency to reach the platform and path length. Search strategies during training were analyzed using numerical parameters from swim tracking data (adapted from (Garthe et al., 2009)), and the respective predominant search strategy for each trial was objectively classified by a criterion-based algorithm. Trials were classified as one of the following ordered strategies (tracking criteria in parentheses): “Direct Swim” characterized by a maintained heading towards the platform (< 15 cm path length, or heading < 22.5° away from platform at each 5 cm point starting at 15 cm path length), “Focal Search” characterized by highly localized search near the platform (> 50% trial in 15 cm radius target zone, centered on the platform location); “Directed Search” characterized by a preference for a corridor towards the platform or platform quadrant (< 50% trial outside a 50 cm wide corridor from start point to platform and > 20% in 25 cm target zone; or > 40% in target quadrant); “Chaining” characterized by searching near the correct radial distance of the platform to the wall (> 75% trial 20-50 cm from pool center, < 15% within 10 cm of wall, and < 10% within 20 cm of pool center); “Scanning” characterized by a preference for the central pool area where distal cue visibility is maximal (> 50% trial within 35 cm of pool center); “Thigmotaxis” characterized by maintaining close proximity to the wall (> 70% trial within 10 cm of wall); “Perseverance” characterized by an erroneous preference for a non-target area (> 60% trial in 1, or > 75% in 2 adjacent non-target quadrant(s) and > 750 cm path length); and “Random Search” characterized by no other discernable strategy (remaining unclassified trials).

Probe test performance was quantified in 3 ways: the amount of time mice searched the target zone (15 cm or 20 cm [for the TMZ experiment]) vs. the average of the 3 other equivalent zones in other areas of the pool, the number of platform location crossings vs. the average of the 3 other equivalent platform locations in other areas of the pool, and the frequency with which mice visited areas of the pool represented as a density plot (or heat map, with hot colors corresponding to more frequent visits) generated using Matlab (MathWorks).
4.3.9 Immunohistochemistry

Mice were perfused transcardially with 0.1 M PBS and 4% PFA. Brains were removed, fixed overnight in PFA, and transferred to 0.1 M PBS. Fifty µm horizontal (for some tracer analyses) or coronal sections were cut using a vibratome (VT1200S; Leica). TUNEL staining was performed using the DeadEnd Calorimetric TUNEL System (Promega) and cresyl violet counterstaining. For other analyses, the following primary antibodies were used: rabbit polyclonal anti-Fos (1:1000; Calbiochem), rat monoclonal anti-BrdU for BrdU and CldU specifically (1:500; Accurate Chemicals), mouse monoclonal anti-BrdU for IdU specifically (1:1000; BD Biosciences), mouse monoclonal anti-NeuN (1:1000; Chemicon), rabbit polyclonal anti-GFP (1:500; Invitrogen), Alexa-488 conjugated mouse monoclonal anti-NeuN (1:1,000; Chemicon). Detailed BrdU/IdU/CldU/Fos/NeuN staining procedures have been previously described (Chapter 3). In brief, sections were washed and incubated for 48 h at 4°C with primary antibodies and then 2 h at 20°C with the following fluorescent-conjugated or biotinylated secondary antibodies: Alexa-488 anti-rat (1:500; Molecular Probes), Alexa-568 anti-mouse (1:500; Molecular Probes), Alexa-568 anti-rabbit (1:500; Molecular Probes), Rhodamine Red-X-conjugated anti-mouse (1:500; Jackson ImmunoResearch), FITC-conjugated anti-rat (1:500; Jackson ImmunoResearch), Biotin-SP-conjugated anti-rabbit (1:500; Jackson ImmunoResearch). Biotinylated antibody signals were visualized using Alexa-Fluor conjugated Streptavidin (Invitrogen). Sections were slide-mounted with Permafluor anti-fade medium (Lipshaw Immunon).

4.3.10 Imaging and Quantification

All images were acquired using epifluorescent (BX61; Olympus) or confocal laser scanning (LSM710; Zeiss) microscopes, and displayed as maximum intensity projections of Z-stack images created using Image-Pro (Media Cybernetics) or ZEN (Zeiss) software. A laser pinhole setting of 1 Airy unit and Z-stack image spacing of 0.5-1.0 µm (spanning the regions of interest)
were used for all confocal imaging. These produced serial optical slices of 0.8 \( \mu \text{m} \) and 1.0 \( \mu \text{m} \) under 100X and 40X objectives, respectively.

Fluorescent tracer distribution was qualitatively assessed from survey images acquired using 10-40X objectives on the epifluorescent microscope. The presence of tracer within neuronal projections was verified using confocal microscopy. DG regional analysis was performed using serial sections covering the entire anterior-posterior extent of the DG, grouped by thirds as anterior, middle, or posterior.

BrdU+, CldU+, IdU+, GFP+, Fos+, and XdU+/Fos+ cells were quantified using a 40X objective on the epifluorescent microscope. DG total cell numbers were estimated stereologically per side using systematic random sampling fractions of 1/4 serially cut coronal sections covering the entire anterior-posterior extent of the DG. The tops of nuclei served as unique characteristic points for exhaustive direct cell counting using Stereo Investigator (MicroBrightField) software. Sample totals were then multiplied by the inverse sampling rate (4) to give total DG estimates. After identification under the epifluorescent microscope, apparent double-labeled cells were confirmed by confocal microscopy.

Area densities of subventricular zone BrdU+ cells were derived using a method modified from Bath et al. (Bath et al., 2008). A systematic random sample fraction of 1/4 serially cut coronal sections, covering approximately 1.54 to 0.50 mm relative to bregma, was used. All BrdU+ nuclei within 0.1 mm of the lateral ventricle ependymal surfaces were counted per side, marking the tops of nuclei as unique characteristic counting points, using a 40X objective on the epifluorescent microscope and Stereo Investigator software. For each section counted, reference lines tracing each ependymal surface were drawn using Stereo Investigator software. Area densities were then represented by dividing total cell counts by total reference line lengths for each side (giving \( \text{BrdU+ cells} / [1.0 \text{ mm reference line} \times 0.1 \text{ mm subventricular zone depth}] \), or \( \text{BrdU+ cells}/0.1 \text{ mm}^2 \)).

To estimate the proportions of IdU+ and CldU+ cells that were co-labeled with NeuN following unilateral surgery, DG regions of interest were randomly-selected from 5 mice and analyzed using confocal microscopy. A total of 300 IdU+ and 300 CldU+ cells were analyzed (i.e., 30 cells per side per mouse). The proportions of BrdU+ cells co-labeled with NeuN following bilateral surgery and drug treatment were identically derived from 5-8 mice/group.
Neuron tracings and spine counts were manually collected from 0.5 µm spaced Z-stack images acquired with a 100X objective on the epifluorescent microscope and using Neurolucida (MicroBrightField) software. In each of 3 mice in the 6 week group, 6 ipsilateral and 6 contralateral neurons were analyzed (for a total of 18 ipsilateral and 18 contralateral neurons from 3 mice). Dendritic architecture and spines were quantified for each neuron in its entirety (i.e., Spines were counted along the entire dendrite length and on all dendrite branches for each neuron). Dendritic architecture was classified as aberrant if there was a dendritic process with spines pointing toward the hilus, and granule cells were considered ectopic if located > 2 cell layers away from the inner border of the granule cell layer (Jessberger et al., 2007b). Total dendrite length, nodes, and linear spine density (total spines divided by total dendrite length for each branch order) were quantified using Neurolucida software. Axon distribution was qualitatively assessed from survey images of the CA3 region using confocal microscopy.

4.3.11 Experimental Procedures

Experiments were conducted as follows (summarized in Table 4-1):

To characterize EC projections to the DG, mice received unilateral injections of fluorescent tracer into the EC (n = 5) and were perfused 7 d later.

Stimulation-induced DG activation was assessed by placing mice (n = 6) under anesthesia for 3 h prior to surgery. Unilateral EC stimulation was delivered for 1 h (50 µA). Anesthesia was maintained for an additional hour before perfusion.

Stimulation-induced effects on DG proliferation and apoptosis were determined after unilateral EC stimulation (50 µA for 1 h). Following stimulation, mice were treated with a single 200 mg/kg BrdU injection 1 (n = 5), 3 (n = 4), 5 (n = 4), or 7 (n = 4) d later, and perfused 24 h post-injection. Additional non-stimulated mice underwent electrode insertion, with no current delivery, followed by BrdU 1 (n = 5), 3 (n = 6), 5 (n = 4), or 7 (n = 4) d post-operatively.

To examine the effects of varied stimulation durations and currents on cell proliferation mice received unilateral EC stimulation. Three d later they received a single 200 mg/kg BrdU injection and were perfused 24 h later. To examine the impact of stimulation duration on
proliferation, 50 µA was delivered for 30 (n = 5), 60 (n = 4), or 120 (n = 5) min. To examine the effect of current intensity on proliferation, mice received 50 (n = 4), 250 (n = 5), or 500 (n = 5) µA stimulation for 1 h.

The proportion of stimulation-induced newly born cells adopting a neuronal phenotype was assessed following unilateral EC stimulation (50 µA for 1 h, n = 5). Mice received IdU injections during the period of stimulation-induced increased proliferation (post-operative days 3-5), CldU injections during a similar period of baseline proliferation (post-operative days 7-9), and were perfused approximately 10 weeks later.

To assess stimulation-induced effects on cell survival, mice were treated twice daily with 100 mg/kg BrdU injections (8 h apart) for 3 consecutive days, either 1, 10, or 30 d (n = 8 each) prior to unilateral EC stimulation (50 µA for 1 h), and perfused 3 weeks post-operatively.

Subventricular zone proliferation following unilateral EC stimulation, and DG proliferation following unilateral cerebellar stimulation, were assessed in separate experiments using single 200 mg/kg BrdU injections 3 d post stimulation (50 µA for 1 h). Both experiments included stimulated and non-stimulated groups (n = 4 each) perfused 24 h post-BrdU injection.

Retroviral-mediated labeling of adult-born DGCs was performed by bilateral viral solution injections into the DG 3 d after unilateral EC stimulation (50 µA for 1h). Mice were perfused 1 or 6 week(s) (n = 3 each) later.

The long-term survival of stimulation-induced newly born neurons and their functional recruitment by spatial memory networks, relative to similarly aged cells, was assessed following unilateral EC stimulation (50 µA for 1 h). Mice received IdU injections during the period of stimulation-induced increased proliferation (post-operative days 3-5) and CldU injections during a similar period of baseline proliferation (post-operative days 7-9). Water maze standard training occurred 6 weeks (n = 17, includes 5 mice used for NeuN phenotype experiment) or 1 (n = 15) week later, followed by probe testing 4 or 9 weeks later respectively. Mice were perfused 90 min after probe testing.

EC stimulation-induced changes in spatial memory performance were assessed in bilaterally stimulated (50 µA for 1 h, n = 25) or non-stimulated (n = 26) mice who underwent water maze (undertraining protocol) 6.5 weeks later and subsequent probe testing 1 h after the last training
trial. In a second experiment stimulated (n = 25) and non-stimulated (n = 26) mice were trained (undertraining protocol) 1.5 weeks after surgery. In the third experiment, mice were first trained (undertraining protocol), followed 1 d later by stimulation (n = 24) or non-stimulation (n = 24) surgery and subsequent probe testing 1 week later. In the final experiment, animals received V or TMZ injections followed on the next day by bilateral stimulation (V-S, n = 24; TMZ-S, n = 24) or non-stimulation (V-NS, n = 17; TMZ-NS, n = 17). Six and a half weeks after surgery, animals were trained in the water maze (undertraining protocol) and received a probe test 1 h after the last training trial. In order to assess the proportion of stimulation-induced newly born cells adopting a neuronal phenotype, BrdU was given IP at a dose of 50 mg/kg/injection 3 times/d (8 h apart) for 3 consecutive days to subsets of animals in each group: on post-operative days 3-5 (V-S, n = 8; TMZ-S, n = 5; V-NS, n = 8; TMZ-NS, n = 5) during the period of stimulation-induced increased proliferation, or for 3 d following the completion of water maze testing (V-S, n = 5; TMZ-S, n = 7; V-NS, n = 7; TMZ-NS, n = 5) approximately ~7 weeks post-operatively.
Table 4-1. Summarized Experimental Procedures.

<table>
<thead>
<tr>
<th>Experiment (Figure)</th>
<th>Treatment</th>
<th>Cell Labeling</th>
<th>Perfusion</th>
<th>Maze Training</th>
<th>Maze Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>anterograde tracer (4-1a-d)</td>
<td>unilateral EC tracer infusion</td>
<td>-</td>
<td>7 d post infusion</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S-induced Fos (4-1e-h)</td>
<td>unilateral EC S</td>
<td>-</td>
<td>1 h post S</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S-induced DG proliferation (4-2), apoptosis (4-4f-h)</td>
<td>unilateral EC S, NS</td>
<td>BrdU 1, 3, 5, or 7 d post S</td>
<td>24 h post BrdU</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S-induced DG survival (4-3d,e)</td>
<td>unilateral EC S, NS</td>
<td>BrdU 1, 10, or 30 d before S</td>
<td>3 w post S</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S-induced SVZ proliferation (4-4a-c)</td>
<td>unilateral EC S, NS</td>
<td>BrdU 3 d post S</td>
<td>24 h post BrdU</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cerebellar S (4-4d,e)</td>
<td>unilateral cerebellar S, NS</td>
<td>BrdU 3 d post S</td>
<td>24 h post BrdU</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>retroviral labeling of DGCs (4-5)</td>
<td>unilateral EC S, NS</td>
<td>bilateral DG retrovirus infusion 3 d post S</td>
<td>1 or 6 w post infusion</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S-induced DG neurogenesis (4-3a-c) and DGC integration (4-6)</td>
<td>unilateral EC S, NS</td>
<td>IdU 3-5 d, CldU 7-9 d post S</td>
<td>~ 10 w post XdU</td>
<td>standard training ~ 1 or 6 w post XdU</td>
<td>~ 10 w post S</td>
</tr>
<tr>
<td>S effects on spatial memory (4-7,8)</td>
<td>bilateral EC S, NS</td>
<td>-</td>
<td>-</td>
<td>undertraining 1.5 or 6.5 w post, or 1 d prior to S</td>
<td>1 h post training or 1.5 w post S</td>
</tr>
<tr>
<td>TMZ/S effects on spatial memory (4-9)</td>
<td>TMZ or V prior to bilateral EC S</td>
<td>BrdU 3-5 d or ~7 w post S</td>
<td>~ 7 w post S</td>
<td>undertraining 6.5 w post S</td>
<td>1 h post training</td>
</tr>
</tbody>
</table>

(BrdU, 5-bromo-2’-deoxyuridine; CldU, 5-Chloro-2’-deoxyuridine; DGC, dentate granule cell; EC, entorhinal cortex; IdU, 5-Iodo-2’-deoxyuridine; TMZ, temozolomide; NS, non-stimulation; S, stimulation; V, vehicle)

4.3.12 Statistical Analyses

Behavioral (excluding swim strategy) and cell count data (excluding scatter plots) are presented as mean ± SEM and were evaluated using ANOVAs or t-tests (2-tailed). Duncan’s MRT was used for post hoc analyses of significant main effects or interactions where appropriate. Swim strategy frequencies were compared across trials within groups using Friedman’s test, and over all trials between groups using Pearson’s chi-square test for independence. Scatter plot data were analyzed using Pearson’s correlation.
4.4 Results

4.4.1 Entorhinal Cortex Projects Ipsilaterally to the Dentate Gyrus

The EC provides the main afferent input to the DG (Amaral and Lavenex, 2007). As neurogenesis is regulated by neural activity (Zhao et al., 2008), stimulation of these afferents provides a direct method to increase adult neurogenesis. To characterize this projection, we first injected a fluorescent, anterograde tracer into the EC (Figure 4-1a). Our injection site was centered on the junction of the ventral intermediate, medial, and caudal entorhinal fields (van Groen, 2001) (Figure 4-1b). Viewed in horizontal sections, labeled axons extended anteromedially from the injection site. They coursed through the angular bundle region to the subiculum, past CA1-3 and around the hippocampal fissure via the stratum lacunosum moleculare, eventually reaching the ML of the DG. Focal dilations and club-shaped terminals were evident along this path, and were especially dense in the outer two-thirds of the ML, a pattern that is consistent with terminal labeling of fibers from both the medial and lateral perforant paths (Amaral and Lavenex, 2007). Fibers also coursed along the alveus, consistent with direct alvear projections to the subiculum and CA1 (Amaral and Lavenex, 2007). In addition, projections also reached the presubiculum, and to a lesser extent, the parasubiculum (van Groen and Wyss, 1990). Labeling was virtually restricted to the side ipsilateral to the injection site, consistent with previous reports that the EC projection to the DG is predominantly ipsilateral (van Groen et al., 2002; van Groen et al., 2003). Furthermore, terminal labeling was densest in the middle and posterior regions of the DG (Figure 4-1c,d), consistent with known topographical organization of EC-DG connections (van Groen et al., 2002; van Groen et al., 2003).
Figure 4-1. Entorhinal Cortex Projects Ipsilaterally to the Dentate Gyrus. (a) Tracer distribution was examined 7 days following unilateral EC target injection (n = 5). (b) NeuN stained (green) horizontal section (upper 2 panels, scale bar = 200 µm) showing tracer (red) distribution ipsi- (I, left panels) and contralateral (C, right panels) to EC target site of injection (dotted oval) (al, alveus; ca1-3, cornu ammonis regions 1-3; hf, hippocampal fissure; lec, lateral EC; mec, medial EC; ml, molecular layer; pa, parasubiculum; pr, presubiculum; s, subiculum; sim, stratum lacunosum moleculare). Squares in upper panels indicate perforant path regions magnified in corresponding lower panels (scale bar = 5 µm). (c) Sagittal schematic indicating anterior (Ant), middle, and posterior (Post) thirds of DG. (d) Representative DG coronal sections ipsi- (I, left panels) and contralateral (C, right panels) to tracer injection. Molecular layer afferents were labeled in the middle and posterior (lower two panels) regions of the ipsilateral DG (gcl, granule cell layer; numbers indicate anteroposterior distance to bregma; scale bar = 50 µm). (e) Fos expression was examined 1 h following unilateral stimulation (n = 6). (f) DAPI (blue) stained coronal section showing DG Fos (red) expression ipsi- (I, left panel) and contralateral (C, right panel) to stimulation (scale bar = 50 µm). (g) Fos expression increased in middle (Mid) and posterior (Post) regions of the ipsilateral (I), but not contralateral (C), DG. (h) Representative coronal sections ipsi- (I, left panels) and contralateral (C, right panels) to stimulation from anterior (upper panels), middle (middle panels) and posterior (lower panels) DG regions (as per schematic (c), scale bar = 20 µm). * P < 0.05
We next asked whether stimulation of the EC would increase activity in the DG. To do this we lowered an electrode into the same EC site, and delivered high frequency stimulation for 1 h under chloral hydrate anesthesia. Our stimulation parameters (50 µA, 90 µs pulse width delivered at 130 Hz) were chosen to model those used for high frequency DBS clinically (Volkmann et al., 2006). Sixty min following the completion of stimulation we assessed expression of the activity-regulated gene, Fos (Figure 4-1e). Consistent with the tracing experiment, Fos expression was elevated in the DG GCL (Figure 4-1f-h), suggesting that stimulation led to sustained activation of DGCs. The increase in Fos expression was limited to the side ipsilateral to the site of stimulation, consistent with the finding that the EC-DG projection is predominantly unilateral. Moreover, increased Fos expression was largely confined to the middle and posterior portions of the DG, a pattern that matches the topographical organization of terminal labeling following injection of anterograde tracer into the same site (Figure 4-1g,h). An ANOVA with Side (ipsilateral, contralateral) and Region (anterior, middle, posterior) as within-subject factors supported the anatomical specificity of these effects. Most critically, there was a significant Side × Region interaction ($F_{2,10} = 6.13, P < 0.05$), reflecting more pronounced changes in Fos expression in the ipsilateral middle and posterior regions of the DG (Duncan’s post-hoc tests comparing ipsi- and contralateral sides, $P’s < 0.05$).

4.4.2 Entorhinal Cortex Stimulation Transiently Increases Adult Neurogenesis in the Dentate Gyrus

We next asked whether stimulation of the EC would increase proliferative activity in the DG. To do this, mice were injected with the proliferation marker, BrdU, either 1, 3, 5 or 7 days after stimulation of the EC. Twenty-four h later, BrdU incorporation was quantified in the DG (Figure 4-2a). EC stimulation increased the number of BrdU-labeled cells (Figure 4-2b,c). An ANOVA, with Side (ipsilateral, contralateral) as a within-subjects variable and Stimulation (stimulated, non-stimulated) and Day (1, 3, 5, 7) as between-subject variables, revealed that these increases were anatomically-specific and delay-dependent (significant 3-way Side × Stimulation × Day interaction, $F_{3,28} = 3.90, P < 0.05$): Increased BrdU incorporation was limited to the ipsilateral side, and to groups injected with BrdU 3-5 days following stimulation (Duncan’s post-hoc tests, $P’s < 0.05$). These results indicate that the pro-proliferative effects of EC stimulation are not
immediate, but rather emerge after a few days. In addition, contralateral to the electrode site, BrdU incorporation was equivalent in both stimulated and non-stimulated mice (Duncan’s post-hoc tests, $Ps > 0.05$). This indicates that stimulation does not modulate proliferation contralateral to the electrode site, and therefore the contralateral side may be used as a within-animal control for basal rates of proliferation following unilateral stimulation of the EC. Finally, in order to explore the topographical specificity of the stimulation-induced changes in proliferation in detail we divided the DG into equal thirds from the anterior to posterior pole. In the 3 day group, we found that increased proliferation was confined to the middle and posterior ipsilateral DG (Figure 4-2d; Side × Region ANOVA, significant interaction, $F_{2,10} = 7.73, P < 0.01$; Duncan’s post-hoc tests comparing ipsi- and contralateral sides, $Ps < 0.01$). This topographical organization is consistent with the tracing and IEG analyses.

**Figure 4-2. Entorhinal Cortex Stimulation Increases Proliferation in Adult Dentate Gyrus.** (a) Separate groups of mice were injected with BrdU at different delays following unilateral stimulation or electrode insertion only ($n = 4-6$ per group). (b), BrdU+ cells (green) ipsi- (I, left panels) and contralateral (C, right panels) to electrode site (gcl, granule cell layer; h, hilus; DAPI counterstain; upper panels scale bar = 50 µm, lower panels scale bar = 10 µm). (c), BrdU incorporation in the ipsi- (I) and contralateral (C) DG in stimulated (S) vs. or non-stimulated (NS) mice at different post-surgery delays. (d), Stimulation increased proliferation in the middle (Mid) and posterior (Post) ipsilateral DG regions. (e), Proliferation was increased following 60 or 120 min of stimulation (BrdU was injected 3 d post-stimulation) ($n = 4-5$ per group). (f), Proliferation was equivalently increased in the ipsilateral DG following stimulation at 50, 250, and 500 µA (BrdU was injected 3 d post-stimulation) ($n = 4-5$ per group). * $P < 0.05$, ** $P < 0.01$
We next evaluated how stimulation duration and intensity impacts DG proliferation. First, to examine the impact of duration, mice were stimulated for 30, 60 or 120 min using the same stimulation parameters as before. They were then injected with BrdU 3 days later, and, as previously, BrdU incorporation was assessed 24 h later. Increasing stimulation duration increased proliferation ipsilateral to the electrode site (Side × Duration ANOVA, significant interaction, \( F_{2,11} = 5.39, P < 0.05 \)) (Figure 4-2e): There were more BrdU-labeled cells following 60 min (compared to 30 min) stimulation (Duncan’s post-hoc test, comparing 30 min and 60 min on ipsilateral side; \( P < 0.01 \)), but no additional benefit of further increasing stimulation duration to 120 min (Duncan’s post-hoc test, comparing 60 min and 120 min on ipsilateral side; \( P = 0.42 \)). Second, to examine the impact of current intensity on proliferation mice received 50, 250 or 500 \( \mu \)A of stimulation for 60 min. They were then injected with BrdU 3 days later, and BrdU incorporation was assessed 24 h later. While stimulation-induced changes in GCL/SGZ proliferation were limited to the ipsilateral side, there were no effects of stimulation intensity (Side × Intensity ANOVA, significant main effect of Side only, \( F_{1,11} = 45.76, P < 0.01 \)) (Figure 4-2f), suggesting that there might be a ceiling on these stimulation-induced changes in proliferation. Indeed, at 250 \( \mu \)A and 500 \( \mu \)A, BrdU-labeled cells were additionally found in the hilus and ML, suggesting that high intensity stimulation may additionally promote proliferation of non-neuronal cell-types (Madsen et al., 2005) and/or aberrant neurogenesis analogous to that seen following seizures (Parent et al., 2006; Parent et al., 1997).

Under basal conditions, about 70-90% of cells generated in the adult SGZ differentiate into neurons (Brandt et al., 2003). To evaluate whether a similar proportion of cells differentiate into neurons after stimulation of the EC, mice were treated with equimolar doses of IdU and then CldU, two chemically-related thymidine analogs. IdU and CldU are recognized by different antibodies and therefore may be used to label separate cohorts of cells in the same animal (Chapter 3; (Tronel et al., 2010; Vega and Peterson, 2005)). In this experiment, IdU injections occurred 3-5 days following stimulation, a post-stimulation time point corresponding to the peak in stimulation-induced changes in proliferation, and CldU injections occurred 7-9 days following stimulation, a post-stimulation time point when proliferation rates have returned to baseline (Figure 4-3a). Ten weeks later, there were no differences in the proportion of IdU- and CldU-labeled cells expressing the neuronal marker, NeuN, either ipsi- or contralateral to the electrode site (Side × Thymidine Analog ANOVA, main effects and interaction \( Ps > 0.05 \)) (Figure 4-3b,c),
indicating that stimulation did not alter the fate of newly-generated cells. Importantly, the number of IdU-labeled cells was increased on the side ipsilateral to the stimulation site (298.40 ± 14.94) compared to IdU-labeled cells in the contralateral DG (186.72 ± 11.74), and CldU-labeled cells in the ipsi- (192.04 ± 12.29) and contralateral (186.53 ± 11.55) DG. The magnitude of the relative increase following this extended survival period (~10 weeks) is comparable to that observed after a short survival period (1 day; Figure 4-2c), suggesting that cells produced as a consequence of stimulation survive for at least several weeks.

**Figure 4-3. Survival and Differentiation of Stimulation-Induced Neurons.**
(a) Following unilateral stimulation (n = 5), mice were injected with IdU (during the period of increased proliferation) and CldU (once proliferation returned to baseline). (b), Representative confocal image of IdU+ and CldU+ DG cells co-labeled with NeuN (scale bar = 20 µm), ipsilateral to stimulation. (c), Similar proportions of IdU+ and CldU+ cells were NeuN+ ipsi- (I) and contralateral (C) to electrode site. (d), Separate groups of mice were injected with BrdU at different delays before stimulation (n = 8 per group). (e), There were more BrdU+ cells ipsilateral to the stimulation site in the mice treated with BrdU 10 d before surgery. **P < 0.01
We found that stimulation of the EC transiently increased the future production of neurons in the DG. To evaluate whether the same stimulation promotes survival of existing, adult-generated cells we next treated mice with the proliferation marker, BrdU, and then stimulated either 1, 10 or 30 day(s) later (Figure 4-3d). The numbers of BrdU-labeled cells were increased ipsilaterally (vs. contralaterally) in the 10 d (planned comparison, paired t-test, $t_8 = 9.31, P < 0.01$) group, but not in the 1 (planned comparison, paired t-test, $t_8 = 0.43, P = 0.68$) or 30 d (planned comparison, paired t-test, $t_8 = 0.38, P = 0.72$) groups (Figure 4-3e; one-way ANOVA, significant effect of Group, $F_{2,21} = 5.46, P < 0.05$; Duncan’s post-hoc tests, $P’s < 0.05$). Consistent with previous reports (Bruel-Jungerman et al., 2006; Kitamura et al., 2010), these data indicate that EC stimulation has a modest pro-survival effect on existing adult-generated cells in the hippocampus. That these effects were specific to the 10 d group is consistent with the idea that 1-3 week-old neurons are especially sensitive to factors that either promote (e.g., environmental enrichment) or reduce (e.g., stress) survival (Zhao et al., 2008).

### 4.4.3 Specificity of Stimulation-Induced Increase in Proliferation

In the above experiments we identified a set of conditions whereby EC stimulation produces an approximate 1.5 to 2-fold increase in adult neurogenesis. We next explored the specificity of the effects of stimulation.

First, the SGZ of the hippocampus is one of two major neurogenic regions in the adult brain. Does stimulation of the EC additionally regulate neurogenesis in the SVZ? To address this, additional groups of mice were treated with BrdU 3 days following stimulation of the EC (Figure 4-4a). Twenty-four h later, BrdU-labeled cells were evident in the subependymal region of the SVZ (Figure 4-4b). There were similar numbers of BrdU-labeled cells in both the stimulation and non-stimulation groups, and no further differences between the ipsi- and contralateral sides (Figure 4-4c; Side × Stimulation ANOVA, main effects and interaction $Ps > 0.05$), suggesting that EC stimulation specifically regulates hippocampal and not olfactory adult neurogenesis. Similarly, the pro-neurogenic effects following stimulation of another limbic target, the anterior
thalamic nucleus, are limited to the DG (Encinas et al., 2011). This contrasts with the bi-regional neurogenic effect seen following generalized neural stimulation by seizures (Parent et al., 2002).

Figure 4-4. Specificity of Entorhinal Cortex Stimulation Effects on Hippocampal Neurogenesis. (a) Mice were injected with BrdU 3 d following unilateral stimulation or electrode insertion only (n = 4 per group). (b), BrdU+ (green) cells in the subventricular zone (DAPI counterstain) ipsi- (I, left panel) and contralateral (C, right panel) to unilateral EC stimulation (scale bar = 50 µm). (c), There were no differences between area density counts of BrdU+ cells in stimulated (S) or non-stimulated (NS) mice either in the ipsilateral (I) or contralateral (C) SVZ. (d), In a separate experiment, mice were injected with BrdU 3 d following unilateral cerebellar stimulation or electrode insertion only (n = 4 per group). (e), There were no differences between the total BrdU+ cell counts in stimulated (S) vs. non-stimulated (NS) groups.

Second, high intensity, generalized electrical activation of the brain (e.g., ECS, seizures) also increases adult neurogenesis in the DG (Ma et al., 2009; Madsen et al., 2000; Parent et al., 2002). Using our lower current intensity, we next evaluated whether localized stimulation elsewhere in the brain might increase adult neurogenesis in the DG. To do this we targeted the 4th and 5th cerebellar lobules, regions that do not send direct afferent input to the DG (Figure 4-4d). BrdU
incorporation 3 days following stimulation was unaltered by cerebellum stimulation (compared to a non-stimulated control group) (Figure 4-4c; Side × Stimulation ANOVA, main effects and interaction Ps > 0.05), suggesting that low intensity stimulation of non-afferent sites does not regulate adult neurogenesis in the DG.

Third, it is possible that stimulation of the EC might induce cell death in the DG, leading to a compensatory increase in adult neurogenesis (Gould and Tanapat, 1997). Therefore, to evaluate the possibility that changes in neurogenesis are secondary to altered levels of DGC death we additionally stained tissue for TUNEL, a marker of programmed cell death, in stimulated or control mice either 2, 4, 6, or 8 days post-stimulation. While there were regional differences in TUNEL counts, with levels highest in the GCL and lowest in the hilus, no such differences were observed between the ipsi- and contralateral sides, stimulated and non-stimulated groups, or days post-stimulation (Figure 4-4f-h; Side × Region × Stimulation × Delay ANOVA, significant main effect of Region only, $F_{2,28} = 193.94, P < 0.01$). Together, these data suggest that our low intensity EC stimulation does not induce apoptotic cell death in the DG, and therefore exclude the possibility that changes in levels of adult neurogenesis are secondary to stimulation-induced cell death.

4.4.4 Following Stimulation, New Neurons Mature Normally

We found that EC stimulation increases adult neurogenesis in the DG. As these newly-generated neurons survive several weeks, this raises the possibility that they become functionally integrated into hippocampal circuits. To evaluate this possibility we used a retroviral vector strategy to label dividing neural progenitors and their progeny with GFP (Tashiro et al., 2006b). As GFP is expressed throughout the cell body and processes, using this strategy it is possible to track morphological changes in newborn cells at different stages following stimulation. Accordingly, mice received unilateral stimulation and then, 3 days later, bilateral infusion of retrovirus encoding GFP into the DG. This 3 day post-stimulation time point corresponds to the peak of stimulation-induced changes in proliferation. Mice were killed either 1 or 6 week(s) later.
In the 1 week group (Figure 4-5a), there were approximately twice as many GFP-labeled cells on the side ipsilateral to the stimulation site (Figure 4-5b,c; paired t-test, $t_2 = 4.64, P < 0.05$), reflecting stimulation-induced changes in proliferation. Both the magnitude and the anatomical specificity of this increase replicate our BrdU incorporation data. Most of GFP-labeled cells were located in the SGZ or innermost part of the GCL, and had short, aspiny, processes running either parallel to or, occasionally, perpendicular to the GCL. In rare instances these perpendicular processes extended through the GCL (but never into the ML). No axon fibers were visible in CA3. Consistent with previous reports (Jessberger et al., 2007b; Zhao et al., 2006), the morphology of these GFP-labeled cells resembled immature neurons. A small minority of cells (~2%) were ectopically located in the hilus or deep within the GCL. Importantly, the frequency of these ectopically located cells was similar ipsi- (1.77%) and contralateral (1.74%) to the electrode site (paired t-test, $t_2 = 0.05, P = 0.96$), indicating that stimulation did not alter the normal migration and early stages of integration of adult-generated neurons in the DG.
Figure 4-5. Morphological Integration of Stimulation-Induced Neurons into Hippocampal Networks. (a) Unilaterally-stimulated mice received bilateral GFP-retrovirus injections into the DG 3 days after surgery (during the period of increased proliferation) and were examined 1 week later (n = 3). (b) Average GFP+ cell counts per DG section were higher ipsi- (I) vs. contralateral (C) to stimulation. (c) Representative images ipsi- (I, left 3 panels) and contralateral (C, right 3 panels) to stimulation at low (upper panels, scale bar = 50 µm) and high (lower panels, scale bar = 10 µm) magnification showing immature-appearing GFP+ (green) neurons (red, NeuN counterstain). (d) A separate group was examined 6 weeks after retroviral injections (n = 3). (e) Average GFP+ cell counts per DG section were higher ipsi- vs. contralateral to stimulation. (f) Representative images ipsi- (I, left 7 panels) and contralateral (C, right 7 panels) to stimulation. Low (upper row panels, scale bar = 50 µm) and high (middle row panels, scale bar = 20 µm) magnification images show examples of mature-appearing neurons. GFP+ axons and terminals were present bilaterally in CA3 (lower row left panels, scale bar = 20 µm). High magnification images of apical dendrites (lower row right panels, scale bar = 10 µm) demonstrate relatively aspiny proximal (P, upper panels), and spiny distal (D, lower panels) portions of apical dendrites. (g-i) Similar total dendrite length (g), average nodes per neuron (h), and progressive increase in linear spine density by branch order (i) ipsi- vs. contralateral to stimulation. * P < 0.05, ** P < 0.01
Six weeks post-infection (Figure 4-5d), there were many GFP-labeled cells resembling mature neurons. As before, there were approximately twice as many GFP-labeled cells on the side ipsilateral to the stimulation site (Figure 4-5e,f; paired t-test, $t_2 = 16.28, P < 0.01$), suggesting that cells, produced as a consequence of stimulation, survive for at least several weeks. The vast majority of GFP-labeled cells were located in the SGZ or innermost part of the GCL. Typically, dendritic processes extended through the GCL with minimal or no branching, but arborized extensively in the ML. Furthermore, there were abundant spines on these apical dendritic processes, especially within the ML, suggesting that these newly-generated neurons had become synaptically integrated into DG circuitry. GFP-labeled axonal fibers were also present in the CA3 region, consistent with previous reports suggesting that by 6 weeks of age adult-generated neurons have established efferent connections with CA3 (Toni et al., 2008; Zhao et al., 2006).

Most importantly, apical dendrite branch morphology was similar on both the ipsi- and contralateral sides to the electrode site, with equivalent total dendrite length (Figure 4-5g; paired t-test, $t_2 = 0.50, P = 0.67$) and nodes per neuron (Figure 4-5h; paired t-test, $t_2 = 0.09, P = 0.94$). Furthermore, spine density on apical dendritic branches was similar both ipsi- and contralateral to the electrode site (Figure 4-5i; Side × Branch Order ANOVA, significant main effect of Branch Order only, $F_{4,2} = 34.24, P < 0.01$). Together, these analyses suggest that stimulation promotes neurogenesis, and that neurons produced as a consequence of stimulation acquire normal morphology and likely establish normal afferent and efferent connections. Similar to the 1 week post-infection group, a small minority of GFP-labeled cells were ectopically located. The low incidence is consistent with previous reports (Jessberger et al., 2007b; Parent et al., 1997) and, importantly, the frequency was similar on both the ipsi- (1.79%) and contralateral (2.08%) sides (paired t-test, $t_2 = 0.19, P = 0.87$) suggesting that stimulation does not promote the aberrant integration of adult-generated neurons into the DG.

### 4.4.5 New Neurons are Functionally Recruited by Hippocampal Memory Circuits

The morphological analyses indicate that new neurons, generated as a consequence of stimulation, integrate into hippocampal circuitry. Using a water maze task, we next asked whether, once integrated, these adult-generated neurons play a functional role in hippocampus-
mediated spatial learning. To address this, mice were treated with equimolar doses of IdU and then CldU at different times after unilateral stimulation of the EC. As before, IdU injections occurred 3-5 days following stimulation (a time point corresponding to the peak in stimulation-induced changes in proliferation) and CldU injections occurred 7-9 days following stimulation (a time point when proliferation rates have returned to baseline). Six weeks later mice were trained in the water maze, and then spatial memory was assessed in a probe test 4 weeks later (Figure 4-6a). Both acquisition and expression of water maze memories engage the DG, and engagement of dentate granule neurons following memory recall may be assessed by quantifying expression of activity-regulated genes such as Fos (Kee et al., 2007a). Therefore, should stimulation increase the pool of functional new neurons, then the proportion of adult-generated neurons included in the population of activated (Fos-labeled) neurons should increase. Such an increase should be specific to neurons generated 3-5 days following stimulation (i.e., IdU-labeled cells), and, as stimulation is unilateral, occur only on the side ipsilateral to the electrode site (For an expanded explanation of this logic using hypothetical scenarios, please see supplemental Section 4.6).
Figure 4-6. Functional Recruitment of Stimulation-Induced Neurons into Hippocampal Memory Networks. (a) Following unilateral stimulation, mice were injected with IdU during the period of increased proliferation, and CldU once proliferation returned to baseline (n = 17). Mice were trained 6 weeks after CldU treatment, and spatial memory was assessed 4 weeks following the completion of training. (b), Mice searched selectively in the probe test. A density plot for grouped data (left), with accompanying color scale, represents the number of visits per mouse per 5 cm × 5 cm area. Mice spent more time (right graph) searching the target zone (T) compared to other (O) zones. (c), Representative confocal images of tissue following water maze testing showing IdU+, CldU+, and Fos+ DGCs, and a DAPI counterstained merge image with a representative IdU+/Fos+ co-labeled cell (scale bar = 20 µm). (d), Numbers of IdU+ and CldU+ cells ipsi- (I) vs. contralateral (C) to stimulation site. (e), Fos expression in the DG following probe testing was similar on both sides. (f), Following the probe test, the probability of Fos+ cells being XdU+ ipsilateral (I) and contralateral (C) to stimulation (Y-axis notation denotes the conditional probability of a cell being XdU+ given it is Fos+, or P(XdU+|Fos+)). (g), As the number of IdU+ cells increases, so does their contribution to the population of activated neurons (i.e., P(IdU+|Fos+)). (h), A separate group of mice underwent identical treatment except were trained 1 week after CldU treatment, and had spatial memory assessed 9 weeks following the completion of training (n = 15). (i), Mice searched selectively in the probe test (left density plot), spending more time (right graph) searching the target zone compared to other zones. (j), Numbers of IdU+ and CldU+ cells ipsi- (I) vs. contralateral (C) to stimulation site. (k), Fos expression in the DG following probe testing was similar on both sides. (l), The probability of Fos+ cells being XdU+ ipsilateral (I) and contralateral (C) to stimulation for IdU+ and CldU+ cells. (m), The stimulation-induced increase in the availability of adult-generated neurons did not produce a proportional increase in their contribution to the population of activated neurons, suggesting neurons 1 week old at the time of training are not functionally integrated. ** P < 0.01
In the probe test, mice searched selectively, spending more time in the region of the pool that formerly contained the platform (Figure 4-6b; paired t-test, $t_{16} = 5.53$, $P < 0.01$). Consistent with our previous experiments, stimulation of the EC led to an increase in adult neurogenesis. As expected, this increase was specific to the ipsilateral side, and corresponded to new cells generated 3-5 days (i.e., IdU-labeled) post-stimulation (Figure 4-6c-d; Side $\times$ Thymidine Analog ANOVA, significant interaction, $F_{1,16} = 167.76$, $P < 0.01$). Following the probe test, Fos expression was equivalent both ipsi- and contralateral to the stimulation site, suggesting equivalent levels of activation (Figure 4-6e; paired t-test, $t_{16} = 0.98$, $P = 0.34$). Within this population of Fos-labeled neurons there were many IdU- or CldU-labeled cells. Most importantly, stimulation increased the proportion of adult-generated neurons included in this population of activated (Fos-labeled) neurons. Critically, this increase was specific to neurons generated 3-5 days following stimulation (i.e., IdU-labeled cells) and occurred only on the side ipsilateral to the electrode site (Figure 4-6f; Side $\times$ Thymidine Analog ANOVA, significant interaction, $F_{1,16} = 11.33$, $P < 0.01$). Furthermore, this increase was proportional to the magnitude of the stimulation-induced increase in adult neurogenesis (Figure 4-6g; $R = 0.58$, $P < 0.05$). Therefore, these data suggest that EC stimulation increases the production of new neurons, and that these new neurons assume functional roles in hippocampal circuits mediating memory.

The integration of adult-generated neurons into hippocampal memory circuits is maturation-dependent, with new neurons not contributing in maximal numbers until they are $> 5$ weeks in age (Chapter 3; (Kee et al., 2007b)). Therefore, stimulation-induced increases in the contribution of adult-generated neurons should not occur if there is only a brief delay between stimulation and training. Accordingly, we repeated the above experiment but trained mice 1 week (rather than 6 weeks) following IdU/CldU injections (Figure 4-6h). In the probe test 9 weeks later mice searched selectively in the region of the pool formerly containing the platform (Figure 4-6i; paired t-test, $t_{14} = 8.16$, $P < 0.01$). As before, stimulation of the EC led to an increase in adult neurogenesis. This increase was specific to the ipsilateral side, and corresponded to new neurons generated 3-5 days (i.e., IdU-labeled) post-stimulation (Figure 4-6j; Side $\times$ Thymidine Analog ANOVA, significant interaction, $F_{1,14} = 33.48$, $P < 0.01$). Following the probe tests, Fos expression was equivalent both ipsi- and contralateral to the stimulation site, suggesting equivalent levels of activation (Figure 4-6k; paired t-test, $t_{14} = 0.14$, $P = 0.89$). However, this Fos-labeled population of dentate granule neurons contained few IdU- or CldU-labeled cells.
Furthermore, there was no relationship between stimulation-induced increases in neurogenesis and the proportion of adult-generated neurons included in this population of activated (Fos-labeled) neurons (Figure 4-6m; \( R = 0.04, P = 0.89 \)). These data are therefore consistent with the idea that 1 week-old adult-generated neurons are insufficiently mature to be integrated into hippocampal memory circuits (Chapter 3; (Kee et al., 2007b)).

Together, these data suggest that stimulation increases the pool of functional newborn neurons. An ANOVA, conducted on data from both experiments, revealed a significant 3-way Delay × Thymidine Analog × Side interaction (\( F_{1,30} = 4.79, P < 0.05 \)). This supports the conclusion that stimulation-induced increases in contribution of adult-generated neurons to water maze memory are maturation-dependent (they are observed only in the 6 week group), as well as anatomically-selective (only occurring on the side ipsilateral to the electrode site) and temporally-specific (corresponding to neurons generated 3-5 days following stimulation). Furthermore, the temporal relationships between stimulation, labeling (IdU and CldU injections) and testing were equivalent in both experiments. Therefore, as IdU and CldU-labeled cells were equivalently aged at the time of testing, these data indicate that stimulation does not simply lower the threshold for activation (and/or Fos induction) of DGCs.

### 4.4.6 Entorhinal Cortex Stimulation Facilitates Spatial Memory Formation in a Delay-Dependent Manner

We next asked whether stimulation-induced increases in neurogenesis would facilitate water maze learning in adult mice. In order to maximize production of new neurons, mice received bilateral (rather than unilateral) stimulation of the EC, and were trained in the water maze 6.5 weeks later. Control mice were treated identically except that no current was delivered (Figure 4-7a). This 6.5 week stimulation-training delay ensures that additional neurons, produced as a consequence of stimulation, are sufficiently mature (~6 weeks old) to contribute to spatial learning (previous experiment; Chapter 3; (Kee et al., 2007b)). During training, latency to find the platform declined similarly in both groups (Figure 4-7b; \( Day \times Stimulation \) ANOVA, significant main effect of Day only, \( F_{2,49} = 51.23, P < 0.01 \)). Immediately following the
completion of training, spatial memory was assessed in a probe test. In this test, both stimulated and non-stimulated mice searched selectively (Figure 4-7c). However, mice in the stimulation group spent significantly more time in the target zone (Figure 4-7d; unpaired t-test, $t_{49} = 2.02, P < 0.05$) and crossed the former platform location more frequently (Figure 4-7e; unpaired t-test, $t_{49} = 3.83, P < 0.01$) compared to non-stimulated mice, indicating that stimulation of the EC facilitated spatial learning.

**Figure 4-7. Stimulation-Induced Enhancement of Spatial Memory.** (a) 6.5 weeks following bilateral stimulation ($n = 25$) or electrode insertion only ($n = 26$), mice were trained in the water maze and spatial memory was assessed 1 h after the completion of training. (b), Latencies to reach the platform declined equivalently for stimulated (S) and non-stimulated (NS) mice during training. (c), Mice searched selectively in the probe test. Density plots for grouped data, with accompanying color scale, represent the number of visits per mouse per 5 cm x 5 cm area. (d,e), Stimulated mice spent significantly more time searching the target zone (d) and crossing the target platform location (e) than non-stimulated mice. (f), Examples of search strategies with corresponding color labels. (g), Percentage of trials classified in each search strategy category across training days for stimulated (left graph) and non-stimulated (right graph) mice. (h), Percentage difference in the frequency of each search strategy in stimulated relative to non-stimulated mice, indicating significantly greater use of “Direct Swim” and “Focal Search” strategies in stimulated mice. * $P < 0.05$, ** $P < 0.01$
The beneficial effect of EC stimulation on spatial learning was detected in the probe test, but not in the training latency data. As the adoption of either localized/spatially-precise (e.g., focal searching) or some non-localized/spatially-imprecise (e.g., chaining) search strategies may contribute to reduced escape latencies across training (Clapcote and Roder, 2004; Gallagher et al., 1993; Lipp and Wolfer, 1998; Wolfer et al., 1998), latency data can be poor predictors of spatial learning (Clapcote and Roder, 2004). To address whether stimulated and non-stimulated mice adopted different search strategies during training we conducted a detailed analysis of search paths. Using tracking data and a criterion-based algorithm (Garthe et al., 2009), search paths were objectively classified into 8 mutually-exclusive categories (Figure 4-7f). At the beginning of training, Thigmotaxis and Random Search strategies predominated in both groups, accounting for > 75% of paths. In contrast, by the end of training these spatially-imprecise strategies were progressively replaced by more localized/spatially-precise strategies (Figure 4-7g) in both stimulated (Friedman’s test, $\chi^2_{8} = 73.89, P < 0.01$) and control (Friedman’s test, $\chi^2_{8} = 66.66, P < 0.01$) groups. Most strikingly, the overall frequencies of search strategies differed between groups (Chi-square test of independence, $\chi^2_{7} = 6.39, P < 0.05$). This difference was most apparent by an elevated prevalence of Direct Swims and Focal Searches in stimulated relative to non-stimulated mice (Figure 4-7h). Therefore, consistent with the probe test, these analyses provide convergent evidence that stimulation of the EC facilitated spatial learning by promoting the adoption of localized/spatially-precise search strategies.

Whether this stimulation-induced facilitation of spatial learning is mediated by a stimulation-induced enhancement of adult neurogenesis or some other mechanism is not clear. Consistent with previous studies (Chapter 3; (Kee et al., 2007b)), we showed that 6 week-old, but not 1 week-old, adult-generated neurons are incorporated into DG circuits engaged during spatial learning (e.g., Figure 4-6). Therefore, the beneficial effects of EC stimulation would be expected to have a delayed onset if they are mediated by a neurogenic mechanism. To test this we next trained mice 1.5 weeks, rather than 6.5 weeks, following stimulation of the EC (Figure 4-8a). As before, both stimulated and control mice required progressively less time to find the platform during training, and there were no differences between groups (Figure 4-8b; Day × Stimulation ANOVA, significant main effect of Day only, $F_{2,49} = 64.14, P < 0.01$). As before, both stimulated and control mice progressed from predominantly non-localized/spatially-imprecise strategies (e.g., Thigmotaxis and Random Search) at the start of training to predominantly
localized/spatially-precise strategies (e.g., *Direct Swim*, *Focal Search* or *Directed Search*) at the completion of training (Figure 4-8c; Friedman’s test for *Stimulated Group*, $\chi^2_8 = 76.34, P < 0.01$; Friedman’s test for *Non-Stimulated Group*, $\chi^2_8 = 94.72, P < 0.01$). In contrast to the mice trained 6.5 weeks after stimulation, our detailed analyses of swim paths during training revealed no differences in the prevalence of various search strategies between groups (Figure 4-8d; Chi-square test of independence, $\chi^2_7 = 3.30, P = 0.86$). Consistent with this, in the probe test 1 day after the completion of training, both stimulated and control mice searched selectively (Figure 4-8e) and the degree of selectivity did not differ between groups: Both stimulated and non-stimulated mice spent equivalent time searching the target zone (Figure 4-8f; unpaired t-test, $t_{49} = 1.11, P = 0.27$) and crossed the former platform location with similar frequency (Figure 4-8g; unpaired t-test, $t_{49} = 0.60, P = 0.55$). Therefore, spatial learning is not facilitated when training takes place 1.5 weeks after stimulation, suggesting that the beneficial effects of stimulation are delayed in their onset.
Figure 4-8. Specificity of Stimulation-Induced Enhancement of Spatial Memory. (a) 1.5 weeks following bilateral stimulation (n = 25) or electrode insertion only (n = 26), mice were trained in the water maze and spatial memory was assessed 1 h after the completion of training. (b) Latencies to reach the platform declined equivalently for stimulated (S) and non-stimulated (NS) mice during the training period. (c), Percentage of trials classified in each search strategy category across training days for stimulated (left graph) and non-stimulated (right graph) mice. (d), Percentage difference in the frequency of each search strategy in stimulated relative to non-stimulated mice, indicating minimal variability between groups. (e), Mice searched selectively in the probe test. Density plots for grouped data, with accompanying color scale, represent the number of visits per mouse per 5 cm × 5 cm area. (f,g), Percent time searching the target zone (T) compared to other (O) zones, and target platform crossings (T) compared to other platform locations (O) for stimulated (S) vs. non-stimulated (NS) mice in the probe test. (h), Separate groups of mice received bilateral stimulation (n = 24) or electrode insertion only (n = 24) 1 d following the completion of water maze training, and spatial memory was assessed 1.5 weeks after surgery. (i), Latencies to reach the platform declined equivalently for both groups during the training period. (j), Percentage of trials classified in each search strategy category across training days for stimulated (left graph) and non-stimulated (right graph) mice. (k), Percentage difference in the frequency of each search strategy in stimulated relative to non-stimulated mice, indicating minimal variability between groups. (l), Mice searched selectively in the probe test. Density plots for grouped data, with accompanying color scale, represent the number of visits per mouse per 5 cm × 5 cm area. (m,n), Both groups spent more time searching the target zone (m) and crossing the target platform location (n) compared to other zones and platform locations respectively; however, there were no differences in these measures between groups.
These data strengthen the causal link between stimulation-induced increases in adult neurogenesis and facilitated spatial learning. Six week-old (but not 1 week-old) adult-generated neurons are engaged during spatial learning. Stimulation increases neurogenesis, and the facilitatory effects of stimulation-induced changes in neurogenesis do not emerge until 6.5 weeks after stimulation. As a further test of this idea, an additional group of mice received stimulation 1 day after (rather than before) training in the water maze (Figure 4-8h). It is unlikely that new neurons would be retroactively integrated into dentate circuits supporting spatial memory. Therefore, it is unlikely that new neurons, generated as a consequence of stimulation, would contribute to spatial learning in this experiment. As expected, during training both groups of mice required progressively less time to locate the platform (Figure 4-8i; Day × Stimulation ANOVA, significant main effect of Day only, $F_{2,46} = 69.96, P < 0.01$) and progressed from predominantly non-localized/spatially-imprecise to localized/spatially-precise strategies (Figure 4-8j; Friedman’s test for Stimulated Group, $\chi^2_8 = 40.43, P < 0.01$; Friedman’s test for Non-Stimulated Group, $\chi^2_8 = 46.02, P < 0.01$) with equivalent tendency (Figure 4-8k; Chi-square test of independence, $\chi^2 = 1.57, P = 0.98$). Similarly, in the subsequent probe test, both groups searched selectively in the region of the pool that formerly contained the platform (Figure 4-8l). As the degree of selectivity did not differ between groups (Figure 4-8m,n; unpaired t-test for Zones, $t_{46} = 0.32, P = 0.75$; unpaired t-test for Platform Crossings, $t_{46} = 0.19, P = 0.85$), these results exclude the possibility that stimulation of the EC non-specifically facilitates performance in the water maze. Moreover, they also indicate that stimulation does not disrupt the expression of a previously acquired memory.

### 4.4.7 Facilitation of spatial memory is prevented by blocking adult neurogenesis

In the preceding section, facilitation of spatial memory occurred only when stimulation-induced neurons reached functional maturity. This effect did not occur when the stimulation-induced increased population of DGCs were present but functionally immature, effectively simulating a knockdown experiment with otherwise identical conditions. In an extension of this control experiment, stimulation after training also did not enhance spatial memory on subsequent testing. A key strength of these experimental designs is that they involved no additional and potentially
confounding manipulations to block the generation of additional mature adult-generated DGCs; rather, they exploited the known maturation-dependent time course of functional integration for adult-generated DGCs to produce an exceptionally confound-free knockdown. These experiments suggest that our observed enhancement of spatial memory necessitated the presence of additional mature adult-generated DGCs, and points to a contributory causal relationship.

Despite the convergent nature of our experimental results, we cannot fully exclude the possibility that a non-neurogenic mechanism with a similar temporal profile to adult-generated DGC maturation was responsible. To more directly evaluate whether EC stimulation facilitates the formation of spatial memory via a neurogenic mechanism we next examined the impact of inhibiting neurogenesis using the DNA-alkylating agent temozolomide (TMZ). TMZ is a prodrug that is converted to the DNA-alkylating agent 3-methyl-(triazen-1-yl)imidazole-4-carboxamide at physiologic pH, readily crosses the blood-brain barrier (Newlands et al., 1997), is well-tolerated (Hart et al., 2008), and causes a partial suppression of adult neurogenesis (Garthe et al., 2009). Although inhibiting neurogenesis is inevitably associated with the introduction of new potential experimental confounds (see Chapter 2), it does enable us to utilize identical timelines in experimental and control groups and compliments our original methodology. In this experiment, stimulated or non-stimulated control mice were pre-treated with V or TMZ. Six and a half weeks later they were trained in the water maze (3 trials/day for 3 days) and then spatial memory was assessed in a probe test 1 h following the completion of training (Fig. 9a). In order to assess neurogenesis at different post-stimulation time-points, mice were additionally treated with BrdU either 3-5 days post-operatively (i.e., a time point corresponding to the peak in stimulation-induced changes in proliferation) or after water maze testing (a time point when proliferation rates would be expected to have returned to baseline or ~7 weeks post-operatively). In mice treated with BrdU soon after training, BrdU incorporation differed between groups (Fig. 9b,c; ANOVA Group effect, $F_{3.22} = 14.91, P < 0.001$), indicating that proliferation was altered at this early post-stimulation time-point. Most notably, in V-treated mice, EC stimulation increased proliferation, as we had previously observed (Duncan’s post-hoc test, $P < 0.01$), and this stimulation-induced increase was attenuated by TMZ treatment (TMZ-S < V-S, Duncan’s post-hoc test, $P < 0.01$). Additionally, TMZ treatment reduced proliferation by approximately 45% in non-stimulated mice (Duncan’s post-hoc test, $P < 0.05$), as expected. There were no group differences in the proportion of BrdU-labeled cells expressing the neuronal
marker, NeuN (Fig. 9d; ANOVA Group effect, $F_{3.22} = 0.25, P = 0.86$), indicating that these treatment combinations did not alter the fate of newly-generated cells.

During training, latency to find the platform declined similarly in all groups (Fig. 9e; $Day \times Group$ ANOVA, significant main effect of Day only, $F_{2.156} = 27.54, P < 0.001$). However, in the probe test following training the V-treated, stimulated group outperformed all other groups, spending more time in the target zone (Fig. 9f,g; ANOVA on target zone data, Group effect, $F_{3.78} = 4.69, P < 0.01$, Duncan’s post-hoc tests $P < 0.05$). These data therefore replicate our previous finding that EC stimulation facilitates spatial memory formation (V-S group > V-NS group) and, most importantly, show that this enhancement is prevented when the stimulation-induced enhancement of neurogenesis is blocked. Both TMZ and stimulation only transiently altered neurogenesis levels as BrdU incorporation was equivalent across groups in mice treated with BrdU after behavioral testing (Fig. 9h; ANOVA Group effect, $F_{3.20} = 0.13, P = 0.94$) and there were no group differences in the proportion of BrdU-labeled cells expressing the neuronal marker, NeuN (Fig. 9i; ANOVA Group effect, $F_{3.20} = 1.06, P = 0.39$).
Figure 4-9. Suppressing the Stimulation-Induced Increase in Neurogenesis Blocks Spatial Memory Enhancement. (a) 1 day after the completion of vehicle (V) or temozolomide (TMZ) injections, 4 groups of mice underwent bilateral stimulation (S) or electrode insertion only (NS) (V-S, n = 24; V-NS, n = 17; TMZ-S, n = 24; TMZ-NS, n = 17). Six and a half weeks following surgery, all mice were trained in the water maze and spatial memory was assessed 1 h after the completion of training. Subsets of these mice received BrdU injections either during an early post-stimulation period (V-S, n = 8; V-NS, n = 8; TMZ-S, n = 5; TMZ-NS, n = 5) or following the completion of probe testing (V-S, n = 5; V-NS, n = 7; TMZ-S, n = 7; TMZ-NS, n = 5). (b) BrdU+ cells (green) labeled during the early post-stimulation period (NeuN counterstain; scale bar = 50 µm). (c) BrdU incorporation following labeling during the early post-stimulation period. The stimulation-induced increase in proliferation was blocked by TMZ treatment. (d) Similar proportions of BrdU+ cells were NeuN+ in each group when mice received BrdU injections during the early post-stimulation period. (e) Latencies to reach the platform declined equivalently for all groups of mice during the training period. (f) Density plots for grouped data, with accompanying color scale, represent the number of visits per mouse per 5 cm × 5 cm area. (g) V-S mice spent significantly more time searching the target (T) zone compared to all other groups. (h) BrdU incorporation following behavioral tests was similar across groups. (i) Similar proportions of these BrdU+ cells co-stained for NeuN across groups. * P < 0.05, ** P < 0.01
4.5 Discussion

While DBS targeting hippocampal afferents may slow or arrest cognitive decline (Laxton et al., 2010), the underlying mechanisms of action are poorly understood. Using a mouse model, the current study provides three lines of evidence that such pro-cognitive effects are mediated, at least in part, by activity-dependent promotion of hippocampal neurogenesis. First, using parameters analogous to clinical high frequency DBS, we show that targeted stimulation of EC in adult mice produces an anatomically- and temporally-specific increase in DGC production. Relative to cells produced under basal conditions, stimulation-induced cells exhibit comparable rates of neuronal differentiation and long-term survival. Second, new neurons produced as a consequence of EC stimulation assume functional roles in hippocampal circuits. Relative to cells produced under basal conditions they show comparable localization in the DG, mature normally, and integrate into hippocampal networks engaged by spatial memory in a maturation-dependent manner. Third, acute, bilateral stimulation of the EC facilitated spatial memory formation. Facilitation only emerged several weeks following stimulation, and not if stimulation occurred approximately 1 week before or after training. This time course tracks exactly the maturation-dependent time course of integration of adult-generated neurons into spatial memory circuits. Fourth, facilitation of spatial memory formation was prevented by blocking neurogenesis, suggesting that the pro-cognitive effects of EC stimulation are most likely mediated by a neurogenic mechanism.

We found that stimulation for 1 h under anesthesia was sufficient to promote proliferation in the DG. The pro-proliferative effects of EC stimulation had distinct temporal and anatomical profiles. Similar to ECS (Ma et al., 2009), induced seizures (Jiang et al., 2003; Parent et al., 1997) and targeted brain stimulation (Bruel-Jungerman et al., 2006; Toda et al., 2008), increased proliferation occurred in a delayed fashion and peaked 3-5 days following stimulation before returning to baseline. Furthermore, following unilateral stimulation, increased proliferation was limited to the side ipsilateral to the electrode site. This is consistent with previous studies (and our own anterograde tracing data; Figure 4-1) showing that the perforant path projection from layer II medial/lateral EC neurons is almost exclusively ipsilateral in the mouse (Amaral and Lavenex, 2007; van Groen et al., 2002; van Groen et al., 2003). We were able to take advantage
of this temporal and anatomical specificity in our experimental designs. By using unilateral stimulation, the contralateral DG provided a within-animal control for basal levels of neurogenesis. Similarly, by injecting different thymidine analogs at different post-stimulation time points, we were able to compare elevated and basal levels of neurogenesis in the same animal.

Stimulation might increase neurogenesis directly by promoting neuronal differentiation or indirectly by altering apoptotic cell death in the DG. Consistent with previous studies, we found that stimulation did not alter rates of neuronal differentiation or apoptosis, suggesting that these effects are most likely mediated instead by neural progenitor cells in the SGZ (Bruel-Jungerman et al., 2006; Encinas et al., 2011; Kitamura et al., 2010; Toda et al., 2008). The SGZ contains cells with varying degrees of multipotency and capacities for self-renewal and proliferation. The most proliferative of these are type 2 progenitor cells, whose progeny progressively differentiate into DGCs. Stimulation of another limbic target, the anterior thalamic nucleus, promotes adult neurogenesis in the SGZ by increasing the number and mitotic activity of type 2 progenitor cells (Encinas et al., 2011), leading to increased DGC production (Encinas et al., 2011; Toda et al., 2008). Similarly, other pro-neurogenic stimuli such as seizures and ECS, primarily increase neurogenesis through increased progenitor cell proliferation suggesting shared underlying mechanisms (Parent and Murphy, 2008; Zhao et al., 2008).

Perforant path axons synapse on apical dendrites of mature DGCs. Our IEG analysis suggested that our stimulation protocol led to a sustained activation of this population of cells, and such activation is known to promote release of factors that stimulate neurogenesis through increased progenitor cell proliferation (Zhao et al., 2008). For example, ECS induces GADD45B expression, which, in turn, leads to the expression of pro-proliferation and survival factors, such as FGF1 and BDNF, via epigenetic region-specific DNA demethylation (Ma et al., 2009). Similarly, high frequency EC stimulation that both induces LTP at perforant path-DG synapses and promotes adult neurogenesis (Bruel-Jungerman et al., 2006; Chun et al., 2006; Kitamura et al., 2010) also increases the expression of GADD45B (Hevroni et al., 1998) and various growth factors (Patterson et al., 1992). While not the focus of the current study, it is plausible that both whole brain ECS and our focal stimulation promote adult neurogenesis via analogous paracrine neurogenic factor release mechanisms. Indeed, a cascade of activity-induced gene expression and
diffusible factor release/action may account for the observed delay between stimulation and increased proliferation.

Under basal conditions, granule cells generated during adulthood take several weeks to fully mature and establish functional afferent and efferent connections (Toni et al., 2008; Toni et al., 2007; Zhao et al., 2006). Using a retroviral strategy to label adult-generated granule cells, our data indicate that neuronal maturation follows an equivalent time course following unilateral EC stimulation: 1 week post-infection, retrovirally-labeled cells typically had short, aspiny, dendritic processes and axons that had not yet reached the CA3 region, whereas 6 weeks post-infection retrovirally-labeled cells had mature neuronal morphology, with spiny, highly arborized dendritic processes extending into the ML and axons extending into CA3. As labeled neurons had similar properties both ipsi- and contralateral to the stimulation site, these data suggest neurons produced as a consequence of stimulation acquire normal morphology and likely establish normal afferent and efferent connections. Other conditions such as epileptic activity (Jessberger et al., 2007b; Parent et al., 1997) may also lead to a sustained increase in adult hippocampal neurogenesis.

However, following epileptic activity, newly-generated neurons develop abnormal morphology and many become ectopically located in the hilus. In contrast, we found no evidence for abnormal morphology or ectopic localization following acute stimulation, suggesting that the low current intensity stimulation that we used does not promote the aberrant integration of adult-generated neurons into hippocampal circuits.

Previous studies have shown that, once sufficiently mature, adult-generated neurons are activated during memory formation and/or expression (Chapter 3; (Kee et al., 2007b; Tashiro et al., 2007; Trouche et al., 2009)). In our experiments, EC stimulation nearly doubled levels of neurogenesis, and there was a corresponding increase in the numbers of adult-generated neurons in the pool of granule cells activated by the expression of a water maze memory. This suggests that stimulation-induced neurons become functionally integrated into hippocampal networks supporting spatial memory. Importantly, this increase was specific to neurons generated 3-5 days after stimulation and limited to the side ipsilateral to the stimulation site, indicating that EC stimulation does not generally alter the responsivity of DGCs. Moreover, this increase was only evident when stimulation preceded training by 6.5 weeks. This finding is consistent with previous studies showing that recruitment of newborn DGCs by hippocampal circuits supporting water maze memory is regulated by cell age, with newborn DGCs not maximally contributing
until they are 5 weeks or older (Chapter 3; (Kee et al., 2007b)). This delayed time course for functional integration follows the establishment and gradual maturation of excitatory connections of newborn DGCs (Piatti et al., 2006; Zhao et al., 2008).

Most strikingly, bilateral stimulation of the EC facilitated the formation of a hippocampus-dependent water maze memory. In the probe test following training, stimulated mice searched more selectively compared to non-stimulated controls. Detailed analyses of swim paths during training revealed that stimulated mice were more likely to use localized/spatially-precise search strategies (e.g., Direct Swim, Focal Search) and that the increased frequency of these more effective strategies very likely accounts for the improved spatial memory formation. Other manipulations that promote adult neurogenesis sometimes (Clark et al., 2008; Kempermann et al., 1998a; Meshi et al., 2006; Pieper et al., 2010; van Praag et al., 1999a) but not always (Kempermann et al., 2002; Kempermann et al., 1997b) facilitate hippocampal memory formation. Whether facilitation is detected likely depends on a number of factors including magnitude and persistence of increased neurogenesis and task difficulty. For example, in our experiment we used a relatively mild training protocol (3 days, 3 trials/day). Whereas with intensive training escape latencies reach asymptotic levels, under these undertraining conditions latencies do not reach asymptotic levels and this may increase the likelihood of detecting the stimulation-induced facilitation. Similarly, in situations where memory formation is compromised (e.g., in a disease model), it may be easier to detect stimulation-induced facilitation of memory.

In these types of experiments, a major challenge is to evaluate whether changes in neurogenesis are causally related to changes in memory formation. For example, while environmental enrichment both promotes adult neurogenesis and facilitates memory formation, its memory effects are independent of those on neurogenesis (Meshi et al., 2006). In our experiments, stimulation of the EC may produce a range of other effects which, in principle, could contribute to improved spatial memory formation. Indeed, while the predominant projection from layer II EC is to the dentate ML via the medial and lateral perforant paths, EC neurons in this region additionally send direct projections to CA1-3, subiculum, presubiculum, and parasubiculum (Amaral and Lavenex, 2007; van Groen et al., 2002; van Groen et al., 2003; van Groen and Wyss, 1990). There might also be effects on developmentally-generated neurons and/or on the differentiation and maturation of existing adult-generated granule cells, including axonal,
dendritic, and synaptic remodelling and synaptogenesis (Manivannan and Terakawa, 1994; Nagerl et al., 2007; Nikonenko et al., 2003). However, EC stimulation only facilitated spatial memory formation if there was a 6.5 week delay between stimulation and training. When a shorter delay was used (1.5 weeks) stimulation had no effect. The delay-dependent nature of these effects matches exactly the time course for functional integration, and strongly suggests that facilitative effects of EC stimulation are mediated by a neurogenic mechanism. In another experiment, when stimulation occurred after training (but before the probe test), stimulated and non-stimulated controls performed at equivalent levels. This indicates that EC stimulation does not simply facilitate spatial memory retrieval. Most importantly, memory facilitation was fully blocked by pharmacological blockade of the stimulation-induced increase in neurogenesis. Since TMZ would predominantly affect proliferation (and not post-mitotic cells), this experiment reduces the likelihood that these non-neurogenic mechanisms independently contribute to the pro-cognitive effects of EC stimulation. We cannot, however, wholly exclude the possibility that stimulation-induced facilitation of memory formation is additionally mediated by non-neurogenic mechanisms. If this were the case, these additional mechanisms are unlikely sufficient in the absence of stimulation-induced increased neurogenesis, perhaps pointing to a key functional significance of new neurons generated following stimulation vs. other proneurogenic stimuli.

Our experiments suggest that promotion of adult neurogenesis is one plausible mechanism by which DBS might exert pro-cognitive effects. In translating these findings to clinical settings several issues are worth considering. First, mechanisms underlying the pro-cognitive effects of DBS likely include, but need not be limited to, activity-dependent promotion of adult neurogenesis. For example, stimulation may induce neurotransmitter release and local or trans-synaptic modulation of neural activity at the cellular level (Kringelbach et al., 2007), as well as restore basal activity levels within dysregulated brain regions at the circuit level (Laxton et al., 2010; Mayberg et al., 2005). Such effects might act in concert with stimulation-induced changes in neurogenesis to promote cognitive recovery. DBS of hippocampal afferents may additionally have immediate effects on memory that are unlikely to be mediated by a neurogenic mechanism. For example, some of our patients experience vivid autobiographical recall upon initiation of stimulation (Hamani et al., 2008; Laxton et al., 2010)). Also, a recently presented abstract provides preliminary evidence that EC stimulation through temporarily implanted depth
electrodes might acutely facilitate spatial memory in human epilepsy patients (Fried et al., 2011). Second, pro-neurogenic effects of stimulation are not limited to the EC. For example, stimulation of other limbic targets increases adult neurogenesis (Derrick et al., 2000; Encinas et al., 2011; Toda et al., 2008). Whether such stimulation has beneficial effects on cognitive function remains to be determined. Indeed, stimulation of the anterior thalamic nucleus using certain parameters may impair, rather than facilitate, cognitive function in rats (Hamani et al., 2010b), and these effects are consistent with memory impairments reported in some DBS-implanted epilepsy patients (Fisher et al., 2010). Third, while we used short duration stimulation in our experiments, in clinical settings DBS typically involves chronic intermittent or continuous stimulation. The main advantage of using acute stimulation was that it allowed us to isolate effects of stimulation-induced neurogenesis on behaviour. Although more sustained stimulation might bring greater cognitive benefit, it is important to recognize that chronic EC stimulation, albeit likely at higher intensity than utilized here, carries a greater risk of toxicity (Sloviter et al., 1996). Indeed, further studies are needed to define optimal target and stimulation parameters for increasing neurogenesis while avoiding potential detrimental side effects. The advent of non-invasive imaging techniques for assessing neurogenesis in live patients (Manganas et al., 2007; Pereira et al., 2007) may enable some of these studies to occur in the clinical setting. Ultimately, we may be able to titrate DBS therapy to optimize both biological and clinical outcomes, providing a novel therapeutic modality in settings where functional hippocampal regenerative therapy is desirable.
### 4.6 Supplemental | Interpreting Memory-Induced Activation Rates in Expanded Populations of Adult-Generated DGCs

Section 4.4.5 presents spatial recall-induced activation rates of adult-generated DGC populations. IEG expression (regulated by neuronal activity and identified here by Fos-labeling) is measured in cohorts of thymidine analogue-labeled (IdU+ or CldU+, collectively referred to as XdU+) DGCs 90 min after spatial recall. The testing-perfusion interval corresponds to the delay in peak Fos expression following testing. The degree of overlap between XdU+ and Fos+ neurons, within the context of the specific experimental designs, provides an indication of adult-generated DGC involvement in hippocampal memory processing (Chapter 3; (Kee et al., 2007a, b; Ramirez-Amaya et al., 2006; Tashiro et al., 2007)). In this chapter, we examined activation rates of adult-generated DGCs when their numbers were increased by EC stimulation.

Five critical aspects of our experimental design warrant reemphasis (see Section 4.4.5 for further details). First, stimulation was performed unilaterally with a resultant increase in adult-generated DGCs ipsilateral, not contralateral, to stimulation. Second, 2 separate thymidine analogues (IdU and CldU, collectively referred to as XdU) were used to label cells produced during the period of stimulation-induced increased proliferation separately from those produced at baseline. Both compounds, however, were injected within days of each other such that XdU+ DGCs were roughly the same age at the end of experimentation. Third, comparisons were made between sides and between thymidine-analogues in a within-animal design with all mice experiencing exactly the same testing procedure immediately prior to sacrifice. Therefore, differences in numbers of Fos+/XdU+ cells are specifically related to spatial memory processing and not related to nonspecific aspects of the testing procedure (e.g., handling, activity, mild stress or arousal). Fourth, groups of animals were trained 1 or 6 week(s) following stimulationXdU labeling. Six weeks, but not 1 week, is a sufficient delay for newly generated XdU+ DGCs to mature and become capable of integrating into spatial memory networks during training. Fifth, XdU+ DGCs were roughly 10 weeks old at probe testing, at which point they are fully capable of activity-induced Fos expression and thus any group differences in Fos expression in XdU+ DGCs are unlikely to be due to differences in maturation.
The following hypothetical example is meant to supplement conceptually our interpretation of the data in Section 4.4.5 (numerical values here are fictitious and generated for demonstrative purposes only). Figure 4-S1 depicts a doubling of available new neurons (XdU+ cells) ipsilateral to stimulation numerically (represented as an increase from 5 to 10) and pictorially (represented by a larger red square ipsilateral vs. contralateral). Spatial memory recall induced Fos expression in a subset of DGCs, arbitrarily depicted numerically as 100 Fos+ DGCs per DG and pictorially as identically sized blue squares on either side. These 100 Fos+ DGCs can include variable numbers of XdU+ and XdU- DGCs. We can now consider several possible outcomes, and 3 extreme examples are illustrated here. If all available XdU+ DGCs participate in processing the spatial memory (Figure 4-S1, outcome a), then more available ipsilateral XdU+ DGCs will account for a greater proportion of those 100 ipsilateral Fos+ DGCs (increasing from 5/100 to 10/100, also shown by the larger red square overlapping (purple) more of the blue square ipsilaterally). In other words, the probability of a DGC being XdU+ “given” it is Fos+ (written in conditional probability as \( P(XdU+ | Fos+) \)) increases. One can also think of the \( P(XdU+ | Fos+) \) as a reflection of the “contribution” of XdU+ DGCs to the memory-induced Fos+ DGC population. If additional DGCs generated following stimulation do not contribute to the Fos+ population (Figure 4-S1, outcome b), for example if they are somehow non-functional, the \( P(XdU+ | Fos+) \) remains stable (as 5/100, or as stable purple overlap of red and blue squares bilaterally with some ipsilateral red square outside the blue square). In the final sample outcome (Figure 4-S1, outcome c), no XdU+ DGCs integrate at all regardless of the number present and thus the \( P(XdU+ | Fos+) \) remains negligible (0/100 or no overlap between red and blue squares bilaterally). This would be expected if, for example, labeled DGCs were too immature (such as in the case of 1 week old DGCs).
Figure 4-S1. Interpreting Memory-Induced Activation Rates in Expanded Populations of Adult-Generated DGCs. Data presented here is entirely hypothetical. Available new neurons (XdU+ cells) are increased ipsilateral (I) vs. contralateral (C) to stimulation from 5 to 10 (left graph, also depicted by a larger ipsilateral red square). Spatial memory recall induces Fos expression in a subset (100) of DGCs on each side (middle graph, also depicted by identically sized blue squares on either side). Fos+ DGCs can include variable numbers of XdU+ and XdU- DGCs. If all available XdU+ DGCs are recruited by the spatial memory network (outcome a), then XdU+ DGCs ipsilateral to stimulation will account for a greater proportion of those 100 Fos+ DGCs. Consequently, the probability of a DGC being XdU+ "given" it is Fos+ (P(XdU+ | Fos+)) increases from 5/100 to 10/100 (also depicted as the larger purple overlap of red and blue squares). If additional DGCs generated following stimulation do not contribute to the Fos+ population (outcome b), the P(XdU+ | Fos+) remains stable at 5/100 (also depicted as stable purple overlap of red and blue squares with additional ipsilateral red square overlapping the blue square). If no XdU+ DGCs are recruited at all (outcome c) regardless of the number present, the P(XdU+ | Fos+) remains 0/100 (also depicted as no overlap between red and blue squares).
Chapter 5  Future Directions: Does Entorhinal Cortex Stimulation Facilitate Spatial Memory in a Model of Alzheimer’s Disease?
5.1 Abstract

Deep brain stimulation (DBS) is being tested in patients suffering from Alzheimer’s disease (AD) as a means of ameliorating cognitive decline, with favorable preliminary results. We have previously demonstrated that activity-dependent regulation of hippocampal neurogenesis is one potential mechanism for DBS-induced enhancement of cognitive function in wild type mice. However, whether similar improvements in spatial memory afforded by entorhinal cortex (EC) stimulation can be obtained in a transgenic (Tg) neuropathological disease model of AD is unknown. Using similar methodology to our previous study, here we address this question in adult TgCRND8-derived (Tg+) mice that express human amyloid precursor protein harboring Swedish and Indiana familial AD mutations. We found that Tg+ animals exhibited similar baseline rates of proliferation in the dentate gyrus (DG) and subventricular zone (SVZ) to that of our historical wild type controls. Furthermore, acute stimulation of the EC promoted proliferation in the DG. Most importantly, deficient spatial memory formation by Tg+ mice in the Morris water maze shows a trend towards improvement 6.5 weeks following bilateral stimulation of the EC. While we are continuing to further characterize this phenomenon, the stimulation-induced enhancement of spatial memory suggested in this disease model corroborates the promising early clinical outcomes in AD patients and is in keeping with stimulation-induced promotion of adult neurogenesis as a possible underlying mechanism.
5.2 Introduction

AD or a related dementia afflicts roughly 1 in 11 North Americans over age 65 and is the 6th leading cause of death (Association, 2011). Healthcare costs to society associated with the disease are estimated at over 180 billion dollars annually and do not include an immeasurable caregiver burden. Current symptomatic treatments fail to cure or even slow the disease, prompting a need for new therapies including regenerative strategies. In a recent phase 1 trial, we reported promising results in patients suffering from mild AD treated with hypothalamic/fornix DBS (Laxton et al., 2010). Limbic stimulation was associated with a possible arrest or slowing of cognitive decline in certain patients, particularly with respect to recall and recognition testing, along with coincident improvements in an AD-specific quality of life measure. Though the mechanisms underlying these effects are unknown, we theorized that activity-dependent increased hippocampal neurogenesis might be a plausible contributor. Previous work (Chapter 4) demonstrated that acute stimulation of the EC, a limbic target sending direct efferents to the DG, promoted hippocampal neurogenesis in mice. These newly generated cells acquired normal DGC morphology and, once sufficiently mature, integrated into hippocampal circuits supporting water maze memory. Most importantly, formation of water maze memory was facilitated in a delayed fashion matching the maturation-dependent integration of adult-generated neurons into dentate circuits supporting maze memory. While these findings in wild type mice suggest a causal relationship between stimulation-induced promotion of adult neurogenesis and enhanced spatial memory, whether similar stimulation-induced memory improvements can be obtained in a neuropathological model of AD is unknown.

AD is predominantly a sporadic neurodegenerative disease with a complex and incompletely understood etiology involving both environmental and genetic determinants. The disease is histopathologically characterized by neuronal loss (particularly in the EC (Gomez-Isla et al., 1996)) and accumulations of extracellular beta-amyloid (Aβ) plaques and intracellular neurofibrillary tangles of hyperphosphorylated tau protein (Braak and Braak, 1991). A minority of cases are associated with the following known heritable or associated genetic abnormalities: autosomal dominant mutations in genes encoding amyloid precursor protein (APP) on chromosome 21 (Goate et al., 1991), presenilin 1 (PS1) on chromosome 14 (Sherrington et al.,
1995), and presenilin 2 (PS2) on chromosome 1 (Levy-Lahad et al., 1995; Rogaev et al., 1995); trisomy 21 (Wisniewski et al., 1985); and the ε4 allele of the apolipoprotein E gene on chromosome 19 (Strittmatter et al., 1993). Since sporadic and genetic forms of the disease show identical neuropathology, and elevated biogenesis or accumulation of Aβ has been associated with all of these genetic abnormalities, it is has been suggested that excessive Aβ plaque formation represents a key pathogenic event in AD (Hardy and Selkoe, 2002). Accordingly, Tg mice that model Aβ pathology have been developed to study the disease in vivo. One such model, the TgCRND8 mouse, carries a human APP transgene harboring Swedish and Indiana familial AD mutations (Chishti et al., 2001). Despite intact locomotor activity, TgCRND8 animals show age-related increases in Aβ production, plaque deposition, Morris water maze impairments, and mortality (Chishti et al., 2001; Fiorentini et al., 2010; Hyde et al., 2005). Although increasing plaque deposition begins around 9 weeks of age and coincides with the onset of memory impairment, brain Aβ protein levels become elevated relative to wild type animals after around 6 weeks of age (Chishti et al., 2001; Hyde et al., 2005); consequently, roughly 6 weeks of age may represent a window of opportunity in adulthood for interventions to modify the future development of neurological and functional pathology. Adult hippocampal neurogenesis is also reduced by at least 3 months of age in TgCRND8 animals (Fiorentini et al., 2010; Herring et al., 2009), though DG proliferative rates in younger adult animals are not known.

Here, we show that 6 week-old Tg+ mice exhibit ongoing proliferation in both the SVZ and DG, and enhanced DG proliferation following EC stimulation using parameters that also promote hippocampal neurogenesis in wild type animals (Chapter 4). As expected, 12 week-old Tg+ animals exhibited profoundly deficient spatial memory formation relative to littermate controls. More importantly, EC stimulation, followed by a 6.5 week stimulation-training delay, led to a partial rescue of this impairment. While ongoing and future characterizations of this phenomenon are needed, collectively, these beneficial effects of stimulation in an AD model mouse complement the promising findings in the human trial and are in keeping with the idea that enhanced neurogenesis is one mechanism via which DBS may have functionally restorative pro-cognitive effects.
5.3 Materials and Methods

5.3.1 Mice

TgCRND8 mice express a human APP695 transgene with both Swedish (K670N-M671L) and Indiana (V717F) familial AD mutations under the regulation of the Syrian hamster prion promoter (PrP) (Chishti et al., 2001) and were obtained from Dr. David Westaway (University of Alberta, Canada). TgCRND8 mice were maintained in a 129Svev [129] (Taconic) genetic background and crossed with wild type C57Bl/6NTacfBr [C57B6] mice to produce F1 hybrid experimental mice (Tg+). Tg+ and genotype negative littermate (Tg-) mice were bred in the colony at The Hospital for Sick Children, maintained on a 12 h light/dark cycle with free access to food and water, included equal numbers of males and females, and were 6 weeks old at the start of experimentation. Behavioral procedures were conducted during the light phase of the cycle, blind to the treatment condition of the mouse and according to protocols approved by the Animal Care Committee at The Hospital for Sick Children.

5.3.2 Stereotactic Brain Electrical Stimulation

Mice underwent bilateral surgical implantation of concentric bipolar electrodes (CBASC75; FHC) into the EC, followed by electrical stimulation or non stimulation as previously described (Chapter 4). Stimulation was delivered for 1 h at 130 Hz frequency, 90 μs square wave pulse width, and 50 μA current using a clinical screener (model 3628; Medtronic), after which electrodes were removed. These settings match those of our previous work and approximate high frequency DBS used in clinical practice (Volkmann et al., 2006).
5.3.3 BrdU Administration

The thymidine analogue BrdU (Sigma) was dissolved in 0.1 M PBS and heated to 50–60 °C, at a concentration of 10 mg/ml. BrdU was administered as a single 200 mg/kg IP injection, with animals perfused 24 h post-injection.

5.3.4 Water Maze Apparatus and Procedures

The apparatus has been previously described (Chapter 3,4; (Teixeira et al., 2006)). A circular water maze tank (120 cm diameter, 50 cm deep), located in a dimly-lit room, was filled to a depth of 40 cm with water (maintained at 28 ± 1 °C and made opaque by adding white, non-toxic paint). A circular escape platform (10 cm diameter) was submerged 0.5 cm below the water surface, in a fixed position in 1 quadrant. White curtains with distinct cues painted on them surrounded the pool, each ≥ 1 m from the pool perimeter.

Prior to training, mice are individually handled for 2 min each day over 7 consecutive days. Mice are trained over 3 days with 3 trials/day (inter-trial interval ~15 s). On each trial mice are placed into the pool, facing the wall, in 1 of 4 start locations (the order of which pseudo-randomly varied throughout training). The trial is complete once the mouse finds the platform or 60 s had elapsed. If the mouse fails to find the platform on a given trial, the experimenter guides the mouse onto the platform. Following the completion of training, spatial memory is assessed in a series of 3 probe tests with an inter-test interval of approximately 3 min. In this test the platform is removed from the pool, and the mouse is allowed 60 s to search for it.

Behavioral data from training trials and probe tests are acquired and analyzed using an automated tracking system (Actimetrics). General training measures include latency to reach the platform and path length. Probe test performance is quantified in several ways: the amount of time mice search the target quadrant or zone (15, 20, or 25 cm radius) vs. the average of the 3 other equivalent quadrants or zones in other areas of the pool, the number of platform location crossings vs. the average of the 3 other equivalent platform locations in other areas of the pool, and the frequency with which mice visit areas of the pool represented as a density plot (or heat
map, with hot colors corresponding to more frequent visits) generated using Matlab (MathWorks).

### 5.3.5 Immunohistochemistry

Mice were perfused transcardially with 0.1 M PBS and 4% PFA. Brains were removed, fixed overnight in PFA, and transferred to 0.1 M PBS. Fifty µm coronal sections were by cryostat. BrdU antigen was exposed by incubating the sections in 1 N HCl at 45°C for 30 min. Sections were incubated for 48 h at 4°C with rat anti-BrdU monoclonal antibody (1:500; Accurate Chemicals) followed by 2 h at 20°C with Alexa-488 goat anti-rat antibody (1:500; Molecular Probes). Antibodies were diluted in blocking solution containing 2% goat serum, 1% bovine serum albumin, and 0.2% Triton X-100 dissolved in PBS. Sections were slide-mounted with Permafluor anti-fade medium (Lipshaw Immunon).

For Aβ immunohistochemistry, sections will be pre-treated with 1% hydrogen peroxide for 30 min at room temperature, incubated with 70% formic acid in methanol for 10 min, and subsequently washed with PBS. Sections will be incubated for 24 h at room temperature in blocking solution (0.5% bovine serum albumin and 0.2% Triton X-100 dissolved in PBS) containing mouse anti-Aβ-6E10 primary antibody (SIG-39320; 1:500; Covance), and then for 2 h at room temperature with Biotin-SP-conjugated anti-mouse (1:500; Jackson ImmunoResearch). Biotinylated antibody signals will be visualized using ABC reagent (Vector Laboratories) treatment for 1 h at room temperature followed by DAB peroxidase (Sigma) development.

### 5.3.6 Imaging and Quantification

Images were acquired using a 40X objective on an epifluorescent (BX61; Olympus) microscope. DG total BrdU+ cell-numbers were estimated stereologically per side using direct cell counting of systematic random sampling fractions of 1/4 serially cut coronal sections covering the entire anterior-posterior extent of the DG. The tops of nuclei served as unique characteristic points for
exhaustive cell counting using Stereo Investigator (MicroBrightField) software. Sample totals were then multiplied by the inverse sampling rate (4) to give total DG estimates.

Area densities of SVZ BrdU+ cells were derived using a modified method described previously (Chapter 4; (Bath et al., 2008)). A systematic random sample fraction of 1/4 serially cut coronal sections, covering approximately 1.54 to 0.50 mm relative to bregma, was used. All BrdU+ nuclei within 0.1 mm of the lateral ventricle ependymal surfaces were counted per side, marking the tops of nuclei as unique characteristic counting points, using a 40X objective on the epifluorescent microscope and Stereo Investigator software. For each section counted, reference lines tracing each ependymal surface were drawn using Stereo Investigator software. Area densities were then represented by dividing total cell counts by total reference line lengths for each side (giving BrdU+ cells / [1.0 mm reference line × 0.1 mm SVZ depth], or BrdU+ cells/0.1 mm²).

Aβ plaque-load will be determined using a systematic random sample fraction of 1/4 serially cut coronal sections covering the entire anterior-posterior extent of the DG. Plaque number, size, and total area will be determined from the hippocampus and other brain atlas-define regions (Paxinos and Franklin, 2000) using using a 40X objective on the epifluorescent microscope and Stereo Investigator software. Detailed methodology will be defined once plaque quantification is attempted, and may employ automated capabilities of the software.

5.3.7 Experimental Procedures

DG and SVZ proliferation rates in Tg+ mice were determined under baseline conditions by treating 6 week-old mice with BrdU followed by perfusion 24 h post-injection (n = 4).

Stimulation-induced effects on DG proliferation were determined after unilateral EC stimulation. 3 days following stimulation, 6 week-old mice (S/Tg+) were treated with BrdU injection and perfused 24 h post-injection (n = 4). Additional non-stimulated mice (NS/Tg+) underwent electrode insertion, with no current delivery, followed by BrdU 3 days post-operatively (n = 4).

EC stimulation-induced changes in spatial memory performance are being assessed (accrual ongoing) in bilaterally stimulated (S/Tg+; n = 12) or non-stimulated (NS/Tg+; n = 10) Tg mice,
and bilaterally non-stimulated Tg- littermates (NS/Tg--; n = 16), who undergo water maze undertraining 6 weeks later and subsequent probe testing 1 h after the last training trial. All animals are 6 weeks old at surgery. Stimulation-induced changes in Aβ plaque-load will be examined in subsets of S/Tg+, NS/Tg+, and NS/Tg- animals perfused at the completion of this experiment.

5.3.8 Statistical Analyses

Behavioral, cell count, and plaque data are/will be presented as mean ± SEM and evaluated using ANOVAs or t-tests (2-tailed). Duncan’s MRT will be used for post hoc analyses of significant main effects or interactions where appropriate.
5.4 Results

The following results illustrate partial findings from this ongoing study. At the time of writing, Aβ plaque-load data is unavailable.

5.4.1 Baseline Dentate Gyrus and Subventricular Zone Proliferation in Adult Transgenic Mice

The SGZ of the hippocampus and the SVZ of the lateral ventricles are two major neurogenic regions in the adult brain (Zhao et al., 2008). Despite their altered genome, adult TgCRND8 reportedly continue to exhibit neurogenesis in the DG, albeit at reduced levels, at both 3 and 7 months of age (Fiorentini et al., 2010). To establish baseline cellular proliferation in Tg+ animals at the beginning of experimentation, 6 week-old Tg+ mice were injected with the proliferation marker BrdU. Twenty-four h later, BrdU incorporation was quantified in the DG and SVZ. BrdU-labeled cells were evident in both the regions of interest (483.75 cells/DG ± 37.21; 139.39 cells/0.1 mm² SVZ ± 14.02). These values were obtained using identical methodology to that of previous wild type experiments (Chapter 4), and interestingly, were similar in both regions to that of wild type animals (comparable data from Chapter 4: 471.58 cells/DG ± 44.25; 126.06 cells/0.1 mm² SVZ ± 21.56). Therefore, these data suggest that adult hippocampal and SVZ-olfactory proliferation remain intact in 6 week-old Tg+ animals and are consistent with ongoing neurogenesis in these regions.

5.4.2 Entorhinal Cortex Stimulation Increases Dentate Gyrus Proliferation

We next asked whether stimulation of the EC would increase proliferative activity in the DG of Tg+ animals. The EC provides the main afferent input to the DG (Amaral and Lavenex, 2007), and as neurogenesis is regulated by neural activity (Zhao et al., 2008), stimulation of EC afferents/perforant path fibers provides a direct method to increase adult neurogenesis (Chapter
4; (Bruel-Jungerman et al., 2006; Chun et al., 2006; Kitamura et al., 2010)). The chosen EC target used in our previous work (Chapter 3,4) centered on the junction of the ventral intermediate, medial, and caudal entorhinal fields (van Groen, 2001), and is known to project predominantly to the ipsilateral DG (Chapter 4; (van Groen et al., 2002; van Groen et al., 2003)). Roughly 6 week-old mice were injected with the proliferation marker BrdU 3 days after unilateral stimulation of the EC (Figure 5-1a). The 3 day stimulation-BrdU interval corresponded to that which produced the largest increase in proliferation during previous wild type experiments (Chapter 4). Twenty-four h later, BrdU incorporation was quantified in the DG (Figure 5-1b,c). EC stimulation increased the number of DG BrdU-labeled cells (Figure 5-1c). An ANOVA, with Side (ipsilateral vs. contralateral) as a within-subject variable and Stimulation (stimulated, non-stimulated) as a between-subjects variable, revealed that this increase was limited to the ipsilateral side of stimulated animals (significant Side × Stimulation interaction, $F_{1,6} = 13.01, P < 0.05$) (Duncan’s post-hoc test, $P < 0.01$). This pro-proliferative effect of EC stimulation in Tg+ mice matches that observed in our previous wild type experiments (Chapter 4), suggesting intact activity-dependent regulation of neurogenesis in Tg+ animals.

**Figure 5-1. Entorhinal Cortex Stimulation Increases Proliferation in Adult Dentate Gyrus.** (a) Mice were injected with BrdU (green syringe) 3 days following unilateral stimulation (S) (n = 4) or electrode insertion only (NS) (n = 4), and sacrificed 24 h later. (b) DG BrdU+ cells (green) were visualized ipsi- (I, left panels) and contralateral (C, right panels) to electrode site (DAPI counterstain; scale bar = 100 µm). (c) BrdU+ cell counts revealed increased proliferation I (solid bars) vs. C (clear bars) in S (red borders), but not NS (blue borders), animals. **$P < 0.01$**
5.4.3 Rescue of Deficient Spatial Memory Formation in Transgenic Mice Following Entorhinal Cortex Stimulation

TgCRND8 mice exhibit impaired spatial memory formation (Chishti et al., 2001; Fiorentini et al., 2010; Hyde et al., 2005). Therefore, we are now testing whether stimulation-induced increases in neurogenesis facilitate water maze learning in adult Tg+ mice. In order to maximize production of new neurons, mice are receiving bilateral (rather than unilateral) stimulation of the EC. Water maze training occurs 6.5 weeks later (Figure 5-2a), matching the design of our previous work (Chapter 4). This 6.5 week stimulation-training delay ensures that additional neurons, produced as a consequence of stimulation, are sufficiently mature (~6 weeks old) to contribute to spatial learning (Chapter 3, 4; (Kee et al., 2007b)). Separate control groups of Tg+ and Tg- mice are being treated identically except with no current delivery (Figure 5-2a). Training latencies to find the platform decline thus far (Figure 5-2b; Day × Group ANOVA, significant main effect of Day only, $F_{2,35} = 20.27, P < 0.01$); moreover, a trend towards a Day × Group interaction is evident ($F_{4,35} = 1.64, P = 0.17$), qualitatively demonstrated by longer latencies in NS/Tg+ mice (Figure 5-2b), suggesting emerging evidence of deficient spatial learning in NS/Tg+ mice vs. the 2 control groups. Immediately following the completion of training, spatial memory is being assessed in a probe test. In this test thus far, group density plots suggest greatest search selectivity in NS/Tg- mice, and least selectivity in NS/Tg+ mice (Figure 5-2c). Various quantitative measures of spatial selectivity suggest stimulation renders the performance of Tg+ mice similar to that of NS/Tg- littermates (Figure 5-2d, Figure 5-S1). For example, using a 15 cm zone analysis, S/Tg+ and NS/Tg- mice trend towards greater searching of the target zone vs. NS/Tg+ animals (Figure 5-2d; $F_{2,35} = 2.10, P = 0.14$). Interestingly, a scatter plot of the same probe data (Figure 5-2e) suggests that NS/Tg+ mice exhibit minimal between-animal variability with clustering of relatively low percent search values. In contrast, the greater variability of S/Tg+ mice percent searches, including the presence of several exceptionally selective values, appears to approach that of NS/Tg- animals. Poorer performance by NS/Tg+ mice could not be explained by differences in swim speed (Figure 5-S1f; $F_{2,35} = 0.88, P = 0.42$) or path length (Figure 5-S1g; $F_{2,35} = 0.89, P = 0.42$), suggesting intact locomotor activity. Collectively, these preliminary training and probe results suggest that stimulation of the EC may, at least in part, rescue deficient spatial learning in Tg+ mice.
Figure 5-2. Stimulation-Induced Partial Rescue of Spatial Memory. (a) Bilaterally stimulated Tg+ (S/Tg+, red, n = 12), non-stimulated Tg+ (NS/Tg+, blue, n = 10), and NS/Tg- (grey, n = 16) mice were undertrained 6.5 weeks following surgery and spatial memory was assessed 1 h after the completion of training. (b) Latencies to reach the platform declined during the training period with a trend towards less decline in NS/Tg+ vs. S/Tg+ and NS/Tg- mice. (c) Density plots for grouped probe test data, with accompanying color scale, represent the number of visits per mouse per 5 cm × 5 cm area (aligned according to (a): S/Tg+ (top), NS/Tg+ (middle), NS/Tg- (bottom)). Mice searched selectively in the probe test, with a suggested gradient of NS/Tg+ > S/Tg+ > NS/Tg- (d) S/Tg+ and NS/Tg- animals trend towards more time searching the 15 cm target zone (T) compared to other (O) zones, and compared to NS/Tg+ mice in both T and O zones. (e) Scatter plot of raw T data from (d) demonstrates constricted variability in the NS/Tg+ group relative to S/Tg+ and NS/Tg- groups.
At the time of water maze application, corresponding to 6.5 weeks following surgery and an animal age of 12.5 weeks (Figure 5-2a), the percentages of overall survival differed between groups (Table 5-1; Fisher's exact test, \( P < 0.01 \)). The largest overall difference was a higher survival rate in Tg- vs. Tg+ mice, in keeping with increased mortality associated with the TgCRND8 genotype (Chishti et al., 2001); however, S/Tg+ animals had an 8% absolute and 14% relative increase in survival over NS/Tg+ mice suggesting stimulation confers a small survival benefit in the disease condition.

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<td>Y (%)</td>
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<tr>
<td>S/Tg+</td>
<td>12 (67)</td>
<td>6 (33)</td>
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<tr>
<td>NS/Tg+</td>
<td>10 (59)</td>
<td>7 (41)</td>
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<tr>
<td>NS/Tg-</td>
<td>16 (100)</td>
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*Fisher's exact test (df = 2), \( P < 0.01 \)*
5.5 Discussion

We have previously described spatial memory enhancement in wild type mice following targeted EC stimulation, and that this behavioral phenotype is likely a product of stimulation-induced increased hippocampal neurogenesis (Chapter 4). It is possible that this mechanism contributes to the slowing or arrest of cognitive decline seen in AD patients following DBS of hippocampal afferents (Laxton et al., 2010). Using a Tg neuropathological mouse model of AD, generated using human familial AD APP mutations, the current study provides disease-related evidence that such pro-cognitive effects could indeed be mediated by activity-dependent promotion of hippocampal neurogenesis. First, BrdU labeling in 6 week-old Tg+ mice revealed ongoing proliferation in the DG and SVZ. This suggested the presence of regulatory mechanisms for adult neurogenesis. Second, using parameters analogous to clinical high frequency DBS, we show that targeted EC stimulation in Tg+ mice produces an increase in DG proliferation analogous to that seen in wild type animals (Chapter 4). Third, ongoing experiments suggest acute, bilateral stimulation of the EC restores deficient spatial memory formation in Tg+ animals. Importantly, the 6.5 week stimulation-training delay is compatible with maturation-dependent integration of stimulation-induced adult-generated neurons into spatial memory circuits (Chapter 3, 4; (Kee et al., 2007b)). Taken together with our previous wild type experiments, these data provide further support for a causal relationship between stimulation-induced increases in neurogenesis and improved spatial memory formation. Moreover, these results can be thought of as a proof of principle, using a disease model, for our promising clinical findings. Finally, the unanticipated suggestion of a slight survival benefit of stimulation in Tg+ animals may indicate a more profound benefit to overall health in these animals, noteworthy given the increased mortality associated with AD (Association, 2011).

Our preliminary findings come on the heels of recent reports that other means of enhancing neurogenesis benefit AD model mice carrying Swedish and Indiana familial AD mutations (Fiorentini et al., 2010; Valero et al., 2011). Fiorentini and colleagues increased neurogenesis in 8 week-old Tg+ mice using 5 weeks of lithium treatment and demonstrated improved Morris water maze learning and recall, and reduced Aβ plaque-load. Similar treatment of 7 month-old animals did not enhance neurogenesis or spatial memory performance, suggesting a lack of
efficacy with advanced pathology. Valero et al. increased neurogenesis using environmental enrichment in 4 month-old Tg mice harboring the same familial AD mutations to that of the TgCRND8 model. Enrichment was associated with increased dendritic length of, and CA3-connecting fibers from, adult-generated DGCs, as well as improved learning and memory in the Morris water maze. To date, neither environmental enrichment or lithium therapy has translated into reliable or highly efficacious therapies for AD beyond symptom control (reflected, for example, by conflicting randomized placebo controlled studies using lithium (Forlenza et al., 2011; Hampel et al., 2009)). In contrast, the present study, suggesting cognitive benefits from targeted brain stimulation, echoes the promising results reported clinically with DBS (Hamani et al., 2008; Laxton et al., 2010). Given the long history of failed therapeutics for this relatively impervious condition (Ballard et al., 2011), cautious ongoing work both in the clinic and laboratory must expand upon these early findings to clarify their ultimate therapeutic value.

The central result of this study is the suggestion that bilateral stimulation of the EC strongly facilitates the formation of hippocampus-dependent water maze memory in Tg+ mice to the extent that their performance approaches that of their Tg- littermates. The magnitude of this stimulation-induced performance improvement appears more pronounced relative to that observed in our previous wild type experiments (Chapter 4). Indeed, the addition of more adult-generated DGCs could be of higher value in the context of the disease model. Widespread neuropathology might impair non-neurogenic compensatory mechanisms such that animals rely more heavily on these neurons, affording stimulated Tg+ animals more potential for improvement with less susceptibility to a ceiling effect. Profoundly deficient baseline spatial learning in non-stimulated Tg+ mice might reflect this lack of “cognitive reserve”. Moreover, the combination of using a relatively mild/challenging training protocol (3 days, 3 trials/day), where escape latencies do not reach asymptotic levels, and a disease model with compromised memory formation at baseline, might synergistically magnify detection of stimulation-induced facilitation of memory. It would be interesting to examine IEG expression rates of adult-generated DGCs in Tg+ animals to see whether they reveal high recruitment rates into networks supporting spatial memory.

Should our early clinical and laboratory results continue to show promise, despite the historical failure of other treatments to produce substantial therapeutic efficacy (Ballard et al., 2011), they could reflect the contributions of neurogenic and non-neurogenic factors. For instance,
successful involvement of stimulation-induced new neurons in functional restoration might point to as yet undefined differences in the functional significance of new neurons generated following different stimuli. In addition, other important non-neurogenic mechanisms of stimulation may act in concert with, or in addition to, potential neurogenic mechanisms. Mechanisms of clinical DBS remain largely uncertain, but as discussed previously (Chapter 4), may include modulation of neural activity locally or trans-synaptically through direct cellular effects or the induction of neurotransmitter release (Kringelbach et al., 2007), as well as normalization of activity levels within dysregulated brain regions at the circuit level (Laxton et al., 2010; Mayberg et al., 2005). In non-disease related laboratory models, electrical stimulation of neurons incites axonal, dendritic, and synaptic remodelling and synaptogenesis via activity-dependent mechanisms (Manivannan and Terakawa, 1994; Nagerl et al., 2007; Nikonenko et al., 2003). Non-neurogenic effects of other pro-neurogenic stimuli in TgCRND8 animals are better understood, and might also be induced by stimulation. For instance, environmental enrichment not only promotes increased neurogenesis in TgCRND8 mice, but also enhances expression of several plasticity-associated molecules, including synaptophysin, Arc, and neuronal growth cone protein GAP43 (Herring et al., 2009). Similar treatment has also been associated with reduced Aβ plaque-load (Adlard et al., 2005; Ambree et al., 2006; Lazarov et al., 2005) (including in the context of improved spatial memory (Adlard et al., 2005)), antagonism of AD-related oxidative damage (Herring et al., 2010), and angiogenic compensation for AD-related vascular dysfunction (Herring et al., 2008). Several ongoing and future experiments are necessary in order to better establish the contribution of neurogenesis promotion to our observed memory restoration. Aside from continued accrual of animals to existing experimental groups, some of our current strategies include examining Aβ plaque-load following stimulation and testing the effects of stimulation under neurogenesis-blocking conditions.

A common theme of many therapeutic interventions across medicine is that of greater symptomatic benefits, and even disease-modifying effects, with treatment initiation early rather than late in the course of disease. In AD patients treated with DBS, patients who were less severely affected (hence earlier in the disease process) demonstrated the most benefit (Laxton et al., 2010). Similarly, Tg+ animals with more advanced disease may not benefit from pro-neurogenic treatments (Fiorentini et al., 2010). Indeed, the fact that the typical age-related decline in neurogenesis may be exaggerated in Tg+ animals, coincident with constantly
accumulating neuropathology, points to a possible therapeutic window of opportunity during early stages of the disease when stimulation-induced rescue of spatial memory might be most efficacious. Pending the completion of our initial experiments in relatively young Tg+ adults, we will stimulate and test aged mice harboring more advanced disease with the prediction of less improvement in spatial memory over their non-stimulated counterparts.

The impetus for this research is the hope that enhanced brain plasticity can be induced under neuropathological conditions, such as AD, in order to restore brain function while potentially also reducing neuropathology and rescuing brain structure. Although the laboratory setting and disease models provide us with greater freedom to study potential mechanisms, such as neurogenesis, their true value relies upon corresponding advances in our knowledge from the clinical setting. To this end, Whether or not DBS promotes neurogenesis in humans remains to be determined; however, recent advances in non-invasive medical imaging both in terms of spatial resolution and the ability to assess neurogenesis in live patients (Manganas et al., 2007; Pereira et al., 2007; Prudent et al., 2010) may enable testing of DBS implanted patients in the near future. We do know that limbic stimulation (in this case fornix) can drive activity in hippocampal regions (Laxton et al., 2010), and is associated with growth in size of the hippocampi (unpublished preliminary data), in patients. Interestingly, a recently presented abstract provides preliminary evidence that EC stimulation might safely facilitate spatial memory in human epilepsy patients (Fried et al., 2011). While further studies are still needed to define optimal target and stimulation parameters for increasing neurogenesis, and whether or not clinical benefit is associated with the degree of enhanced neurogenesis, we now have favourable early clinical and laboratory evidence upon which to proceed.
5.6 Supplemental | Additional Water Maze Measures

The trend towards improved spatial memory of Tg+ mice following stimulation, depicted in the “Results” section using a 15 cm zone analysis (Figure 5-2d), is also evident using several additional water maze measures (Figure 5-S1a-e; multiple ANOVAs, Ps > 0.05): quadrant analyses, 20 and 25 cm zone analyses, and platform crossings. All 3 groups had similar mean swim speeds (Figure 5-S1f; $F_{2,35} = 0.88, P = 0.42$) and total path lengths (Figure 5-S1g; $F_{2,35} = 0.89, P = 0.42$) during probe testing.

![Figure 5-S1. Additional Measures of Stimulation-Induced Partial Rescue of Spatial Memory.](image)

Bilaterally stimulated Tg+ (S/Tg+, red, n = 12), non-stimulated Tg+ (NS/Tg+, blue, n = 10), and NS/Tg- (grey, n = 16) mice were undertrained 6.5 weeks following surgery and spatial memory was assessed 1 h after the completion of training (Figure 5-2a). (a,b) S/Tg+ and NS/Tg- animals trend towards spending more time (a) and percent time (b) searching the target quadrant (T) compared to other quadrants (O), and compared to NS/Tg+ mice in both T and O quadrants. (c,d) S/Tg+ and NS/Tg- animals trend towards spending more percent time searching the 20 cm (c) and 25 cm (d) T zones compared to O zones, and compared to NS/Tg+ mice in both T and O zones. (e) S/Tg+ and NS/Tg- animals trend towards crossing the T platform location more frequently compared to O equivalent platform locations, and compared to NS/Tg+ mice in both T and O platform locations. (f,g) During probe testing, average swim speeds (f) and total path lengths (g) did not differ between groups. All ANOVAs $P > 0.05$.
Chapter 6  Concluding Summary, General Discussion, and Future Directions
6.1 Concluding Summary

The fact that neurogenesis persists in the hippocampus beyond development, is somehow related to learning and memory, and responds to multiple influences, engenders hope amongst clinicians and scientists alike that therapeutic interventions might be able to appropriately promote this process as a means of restoring mnemonic function. Decades of scientific investigation have greatly expanded our understanding of adult hippocampal neurogenesis, but have also continued to generate questions regarding the functional importance of these adult-born neurons. The objectives of this thesis were to expand our basic knowledge concerning the operational significance of adult-generated dentate granule cells (DGCs) in learning and memory and to examine the therapeutic potential of DGCs generated in the context of targeted brain stimulation. To this end, we initially examined the functional significance of adult-generated DGCs relative to their developmentally-generated counterparts in memory processes. This set the stage for the subsequent larger and expanding share of content dealing with the functional significance of focal brain stimulation-induced increased neurogenesis both at the DGC- and behavioral-levels in wild type and Alzheimer’s disease (AD) model mice.

The adult dentate gyrus (DG) is composed of a heterogeneous pool of developmentally- and adult-generated granule cells, both of which are believed to contribute to hippocampal memory processing. However, whether these populations represent functionally equivalent or distinct pools of neurons at the network level has not been definitively established. Should differences exist, they should be evident through different activation rates at the network level that reflect greater recruitment of one over the other during learning/recall. By visualizing this activation of developmentally- and adult-generated DGCs following memory recall, we obtained several lines of evidence to suggest that developmentally- and adult-generated DGCs are equally likely to contribute to hippocampal memory formation. First, using the Morris water maze task, activation rates of adult-generated DGCs stabilized by 5 weeks of age without temporary ripples that might correspond with varied plasticity in maturing, adult-generated DGCs. Second, embryonically-, postnatally-, and adult-generated DGCs, all 7.5 weeks-old, were functionally recruited by circuits supporting water maze memory at similar rates. Third, corresponding recruitment rates for embryonically-, postnatally- and adult-generated DGCs were maintained using a within-
animal comparison. Finally, contextual fear conditioning generated similar results, suggesting these findings can be generalized to other hippocampus-dependent tasks. These experiments jointly suggest that developmentally- and adult-generated DGCs integrate at similar rates into hippocampal memory networks and constitute functionally equivalent neuronal populations with neither favored over the other during memory-related functional recruitment.

Activity-dependent regulation of hippocampal neurogenesis is a potential mechanism underlying the pro-cognitive effects seen in AD patients receiving DBS of the limbic system (Laxton et al., 2010). Given their functional equivalence to developmentally-generated DGCs, mature adult-generated DGCs present in larger numbers might facilitate hippocampus-dependent memory formation. By delivering EC stimulation in mice, using parameters analogous to clinical high frequency DBS, we obtained several lines of evidence to suggest that such pro-cognitive effects are mediated by activity-dependent promotion of hippocampal neurogenesis. First, stimulation increased DG proliferation in an anatomically- and temporally-specific fashion, culminating in a greater production of DGCs. Second, EC stimulation-induced DGCs displayed DG localization, maturation, and maturation-dependent functional recruitment by hippocampal networks supporting spatial memory that were comparable to DGCs produced at baseline. Third, acute, bilateral EC stimulation facilitated spatial memory formation in a DGC maturation-dependent pattern. The fact that spatial memory was facilitated when stimulation-induced neurons reached functional maturity, but not when present yet functionally immature, effectively constituted a knockdown experiment that underscored the functional significance of the additional mature DGCs. Fourth, a series of analogous actual knockdown experiments using temozolomide (TMZ) treatment to inhibit stimulation-induced neurogenesis prevented stimulation-induced facilitation of spatial memory. Collectively these wild type animal stimulation experiments suggest that new neurons produced as a consequence of EC stimulation assume functional roles in hippocampal circuits and can lead to improved spatial memory formation.

To further explore stimulation-induced increased hippocampal neurogenesis as a potential mechanism underlying the slowing or arrest of cognitive decline seen in DBS-treated AD patients (Laxton et al., 2010), we are attempting to replicate findings using a neuropathological transgenic (Tg) mouse model of AD. Our accumulating data suggest that these animals, which harbor human familial AD APP mutations, respond to EC stimulation in a similar manner to their wild type counterparts. First, young adult Tg+ mice exhibited intact proliferation in the DG and
subventricular zone (SVZ) despite their altered genome. Second, EC stimulation using our same clinically based parameters produced an increase in DG proliferation approximating that seen in wild type animals. Finally, acute, bilateral EC stimulation appears to facilitate spatial memory formation in otherwise deficient Tg+ animals. This mounting disease-related evidence suggests that activity-dependent promotion of hippocampal neurogenesis could indeed contribute to pro-cognitive effects of limbic DBS and underscores the clinical relevance of the preceding wild type experiments. Furthermore, an emerging trend towards improved survival of Tg+ animals receiving stimulation points to the potential broader implications to health and wellbeing of modulating activity-dependent hippocampal neurogenesis through targeted brain stimulation in the disease setting.
6.2 Unifying Discussion

The “multiple paper format” of this thesis permits specific discussion sections for each chapter that contained new data (see sections 3.5, 4.5, 5.5). In the hopes of avoiding redundancy, these brief final unifying thoughts are limited to considering how our findings impact the two overall themes that permeate this research: the role of adult-generated DGCs in learning and memory, and the potential to enhance adult hippocampal neurogenesis clinically in order to facilitate memory function.

The most constant theme throughout all sections is the role of adult hippocampal neurogenesis in learning and memory. This is the main focus of the overall introductory and background material of Chapter 2, and is most directly addressed experimentally in Chapter 3. The conclusion suggested by data from Chapter 3, namely that adult and developmentally-generated DGCs are functionally convergent with respect to hippocampal dependent learning and memory, provides some added perspective on how these new neurons might impact theorized cognitive/psychological processes (For an overview of the main theories proposed in the literature, the interested reader is encouraged to see Appendix A1). For instance, theories that ascribe unique functional roles to adult-generated cells in learning and memory, such as encoding temporal associations between stimuli (Aimone et al., 2006, 2009), predict that newly generated DGCs must out compete their developmentally-generated counterparts for integration into memory networks. In other words, cells bestowing a unique and necessary aspect to the memory that cannot be provided by alternative cell populations must be preferentially integrated. The findings here, which for the first time examine truly comparable cohorts of adult- and developmentally-generated DGCs, do not support this prediction. While the absence of heightened recruitment rates for adult-generated DGCs by larger memory networks still does not tell us what roles they may be capable of assuming, it does suggest that these roles are not manifest at the cellular integration level and that some theoretical models consequently require clarification (Appendix A1; (Alme et al., 2010)). One possible perspective that cannot be critically evaluated by this current research is that comparable recruitment would be expected if adult-generated DGCs uniquely support some functions that only apply to special circumstances, such as discriminating between highly similar contexts (Appendix A1; (Clelland et al., 2009;
Creer et al., 2010; Sahay et al., 2011a)). However, since numerous studies using different tasks and protocols suggest adult-generated DGCs are involved in many forms of hippocampus-dependent memory (Chapter 2), it is perhaps currently more reasonable to view similar recruitment rates for adult- and developmentally-generated DGCs as supporting the idea that new and existing DGCs generally operate as fairly equivalent participants in hippocampus-dependent memory processes. Adult-generated neurons could thus harbor the potential to assume the same full range of functions as developmentally-generated neurons. Indeed, this latter perspective bolsters their potential utility as a source of new cellular substrate in memory processes when more are needed to maintain function.

The overarching goal of enhancing mnemonic function in clinical dementia progressively becomes the dominant theme of this thesis. This is directly reflected by the contents of two data-driven chapters (see Chapters 4 and 5, including discussion sections 4.5 and 5.5) and by the fact that continuing aspects of the project are entirely concerned with this theme. While I acknowledge the fact that translating and interpreting findings between the clinical and laboratory settings is a formidable challenge that this thesis by no means fully accomplishes, this does represent a concerted attempt to examine one underlying mechanism that could mediate an important clinical phenotype. Modern advancements in neurosurgical techniques and DBS, functional neuroimaging, and our understanding of neurological disease processes at the molecular, cellular, and network levels are now enabling clinicians to increasingly intervene with targeted neuromodulatory therapy for the betterment of diseased brain function. An important caveat to these exciting developments is that therapeutic hopes must remain tempered by acknowledging the fact that we are currently only able to address portions of disease processes. In the case of augmenting hippocampal neurogenesis, our manipulations are targeting only one region within one, albeit critical, brain area. While improved hippocampal functionality might provide the means to further compensate for other impaired brain regions, it is undoubtedly incapable of rectifying all of the negative effects of more global degeneration. Should stimulation-induced neurogenesis become a real therapeutic entity, it will need to be part of a larger therapeutic armamentarium so that patients realize the most benefits possible. To this end, it is critical that continued neuroscientific and clinical research be conducted via a multimodal approach to searching for important synergistic effects of multiple disease component-targeted therapies.
6.3 Future Directions

Several experiments that are ongoing or planned for the near future are detailed in Chapter 5, reflecting the general aim to further evaluate the importance, and potential therapeutic utility, of stimulation-induced increased hippocampal neurogenesis to learning and memory. In keeping with this general aim, numerous avenues for further study could be explored moving forward. Possibilities include the use of higher fidelity DBS modeling, examination of other potential underlying mechanisms, application to other therapeutic indications, expansion of testing to other cognitive tasks and measures of mood, and translation and extension of experiments into the clinical environment through opportunities provided by the neurosurgical management of patients.

Clinical DBS is generally performed using chronic continuous or intermittent delivery of electrical stimulation to precisely targeted areas in the brain, as was the case in reported DBS-treated AD patients (Laxton et al., 2010). In contrast, our animal stimulation protocol utilizes temporary electrode implantation and acute stimulation. Our acute protocol was specifically chosen to isolate effects of stimulation-induced neurogenesis on behaviour from that of other potential acute surgical- or stimulation-induced mechanisms. This was primarily accomplished by testing mice after an extended implant- and stimulation-free period. As a consequence, animal stimulation did not reflect the typical chronicity of human DBS. In reality, more sustained stimulation might bring greater cognitive benefit via potential pro-neurogenic mechanisms, assuming stimulation-induced increased neurogenesis is maintained over time and possibly even compounded by resultant enhanced survival of immature DGCs (Chapter 4; (Bruel-Jungerman et al., 2006; Kitamura et al., 2010)). This possibility, along with any potential toxicity related to chronic stimulation, warrants further study and is becoming increasingly feasible from a technical standpoint due to the continued development of portable and implantable stimulation units for use in rodent experiments.

As outlined in Chapter 4, our experiments were designed to focus on altered neurogenesis, and our results strongly point to a behavioural manifestation arising due to a neurogenic mechanism. However, questions still remain as to how stimulation might also incite non-neurogenic influences that could contribute to altered hippocampus-dependent memory function. For
instance, while no morphologic differences were noted between adult-generated DGCs induced by stimulation or generated at baseline (Chapter 4), stimulation could prompt dendritic/spine remodelling in pre-existing DGCs or neurons within other hippocampal or brain regions (Desmond and Levy, 1986; Maletic-Savatic et al., 1999). A myriad of other factors, such as alterations in regional blood supply, inter-regional synaptic connectivity/excitability, and intrinsic excitability could manifest in nearby or remote brain regions and prove functionally relevant to our observations (Feldman, 2009; Girouard and Iadecola, 2006). Indeed, metabolic imaging studies demonstrate dramatic alterations in the activity of brain regions immediately connected to, and separate from, DBS targets (Laxton et al., 2010; Mayberg et al., 2005).

Evaluating all mechanisms, including their potential inter-relations, is a tremendous scientific challenge. Our general strategy here is to continue to target individual components that appear to hold significant promise using experimental designs, interventions, and Tg models. Optimal use of constantly advancing physiological and anatomical techniques may permit further isolation of specific biochemical elements, in particular through the use of methods applicable in vivo such as optogenetic neurostimulation (Gradinaru et al., 2010). Once sufficient gains are made via this reductionist approach, we may then be able to appreciate how they combine to manifest at higher levels of analysis and produce holistic effects on behavior. Indeed, advances in areas such as high resolution voxel- and deformation-based morphometric brain imaging (Ashburner and Friston, 2000; Ashburner et al., 1998) in vivo are increasing our ability to measure phenotypes for phenomena at the system level.

AD, the clinical entity indirectly examined in this thesis and directly studied in the related human clinical trial, is by no means unique as a disease process characterized, in part, by memory impairments. The long list of conditions in which poor mnemonic function remains largely or completely resistant to available treatments includes stroke, traumatic brain injury, CNS infections, chronic epilepsy, congenital brain diseases, other neurodegenerative diseases, chronic substance abuse, chronic brain malignancies, chronic hydrocephalus etc. Some of these conditions have been associated with acute alterations in neurogenesis that appear directly driven by the disease pathology. For example, epilepsy has been associated with increases (Crespel et al., 2005) or decreases (Hattiangady et al., 2004) in the production of adult-generated DGCs. In studies showing seizure-induced increased neurogenesis, resultant new DGCs appear significantly abnormal (Jessberger et al., 2007b; Parent et al., 1997) and may even negatively
contribute to cognitive function (Jessberger et al., 2007a; Parent and Lowenstein, 2002). Moreover, pathology incited by chronic epilepsy even extends to pre-existing DGCs (Fahrner et al., 2007). Based on this example, it is conceivable that a means of promoting repopulation of the DGC pool with functionally normal adult-generated DGCs, that are capable of assuming the roles of their developmentally-generated counterparts, might be beneficial. Studies examining neurons generated by therapeutic stimulation in models of these conditions are needed to further understand these issues and whether or not our observed cognitive benefits can be generalized to other neuropathologies.

Behavioral experiments conducted here utilized only two well-described hippocampus-dependent memory tasks, contextual fear conditioning and the Morris water maze (with stimulation-induced effects examined in the latter only), and did not examine putative roles of adult hippocampal neurogenesis in the regulation of mood (Jacobs et al., 2000). In terms of memory, it would be interesting to establish whether or not our observations generalize to other hippocampus-dependent protocols (see Chapter 2), especially those requiring efficient differentiation between highly similar contextual representations (i.e. pattern separation) which have recently demonstrated sensitive behavioral responses to perturbations of adult neurogenesis (Clelland et al., 2009; Creer et al., 2010; Sahay et al., 2011a). With respect to mood, a large body of literature proposes possible mechanistic involvements of adult neurogenesis in depression (reviewed extensively by (Zhao et al., 2008)). Chronic stress, considered to precipitate and aggravate depression (Hammen, 2005), and elevated corticosteroids, prevalent in depressed patients and a by-product of stress (Holsboer, 2000), depress neurogenesis (Gould et al., 1992; Mirescu and Gould, 2006). Moreover, studies report that certain antidepressant medications promote neurogenesis, and radiation-induced suppression of neurogenesis blocks their behavioral effects in rodents (Airan et al., 2007; Santarelli et al., 2003). Although an etiological link between impaired neurogenesis and depression has been increasingly challenged (Sahay and Hen, 2007), most data is at least compatible with neurogenesis acting as a substrate for many successful therapeutics. Subgenual cingulate area DBS is a novel therapy for otherwise treatment refractory depression (Lozano et al., 2008; Mayberg et al., 2005) that can be effectively modeled in rodents (Hamani et al., 2010a). While the subgenual cingulate area, like the EC, is part of the limbic system, it remains undetermined whether activity-dependent neurogenesis could be an underlying mechanism. Applying our stimulation protocol and cellular analyses to experiments
using behavioral tests that predict antidepressant action clinically (such as the forced swim test) might serve as a platform for seeking contributions of stimulation-induced increased neurogenesis to the treatment benefits of limbic DBS.

Finally, neurosurgical interventions offer unique opportunities to assess stimulation-induced effects, such as increased neurogenesis, in humans. The operating room provides unparalleled access to the viable human CNS, and historically, many major advances in our understanding of human neuroscience are rooted in findings from live human subjects undergoing surgical procedures. Examples include the famous neurosurgeon Wilder Penfield’s reports of vivid recollections by epilepsy patients during awake temporal lobe stimulation (Penfield, 1952), the findings of William Scoville and Brenda Milner concerning memory as a result of bilateral mesial temporal lobe resections for epilepsy (Scoville and Milner, 1957), and the discovery by Lozano and colleagues of human cortical neurons that directly respond to pain via intraoperative microelectrode recordings (Hutchison et al., 1999). Today and in the future, we are increasingly examining new neuromodulatory targets in the brain with DBS, including limbic regions such as the fornix, anterior thalamic nucleus, cingulate gyrus, hippocampus, and EC, giving us expanding opportunities to perform recording and stimulation studies. The chemical environment of the brain can be monitored through microdialysis- and voltammetry-based systems, and altered using targeted infusion systems. Through research protocols we can access fresh en bloc surgical resections of such regions as the mesial temporal lobe, which can then be studied in the laboratory. Noninvasive neuroanatomical and functional imaging is allowing unprecedented monitoring of real-time changes in brain physiology. Indeed, the potential for ethically sound neuroscientific investigations of neurogenic and non-neurogenic processes within the human brain has never been greater. Advances in tools and methodology are closing the gaps between clinicians, scientists, and clinician-scientists, and more than ever before the future limits of what we can achieve neuroscientifically and therapeutically through collaboration will be determined by the bounds of our investigative creativity.
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Appendix

A1 Theorized Contributions of Neurogenesis to Learning and Memory at the Cognitive Level

This section is modified from the following:


A1.1 Introduction

Decades of research have demonstrated that dentate granule cells (DGCs) are generated throughout mammalian adulthood. Moreover, their assumption of neural function within the hippocampal circuit and involvement to some degree in hippocampal memory processing is reasonably established. In spite of these monumental achievements, the specific purpose and overall significance of this phenomenon remain somewhat elusive.

In this section, relating to the discussion in Chapter 2 (section 2.3.5), several theorized roles for new DGCs at the network or cognitive level are discussed based on a review of the literature. Prior to discussing these theories, it must be re-emphasized that several general complicating factors should be considered. First, our biological understanding of adult neurogenesis continues to evolve. Second, adult-generated neurons may contribute to multiple cognitive processes during different developmental stages. Finally, the roles of mesial temporal lobe structures in memory, and their interaction with other brain regions, continue to be debated.

The following subsections discuss potential cognitive roles for adult-generated DGCs in increasing the capacity for information processing, providing memory storage, clearing memory traces, associating memories, and discriminating between similar patterns.
A1.2 Increasing Information Processing Capability

Regardless of their details, most published theories propose that generating more DGCs in adulthood is beneficial to learning and memory. One such theory asserts that the primary purpose of adult neurogenesis is to allow the brain to increase mnemonic capacity by assisting the hippocampus to accommodate greater levels of novelty and complexity (Kempermann, 2002, 2008). In doing so, new neurons could act as new “gatekeepers” (Kempermann, 2002), increasing the processing ability of the dentate gyrus (DG) to prepare information for memory storage. A “neurogenic reserve”, or greater potential to remain flexible and plastic, is created when neurogenesis increases in response to functional stimulation (such as enrichment) that signifies probable future increased cognitive demand (Kempermann, 2008). Accordingly, increased neurogenesis associated with certain experiences, such as enrichment and exercise, might non-specifically contribute to learning through a positive feedback loop (Kempermann, 2002). This would predict that the functional benefit of increasing adult hippocampal neurogenesis develops in a delayed fashion, because it takes time for adult-generated DGCs to mature and functionally integrate into hippocampal circuits (Kee et al., 2007b; van Praag et al., 2002). While nicely accounting for the activity-dependent regulation of neurogenesis rates and maturational-timeline of adult-generated DGCs, this theory has yet to be extensively tested directly. For instance, it is not well-established if manipulations that increase neurogenesis only improve performance in memory tasks after a sufficient cell maturational delay (though supported by our results from Chapters 3 and 4). In addition, one would need to demonstrate that increasing or decreasing neurogenesis expands or contracts memory processing capacity in the long-term through an experimental design that reliably stresses information input capacity.

A1.3 Providing Memory Storage

A changing population of adult-generated neurons might provide transient storage of information (Barnea and Nottebohm, 1996; Gould et al., 1999c; Gross, 2000). This possibility is analogous to the annual renewal of song repertoire in adult canaries, linked to the temporary incorporation of new neurons into their high vocal center (Kirn et al., 1991). The plasticity features of newborn neurons might support flexible roles in temporary memory storage that older cells are less able to
perform, such as the detection and encoding of novel stimuli (Leuner et al., 2006a).

Computational simulations have elaborated upon this role for adult hippocampal neurogenesis in short-term information coding, namely by providing distinct codes for highly similar events that ultimately permit an increase in memory capacity (Becker, 2005). Using another network model, Weisz & Argibay demonstrated that a lump increase in the DGC population of 30% significantly improved the model’s ability to accurately retrieve a specific recently learned input pattern, and this benefit was enhanced when the system was exposed to greater numbers of competing input patterns that simulate increasing storage requirements (Weisz and Argibay, 2009). In keeping with concepts of memory reorganization and eventual transfer to extra-hippocampal regions (Squire and Zola, 1997), turnover of some new DGCs could also be linked to early transient processes in memory formation, after which those neurons may no longer be required to support consolidated memories (Barnea and Nottebohm, 1996; Gould et al., 1999c; Gross, 2000). However, the long-term survival of some adult-generated DGCs suggests that they could then still serve as yet undetermined longer-lasting functions.

Another potential role for adult-generated DGCs is in providing additional plastic substrate for long-term memory storage. Continued modification of existing neural elements, as they become increasingly shared amongst different representations, could eventually create interference between those representations and render them indistinguishable from one another (Carpenter and Grossberg, 1987; Nottebohm, 2002). The necessity to avoid such “catastrophic interference” (McCloskey and Cohen, 1989) is particularly relevant to the DG in most computational simulations of hippocampal function (Bakker et al., 2008; Leutgeb et al., 2007; O'Reilly and McClelland, 1994; Treves and Rolls, 1994). Overlapping entorhinal cortex (EC) inputs must be highly separated in order to encode different memories in CA3, and it is believed that this separation task occurs primarily in the DG. Continually adding new neurons to the DG could provide additional structural plasticity, thus increasing the potential for generating non-interfering memory traces when creating new long-term memory stores. Nottebohm suggests that the newly born neurons may be preferentially selected for storing new memories (contrary to our results from Chapter 3), thereby protecting old memories from interference (Deng et al., 2010; Nottebohm, 2002). Consistent with this hypothesis, both simplified neural network and hippocampal memory models demonstrate that adding highly plastic new neurons to encode novel information can prevent new learning from interfering catastrophically with older
memories, in-turn preserved by stable older neurons (Appleby and Wiskott, 2009; Wiskott et al., 2006). In a recent report (Winocur et al., 2011), suppression of neurogenesis using low-dose irradiation had no effect on memory for a visual discrimination task under low interference conditions. However, high interference caused memory impairment that was rescued when neurogenesis was restored by running. This would also predict that animals who learnt a given task in the remote past, underwent neurogenesis suppression in the present, and subsequently learnt a new highly similar task would experience a significant disruption of the older memory because the new memory would have no alternative but to use and modify some neurons critical to maintaining the older memory.

Despite the potential utility of new neurons in reducing interference between long-term memories, some data suggests that adult neurogenesis can actually increase interference under certain short-term memory conditions. In a relevant study, focal hippocampal irradiation or an inducible transgenic (Tg) knockout of hippocampal neurogenesis actually improved performance in a radial maze task (Saxe et al., 2007). This task was designed to promote interference, requiring the animal to disregard conflicting non-relevant information from previous trials. Unlike controls, neurogenesis deficient animals were relatively insensitive to this interference. The authors speculated that blocking neurogenesis may reduce short-term memory capacity sufficiently to prevent the formation of interfering memories.

### A1.4 Clearing Memory Traces

In contrast to increasing processing or storage capacity, other models propose that DG neuronal turnover clears older memories and in so doing potentially increases capacity for new learning (Chambers et al., 2004). Some animal data supports this concept, including a first study using presenilin-1 conditional knockout mice (Feng et al., 2001). These Tg Alzheimer’s disease (AD) model animals exhibit normal baseline hippocampal neurogenesis, deficient enrichment-induced increased neurogenesis, and no identified alterations in basal synaptic function or plasticity. Knockout mice suffered no deficits during hippocampus-dependent contextual fear learning relative to controls; however, when subjected to a sequential paradigm of learning, enrichment for 2 weeks, and retrieval, knockout mice outperformed controls in the retention test. Feng et al. postulated that enrichment-induced increased neurogenesis in the DG of controls facilitated the
clearance of hippocampal memory traces (Feng et al., 2001). In the absence of this clearance, memory retention was superior in knockout mice. They also propose that DGC turnover could purge hippocampal memory traces after they are consolidated into long-term memory, enabling the system to process new memories and avoid interference. Indeed, the time it takes for hippocampal-cortical transfer of contextual fear memories in rodents matches the baseline survival period for the majority of their adult-generated DGCs (Cameron et al., 1993; Kim and Fanselow, 1992). Increasing the rate of adult neurogenesis, and presumably therefore DGC turnover, could accelerate this clearance process. In a more direct test of this theory, in vivo radiation-induced depression of hippocampal neurogenesis promoted the maintenance of DG LTP and extended the period of hippocampal dependency for contextual fear memories in rodents (Kitamura et al., 2009). Pharmacological hippocampal inactivation during retrieval impaired remote memory expression specifically in animals with previously suppressed neurogenesis (either by radiation or Tg means) (Kitamura et al., 2009). Moreover, exercise-induced enhanced neurogenesis accelerated the decay of hippocampal dependency without altering retrieval. Collectively these studies not only point to a role for adult-generated DGCs in degrading existing memories, but also suggest that such degradation may promote the transformation of memories from hippocampus-dependent to hippocampus-independent forms in the cortex.

Ascribing a role for memory clearance to adult-generated DGCs warrants caution for at least two reasons. First, broad assertions regarding hippocampal memory processing from these well-designed studies are inherently hampered by many potential confounds. Effects of presenilin-1 knockout on memory could be mediated through several known non-neurogenesis-related mechanisms, including modulation of neuronal calcium entry (Yoo et al., 2000), alterations in amyloid precursor protein processing or accumulation (McGuire and Davis, 2001), and secondary effects of presenilins on other proteins via their secretase activity (Brown et al., 2000). Likewise, as previously discussed in Chapter 2, radiation-induced brain inflammation may impact memory performance (Monje et al., 2002; Rola et al., 2004). Second, balancing the aforementioned memory-promoting influence of neurogenesis with this idea of memory disruption likely necessitates some form(s) of homeostatic control mechanism(s) that adaptively regulate(s) the system (Chambers and Conroy, 2007; Chambers et al., 2004; Meltzer et al.,
Further studies will be required in order to establish the generalizeability and biological control of this hippocampal memory clearance phenomenon.

A1.5 Encoding Associations

A suggested critical learning and memory function of the hippocampus is to construct new associations between items separated in time or space (Eichenbaum et al., 1990; Rawlins, 1985; Wallenstein et al., 1998). Shors et al. proposed a specific role for adult-generated DGCs in forming a subset of associations, namely trace memories (Shors et al., 2001; Shors et al., 2002). Trace memories are maintained associations between discontinuous stimuli that were experienced at different times (Pavlov, 1927). These associations are studied using trace conditioning (TC), a type of classical conditioning where an interval of time or a “trace” separates the unconditioned and conditioned stimuli. In contrast, delay conditioning (DC) occurs when the unconditioned and conditioned stimuli overlap. Unlike DC, evidence suggests that TC is dependent on the hippocampus (McEchron et al., 1998; Moyer et al., 1990; Shors, 2004; Solomon et al., 1986). While traditional mechanisms of hippocampal neural plasticity have been implicated in TC, including postsynaptic density remodeling and increased dendritic spine density of CA1 pyramidal neurons (Geinisman et al., 2000; Leuner et al., 2003), so too has adult hippocampal neurogenesis (Shors et al., 2001). Shors et al. partially reduced neurogenesis and demonstrated a selective impairment for TC, not DC, which was subsequently rescued as neurogenesis recovered. Related studies demonstrate that associative learning in general increases the survival of immature new neurons in the adult hippocampus, regardless of stimulus timing, as long as a hippocampus-dependent behavioral paradigm is employed (Gould et al., 1999a; Leuner et al., 2006b).

Another theoretical associative function for new hippocampal neurons is in encoding time in new episodic memories (Aimone et al., 2006, 2009). Although there is general consensus that time is associated with episodic memories, how this occurs remains undetermined (Friedman, 1993). It is plausible that the hippocampus encodes temporal associations between new episodic memories given its central role in their formation (Moscovitch et al., 2005; Scoville and Milner, 1957; Squire and Zola-Morgan, 1991). Aimone et al.’s theory presupposes that immature adult-generated DGCs preferentially integrate into memory networks (Aimone et al., 2006). Assuming
that mature granule neurons encode events by converting EC inputs into sparse and distinct downstream representations (McNaughton and Morris, 1987; Treves and Rolls, 1994). Aimone et al. hypothesized that the constantly renewing population of immature DGCs encode inputs in a relatively less distinct manner (Aimone et al., 2006). Temporally associated representations could harbor partially overlapped immature granule cell-derived code, computationally modeled as pattern integration. This fits with the suggestion that experiences during a critical developmental period can promote experience-specific functional integration (Tashiro et al., 2007; Trouche et al., 2009). This could also account for the temporal properties of recency judgments in humans that are consistent with the time course for the maturation of newborn granule cells (Friedman, 1993, 2001). There is a rapid increase in the subjectively determined ages of events in the first weeks following their experience, when immature DGCs are undergoing considerable developmental changes, but small changes in perceived age beyond about 1–2 months when cells become less distinct from each other and developmentally-generated DGCs.

Preferential incorporation of adult-generated DGCs into hippocampal memory networks is critical to the temporal association theory for adult hippocampal neurogenesis. As previously discussed, preferential selection of these neurons during encoding is suggested by their transiently enhanced plasticity relative to their developmentally-generated counterparts (Ge et al., 2007; Schmidt-Hieber et al., 2004). While previous behavioral studies have also supported preferential integration by indirectly comparing functional incorporation of BrdU-labeled adult-generated DGCs to that of NeuN-labeled cells in the same animals (Kee et al., 2007b; Ramirez-Amaya et al., 2006; Tashiro et al., 2007), a direct comparison of equivalently labeled adult- and developmentally-generated DGCs finds similar integration rates into hippocampal memory networks, suggesting a functional equivalence between DGCs generated at different developmental stages (Chapter 3). Moreover, mature adult-generated DGCs exhibit similar neuronal connectivity, morphology, and electrophysiologic properties as developmentally-generated DGCs (Ge et al., 2007; Laplagne et al., 2006; Laplagne et al., 2007; Toni et al., 2008; Toni et al., 2007; Zhao et al., 2006). Although this evidence for functional convergence does not support a critical contribution of adult neurogenesis through outcompeting neighbors, it is not incompatible with a critical role via some other mechanism. Important contributions are still possible, especially in light of evidence that a normal neurogenesis level is necessary for
hippocampal memory function in some conditions (Deng et al., 2009; Shors, 2008). Incorporation studies solely indicate the number of adult-generated DGCs involved in memory processes, thus only permitting assessment of theories that hinge upon alterations in cellular incorporation rates. The absence of unique recruitment rates for adult-generated DGCs into larger memory networks still does not tell us what qualitative or content aspects of memory might be specifically mediated by these neurons.

The specific idea that cohorts of immature postnatally-generated DGCs are permanently tuned to temporally linked events is also challenged by a series of experiments exposing rats to several environments at different times (Alme et al., 2010). If adult-generated DGCs preferentially integrate into memories during a highly plastic period of development, experiencing multiple environments at separate points in time should generate network representations for each experience that include distinct cohorts of adult-generated DGCs. These representations should overlap less than those created by the same environments if experienced around the same time, because these temporally-linked experiences should share temporally tuned adult-generated DGCs. Accordingly, the total population of DGCs activated upon re-exposure to those experiences should be larger if they were initially separated by time. Both expression of the IEG Arc in, and electrophysiologic recordings from, putative DGCs indicate no such cumulative recruitment (Alme et al., 2010). In addition, the authors suggest that instead of permanently tagging memories with a temporal context through integrating into networks, recently born DGCs might preferentially respond to new stimuli in a temporary fashion in order to sculpt CA3 activity and generate time-encoded downstream representations. New DGCs would then become less excitable with age and respond minimally either to reinstatement of remote experiences or to new, even highly similar, events. This functional “retirement” of DGCs would lessen network overlap between temporally dispersed events because their respective CA3 representations would be created by different cohorts of adult-generated DGCs. Collectively, these findings suggest that if adult neurogenesis specifically provides a mechanism for encoding time in new memories, this likely does not occur at the cellular integration level.

Despite the aforementioned links between immature adult-generated-neurons and creating new memory associations, their role once mature remains poorly understood. For instance, the function of long-term survivors beyond the 1-week period of apparent hippocampal dependency for TC is unclear (Shors, 2004; Takehara et al., 2003). Whether this indicates a transient purpose
for these new neurons in trace associations, followed by other functions, remains unknown. Similarly unanswered is if immature granule neurons encode time, would they do so permanently or would downstream representations produced by these cells eventually do so independently. Aimone et al. theorizes that early pattern integration functions could eventually be complemented by the development of pattern separation capabilities (Aimone et al., 2009). They propose that adult-generated DGCs form specialized dimensions based on environmental experiences during maturation and eventually encode new information using those pre-defined dimensions, allowing the network to processes new information in the context of past experiences.

A1.6 Discriminating Between Similar Patterns

Pattern separation, the process of transforming similar representations or memories into highly dissimilar, non-overlapping representations, is considered a critical step in information processing within the hippocampal formation (McNaughton and Morris, 1987). Computational and behavioral studies assign this role to the DG given its relatively large neuronal population, sparse encoding, and ability to activate specific CA3 pyramidal neurons using single DGCs (Bakker et al., 2008; Gilbert et al., 2001; Leutgeb et al., 2007; O'Reilly and McClelland, 1994; Treves and Rolls, 1994). Not surprisingly, adult hippocampal neurogenesis has recently been implicated in this process.

In the first direct experimental evaluation of adult hippocampal neurogenesis in pattern separation, mice receiving focal hippocampal brain irradiation suffered a specific depression of DG neurogenesis and impaired performance in a delayed nonmatching-to-place radial arm maze task in which locations were closely spaced (Clelland et al., 2009). The radial arm maze findings were also verified substituting radiation with DG lentiviral vector delivery of dominant negative Wnt protein to partially knock down neurogenesis. Furthermore, this behavioral phenotype, with low but not high degrees of spatial separation, generalized to a touch screen spatial task only when a pattern separation paradigm was employed. More recently, enhanced neurogenesis following voluntary running improved spatial pattern discrimination performance (Creer et al., 2010), and using inducible genetic means of inhibiting adult-generated DGC apoptosis improved contextual discrimination performance (Sahay et al., 2011a). An important confound, however, is
the difference in task difficulty between low and high spatial separation tasks. It is possible that the low spatial separation tasks were sufficiently challenging to reveal a small memory deficit, but the less difficult high separation trials were insensitive to any difference.

Despite mounting behavioral evidence that adult-generated DGCs contribute to pattern separation, how this relatively small population of new neurons might assist a process that is seemingly reliant upon sparse encoding by a large DGC population remains speculative. One theory suggests adult-generated DGCs individually provide a constantly replenishing substrate for encoding subtle distinguishing features of stimuli and thus better resolve otherwise similar representations (Aimone et al., 2010, 2011). Alternatively, due to their hyper-excitability, young adult-generated DGCs might be more sensitive to subtle (or weak) inputs that serve to distinguish stimuli and ultimately function to modulate the larger DGC population (Sahay et al., 2011b). This modulation could take the form of feedback inhibition by young DGC-mediated activation of local inhibitory interneurons or mossy fibers. Feedback inhibition could enhance the sparseness of DG encoding and thus effectiveness of pattern separation. Given the high degree of recent interest in these concepts, it is likely that studies will begin to clarify this issue in the near future.