REGULATION OF DEVELOPMENTALLY-BORN AND ADULT-BORN NEURONS IN
THE DENTATE GYRUS

by

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Abstract

The discovery that neurons are added to the adult brain of nearly all mammals examined, including humans, has had profound effects on our understanding of the potential for plasticity in the brain. There has been an overwhelming focus on understanding the unique properties of adult-born neurons, often at the expense of another important cell population: the already present developmentally-born neurons. While the stages and integration of neurons in adulthood have been relatively well characterized, less is known about how developmentally-born cells integrate and survive over time and are regulated by experience. Since the dentate gyrus (DG) is comprised of large numbers of both populations, identifying the properties of these two populations and their relationship is essential for understanding how the dentate gyrus contributes to memory and behaviour. In chapter 2 we show that developmentally-born neurons die in early adulthood, unlike adult-born cells, which are known to remain stable (after reaching maturity). While adult-born neurons are unique during their immature stages, many reports indicate that they may become functionally equivalent to neurons born in early postnatal development once they have reached maturity. These data collectively suggest that adult neurogenesis may serve to replace lost developmentally-born cells. In chapter 3 we show that alternating 4-week blocks of running and memantine, an NMDA antagonist, produces sustained increases in adult neurogenesis in males, while in females interval running increased adult neurogenesis. In chapter 4 the relationship between the two populations was investigated using either neurogenesis-promoting or suppressing treatments during early adulthood. We found that increasing adult neurogenesis decreases the activity in the DG and specifically in
developmentally born neurons, indicating that there is a functional relationship between the two populations, and that adult-born neurons may act to inhibit older neurons. This thesis set out to better describe both the developmentally-born and adult-born neuronal populations and investigate the relationships between these two populations. Collectively, these data hope to clarify whether there are interactions between neurons born throughout the lifespan, which may shape how information is retained in the hippocampus and could prioritize treatments that are aimed at generating new cells vs. preserving older cells.
Lay Summary

The discovery that the brain can add new neurons in adulthood of nearly all mammals, including humans, has increased awareness of the ability of the brain to change. Specifically, in the hippocampus, a brain structure involved in learning, the addition of new neurons is important for forming memories. We do not know if these newly-born adult cells are more important or different from cells born around birth. In this dissertation, we looked at cell born in adulthood and development to understand their differences and if there is a relationship between them. Developmental cells showed a different pattern of survival than cells born in adulthood and adult-born cells also inhibited them. It is possible that adult cells could replace older developmental cells, which could improve the storage of new memories. This research allows some insight into where importance should be placed on finding treatments for memory loss.
Preface

**CHAPTER 2:** A version of the chapter has been published. Cahill, S. P., Yu, R. Q., Green, D., Todorova, E. V., & Snyder, J. S. (2017). Early survival and delayed death of developmentally-born dentate gyrus neurons. *Hippocampus, 27*(11), 1155–1167. S. Cahill conducted the experimental work with additional assistance from R. Yu and D. Green. S. Cahill and J. Snyder analyzed data and S. Cahill wrote manuscript with input from J. Snyder. S. Cahill performed all histology, counted BrdU and caspase for the 2-6 month groups, performed BrdU categorization of DCX, NeuN, and PCNA, and cellular location analyses and DAPI+ IEG activity. E. Todorova performed IEG activity of P6 Brdu, D. Green and R. Yu performed P6 BrdU counts for 1hr-8 week groups. J. Snyder and S. Cahill conceived and planned manuscript.

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**Chapter 4:** This data is unpublished. S. Cahill conducted the experimental work with additional assistance from J. Cole and A. Martinovic. S. Cahill analyzed data and wrote manuscript with input from J. Snyder. S. Cahill and A. Martinovic performed histology. S. Cahill performed
DCX, volume, and location analyses. A. Martonovic performed BrdU/cFos/zif and DAPI/cFos/zif analyses. J. Cole and S. Cahill complied running data. S. Cahill and J. Snyder conceived and planned manuscript.

All animal testing was in accordance with ethical guidelines set by Canada Council for Animal Care and were approved by the University of British Columbia Animal Care Committee, certificate numbers A13-0065 and A12-0339.
Table of Contents

Abstract ............................................................................................................................................... iii
Lay Summary ......................................................................................................................................... v
Preface .............................................................................................................................................. vi
Table of Contents ............................................................................................................................... viii
List of Tables ....................................................................................................................................... xiv
List of Figures ....................................................................................................................................... xv
List of Abbreviations ........................................................................................................................ xvii
Acknowledgements .......................................................................................................................... xx
Dedication ........................................................................................................................................ xxii

Chapter 1: Introduction ........................................................................................................................1

1.1 The Hippocampus ............................................................................................................................ 1

1.1.1 Connectivity of the hippocampus ................................................................................................. 2

1.1.1.1 The Dentate Gyrus .................................................................................................................. 5

1.1.1.2 CA3 ......................................................................................................................................... 7

1.1.1.3 CA1 ......................................................................................................................................... 8

1.1.2 Development of the hippocampal structure .............................................................................. 9

1.2 Neurogenesis .................................................................................................................................. 12

1.2.1 Definition of adult neurogenesis ............................................................................................... 14

1.2.1.1 Markers used to study adult neurogenesis – a selection ..................................................... 16

1.2.1.2 Characteristics of adult-born neurons .................................................................................. 20

1.2.2 Factors affecting adult neurogenesis ...................................................................................... 22
1.2.2.1 Increasing adult neurogenesis .......................................................... 23
  1.2.2.1.1 Exercise: wheel running .......................................................... 25
  1.2.2.1.2 NMDA receptors ................................................................. 27
  1.2.2.1.3 Memantine ........................................................................... 31
1.2.2.2 Decreasing adult neurogenesis ......................................................... 33
  1.2.2.2.1 Ageing and natural decline .................................................... 34
1.2.3 Augmenting adult neurogenesis and sex differences ....................... 36
1.3 Comparison of adult and developmental born cells ............................ 38
  1.3.1 Cellular development and survival .................................................. 38
  1.3.2 Relationship between two cell populations in adulthood .................. 42
  1.3.3 Cellular activity and functionality ................................................... 46
1.4 Hippocampus and human mental health ............................................. 48
  1.4.1 Neurogenesis, exercise and antidepressants .................................. 50
1.5 Thesis overview and objectives .......................................................... 53

Chapter 2: Early survival and delayed death of developmentally-born dentate gyrus neurons ........................................................................................................... 57

2.1 Introduction ............................................................................................ 57
2.2 Methods .................................................................................................... 59
  2.2.1 Animals and treatments ................................................................. 59
  2.2.2 Tissue processing and immunohistochemistry ............................... 60
  2.2.3 Microscopy and sampling .............................................................. 62
  2.2.4 Statistical analyses ........................................................................ 64
2.3 Results ..................................................................................................... 64
2.3.1 Early survival of developmentally-born DG neurons ............................................... 64
2.3.2 Expression of doublecortin and NeuN. ................................................................. 66
2.3.3 Expression of activity-dependent immediate-early genes ........................................ 68
2.3.4 Late death of developmentally-born cells ............................................................... 71
2.4 Discussion ................................................................................................................... 74
2.4.1 Alternative explanations for early survival and delayed death ............................... 76
2.4.2 Maturation and early zif268 expression relative to adult-born dentate gyrus neurons .............................................................. 77
2.4.3 Neuronal persistence, turnover and memory ........................................................... 78
2.4.4 Relevance for mental health .................................................................................. 80
2.5 Conclusions ............................................................................................................... 81

Chapter 3: Differential effects of extended exercise and memantine treatment on adult neurogenesis in male and female rats .................................................................................83

3.1 Introduction ................................................................................................................. 83
3.2 Methods ...................................................................................................................... 86
3.2.1 Animals and Treatments ...................................................................................... 86
3.2.2 Tissue processing and immunohistochemistry ....................................................... 89
3.2.3 Microscopy and sampling .................................................................................... 92
3.2.4 Running distance calculations ............................................................................. 92
3.2.5 Statistical Analyses ............................................................................................. 93
3.3 Results ......................................................................................................................... 93
3.3.1 Experiment 1 – Short term continuous RUN and a single MEM injection ............ 93
3.3.1.1 Short-term continuous running behavior ....................................................... 93
3.3.1.2 Five weeks of continuous running transiently increases neurogenesis........... 95
3.3.1.3 A single MEM injection transiently increases neurogenesis ....................... 97
3.3.2 Experiment 2 – Long-term continuous RUN.................................................. 99
  3.3.2.1 Long-term continuous running behavior ................................................. 99
  3.3.2.2 Effects of long-term continuous running on neurogenesis ...................... 99
3.3.3 Experiment 3 – Long-term interval RUN and multiple MEM injections ......... 101
  3.3.3.1 Long-term interval running behavior..................................................... 101
  3.3.3.2 Effects of long-term mMEM, iRUN and mMEM+iRUN treatments on neurogenesis............................................................. 103
3.4 Discussion........................................................................................................ 107
  3.4.1 Transient, early neurogenic effects of cRUN and sMEM............................. 108
  3.4.2 Differential effects of iRUN and mMEM..................................................... 109
  3.4.3 Neurogenic effects of RUN in males and females...................................... 110
  3.4.4 Neurogenic effects of MEM in males and females..................................... 112
  3.4.5 Implications for neurogenic therapies....................................................... 114

Chapter 4: Adult neurogenesis and the regulation of dentate gyrus neurons born in early postnatal development ............................................................................................................. 116
4.1 Introduction....................................................................................................... 116
4.2 Methods.......................................................................................................... 120
  4.2.1 Animals and Treatments............................................................................ 120
  4.2.2 Tissue processing and immunohistochemistry ......................................... 123
  4.2.3 Microscopy and sampling......................................................................... 125
  4.2.4 Running distance calculations................................................................. 127
4.2.5 Statistical analyses ................................................................. 127

4.3 Results.......................................................................................... 127

4.3.1 Changes in DCX and DG volume after manipulations of adult neurogenesis .... 127

4.3.2 Manipulating the adult-born population alters cellular activity......................... 130

4.3.2.1 Increasing adult neurogenesis decreases IEG activity in the DG ............... 132

4.3.2.2 Decreasing neurogenesis during early adulthood increases IEG activity in
developmentally-born cells ........................................................................... 133

4.3.3 Blocking adult neurogenesis prevents the pushing of developmentally-born neurons into more superficial layers ................................................................. 133

4.3.4 IEG expression in BrdU+ and DAPI+ cells is found in similar locations after
increasing neurogenesis ................................................................................... 135

4.4 Discussion....................................................................................... 137

4.4.1 Effects of long-term manipulations of neurogenesis on cellular activity and cell
location ........................................................................................................... 139

4.4.2 Role new neurons may play in inhibition .................................................... 141

4.4.3 Possible limitations .................................................................................. 143

4.4.4 Implications for neurogenic therapies: importance of cellular age............... 145

Chapter 5: General discussion ........................................................................ 147

5.1 Overview of findings ............................................................................. 147

5.2 Implication for memory turnover ............................................................. 149

5.3 Insights into the role of new neurons: activity in DG after alterations of adult
neurogenesis ...................................................................................................... 152

5.4 Why include cellular age? ........................................................................ 154
List of Tables

Table 1-1 Comparing developmentally-born and adult-born neurons................................. 42
Table 5-1: Updated figure from section 1-3: comparison of developmental and adult populations.
........................................................................................................................................ 161
List of Figures

Figure 1-1 Visual representation of the Tri-synaptic circuit ........................................................... 4
Figure 1-2 Visual representation of the process of adult neurogenesis ............................................. 16
Figure 2-1: Dynamics of developmental neurogenesis and early cell survival. .............................. 65
Figure 2-2: Early timecourse of neuronal marker expression .......................................................... 67
Figure 2-3: Timecourse of activity-dependent immediate-early gene expression in P6-born DG neurons and the general population of DG neurons ......................................................... 70
Figure 2-4: Delayed death of developmentally-born neurons. .......................................................... 73
Figure 2-5: Comparison of early vs. late survival in developmentally-born and adult-born DG neurons ........................................................................................................................................ 75
Figure 3-1: Immunohistochemistry for neurogenesis markers used in this study. ............................ 91
Figure 3-2: Experiment 1: Short-term treatment with cRUN and sMEM ......................................... 94
Figure 3-3: Experiment 1: Transient effects of running on neurogenesis. ...................................... 96
Figure 3-4: Transient effects of a single memantine injection on neurogenesis .............................. 98
Figure 3-5: Experiment 2: Long-term continuous running .............................................................. 100
Figure 3-6: Experiment 3: long-term treatment with iRUN and mMEM ......................................... 102
Figure 3-7: Experiment 3: Effects of long-term iRUN and mMEM treatments on neurogenesis. ............................................................................................................................................. 106
Figure 4-1: Study designs of both manipulations of adult neurogenesis ........................................ 122
Figure 4-2: Effects of long-term manipulations of neurogenesis on the DG ................................. 129
Figure 4-3: Effects of manipulating adult neurogenesis on activity in DG in both developmentally born cells (BrdU) and the total granule cell (DAPI) population .................................... 131
Figure 4-4 Effects of manipulating adult neurogenesis on locations of developmentally-born cells within the GCL ................................................................. 134

Figure 4-5: Effects of manipulating adult neurogenesis on the locations of activity in DG in both developmentally born (BrdU) and overall granule cell (DAPI) population ...................... 137

Figure 5-1: Effects of long-term manipulations of neurogenesis on the survival of developmentally-born neurons ..................................................................................... 158
List of Abbreviations

Abeta- amyloid beta
AD- Alzheimer’s disease
ANOVA- analysis of variance
APP- amyloid precursor protein
BDNF- brain-derived neurotrophic factor
BrdU- Bromodeoxyuridine
CA- cornus ammonis
CBV- cerebral blood volumes
CldU- 5-chloro-2’-deoxyuridine
CON- control
cRun- continuous RUN
DAB- 3,3’-diaminobenzidine
DAPI- 4’,6-diamidino-2-phenylindole
DCX- doublecortin
DG- dentate gyrus
E- embryonic
EC- entorhinal cortex
ECL- lateral entorhinal cortex
ECM- medial entorhinal cortex
EE- environmental enrichment
EPSC- excitatory postsynaptic potential
GABA- gamma-aminobutyric acid
GAD- Glutamic acid decarboxylase
GCL- granule cell layer
GFAP- glial fibrillary acidic protein
GFP- green fluorescent protein
I.P.- intraperitoneal
IDU- 5-iodo-2’-deoxyuridine
IEG- immediate early genes
IGF- insulin-like growth factor
IPSC- inhibitory postsynaptic potential
iRUN- interval RUN
LTP- long-term potentiation
MCI- mild cognitive impairment
MDD- major depressive disorder
MEM- memantine
mMEM- multiple MEM injections
MRI- magnetic resonance
NeuN- neuronal specific nuclear protein
NMDA- N-methyl-D-aspartate
NMDAR- N-methyl-D-aspartate receptor
NSC- neural stem cells
OVX- ovariectomized
P- postnatal
PBS- phosphate buffered saline
PCNA- proliferating cell nuclear antigen
RUN- wheel running
S.E.M- standard error of the mean
SGZ- subgranular zone
sMEM- single MEM injection
TK- GFAP-TK transgenic rats
VEGF- vascular endothelial growth factor
Zif268- also known as ERG1
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Dedication

This thesis is dedicated to all those I have lost during my time in graduate school.

To my father, nephew and grandmother.
Chapter 1: Introduction

1.1 The Hippocampus

The hippocampus is best known for the role it plays in memory, which was most famously brought to light with the findings from patient H.M.’s loss of the ability to form new declarative memories after surgery which resected a large portions of his medial temporal lobes, including large portions of his hippocampus (Milner et al., 1968; Squire, 2009). Further data linking the hippocampus to memory has been found experimentally in humans, other primates, and rodents (Murray and Mishkin, 1998; Burgess et al., 2002; Madsen et al., 2003; Wirth et al., 2003; Bakker et al., 2008; Winocur et al., 2012). The hippocampus has been shown to be involved in a wide variety of learning and memory tasks in rodents including spatial learning and memory, novelty detection, and fear memory to name a few (Gould et al., 1999; Guzowski et al., 1999; Drapeau et al., 2003; Chawla et al., 2005; Saxe et al., 2006; Epp et al., 2011; Winocur et al., 2012; Bolz et al., 2015; Wu et al., 2015). While the hippocampus has been thought to play an important role in memory, studies have shown that there may be a consolidation gradient, whereby some memories gradually become independent of the hippocampus as they are consolidated in other brain regions or are forgotten (Mumby et al., 1999; Clark et al., 2005; Kitamura et al., 2009; Kitamura and Inokuchi, 2014).

The structure of the hippocampus is known to be somewhat heterogeneous with dorsal and ventral subdivisions with diverse functions (Swanson and Cowan, 1977). The dorsal hippocampus has been implicated in cognition and spatial memory (Jung et al., 1994; Moser et al., 1995; Bannerman et al., 2004), while the ventral hippocampus has been tied to stress responses, emotional and feeding behaviour (Ferbinteanu and McDonald, 2001; Maren and Holt,
2004). The hippocampus is highly connected (covered in section 1.1.2), shown to have a diverse assortment of functions, play a part in various tasks and processes large amounts of incoming information. This may be possible due to the duality of the structure, (though the functions of each subdivision of the hippocampus are not exclusive) and its highly plastic ability in adulthood (covered in section 1.2).

1.1.1 Connectivity of the hippocampus

The hippocampal formation has been extensively studied and the connectivity of this structure has been clarified possibly to a greater extent than any other cortical area (Cajal, 1911; Blackstad, 1956; Hjorth-Simonsen, 1972; Swanson, 1979; Bayer, 1985). This may be in part to the relatively simple organization and structural arrangements of the hippocampal formation. While connectivity within the hippocampal formation may be largely understood, how it contributes to the functions of the hippocampus, which are complex and wide, is far from clear. Swanson (1979) noted that part of the difficulty with relating connectivity to function within the hippocampus is that fibres entering and leaving hippocampal fields often pass through adjacent areas. This innervation of fibres can confound the interpretation of anatomical, physiological, and behavioural studies that use stimulation and ablation methods, as fibres-of-passage may be activated or disrupted incorrectly. This section will highlight the connectivity within the hippocampal formation and our understanding of how this structure connects with other areas of the brain.

The hippocampal formation is composed of the following subfields: the Ammon’s horn (containing Cornus Ammonis (CA) 1, CA2 and CA3), the dentate gyrus (DG), the entorhinal cortex (EC) and subiculum. It can be convenient to divide the hippocampal formation into 2 major divisions: the hippocampal region containing the DG, CA1, CA2, and CA3 and the
retrohippocampal region containing the subiculum and EC (Cenquizca and Swanson, 2007). Intra-hippocampal activity consists of sequential excitatory projections forming the tri-synaptic loop; this circuit has the unique feature that many of its connections are unidirectional (Swanson, 1979, see Figure 1-1). The dentate gyrus can be viewed as the first step in the hippocampal circuit. It receives its major input from the lateral EC (ECL) and medial EC (ECM) (Cajal, 1911; Nafstad, 1967; Hjorth-Simonsen and Jeune, 1972). These afferent inputs from the EC called the perforant path travel to the outer two-thirds of the molecular layer of the DG where they form contacts with dendrites of granule cells of the DG (Nafstad, 1967; Hjorth-Simonsen and Jeune, 1972). The axons of granule cells in the dentate gyrus, in turn, project heavily to the pyramidal cells of the hippocampal CA3 region via the mossy fibres (Blackstad, 1956; Blackstad et al., 1970). The pyramidal cells of CA3 give rise to a projection (Schaffer collaterals) that terminates predominantly in stratum radiatum of the hippocampal field CA1(Swanson et al., 1978). The CA1 then projects heavily to the subiculum (Swanson et al., 1978; O'Mara, 2006). To complete the circuit, both the CA1 and subiculum project to the EC (Swanson and Cowan, 1977; Beckstead, 1978; Swanson, 1981; O'Mara, 2006; Cenquizca and Swanson, 2007; Amaral, 2011, see figure 1-1 for visual).
Computational studies have begun to model the distinct functions of the sub-regions of the hippocampus. The DG has been described to filter the large volumes of information it received from the entorhinal cortex (EC), the perforant path, and acts to allow overlapping inputs to be separated (Bayer, 1985; Kesner et al., 2004; Rolls and Kesner, 2006). The DG sends axons, the mossy fibres, to CA3, an area defined to play a role in pattern completion (Rolls and Kesner, 2006). The Schaffer collaterals then connect the CA3 and CA1 regions, with the CA1 region being reported to processes inputs and outputs from downstream regions, connecting the hippocampus directly to cortical structures such as the prefrontal cortex (Swanson, 1981; Rolls and Kesner, 2006; Cenquizca and Swanson, 2007). The hippocampus is well-integrated with the EC, subiculum and CA1 connecting to many structures within the brain including the amygdala,
prefrontal cortex, nucleus accumbens, and brainstem structures (Swanson, 1981; Bayer, 1985; O'Mara, 2005; Cenquizca and Swanson, 2006; O'Mara, 2006; Cenquizca and Swanson, 2007).

1.1.1.1 The Dentate Gyrus

The dentate gyrus (DG) contains three layers (granule cell layer, molecular layer and hilus) and is the first step in the trisynaptic circuit (Bayer, 1985; Amaral et al., 1987; Amaral, 2011). The DG major cell type is the granule cell which resides in the granule cell layer (GCL), which consists of densely packed granule cells stacked 4-10 cells deep (Bayer, 1985). The GCL is divided into two blades; the portion of the GCL adjacent to CA1 is called the suprapyramidal blade and the other is the infrapyramidal blade (Cappaert, Van Strien, & Witter, 2015). While adult neurogenesis is shown to be higher in the infrapyramidal blade than in the suprapyramidal blade (Snyder et al., 2009c), a greater proportion of granule cells express immediate early gene (IEG) activity in the suprapyramidal blade. Looking at pattern separation in the DG, the process of reducing interference among similar inputs by using non-overlapping representations (Aimone et al., 2014), when animals are placed in two distinct environments the suprapyramidal blade is maximally responsive, showing recruitment of different neurons (Marrone et al., 2011; Satvat et al., 2011). When animals are placed in the same environment twice there is little change in IEG expression in suprapyramidal blade during the second exposure. This change in activation pattern based on environmental exposure is not displayed in the infrapyramidal blade which remains constantly inactive regardless of the manipulation (Marrone et al., 2011; Satvat et al., 2011).

There are approximately 1,000,000 granule cells in the rat DG (Bayer, 1982; Boss et al., 1985) though this number can range from 630,000 to 1.5 million (Schlessinger et al., 1975; Gaarskjaer, 1978; West and Andersen, 1980; Seress and Pokorny, 1981; Bayer, 1982; Boss et al.,
depending on age, strain and counting method, while the mouse granule cell estimation has been approximated at between 350,000 - 500,000 (West and Andersen, 1980; Amrein et al., 2004). With a large population of cells compared to the input from EC and downstream output CA3 (Boss et al., 1987; Merrill et al., 2001), and low levels of activation, with 2-6% of DG granule cells shown to be active after exposure to a given context (Vazdarjanova, 2004; Schmidt et al., 2012), the DG has been typically associated with a pattern separation function. This means that more neurons are available overall, which would allow for a small proportion of neurons to be used to represent information (Kubik et al., 2007; Satvat et al., 2011; Deng et al., 2013).

Another reason is the low activity of granule cells and high tonic inhibition within the DG, which would allow for sparse coding needed for pattern separation (Jung and McNaughton, 1993; Leutgeb et al., 2007).

The molecular layer contains the dendrites of the granule cells. The unipolar dendrites of the granule cells receive its major input from the EC, via the perforant pathway. These afferents from EC are confined to the outer two-thirds of the molecular layers (Nafstad, 1967; Hjorth-Simonsen and Jeune, 1972). The lateral EC terminates in the superficial third of DG molecular while the medial EC terminates in the middle third of the DG molecular layer (Hjorth-Simonsen and Jeune, 1972). The hilus contains the axons of the granule cells called the mossy fibres, which project heavily to the proximal dendrites of the pyramidal cells of the hippocampal CA3 region. The layer of mossy fiber termination is located just above pyramidal cells layer of CA3, the ratio of innervation is about 12:1, as the DG has a much higher cell density compared to the CA3 (Acsády et al., 1998; Gonzales et al., 2001). Each granule cell likely influences between 14-28 CA3 cells and each of these CA3 pyramidal cells can receive contact from up to 50 granule cells (Acsády et al., 1998). This sparse connectivity and location of the mossy fibers are thought to
play a role in efficient information storage in the CA3 (Jung and McNaughton, 1993; Treves and Rolls, 1994; Rolls and Kesner, 2006).

1.1.1.2 CA3

One of three regions of the Ammon’s horn, the CA3 is the second step in the trisynaptic loop. It can be distinguished from its neighbouring region CA2 by the innervation from the mossy fibres of DG (Bayer, 1985; Amaral et al., 1987; Amaral, 2011). The proximal dendrites containing large complex spines which reside in the stratum lucidum and provide the primary site of connection for the mossy fibres from the DG granule cells (Cajal, 1911; Blackstad et al., 1970). The CA3 also receives afferent connections from both the lateral EC and the medial EC which terminate in the stratum lacunosum-moleculare (Van Groen et al., 2003). The CA3 has two primary projections those to CA1 and those connections back onto CA3 pyramidal cells. Pyramidal cells of the CA3 project back to CA3 creating recurrent collaterals, residing primarily in the stratum radiatum (Amaral and Witter, 1989). Each CA3 pyramidal cell can connect with up to 50 other CA3 neurons, this recurrent connection via these axon collaterals could cause amplification in the information from the DG to the CA3 (Acsády et al., 1998). The CA3 is predicted to be important for pattern completion, the retrieval of hippocampus-dependent information with incomplete retrieval cue (Rolls and Kesner, 2006).

Episodic memories are formed and stored within the CA3 network and the extensive recurrent collateral connectivity allows for the retrieval of a whole representation of a memory to be recalled with only some parts of the same representation presented (Treves and Rolls, 1994; Rolls and Kesner, 2006). When looking at the overall activation of CA3, it has been shown that 20-40% of neurons are activated during exploration of a novel environment, much greater than which has been reported in the DG (Vazdarjanova, 2004). The CA3 axons project to CA1
creating the Shaffer Collateral, these projections travel in the stratum oriens and stratum radiatum layers (Cappaert et al., 2015).

1.1.1.3 CA1

CA1 contains three layers that are common across the Ammon’s horn, stratum oriens, stratum radiatum, and stratum lacunosum as well as the principle cell layer, the pyramidal cell layer. CA1 is the last step in the trisynaptic circuit receiving projections from CA3 via the Schaffer collateral and sending projections out primarily via the EC and subiculum (Bayer, 1985). CA1 sends projections to all regions of subiculum, which in turn projects to many cortical and subcortical regions, though CA1 does have some direct projects to these regions (Swanson, 1981; Cenquizca and Swanson, 2006; 2007). It has been suggested that the subiculum may act to gate the output of the hippocampal formation, as the most major output of the CA1 is to the subiculum (Amaral et al., 1991; O’Mara et al., 2001). Like both the DG and CA3, CA1 does receive projections from EC, but these projections are sparse and project into the lacunosum moleculare (Steward and Scoville, 1976; Swanson et al., 1978; Swanson, 1979; 1981). With regards to activation, it has been shown the exposure to a new environment causes 40-70% of CA1 pyramidal cells to become active, which is the most active between the DG, CA3 and CA1 (O’Keefe, 1976; Wilson and McNaughton, 1993; Vazdarjanova, 2004).
1.1.2 Development of the hippocampal structure

The hippocampal formation is formed both in the prenatal and postnatal periods with the Ammon horn (CA1, CA2, CA3) completely forming during the prenatal period completing its formation before birth. Ammons horn contains three layers: the stratum radiatum, stratum oriens and stratum lacunosum moleculare, while CA3 also contains stratum lucidum. The three layers are generated between embryonic day (E)15- E17 (Altman and Bayer, 1990a). The stratum radiatum and stratum oriens neuronal production begin at E15 and the migration and formation for the stratum radiatum and stratum oriens occurs between E16-E19 (Bayer, 1985; Altman and Bayer, 1990a). The pyramidal cells are generated between E16 – E21, with a regional difference from CA1 and CA3 (Bayer, 1980; Altman and Bayer, 1990b; Hayashi et al., 2015). While the CA3 cells show an earlier peak in pyramidal cells generation at E17 compared to CA1 peaking at E19, the CA1 is completed its formation before the CA3 at E19 vs E22 (Bayer, 1980; Altman and Bayer, 1990b). This delay in the formation of the CA3 is related to the late formation of the curved portion of the CA3, which is delayed due to the late formation of the DG, with this curved portion of the CA3 forming at E21-E22, occurring after the DG migration has entered its later stages (Altman and Bayer, 1990b).

The DG is truly unique as its developmental process spans both the prenatal and postnatal periods, continuing into adulthood through the addition of new cells to this region (Altman and Das, 1965; Schlessinger et al., 1975; Altman and Bayer, 1990a; 1990b). During the prenatal period, the migration of cells that will later form the DG begin in the primary dentate neuroepithelium located at the dentate notch at the ventricular surface, begins substantial division at E17 in the rat (Altman and Bayer, 1990a). These newly divided cells migrate through
the secondary dentate matrix, which is connected by the DG migration to the tertiary dentate matrix by E19 (Altman and Bayer, 1990a). The tertiary dentate matrix is the final stage of the migratory stream, consisting of a large body of proliferative cells located in the DG (Altman and Bayer, 1990a; 1990b).

These migratory streams host a mixture of cellular ages including those originating in the primary dentate neuroepithelium and those multiplied locally (Schlessinger et al., 1975; Altman and Bayer, 1990a; 1990b). Following an outside-in formation pattern, the oldest cells are found on the outer portion of the suprapyramidal blade which begins to form around E21 (Schlessinger et al., 1975; Altman and Bayer, 1990a). The formation of the DG follows three distinct patterns which may be related to the efferent and afferent connections and their formation (Schlessinger et al., 1975). These patterns are as follows: 1. the formation along the temporal to septal pole; 2. the formation from the suprapyramidal blade to the infrapyramidal blade; and 3. the outside-in formation as referenced above (Schlessinger et al., 1975).

As established, the formation of the migratory streams occurs prenatally, while the formation of the DG occurs almost completely postnatal, with greater than 80% of dentate granule cells being generated after birth in rats (Altman and Bayer, 1990b). Neurogenesis peaks between postnatal days (P) 6-9 (Schlessinger et al., 1975; Altman and Bayer, 1990b). The subgranular zone (SGZ), which is the main site of proliferation after P30, also forms in the DG during the postnatal period (Altman and Bayer, 1990b). The SGZ originates at the deep aspect of the granular layer of the suprapyramidal blade as early as P1, reaching both blades by P10 (Altman and Bayer, 1990b). By P30 there is a reduction in the migratory streams that have supported the growth of the DG, and granule cell production shifts to the SGZ establishing a more adult-like DG (Altman and Bayer, 1990b). Groups have shown that the formation of the
adult-like SGZ occurs as an extension of DG development with the granule cells shifting toward a mature neuronal phenotype between P7 and P14 (Nicola et al., 2015; Radic et al., 2017).

As the structure of the DG forms both prenatally and postnatally through a series of timed processes, so too does the trisynaptic circuit of the hippocampus. The axons of the Ammon's horns are present are birth, and become fully developed by P14 (Singh, 1977). The perforant path from the EC develops its first fibres reaching the hippocampus at P9, reaching adult distribution by P13 and adult density by P15 (Singh, 1977). Developing at a similar time are the mossy fibres from the DG, arriving at CA3 at P10 and achieving adult density by P16 (Singh, 1977). There is also an interesting pattern for dendritic development at the afferent terminals in the hippocampal regions (specifically: CA1, CA3, and DG) (Bayer, 1985). The more proximal parts of the dendrites receive input from the latest forming (or youngest) afferents, while progressively older inputs are found more distally (Bayer, 1985). The afferent terminals form in the following timescale as described by Bayer (1985): Lateral EC (E15-E16), Medial EC (E16-17), CA3 (E17-E19), DG (P0-14).

It is important to note that while DG development is similar in rats and mice, there are clear species differences with mice showing an earlier time course than rats (described above). In mice, neurons of the DG arise as early as E10 (rats E17), the blades of the DG are formed by birth and the peak in cell addition occurs at P1 (birth) (rats P6-P9) (Angevine, 1965; Schlessinger et al., 1975; Altman and Bayer, 1990a; 1990b). It is also important to take into account that the granule cell population in rats is 3 times larger than that of mice (West and Andersen, 1980). These distinctive differences between mice and rat extend beyond development as new adult-born cells are more likely to survive and be recruited in rats than in mice, with new cells in mice showing a delayed maturation and formation of functional synapses(Snyder et al., 2009a). These
important difference between mice and rats must be considered when comparing both
developmental studies as well as and those comparing developmentally-born cells with those in
adulthood

1.2 Neurogenesis

It was a long-held belief for almost a century in neuroscience that no new neurons were
added to the brain after the developmental period, this idea stemming from the seminal work for
Santiago Ramon y Cajal (1913) who concluded that neurons are generated exclusively during the
prenatal phase of development (Cajal, 1913; Gross, 2000). Joseph Altman’s pioneering studies
provided the first anatomical evidence that neurogenesis persists beyond development in
mammalian brains (Altman and Das, 1965). Altman injected young rats with H³-thymidine and
found that new dentate granule cells were labeled in the postnatal rat hippocampus, using aged
rats, he showed that the number of labelled cells quickly decreased with age (Altman and Das,
1965).

Most neurons of the adult central nervous system are terminally differentiated during the
developmental phase, exist through the life of the organism, and are not replaced when they die
(Gage et al., 1998). However, there are two areas of the adult brain that continue to generate
neurons in appreciable numbers throughout adult life: the sub-ventricular zone of the anterior
lateral ventricles with cells migrating to the olfactory blub and the subgranular zone in the
dentate gyrus of the hippocampus (Gage et al., 1998). Here the focus will be on the subgranular
zone in the dentate gyrus [though new neurons have also been reported in other areas including
the neocortex (Gould et al., 1999; Gould, 2007; Cameron and Dayer, 2008) and hypothalamus
(Kokoeva et al., 2005) but this remains controversial.] Since the discovery that the adult brain
could create new neurons in the rodent hippocampus, hippocampal neurogenesis has been
observed in adult animals from birds to humans (Rat: (Altman and Das, 1965; Cameron et al., 1993), Mice: (Kempermann et al., 1997a), Macaques: (Kornack and Rakic, 1999; Gould et al., 2001), Birds: (Barnea and Nottebohm, 1996), Humans: (Eriksson et al., 1998).

This discovery of neurogenesis in the adult brain confronted the persistent assumption that adult brain was static and that the cells in the adult brain lacked the ability to undergo proliferation to create new adult-born neurons. The significance of the birth of these new cells and their abundance have been controversial since their discovery (Rakic, 1985; Abrous et al., 2005; Kempermann et al., 2018). Three major factors have provided convincing evidence and sparked increased interest for adult neurogenesis. First, the improvement of techniques for labelling dividing cells with nucleotide analogs [bromodeoxyuridine (BrdU)], protein markers specific to neurons [neuronal specific nuclear protein (NeuN)] and the ability to retrovirally label new neurons [green fluorescent protein (GFP)] allowed for unambiguous identification of adult-born neurons (Cameron and McKay, 2001; van Praag et al., 2002). Second, studies showing that young neurons did not simply occur at a persistent low rate of residual development; rather, it was heavily regulated by behavioural factors such as age, exercise, and enrichment (Kuhn et al., 1996; Kempermann et al., 1997a; van Praag et al., 1999a; Kempermann, 2015). Third, the confirmation of adult-born neurons in the human hippocampus, even in aged individuals, though the existence of olfactory bulb neurogenesis in humans is controversial (Eriksson et al., 1998; Curtis et al., 2007; Bergmann et al., 2012).

While human neurogenesis in the DG has been generally accepted, it has become a topic of hot debate and controversy as of late. Human neurogenesis was originally confirmed using BrdU, though sample sizes were low (Eriksson et al., 1998). Using the $^{14}$C technique, estimating the overall levels of neurogenesis, studies have shown that about 700 neurons are added to the
adult human brain and the turnover rate of granule cells is about 1.75% a year, showing that the process of adult neurogenesis is active and substantial in humans (Spalding et al., 2013). However, in 2018, two studies have provided contradictory evidence about the existence of adult neurogenesis in humans. While Sorrells and colleagues (2018) have shown that human hippocampal neurogenesis is undetectable in adulthood, Boldrini and colleagues (2018) showed the presence of adult neurogenesis (Boldrini et al., 2018; Sorrells et al., 2018; for review see Kempermann et al., 2018). The debate about human adult neurogenesis is an important one, but it is important to remember that human hippocampal plasticity, such as alterations in hippocampal volume, have been confirmed and have been shown to play an active role in human health such as improvements with antidepressants and exercise (Erickson et al., 2009; 2011; Boldrini et al., 2013). There is a need for better techniques to allow for the analysis of human adult neurogenesis to better address this debate (see section 1.4).

1.2.1 Definition of adult neurogenesis

Neurogenesis is the process of creating new and functionally integrated neurons, a process consisting of proliferation, differentiation, migration, maturation, synaptic integration, and survival (Gage et al., 1998; Zhao et al., 2008; Ming and Song, 2011; Aimone et al., 2014). During embryonic life neurogenesis expands the number of neuronal cells throughout the brain, while adult neurogenesis remains active in two spatially restricted neurogenic brain regions, the subgranular zone (SGZ) in the dentate gyrus of the hippocampus, where new dentate granule cells are generated; and the subventricular zone of the lateral ventricles, where new neurons migrate through the rostral migratory stream to the olfactory bulb to become interneurons (Gage, 2000; Ming and Song, 2005).
In the subgranular zone of the DG, activation of quiescent radial glial-like cells (Type 1 cells) generates intermediate progenitors (Type 2 cells), which sequentially generate neuroblasts (Type 3 cells). Undifferentiated immature neurons travel to the inner granule cell layer and differentiate. New neurons must then integrate into the existing hippocampal circuitry via the spread of dendrites towards the molecular layer and axons towards CA3 (Overstreet-Wadiche and Westbrook, 2006; Zhao et al., 2006, see figure 1-2 for visual representation). Type 1 cells possess a radial process that extends into the molecular layer, they express glial fibrillary acidic protein (GFAP) and Nestin and they are deemed to be relatively quiescent (Gonçalves et al., 2016; Toda and Gage, 2018). Type 1 neural stem cells divide asymmetrically, creating a neuronal lineage-restricted progenitor cell (type 2) and a glial lineage-restricted progenitor cell (type 1) (Gage et al., 1998; Seri et al., 2001; Abrous et al., 2005). Interestingly, the proliferation of type 2 cells has been shown to be subject to activity-dependent regulation through a number of physiological stimuli such as exercise (van Praag et al., 2005; van Praag, 2008) and pharmacological stimulation (Malberg et al., 2000; Lipton, 2004; Maekawa et al., 2009). Type 2 cells can give rise to neuroblasts (type 3) that express neuronal lineage markers and subsequently differentiate into mature dentate granule neurons (Gonçalves et al., 2016). Adult-born cells are generated in larger numbers than those that survive to maturity, with cells dying off at a steady rate between 1-4 weeks after birth, with reported losses of about 50% (Dayer et al., 2003; Kempermann et al., 2003). The adult-born cells that do survive and integrate into existing circuits become very stable, living for many months or possibly indefinitely (Dayer et al., 2003; Kempermann et al., 2003).
1.2.1.1 Markers used to study adult neurogenesis – a selection

The most common method for labelling dividing cells involves the incorporation of a traceable molecule into DNA. As DNA synthesis is generally limited to mitosis, at least at measurable levels, it has been used as a marker of neurogenesis (Aimone et al., 2014). During DNA replication in the S-phase of the cell cycle, exogenous nucleotides such as [H3]-thymidine or BrdU are incorporated into newly synthesized DNA and then passed on to cell progeny (Cameron and McKay, 2001; Hayes and Nowakowski, 2002; Taupin, 2007). In the late 1950s, [H3]-thymidine was developed to label dividing cell with autoradiography, this method was used to aid in the discovery of adult neurogenesis (Sidman et al., 1959; Altman, 1962; Altman and
Das, 1965). In the 1990s, the introduction of bromodeoxyuridine (BrdU), a synthetic thymidine analogue enhanced the study of adult neurogenesis as this molecule could be detected using immunohistochemistry (Gratzner, 1982; Aimone et al., 2014). By varying injection and examination time points, it has been shown that the use of these traceable exogenous nucleotides allows quantitative analysis of proliferation, differentiation, migration and survival of newborn cells (Crespo et al., 1986; Miller and Nowakowski, 1988; Kempermann et al., 1997a; Dayer et al., 2003). While the use of these traceable exogenous nucleotides is extremely valuable, these markers alone can only identify cells undergoing DNA replication and those that survive but cannot provide evidence of the phenotype of the cell. It is because of this limitation that these markers are used in combination with other antigens present in cycling cells (Abrous et al., 2005).

Newly born adult neurons fall into four general categories as they progress from neural stem cell (NSC) to mature neuron: stem cells, proliferative cells, immature neurons and mature neurons. As they pass through different developmental phases, different antigens are present at these different stages of the cells maturation and are used to categorize and understand the functional role of these new neurons. The most widely used stem cell marker is nestin, which is expressed in quiescent radial glia-like cells (Type 1) as well as intermediate progenitors (Type 2) (Ma et al., 2005; Zhao et al., 2008; Ming and Song, 2011). Glial fibrillary acidic protein (GFAP) can also be used to differentiate between type 1 and type 2 cells, as it is only present in type 1 cells which are radial glia-like, thought traditionally GFAP is used as an astrocyte marker (Ma et al., 2005; Zhao et al., 2008). GFAP has been utilized by numerous studies in combination with BrdU to identified newly divided astrocytic cells (Seri et al., 2001; Balu and Lucki, 2009; Bednarczyk et al., 2011;
Bonaguidi et al., 2016; Gonçalves et al., 2016; Sibbe and Kulik, 2016; Vivar and van Praag, 2017).

The most commonly used proliferative marker is Ki-67, a nuclear protein expressed in dividing cells for the entire duration of their mitotic activity, and is not linked to DNA repair nor to apoptosis (Kee et al., 2002). Another markers of cell proliferation is proliferating cell nuclear antigen (PCNA), which is increasingly expressed throughout a larger part of the cell cycle that Ki-67, expressed through G1, peaks at the G1/S interface, and decreases through G2 (Kurki et al., 1986; Galand and Degraef, 1989).

For the identification of immature neurons, doublecortin (DCX), which encodes a microtubule-associated protein, has most commonly been used as it has been shown to be expressed between the stages of neuroblasts (Type 3) and differentiated neurons (Francis et al., 1999; Gleeson et al., 1999; Nacher et al., 2001a; Brown et al., 2003; Ming and Song, 2011). Specifically, DCX has been observed 2 hours after cell labelling (S-phase), with high co-labelling with proliferative markers such as Ki67 (Brown et al., 2003). DCX expression continues as new neurons transition into their mature phases, with DCX and neuronal specific nuclear protein (NeuN) coexpressed between day 10 and 14, and then DCX becoming undetectable in these cells as they reach full maturity (Brown et al., 2003). NeuN is a marker for fully-differentiated or mature neurons and serves as an excellent marker for the identification of neuronal phenotypes of the adult brain (Mullen et al., 1992; Ming and Song, 2011). NeuN is expressed in the nucleus of neurons and is first detected around 10 days after cell division, and it has been shown that approximately 85% of newly dividing cells in the DG are NeuN+ (Brown et al., 2003).
To look at the functional role of both new adult-born and all granule neurons immediate early genes (IEGs) have been used as a proxy for functionality and a marker of neuronal activity. Changes in IEG expression are correlated with neuronal firing and IEGs are shown to be up-regulated when a neuron fires an action potential all indicating that IEGs are a good proxy for neuronal activity (Sheng and Greenberg, 1990; Guzowski et al., 1999; Chawla et al., 2005; Guzowski et al., 2005; Kovács, 2008; Minatohara et al., 2015). IEGs are dynamically regulated in response to neuronal activity in the brain, with increased expression after learning and experience-dependent expression in populations of cells depending on the environmental context (Guzowski et al., 1999; Ramirez-Amaya, 2005). There are a number of IEGs that have been studied within the hippocampus but here c-fos and zif268 have been selected. Most of our understanding of the types of neuronal activity being labelled by both c-fos and zif268 has been demonstrated by genetic knockout studies. While both c-fos and zif268 have been shown to be expressed in newborn granule neurons during the critical periods of maturation, the survival of these new neurons during this critical period of integration between 2-3 weeks after birth seems in part to be regulated by zif268, as zif268 knockout mice show increased cell death of this population (Veyrac et al., 2013). Both c-fos and zif268 have been associated with memory in the hippocampus, and c-fos expression is shown to increase following spatial learning (Jessberger and Kempermann, 2003; Kee et al., 2007). While c-fos expression has been shown to increase in relation to memory, c-fos expression may not be necessary for spatial learning as c-fos knockout mice demonstrated normal spatial learning (Zhang et al., 2002). Hippocampal-dependent learning rapidly induces zif268 and zif268 knockout mice show impairments in long-term memory in various learning tasks; zif268 involvement in hippocampal memory seems to be a role in the transition from short-to-long term memory (Jones et al., 2001; Katche et al., 2012).
1.2.1.2 Characteristics of adult-born neurons

New neurons follow a stereotypic process as they integrate into the existing hippocampal circuit. First they possess silent synapses, then excitatory gamma-aminobutyric acid (GABA) responses, then glutamatergic inputs, and finally GABA inhibitory responses (Espósito et al., 2005). Initial activation of newborn neurons is nonsynaptic: ambient GABA released from local interneurons causes tonic activation of new neurons (Espósito et al., 2005; Ge et al., 2006). Newborn neurons then become activated by input-specific GABAergic signaling through synaptic transmission from local interneurons and excitatory glutamatergic synaptic inputs begin to activate the new neurons through dendritic inputs (Espósito et al., 2005; Ge et al., 2006; Overstreet-Wadiche and Westbrook, 2006). Finally, GABAergic synaptic inputs are converted from excitatory to inhibitory (Espósito et al., 2005; Ge et al., 2006).

A unique characteristic of new neurons is the switching of GABA influence of neuronal polarity over neural development. Through the stage of type 2 cells to neuroblasts (type 3), there is tonic GABA activation resulting in depolarization of newborn neurons (Ge et al., 2006). This depolarization is caused by chloride efflux due to NKCC1 transporter expression in progenitor cells and immature neurons which causes GABA to depolarize instead of hyperpolarizing cells (Li et al., 2002). This initial phase of GABA depolarization has been shown to be critical for the maturation new adult-born neurons, as knockdown of the NKCC1 transporter expression in immature neurons has been shown to lead to defects in synapse formation and dendritic development (Ge et al., 2006).

Other unique characteristics exhibited by new adult-born neurons are their hyperexcitability and enhanced synaptic plasticity during a critical period of their development between 1 and 1.5 months (Schmidt-Hieber et al., 2004; Ge et al., 2007). As well these newborn
cells display a high input resistance (Espósito et al., 2005), receive less inhibition (Li et al., 2012; Xiao et al., 2013), and decreased threshold for long-term potentiation (LTP) (Schmidt-Hieber et al., 2004). While adult-born neurons have enhanced plasticity and unique circuitry during their immature stages, many reports indicate that they become functionally equivalent to neurons born in early postnatal development once they have reached maturity (Liu et al., 2000; van Praag et al., 2002; Laplagne et al., 2006; 2007),

With the discovery that the adult brain could create new neurons two main lines of research have evolved, first to understand the fundamental properties of adult proliferating neural stem-like cells and how they integrate into preexisting networks and second to understand the functional relevance of these adult-born neurons (Abrous et al., 2005). Although the number of neurons added via adult neurogenesis is minimal compared to the total population of DG neurons, taking into account the finding of about 700 cells per day in humans added, the continuous addition over a lifetime implies that adult neurogenesis could have substantial effects on both structural and functional aspects of the hippocampal circuit (Gage, 2000; Abrous et al., 2005; Ming and Song, 2005; Spalding et al., 2013) There have been 2 primary mechanisms suggested by which adult-born neurons contribute to information processing within the hippocampal circuit. The first is that young neurons function as encoding units, with studies showing that adult-born neurons are selectively recruited by specific inputs (Ramirez-Amaya et al., 2006; Kee et al., 2007). These new adult-born neurons are shown to display hyperexcitability, enhanced plasticity (Ge, Yang, Hsu, Ming, & Song, 2007; Schmidt-Hieber, Jonas, & Bischofberger, 2004) and are better able to store information (van Praag et al., 1999a; Anderson et al., 2000; Schmidt-Hieber et al., 2004; Creer et al., 2010; Sahay et al., 2011). The second mechanism is that adult-born neurons can inhibit local hippocampal circuitry modulating
the activity in the DG (see Chapter 4, Scharfman, 2007; Drew et al., 2016; Adlaf et al., 2017). New adult-born neurons could play a role in the overall sparse activity levels seen in the DG, as they are shown to regulate local GABA interneurons activity, which act to inhibit granule cell activation (Ikrar et al., 2013; Drew et al., 2016; Adlaf et al., 2017). This sparse pattern of activity, which promotes pattern separation, is also enhanced with adult neurogenesis, it may be the role of adult-born neurons to inhibit older neurons (Burghardt et al., 2012; Nakashiba et al., 2012; McAvoy et al., 2016, see section 1.3 for a look at the interaction between adult-born and developmentally-born cells).

1.2.2 Factors affecting adult neurogenesis

Since the discovery that the brain has the ability to create new neurons into adulthood, there has been a dominating focus on adult neurogenesis and understanding the unique properties of these adult-born neurons. These new adult-born neurons do integrate and contribute significantly to the synaptic circuitry in the dentate gyrus (DG), showing stable long lasting survival once reaching maturity (van Praag et al., 2002; Jessberger and Kempermann, 2003; Laplagne et al., 2006; 2007). External manipulations have been shown to both positively and negatively impact the levels of neurogenesis. Understanding the effects of these alterations is important as these new adult-born neurons have been shown to play an important role in the cognitive and emotional functions of the hippocampus (Jacobs et al., 2000; Madsen et al., 2003; Drew and Hen, 2007; Martinez-Canabal et al., 2012; Winocur et al., 2012). The most prominent positive external manipulations affecting adult neurogenesis are environmental enrichment (EE), voluntary exercise, antidepressants, and learning (Kempermann et al., 1997a; Gould et al., 1999; van Praag et al., 1999a; Malberg et al., 2000). The external manipulations negatively affecting adult neurogenesis is less clear, while genetic manipulations and irradiation can be used to ablate
the addition of new neurons and in aging adult neurogenesis naturally declines (Kuhn et al., 1996), both stress (Gould and Tanapat, 1999; Mirescu and Gould, 2006) and seizures (Jessberger et al., 2007b) can have both negative and positive effects on adult neurogenesis, making the point that not all additions of cells may be beneficial for the circuit.

1.2.2.1 Increasing adult neurogenesis

Increases in the rate of adult neurogenesis have been shown to play an important role in various cognitive processes as well as the neuroplasticity of the DG (van Praag et al., 1999a). Many factors have been shown to increase the rate of adult neurogenesis including EE (Kempermann et al., 1997a), exercise (van Praag et al., 1999a; Kennard and Woodruff-Pak, 2012), antidepressant treatment (Malberg et al., 2000, this topic is covered in section 1.4), seizures (Jessberger et al., 2007a) and learning and memory (Gould et al., 1999), each playing a role in the proliferation and/or survival of new neurons and their integration into the functional circuit of the hippocampus.

Environmental enrichment (EE) has been shown to increase the survival of new adult-born cells. When compared to voluntary running, EE was shown to have similar increase in survival of new adult-born cells, while voluntary running was shown to increase proliferation to a greater degree than EE (Kempermann et al., 1997a; van Praag et al., 1999b). The similarity in survival between EE and voluntary running was found to be driven by the presence of running wheels in many enrichment protocols; while EE without running wheels still increases survival, the levels are lower than that of voluntary running (Ehninger and Kempermann, 2003).

It is also important to note, that while voluntary running has been shown to increase adult neurogenesis and affects both neuronal morphology and cognition (van Praag et al., 1999b; 2005; Redila and Christie, 2006; Zhao et al., 2006), the effects of forced treadmill running has
shown both negative and positive effects. Forced treadmill running has been shown to elevate corticosterone (Griesbach et al., 2012) and both improve spatial learning, memory and retention (Fordyce and Wehner, 1993; Uysal et al., 2005; Huang et al., 2006; Liu et al., 2009; Aguiar et al., 2011; Kennard and Woodruff-Pak, 2011) as well as lead to impairments (Barnes et al., 1991; Blustein et al., 2006) depending on the intensity of training (Kennard and Woodruff-Pak, 2012). Kennard & Woodruff-Pak (2012) compared the effects of low and high intensity forced treadmill running and found that while the high-intensity group did show increased improvements on physical fitness, this group was impaired in spatial memory acquisition while the low-intensity group showed increased spatial memory retention compared to the high-intensity group (Kennard and Woodruff-Pak, 2012).

Increasing hippocampal neurogenesis has been shown to improve hippocampal functions such as pattern separation, learning and retention of spatial memories (van Praag et al., 1999a; Anderson et al., 2000; Creer et al., 2010; Sahay et al., 2011). Studies have shown that this increase in hippocampal neurogenesis is related to the increasing demand on the hippocampus, as there is an interaction between learning hippocampal-based tasks and the increase in neurogenesis seen in these animals. Animals trained on a hippocampal-dependent task show increases in hippocampal neurogenesis and survival of new adult-born neuronal compared to animals performing a hippocampal-independent task (Gould et al., 1999; Epp et al., 2010). Learning has been shown not only to affect survival but also the maturation of new adult-born neurons, with training in the water maze, a spatial memory task, shown to increase the complexity of dendritic arborization and spine density (Tronel et al., 2010; Lemaire et al., 2012).

With regards to the seizures, while there is research showing large increases in hippocampal proliferation as well as long-term survival of these newly added cells, these
additions are not known to be positive (Parent et al., 1997; Jessberger et al., 2007a; 2007b). Seizures have been shown to change the morphology of adult-born neurons with both ectopic migration into the hilus and the extension of additional dendrites deep into the hilus making synaptic connections with hilar structures (Jessberger et al., 2007b). Along with the aberrant increase in adult-born cells and migration into the hilus, seizures have been shown to cause impairments on hippocampal-dependent tasks (Jessberger et al., 2007a).

1.2.2.1.1 **Exercise: wheel running**

Wheel running has been extensively studied because of its known ability to increase both the proliferation and survival of new adult neurons two-fold (van Praag et al., 1999b). The effect of running on these newly created cells is widespread with increased dendritic complexity, spine density of granule cells and alteration in LTP within the DG (van Praag et al., 1999a; Eadie et al., 2005; Redila and Christie, 2006; Dostes et al., 2016; Vivar et al., 2016). Wheel running has been shown to not only affect cellular properties but to also cause behavioural changes such as increases in spatial learning and memory on the water maze task (van Praag et al., 2005).

Increases in neurogenesis can be seen almost immediately in animal models with increases in proliferation seen as quickly as 24 hours after the introduction of running wheels. These increases in proliferation of newly added cells are often studied in short-term, studies that have looked into longer periods have shown that proliferation returns to baseline after 32 days (van Praag et al., 1999b; Kronenberg et al., 2006). While there is some debate about the length of initial enhancement in proliferation caused by running, the survival of these newly born cells is robust (van Praag et al., 1999b; Dostes et al., 2016). There have been groups that have shown that some effects of the running that may be prolonged past 30 days (van Praag et al., 2005; Patten et al., 2013), but many have found decreased effects over time or, as many have done,
only chose to look at the initial increases in neurogenesis (van Praag et al., 1999a; Naylor et al., 2005; van Praag et al., 2005; Kronenberg et al., 2006; Clark et al., 2010). It was this debate about the effectiveness of running that prompted us to investigate its neurogenic properties over short and long period in Chapter 3.

While the proliferative effects of running in animal models may be transient, findings in humans ranging from structural changes to cognitive and health-related improvements after physical exercise highlighted the importance of exercise on the hippocampal formation (covered in section 1.4.1). Physical exercise has been shown in humans to increase hippocampal volume and prevent age-related atrophy, which seems to be dependent on continued exercise as removal of exercise returns volume to baseline after an equal period of time (Colcombe et al., 2003; Erickson et al., 2011; Thomas et al., 2016).

The changes in neurotrophic factors within the brain associated with exercise are paralleled in animals and humans, where there is a connection between physical exercise, increased neurotrophic factors and increased hippocampal plasticity and adult neurogenesis (Tang et al., 2008; Zoladz and Pilc, 2010; Griffin et al., 2011; Marlatt et al., 2012; Lee and Soya, 2017). Running has been shown to enhance neurogenesis in part through the modulation of neurotrophic factors such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor (VEGF) (McAllister et al., 1999). BDNF has been shown to be the most downstream factor mediating the upregulation of adult neurogenesis by exercise with both short and long periods of exercise shown to increase BDNF (Marlatt et al., 2012; Abel and Rissman, 2013; Yau et al., 2014). BDNF has also been shown to play an essential role in other enhancements, such as the cognitive function and synaptic plasticity associated with exercise (Messaoudi et al., 2002; Vaynman et al., 2004).
1.2.2.1.2  NMDA receptors

One of the key mediators of plasticity is the N-methyl-D-aspartate (NMDA) receptor. The NMDA receptor is an ionotropic glutamate receptor which is composed of subunit families of NMDA receptors (NMDARs) NR1 and NR2 (A-D) (Cull-Candy and Leszkiewicz, 2004; Nacher et al., 2007). NMDARs are known mediators not only of neuronal birth, development, and survival (Cameron et al., 1995; Tashiro et al., 2006) but as well these receptors have been shown to play an important role in neuronal plasticity and connectivity of adult-born neurons (Ge et al., 2006; Tashiro et al., 2006). During the early phases of neuronal development there does appear to be significant alterations in the expression of NMDAR subtype expression, with NR1 and NR2B both being expressed in type 1 cells, absent from transiently amplifying progenitors (type 2–3 cells), and present in most cells by 14 days of age (immature neurons), indicating that dividing progenitor cells in the adult dentate gyrus express NR1 and NR2B receptors (Nacher et al., 2007). NR1 subunit is the obligatory subunit and appears to be necessary for the formation of functional channels (Schoepfer et al., 1994; Tashiro et al., 2006).

While the NR1 subunit is necessary for function, the NR2 subunit goes through a developmental switch in which the NR2B subunit is expressed first and appears to be associated with enhanced synaptic plasticity but NR2A becomes the dominant subtype as neurons enter mature phases, when there are increases in the threshold for activity-dependent modification of synapses (Kirkwood et al., 1996; Ge et al., 2006; Yashiro and Philpot, 2008). This switch from NR2B to NR2A subunit occurs around the time of the closure of the period of enhanced synaptic plasticity found in new adult-born neurons and when then new neurons are more similar to their mature and developmentally-born counterparts (van Praag et al., 2002; Ge et al., 2006).
A critical window between 4 and 6 weeks of the cell age exists when adult-born neurons exhibit enhanced synaptic plasticity, these adult-born cells transiently exhibit increases in the LTP amplitude and a decrease in the LTP induction threshold (Ge et al., 2007). This time window corresponds with a period of high anatomical plasticity for adult-born neurons, as dendritic spines and the formation of excitatory synapses begin to appear during this critical window (Espósito et al., 2005; Ge et al., 2006; Zhao et al., 2006; Toni et al., 2007). This critical window of enhanced synaptic plasticity in adult-born neurons has been shown to be dependent on the expression of NR2B subunits (Ge et al., 2007). Blocking NR2B subunits during this critical window has been shown to reduce LTP in adult-born neurons, showing that these newborn neurons have a higher dependence on the NR2B subtype during early maturation (Snyder et al., 2001; Ge et al., 2007). While the importance of NR2B subunit has been highlighted during the critical period, blocking NMDAR with APV, a specific antagonist of NMDARs, abolished LTP from both mature and new adult-born neurons, so NMDAR activation has been shown to be required for LTP regardless of the cell age (Ge et al., 2007; Kheirbek et al., 2012).

The critical role that NMDAR play in the survival of new neurons has been investigated by genetic knockdown of the obligatory subunit, NR1, during this critical period. While the role of NMDAR has been shown not to play a role in either the overall cell morphology (dendritic length or the number of branch points) or the temporal pattern of neuronal marker expression, NMDARs have been shown to play important roles in signalling cell survival (Tashiro et al., 2006; Mu et al., 2015). Tashiro et al (2006) found that knocking out the NR1 subunit decreases the density of new adult-born neurons and that this decrease in density occurred when cells were between 2 and 3 weeks of age, when spinogenesis is occurring, indicating the existence of a
survival mechanism during this period (Espósito et al., 2005; Tashiro et al., 2006; Zhao et al., 2006). They also showed that the survival of these new adult-born neurons is competitively regulated during this 2-3 week period by NMDARs: by globally blocking NMDAR activation using the competitive antagonist, CPP, they increased the survival of NR1-deficient neurons by 50%, indicating that the survival of new neurons is competitively regulated depending on the relative levels of NMDAR activation (Tashiro et al., 2006).

The increased loss of adult-born neurons lacking NR1 subunit between 2 - 3 weeks of age was suggested to be caused by insufficient spinogenesis during the critical time window for spine grown. Using an NR1 subunit knockout model, Mu and colleagues (2015) found that the density of new adult neurons was decreased and spine density was reduced in NR1 deficient cells. They concluded that NMDARs appear to promote the initial spine formation and that this may play a role in cell survival (Mu et al., 2015). They further tested the idea that insufficient spinogenesis alters cell survival and the role NMDAR plays by increasing cell survival in both wild-type and NR1 knockout animals. Increasing cell survival in wild-type animals caused a decrease in total spine density compared to the cells that were selected to survive under natural conditions and NR1 deficient animals had defects in spine growth and synaptogenesis. This suggests that natural death of adult-born cells might be a result of the failure of spine formation. It is possible to deduce that there is a role for NMDAR-dependent cell survival as the critical window for spine growth and synapse formation seems to line up with the defect in cell survival and spinogenesis see in the NR1 deficient animals (Espósito et al., 2005; Tashiro et al., 2006; Zhao et al., 2006; Mu et al., 2015).

While the knockout studies have indicated the importance of NMDARs for survival during the critical period and its role in circuit connectivity, these studies clearly indicate that
NMDARs plays a role during periods of specific maturation. Pharmacological alteration of NMDARs tell a somewhat different story for the role of NMDARs in the time course of cellular maturation, with these alterations looking at periods when cells are proliferating and the later survival of these newly born cells. Acute pharmacological blockade of NMDARs by antagonists has been shown to increase proliferation in the DG (Cameron et al., 1995; Nacher et al., 2001b). The increase in newly born adult neurons have been shown to be sustained from 7 to as long as 14 days post a single injection of both competitive (e.g. CGP37849) and non-competitive (e.g. MK801) NMDAR antagonists. The increased proliferation stimulated by NMDAR blockade is paralleled by an increase in the number of immature PSA-NCAM-expressing granule neurons and nestin-immunoreactive radial glia-like cells, which indicates that there is a possible stimulation of both maturation of these newly proliferating cells and the boosting of new type 1 radial glia-like cells to begin to divide, coming out of quiescence (Nacher et al., 2001b). This possibility is supported by findings in aged rats, showing that administration of competitive NMDAR antagonist increased proliferation and that these new neurons survived 21 days post-injection (Nacher et al., 2003). These aged animals also showed an increase in the number of nestin-immunoreactive radial glia-like cells in the DG. This increase could suggest that residual radial glia-like cells not only exist in the aged hippocampus and that its number can be enhanced by NMDAR antagonist administration, but that NMDAR blockade could act to stimulate these residual radial glia-like cells out of quiescent to act as neuronal progenitors (Nacher et al., 2003). While the blockade of NMDAR have been shown to increase proliferation it is important to note that this enhancement is not accompanied by a rise in cell death, or a microglial or astrocyte activation and that these cells have been shown to survive in young animals up to 14 days and in aged animals up to 21 days, showing that with acute treatment NMDARs by antagonists seems to
increase proliferation with no harm to the newly born cells or the structure itself (Cameron et al., 1995; Nacher et al., 2001b; 2003).

While blocking NMDAR pharmacologically has been shown to increase proliferation and these studies have shown that these cells survive for weeks, these have looked mainly during the period of time when cells are still transitioning to full maturity a period when many new cells are still lost (Kempermann et al., 2003). The role of NMDARs changes throughout the period of cell maturation with changes in action on adult-born cells depending on time, which has been clearly highlighted by pharmacological administration of NMDA. When NMDA is administered 1 hour or 3 days earlier, proliferation is decreased, while blocking NMDAR showed increases in proliferation within these timeframes (Cameron et al., 1995; Joo et al., 2007). During short periods, the actions on proliferation by NMDA are not surprising; it is when you look after longer periods of time (28 days) that the story becomes more surprising and complicated. When NMDA is administered 28 days before visualization, there is an increase in cell survival, while blocking NMDAR showed a decrease in cell survival compared both to controls and NMDA injected groups (Cameron et al., 1995; Joo et al., 2007). While NMDA administration does not increase initial proliferation, it does increase survival of these cells over longer periods. The actions of NMDAR blockade are not clear but the utilization of this method is of interest, but the relationship between blocking NMDARs and pharmacological administration of NMDA seem to further complicate the story.

### 1.2.2.1.3 Memantine

Memantine (MEM) is an open-channel uncompetitive low-affinity NMDA receptor antagonist, which has been shown not to accumulate in NMDA channels and does not interfere with overall normal synaptic transmission (Lipton, 2004). The neuroprotective effects of MEM
possibly come from its ability to block NMDAR, and decrease over excitation of NMDAR by glutamate, allowing for inhibition of excitation toxicity in diseased neurons (Lipton, 2004; Johnson and Kotermanski, 2006). MEM is an FDA-approved treatment for moderate to severe Alzheimer’s disease, and because of its ability to allow the physiological activation of NMDAR while inhibiting over-activation there are other possible benefits and applications (Johnson and Kotermanski, 2006; Thomas and Grossberg, 2009). MEM treatments have been shown to improve memory and decrease both amyloid precursor protein (APP) and amyloid beta (Abeta) in the hippocampus in animal models of Alzheimer’s disease (AD) while in humans cognitive and functional improvement have been seen in moderate to severe Alzheimer’s patients, but these effects are not seen in mild cases and improvements are small (Thomas and Grossberg, 2009; Di Santo et al., 2013; Yang et al., 2013; Liu et al., 2014; Sun et al., 2015).

Memantine, similar to other NMDAR antagonists, has been shown to influence adult neurogenesis. MEM has been shown to increase the proliferation of adult-born cells over both short and long periods with a single injection (Maekawa et al., 2009). Single injections of MEM have been shown to increased proliferation of adult neurons as quickly as 3 days post injection and a subsequent 3 fold neuronal increase is seen 7 days post injection (Maekawa et al., 2009; Ishikawa et al., 2014). This proliferation is followed by differentiation into mature granule neurons and this increase in new neurons is correlated with increased spatial and trace memory (Maekawa et al., 2009; Namba et al., 2010; Ishikawa et al., 2014). New adult-born neurons generated by an injection of MEM are incorporated into memory circuits similarly to naturally generated neurons, indicating normal functioning (Maekawa et al., 2009; Ishikawa et al., 2014).

The mechanisms by which blocking NMDAR increase adult neurogenesis are not well defined but MEM has been shown to not only promote proliferation of radial glia-like progenitor
cells, it has been shown to have the unique property of stimulating symmetric division, which could induce the expansion of the primary progenitor cell pool, which is not shown in other methods to increase adult neurogenesis (such as running or EE) (Namba et al., 2009). MEM treatment has also been shown to have effects on increasing BDNF, similar to running, but this effect on BDNF has been shown only with acute and not chronic treatment of MEM (Réus et al., 2007; Namba et al., 2009).

1.2.2.2 Decreasing adult neurogenesis

By ablating adult neurogenesis through irradiation, antimitotic agents and transgenic models, a greater understanding of the function adult-neurogenesis plays in the hippocampus has been achieved. While the literature on these ablation studies is somewhat divided, support exists for the role of adult neurogenesis in hippocampal memory based task with findings showing that a loss of adult-born cells leads to memory impairments (Winocur et al., 2006; Jessberger et al., 2009).

Pattern separation, or the ability to distinguish similar inputs, has been shown to be impaired through both irradiation and transgenic models (Clelland et al., 2009). This impairment reflects the inability to distinguish nearby cues, as animals demonstrated a greater impairment the closer the cues were placed to each other (Clelland et al., 2009). Madsen and colleagues (2003) compared the performance of irradiated animals showing no impairment on a hippocampal-independent, object recognition task, while irradiated animals were severely impaired on the hippocampal-dependent task, showing that disruption of neurogenesis was associated with defects in hippocampal-dependent memory only (Madsen et al., 2003). Martinez-Canabal, Akers, Josselyn, & Frankland, (2012) used an antiproliferation agent, to reduce neurogenesis and found that animals with reduced neurogenesis showed poorer acquisition and
retention of platform location in the water maze task, highlighting the importance of new adult-born neurons in spatial memory (Martinez-Canabal et al., 2012). The ablation of adult neurogenesis has also shown to affect spatial memory, impairing the acquisition and memory of platform location in the hidden platform version of the water maze task (Dupret et al., 2007). This impairment was absent if the water maze task could be solved without a spatial strategy, indicating that adult neurogenesis plays a role in spatial memory and possibly cognitive flexibility (Dupret et al., 2007). Studies that have found impairments caused by a loss of adult-born cells have led to an early understanding of how these cells function at a behavioural level. Despite these findings, there is much work to be done to fully understand the role of adult neurogenesis, as similar learning and memory impairments have been found with the inhibition of adult neurogenesis (Winocur et al., 2006) and globally disrupting the DG using lesions (Gilbert et al., 2001). As well both the type of memory and the delay for memory recall play a role in if an impairment is seen after adult neurogenesis is reduced or ablated, indicating that there is a complicated role for adult neurogenesis in hippocampal learning (Snyder et al., 2005; Winocur et al., 2006; Wojtowicz et al., 2008).

1.2.2.2.1 Ageing and natural decline

There is a age-related decline in the production of new adult-born neurons in the dentate gyrus, with Kuhn et al (1996) showing that from 6 – 27 months in rats there is a steady decrease in neurogenesis with levels at 50% by 9 months and 17% at 2 years of age compared to 6 months (Kuhn et al., 1996). A comparable finding has been shown in mice, showing an age-related decrease in new neurons 3 – 12+ months, as well as a reduction in the differentiation of new granule cells into neurons in the hippocampus (Hamilton and Holscher, 2012; Kuipers et al., 2015). A decrease in neuronal differentiation with age has been shown to be offset by an increase
in glial differentiation, as cell survival is not decreased with ageing, and gliogenesis is shown to be higher in aged animals (van Praag et al., 2005; Kuipers et al., 2015). As well the proportion of SOX2+ cells, a stem cell marker, is unchanged in aged animals but there is a clear reduction in proliferation of NSC, it seems there is a change both in increased differentiation of cell into glia as well as increased quiescence in ageing (Kuhn et al., 1996; van Praag et al., 2005; Hattiangady and Shetty, 2008; Kuipers et al., 2014). Another suggested explanation for the decline in hippocampal neurogenesis has been an increase in the circulating levels of glucocorticoids that occurs with ageing, as decreasing glucocorticoid levels through adrenalectomy was shown to increase adult-born granule cells in aged animals (Cameron and McKay, 1999).

Studies have shown that ageing not only leads to clear decreases in adult neurogenesis but is also associated cognitive decline (Kuhn et al., 1996; Martinez-Canabal et al., 2012). Martinez-Canabal et al (2012) examined groups of mice ranging from juvenile to middle-aged and found that middle-aged mice show both a decreased levels of adult neurogenesis as well as an impairment on both acquisition and retrieval of spatial water maze task (Martinez-Canabal et al., 2012). Aged animals shown impairments not only in spatial learning and memory but pattern separation as well as short-term memory (Kim et al., 2010; Wu et al., 2015).

Additional work reinforces the link between reduced levels of neurogenesis and impairments in learning and memory during ageing. A relationship has been demonstrated between the extent of spatial memory dysfunction in aged rats and the levels of adult-born neurons (Drapeau et al., 2003). Aged animals with preserved spatial memory have a higher level of cell proliferation and a higher number of new neurons compared with rats showing spatial memory impairments, results reinforce the idea that neurogenesis is involved in learning and memory and age-related cognitive functions (Drapeau et al., 2003).
Ageing leads to both morphological and synaptic changes in the hippocampus which may be related to the reductions in memory and learning that have been associated with ageing (Siette et al., 2013; Trinchero et al., 2017). The presynaptic densities in both the DG and CA3 have been shown to be reduced by ageing, and these reductions have been correlated with place recognition memory defects (Siette et al., 2013). As well, new adult-born cells in the ageing rodent brains have been shown to exhibit a slowed development, with shorter and simpler dendrites, as well as a delay in glutamatergic connectivity, as shown by a delay in the presence of dendritic spines (Trinchero et al., 2017).

The production of new granule cells can be enhanced in ageing through exercise or enriched environment, which has also been shown to improve other impairments found in ageing such as the slowed development of new adult-born neurons and cognitive declines (Kempermann et al., 1998; van Praag et al., 2005; Trinchero et al., 2017). Voluntary exercise in aged animals was shown not only to increase adult neurogenesis to 50% that of young animals but also to accelerate the development of new adult-born neurons, increasing the glutamatergic connectivity and dendritic growth, and presynaptic density to that of younger animals (van Praag et al., 2005; Siette et al., 2013; Trinchero et al., 2017). Exercise in ageing reverses impairments in both acquisition and retention of spatial memory, improves place recognition, and improves context discrimination (van Praag et al., 2005; Siette et al., 2013; Wu et al., 2015).

1.2.3 Augmenting adult neurogenesis and sex differences

Proliferation has been shown to be 45% greater in females than males, while there is some disagreement about the sex difference in basal rates of adult neurogenesis (Tanapat et al., 1999). Tanapat and colleagues (1999) showed with BrdU labelling that females show an increased level of proliferation but this increase is not carried over into survival as the sex
difference in neurogenesis is gone 14 days after cells are labelled (Tanapat et al., 1999). Others have shown that in young adults (increased basal adult neurogenesis), neurogenesis is similar between males and females, but as basal adult neurogenesis decreases with age, females maintain a higher level of both proliferation and neurogenesis (Roughton et al., 2012). As the difference in proliferation between male and females appears to be altered with age as well as the different patterns in the survival of newly born adult neurons, in chapter 2 we included both sexes to evaluate where there are differences in the developmentally-born population.

With respect to exercise, female rodents show increased running wheel activity compared to males (Asdell et al., 1962; Tokuyama et al., 1982; Berchtold et al., 2001). It has also been shown that the addition of new neurons related to running is greater in females than in males (Clark et al., 2008). There are also known sex differences in the neurotoxic effects of NMDA receptor antagonists, with adult female rodents showing neurotoxic reactions even at low doses, while males show no or lower reactivity (Fix et al., 1995; Jevtovic-Todorovic et al., 2001). Specific to MEM the sex differences are less clear; in female rats, pretreatment with low doses of MEM have been shown to impair 24-hour memory retention, while in males retention of a 24-hour memory is enhanced with pretreatment of MEM, though there have been findings of MEM increasing learning in females at higher doses (Zajaczkowski et al., 2000; Creeley et al., 2006; Zoladz et al., 2006). Based on the sex differences shown in both running and NMDAR blockade, in chapter 3 both males and females were included to look at how sex affects the rates of adult neurogenesis with the treatment of running, MEM or both.

There are known sex difference in spatial learning with females showing a preference for cued strategies. On a standard water maze task, where cued strategies cannot be employed, males outperform females (Williams et al., 1990; Chow et al., 2013). Chamizo and colleagues (2016)
did find that when given running wheels in an enriched environment males and females performed the same on a task where both a cued and spatial strategy could be used, though females still preferred a cue-based strategy (Chamizo et al., 2016). The activation of new neurons by spatial learning is also different between the sexes, though males outperform females on spatial tasks, female but not male performance is related to the activation of new neurons, which may have to do either with task difficulty or activation based solely on level of learning (Chow et al., 2013).

1.3 Comparison of adult and developmental born cells

Once adult-born cells fully integrate and become mature they are morphologically, functionally and physiologically the same or similar to the developmentally-born population (Dayer et al., 2003; Kempermann et al., 2003; Laplagne et al., 2006; Zhao et al., 2006; Stone et al., 2011). This has led many studies to use this general mature population as a comparison group for new adult-born and immature neurons (van Praag et al., 2002; Espósito et al., 2005; Ge et al., 2007). While the developmentally-born and adult-born populations are quite similar once mature, there are various difference between the early maturation of the populations which may have lasting effects, though little is known about whether maturation is different between these populations (see section 1.3.1). To better understand the role of newly added neurons it is important to have information about which populations are interacting and are most affected by manipulations.

1.3.1 Cellular development and survival

While these two populations do become functionally similar once mature, their developmental patterns are quite distinct (see Table 1-3). Adult-born cells mature at a delayed rate compared to their developmental counterpart staying in a immature state longer and taking
longer to express mature neuronal proteins (Laplagne et al., 2006; Overstreet-Wadiche et al., 2006; Zhao et al., 2006). Adult-born neurons have a delayed presentation of dendritic spines, which are delayed by about 4 days in adulthood (Zhao et al., 2006). The timing of spine formation may reflect an important physiological transition, as spines are the major postsynaptic site for neurons to receive glutamate inputs, with 90% of excitatory synapses occurring on dendritic spines (Zhao et al., 2006; Toni et al., 2007). Dendritic growth is also delayed in adulthood, this delay is correlated with spine growth. The more accelerated development of glutamatergic and afferent connections in neurons born in development is related to the active and enriched GABAergic environment found during this time, as deleting glutamic acid decarboxylase (GAD) 65, reduces GABA activity, causing developmentally-born new neurons to develop at the rate of adult-born cells (Overstreet-Wadiche et al., 2006; Zhao et al., 2006). Not only are the afferent connections delayed during the maturation of adult-born cells, so are the efferent connections to the CA3 region when compared to developmentally-born cells (Zhao et al., 2006).

These differences in maturation, which influence cellular integration, may be related to differences in the local environment between the adult and infant brain. In development, two differences may influence the faster maturation of cells. The developmental brain is more depolarizing, with robust depolarizing activity, showing increased spontaneous and synchronized network activity that is absent in the adult brain (Overstreet-Wadiche et al., 2006; Pedroni et al., 2014). This difference in early network activity may play a crucial role in the early refinement of local neuronal circuits, as the embryonic and developing brain must generate most of its neurons and glia in a fixed period of time and create various connections to create the brain (Götz et al., 2016).
Another important factor that may influence the maturation rates of neurons born during adulthood and development is the gliogenic environment. Glial cells are largely generated later in the postnatal window, after the peaks in neuronal cell birth (Schlessinger et al., 1975; Götz et al., 2016). This means that early developmental neurogenesis takes place in a privileged environment, where the default fate for cells is neuronal and gliogenesis is inhibited (Miller and Gauthier, 2007; Götz et al., 2016). Adult neurogenesis, on the other hand, occurs in a gliogenic environment, where adult neural stem cells (NSC) in the DG are surrounded by glial cells (Götz et al., 2016). This difference in environment means that while adult NSC are primed to become neurons, they will readily differentiate into either a neuron or a glia, while developmental NSCs readily differentiate into neurons (Ninkovic et al., 2013; Götz et al., 2016).

Another important factor in the relationship between developmentally-born and adult-born cells is the survival rates of these two populations. Adult-born cells are generated in larger numbers than those that survive to maturity, reportedly as high as 9,000 per day (Cameron and McKay, 2001), however, dying off at a steady rate between 1-4 weeks after birth, with reported losses of about 50% (Dayer et al., 2003; Kempermann et al., 2003). The adult-born cells that do survive and integrate into circuits become very stable, living indefinitely (Dayer et al., 2003; Kempermann et al., 2003). While the initial survival patterns of developmentally-born cells are not understood (investigated in chapter 2), it has been shown that these cells die off late into adulthood when cells are mature (Dayer et al., 2003). This cellular death indicates a transient function for at least some of these developmentally-born cells which differs from the finding that adult-born cells become permanent in the DG after 4 weeks (Kempermann et al., 2003). The differences between these two cell populations and their life cycles further suggest the possible variation in their function and relationships to each other, with the possibility that the loss of
developmentally-born cells may play a role in memory transience within the hippocampus and adult-born neurons may act to replace these older developmental neurons (Mumby et al., 1999; Toni et al., 2008; Kitamura et al., 2009; Kitamura and Inokuchi, 2014; Drew et al., 2016).

Although these cells differ in their early maturation processes and their later survival, they still go through many of the same functional steps towards maturity. First, cells are silent, then they receive slow (tonic) GABA, fast glutamate and fast GABA, leading to mature neurons which form a homogeneous population (Espósito et al., 2005; Laplagne et al., 2006; Stone et al., 2011; Pedroni et al., 2014). This functionally seemingly homogeneous neuronal population both receive excitatory afferents from the perforant path with similar functional characteristic, showing similar GABA and glutamate afferent connectivity, and inhibitory GABA inputs (Laplagne et al., 2006; 2007). The seamless integration of these two cell populations through substantially different environments suggests that adult-born cells may functionally replace developmentally-born cells. Studies comparing the functional interaction between adult-born and developmentally-born, and not just the mature granule cell population are lacking, so the intricacies of this relationship remain unclear (see section 1.3.2-1.3.3).
<table>
<thead>
<tr>
<th>Developmentally-born</th>
<th>Adult-born</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Appearance of spine</strong></td>
<td>Start: 12 days</td>
</tr>
<tr>
<td><strong>Axons at CA3</strong></td>
<td>Reach CA3 before adult-born</td>
</tr>
</tbody>
</table>
| **Dendrites** |  | • Delayed dendritic growth  
  • Similar dendritic complexity between 12 day developmental and 16 day adult |
| **Overall Maturation** |  | • Delayed maturation  
  • Lower expression of NeuN at 12 days (47% adult vs 67% in developmental) |
| **Cellular properties once mature** | • Lower IR at maturation  
  • Increased threshold current for spiking  
  • Lower excitability |  
| **Cellular environment** | • Increased GABAeric network activity  
  • Increased transcription factor activation  
  • Show random bursts of depolarizing activity (GDPs) |  
| **Activation** | Unknown | • No IEG before 2 weeks of age  
  • Peak IEG at 3 weeks |
| **Cell survival** | • Early survival unknown  
  • Cell shown to die off in early adulthood | • Large cell loss between 1-4 weeks when cells are immature  
  • Cells show stable survival after 4 weeks up to 11 months |

**Table 1-1 Comparing developmentally-born and adult-born neurons**

### 1.3.2 Relationship between two cell populations in adulthood

Contacting pre- and post- synaptic sites and creating stable connections is a crucial step for newborn neurons to functionally integrate into the hippocampal network. The relationship between the new adult-born population and the mature population (possibly developmentally born) has been investigated in terms of the integration of new neurons at both pre- and post-synaptic sites, how new cells acquire connections, the regulation of activity within DG, and how stable these connections are.

Information enters the DG via the EC; these connections form between axonal synapse boutons from the EC and post-synaptic connections from dendritic spines on granule cells.
Dendritic spines of new adult-born neurons preferentially synapse with multiple-synapse boutons, making contact with boutons that have already formed connections with mature granule cells already present (Toni et al., 2007). These immature new neurons form multiple-synapse boutons over 64% of the time but this preference for shared synaptic connection decreases as neurons mature over 2 months and become similar to mature neurons (Zhao et al., 2006; Toni et al., 2007). This switch from multiple to single synapse boutons could possibly be caused by the enlargement of spines, which causes older spines in the multiple-synapse boutons (from mature granule cells) to retract, indicating a possible competition at the site of EC axonal synapse boutons between new adult born and mature, possibly developmentally born cells (Toni et al., 2007).

Sparse activity and the addition of new neurons in adulthood are hallmarks of the DG and both are thought to be pivotal to its function. As such, the relationship between adult-born neurons and the larger mature granule cell population has been studied by altering the activity of each population or by manipulating the rate of adult neurogenesis. When spines on mature neurons were reduced there was an increase in adult neurogenesis both in terms of proliferation and immature neurons. As well, the competitive survival of adult-born cells at 3 weeks was increased, which is when new neurons undergo synaptic integration and spinogenesis (Tashiro et al., 2007; Mu et al., 2015; McAvoy et al., 2016).

While altering the mature population showed that there is competition for survival and integration, Adlaf and colleagues (2017) looked at the effects of manipulating adult neurogenesis and the repercussions on synaptic density and connectivity of mature granule cells. Increasing the rate of adult neurogenesis by increasing the survival of these cells lead to a compensatory decrease in spines on mature granule cells, while decreasing the adult population lead to no
change in the density of spines on mature granule cells, in both manipulations the total number of dendritic spines remained constant, suggesting balance (Adlaf et al., 2017). The addition of new adult-born neurons does not alter total dendritic spine density, there seems to be a redistributions of spines from old mature granule cells to new adult-born neurons, with new neurons first competing at axonal synapse boutons with both old dendritic spines and if replaced the old dendritic spines retract and are replaced by new dendritic spines of new adult-born neurons (Toni et al., 2007; Adlaf et al., 2017).

Adult-born neurons have also been shown to regulate local GABA interneuron activity, which acts to inhibit the mature granule cells population and could lead to the overall sparse activity seen in the DG. Drew et al (2016) looked that this relationship between adult-born neurons and the activation of GABA interneurons by manipulating both in vivo and in vitro the adult-born population using both optical stimulation and direct methods (EE, irradiation and aging) and looking at the inhibition of activity within the DG. They found that increasing the activity in adult-born cells decreased the activity of both mature granule cells and the total DG population, while increasing the activity of mature granule cells did not cause a decrease in activity. It seems that activation of adult-born cells leads to a net inhibitory effect on mature granule cells which is further supported by the finding that blocking glutamate blocked the increased inhibition (Drew et al., 2016). The finding that adult neurogenesis acts to inhibit mature granule cells, and that these new cells act not only as encoding units of new memories but also to modulate overall activity was displayed when the effects of modulating adult neurogenesis on synaptic transmission were investigated. Increasing adult neurogenesis lead to a decreased excitatory synaptic transmission and an increase in inhibition in mature granule cells. In contrast, reducing, either the number or activity of the adult population increased excitatory
synaptic transmission and decreased inhibition on mature cells by altering the IPSC/EPSC ratio to favor EPSCs (Drew et al., 2016; Adlaf et al., 2017). These findings seem to indicate a possible homeostatic relationship between the adult-born and older mature neurons in the DG.

At the mossy fibres, the competition for connections with CA3 neurons between the young and old neuronal populations shows both similarities and differences to what is observed at the EC to DG synapse. New adult-born neurons make axonal connections with hilar cells, granule cell layer (GCL) interneurons, and CA3 pyramidal cells. The sequence of connections follow the same steps as in development, first making contact with dendritic shafts and then spines (known as thorny excrescences). However, in adulthood, new neurons synapse with thorny excrescences that are also contacted by mature cells (Toni et al., 2008). This is similar to that of the multiple-synapse boutons found in dendritic spines, where new adult neurons first form spines on synapse containing older mature neurons, then switch during maturation from multiple to single synapse boutons. Here axonal boutons of new adult neurons recruit synapse with preexisting targets (older mature neurons), and compete for thorny excrescences, as is seen in new adult-born neurons on dendritic spines (Toni et al., 2007; 2008).

The role of synaptic transmission in the refinements of mossy fibres as well as the stability of the connection between young and old neurons and CA3 pyramidal neurons has also been investigated. When the synaptic output of mature cells (<6wks) was silenced for extended periods, no mossy fibre retractions was found, with no changes in mossy fibre density in the total population found, indicating that the mature cells, once connected seem to be stable (Lopez et al., 2012). In new immature neurons (~2-3weeks), when synaptic release is blocked, axons still reach CA3 but because these new cells are ‘silent’ no boutons form and this lack of connection causes the axons to retract, showing that synaptic transmission plays a role in refinement and
maturation of mossy fibres (Yasuda et al., 2011). By blocking neurogenesis, to reduce the addition of additional younger neurons, the axonal retraction found in the silenced new immature neurons was abolished; removal of neurogenesis inhibits inactive DG axonal elimination, showing that there is competition between young and more mature cells for connections at CA3 (Yasuda et al., 2011).

1.3.3 Cellular activity and functionality

The DG is made up of a population of cell born in development and those added throughout adulthood. While both are known to contribute to memory, it is less clear if these populations are functionally equivalent or are distinct pools that contribute to memory processing in different ways. It has been shown that adult-born cells must reach maturation to be incorporated into hippocampal circuits (Huckleberry et al., 2015). Once maturation is reached newly mature adult-born cells (5-8 weeks) have been shown to be twice as likely compared with the existing mature (NeuN+) granule cell population to be recruited into memory circuits as measured by immediate early genes (IEGs) (Kee et al., 2007). This finding of increased recruitment of adult-born cells into memory circuits was further supported by findings that although both new mature 5-month-old adult-born cells and the existing mature (NeuN+) granule cell population respond to spatial exploration, a greater proportion of new adult-born cells (~2.8%) respond to spatial exploration compared to the existing population of granule cells (~1.6%) (Ramirez-Amaya et al., 2006). While both studies indicate an increased recruitment in adult-born cells, these studies used the proportion of IEG expression in the labelled adult-born population compared to the general mature granule cell population (Ramirez-Amaya et al., 2006; Kee et al., 2007). This general mature population would consist of not only of developmentally-born and mature adult-born neurons but also adult-born cells that are very immature and may not
express IEGs. It has been shown that immature 1-week old adult-born cells do not express IEG markers and until early maturation at 4 weeks is reached suboptimal recruitment of these newly added adult-born cells is reported (Kee et al., 2007; Stone et al., 2011; Huckleberry et al., 2015). As well, NeuN is expressed in cells still expressing DCX, which is expressed in immature neurons until 4 weeks of age and NeuN has been shown to be expressed in cells as early as 1 week of age in adult-born cells (Brown et al., 2003; Snyder et al., 2009a). To rectify these issues Stone and colleagues (2011) used BrdU to specifically analyze cells activated by spatial memory tasks, labelling cells born in embryogenesis, early postnatal life, or adulthood. They found that the activation rates were equivalent regardless of when the cells were born and that overall activity measured by IEGs was the same, even though the age of the animals varied (Stone et al., 2011). These findings highlight two important things. The first is the importance of birth dating populations of dentate gyrus neurons. Earlier studies have suggested that there is an increased recruitment of adult-born over developmentally-born cells which turns out to be incorrect since NeuN labels cells that were born at many stages of life and not just adulthood (Ramirez-Amaya et al., 2006; Kee et al., 2007). The second is that, once mature, adult-born cells are similar to developmentally-born cells and seem to be recruited in a similar fashion (Laplagne et al., 2006; Zhao et al., 2006; Laplagne et al., 2007; Stone et al., 2011).

While knowledge about the rates at which cells are recruited is important, to fully understand if the developmentally-born and adult-born populations are functionally equivalent or distinct it is important to understand the type of information which recruit these two populations of granule cells. Tronel et al (2015) found that when a neuron is born affects the type of memory process it is active during showing that adult-born cells are more involved in spatial memory formation, task novelty, and are affected by prior learning experience as learning a new spatial
task increased to the recruitment of adult-born cells. Developmentally-born cells, on the other hand, were shown to be recruited when rats had to discriminate between dissimilar contexts (Tronel et al., 2015). This increased recruitment in developmentally-born cells during discrimination of dissimilar contexts is similar to findings by Nakashiba and colleagues (2012) that showed that older (~6wk) granule cells are required for pattern completion which included the ability to compare distinct pairs of experiences, while young adult-born neurons were crucial for contextual discrimination of similar contexts (Nakashiba et al., 2012).

1.4 Hippocampus and human mental health

The hippocampus has been shown to be affected in various disorders with reductions in hippocampal volume, alterations in hippocampal-dependent learning and memory as well as reductions in neurogenesis seen in both human and rodent models of depression, mild cognitive impairment (MCI) and Alzheimer's disease (AD) (Bremner et al., 2000; Malberg et al., 2000; Czéh et al., 2001; Campbell et al., 2004; Videbech and Ravnikilde, 2004; Dickerson et al., 2005; Schuff et al., 2008; Erickson et al., 2011; Cunha et al., 2013; Zeng et al., 2016; Fang et al., 2018). While there is a natural decline in the total volume of the hippocampus with age, beginning in early adulthood and stabilizing in the EC and DG around age 50 in healthy individuals (Daugherty et al., 2016). This loss in hippocampal volume in individuals suffering from depression, MCI and AD seem to continue to atrophy and the earliest pathology of AD, is the degeneration of EC neurons which has been shown to continue past 50 years (Gómez-Isla et al., 1996; Jack et al., 1998; Bremner et al., 2000; Mungas et al., 2005; Schuff et al., 2008; Brinke et al., 2015; Roddy et al., 2018).

Alzheimer's disease is the most common cause of dementia; currently, dementia affects around 30 million people worldwide (Selkoe and Schenk, 2003; Ferri et al., 2005). Alzheimer’s
disease (AD) is characterized by progressive memory loss and cognitive dysfunction, such as dysfunction in semantic and episodic memory, language, and spatial orientation (Dickerson et al., 2005; Goedert and Spillantini, 2006; Irvine et al., 2012). Both the prevalence and the incidence of AD are greater amongst women than men; as well males with AD show savings of cognitive dysfunctions compared to females (Anderson et al., 2000; Lobo et al., 2000; Irvine et al., 2012). In the progression of AD, the hippocampus is particularly vulnerable and is one of the earliest areas to be affected, and the pathological changes are particularly evident in this region (Braak and Braak, 1997; Silverman et al., 1997; Ikonomovic et al., 2008). The hippocampus has therefore become a primary target of magnetic resonance (MRI) studies and rodent models in Alzheimer’s disease, as both AD and MCI patients show reductions in hippocampal volume and the number of new neurons is decreased in both humans and animal models with AD (Schuff et al., 2008; Ekonomou et al., 2015). Current therapeutics temporarily ameliorate the symptoms of AD but few affect the underlying disease mechanism (Selkoe and Schenk, 2003; Thomas and Grossberg, 2009; Di Santo et al., 2013; Yang et al., 2013).

Depression is a prominent global public health issue, estimated to affect 12–17% of the population at some point during the lifetime of an individual (Kessler et al., 1994; 2003; Alonso et al., 2004). Major depressive disorder (MDD), a more-severe form of depression, is estimated to affect more than 300 million people worldwide. This form of depression is associated with increased individual suffering and risk of suicide (Eaton et al., 2008; Mrazek et al., 2014). There is a clear sex ratio in the incidence and prevalence of depression, with women showing a 2:1 ratio of depression incidence to men irrespective of age, while there have been studies showing that marital status and employment do impact the incidence of depression, with a 1:1 ratio around 55-60 years in some groups (Angst et al., 2002; Gutiérrez-Lobos et al., 2002).
Hypotheses on the neurobiology of depression include alterations in various mechanisms related to the hippocampus such as neuroplasticity, neurogenesis and stress (Kempermann and Kronenberg, 2003; Hanson et al., 2011). Patients with major depression are shown to have decreased hippocampal volume and impaired hippocampal learning and memory as well as impairments in episodic memory during depressive episodes (Burt et al., 1995; Zakzanis et al., 1998; Bremner et al., 2000; Wang et al., 2010; Boldrini et al., 2013; Huang et al., 2013; van Eijndhoven et al., 2013). Also familial risk for depression is associated with changes in hippocampal volume, as young girls with mothers with recurrent depression are shown to have reduced volumes (Chen et al., 2010). Another clear indicator of the role in the hippocampus in depression is that the response to treatment is related to increase hippocampal volume in humans (Sheline et al., 2003; MacQueen et al., 2008) and is neurogenesis dependent in rodents (Malberg et al., 2000; Czéh et al., 2001; Santarelli et al., 2003).

1.4.1 Neurogenesis, exercise and antidepressants

What has been shown is the adult human brain’s capacity to respond to methods known in rodents to increase new neurons (i.e. physical exercise, memantine, antidepressants) by measure of increases in hippocampal volume and improvements in cognition in healthy individuals rather than increase in adult neurogenesis (Erickson et al., 2011; Killgore et al., 2013). The clear similarities between the effects of physical exercise in rodent models and humans was shown by Pereira and colleagues (2007) showing that cerebral blood volumes (CBV) in exercising mice was increased in the DG and was correlated with neurogenesis. They then looked at CBV in exercising humans and saw that CBV was increased in the DG and this correlated with cognitive function (Pereira et al., 2007).
Physical exercise has been shown in humans to increase hippocampal volume and prevent age-related atrophy, which seems to be dependent on exercise as removal of exercise returns volume to baseline after an equal period of time (Colcombe et al., 2003; Erickson et al., 2011; Thomas et al., 2016). Studies evaluating the effects of physical exercise on young populations have shown that changes in the hippocampus happen throughout life and not just in response to age-related atrophy, as highly fit children are shown to increased hippocampal volumes and improved performance on specific memory tasks (Chaddock et al., 2010). Young adults are shown to respond to aerobic exercise both in the short and long term, with short exercise bouts ranging from 15-30 minutes associated with increased BDNF and increased hippocampal-dependent memory (Tang et al., 2008; Griffin et al., 2011). Longer exercise regimens have similar improvements in BDNF and hippocampal memory but also show increases in hippocampal volume (Griffin et al., 2011; Thomas et al., 2016; Stillman et al., 2018).

Ageing is associated with the shrinking of the hippocampus, which has been shown to be related to memory impairments and increased risk of dementia (Kramer et al., 1999; Erickson et al., 2011). Aerobic exercise has been shown in the elderly to increase hippocampal volumes, reversing hippocampal atrophy by about 2 years (Erickson et al., 2011). In the aged population, aerobic exercise has also been shown to increase BDNF levels and improve various forms of cognitive functions including spatial memory, executive control and delayed word recall (Kramer et al., 1999; Lautenschlager et al., 2008; Erickson et al., 2009; 2011; Ruscheweyh et al., 2011). Physical exercise has been associated with other improvements in humans outside of morphological changes such as decreased risk of dementia, improved cognitive decline with aging, as well the antidepressant effect of exercise have been compared to those of
antidepressant medications (Kramer et al., 1999; Babyak et al., 2000; Yaffe et al., 2001; Hamer and Chida, 2008; Ahlskog et al., 2011; Ruscheweyh et al., 2011; Chapman et al., 2013; For review see Ernst et al., 2006; Baek, 2016).

Improvements in depression and cognition have also been shown with the treatment of both traditional antidepressants and physical exercise in both animal models and humans. In animals models chronic antidepressant treatment leads to 20-40% increase in proliferation and increased hippocampal volume (Malberg et al., 2000; Czéh et al., 2001). Since animal models given physical exercise have also shown increases in adult neurogenesis as well as antidepressant-like behaviour, these findings have led to the belief that these new neurons may play a role in the improvement (Duman, 2004; Parachikova et al., 2008; Cunha et al., 2013). In humans, antidepressants are thought to have neuroprotective effects with longer durations of untreated depression shown to be associated with reductions in hippocampal volume (Sheline et al., 2003). Physical exercise as a treatment for MDD has been shown to increase BDNF in patients with MDD and, compared to patients treated with an antidepressant, physical exercise reduces relapse rates and continued exercise decreases depression rates in MDD patients (Babyak et al., 2000; Kerling et al., 2017).

Physical activity has been shown in humans to be inversely associated with risk of dementia, with one meta-analysis showing that increased activity was related to a 28% decrease in the risk of dementia and a 45% decrease in the risk of Alzheimer's disease (AD) (Hamer and Chida, 2008). It is true that for both men and women increased activity by walking distance is related to lower risk, with men showing 1.8X higher risk of dementia with low walking rates and women presenting with 20% lower risk of cognitive impairments with increased activity (Abbott et al., 2004; Weuve et al., 2004). In terms of animals models of AD and physical activity, the
duration of treatment seems to play an important role as shorter running regiments lead to improvements in cognitive function and increases in neurogenesis, with no or few changes in pathology and neuronal numbers (Adlard et al., 2005; Parachikova et al., 2008; Maliszewska-Cyna et al., 2016). Applications of long-term running (2+months) are shown in many Alzheimer's models to decrease AD pathology, improve cognition, increase neuronal number as well as proliferation, and to decrease neuronal loss in CA1. It seems that increased physical activity counteracts neuronal loss, AD pathology and behavioural deficits, but the length of exposure to exercise plays an important role (Adlard et al., 2005; Belarbi et al., 2011; Tapia-Rojas et al., 2015; Hüttenrauch et al., 2016; Maliszewska-Cyna et al., 2016).

1.5 Thesis overview and objectives

This thesis set out to characterize developmentally-born neurons in the DG, to gain an understanding of their short and long-term survival, maturation and functionality as measured by cell activity. These properties have previously been investigated in the adult-born population but neglected in the population born during the developmental period. We additionally investigated the ability of exercise and NMDA receptor blockade to increase the birth of new cells in adulthood. We assessed the short and long-term effects of these treatments and investigated how using them in conjunction may allow for increases in adult neurogenesis over longer treatment intervals. Finally, using exercise and NMDAR blockade to increase, or transgenic model to decrease, adult neurogenesis, we investigated the relationship between adult-born and developmentally-born neurons. To investigate this relationship we evaluated cellular activity in the developmentally-born population after manipulations of the adult-born population, hypothesizing that altering the number of new adult-born neurons in the DG should directly affect the activity of the developmentally-born population. Specifically we hypothesize
that decreasing the number of adult-born neurons will lead to an increase in IEG activity in developmentally-born cells in rats that have long-term reductions in adult neurogenesis while increasing the number of adult-born neurons we predict that there will be less IEG activity in developmentally-born cells, as adult-born cells are more likely to be activated and there will be increased inhibition with more adult-born cells. Our overreaching hypothesis of this thesis is that the two populations in the DG, the adult-born and developmentally-born populations, have different characteristics and that the developmental population is distinct from the adult-born population. Also, that there is a relationship between these two populations, where adult-born neurons regulate the activity of developmentally-born neurons and may act to replace or inhibit these older neurons.

Chapter 2 examines the natural survival and maturation of developmentally-born cells during early postnatal development and adulthood. This project examined how developmentally-born DG cells born during the first postnatal week survive and mature during both the developmental and juvenile window. The survival of cell born during the first postnatal week was also examined during adulthood, to both confirm and extend previous findings showing that the death of developmentally-born cells occurs after cells are mature and into adulthood (Dayer et al., 2003). We hypothesize that a higher proportion of developmentally-born cells will survive comparatively to what has been seen in adult-born populations. This is based on the idea that during development a set number of cells are generated and due to the high demands during the early postnatal period, complete survival would be needed to form new memories and connections. As well we expect that developmentally-born cells will die off during adulthood after cell maturity unlike what has been seen in adult-born cells (Dayer et al., 2003; Kempermann et al., 2003). This is based on findings of that some memories within the
hippocampus are transient and cellular turnover may underlie this, and that new adult neuron may compete with older DG neurons for synaptic connection, possibly replacing these older neurons (Mumby et al., 1999; Toni et al., 2007; 2008).

Chapter 3 will examine ways of increasing adult neurogenesis in both males and females. By identifying new ways of increasing adult neurogenesis over longer periods of time we may be able to develop new protocols that allow for a heightened impact of adult neurogenesis. This experiment first looks at methods (exercise and NMDA receptor blockade) to increase adult neurogenesis in both males and females over short and long intervals. While the benefits that running has on adult neurogenesis have been shown (Baek, 2016), running’s proliferative effects display an overall decrease towards baseline levels after a month of exposure (Kronenberg et al., 2006). The limitation that this presents led us to consider the possible benefits of alternating treatments between running and another neurogenic treatment, the NMDA receptor antagonist Memantine. While both our chosen pharmacological and non-pharmacological methods have been shown to increase adult neurogenesis, we hypothesized that these effects would be transient during long-term treatment periods. We hypothesized that by combining both our pharmacological and non-pharmacological treatment methods, seeing an elevation in neurogenesis, demonstrating a greater proliferation of cells at the conclusion of the treatment periods, comparatively to single method treatments.

Chapter 4 will investigate a possible homeostatic balance by manipulating the adult-born population and examining its effects on the cellular activity of the developmentally-born population. This experiment will examine how increasing or decreasing adult neurogenesis will affect the activity of the developmentally-born population. We hypothesized that, as the number of adult-born cells is increased, there will be a decrease in the activity of
developmentally-born cells. Conversely, when adult neurogenesis is decreased we expect that developmentally-born cells would show an increase in activity.
Chapter 2: Early survival and delayed death of developmentally-born dentate gyrus neurons.

2.1 Introduction

Many hippocampal dentate gyrus (DG) granule neurons are born postnatally in rodents (Crespo et al., 1986; Cameron and McKay, 2001; Rao and Shetty, 2004). The adult-born population alone ultimately comprises up to 40% of the DG population in rats (Snyder and Cameron, 2012), and a large number in mice as well (DeCarolis et al., 2013). Recent data suggests that cumulative neurogenesis, over decades, may produce similarly large proportions of DG neurons in adult humans (Spalding et al., 2013). Despite the substantial number of neurons added in adulthood, many DG neurons are generated during the perinatal period and yet comparably little is known about their properties. A better characterization of developmentally-born neurons is necessary to understand how different populations of neurons, born at different periods of life, contribute to DG function as a whole.

The basic pattern of DG development has been described. Precursor cells migrate from the dentate notch in the late embryonic period and form a germinal zone in the hilus that gives rise to granule neurons (Altman and Bayer, 1990a; 1990b). In the rat, 1-10% of DG neurons are added daily between E14 and P14 (Schlessinger et al., 1975). Thus, at any given time in the first few weeks of life the DG is made up of a heterogeneous population of neurons that span all stages of cellular development. This heterogeneity is reflected in patterns of electrophysiology, morphology and immediate-early gene (IEG) expression (Liu et al., 2000; Jones et al., 2003;
Montes-Rodríguez et al., 2013). However, in the absence of cellular birthdating methods, it is impossible to identify how anatomical and functional properties relate to neuronal age.

Markers of dividing cells, such as tritiated thymidine, BrdU and retroviruses, have been used extensively to characterize the birth, survival and cellular properties of DG neurons, particularly those born in adulthood. These studies have revealed that adult-born neurons go through a sensitive period of 4 weeks during which many neurons die (Cameron et al., 1993; Kempermann et al., 2003; McDonald and Wojtowicz, 2005; Tashiro et al., 2006; Mandyam et al., 2007; Snyder et al., 2009a), after which neurons survive indefinitely (Dayer et al., 2003; Kempermann et al., 2003). During immature stages adult-born neurons form afferent and efferent synapses according to a specific pattern (Espósito et al., 2005), they display critical periods for synaptic plasticity and memory (Ge et al., 2007; Gu et al., 2012), and their survival can be modulated by learning, stress, exercise and enriched environment, often at very specific cell ages (Döbrössy et al., 2003; Olariu et al., 2005; Dupret et al., 2007; Epp et al., 2007; Tashiro et al., 2007; Snyder et al., 2009b; Alvarez et al., 2016).

In contrast to adult neurogenesis, such extensive and detailed analyses of developmental DG neurogenesis are lacking. Studies that have employed birthdating methods indicate that developmentally-born neurons mature faster than adult-born neurons (Overstreet-Wadiche et al., 2006; Zhao et al., 2006). Electrophysiological properties may ultimately be similar once cells have reached maturity (Laplagne et al., 2006). However, different immediate-early gene responses to experience (Tronel et al., 2015) and enhanced morphological plasticity in old adult-born cells (Tronel et al., 2010) suggest that there may be persistent differences between DG neurons born at different ages. Understanding the behavioral contribution of developmentally-born neurons also depends on their patterns of survival. For example, neuronal survival may
contribute to persistent information storage and yet there is no detailed quantification of the initial lifespan of developmentally-born neurons, though there is evidence that they may die in adulthood (Dayer et al., 2003).

To better understand the maturation and survival of developmentally-born DG neurons we used the thymidine analog BrdU to label DG neurons born at postnatal day 6 (P6). We found that, unlike neurons born in adulthood, P6-born neurons do not undergo appreciable cell death during their immature stages but rather undergo delayed cell death between 2-6 months of age. Furthermore, patterns of immediate-early gene expression suggest P6-born neurons mature faster than adult-born neurons and show peak zif268 expression when they are 2 weeks old. Collectively, these unique patterns of neuronal maturation and turnover suggest that developmentally-born neurons may contribute unique forms of plasticity to the DG throughout the lifespan, which could play a role in the dynamic nature of hippocampal memory.

2.2 Methods

2.2.1 Animals and treatments

All procedures were approved by the Animal Care Committee at the University of British Columbia and conducted in accordance with the Canadian Council on Animal Care guidelines regarding humane and ethical treatment of animals. Experimental Long-Evans rats were generated in the Department of Psychology animal facility with a 12-hour light/dark schedule and lights on at 6:00 am. Breeding occurred in large polyurethane cages (47 cm × 37 cm × 21 cm) containing a polycarbonate tube, aspen chip bedding and ad libitum rat chow and water. The day of birth was designated postnatal day 1. Litters ranged from 8-18 pups, and pups from each litter were distributed equally amongst experimental groups. Breeders (both male and female)
remained with the litters until P21, when offspring were weaned to 2 per cage in smaller polyurethane bins (48 cm × 27 cm × 20 cm).

This study is comprised of 2 experiments that examine developmentally-born neurons at early vs. late intervals. In both experiments rats were injected with the thymidine analog BrdU (50 mg/kg, intraperitoneal) at P6, to label neurons born at the peak of granule cell birth (Schlessinger et al., 1975; Snyder et al., 2009a). In experiment 1, to track early neuronal survival and development, equal numbers of male and female rats were killed at the following post-BrdU injection timepoints: 1 hour, 1 day, 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 8 weeks. One hour before being euthanized, rats in the 1-8 week groups were exposed to a novel environment to induce activity-dependent IEG expression. The novel environment exposure consisted of 1 hour in an empty cage filled with corncob bedding in an unfamiliar room. Rats were picked up and briefly handled at least twice during the exposure. Immediately after the novel environment exposure rats were anaesthetized with isoflurane and perfused with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4). Brains remained in paraformaldehyde for 48 hours and were then stored in 0.1 % sodium azide in PBS until processed. In experiment 2, to examine cell death and the long-term survival of cells born on P6, male rats were killed at 2 months and 6 months of age (when BrdU+ cells were 8 weeks old and 26 weeks old, respectively; for clarity we subsequently refer to these timepoints as 2 and 6 months). These rats did not receive any environmental exposure for immediate-early gene analyses, but were perfused directly from their home cage.

2.2.2 Tissue processing and immunohistochemistry

Brains were immersed in 10% glycerol solution for 1 day, 20% glycerol solution for 2 days and then sectioned coronally at 40 µm on a freezing microtome. Sections were stored in
cryoprotectant at -20°C until immunohistochemical processing. For stereological quantification of BrdU+ cells a 1 in 12 series of sections throughout the entire dentate gyrus were mounted onto slides, heated to 90°C in citric acid (0.1 M, pH 6.0), permeabilized in PBS with 10% triton-x for 30min and then incubated overnight with goat anti-Prox1 (1:1000 in 10% triton-x and 3% horse serum; R&D systems, AF2727). Sections were washed, incubated in biotinylated donkey anti-goat secondary antibody for 1 hour (1:250; Jackson, 705-065-147), 0.3% hydrogen peroxide for 30 min and Prox1+ cells were visualized with an avidin-biotin-horseradish peroxidase kit (Vector Laboratories) and vector nova red HRP substrate (Vector Laboratories). Sections were washed, permeabilized with trypsin, incubated in 2N HCl for 30 min to denature DNA, and incubated overnight with mouse anti-BrdU (1:200 in 10% triton-x and 3% horse serum, BD Biosciences; 347580). Sections were washed and incubated in biotinylated goat anti-mouse secondary antibody for 1 hour (1:250; Sigma, B0529), and BrdU+ cells were visualized with an avidin-biotin-horseradish peroxidase kit (Vector Laboratories) and cobalt-enhanced DAB (Sigma Fast Tablets). Sections were then rinsed in PBS, dehydrated, cleared with citrisolv (Fisher) and coverslipped with Permount (Fisher).

Immunohistochemical analyses of neuronal phenotype were performed on free-floating sections with fluorescent detection. Sections were treated with 2N HCl for 30 minutes, incubated at 4°C for 3 days in PBS with 10% triton-x, 3% horse serum and combinations of the following antibodies: rat anti-BrdU (1:200; AbD Serotec, OBT0030G), goat anti-doublecortin (1:250; Santa Cruz, sc-8066), mouse anti-NeuN (1:200; Millipore, MAB377), rabbit anti-zif268 (1:1000; Santa Cruz, sc-189), goat anti-c-fos (1:250; Santa Cruz, sc-52G), rabbit anti-active caspase3 (1:200 BD Biosciences, 559565) and mouse anti-PCNA (1:200 Santa Cruz, sc-56). Visualization was performed with Alexa488/555/647-conjugated donkey secondary antibodies.
(Invitrogen/Thermofisher) diluted 1:250 in PBS for 60 minutes at room temperature. Sections were counterstained with DAPI, mounted onto slides and coverslipped with PVA-DABCO.

2.2.3 Microscopy and sampling

Quantification of total DAB-stained BrdU+ cells was performed under brightfield microscopy using stereological principles. A 1 in 12 series of sections spanning the entire dentate gyrus was examined with a 40x objective and an Olympus CX41 microscope. All BrdU+ cells located within the granule cell layer or its 20 µm hilar border (the subgranular zone) were counted in each section and counts were multiplied by 12 to estimate the total number of BrdU+ cells per DG (bilaterally). At the 1hr, 1d and 3d timepoints (postnatal days 6-9) the DG was not yet fully formed and many BrdU+ cells resided in the tertiary dentate matrix, located in the hilus (Crespo et al., 1986; Altman and Bayer, 1990a; Muramatsu et al., 2007; Mathews et al., 2010). To accurately capture BrdU+ cells from all regions that will ultimately form the DG granule cell layer we used the dentate granule neuron-specific marker Prox1 to delineate the boundary of the DG and its germinal zones and counted all BrdU+ cells in this region (Fig. 2-1A).

Fluorescent tissue was examined on a confocal microscope (Leica SP8). For PCNA, DCX and NeuN approximately 25 BrdU+ cells per animal located in the suprapyramidal blade of the dorsal DG were examined for marker co-expression using a 63X objective (NA 1.4) and 1 µm z-sections throughout each cell. Activity in P6-born neurons was assessed by examining the immediate-early genes Fos and zif268 in 200 BrdU+ cells per animal, also sampled from the suprapyramidal blade of the dorsal DG, using a 40X oil-immersion lens (NA 1.3) and offline analyses of image stacks. Since zif268 and Fos staining intensity in immature neurons is graded, fluorescence intensity for each cell was measured and compared to background levels (regions in the hilus devoid of DAPI+ cell bodies) within each field. Cells were counted as positive if
staining intensity was twice background, a threshold that captures cells with at least moderate levels of immediate-early gene immunostaining (Snyder et al., 2009b). Activity in the overall population of DG neurons was assessed by examining Fos and zif268 expression in ~300 DAPI+ granule neurons from the same image stacks as the BrdU analyses. Approximately equal numbers of DAPI+ cells were sampled from two regions of the suprapyramidal blade, located 1/3 and 2/3 along its medial-lateral extent, and spanning layers of the granule cell layer (to ensure cells of all ages were sampled equally). As with zif268 and Fos, NeuN expression increases with cell age/maturity (Snyder et al., 2009a). We therefore quantified both weak NeuN expression (2x background, to quantify all neurons) and strong NeuN expression (4x, to quantify relatively mature neurons; see Fig. 2-2B for examples). For cell death measurements in 2 and 6-month-old rats we found that expression of caspase3 was widespread, graded and only cells that strongly expressed caspase3 were also pyknotic. To avoid overestimating numbers of dying cells we therefore only quantified cells that had intense immunostaining for caspase3 and were also pyknotic. A 1 in 12 series of sections spanning the entire dentate gyrus was examined for dying cells and dying BrdU+ cells. Immature neurons tend to reside in the deep layers near the hilus/subgranular zone and older neurons reside in the superficial layers, near the molecular layer (Crespo et al., 1986; Wang et al., 2000; Muramatsu et al., 2007; Mathews et al., 2010). To estimate the age of dying cells, each pyknotic caspase3+ cell was characterized according to its anatomical position within the granule cell layer. The thickness of the granule cell layer was normalized to 100, with the hilar border being 0 and the molecular layer border being 100. The position of dying cells was measured as the relative distance from the hilar border to the middle of the cell body. Cells located in the subgranular zone were assigned a score of 0. To compare the anatomical distribution of dying cells with the distribution of developmentally-born and
adult-born cells, we analyzed the distribution of 25 P6-labeled BrdU$^+$ cells and 25 DCX$^+$ cells from each rat.

**2.2.4 Statistical analyses**

Developmental timecourse analyses (1hr to 8w groups) were performed using ANOVA with Holm-Sidak post hoc tests corrected for multiple comparisons. Differences between the long-term survival groups (2mos and 6mos) were assessed by unpaired t test or Mann Whitney test. In all cases significance was set at $p = 0.05$.

**2.3 Results**

**2.3.1 Early survival of developmentally-born DG neurons**

A single BrdU injection at P6 labeled many DG neurons. At early timepoints (1hr, 1d and 3d post-injection) BrdU$^+$ cells could be observed throughout the proliferative tertiary dentate matrix in the hilus and in the deep layers of the granule cell layer (Fig. 2-1). At longer survival intervals BrdU$^+$ cells were found throughout the granule cell layer but most were observed in the middle layers. Quantitatively, there was a steady increase in BrdU$^+$ cells from 1hr to 1w, resulting in a doubling of cells from ~50,000 cells to just over 100,000 cells (Fig. 2-1C). Co-labeling with PCNA, a marker of cell division, revealed that 100%, 78% and 25% of BrdU$^+$ neurons were undergoing cell division at the 1hr, 1d and 3d timepoints, respectively (Fig. 2-1D, E). Thus, growth of the BrdU$^+$ cell population is due to the continued division of BrdU-labeled precursor cells until BrdU is diluted beyond the limits of detection, as has been observed in adult animals (Dayer et al., 2003). At 1 week and beyond, a negligible proportion of BrdU$^+$ cells were labeled with PCNA. It is well established that adult-born neurons transition through a critical period for survival, when neurons are between 1 and 4 weeks of age and many undergo cell death (Cameron et al., 1993; Gould et al., 1999; Brandt et al., 2003; Snyder et al., 2009a). In
contrast to the adult pattern, we found that BrdU\(^+\) cell numbers remained constant from 1-8 weeks of age, suggesting developmentally-born neurons do not undergo appreciable cell death during this period (and may be culled primarily at earlier stages of cellular development (Gould et al., 1994). We observed no obvious differences in developmental neurogenesis between males and females but our small sample precludes any definitive conclusions (n=3-4/sex/timepoint).

**Figure 2-1: Dynamics of developmental neurogenesis and early cell survival.**

* A) Confocal images of the dentate gyrus 1 hour after BrdU injection at P6. At this early stage of dentate gyrus formation many BrdU\(^+\) cells can be found within the granule cell layer (gcl) and the proliferative tertiary dentate
Figure 2-1: Dynamics of developmental neurogenesis and early cell survival.

matrix in the hilus. Granule neuron precursors and granule neurons can be identified by immunoreactivity for the granule neuron-specific marker Prox1. Inset shows BrdU+ and weakly Prox1+ cells in the granule cell layer and hilus (arrows). Scale bars, 100 µm and 10 µm for the low and high magnification images, respectively. B) Confocal image of the dentate gyrus 8 weeks after P6 BrdU injection. BrdU+ granule neurons are limited to the granule cell layer. Scale bar, 100 µm. C) The total number of BrdU+ cells labeled at P6 doubled from 1 hour (52,407) to 1 week (108,593) and remained stable thereafter (n = 7-8/timepoint; ANOVA F7,54 = 10.8, P < 0.0001). BrdU+ cell number was significantly greater at 3 days and 1 week compared to 1 hour (Ps < 0.001). BrdU+ cell number did not differ between any of the 3 day to 8 week timepoints (all Ps > 0.4). D) Confocal images of the dentate gyrus immunostained for BrdU and the proliferation marker PCNA reveal many double labeled cells at 1 hour but not 4 weeks. Scale bar, 10 µm. E) There was a significant decline in BrdU+ cells that expressed PCNA, where all BrdU+ cells expressed PCNA at 1 hour, 78% at 1 day and 25% at 3 days (ANOVA F7,54 = 429, P < 0.0001; post hoc comparisons all P < 0.0001). Data for males and females are indicated in the graphs but were pooled for statistical analyses. Mol, molecular layer; gcl, granule cell layer.

2.3.2 Expression of doublecortin and NeuN.

To assess the rate of maturation of P6-born DG neurons we first quantified expression of the immature neuronal marker DCX and the mature neuronal marker NeuN, both of which have been extensively characterized in adult-born neurons. At the 1hr to 3d timepoints DCX was expressed at very high levels throughout the granule cell layer and the tertiary dentate matrix in the hilus (Fig. 2-2). Given the density of DCX+ processes it was difficult to unambiguously characterize all neurons as either DCX positive or negative. However, many cells were surrounded by DCX immunoreactivity and even expressed weak levels of NeuN at these early timepoints. Approximately ~60% of BrdU+ cells expressed DCX at the 1hr and 1d timepoints, when the majority of cells were also PCNA+. Thus, as in the adult brain (Brown et al., 2003; Kee et al., 2007; Snyder et al., 2009a), a substantial proportion of actively dividing precursor cells are...
committed to a neuronal lineage in the developing brain. The proportion of BrdU\(^+\) cells that were DCX\(^+\) rose to \(\sim 80\%\) at both 3d and 1w as daughter cells matured into a post-mitotic neuronal phenotype. Thereafter, DCX expression waned but remained at 20-25\% at 3-4 weeks. As previously observed in adult-born cells, NeuN expression increased with cell age and distinct patterns were observed for weak vs. strong levels of NeuN immunoreactivity (Brown et al., 2003; Snyder et al., 2009a). Whereas strong NeuN expression was not observed at the earliest timepoints, and increased over several weeks, weak NeuN expression was observed as early as 1 hour post-BrdU injection and reached near-peak levels by 2 weeks.

**Figure 2-2: Early timecourse of neuronal marker expression.**

A) The vast majority of BrdU\(^+\) cells were neuronal from 3 days (82\% DCX\(^+\)) until 8 weeks (95\% 2x background NeuN\(^+\)). Just over half of BrdU\(^+\) cells expressed DCX at the 1 hour and 1 day timepoints before reaching maximal expression at 3 days (ANOVA \(F_{7,52} = 38, P < 0.0001; 1d\) vs 3d \(P = 0.04, 3d\) vs 2w \(P = 0.047, 2w\) vs 3w \(P < 0.0001, 2w\) vs 8w \(P = 0.06\)). 2x NeuN expression increased rapidly during the first week and then stabilized (ANOVA \(F_{7,52} = \ldots\).
Figure 2-2: Early timecourse of neuronal marker expression.

Figure 2-2: Early timecourse of neuronal marker expression.

= 17, P < 0.0001; 1h and 1d vs 1w Ps < 0.001, differences between 1w-8w timepoints all P > 0.4). Stronger 4x NeuN expression increased markedly over the first two weeks and continued to rise to 80% by 8 weeks (ANOVA F7,52 = 20, P < 0.0001; 1d vs 3d P = 0.04, 1w vs 2w P = 0.0005, differences between 2-8w timepoints all P > 0.16).

B) Raw confocal images (single z-plane) illustrating NeuN expression levels. DG neuron ROIs and corresponding NeuN intensities relative to background (“b”) are indicated.

C) Confocal images of P6-born neurons immunostained for BrdU, DCX and NeuN. One day after BrdU injection, on P7, the granule cell layer was dispersed and BrdU+ cells were scattered throughout the hilus bordering the granule cell layer. More mature granule neurons, in the superficial layers near the molecular layer, had stronger NeuN immunoreactivity. The entirety of the dentate gyrus showed DCX+ immunoreactivity. The arrow indicates a BrdU+ cell that expresses DCX and NeuN. The open arrowhead indicates a BrdU+ cell that did not stain for NeuN. One week after BrdU injection (P13) there was a strong gradient of NeuN expression across cell layers and most BrdU+ cells expressed both DCX and NeuN (arrows). Two weeks after BrdU injection some BrdU+ cells expressed both DCX and NeuN (arrows) and some only expressed NeuN (arrowhead). At four weeks post-BrdU injection the granule cell layer was adult-like, with a thin, tight layer of DCX+ cells bordering the hilus and no gradient of NeuN expression; BrdU cells overwhelmingly expressed NeuN and not DCX (see arrowhead). Mol, molecular layer; gcl, granule cell layer; hil, hilus.

2.3.3 Expression of activity-dependent immediate-early genes

Immediate-early gene expression is upregulated by synaptic activity and is often used as a proxy for neuronal activity, to identify experience-dependent recruitment of neuronal ensembles (Worley et al., 1991; Guzowski et al., 1999; Satvat et al., 2011). In adult-born neurons, expression of genes such as zif268, Fos and Arc develops over several weeks as new neurons integrate into circuits (Cameron et al., 1993; Gould et al., 1999; Kee et al., 2007; Snyder et al., 2009a). To identify the rate at which P6-born neurons are integrated into circuits and recruited during exploration of a novel environment, we quantified zif268 and Fos expression at the 1, 2, 3, 4 and 8 week timepoints (Fig. 2-3). We found that zif268 was only expressed in 2%
of 1-week-old BrdU$^+$ cells but there was a dramatic but transient peak in expression at the 2 week timepoint, to 16% (Fig. 2-3B). Expression then dropped to 7%, 10%, and 12% at 3, 4 and 8 weeks, respectively. Notably, while P6-born cells had transient peak expression at 2 weeks, this pattern was not observed in the overall population of DG neurons, which gradually increased zif268 expression from 4% at the 1 week timepoint to 9% at the 4 week timepoint (corresponding to animal ages of ~2 to 5 weeks). In contrast to zif268, Fos expression was much lower and increased to ~1% of BrdU$^+$ cells by the 4 week timepoint (Fig. 2-3C). Fos expression in the general DG population followed a similar pattern, though expression levels were significantly greater than in BrdU$^+$ cells, possibly because the overall population of DG neurons was, on average, older than BrdU$^+$ cells. To investigate whether zif268 and Fos identified different populations of cells activated by experience we quantified the amount of overlapping immediate-early gene expression in BrdU$^+$ cells (Fig. 2-3D). Since both zif268 and (especially) Fos are expressed in only a fraction of cells, counts were pooled across animals within each timepoint. Generally, BrdU$^+$ cells that expressed Fos also expressed zif268 (86% of cells across 2-8w timepoints, range 67-100%). In contrast, only 4% of BrdU$^+$zif268$^+$ cells also expressed Fos (range 1-8% across 2-8w timepoints). This was driven by the large number of 2-week-old BrdU$^+$zif268$^+$ neurons that did not express Fos (only 2/266 co-expressed Fos, < 1%). Older BrdU$^+$zif268$^+$ cells were more likely to also express Fos but the pattern of partial overlap suggests that zif268 may identify a population of active neurons that are not captured by Fos immunostaining.
Figure 2-3: Timecourse of activity-dependent immediate-early gene expression in P6-born DG neurons and the general population of DG neurons.

A) Confocal image of an 8-week-old BrdU+ dentate gyrus neuron expressing zif268 and Fos following exploration of a novel environment. Vertical white line indicates the width of the granule cell layer (gcl). Scale bar = 10 µm. mol, molecular layer; sgz, subgranular zone. B) zif268 expression was virtually absent in 1-week-old cells, peaked at 2 weeks (16%), and then stabilized at 3-8 weeks (7-10%; ANOVA F_{4,33} = 11, P < 0.0001). †zif268 expression at 2 weeks was greater than all other time points (all Ps < 0.05) and expression levels at 3, 4 and 8 weeks were not different from each other (Ps > 0.6). ****P < 0.0001, **P < 0.01, * P < 0.05 vs 1 week. Inset: Comparison of zif268 expression in P6-born BrdU+ cells and DAPI+ cells (overall population). zif268 expression was greater in BrdU+ cells than in DAPI+ cells at the 2 week timepoint (2 way repeated measures ANOVA effect of time F_{4,32} = 8, P = 0.0002, effect of cell population F_{1,32} = 5, *P < 0.05, interaction F_{4,32} = 7, P = 0.0005; ****P < 0.0001). C) Fos
Figure 2-3: Timecourse of activity-dependent immediate-early gene expression in P6-born DG neurons and the general population of DG neurons.

expression in BrdU+ cells gradually increased, reaching 1% by 4 weeks (ANOVA F_{4,33} = 5, P < 0.01), and occurred at much lower rates than zif268. Fos expression at 4 weeks, but not the other timepoints, was significantly greater than at 1 week (***P < 0.01). Inset: Fos expression increased over time and was greater in the general population of DG neurons (DAPI+ cells) than in P6-born BrdU+ cells. Fos expression was not significantly different between BrdU+ cells and DAPI+ cells at any timepoint (2 way repeated measures ANOVA, effect of cell population F_{1,32} = 10, P = 0.004; effect of time F_{4,32} = 4, P = 0.006; interaction F_{4,32} = 0.2, P = 0.9). D) Co-expression of zif268 and Fos in BrdU+ cells. Most BrdU+Fos+ cells also expressed zif268 but few BrdU+zif268+ cells also expressed Fos. Numbers above each bar indicate the fraction of cells expressing both immediate-early genes (pooled across animals). Sexes are pooled for all statistical analyses; lines indicate group means; error bars indicate S.E.M.

2.3.4 Late death of developmentally-born cells

The early timecourse data indicate that P6-born neurons are remarkably stable during their immature stages, unlike adult-born neurons. Adult-born neurons do not die after reaching maturity (once 4 weeks old) (Dayer et al., 2003; Kempermann et al., 2003) but less is known about the long-term survival of developmentally-born neurons. We therefore injected male rats with BrdU at P6 and compared the number of BrdU+ cells at 2 and 6 months of age. In contrast to the initial stability of P6-born neurons, we found a significant loss of BrdU+ cells (17% or 29,000 cells) over this 4 month period of early adulthood (Fig. 2-4B).

To obtain positive evidence for cell death we quantified cells that were both pyknotic and expressed activated caspase3, and found an average of 217 total dying cells per rat at 2 months of age, and 70 cells at 6 months of age (Fig. 2-4C). Furthermore, we observed on average ~10 pyknotic BrdU+caspase3+ cells per rat at 2 months of age, indicating death of P6-born cells (Fig. 2-4D). Since dying BrdU+ cells were infrequent and difficult to sample, we also analyzed the
anatomical distribution of dying cells within the granule cell layer, where older neurons are located in more superficial regions (Fig. 2-4E). We first examined the distribution of DCX$^+$ cells, since this marker labels immature neurons and can be used to identify regions of the granule cell layer where adult-born cells are dying. As expected, nearly all DCX$^+$ cells were located in the deepest 25% of the granule cell layer, near the hilus. P6-born BrdU cells were scattered throughout the granule cell layer and were primarily located in the middle regions. Finally, the majority of pyknotic caspase3$^+$ cells were located within the deepest 25% of the granule cell layer, consistent with the death of immature adult-born neurons (Sierra et al., 2010). However, pyknotic caspase3$^+$ cells (with and without BrdU) could be found throughout the full width of the granule cell layer at both 2 and 6 months, providing additional evidence that developmentally-born neurons die in young adulthood.

We next quantified the proportions of cells in the superficial 75% of the granule cell layer, i.e. where cells are mature and largely born in early postnatal development (Fig. 2-4F). As expected, P6-born BrdU$^+$ cells were mainly found in this region. However, a significant proportion of pyknotic caspase3$^+$ cells were also observed in the superficial granule cell layer region at 2 months (18%; 13% at 6 months, not significantly different) providing additional evidence that death of developmentally-born neurons reflects a modest but significant proportion of overall cell death within the dentate gyrus.

Notably, the distribution of P6-born BrdU$^+$ neurons shifted towards more superficial regions of the granule cell layer from 2 to 6 months (Fig. 2-4E, F). This could reflect ongoing adult neurogenesis in the deeper regions, or preferential death of P6-born neurons in the deeper layers.
Figure 2-4: Delayed death of developmentally-born neurons.

A) Confocal image of a pyknotic caspase3\(^+\)BrdU\(^+\) cell, undergoing apoptosis (arrow). Lines indicate the superficial and deep borders of the granule cell layer that were used to calculate the anatomical distribution of dying dentate gyrus neurons. B) Between 2 and 6 months of age there was a 17% loss of BrdU\(^+\) cells that were born on P6 (166,000 cells at 2 months, 137,000 cells at 6 months; \(T_{14} = 2.2, P = 0.047\)). C) There were significantly fewer pyknotic, caspase3\(^+\) cells in the DG at 6 months compared to 2 months of age (\(T_{13} = 6.4, P < 0.0001\)). D) Pyknotic
Figure 2-4: Delayed death of developmentally-born neurons.

caspase3^BrdU^ cells were observed at 2 months but not 6 months of age (Mann-Whitney test, P = 0.026). E) Cell
distribution within the granule cell layer: box and whisker plots indicate quartiles, plus sign indicates mean. The
oldest neurons typically are found in the superficial-most layer (maximum 100) and the youngest neurons are
typically found in the deep layers near the hilus (minimum 0). Nearly all immature DCX^+ cells are found within the
deepest 25% of the granule cell layer. P6-born dentate gyrus neurons are distributed throughout, but are most
centralized in the middle of the granule cell layer. There was a significant shift in distribution towards the
superficial layers at 6 months of age (T_{13} = 3.2, P = 0.0014). The distribution of DCX^+ and caspase3^+ cells did not
change between 2 and 6 months of age. While most pyknotic caspase3^+ cells were found in the deep (neurogenic)
layers, dying cells could also be observed in the superficial layers inhabited by P6-born neurons. The location of
pyknotic caspase3^BrdU^ cells are indicated by the filled black symbols. F) Analyses of cells in the superficial
layers (superficial 75%). Out of 192 cells examined, only 1 DCX^- cell was observed in the superficial granule cell
layer, indicating that relatively mature dentate gyrus neurons reside in these layers. The majority of P6-born BrdU^+
neurons were found in the superficial granule cell layer, with a greater proportion observed at 6 months than at 2
months (T_{13} = 2.7, P = 0.017). At 2 months of age 18% of pyknotic caspase3^+ cells were observed in the superficial
granule cell layer (significantly greater than zero, one sample t-test, T_6 = 3.2, P = 0.018). The proportion of pyknotic
caspase3^+ cells that were found in the superficial granule cell layer did not differ between 2 and 6 months of age (T_{13}
= 0.6, P = 0.5).

2.4 Discussion

Our principal finding is that the survival pattern of DG neurons born in early postnatal
development is essentially the opposite of DG neurons born in adulthood (summarized and
compared with previously published findings in Fig. 2-5). In adult rodents approximately 40-
80% of adult-born cells die between 1-4 weeks of age (Cameron et al., 1993; Gould et al., 1999;
Brandt et al., 2003; McDonald and Wojtowicz, 2005; Tashiro et al., 2006; Mandyam et al., 2007;
Snyder et al., 2009a). Remaining neurons continue to survive to at least 6 months in rats (Dayer
et al., 2003), 11 months in mice (Kempermann et al., 2003), and likely persist for the life of the
animal. In contrast, here we observed no early loss of developmentally-born BrdU\(^+\) cells between 1-8 weeks but we did find that 17\% died after reaching maturity, between 2-6 months of age. A steady loss of P6-born DG neurons between 1-6 months of age has been previously demonstrated in Sprague Dawley rats (Dayer et al., 2003). Here, we confirm these data in another strain (Long Evans), within a more restricted window of development (2-6 months of age), and with specific markers for dying cells. Using behavioral immediate-early gene induction as an indirect measure of circuit integration we found that Fos steadily increased, reaching maximal levels when cells were 4-weeks-old. In contrast, zif268 expression showed a transient peak when cells were 2-weeks-old, suggesting a potential critical period for plasticity in immature DG neurons. While our group sizes were too small for a properly powered comparison between males and females, no obvious sex differences were observed for any measure.

![Figure 2-5: Comparison of early vs. late survival in developmentally-born and adult-born DG neurons.](image)

**A)** Developmentally-born neurons survive early, immature stages of cellular development but many adult-born DG neurons die. **B)** Roughly 20\% of developmentally-born neurons die after reaching maturing but adult-born neurons are relatively stable. *Asterisk indicates data from the current study, other data are from \(^2\)Dayer et al., (2003), \(^3\)Snyder et al., (2009a) and \(^4\)Kempermann et al., (2003). Where raw data were not available, values were extracted from published graphs with Plot Digitizer.
2.4.1 Alternative explanations for early survival and delayed death

When examining the birth and persistence of cohorts of neurons, it is important to consider possible confounds associated with labeling methods. For example, BrdU that is taken up by precursor cells will continue to label daughter cells with each division until it is diluted below the limits of detection. Redivision of BrdU$^+$ cells could therefore give the false impression that P6-labeled neurons are stable between 1-8 weeks after injection, for example if the addition of new cells offset the death of immature cells. Likewise, infrequent/delayed division between 2-6 months could lead to BrdU dilution below the detection threshold, giving the appearance of cell loss. However, these alternative explanations are unlikely to explain our results for several reasons. First, while many BrdU$^+$ cells expressed the cell division marker PCNA between 1 hour and 3 days after BrdU injection, when there was corresponding growth in the BrdU$^+$ population, there was negligible expression of PCNA from 1 week onwards. Thus, continued division of BrdU$^+$ cells cannot explain the early stability (1-8w) or delayed loss (2-6mos) of BrdU$^+$ cells. Second, BrdU$^+$ adult-born cell numbers remain stable over durations that are significantly longer than those examined here (6-11 months), indicating that incorporated BrdU remains a persistent label throughout the life of postmitotic cells (Dayer et al., 2003; Kempermann et al., 2003). Third, only 5% of 8-week-old cells were non-neuronal (NeuN$^-$). Even if this entire population consisted of slowly-dividing stem cells that diluted their BrdU between 2-6 months, this cannot account for the observed 17% drop in P6-born neurons. Fourth, in 2-month-old rats, 18% of dying pyknotic caspase3$^+$ cells were located in the superficial granule cell layer, which was devoid of DCX immunoreactivity, strongly suggesting that mature neurons were actively undergoing apoptosis. Fifth, the presence of pyknotic caspase3$^+$BrdU$^+$ cells provides direct evidence that P6-born neurons undergo apoptosis in young adult rats. Assuming a linear decline
in cell death from 10 BrdU$^+$ cells at 2 months to 0 BrdU$^+$ cells at 6 months (5 cells on average) and 1 hour to clear apoptotic DG neurons (Sierra et al., 2010): 5 dying BrdU$^+$ cells x 2880 hours = 14,400 cells predicted to die between 2-6 months (we observed 28,538 BrdU$^+$ cells lost).
Sampling limitations and insufficient knowledge about the kinetics of cell clearance make precise calculations difficult, but the BrdU and pyknotic/caspase data are broadly consistent and indicate that substantial numbers of developmentally-born neurons die throughout young adulthood.

2.4.2 Maturation and early zif268 expression relative to adult-born dentate gyrus neurons

Morphological and electrophysiological studies have found that developmentally-born neurons mature faster than adult-born neurons (Overstreet-Wadiche et al., 2006; Zhao et al., 2006). While expression patterns of DCX, NeuN and Fos did not suggest obviously different maturation rates compared to adult-born neurons, the zif268 profile was shifted 1 week earlier than we have previously observed in adult-born neurons. Specifically, we have previously observed a sharp peak in zif268 expression in 3-week-old adult-born neurons (Snyder et al., 2009a); here we observed a similar peak in 2-week-old developmentally-born DG neurons. This peak was not observed in the overall DG population, indicating that it is related to cell age rather than the developmental stage of the animal. Zif268 is an immediate-early gene that is critical for long-term plasticity and long-term memory (Jones et al., 2001). Moreover, zif268 promotes the survival and experience-dependent activation of immature adult-born neurons (Veyrac et al., 2013). If zif268 plays a similar role in developmentally-born neurons, our findings suggest that neurogenesis around the peak of postnatal DG development (~P6) would result in a very large population of highly plastic neurons 2 weeks later when rats are ~3 weeks old and just beginning
to display hippocampal-dependent memory (Rudy, 1993; Akers and Hamilton, 2007; Raineiki et al., 2010). While the immediate-early genes zif268, Fos and Arc are often used as cellular activity markers, few studies have compared patterns of expression. All 3 immediate early genes are upregulated by spatial water maze training, but Arc has been found to correlate best with performance and task demands (Guzowski et al., 2001). Arc and Fos are largely expressed by similar DG cell populations (Stone et al., 2011) but here we find that Fos and zif268 are only partially co-expressed within cells. While most Fos$^+$ cells also expressed zif268, zif268 was present in a much larger population of cells that did not express Fos, possibly because zif268 has a lower threshold for activity-dependent expression (Worley et al., 1991; 1993). It is worth noting that since we did not include an unstimulated, caged control group, we cannot conclude with certainty that zif268 and Fos were induced by novel context exposure, particularly in light of evidence that immature adult-born DG neurons express high levels of zif268 in the home cage (Snyder et al., 2012; Huckleberry et al., 2015). However, the expression timecourse is consistent with the formation of synapses, and others have shown that resting levels of immediate early genes are activity-dependent (Worley et al., 1991) and experience-specific (Marrone et al., 2008). We therefore believe that these markers are valid activity indicators, but future studies are required to elucidate their precise functions.

**2.4.3 Neuronal persistence, turnover and memory.**

The initial persistence and delayed death of developmentally-born DG neurons raises fundamental questions about their role in hippocampal function. Clearly, the early postnatal period is experientially rich; high mnemonic demands may require the complete survival of developmentally-born neurons during their first few weeks and months. That experience can increase the survival of adult-born neurons is consistent with this idea (Kempermann et al.,
but the extent to which developmentally-born neurons are hard wired for survival, or survive based on sensory experience and mnemonic demands is not clear. Additional investigation into the early stability of developmentally-born neurons is therefore warranted, particularly in light of intriguing evidence that behavioral stimuli can induce death of superficially-located (presumably developmentally-born) granule cells (Olariu et al., 2005).

In our late survival experiment, P6-born DG neurons died between 2 and 6 months of (cell) age. While death of mature neurons is observed in neurodegenerative disorders, it is generally not believed to occur in the healthy young adult brain. Our findings suggest that death of mature functional DG neurons may be a part of normal development/aging. The consequences of dying developmentally-born neurons would be very distinct from death of immature adult-born cells, as they would have presumably participated in memory processes and their removal could result in loss of information from hippocampal circuits. In contrast, dying immature adult-born cells would have had fewer, if any, opportunities to store memories or process information prior to their death.

Computational models predict that adult neurogenesis coupled with neuronal turnover benefits learning (Becker, 2005) beyond what can be achieved with adult neurogenesis alone (Meltzer et al., 2005). The addition of new neurons and removal of old neurons might preferentially enhance new learning at the expense of retaining older information (Meltzer et al., 2005; Chambers and Conroy, 2007). Indeed, there is evidence to support these predictions: in songbirds, the death of mature neurons promotes the birth of new adult-born neurons, and neuronal turnover contributes seasonal changes in song repertoire (Alvarez-Buylla and Kirn, 1997; Larson et al., 2014). In mammals, adult neurogenesis causes forgetting of hippocampal
memories (Akers et al., 2014) and consolidation of memory into extra-hippocampal structures (Kitamura et al., 2009). Our data raise the question of whether death of developmentally-born neurons might also contribute to the loss and consolidation of hippocampal memory.

While the number of dying developmentally-born cells is a considerable proportion of the total DG population, the majority of P6-born DG neurons did not die between 2-6 months of age. This fits with behavioral findings that, while some memories may transform or be forgotten over time, episodic-like memories can persist indefinitely in the hippocampus (Moscovitch et al., 2016). The combination of survival and death could therefore be highly adaptive, enabling detailed memory for important events while minimizing overreliance on obsolete information (Richards and Frankland, 2017).

2.4.4 Relevance for mental health.

Animal models indicate that genetic factors and experience in both early life and adulthood can impact total DG cell number (Kempermann et al., 1997b; 1997a; Fabricius et al., 2008; Oomen et al., 2011). Our findings are therefore relevant for a number of psychiatric disorders. For example, the restricted window of zif268 expression might render specific cohorts of neurons vulnerable to neurodevelopmental insults to the hippocampus (Alberini and Travaglia, 2017). Altered production and survival of developmentally-born DG neurons is also relevant for a number of disorders that are associated with large-scale structural changes in the hippocampus, and sometimes the DG in particular, such as autism (Saitoh et al., 2001; Schumann et al., 2004), schizophrenia (Tamminga et al., 2010; Lodge and Grace, 2011) and depression (McKinnon et al., 2009). For example, recent reports indicate that depressed patients have fewer total DG neurons in the anterior hippocampus, which can be restored by SSRI antidepressants (Boldrini et al., 2013; 2014). While antidepressant treatments are often cited for their
proneurogenic properties, our findings indicate that changes in the developmentally-born cell population could also contribute to changes in total granule cell number.

Whether it is in fact desirable to rescue developmentally-born neurons is another question, as they appear to be fundamentally different from (even relatively old) adult-born neurons. Adult-born neurons have greater experience-dependent morphological plasticity (Tronel et al., 2010; Lemaire et al., 2012), display unique experience-dependent patterns of immediate-early gene expression (Snyder et al., 2009c; 2011; Tronel et al., 2015), and have distinct functions in contextual encoding (Nakashiba et al., 2012; Danielson et al., 2016). Thus, culling developmentally-born neurons and replacing them with new neurons may in fact be beneficial, particularly if the developmentally-born neurons are less plastic and have formed maladaptive associations.

2.5 Conclusions.

Lifelong neurogenesis in the DG results in a degree of cellular heterogeneity that has only begun to be explored. Here, we examined how cells born at a single time point survive from infancy through young adulthood. How do our results generalize to neurons born at, and surviving to, other stages of development? Assuming there is delayed death of other DG neurons born in the first postnatal week, there could be hundreds of thousands of cells lost in young adulthood in rats, and perhaps even more in older age. The survival and death of different cohorts of adult-born neurons are interrelated processes (Dupret et al., 2007) and it is known that mature DG neurons regulate the recruitment of adult-born DG neurons (Alvarez et al., 2016; McAvoy et al., 2016). Thus, there may be a functional link between the loss of developmentally-born neurons and the addition of adult-born neurons. Identifying such relationships may help resolve longstanding conflicting reports that DG neurons accumulate (Bayer, 1982; Bayer et al.,
1982; Amrein et al., 2004), remain constant (Boss et al., 1985; Rapp and Gallagher, 1996; Amrein et al., 2004) or fluctuate throughout the lifespan (Boss et al., 1985).
Chapter 3: Differential effects of extended exercise and memantine treatment on adult neurogenesis in male and female rats

3.1 Introduction

In the dentate gyrus subregion of the hippocampus, adult-born neurons have enhanced plasticity and unique connectivity relative to older neurons (Snyder and Cameron, 2012; Toni and Schinder, 2015), and they play an important role in memory and emotional behavior (Abrous and Wojtowicz, 2015; Cameron and Glover, 2015). Their functional role has stimulated much research on regulatory factors that could be harnessed, typically to promote neurogenesis, in order to enhance cognitive function or recovery from neurological disorders (Toda et al., 2018). However, most studies have examined neurogenesis regulation over hours, days or weeks. Since most disorders of hippocampal function are chronic, it is important to identify whether neurogenesis can be increased over extended intervals to potentially offset long-term dysfunction (e.g. months in rodents, years in humans).

In humans, reduced hippocampal volume is typically interpreted as a sign of damage and is observed in a number of disorders including depression (McKinnon et al., 2009), schizophrenia (Harrison, 2004), mild cognitive impairment (Yassa et al., 2010), and Alzheimer’s disease (Jack et al., 2000). While the mechanisms underlying hippocampal volume changes are multifaceted, and certainly not wholly reflective of neurogenesis (Schoenfeld et al., 2017), changes in adult neurogenesis could contribute to structural damage as well as recovery. Neurogenesis is difficult to measure in humans, and currently can only be assessed in post mortem tissue. However, a number of known neurogenic treatments (identified in animal studies)
are associated with reversal of hippocampal structural deficits in humans. Antidepressant treatment restores dentate gyrus granule cell number in depressed patients (Boldrini et al., 2013; Mahar et al., 2017), possibly by increasing adult neurogenesis (Boldrini et al., 2009). Exercise increases hippocampal volume in healthy individuals (Erickson et al., 2011; Killgore et al., 2013), women with mild cognitive impairment (Brinke et al., 2015) and schizophrenic patients (Pajonk et al., 2010), though effects in schizophrenia have been inconsistent (Kim et al., 2018). Moreover, there are sex differences in the prevalence of disorders that impact the hippocampus, with depression (Seedat et al., 2009; Bangasser and Valentino, 2014) and Alzheimer’s disease (Gao et al., 1998) more common in females, and schizophrenia more common in males (Aleman et al., 2003). Thus, it is important to determine which factors can effectively increase adult neurogenesis in males and females, and potentially improve behavioral outcomes.

Neurogenesis is a multistep process whereby precursor cells undergo lineage-directed cell division to produce immature neurons, of which only a fraction are selected to survive and contribute to hippocampal function. Animal models have identified a number of factors that increase neurogenesis, either by promoting precursor proliferation or enhancing immature neuronal survival. For example, factors that can increase proliferation include exercise (Eadie et al., 2005; Kronenberg et al., 2006), antidepressant drugs and electroconvulsive shock (Malberg et al., 2000), synthetic chemicals (Petrik et al., 2012), learning (Dupret et al., 2007) and NMDA receptor antagonists (Cameron et al., 1995; Maekawa et al., 2009). Survival of immature neurons is enhanced by exercise (Snyder et al., 2009b) and learning (Gould et al., 1999; Dupret et al., 2007; Epp et al., 2007). Importantly, regulatory factors can be highly dose and time-dependent, with some factors increasing or decreasing neurogenesis depending on the conditions (e.g.
similar experiences can increase or decrease neurogenesis depending on the age of the cell and the extent of learning (Olariu et al., 2005; Dupret et al., 2007).

Here we focus on two treatments that have been shown to increase neurogenesis in rodents: running (RUN) and memantine (MEM). RUN is likely the most well-studied method for increasing neurogenesis. In addition to increasing proliferation and survival, it also increases the dendritic complexity of newborn neurons, promotes spine formation, and accelerates their functional maturation (van Praag et al., 1999b; Redila and Christie, 2006; Piatti et al., 2011; Dostes et al., 2016; Vivar et al., 2016). However, RUN does not increase neurogenesis in socially isolated rats (Stranahan et al., 2006; Leasure and Decker, 2009), in wild mice (Hauser et al., 2009), in mice that run only in the light phase (Holmes et al., 2004), or in animals that run at high intensities (Naylor et al., 2005; Grégoire et al., 2014; So et al., 2017). Furthermore, a number of studies have found that RUN-induced increases in neurogenesis are transient, raising questions about the extent to which it may be used as a strategy for long-term enhanced production of new neurons (Naylor et al., 2005; Kronenberg et al., 2006; Snyder et al., 2009b; Clark et al., 2010). Interestingly, there is evidence that neurogenesis may be sustained for extended durations if RUN amount is restricted (Naylor et al., 2005; Nguemeni et al., 2018). However, since these studies only investigated cells born at a single timepoint in male rats, it remains unclear whether restricted RUN enhances neurogenesis consistently across sexes and over extended periods of time.

MEM is a low-affinity NMDA receptor antagonist that has neuroprotective effects and has been approved as an Alzheimer’s disease treatment (Lipton, 2004). In mice, MEM increases cellular proliferation, the size of the stem cell pool, and the production of new neurons by 2-3 fold (Maekawa et al., 2009; Namba et al., 2009; Akers et al., 2014; Ishikawa et al., 2014).
Notably, two other NMDA receptor antagonists, MK-801 and ketamine, have also been found to increase cell proliferation in the dentate gyrus (Cameron et al., 1995; Soumier et al., 2016). While repeat dosing of MEM has been found to broadly increase neurogenesis (Ishikawa et al., 2016), the efficacy of single vs. multiple doses of MEM remain unknown.

Here, we investigated the long-term efficacy of neurogenic treatments in male and female rats. Rats were subjected to RUN, MEM, or alternating blocks of RUN and MEM and multiple immunohistochemical markers (Fig. 3-1) were used to quantify neurons born at the beginning, middle and end of treatments. While a single MEM injection (sMEM) and continuous RUN (cRUN) only transiently increased neurogenesis, extended treatments were capable of increasing neurogenesis at later timepoints. Neurogenic efficacy depended on sex and treatment: in females, 2 months of interval RUN (iRUN) increased DCX⁺ cells; in males, DCX⁺ cells were elevated after 1 month of iRUN followed by 1 month of multiple MEM injections (mMEM). However, thymidine analog labeling revealed that all extended treatments were relatively ineffective at increasing numbers of neurons born in the earlier phases of treatment.

3.2 Methods

3.2.1 Animals and Treatments

All procedures were approved by the Animal Care Committee at the University of British Columbia and conducted in accordance with the Canadian Council on Animal Care guidelines regarding humane and ethical treatment of animals. Experimental Long-Evans rats were generated in the Department of Psychology’s animal facility with a 12-hour light/dark schedule and lights on at 6:00am. Breeding occurred in large polyurethane cages (47cm × 37cm × 21cm) containing a polycarbonate tube, aspen chip bedding and ad libitum rat chow and water. Breeders (both male and female) remained with the litters until P21, when offspring were weaned to 2 per
cage in smaller polyurethane bins (48cm × 27cm × 20cm) with a single polycarbonate tube, aspen chip bedding and ad libitum rat chow and tap water. In Experiment 3, animals housed on a reverse light dark cycle (beginning at weaning) in order to study running effects during the active cycle, when rats are most active and neurogenic effects are greatest (Holmes et al., 2004; van der Borght et al., 2006). Running wheel cages consisted of 24” x 18” x 15” plastic tub containing aspen chip bedding, ad libitum rat chow, water and a 12” running wheel (Wodent Wheel, Exotic Nutrition). Running distance was measured by attaching a neodymium magnet to the running wheel, which allowed each revolution to be detected by a bicycle odometer positioned on the outside of the cage.

This study is comprised of 3 experiments examining the effects of different methods of increasing adult neurogenesis in both males and females. Treatments started at 2 months of age for all experiments.

In Experiment 1 animals were either given continuous running wheel access (cRUN group) or a single memantine treatment (sMEM group; see timeline in Fig. 3-2A) and compared to their respective controls. cRUN animals were pair housed and given free access to running wheels in their home cages. cRUN animals were compared to sedentary controls that were pair housed without access to a running wheel. Seven days after cRUN treatment began, animals were given a single BrdU injection (200 mg/kg, I.P.; Sigma, cat #B500205). sMEM animals were given a single memantine injection (35 mg/kg, I.P; Toronto Research Chemicals, cat #M218000100) at 2 months of age followed 3 days later by a single BrdU injection (200 mg/kg, I.P). sMEM animals were compared to vehicle-injected controls. All animals were perfused with 4 % paraformaldehyde 4 weeks after BrdU injection and brains were extracted and post-fixed for an additional 48 hours.
In Experiment 2 we assessed the long-term effects of continuously housing male rats with access to a running wheel. These animals were injected with BrdU (50 mg/kg, I.P.) on postnatal day 6 as a part of a separate study of male rats, and this is the only experiment that did not include female rats. At 2 months of age rats were pair housed with constant access to running wheels for 4 months, and were perfused with 4% paraformaldehyde at 6 months of age.

In Experiment 3 groups were given 2 x 4-week treatment blocks according to 5 possible combinations: CON, iRUN/iRUN, mMEM/mMEM, iRUN/mMEM, mMEM/iRUN (see timeline in Fig. 3-6A). CON rats remained in their home cages throughout both treatment blocks, and were handled by an experimenter on MEM injection days. A block of mMEM treatment consisted of 4 weekly MEM injections (35 mg/kg each). A block of iRUN treatment consisted of rats being placed individually in running wheel cages for 4 hours on weekdays. The iRUN was counterbalanced, so that rats would run for the first four hours of the dark phase on one day and the middle four hours of the dark phase on the next day. On weekends (3 nights/week), iRUN rats were pair housed in the running wheel cage, with free access to the wheels. Thymidine analogues (CldU and IdU, see below Fig 3-6A) were used to label neurons born at the beginning of the first and second block of treatments, respectively. For mMEM blocks, the thymidine analog was injected 3 days after the first MEM injection. For the iRUN blocks, the thymidine analogue was injected 5 days after first 4-hour block of running. For the first treatment block, whether iRUN or mMEM, rats were injected with CldU (42.5 mg/kg, IP; Toronto Research Chemicals, cat #2105478) and for the second treatment block, whether iRUN or mMEM, rats were injected with IdU (57.5 mg/kg, IP; Toronto Research Chemicals, cat #2100357)(Vega and Peterson, 2005). Immediately following the second block of treatment animals were perfused with 4% paraformaldehyde and brains were extracted and post-fixed for an additional 48 hours.
3.2.2 Tissue processing and immunohistochemistry

Brains were immersed in 10% glycerol solution for 1 day, 20% glycerol solution for 2 days and then sectioned coronally at 40 μm on a freezing microtome. Sections were stored in cryoprotectant at -20°C until immunohistochemical processing. To detect BrdU\(^+\), CldU\(^+\) or IdU\(^+\) cells in a 1 in 12 series of sections throughout the entire dentate gyrus were mounted onto slides, heated to 90°C in citric acid (0.1M, pH 6.0), permeabilized with trypsin, incubated in 2N HCl for 30 min to denature DNA, and incubated overnight at 4°C with mouse anti-BrdU antibody (to detect BrdU and IdU; BD Biosciences, cat # 347580) or rat anti-BrdU antibody (to detect CldU; BioRad, cat # OBT0030G). Anti-BrdU antibodies were diluted 1:200 in 10% triton-x and 3% horse serum. Sections were washed and incubated in biotinylated goat anti-mouse (Sigma, cat #B0529) or biotinylated donkey anti-rat (Jackson, cat #712065153) secondary antibody for 1 hour (1:250), tissues was blocked in 0.3% H\(_2\)O\(_2\) for 30 min, and cells were then visualized with an avidin-biotin-horseradish peroxidase kit (Vector Laboratories, cat #OK-6100) and cobalt-enhanced DAB (Sigma Fast Tablets, cat #DO426). Sections were then rinsed in PBS, dehydrated, cleared with citrisolv (Fisher, cat #22143975) and coverslipped with Permount (Fisher, cat #SP15500). To detect PCNA\(^+\) cells a 1 in 12 series of sections throughout the entire dentate gyrus were mounted onto slides, heated to 90°C in citric acid (0.1 M, pH 6.0), permeabilized with trypsin, incubated in 2N HCl for 30 min, incubated overnight at 4 °C with mouse anti-PCNA antibody (1:200, Santa Cruz Biotechnology, cat #sc-56) and then processed as per thymidine analogs. Two sections containing the dorsal hippocampus were stained for the immature neuronal marker doublecortin (DCX) to detect immature neurons. In Experiment 1, staining was performed on free-floating sections with fluorescent detection. Sections were treated with PBS with 10% triton-x, 3% horse serum for 30 minutes, incubated at 4°C for 3 days.
in PBS with 10% triton-x, 3% horse serum and goat anti-DCX (1:250; Santa Cruz Biotechnology, cat #sc-8066). Visualization was performed with Alexa 555-conjugated donkey secondary antibody (Invitrogen/Thermofisher, cat #A21432) diluted 1:250 in PBS for 60 minutes at room temperature. Sections were counterstained with DAPI (Life Technologies, cat #D1306), mounted onto slides and coverslipped with PVA-DABCO. In experiments two and three, sections were mounted on slides, heated to 90°C in citric acid (0.1 M, pH 6.0), sections were washed, permeabilized in PBS with 10% triton-x for 30 min and incubated for three days at 4 °C with goat anti-DCX (1:250 in 10% triton-x and 3% horse serum). Sections were washed and incubated in biotinylated donkey anti- goat secondary antibody for 1 hour (1:250, Jackson, cat #705065147) and processed for peroxidase-DAB as above.
Figure 3-1: Immunohistochemistry for neurogenesis markers used in this study.

Thymidine analogs (BrdU, CldU, IdU) were used to detect neurons born well before the experimental endpoint, DCX was used to detect neurons born in the few weeks preceding the experimental endpoint, and PCNA was used to detect cells that were proliferating at the experimental endpoint. Since CldU and IdU were used in the same animals, and they have the potential to cross react, control tissue was stained to ensure antibody-antigen specificity.
Figure 3 1: Immunohistochemistry for neurogenesis markers used in this study.

(“CldU control” was injected with only IdU and stained for CldU, ie with rat anti-BrdU antibody; “IdU control” was injected with only CldU and stained for IdU, ie with mouse anti-BrdU antibody). Scale bar = 200 µm.

3.2.3 Microscopy and sampling

Quantification of all DAB-stained cells was performed using a brightfield Olympus CX41 microscope and a 40x objective. For BrdU, IdU, CldU and PCNA, a 1 in 12 series of sections spanning the entire dentate gyrus was examined and all cells that were located within the granule cell layer or its 20 µm hilar border (the subgranular zone) were counted and multiplied by 12 to estimate the total number of cells per dentate gyrus (bilaterally). DAB-stained DCX⁺ cells were quantified across the entire granule cell layer and subgranular zone (~20 µm wide) from 2 dorsal sections (4 hemispheres). Due to the large numbers of DCX⁺ cells they were not analyzed along the full dorsoventral axis but from a restricted portion of the DG. The granule cell layer volume was calculated by multiplying the section thickness (40 µm) by the 2D area (measured from 2x images with ImageJ (NIH), which was then used to calculate DCX⁺ cell densities.

3.2.4 Running distance calculations

For all experiments, running wheel revolutions were tracked and converted to distances using $2\pi r$. To facilitate comparisons between experiments, all running data is presented as distance run per rat per week. For Experiments 1 and 2 this was calculated by dividing the running distance for the cage by 2, since animals were continuously pair-housed. For Experiment 3, individual distances were tracked on weekdays and the relative amount of running by each rat in a pair was calculated. This proportional value was applied to the distance run by the cage on weekends, when rats were pair housed, to estimate each rat’s contribution to the weekend
running distance. Weekday and weekend running distances were then summed to determine total weekly running distances per rat.

Purely behavioral (running) analyses were at the level of cage when individual data were unavailable (Experiments 1 and 2), and animal when individual running data were available (Experiment 3). Since individual data on neurogenesis rates were available for all experiments, running-neurogenesis correlations were explored at the individual level for all experiments. For Experiments 1 and 2 the average running distance per rat was used for correlational analyses and for Experiment 3 the individual running distances were used (with corrections to estimate individual weekend distances, described above).

3.2.5 Statistical Analyses

Unpaired t-tests and two-way (sex x treatment) ANOVAs were used to detect effects of treatment and sex on measures of neurogenesis. Where significant interactions were observed, Sidak’s (Experiment 1) or Dunnett’s tests (Experiment 3) were used to compare treatment groups to controls. In cases where distributions failed tests of normality and homogeneity of variance, analyses were run on log transformed data or non-parametric tests were used. In all cases statistical significance was set at \( p = 0.05 \).

3.3 Results

3.3.1 Experiment 1 – Short term continuous RUN and a single MEM injection

3.3.1.1 Short-term continuous running behavior

Rats housed with running wheels ran progressively more over time (Fig. 3-2B; effect of time: \( F_{4,24} = 3.8, P=0.015 \)). There were no differences in distance run between males and females, but sample sizes were small since rats were pair-housed and we could only determine the
distance by the cage rather than per individual rat (but Fig. 3-2 shows cage distance divided by 2 to facilitate comparisons with later experiments; effect of sex $\text{F}_{6,24}=4.6$, $P=0.09$).

Figure 3-2: Experiment 1: Short-term treatment with cRUN and sMEM.

A) Experimental timeline: cRUN rats were given continuous access to running wheels and sMEM rats were given a single injection of memantine. A single BrdU injection labelled a discrete population of cells born in the early phase of treatment, PCNA immunostaining was used to visualize cells that were actively proliferating at the end of the
Figure 3-2: Experiment 1: Short-term treatment with cRUN and sMEM.

experiment when the animals were killed, and DCX immunostaining was used to visualize neurons born in the (primarily 2) weeks prior to death. B) Running distance increased over time (symbols indicate average distance per rat in a cage, bars indicate standard error; *P<0.05 vs 1w).

3.3.1.2 Five weeks of continuous running transiently increases neurogenesis

Neurogenesis was measured with 3 immunohistochemical markers that label complementary populations of cells born at different phases of the cRUN treatment. BrdU+ cells, born after the first week of running, were increased in both males and females (effect of cRUN F1,26=26.8, P<0.0001; Fig. 3-3A). The cRUN-induced increase in BrdU+ cells was larger in females than males (84% vs 42%), but sex differences were not statistically significant (effect of sex F1,26=3.4, P=0.08; interaction F1,26=2.4, P=0.13). To investigate possible relationships between running distance and neurogenesis, we correlated neurogenesis rates with the average distance run per rat. Here, BrdU+ cell numbers correlated with running distance in males but not females (males: Pearson r=0.87, P=0.0053; females: Pearson r=0.48, P=0.23; Fig. 3-3B,C). A limitation of this analysis is that it assumes equal distances were run by both rats in a cage (see methods). However, we observed no evidence to suggest that rats had differential access to the wheel. Rats were frequently observed in the wheel together during both active and inactive phases of the day, there were no overt signs of aggression, and we found that cagemates ran similar amounts in Experiment 3 (see below). Collectively, this suggests that pair housing likely did not result in a significant imbalance in running between cagemates. DCX+ cells were quantified to examine cumulative cRUN effects on neurons mainly born during the last 2 weeks of running (Brown et al., 2003; Rao and Shetty, 2004; Snyder et al., 2009b). Here, we also found
that DCX was higher in runners compared to sedentary rats (effect of cRUN $F_{1,28}=4.6$, $P=0.04$; Figure 3-3D). However, the neurogenic effect of running was weaker than for BrdU$^+$ cells, with only a 14% increase in males and 12% increase in females. PCNA immunostaining, to identify cells that were proliferating at the end of the 5 weeks of running were not significantly increased in runners compared to sedentary controls (effect of running, $F_{1,27}=3.8$, $P=0.06$; Fig. 3-3E). DCX$^+$ and PCNA$^+$ cells did not correlate with running distance in either sex (not shown). This experiment demonstrates that running initially increases neurogenesis but any effects remaining after 5 weeks are marginal.

**Figure 3-3: Experiment 1: Transient effects of running on neurogenesis.**

A) Five weeks of running increased neurogenesis as measured by BrdU labelling of neurons born after the first week of running. B) In females, BrdU cell numbers did not correlate with running distance. C) In males, BrdU cell
columns correlated with running distance. D) Numbers of DCX$^+$ cells, reflecting neurons born in the last ~3 weeks, were increased in runners by $<15\%$. E) PCNA$^+$ cells, proliferating at the end of 5 weeks of cRUN, were not different in runners compared to sedentary controls. ****$P<0.0001$, *$P<0.05$. Graphs indicate group means ± standard error. Numbers embedded in cRUN bars indicate running effect size (%) relative to sedentary controls.

3.3.1.3 A single MEM injection transiently increases neurogenesis

MEM injection increased the number of BrdU$^+$ cells that were born 3 days later and survived for an additional 4 weeks (treatment effect $F_{1,18}=14.5$, $P=0.0013$; Fig. 3-4A). The relative increase in BrdU$^+$ cells was identical in males and females (60% over controls). There were no differences in numbers of DCX$^+$ or PCNA$^+$ cells due to sMEM or sex (Fig. 3-4B,C). Thus, as with running, the neurogenic effects of sMEM were transient.
Figure 3-4: Transient effects of a single memantine injection on neurogenesis.

A) sMEM increased the number of BrdU⁺ cells born 3 days later and surviving for ~ 4 more weeks. B) Four weeks after sMEM, numbers of immature DCX⁺ cells were not different from vehicle-injected controls. C) Four weeks after sMEM, numbers of proliferating PCNA⁺ cells were equivalent to vehicle controls. **P<0.01. Graphs indicate...
Figure 3-4: Transient effects of a single memantine injection on neurogenesis.

Group means ± standard error. Numbers embedded in sMEM bars indicate effect size (%) relative to vehicle-injected controls.

3.3.2 Experiment 2 – Long-term continuous RUN

3.3.2.1 Long-term continuous running behavior

Rats were continuously pair-housed with a running wheel as in Experiment 1. Tracking for one cage was incomplete and therefore not included. For the remaining 3 cages, running increased over the first 3 weeks and then subsided to stable levels for the remaining weeks (Fig. 3-5A; repeated measures ANOVA, effect of time $F_{2,56}=2.4$, $P=0.011$; week 3 vs week 1: $P=0.0013$, week 3 vs week 14: $P=0.042$, week 3 vs week 16: $P=0.047$, week 3 vs week 17: $P=0.048$, week 3 vs week 18: $P=0.0028$, week 3 vs week 19: $P=0.013$).

3.3.2.2 Effects of long-term continuous running on neurogenesis

To investigate whether the marginal increase in DCX$^+$ cells observed after 1 month of cRUN (Experiment 1) persists if cRUN is extended to 4 months, we quantified DCX$^+$ cells in cRUN and sedentary rats. We observed no difference between groups, indicating that cRUN treatment does not result in sustained increases in neurogenesis (Fig. 3-5B; $T_{14}=0.51$, $P=0.6$). Running did not correlate with DCX$^+$ cell densities (not shown).
Figure 3-5: Experiment 2: Long-term continuous running.

A) Average weekly running distance per rat increased over the first 3 weeks and then decreased to stable levels. B) Numbers of immature DCX$^+$ cells were similar in runners and sedentary controls. Symbols and bars indicate mean ± standard error.
3.3.3 Experiment 3 – Long-term interval RUN and multiple MEM injections

3.3.3.1 Long-term interval running behavior

To test whether other paradigms were capable of leading to sustained increases in neurogenesis, rats were subjected to interval RUN (iRUN), multiple MEM injections (mMEM), or combined iRUN + mMEM treatment (Fig. 3-6A). Most groups ran significantly greater distances with each week of iRUN (Fig. 3-6B), as detected by within-sex repeated measures ANOVAs and post tests for linear increases in running with time (female iRUN/iRUN: F7,49=7.6, P<0.0001, linear trend P<0.0001; female iRUN/mMEM: F3,27=10.6, P<0.0001, linear trend P<0.0001; female mMEM/iRUN: F3,21=6.8, P=0.0022, linear trend P=0.0002; male iRUN/iRUN: F7,77=1.5, P=0.16; male iRUN/mMEM: F3,33=4.0, P=0.016, linear trend P=0.0019; male mMEM/iRUN: F3,21=5.7, P=0.0051, linear trend P=0.0046). An analysis of average weekly running distance across treatment groups and sexes revealed no differences between treatment groups but significantly greater distances run by females (treatment effect F2,52=0.98, P=0.38; sex effect F1,52=17, P=0.0001; Fig. 3-6C). Since rats were pair-housed but ran individually we were able to examine whether cagemates ran similar distances. Examining all female and male pairs revealed that cagemates’ running distances were correlated (females: r=.57, P=0.04; males: r=0.68, P=0.054).
Figure 3-6: Experiment 3: long-term treatment with iRUN and mMEM.
Figure 3-6: Experiment 3: long-term treatment with iRUN and mMEM.

A) The experimental design consists of 2 x 4-week treatment blocks. Four neurogenesis markers were used to identify neurons born at different stages of the 2 treatment blocks: CldU (neurons born at the beginning of block 1), IdU (neurons born at the beginning of block 2), DCX (neurons born during the latter weeks of block 2) and PCNA (neurons born at the end of block 2). CON rats remained in standard cages and were handled on MEM injection days. iRUN/iRUN rats were given 8 weeks of interval running. mMEM/mMEM rats received 8 weekly injections of memantine. iRUN/mMEM rats received 4 weeks of interval running and then 4 weeks of memantine injections. mMEM/iRUN rats received 4 weeks of memantine injections and then 4 weeks of interval running. B) Average weekly running behavior per rat, broken down by sex. Most groups ran significantly more with time (see text for details). C) Weekly running behavior averaged over the entire treatment period. Females ran significantly more than males (****P<0.0001). Symbols and bars indicate mean ± standard error.

3.3.3.2 Effects of long-term mMEM, iRUN and mMEM+iRUN treatments on neurogenesis

To investigate the efficacy of long-term neurogenic manipulations, we used complementary immunohistochemical markers to quantify: neurons born at the beginning of treatment block 1 (CldU), neurons born at the beginning of treatment block 2 (IdU), immature neurons born during the latter portion of treatment block 2 (DCX) and cell proliferation at the end of treatment block 2 (PCNA) (Fig. 3-7A). To ensure specificity of the CldU and IdU labelling, CldU-only tissue was stained for IdU and IdU-only tissue was stained for CldU (2 animals for each). No thymidine-labelled cells were observed in this control tissue (Fig. 3-1). Numbers of CldU+ cells, born at the beginning of block 1, were 30% greater in males than females (sex effect F_{1,80}=15, P=0.0002; Fig. 3-7B). There was no main effect of treatment (F_{4,80}=2.3; P=0.07) but a sex x treatment interaction (F_{4,80}=3.6; P=0.009) where mMEM/iRUN increased CldU+ cells relative to controls in males only (79% increase in males, P=0.01; 16%
increase in females). However, the individual data points clearly reveal that mMEM/iRUN effects on CldU+ cells were variable: 3 of 7 rats had CldU+ cell numbers that were approximately twice control levels, the other 4 rats were within the range of controls, and re-analysis with a non-parametric test revealed no difference from controls (Dunn’s test: mMEM/iRUN vs control, P=0.09).

To examine cells born at the beginning of the 2nd treatment block, we quantified IdU+ cells (Fig. 3-7C). The total number of IdU+ cells was (17%) greater in males than in females (effect of sex F1,81=4.1, P=0.046). However, there were no effects of treatment on IdU+ cell numbers (effect of treatment F4,81=1.6, P=0.17; treatment x sex interaction F4,81=0.7, P=0.6). Since these data were not normally distributed, even after transformation, we additionally examined the overall sex difference with non-parametric tests and confirmed that males had more IdU+ cells than females (Mann Whitney test, P=0.0003).

To examine a broad-aged population of cells born in the 2nd block of the extended treatments, we quantified immature DCX+ neurons (Fig. 3-7D). Here, we found an effect of treatment, where only the combined iRUN+mMEM treatments increased DCX+ cell density (across sexes, iRUN/mMEM 26% over controls, P=0.005; mMEM/iRUN 25% over controls, P=0.015). A sex x treatment interaction (F2,82= 3.5, P=0.012) revealed distinct patterns in males and females: in females, iRUN/iRUN selectively increased DCX+ cell density (30% increase over controls, P=0.03) whereas in males iRUN/mMEM selectively increased DCX+ cell density (42% increase, P=0.002).

To identify whether extended iRUN and mMEM treatments had a sustained impact on neurogenesis, we examined PCNA+ cells to determine cellular proliferation levels at the end of the treatment paradigms (Fig. 3-7E). We found no effect of treatment or sex on proliferating
PCNA$^+$ cells (treatment effect $F_{4,82}=0.9$, $P=0.44$; sex effect $F_{1,82}=0.1$, $P=0.7$). There was a significant sex x treatment interaction ($F_{4,82}=4.1$, $P=0.005$) but no treated groups were significantly greater than controls.

To determine whether iRUN-related changes in neurogenesis depended on running distances, we correlated cell counts with the weekly distance run by each rat. Consistent with a stronger group effect of iRUN/iRUN in females, we found that the distance run in treatment block 2 positively correlated with DCX$^+$ cell density in females but not males (females: Pearson $r=0.83$, $P=0.0081$; males: Pearson $r=-0.08$, $P=0.83$; Fig 3-7F-G). In contrast, block 2 running distances negatively correlated with PCNA$^+$ cell numbers in females, with no correlation observed in males (females: Pearson $r=-0.76$, $P=0.047$; males: Pearson $r=0.11$, $P=0.76$; Fig 3-7F-G). No other correlations between running and neurogenesis markers were significant, with the exception that the distance run by females in the first block of iRUN/iRUN also negatively correlated with numbers of PCNA$^+$ cells (not shown).
Figure 3-7: Experiment 3: Effects of long-term iRUN and mMEM treatments on neurogenesis.

A) Experimental timeline indicating populations of cells identified by immunohistochemical markers. B) CldU+ cells, born at the beginning of block 1, were significantly greater in mMEM/iRUN animals relative to controls. This effect was mainly driven by effects in males. There were also more CldU+ cells in males than in females. C) IdU+ cells, born at the beginning of block 2, were not different from controls following any of the treatments. There were more IdU+ cells in males than in females. D) The density of immature DCX+ cells, born during block 2, was greater in the iRUN/mMEM and mMEM/iRUN treatment groups. In males, iRUN/mMEM significantly increased DCX+...
Figure 3-7: Experiment 3: Effects of long-term iRUN and mMEM treatments on neurogenesis.

cell density and, in females, iRUN/iRUN significantly increased DCX<sup>+</sup> cell density. E) PCNA cell counts revealed that no treatment regimen altered cell proliferation at the end of the experiment. F) DCX<sup>+</sup> cell densities positively correlated, and PCNA<sup>+</sup> cells negatively correlated, with distance run by iRUN/iRUN females in treatment block 2. G) DCX<sup>+</sup> cell densities and numbers of PCNA<sup>+</sup> cells did not correlate with block 2 running distances in males.

***P<0.001, **P<0.01, *P<0.05). Bars indicate group means ± standard error. Numbers embedded in bars indicate effect size (%) relative to controls.

3.4 Discussion

Here we examined the effects of two neurogenic treatments, RUN and MEM, on adult neurogenesis in the dentate gyrus of male and female rats. Our general strategy was to immunostain for thymidine analogs to detect treatment effects on cells born long before the experimental endpoint, DCX to detect effects on neurons born in the weeks prior to endpoint, and PCNA to detect effects on cell proliferation at the very end of each experiment. We have two main findings. First, neurogenic effects were temporally limited: cRUN and sMEM increased neurogenesis only at early timepoints and iRUN and mMEM increased neurogenesis only at the later timepoints. Second, there were sex differences in the neurogenic treatment efficacy: iRUN increased neurogenesis in females and iRUN/mMEM increased neurogenesis in males.

A primary goal of the study was to identify long-lasting neurogenic manipulations. Given the transient effects of cRUN and sMEM in Experiment 1, we therefore adjusted the treatment regimens. For MEM, we investigated whether repeated dosing was capable of consistently upregulating neurogenesis. Repeated MEM dosing has been found to increase DCX<sup>+</sup> cells (i.e. neurons born primarily near the end of treatment) (Akers et al., 2014). BrdU labelling has also revealed neurogenic effects of repeated MEM dosing (Ishikawa et al., 2016), but this study
labelled cells born throughout treatment, precluding identification of effects on cells born early vs late in the treatment regimen. Due to the limited neurogenic efficacy of cRUN, we switched to an intermittent RUN paradigm in Experiment 3, since others have shown that restricted RUN is more effective at inducing long-lasting changes in neurogenesis (Naylor et al., 2005; Grégoire et al., 2014; Inoue et al., 2015; So et al., 2017; Nguemeni et al., 2018). Additionally, we reasoned that neurogenic mechanisms might habituate or desensitize with repeated treatment. To avoid this we therefore included groups in which iRUN and mMEM alternated. While little is known about how RUN and MEM increases neurogenesis, others have shown that MEM promotes symmetric division, and therefore expansion, of radial glial stem cells (Namba et al., 2009). In contrast, RUN increases division of transit amplifying cells (neuronal precursors that are downstream of radial stem cells) (Kronenberg et al., 2003) and enhances neuronal survival (Snyder et al., 2009b). We hypothesized that, by targeting distinct and complementary mechanisms, combined RUN+MEM treatment might be more effective than either treatment in isolation. For example, MEM could increase the size of the stem cell pool, which in turn generates more transit amplifying cells that can be targeted by the proliferative effects of RUN.

### 3.4.1 Transient, early neurogenic effects of cRUN and sMEM

In Experiment 1 we confirmed previous reports that both cRUN and sMEM can have potent neurogenic effects, increasing numbers of surviving BrdU+ cells by 40-80%. While we did not phenotype these cells, using similar methods we have found that the vast majority of new cells in the granule cell layer and subgranular zone become neurons (Snyder et al., 2009a) and others have reported that RUN and MEM specifically increase the production of neurons (van Praag et al., 1999b; Maekawa et al., 2009). BrdU was injected 1 week after cRUN onset, to capture combined neurogenic effects on proliferation and survival (Snyder et al., 2009b). While
this maximized our ability to detect cRUN-induced increases in BrdU+ cell numbers, we cannot conclude whether effects were due to enhanced proliferation, survival, or both. Regardless, cRUN-induced elevations in DCX+ and PCNA+ cells were minor/absent at the end of 5 weeks. Since many previous studies have found that markers of proliferative and immature cells are effective at detecting running-induced enhancements in neurogenesis (Kronenberg et al., 2006; Snyder et al., 2009b; Patten et al., 2013; Akers et al., 2014; Nokia et al., 2016), this strongly suggests that the neurogenic effects of cRUN were transient. Extending cRUN for 4 months in Experiment 2 revealed that any residual enhancements in DCX+ cells dissipated with time. While cRUN may increase neurogenesis beyond 30 days in some circumstances (Patten et al., 2013), our findings are consistent with temporally-limited effects of cRUN (Naylor et al., 2005; Kronenberg et al., 2006; Snyder et al., 2009b; Clark et al., 2010).

In the case of sMEM, BrdU was injected 3 days after MEM injection, to capture the delayed effects of MEM on proliferation (Maekawa et al., 2009). sMEM effects were also transient, increasing BrdU+ cell numbers but without effects on DCX+ cells or PCNA+ cells that were born days and weeks later. This experiment provides new data on the transient nature of MEM effects, since previous studies have only examined neurogenic effects at short post-MEM intervals (Maekawa et al., 2009; Namba et al., 2009; Ishikawa et al., 2014; Sun et al., 2015).

3.4.2 Differential effects of iRUN and mMEM

DCX+ cell densities revealed that, unlike the sMEM and cRUN paradigms, elevated neurogenesis could be induced at the end of extended treatment regimens. The only manipulations that increased DCX+ cells when males and females were pooled (i.e. main effect) were the combined iRUN+mMEM treatments. This parallels a report that exercise and dietary supplementation can promote neurogenesis when delivered in combination, but not isolation.
(Hutton et al., 2015). Here, combined treatments were most effective in males, which were particularly responsive to iRUN followed by mMEM. Notably, males subjected to mMEM followed by iRUN also showed signs of more DCX$^+$ and PCNA$^+$ cells (increased by 28% and 44%, respectively). Since these changes did not reach statistical significance, future studies might use larger sample sizes to investigate whether efficacy and/or mechanisms depend on the order of treatment. Likewise, resource limitations prevented us from including CON/iRUN and CON/mMEM groups in the current study. Future analyses of these conditions would more conclusively determine whether the late-stage effects require a combination of treatments.

Whereas males responded to combined treatments, only females displayed increased DCX$^+$ cell densities in the iRUN/iRUN condition. Thus, Experiment 3 successfully identified sex-specific strategies for increasing neurogenesis in the final weeks of a 2-month treatment regimen. However, strategies were relatively ineffective at earlier stages of treatment, since CldU$^+$ cells (born ~1w into treatment) and IdU$^+$ cells (born 5w into treatment) were generally not different between treated groups and controls. Collectively, we found that neurogenesis was elevated only early (Experiments 1) or only late (Experiment 3), highlighting the difficulty of sustaining elevated levels of neurogenesis. Below, we discuss in more detail the possible differential effects of RUN and MEM treatments in our experiments.

3.4.3 Neurogenic effects of RUN in males and females

Sex differences in the neurogenic effects of RUN may be due to differences in activity levels, with females typically being more active than males (Lightfoot, 2008). Consistent with this possibility, iRUN/iRUN did not increase numbers of CldU$^+$ cells that were born when females ran less than 10 km/week and iRUN/iRUN did not increase neurogenesis (as measured by any marker) in males, which consistently ran \( \leq 10 \text{ km/week} \). However, when rats consistently
ran ~20 km/week, neurogenesis was increased (DCX+ cells in female iRUN/iRUN, BrdU+ cells in male cRUN). Our correlational analyses also point to an optimal range, centered around 20 km/week, whereby running modulates neurogenesis levels. Rats that ran less than this did not show any correlations between running and neurogenesis, presumably because all rats ran below threshold. Groups of rats that ran ~20 km/week displayed correlations (male cRUN BrdU, female iRUN/iRUN DCX). But rats that ran substantially more than 20 km/week did not show any correlations (female cRUN), possibly due to ceiling effects. Sex differences in activity levels therefore appears to be a critical factor that regulates the neurogenic response to exercise. Future studies could test this hypothesis directly by controlling running distances in males and females. This could be accomplished by restricting access to the running wheel in a voluntary exercise paradigm. Alternatively, forced running could be used to equalize exercise across sexes, with the caveat that this paradigm impacts neurogenesis and anxiety levels differently than voluntary paradigms (Leasure and Jones, 2008). As noted by others, these types of findings warrant additional research into the effects of exercise in males vs. females and they indicate that, as a therapy, exercise likely needs to be tailored to individual needs (Barha et al., 2017).

Analyses of the running data also suggest that the amount of running is a key difference between the cRUN and iRUN protocols. In the cRUN paradigm rats ran greater distances beginning earlier in the treatment but in the iRUN paradigm running increased over many weeks. Thus, the early neurogenic efficacy of cRUN and the late efficacy of iRUN/iRUN may simply reflect the timecourse when rats ran sufficient distances to increase neurogenesis. In any case, given that more restricted and less intense forms of exercise have longer-lasting neurogenic effects (Naylor et al., 2005; Grégoire et al., 2014; Inoue et al., 2015; So et al., 2017; Nguemeni et al., 2018), iRUN/iRUN may be suitable for sustaining higher levels of neurogenesis beyond the 2
months tested in this study. The PCNA data are equivocal on this point: on one hand, many iRUN/iRUN female rats had greater numbers of proliferating cells than controls. On the other hand, proliferating cells negatively correlated with running distance, suggesting that there may be compensatory reductions in proliferation to maintain a balanced number of new neurons. Future studies, employing thymidine analogs to parse out effects on proliferation vs survival, may be needed to make firmer conclusions about the exact timecourse and duration of RUN effects on various aspects of neuron production.

3.4.4 Neurogenic effects of MEM in males and females

Surprisingly, while sMEM increased neurogenesis in both females and males, mMEM less effective in males and completely ineffective in females. Specifically, in males, MEM increased BrdU⁺ neurons that were born 3 days after a single MEM injection in Experiment 1 but it less reliably increased CldU⁺ neurons that were born at a similar timepoint in Experiment 3 (i.e. born 3 days after the first MEM injection). Some males in the mMEM/iRUN condition (3/7) clearly had elevated CldU⁺ cells. It is possible that iRUN in the 2nd block was more effective at rescuing these cells. This explanation seems unlikely, however, since once adult-born neurons reach ~4 weeks of age (i.e. when iRUN began) they are beyond their critical period for survival and do not undergo further cell death (Dayer et al., 2003; Kempermann et al., 2003; Snyder et al., 2009a). Instead, these data collectively suggest that a single MEM injection increases the birth of new neurons but subsequent MEM injections reduce the survival of those neurons. Indeed, whereas 3 additional MEM injections (male mMEM/iRUN) only partially offset the neurogenic effects of a single injection, no rats had elevated CldU⁺ cells after 7 additional MEM injections (male mMEM/mMEM).
A MEM-induced loss of early-born CldU\(^+\) cells could result from homeostatic mechanisms whereby later injections of MEM increase proliferation, which in turn reduces the survival of earlier-born cells. Such a mechanism would fit with findings that learning balances net neurogenesis by increasing both the addition and loss of adult-born neurons depending on cell age (Olariu et al., 2005; Dupret et al., 2007). The fact that iRUN/mMEM increased DCX\(^+\) cell densities in males is consistent with the possibility that subsequent/multiple MEM injections retains some neurogenic effects. However, proliferative effects of MEM must wane between the 1\(^{st}\) and 4\(^{th}\) injections because neither the iRUN/mMEM nor mMEM/mMEM groups had elevated PCNA\(^+\) cell counts.

Alternatively, while MEM is therapeutically attractive given its positive effects on hippocampal-dependent learning (Zajaczkowski et al., 1996; Zoladz et al., 2006; Ishikawa et al., 2014) and neuroprotective functions (Creeley et al., 2006), it alters the migration of adult-born neurons (Namba et al., 2011) and mMEM treatments may lose their neurogenic effects due to off target effects. A different NMDAR antagonist, MK-801, dose-dependently increases necrosis in the neocortex and these effects are particularly pronounced in females (Fix et al., 1995). While MEM has been reported to be relatively innocuous compared to MK-801, MEM concentrations in serum and brain are twice as high in females as compared to males after intraperitoneal injection (Zajaczkowski et al., 2000). Indeed, females were virtually unresponsive to the neurogenic effects of mMEM treatments. These observations collectively highlight the need to include both sexes in studies of neurogenic treatments because the outcomes, whether physiological or pathological, can be quite different.
3.4.5 Implications for neurogenic therapies

Our findings have a number of implications for disorders that impact the structural integrity of the hippocampus. Alzheimer’s disease, schizophrenia and depression differentially impact males and females (Gao et al., 1998; Aleman et al., 2003; Seedat et al., 2009) and all are associated with hippocampal volume loss (Jack et al., 2000; Harrison, 2004; McKinnon et al., 2009). Our results clearly show that there are sex differences in the efficacy of treatments that increase neurogenesis and could potentially offset structural deficits. While both RUN and MEM were capable of increasing neurogenesis in both sexes, iRUN was more effective in females and combined RUN+MEM treatments were more effective in males. However, both short and long treatments were only partially effective, with short treatments increasing neurogenesis only at early timepoints and longer treatments increasing neurogenesis only at later timepoints. It will be important for future work to determine whether extended treatment paradigms can increase neurogenesis beyond 2 months. If not, this may suggest that there are constraints on the extent to which neurogenesis can be increased, which could be due to a limited capacity of the stem cell pool (Encinas et al., 2011).

Additional considerations and future work will also be necessary to translate our findings to treatments that can offset behavioral deficits in human disorders. For example, we did not include behavioral testing since learning experiences can alter neurogenesis and potentially confound interpretations about treatment effects. But this will be important to determine whether differential effects on neurogenesis result in relevant behavioral improvements. Likewise, RUN and MEM could impact functional properties of newborn neurons and enhance behavioral outcomes independently of changes in neuronal number. The applicability of RUN and MEM treatments to humans also needs to be considered. Species running comparisons have suggested
that humans run ~10x more than rodents, per time (Hatchard et al., 2014). Since we found that running had neurogenic effects when rats ran ~20 km/week, this suggests humans may need to run ~200 km/week (~30 km/day) to achieve similar neurogenic effects. Alternatively, it may be that the time spent exercising is more important than the distance travelled. If we assume that rats ran at a rate of ~1 km/hr (Hatchard et al., 2014), beneficial effects might therefore be observed with ~20 hrs of exercise per week (~3 hrs/day). These amounts of exercise are unsustainable, however, and so it will be important to determine whether even more restricted running paradigms are neurogenic. A direct comparison using a shorter and more intense running paradigm revealed that humans only run ~4x faster than rats, and the 2 species have very similar metabolic responses to exercise (Goutianos et al., 2015). While neurogenesis was not examined, this suggests that rodent studies do have the potential to translate to realistic exercise paradigms for humans. With respect to MEM, whereas rodent neurogenesis studies typically administer high doses (25-50 mg/kg) once or on a weekly schedule, patients in clinical trials receive much lower doses (~0.3 mg/kg assuming 70 kg body weight) on a daily schedule (Reisberg et al., 2003; Okuizumi et al., 2018). Given that the half-life of MEM is ~20x longer in humans (Parsons et al., 1999), this means that clinical protocols induce chronic, low levels of MEM whereas our protocol induced transient, high levels of MEM. Given the limited success of mMEM treatment in the current study, it would therefore be valuable to test whether more therapeutically-relevant doses have neurogenic effects in rodents.
Chapter 4: Adult neurogenesis and the regulation of dentate gyrus neurons born in early postnatal development

4.1 Introduction

The dentate gyrus, a subregion of the hippocampus, is composed of cells born both in development and adulthood. The discovery that neurons are added to the adult brain of nearly all mammals examined, including humans (Rat: (Altman and Das, 1965; Cameron et al., 1993), Mice: (Kempermann et al., 1997a), Macaques: (Kornack and Rakic, 1999; Gould et al., 2001), Birds: (Barnea and Nottebohm, 1996), Humans: (Eriksson et al., 1998)), has had profound effects on our understanding of the potential for plasticity in the brain, and has lead to a focus on these newly added adult-born cells. These new adult-born neurons have enhanced synaptic plasticity (Snyder et al., 2001; Schmidt-Hieber et al., 2004; Ge et al., 2007) and are required for learning and memory (Snyder et al., 2005; Saxe et al., 2006; Clelland et al., 2009). It is estimated that 40% of the total neuronal population is added in adulthood, a much greater amount than is generally appreciated (Snyder and Cameron, 2012). With such a large proportion of neurons added during the adult period, it is important to understand the relationship between the developmentally-born and adult-born populations, not only to understand how this adult-born population may affect the developmental population but also how they interact.

Alterations have been found in humans and rodents in hippocampal volume and adult neurogenesis in response to aging and in a number of mental health disorders such a depression and Alzheimer’s disease (AD) (Bremner et al., 2000; Malberg et al., 2000; Czéh et al., 2001; Campbell et al., 2004; Videbech and Ravndalde, 2004; Dickerson et al., 2005; Schuff et al., 2008;
Erickson et al., 2011; Cunha et al., 2013; Zeng et al., 2016; Fang et al., 2018). In humans, reduced hippocampal volume and cognitive defects have been interpreted as signs of damage, and while the mechanisms underlying these changes are multifaceted, external manipulations known to increase adult neurogenesis in rodents have been shown to improve cognition and reverse hippocampal atrophy (Pereira et al., 2007; Erickson et al., 2011; Killgore et al., 2013).

Improvements in depression, cognition, and hippocampal plasticity have also been shown with the treatment of both traditional antidepressants and physical exercise in both animal models and humans (Babyak et al., 2000; Malberg et al., 2000; Czéh et al., 2001; Sheline et al., 2003; Parachikova et al., 2008; Cunha et al., 2013; Kerling et al., 2017). Physical exercise as a treatment for depression in humans has been shown to reduce relapse rates comparable to traditional antidepressants, continued exercise decreases depression rates beyond traditional treatments, and in rodents it increases adult neurogenesis and reverses depressive-like behaviours (Babyak et al., 2000; Duman et al., 2008). With regards to Alzheimer’s disease (AD) and related dementias, physical activity has been shown in humans to be inversely associated with the risk of dementia (Hamer and Chida, 2008). The amount of physical activity has been shown to play a role, as men show a higher risk of dementia with decreased walking rates and women present with 20% lower risk of cognitive impairments with increased activity (Abbott et al., 2004; Weuve et al., 2004). In terms of animals models of AD, short access to a running wheel has been associated with improvements in cognitive function and increases in neurogenesis (Adlard et al., 2005; Parachikova et al., 2008; Maliszewska-Cyna et al., 2016), while longer treatment regiments are shown in many Alzheimer's models to decrease AD pathology in the hippocampus, improve cognition, increase neuronal number as well as proliferation, and to decrease neuronal
loss in CA1 (Adlard et al., 2005; Belarbi et al., 2011; Tapia-Rojas et al., 2015; Hüttenrauch et al., 2016; Maliszewska-Cyna et al., 2016).

Another treatment known to increase adult neurogenesis that has also been used to treat AD and has recently begun trials as a treatment for depression is NMDA receptor blockade. One such NMDA receptor antagonist is memantine (MEM), which is known for its neuroprotective effects and its approval for use as an AD treatment (Lipton, 2004). MEM, as well as other NMDA receptor antagonists (MK-801 and ketamine), have been shown to increase adult neurogenesis (Cameron et al., 1995; Akers et al., 2014; Ishikawa et al., 2014; Soumier et al., 2016), while MEM alone has been shown to increase the size of the stem cell pool by increasing symmetrical division (Namba et al., 2009). In the treatment of Alzheimer’s disease MEM has been shown in animal models to improve memory and decrease AD pathology in the hippocampus, while in humans, cognitive and functional improvement have been seen in only moderate to severe Alzheimer's patients though improvements are small (Thomas and Grossberg, 2009; Di Santo et al., 2013; Yang et al., 2013; Liu et al., 2014; Sun et al., 2015). MEM has been shown as an effective antidepressant, and in animal models has been shown to improve memory impairments, increase BDNF, and decrease depressive-like behaviours (Muhonen et al., 2009; Réus et al., 2010; Amidfar et al., 2017).

While these positive changes associated with physical exercise and MEM, the question remains if new adult-born cells may play an important role in these positive effects. Both treatments are known to alter adult neurogenesis as well as alleviate or reverse some symptoms of mental health disorders associated with the hippocampus. Understanding how these new neurons may interact with, affect and become integrated with older developmentally-born cells,
and what affect manipulating these new adult-born cells may have on these developmentally-born cells is an important question.

Studies have found that increasing either the number or the activity of adult-born neurons decreases the activity in dentate gyrus, via an increase in inhibition (Drew et al., 2016; Adlaf et al., 2017). While decreasing adult neurogenesis either naturally or genetically is shown to increase granule cell activity (Drew et al., 2016; Adlaf et al., 2017). Adult-born neurons may also regulate pre-existing neurons through competition for both afferent and efferent connections (Toni et al., 2007; 2008). Increasing adult neurogenesis was shown to decrease both activity and the number of spines on mature granule cells, while decreasing spines on mature neurons was shown not only to decrease activity but also increase adult neurogenesis and the survival of these newly added cells (McAvoy et al., 2016; Adlaf et al., 2017).

While these studies do show that adult-born neurons impact the larger mature granule cell population, by not birthdating neurons it is impossible to know which population the adult-born neurons interact or impact the most, the mature adult-born population or the developmentally-born population, leaving a crucial piece of the puzzle missing. While adult-born neurons are known to become functionally equivalent to neurons born in early postnatal development once they have reached maturity (Liu et al., 2000; Laplagne et al., 2006; 2007), we have previously shown that developmentally-born neurons die in early adulthood after reaching maturity, unlike adult-born cells, which are known to remain stable after reaching maturity (see chapter 2, Dayer et al., 2003; Kempermann et al., 2003; Cahill et al., 2017).

To investigate the relationship between the adult-born and developmentally-born populations we chose to label the developmentally-born population in the early postnatal period (postnatal day (P) 6) during a peak period in dentate development and then manipulated the
adult-born populations throughout early adulthood (2-6 months). To increase adult neurogenesis we used alternating methods of wheel running (RUN) and memantine (MEM), as we recently demonstrated (see chapter 3, Cahill et al., 2018). To decrease adult neurogenesis we used GFAP-TK rats, in which neurogenesis can be arrested upon treating with an antiviral drug (valganciclovir) that interferes with DNA replication during cell division, killing the GFAP+ radial cells that give rise to new neurons (Snyder et al., 2016).

Here we show that by increasing adult neurogenesis we decrease the activity of both developmentally-born neurons and the general granule cell population within the dentate gyrus. When adult neurogenesis was decreased we found that there was an increase in activity in developmentally-born cells though, compared to animals with intact adult neurogenesis, there was only a trend towards an increase in activity after novel context exposure.

4.2 Methods

4.2.1 Animals and Treatments

All procedures were approved by the Animal Care Committee at the University of British Columbia and conducted in accordance with the Canadian Council of Animal Care guidelines regarding humane and ethical treatment of animals. Experimental Long-Evans and GFAP-TK transgenic rats were generated in the Department of Psychology's animal facility with a 12-hour light/dark schedule and lights on at 6:00 AM. Breeding occurred in large polyurethane cages (47cm × 37cm × 21cm) containing a polycarbonate tube, aspen chip bedding and ad libitum rat chow and water. The day of birth was designated postnatal day 1. Breeders (both male and female) remained with the litters until P21, when male offspring were weaned into 2 per cage in smaller polyurethane bins (48cm × 27cm × 20cm) with a single polycarbonate tube, aspen chip bedding, and ad libitum rat chow and tap water. In experiment 1, animals were weaned into and
continuously housed in a reverse light-dark cycle to analyze running effects during the active
cycle of rats (Holmes et al., 2004; van der Borght et al., 2006). Running wheel cages consisted of
a 23” x 18” x 15” plastic tub containing aspen chip bedding, ad libitum rat chow, water, and a
12” running wheel (Wodent Wheel, Exotic Nutrition). Running distance was measured the same
as in Cahill et al (2018) with each revolution of the wheel detected by a bicycle odometer
positioned outside of the cage. In experiment 2, animals were weaned and housed in standard 12-
hour light/ dark cycle.

This study is comprised of two experiments that investigate how increasing and
decreasing adult neurogenesis will affect the balance of cellular activity in the dentate gyrus. In
both experiments, only male rats were used. Animals were injected with the thymidine analog
BrdU (50 mg/kg, I.P.; Sigma, B500205, St. Louis, MO, USA) at P6, to label neurons born at the
peak of granule cell birth (Schlessinger et al., 1975). At 2 months of age, treatments to
manipulate adult neurogenesis began (see below) and continued until rats were 6 months of age.
At 6 months of age, rats were assigned to either a novel context exposure, to induce activity-
dependent Immediate-early gene (IEG) expression, or a cage control condition. For the novel
context group rats were exposed to an opaque polyurethane cage (47cm × 37cm × 21cm) filled
with a mix of aspen chip and corn cob bedding, 2 polycarbonate tubes, 2 paper towels, and 3ml
of white vinegar, which was pipetted on the outer perimeter of the cage. Rats underwent novel
context exposure in an unfamiliar room and were sacrificed 60 minutes after novel context
exposure. Rats assigned to the home cage condition did not receive any environmental exposure
but were perfused directly from their home cage.

In Experiment 1 to investigate the effects on activity after increasing adult neurogenesis,
31 wild-type Long-Evans male rats were used. At 2 months of age, treatment to increase adult
neurogenesis began. Animals were administered 4 X 4-week blocks of treatment. MEM treatment blocks consisted of 4 weekly MEM injections (35 mg/kg each) and RUN blocks consisted of rats being placed individually in running wheel cages for 4 hours on weekdays (see Fig 4-1). For the other 2 days per week, animals were pair housed in the running wheel cage with their cage mate, with free access. The RUN treatment was counterbalanced, so that rats would run for the first four hours of the dark phase on one day and the middle four hours of the dark phase on the next day. Controls consisted of animals housed in the same colony as the increasing neurogenesis group but who remained in their home cages throughout the 4-month treatment period. Each group consisted of 15-16 male Long-Evans rats.

**Figure 4-1: Study designs of both manipulations of adult neurogenesis.**

A) Increasing adult neurogenesis: Wild-type long Evans rats were given a single 50mg/kg BrdU injection at postnatal day 6 followed by treatment to increase adult neurogenesis from 2-6 months of age. Treatment consisted of two cycles of alternating 4-week regimens daily interval running followed by weekly memantine (MEM) injections. Interval running consisted of 4-hour blocks of single-housed unrestricted access to a running wheel. MEM treatment blocks consisted of weekly injections (35 mg/kg IP), with animals receiving a total of 4 MEM injections. B) Decreasing adult neurogenesis: GFAP-TK rats were given a single 50mg/kg BrdU injection at postnatal day 6.
Figure 4-1: Study designs of both manipulations of adult neurogenesis.
followed by treatment from 2-6 months of age. Treatment to knock down neurogenesis consisted of GFAP-TK rats receiving Valganciclovir, the antiviral drug that is phosphorylated by the HSV-TK and interferes with DNA replication during cell division; killing GFAP+ radial cells which give rise to new neurons.

In Experiment 2 in order to decrease adult neurogenesis, 21 male Long-Evans GFAP-TK (TK) transgenic rats were used, whereby neurogenesis was selectively inhibited in adulthood via antiviral drug treatment (Snyder et al., 2016). At 2 months of age, treated TK group (11 males) were orally administered 4 mg of valganciclovir (Hoffman La-Roche; delivered in 0.5 g peanut butter + chow pellets) twice per week for 16 weeks, for a total of 32 doses (see Fig. 4-1). Untreated animals (10 males) received a vehicle treatment in the form of a 0.5g pellet of powdered chow and peanut butter.

4.2.2 Tissue processing and immunohistochemistry

Immediately following novel context exposure rats were anesthetized with isoflurane and perfused with 4% paraformaldehyde in a phosphate buffered saline (PBS, pH 7.4). Brains remained in paraformaldehyde for 48hr and were then stored in 0.1% sodium azide in PBS until processed. Before processing brains were immersed in 10% glycerol solution for 1 day, 20% glycerol solution for 2 days for cryoprotection, and then sectioned coronally at 40 µm on a freezing microtome. Sections were stored in cryoprotectant at -20°C until immunohistochemical processing. To detect BrdU+ and c-fos+ cells, immunofluences on free-floating sections were performed on two dorsal DG in a 1 in 12 series. Sections were treated in 2N HCL for 30 minutes, incubated at 4°C for 3 days in PBS with 10% triton-x, 3% horse serum, and goat anti-c-fos antibody (1:250; Santa Cruz, sc-52G, Dallas, TX, USA). The second day of staining was
designated for amplifying c-fos. Sections were incubated in Alexa 555-conjugated donkey anti-goat secondary antibody (1:250, Invitrogen/Thermofisher, A21432, USA) for 60 minutes at room temperature. Sections were incubated in 5% TSA blocking reagent (Perkin-Elmer, FP1020, Walmam, MA, USA), followed by Streptavidin-HRP conjugate (Perkin-Elmer, NEL750001EA, Walmam, MA, USA) for 60 minutes. Sections were treated with Rhodamine (1:2000, Fisher, PI-46410) in PBS with hydrogen peroxide (1:20,000) for 30 minutes. Sections were incubated for 3 days in PBS with 10% triton-x, 3% horse serum, and combinations of the following antibodies: mouse anti-BrdU (1:200, BD Biosciences; 347580, SanJose, CA, USA) and rabbit anti-zif268 (1:1000, Santa Cruz, sc-189, Dallas, TX, USA {Data not shown}). Visualization was performed with Alexa 488- conjugated donkey anti-mouse secondary antibody (1:250, Invitrogen/Thermofisher, A21202, USA) for 60 minutes at room temperature. Sections were counterstained with DAPI, mounted onto slides and cover slipped with PVA-DABCO.

For DCX analysis four sections, two dorsal and two ventral hippocampus, were stained for the doublecortin (DCX) to detect immature neurons. Sections were mounted on slides, heated to 90°C in citric acid (0.1M, pH 6.0). Sections were washed, permeabilized in PBS with 10% triton-x for 30 min and incubated for three days at 4 °C with goat anti-DCX (1:250 in 10% triton-x and 3% horse serum (sc-8066; Santa Cruz Biotechnology, USA). Sections were washed and incubated in biotinylated donkey anti-goat secondary antibody for 60 minutes (1:250, Jackson, 705065147, West Grove, PA). Cells were then visualized with an avidin-biotin-horseradish peroxidase kit (Vector Laboratories, cat #OK-6100) and cobalt-enhanced 3,3’-diaminobenzidine (DAB) (Sigma Fast Tablets, cat #DO426). Sections were then rinsed in PBS,
dehydrated, cleared with citrisolv (Fisher, cat #22143975) and cover slipped with Permount (Fisher, cat #SP15500).

4.2.3 Microscopy and sampling

An analysis of P6 BrdU+ cells was performed by examining fluorescent tissue on a confocal microscope (Leica SP8). The expression of the immediate early gene c-fos was examined in approximately 200 BrdU+ cells per animal, sampled from the suprapyramidal blades of the dorsal dentate gyrus of 2 sections, using a 40X oil-immersion lens (NA 1.3) and 1.5µm z-sections throughout each tissue section. An offline analysis of image stacks was done subsequently. As c-fos staining intensity in neurons is graded, fluorescence intensity for each cell was measured and compared to background levels within each field (as done in Cahill et al., 2017). C-fos cells were marked positive if staining intensity was three times the background. These thresholds captured cells with at least moderate levels of IEG immunostaining detectable by eye.

To analyze the overall IEG expression in the entire granule cell population, approximately 350 DAPI+ cells were selected from the same image stacks as the BrdU analyses. The suprapyramidal blade was divided into 3 equal boxes that included approximately the same number of cells along its medial-lateral extent, spanning the layers of the granule cell layer (to ensure cells of all ages were sampled equally, as done in Cahill et al., 2017). Each cohort of cells was measured and compared to background levels near each box, with the same intensity threshold set as was used for BrdU cell analyses. This analysis was done twice; once for the middle plane of the stack of each animal, and a second plane, 7.5um away to ensure there were no overlapping cells.
Locations analyses were done for DAPI+/fos+, BrdU+/Fos+ and BrdU+ cells to estimate the age of active cells as was previously done with pyknotic caspase3+ cells in Cahill et al (2017). Each evaluated BrdU+ cell and all Fos+ cells were characterized according to their anatomical position within the granule cell layer. Older neurons are known to reside in the superficial layers, near the molecular layer while immature neurons reside in the deep layers near the hilus/subgranular zone (Crespo et al., 1986; Wang et al., 2000; Brown et al., 2003; Muramatsu et al., 2007; Mathews et al., 2010). The thickness of the granule cell layer was normalized to 100, with the SGZ border being 0 and the molecular layer border being 100. A Python code was used to calculate cellular position, with the position of Fos+/BrdU+, Fos+/DAPI+ or BrdU+ cells measure using FIJIJ (though some files were lost and not all animals were included in this analyses). Position within the GCL was measured as the relative distance from the SGZ border to the middle of the cell body divided by GCL length measured as the distance from the SGZ border to the molecular layer border for each cell location. We then compared the anatomical distribution of the three populations to determine if similarly aged groups were being measured.

To evaluate the effectiveness of our neurogenic manipulations, DCX+ cells were quantified. The quantification consisted of all DCX+ cells across the entire granule cell layer and subgranular zone (~20 µm wide) from 2 dorsal and 2 ventral sections (8 hemispheres) being counted using a bright field Olympus CX41 microscope and a 40x objective. The granule cell layer volume was calculated by multiplying the section thickness (40 µm) by the 2D area (measured from Stereoinvestigator, MBF systems, Vermont, USA), which was then used to calculate DCX+ cell densities.
4.2.4 Running distance calculations

Running distance was calculated similarly to Cahill et al (2018) with individual distances tracked on weekdays during 4-hour blocks. The proportional distance of total weekend distance was calculated by the relative amount of running by each rat in a pair during the week and applied to weekend total.

4.2.5 Statistical analyses

Two-way (context x treatment) ANOVAs were used to detect treatment effects on cell activity. Two-way repeated measures ANOVA was used to analyses weekly running behaviour. Three-way (context X cell age X treatment) ANOVA was used to detect the treatment effect on the location of active cell. A one-way ANOVA was performed to analyze the effect of increasing adult neurogenesis on the volume of the GCL over the 4-month treatment window. Where significant main effects or interactions were observed, post hoc comparisons were performed using Sidaks or Tukey multiple comparisons. A two-tailed unpaired t-test was used to analyze DCX and BrdU location differences between treatment and control conditions. An a priori two-tailed unpaired t-test was used to evaluate the effect of decreasing adult neurogenesis on cell activity in P6 cells. In all cases significance was set at p=0.05, but we note statistical trends where 0.05 < p < 0.1.

4.3 Results

4.3.1 Changes in DCX and DG volume after manipulations of adult neurogenesis

DCX$^+$ cells were quantified to evaluate the long-lasting effects of both our ability to increase and decrease adult neurogenesis. DCX is known to have a long labelling window ranging between 2hrs to 4 weeks (Brown et al., 2003), allowing us to evaluate how effective our
treatments were at the end of the 4-month treatment window. In GFAP-TK rats (TK), 4 months of valganciclovir treatment resulted in a significant 68% decrease in DCX$^+$ cells compared to untreated TK animals (Fig. 4-2A; $T_{18}=8.796$, $P<0.001$). For our increasing group, our RUN/MEM treatment has previously been shown to increase DCX after 2 months of treatment (see chapter 3, Cahill et al., 2018). Here, our RUN/MEM treatment group showed a 17% increase in DCX$^+$ cells compared to controls (Fig. 4-2B; $T_{29}=2.065$, $P=0.048$). This extends our previous finding, showing that our treatment for increasing adult neurogenesis (RUN/MEM) does significantly increase immature neurons at the end of the 4-month treatment window, twice the length of time previously shown (see chapter 3, Cahill et al., 2018).

In human studies using physical exercise, one of the indicators of improvement and neuroplasticity is increased hippocampal volume (Erickson et al., 2009; 2011). Here we show in our increasing adult neurogenesis groups that from pretreatment (2 months) to end of treatment (6 months) there is a significant (20%) increase in hippocampal volume that is not seen in our sedentary animals (Fig. 4-2C; ANOVA $F_{2, 43}=6.738$, $P=0.0028$, Tukey multiple comparison: 2 months vs. 6 month RUN/MEM, $P=0.0019$).

We analyzed running behaviour by measuring weekly running distance per rat during the two running sessions. Weekly running data per rat showed that animals ran significantly more during session one than session two during weeks 3 and 4 (Fig 4-2D; repeated measures ANOVA, effect of block $F_{1, 15}=24.59$, $P<0.001$ and effect of interaction $F_{3, 45}=5.811$, $P=0.0019$; Sidak multiple comparison of session 1 vs. session 2: week 3: $P=0.0149$, week 4: $P<0.001$) and that that weekly running distance-increased overtime during session one but these increases were not seen during session two (Fig 4-2D; repeated measures ANOVA, effect of week $F_{3, 45}=4.583$, $P=0.0019$).
P=0.007; Tukey multiple comparison block one week 1 vs. 3: P=0.0016, week 1 vs. 4 P <0.001, week 2 vs. 4 P <0.001).

Figure 4-2: Effects of long-term manipulations of neurogenesis on the DG

A) Decreasing Adult Neurogenesis: GFAP-TK rats treated with Valganciclovir, showed a 68% decrease in DCX, a measure of adult neurogenesis compared to our untreated GFAP-TK rats, indicating we effectively decreased the number of new neurons over the 4 month period. B) Increasing Adult Neurogenesis: Our RUN/MEM treatment increased the number of new immature neurons (DCX+) by 17% compared to control animals after 4 months of treatment, indicating the neurogenesis was still elevated by our treatment. C) Total DG granular cell volume was measured in a 1:12 series. RUN/MEM treatment increased DG granular cell volume from the start of treatment (2
Figure 4-2: Effects of long-term manipulations of neurogenesis on the DG

months) to the end of treatment (6 months), this increase is not seen in control animals. D) Weekly running data per rat showing both RUN treatment blocks: session one and session two. Running distance-increased overtime during session one with significant increases from week 1 to weeks 3 and 4 as well as week 2 to week 4, these increases were not seen during session two. As well running was shown to be significantly greater in session one on weeks 3 and 4 compared to session 2 (see text for details). E/F) Representative immunohistochemistry images of DXC at 10X and 40X magnification E) Doublecortin staining in RUN/MEM treated animals. F) Doublecortin staining in TK treated animals.

4.3.2 Manipulating the adult-born population alters cellular activity

Immediate-early gene (IEG) expression is often used as a proxy for neuronal activity as IEG expression has been shown to be correlated with neuronal firing (Sheng and Greenberg, 1990; Guzowski et al., 1999; Chawla et al., 2005; Guzowski et al., 2005). To identify the effect that manipulating adult neurogenesis would have on the rate at which P6-born neurons are recruited during exploration of a novel environment, we quantified c-Fos expression in P6 labelled BrdU+ cells. We also evaluated the c-Fos expression in DAPI+ cells to look at the general cell population and understand which populations are being recruited and if there were differences in the rate of recruitment of our P6-born neurons and the overall DG population after our manipulations on adult neurogenesis.
Figure 4-3: Effects of manipulating adult neurogenesis on activity in DG in both developmentally born cells (BrdU) and the total granule cell (DAPI) population.

A/B) Increasing Adult Neurogenesis: A) Developmentally-born (BrdU+) cells show a decrease in c-fos activity in the RUN/MEM group compared to sedentary controls when animals were exposed to a novel context. B) A similar pattern of activity was found in the overall granule cell population (DAPI): decreased c-fos activity after exposure to a novel context in RUN/MEM group compared to sedentary controls.

C/D) Decreasing Adult Neurogenesis: C) Developmentally-born cells showed an increase in c-fos activity after exploring a novel context, compared to home cage controls. C-fos expression in P6 BrdU+ cells shows a trend towards an increase in activity between TK-treated and TK-untreated animals after exposure to a novel environment. D) C-fos activity in the overall granule cell population (DAPI) was significantly increased in the novel context condition.

E) Representative confocal
Figure 4-3: Effects of manipulating adult neurogenesis on activity in DG in both
developmentally born cells (BrdU) and the total granule cell (DAPI) population.

immunofluorescent images of DAPI, BrdU, c-fos. F) Confocal image of a P6 labelled a BrdU+ dentate gyrus neuron
expressing c-fos following exploration of a novel environment.

4.3.2.1 Increasing adult neurogenesis decreases IEG activity in the DG

Increasing adult neurogenesis (RUN/MEM) lead to an overall reduction in c-fos
expression in P6 BrdU+ cells compared to sedentary animals (Fig 4-3A Two-way ANOVA,
effect of treatment $F_{1,26} = 5.572, P=0.0260$). There was significantly more activity in the novel
context compared to the home cage (Fig 4-3A Two-way ANOVA, effect of context $F_{1,26} = 7.405,
P=0.0114$, Tukey multiple comparisons: home cage sedentary vs. novel context sedentary
$P=0.0028$, home cage RUN/MEM vs. novel context sedentary $P=0.0069$). After novel context
exposure sedentary animals showed a significant increase in c-fos+/BrdU+ cell compared to the
RUN/MEM group, illustrating the reduction of activity in the RUN/MEM group (Fig 4-3A Two-
way ANOVA context X treatment interaction $F_{1,26} = 8.273, P=0.0079$; Tukey multiple
comparison novel context sedentary vs. novel context RUN/ MEM $P=0.0038$).

A similar pattern of findings was seen in the overall activity of the dentate gyrus,
following novel context exposure. Animals in the sedentary group showed a significant increase
in DAPI+/c-fos+ cells after novel conext exposure while the RUN/MEM animals failed to show
an increase in DAPI+/c-fos+ activity (Fig 4-3B Two-way ANOVA, effect of treatment $F_{1,26} =
5.346, P=0.0289$). After novel context exposure sedentary animals showed a significant increase
in c-fos expression compared to the RUN/MEM group, illustrating that the reduction in activity
in the RUN/MEM group affects more than just the P6 BrdU labelled cells (Fig 4-3B Two-way
ANOVA context X treatment interaction $F_{1,26} = 7.965, P=0.009$; Tukey multiple
comparisons novel context sedentary vs. novel context RUN/MEM P=0.0046, home cage sedentary vs. novel context sedentary P=0.0212).

4.3.2.2 Decreasing neurogenesis during early adulthood increases IEG activity in developmentally-born cells

Developmentally-born (P6 BrdU+) cells showed an increased in c-fos activity during novel context exposure, and this effect was greatest in the treated TK rats that lacked adult neurogenesis (Fig 4-3C Two-way ANOVA, effect of context F1, 17 = 10.11, P=0.0055, Sidak multiple comparisons of novel context vs. home cage: TK-treated: P=0.0061). A priori comparison based on our hypothesis showed that TK-treated animals showed a trend toward more c-fos+/BrdU+ cell during novel context than TK untreated animals, indicating that decreasing adult neurogenesis may increase the recruitment of developmentally-born cells (Fig 4-3C t-test T13= 1.895, P=0.0806). Cell activity in the novel context condition was found to be increased in DAPI+ cells as expressed by the increased DAPI+/c-fos+ cells in the novel context over home cage condition (Fig. 4-3D Two-way ANOVA, effect of context F1, 17 = 4.757, P=0.0435).

4.3.3 Blocking adult neurogenesis prevents the pushing of developmentally-born neurons into more superficial layers

Decreasing adult neurogenesis (GFAP-TK treated) prevented the movement of the developmentally-born cells into more superficial layers of the GCL compared to all other treatment groups animals, with a significant effect of treatment on location of BrdU+ cells (Fig. 4-4A one-way ANOVA, F3, 45= 12.87, P<0.0001). Both RUN/MEM (P=<0.0001) and sedentary (P=<0.0001) animals had more developmentally-born cells in the superficial layers of the GCL compared to the GFAP-TK treated group. As well, the GFAP-TK untreated group also showed
developmentally-born cells into more superficial layers of the GCL compared to GFAP-TK treated (P=0.0016). Increasing adult neurogenesis did not affect the location of the developmentally-born cells with both RUN/MEM and sedentary animals showing a similar pattern of cells in the more superficial layers of GCL as shown previously (see chapter 2, Cahill et al., 2017). These finding suggest that, without adult neurogenesis, developmentally-born neurons are not pushed, by new neurons, out into the more superficial layers of the GCL or that without the addition of new adult-born neurons new deep layers below these developmentally-born cell are not created, in both cases by blocking adult neurogenesis developmentally-born neurons are found in deeper layers.

Figure 4-4 Effects of manipulating adult neurogenesis on locations of developmentally-born cells within the GCL
Figure 4-4 Effects of manipulating adult neurogenesis on locations of developmentally-born cells within the GCL

A) When comparing the effects of increasing and decreasing neurogenesis on BrdU location RUN/MEM, sedentary and TK-untreated animals showed cells in more superficial layers of the GCL compared to TK-treated animals, suggesting that the addition new adult-born cells help pushed developmentally-born out into the more superficial layers. Increasing adult neurogenesis did not affect the location of the developmentally-born cells with cells found mostly in the more superficial layers of GCL. B) The proportion (relative fraction) of developmentally-born cells found across the GCL (SGZ=0, Molecular layer border = 100). C) Confocal image of a P6 BrdU+ cell within the GCL with a visual representation of how cell location was measured. Each cell was identified and the distance from the cell center to the SGZ borderline was divided by the width of the granule cell layer to calculate cell location (% of width of GCL).

4.3.4 IEG expression in BrdU+ and DAPI+ cells is found in similar locations after increasing neurogenesis

Since the cellular activity patterns (as measured by c-fos) were quite similar for the increasing neurogenesis project, and to better understand the anatomical distribution of cellular activity patterns after manipulations of adult neurogenesis, we examined the distribution of c-fos+/BrdU+ and c-fos+/DAPI+ cells within the GCL. There was no effect of treatment found after performing a three-way ANOVA in either our increasing or decreasing adult neurogenesis groups; we therefore collapsed this factor for the location analysis (Three-way ANOVA (Increasing) effect of treatment $F_{1, 45} = 0.271, P=0.605$; effect of cell age $F_{1, 45} = 1.281, P=0.264$; effect of context $F_{1, 45} = 1.309, P=0.259$; Three-way ANOVA (decreasing) effect of treatment $F_{1, 27} = 0.002, P=0.969$; effect of cell age $F_{1, 27} = 9.978, P=0.004$; effect of context $F_{1, 27} = 4.658, P=0.048$).
In our increasing adult neurogenesis experiment the location of both BrdU+/Fos+ (developmentally-born) and DAPI+/Fos+ (overall population) were similar (Fig 4-5 A&B). While we have shown that c-fos activity is decreased after increasing adult neurogenesis (Fig 4-3A&B), there is no effect of either context or treatment on the location of either BrdU+/Fos+ and DAPI+/Fos+ cells. It is possible that cell activation in both BrdU+ and DAPI+ cells are from similarly aged (developmental) populations based on their locations, as we have previously shown the majority of developmentally-born neurons are found in the superficial granule cell layer (75% GCL) (Fig 4-5B and see chapter 2, Cahill et al., 2017).

In our decreasing neurogenesis experiment DAPI+/Fos+ cells were found in more superficial layers of the GCL than BrdU+/Fos+ cells (Fig 4-5C&D, Two-way ANOVA effect of cell age $F_{1, 31}= 12.07$, $P=0.0015$; Sidak multiple comparison of BrdU+/Fos+ vs. DAPI+/Fos+: novel context: $P=0.0022$, home cage: $P=0.1001$), indicating recruitment of cells born prior to the first postnatal week. There was also an effect of context, with animals after novel context exposure found to have c-fos activity in deeper layers (Fig 4-5C&D Two-way ANOVA effect of context $F_{1, 31}= 5.802$, $P=0.0221$). This suggests that even older, possibly embryonic, cells are recruited, and that with long-term ablation of adult neurogenesis there is no significant change in the reliance on older superficially located cells.
Figure 4-5: Effects of manipulating adult neurogenesis on the locations of activity in DG in both developmentally born (BrdU) and overall granule cell (DAPI) population

A/C) Average c-fos+ cell location by animal: A) Increasing adult neurogenesis: There was no significant difference found between the location of BrdU+/Fos+ and DAPI+/Fos+ populations between the home cage and novel context exposure, as well there was no difference between RUN/MEM or sedentary. It is possible that cell activation in both BrdU+ and DAPI+ cells are from similarly aged populations based on their locations (see text). C) Decreasing adult neurogenesis: DAPI+/Fos+ cells were located in more superficial layers of the GCL than BrdU+/Fos+ cells. BrdU+/Fos+ cells activated by novel exposure were found in the deeper layers of the GCL compared to DAPI+/Fos+ cells. B/D) All active cells and their distribution within the granule cell layer. Box and whisker plots indicate quartiles. The oldest neurons are typically found in the most superficial layer (maximum 100) and the youngest neurons are typically found in the deep layers near the hilus (minimum 0).

4.4 Discussion

Here we examined the effects of manipulating neurogenesis during early adulthood on activity in both developmental and total granule cell populations in the dentate gyrus. Our
general strategy was to label developmentally-born cells with thymidine analogs and manipulate, either increasing or decreasing, adult neurogenesis from 2-6 months of age so that these alterations would last throughout the period when developmentally-born cells have been shown to be lost (see chapter 2, Dayer et al., 2003; Cahill et al., 2017). We found that both our treatments successfully altered adult neurogenesis, with our increasing group showing not only an 17% increase in DCX but also an increase in granule cell layer volume. Our treatment to decrease adult neurogenesis was found to decrease DCX by 68%. Our principal finding is that adult neurogenesis has effects on the cellular activity of both developmentally-born and the total granule cell population.

We found that when adult neurogenesis was increased there was an overall decrease in the cellular activity in the DG both in the developmentally-born and total granule cell populations. When adult neurogenesis was decreased we found a trend towards an increase in activity during exposure to a novel environment found only in developmentally-born cells, while no effect was found in total granule cell activity, indicating a possible compensation by cells of other ages. It is important to note that our alterations to manipulate neurogenesis happened over a 4 month time period; this is a longer treatment than the average duration of 3 weeks to 2 months (van Praag et al., 1999b; Anderson et al., 2000; van Praag et al., 2005; Patten et al., 2013; Yeung et al., 2014; Schoenfeld et al., 2016). Specifically our extended period of reduced adult neurogenesis may have allowed for some compensation within the DG, a caveat that may be less prevalent in studies using shorter treatment windows (Drew et al., 2016; Schoenfeld et al., 2016; Adlaf et al., 2017).
4.4.1 Effects of long-term manipulations of neurogenesis on cellular activity and cell location

Here we showed that increasing adult neurogenesis using a combination of interval running (RUN) and memantine (MEM) administration reduces the activity of granule cells in the DG. Specifically, we found that both the developmentally-born and the total granule cell population show a decreased c-fos expression after exposure to a novel environment in animals where adult neurogenesis had been increased. IEG activity is used as a proxy of cellular activity, and has been shown in the DG to be increased after hippocampal-dependent situations such as exposure to novel environments (Guzowski et al., 1999; Chawla et al., 2005; Guzowski et al., 2005). Previous in vitro work has shown that increased adult neurogenesis using both natural and genetic means decreases the electrophysiological activity in the mature granule cell population (Drew et al., 2016; Adlaf et al., 2017). Here we show a similar pattern of findings in an in vivo model, that increasing adult neurogenesis decreases total DG activity, including the developmentally-born population. While there is a reduction in the total DG activity, what c-fos activity that remains in both the developmentally-born and total granule cell population are found in the superficial granule cell layer, indicating that the remaining active total granule cell population are possibly developmentally-born (see chapter 2, Cahill et al., 2017; Imura et al., 2018). One would expect that after a long period of increased adult neurogenesis there would be a increased population of mature, integrated adult-born cells, which would be reflected by increased activity in the deep portions of the GCL (van Praag et al., 1999b; 2002; Jessberger and Kempermann, 2003). However, here we did not see an increase in c-fos activity in the deeper layer of the GCL, where these adult-born neurons would be located, which would be expected if these new neurons were replacing the activity of the old developmentally-born cells. Our
findings suggest that while increasing adult neurogenesis does decrease c-fos activity in the GCL, the remaining activity is still developmentally-born or in more superficially located cells.

When we genetically altered adult neurogenesis using the GFAP-TK model we showed that decreasing neurogenesis throughout the early adult period did cause an increase in activity, but this increase was small, found only in the developmentally-born cells and was not significantly different after novel exposure from our neurogenesis intact GFAP-TK animals, while there was a trend. The limited animal numbers and the extended length of treatment hamper our findings in our decrease neurogenesis groups. As we only had ~10 animals per treatment condition and only 3 animals in the home cage control condition, our findings are limited. The extended length of our knockdown of adult neurogenesis is double what is normally used in both genetic and irradiation studies (Winocur et al., 2006; Yeung et al., 2014; Drew et al., 2016; Adlaf et al., 2017). This extended period without adult neurogenesis may have lead to compensation within the DG. Singer and colleagues (2011) showed that while decreasing adult neurogenesis causes deficits in LTP during the early phases of ablation, after longer periods LTP recovers to control levels within the DG. After prolonged ablation of adult neurogenesis there may have been compensatory changes in the DG which function to restore homeostasis in the network (Singer et al., 2011).

A previous study showed that extended removal of adult neurogenesis (3 month) in GFAP-TK mice resulted in similar patterns of c-fos activity in both GFAP-TK and wild type mice after anxiety testing as well as similar behavioural phenotype (Schoenfeld et al., 2016). While no difference were found in both behavioural and activity patterns after extended removal of adult neurogenesis, the same group showed that increasing adult neurogenesis was found to both improve anxiety after stress and increase inhibition compared to control animals where
stress increased c-fos activity (Schoenfeld et al., 2013). This increased inhibition after increasing adult neurogenesis as well as the similarities in activity patterns after reducing adult neurogenesis are in agreement with our findings, that there may be compensation after extended removal of adult-born neurons, while adding new neurons seems to increase inhibition. Modest findings, similar to ours, have been found using of an extended (~3+ month) periods of neurogenesis reduction, decreasing adult neurogenesis has been shown to increase activity in the DG both in vivo and with stimulation in studies using shorter ablation periods, indicating that the length of neurogenesis reduction may have an affect (Ikrar et al., 2013; Schoenfeld et al., 2016).

We also examined the location of the activity in both the developmentally-born and the total granule cell populations finding that developmental cells are found in deeper locations compared to the total granule cell activity, indicating that decreasing adult neurogenesis may cause a recruitment of cell born earlier in the developmental window.

4.4.2 Role new neurons may play in inhibition

The reduction in activity of the total and developmental granule cell populations seen after long-term increases in adult neurogenesis leads to the question of the role new adult-born neurons are playing in the functioning of the DG. Two theories about the role of adult-born neurons and their widespread effects on granule cell activation have been supported by previous work: increased inhibition and synaptic redistribution. After increasing adult neurogenesis or activation of adult-born neurons various studies have shown decreased activity of the total or the mature granule cell populations (Lacefield et al., 2012; Ikrar et al., 2013; Drew et al., 2016; Adlaf et al., 2017). This decrease in neuronal activation has been accompanied by an increase in the number of excitatory synaptic connections onto hilar interneurons by adult-born neurons and
increased recruitment of GABA interneurons showing that new adult-born neurons are not just encoding units but may act to synaptically inhibit mature granule cells (Ikrar et al., 2013; Drew et al., 2016). Adult-born cells may act to inhibit recurrent network activity, as decreasing adult neurogenesis is shown to increase spontaneous DG-hilus activity and decrease inhibition within the DG (Lacefield et al., 2012; Drew et al., 2016).

Another possible role of adult-born neurons in the alteration of DG activity is synaptic redistribution. Dendritic spines of new adult-born neurons are known to preferentially make synapses with boutons that have mature granule cells already present (Toni et al., 2007; 2008). As new adult neurons mature there is a switch from sharing synapse boutons with older neurons to having solo connections, with older spines from mature granule cells retracting. This indicates a possible competition between new adult born and mature, possibly developmentally born, cells (Toni et al., 2007; 2008). Adlaf et al (2017) showed that the total number of spines remains stable within the DG, and that spines are redistributed to new neurons. Increasing adult neurogenesis decreases spines as well as activity within the mature granule cell population, while decreasing the adult population lead to no change in the density of spines on mature granule cells (Adlaf et al., 2017). When the number of spines on mature neurons in the DG is decreased both immature and proliferative cells increased, as well survival of 3-week old cells increased (McAvoy et al., 2016), a time period known for spinogenesis (Espósito et al., 2005; Tashiro et al., 2006; Zhao et al., 2006). This highlights the competition for survival and integration between mature and adult-born cells.

While the reduction in activity found with increased adult neurogenesis may be caused by either increased inhibition and synaptic redistribution or both, the outcome is that adult neurogenesis reduces interference between overlapping contexts or spatial information (Clelland
et al., 2009; Nakashiba et al., 2012). Decreasing adult neurogenesis has been shown to not affect initial learning of a location, but to affect the learning of a new location if there is conflicting spatial information with the original location (Burghardt et al., 2012). Animals with reduced adult neurogenesis cannot suppress the originally learned spatial location and there is increased activity found in the DG; it seems the increased activity in the DG impaired the ability to segregate between the two conflicting memories (Burghardt et al., 2012). It may be the role of adult-born neurons is to inhibit the older neurons, which has been shown itself to increase pattern separation between similar contexts (Burghardt et al., 2012; Nakashiba et al., 2012). Alterations in spine density in mature cells have been found even in aged animals to increase adult neurogenesis, a time period when neurogenesis is known to be naturally reduced (Kuhn et al., 1996; McAvoy et al., 2016). Aged animals with increased neurogenesis showed increased pattern separation in a context fear conditioning task compared to middle-aged animals, showing less overlap of activation between contexts (McAvoy et al., 2016).

4.4.3 Possible limitations

There are some possible limitations of our findings based on our design and our results. First, we chose to use a mix of light and dark cycle during our novel context exposure. Our increasing adult neurogenesis groups were run during the dark cycle, to study the effects of running when rats are most active and neurogenic effects are greatest (Holmes et al., 2004; van der Borkht et al., 2006). Our decreasing adult neurogenesis groups were instead given novel context exposure during the day. We chose not to make any comparisons based on c-fos activity level between the two groups as animals are known to be more active during the dark phase which may have affected IEG expression.
Reduced running over time may have an effect on the positive effects of this treatment block, as we do find significantly less running between the first and second session of running wheel exposure. This is important as we have previously noted that when animals run less than 10 km/wk there is no relationship between running behaviour and neurogenesis (see chapter 3, Cahill et al., 2018), though we do see increases in both DG volume and DCX at the end of our treatment window. Our use of a more restricted and less intense form of exercise may have allowed for our prolonged increases in DCX, as these two factors have been associated with longer-lasting neurogenic effects of running behaviour (Naylor et al., 2005; Grégoire et al., 2014; Inoue et al., 2015; Nguemeni et al., 2018). Another important limitation based on our treatment paradigm is our use of MEM. While MEM has known positive effects on learning (Zajaczkowski et al., 1996; Zoladz et al., 2006; Ishikawa et al., 2014) and it increases neurogenesis (Maekawa et al., 2009; Namba et al., 2009; 2010), it has been shown to alter the migration of adult-born neurons (Namba et al., 2011), and be less effective in females (see chapter 3, Zajaczkowski et al., 2000; Cahill et al., 2018). While these caveats do not seem to effect our results based on the similarities in the decrease in total granule cell activity in our RUN/MEM group to others using other manipulation methods (Drew et al., 2016; Adlaf et al., 2017), it is important to note that these new adult-born cells may be affected by treatment type and that these effect may differ in females (see chapter 3, Cahill et al., 2018).

Future work to translate our findings to disorders and treatments outcomes that can offset behavioural deficits in human disorders will need to look further into cognitive testing as well as age as a factor. Here we did not include any behavioural or cognitive testing since our primary goal was to understand activity relationships between populations within the DG. Also, learning experiences can alter neurogenesis and cellular death, which could affect our interpretations.
about manipulations of adult neurogenesis on the granule cell populations. We also used relatively young rats, starting at the beginning of adulthood. While we did use a prolonged treatment window, further research should also take age into account as many diseases impacting the hippocampus are affected by age.

4.4.4 Implications for neurogenic therapies: importance of cellular age

Adult-born and developmentally-born neurons have been shown to become ultimately similar once cells have reached maturity (van Praag et al., 2002; Espósito et al., 2005; Laplagne et al., 2006; Ge et al., 2007). However, different immediate-early gene responses to experience (Tronel et al., 2015), enhanced morphological plasticity in older adult-born cells (Tronel et al., 2010) and different survival patterns (see chapter 2, Dayer et al., 2003; Kempermann et al., 2003; Cahill et al., 2017) suggest that there may be persistent differences between DG neurons born at different ages. Understanding how each neuronal population in the DG contributes to persistent information storage is important and is particularly intriguing since experiences such as learning can increase the survival of adult-born neurons and induce the death of superficially-located (presumably developmentally-born) granule cells (Olariu et al., 2005; Epp et al., 2007; Tashiro et al., 2007). Adult neurogenesis has been shown to not only enhance learning and memory (Anderson et al., 2000; Creer et al., 2010; Sahay et al., 2011) but can also to causes forgetting of hippocampal memories (Akers et al., 2014) and it plays a role in the consolidation of memory into extra-hippocampal structures (Kitamura et al., 2009). While computational models have predicted that adult neurogenesis in conjunction with the turnover of older neurons can benefit learning (Becker, 2005) beyond adult neurogenesis alone (Meltzer et al., 2005), it may be that there is a silencing of the mature neuronal population with increased adult neurogenesis which leads to improvements in cognition.
It is important to note that reduced hippocampal volume and fewer total DG neurons are related to a number of mental health disorders in humans (McKinnon et al., 2009; Yassa et al., 2010; Boldrini et al., 2013). While we and others have shown that increasing adult neurogenesis alters activity in the DG (Ikrar et al., 2013; Drew et al., 2016; Adlaf et al., 2017), it is important to understand which populations are most highly affected when neurogenesis is manipulated. This understanding will allow for insights into treatments that alter new neurons, knowing which populations are either lost during disease or malfunction and if new adult-born cells can replace the function of these populations to restore function. It is promising that antidepressant treatment restores dentate gyrus granule cell number in depressed patients (Boldrini et al., 2013; Mahar et al., 2017), and exercise increases hippocampal volume in individuals with mild cognitive impairment (Brinke et al., 2015). As well treatments shown to improve hippocampal atrophy have been shown to also improve memory (Kramer et al., 1999; Yaffe et al., 2001; Adlard et al., 2005; Ahlskog et al., 2011; Belarbi et al., 2011; Ruscheweyh et al., 2011; Chapman et al., 2013).

Finally many neurological diseases, including depression (Campbell and Macqueen, 2004; Milne et al., 2012), Alzheimer’s disease (Schmitt, 2005; Gorman, 2008), and mild cognitive impairment (Kircher et al., 2007) are associated with increased activity in the hippocampus. This increased activity could possibly lead to excitotoxic cell death (Lipton, 2004; Johnson and Kotermanski, 2006), which may lead to the neuronal loss and reduced hippocampal volume seen in these diseases (McKinnon et al., 2009; Yassa et al., 2010; Boldrini et al., 2013). With this context in mind, our findings of decreased activity in the DG with increased neurogenesis, it is possible to see that the addition of new neurons could minimize aberrant levels of activity seen in these neurological disorders.
Chapter 5: General discussion

5.1 Overview of findings

Since the discovery that the brain has the ability to create new neurons into adulthood, there has been an overwhelming focus on understanding the unique properties of adult-born neurons. This is often at the expense of another important cell population: the already present developmentally-born cells. Studying these two populations together is necessary to identify whether adult neurogenesis serves to replace developmentally-born cells, either functionally or physically, or leads to overall growth in the dentate gyrus subregion of the hippocampus. The overall objective of this thesis was to both characterize the two populations in the DG and to determine if a relationship between developmentally-born and adult-born DG neurons exists. We looked first in chapter 2 at how developmentally-born cells mature and survive during both development and adulthood. We then looked at how to manipulate adult neurogenesis long-term in both sexes in chapter 3. Finally in chapter 4 we investigated the relationship between the two populations by manipulating adult neurogenesis. The central hypothesis is that while the two populations are different, a relationship does exist between the adult-born and developmentally-born neurons, where adult-born neurons may act to regulate the activity of developmentally-born neurons, possibly by increased inhibition and/or synaptic redistribution.

The major findings of this thesis are: 1) Cells born in development show stable survival until young adulthood, where they undergo a delayed period of cell death (see chapter 2, Cahill et al., 2017). This pattern is opposite to that seen in adult-born neurons, where 40-80% of cells die before cell reach maturity, but once mature neurons are shown to likely persist for the life of the animals (Cameron et al., 1993; Gould et al., 1999; Brandt et al., 2003; Dayer et al., 2003; Kempermann et al., 2003; McDonald and Wojtowicz, 2005; Tashiro et al., 2006; Mandyam et
al., 2007). 2) We found a transient peak in Zif268 expression in 2-week old developmentally-born cells in rats, suggesting a potentially critical period for plasticity and integration of these neurons which is shown to be one week earlier than previously found in adult-born neurons in rats (see chapter 2, Snyder et al., 2009a; Cahill et al., 2017). 3) Transient effect of methods often used to increase adult neurogenesis, constant free running and single memantine injections, in both males and females when used in isolation. Alternation of two neurogenic treatments showed longer increases in neurogenesis at the end of a 2-month treatment window. Females showed a positive treatment effect with interval running, while for males the use of two treatments in alternation was most effective, showing a clear sex difference in treatment efficacy (see chapter 3, Cahill et al., 2018). 4) Adult neurogenesis affects the activity of the DG and developmental populations. Increasing adult neurogenesis decreased activity in both developmental and total DG neuronal activity. Decreasing adult neurogenesis increased activity, though somewhat tentatively, only in the developmental population (see Chapter 4). These data suggest that adult-born neurons inhibit mature, possibly developmentally-born, neurons, and there is a homeostatic regulation of activity levels in the DG (Drew et al., 2016).

The developmentally-born population may possibly be more at risk of insult than originally appreciated. This based on our findings of loss of mature developmentally-born neurons in the adult period and previous work showing that behavioural experiences can induce death of superficially-located (presumably developmentally-born) granule cells (Olariu et al., 2005), that this neuronal population is lost in normal ageing (Colcombe et al., 2003; Erickson et al., 2011), and increased volume loss in the DG has been associated with mental health disorders (McKinnon et al., 2009; Yassa et al., 2010; Boldrini et al., 2013, see section 1.4 for more detail). The consequence of excess loss of developmental neurons, and if new adult-born
neurons can function to replace these lost neurons is still not clear. More research on the relationship between these two populations and how they are impacted in both normal and diseased ageing is important.

5.2 Implication for memory turnover

The early postnatal period contains high demands on the brain, with numerous learning experiences and exposures to stressors. During the embryonic and early postnatal period neural stem cells are set to generate a certain number of neurons and glia to create the brain (Götz et al., 2016). Along with the high demands on the brain found during the early postnatal period, complete survival of developmentally-born neurons during their first few weeks and months seem to support the importance of these newly formed neurons in these processes (see chapter 2, Götz et al., 2016; Cahill et al., 2017). At 3 weeks of age, in rodents, hippocampal-dependent memory is beginning to be displayed (Rudy, 1993; Akers and Hamilton, 2007; Rainekei et al., 2010). It is at 3 weeks of animal age when P6-born cells, which are critical for the formation of the DG (Schlessinger et al., 1975; Altman and Bayer, 1990a; 1990b), show a peak in IEG expression. Since, in adult-born neurons, experiences can increase the survival of DG neurons, this suggests that the complete survival of these early postnatal neurons may be due to the high demands during this time period (Kempermann et al., 1997a; Gould et al., 1999; Epp et al., 2007). There has been little investigation into the early stability of developmentally-born neurons, so a complete understanding of the how likely they are to survive, or how survival is truly affected based on sensory or learning experiences is not clear.

Adult-born neurons have enhanced synaptic plasticity (Schmidt-Hieber et al., 2004; Ge et al., 2007) and are required for learning and memory (Snyder et al., 2005; Saxe et al., 2006; Clelland et al., 2009), and many studies focus on the unique properties of adult-born neurons.
assuming that they make greater contributions to hippocampal function. Less attention is paid to
the developmentally-born population of neurons, the role both populations may play in the
transience of some forms of hippocampal memory and the possible role of adult-born neurons in
replacing the older developmentally-born population. The finding that many developmentally-
born neurons die off over the first 6 months of life (in rodents), which was generally not believed
to occur in the healthy young adult brain, gives new insight into a possible loss of these older
mature cells during normal ageing. Since some forms of hippocampal memory are transient
(Mumby et al., 1999; Frankland and Bontempi, 2005; Kitamura et al., 2009; Kitamura and
Inokuchi, 2014), this raises the question of whether cellular turnover might underlie memory
turnover. Loss of developmentally-born neurons compared to immature adult-born neurons
would, based on the differences in integration of these neurons into memory processes, have
distinct effects. The removal of older developmentally-born neurons may result in loss of
information from hippocampal circuits, while removal of new immature neurons that are non-
inegrated and have yet to form functional synapses would have few consequences in terms
information loss. While many have shown that once adult-born cells reach maturity they become
ultimately similar to the developmentally-born population (Laplagne et al., 2006; Zhao et al.,
2006; Stone et al., 2011), the adult-born population may remain fundamentally different from the
developmentally-born population. Adult-born neurons, even relatively old, have greater
experience-dependent dendritic plasticity (Tronel et al., 2010; Lemaire et al., 2012), immature
adult-born newborn granule cells shown increased synaptic plasticity and excitability (Schmidt-
Hieber et al., 2004; Ge et al., 2007) while mature granule cells are shown to be more resistant to
excitatory inputs (Lopez-Rojas and Kreutz, 2016), though it is important to note that it has been
shown that all age neuronal populations are as likely to be recruited for spatial memory in
maturity (Stone et al., 2011).

While our finding of a loss of 17% of the P6-born DG neurons is a considerable proportion of the total DG population, it is important to know that a majority of the developmentally-born neuronal population would remain intact. With this in mind looking at memory storage, studies have found that episodic-like and hippocampal-dependent memories can persist indefinitely in the hippocampus (Mumby et al., 1999; Clark et al., 2005; Moscovitch et al., 2016), while other memories can be removed from the hippocampus (Frankland and Bontempi, 2005; Kitamura and Inokuchi, 2014). The mixture of neuronal survival and death could allow for the removal of obsolete information while retaining only memories of importance (Richards and Frankland, 2017). The role of adult neurogenesis and cellular turnover have been investigated via computational models which have shown that mixed model of adult neurogenesis with neuronal turnover is beneficial to learning (Becker, 2005) beyond what can be achieved with adult neurogenesis alone (Meltzer et al., 2005). The replacement of older neurons, most likely developmentally-born based on our findings, with new adult-born neurons, may allow for the enhancement of new learning experience.

Adult neurogenesis has been shown to enhance learning and memory (Anderson et al., 2000; Creer et al., 2010; Sahay et al., 2011) as well as cause forgetting of hippocampal memories (Akers et al., 2014). Studies have also shown a role for adult neurogenesis in memory consolidation into extra-hippocampal structures (Kitamura et al., 2009). It would seem that the process of memory consolidation or alteration from hippocampal dependency is an active one with adult neurogenesis playing a role in the clearing of old and remote memories out of the hippocampus (McClelland et al., 1995). Kitamura and colleagues (2009) highlight this finding
showing that increasing adult neurogenesis accelerates the independence of contextual fear from the hippocampus, without any loss of memory. It seems the role of new adult-born neurons may be to not only facilitate acquisition of memory, based on their enhancement of learning and increased synaptic properties (Gould et al., 1999; Schmidt-Hieber et al., 2004; Espósito et al., 2005; Overstreet-Wadiche and Westbrook, 2006; Ge et al., 2007; Martínez-Canabal et al., 2012; Winocur et al., 2012), but also to aid in the consolidation and clearance of memories from the hippocampus (Frankland et al., 2013).

5.3 Insights into the role of new neurons: activity in DG after alterations of adult neurogenesis

Our findings indicate that adult neurogenesis may play a role in inhibiting older (most likely) developmentally born neurons (see Chapter 4). Our findings are in agreement with in vivo and in vitro findings that increasing adult neurogenesis decreases activity in the DG, and can have effects on the synaptic density of the mature populations (Drew et al., 2016; Adlaf et al., 2017). When we looked at the effect of removing adult-born neurons we found a small increase in activity in the developmentally-born cells, agreeing with previous work which indicated that adult-born cells may act to inhibit the older developmentally-born cells (Drew et al., 2016, see Chapter 4). The alterations in the activity within the DG after manipulations of the adult-born population lead to two possible relationships adult-born neurons may have in the functioning of the DG: increased inhibition or synaptic competition.

Adult-born neurons have been shown to inhibit the mature neuronal population through increased connections and recruitment of GABA interneurons which may lead to the overall sparse activity seen in the DG (Ikrar et al., 2013; Drew et al., 2016). Activation or increased number of adult-born neurons seems to lead an overall inhibitory effect on mature granule cells
(Drew et al., 2016; Adlaf et al., 2017, see chapter 4). This increased inhibition by the adult population plays a role in reducing overlap between contexts or improving pattern separation (Nakashiba et al., 2012). Burghardt and colleagues (2012) showed this need for new adult neurons in learning two similar contexts on a revolving shock disk task. While animals could learn the initial location of the shock zone, animals with reduced adult neurogenesis could not learn the new shock zone if there was conflicting spatial information (Burghardt et al., 2012). It seems that without adult-born neurons, animals cannot suppress the originally learned shock zone location. As well these animals show an overactivation in the DG, it is possible that this increased activity in the DG impaired the ability to segregate between the two conflicting memories (Burghardt et al., 2012).

It is also possible that the addition of new adult-born neurons may cause redistribution synaptic connections within the DG, where new neurons compete for and eventually take over synaptic sites of older neurons (Toni et al., 2007; 2008). While new adult-born neurons have been shown to compete for both afferent and efferent connections with mature neurons, total spine number in the dentate gyrus appears to be constant (Toni et al., 2007; 2008; Adlaf et al., 2017). The addition of new adult-born neurons does not alter dendritic spine density; spines are redistributed from mature old granule cells to new adult-born granule cells (Toni et al., 2007; Adlaf et al., 2017). Reducing spines on mature neurons increases adult neurogenesis and survival of 3-week old cells (McAvoy et al., 2016). As the competition for synaptic connections was reduced, adult-born cell survival increased, leading to an inhibition of the DG, indicating that both synaptic redistribution and increased inhibition may be roles of adult-born neurons in the DG (McAvoy et al., 2016; Adlaf et al., 2017).
While the reduction in activity found with increased adult neurogenesis may be caused by either increased inhibition and synaptic redistribution or both, increased adult neurogenesis is known to play a role in mismatch detection, pattern separation, and suppression or inhibition of past information (Burghardt et al., 2012; Lacefield et al., 2012; Nakashiba et al., 2012; McAvoy et al., 2016). Overall both increased inhibition and synaptic redistribution may explain our findings of decreased activity in the DG with increased adult neurogenesis (see chapter 4).

5.4 Why include cellular age?

5.4.1 Differences in survival

An important factor in the relationship between developmentally-born and adult-born cells is the survival rates of these two populations, and the potential that each population may influence the survival of the other. Previous work has shown that adult-born neurons die off at a steady rate between 1-4 weeks after birth, with reported losses of about 50% (Dayer et al., 2003; Kempermann et al., 2003). The surviving adult-born neurons that do reach maturity become very stable and are thought to likely persist for the life of the animal (Dayer et al., 2003; Kempermann et al., 2003). Here we have shown that the survival pattern of developmentally-born DG neurons is essentially the opposite of DG neurons born in adulthood (see chapter 2, see table 5-1). We found that there was no loss of developmentally-born cells between 1-8 weeks and that 17% died after reaching maturity, between 2-6 months of age (see table 5-1). These differences between these two cell populations and their life cycles further suggest the possible variances in their function and relationships to each other. It is possible that the importance of each population is time-dependent, as early postnatal period may require the complete survival of developmentally-born neurons during these months (see section 5.2). Another possibility is there may be a functional link between the loss of developmentally-born neurons and the addition of adult-born
neurons, either as a functional replacement within the circuit or physical cellular replacement or a mixture of both (see sections 5.3). Our data also raise the question of whether the death of developmentally-born neurons might also contribute to the loss and consolidation of hippocampal memory (See section 5.2).

5.4.2 Turnover or addition

How adult neurogenesis contributes to the makeup of the total granule cell population within the DG remains somewhat controversial, surrounding two main ideologies: 1. the turnover hypothesis, where new adult-born cells replace older cells; or 2. the addition hypothesis, where there is a net increase to the total number of cells in DG (Bayer, 1982; Bayer et al., 1982; Boss et al., 1985; Crespo et al., 1986; Nottebohm, 2002; Spalding et al., 2013). The addition hypothesis originally gained support from findings showing that during the juvenile to adult period there is an overall growth in the cellular volume of the DG, with increases in cell numbers and growth ranging from 30-43% during this period (Bayer, 1982; Bayer et al., 1982; Crespo et al., 1986). Despite the early support this hypothesis received, as adult neurogenesis was further studied and techniques became more refined, the idea of cell turnover gained greater support (Nottebohm, 2002; Spalding et al., 2013).

Strong support for the turnover theory stems from findings in songbirds that demonstrate large seasonal fluctuations in cellular numbers (Nottebohm, 2002). For example, males exhibited an increase in the number of cells in the area of the brain associated with song learning during breeding season (Kirn et al., 1994; Nottebohm, 2002). As the breeding season ends, and the animals switch from being reliant on singing for mating purposes, these cells die off (Kirn et al., 1994; Nottebohm, 2002). Despite the extreme nature of this specific example of seasonal turnover, it provides a sound model for cellular turnover. Though cellular turnover has been
difficult to prove in mammals, the existing relationship between the developmentally-born and adult-born populations as they integrate and survive in the DG provides possible insight into cellular turnover. Unlike developmentally-born cells which die off in the adult period, as adult-born cells reach early maturity, their survival is stable (see chapter 2, Dayer et al., 2003; Kempermann et al., 2003; Cahill et al., 2017). This loss of developmentally-born cells, as well as the similar mature functional patterns and stability of the adult-born cells, seems to indicate that adult-born cells may be directly replacing developmentally-born cells (Dayer et al., 2003; Kempermann et al., 2003; Espósito et al., 2005; Ge et al., 2007). Furthermore, it has been shown in humans that approximately 700 neurons are added daily in the adult brain showing an almost 2% annual turnover rate of these new adult-born cells (Spalding et al., 2013). Using mathematical modelling, this annual turnover reflects two interacting cellular populations, one which demonstrates a continual renewal of cells which feeds into the second proportion of cells that are reflectively replaced (Spalding et al., 2013).

To investigate if the relationship within the hippocampus was either based on addition or replacement (turnover) we looked at two methods to increase adult neurogenesis between 2-6 months, the first was constant access to a running wheel (see chapter 3 experiment 2) and a combination of interval running and memantine (see chapter 3 experiment 3 and chapter 4). This treatment window was chosen based on findings that developmentally-born neurons die in early adulthood, unlike adult-born cells, which are known to remain stable (after reaching maturity) (Dayer et al., 2003; Kempermann et al., 2003). While adult-born neurons have enhanced plasticity and unique circuitry during their immature stages (Ge et al., 2007), many reports indicate that they may become functionally equivalent to neurons born in early postnatal development once they have reached maturity (Liu et al., 2000; Laplagne et al., 2006; 2007).
These data collectively suggest that adult neurogenesis may serve to directly replace lost developmentally-born cells. Whether there is a causal, homeostatic relationship between numbers of developmentally-born and adult-born neurons remains unknown. We measured the number of BrdU+ postnatal day 6 cells as well as the addition of adult-born neurons in the same animals, using doublecortin. If there is a homeostatic balance of cell number in the dentate gyrus, and the death of developmentally-born neurons depends on replacement by adult-born neurons, increasing adult neurogenesis should lead to compensatory increases in the death of developmentally generated cells. Our findings suggest that increasing adult neurogenesis above natural levels, using either constant running wheel access or alternating RUN/MEM treatment, does not increase the death of developmentally-generated cells. Only one of our methods to increase adult neurogenesis long-term lead to increased levels of immature neurons at the end of the treatment period, while both groups showed similar levels of survival of developmentally-born cells at the end of treatment (Fig 5-1). Our findings are inconclusive about the role of turnover or addition based on our limited ability to increase adult neurogenesis over long period in a controlled manner, and therefore these data warrant further testing and were not included in the data chapters. These findings seem to indicate that there may not be a homeostatic replacement by adult-born neurons, it may be that the loss of developmentally-born cells in the healthy brain happens in a predetermined pattern based possibly on the decline in adult neurogenesis in ageing-related to the rate of developmental cell loss (Kuhn et al., 1996). Our findings of altered activity within the DG after manipulations of adult neurogenesis show that adult neurogenesis can affect the DG circuit and that cellular replacement may not be the only avenue (see section 5.3 and chapter 4).
Figure 5-1: Effects of long-term manipulations of neurogenesis on the survival of developmentally-born neurons

A) Constant access to running wheel: Our constant running wheel treatment did not increase adult neurogenesis above control levels at the end of the four-month treatment period (insert). There was a 17% loss in P6 BrdU+ cells during the 4-month period in the 6-month sedentary group, while the cRUN treatment leads to a 13% loss of P6 BrdU+ cell during this same period. While there was no significant effect found, comparisons of the 2-month and sedentary controls show the 17% loss was significant (this group was used in Chapter 2; one-way ANOVA, F2, 21= 3.100, P=0.0661, Compared 2 month vs. Sedentary using t-test: t-test T14= 2.180, P=0.0468). 

B) Alternating treatments RUN/MEM: Our RUN/MEM treatment increased the number of new immature neurons (DCX+) by 17% compared to control animals after 4 months of treatment, indicating the neurogenesis was still elevated by our treatment (T29=2.065, P=0.048, insert). Developmentally-born (P6) cells show a significant 17% loss from 2 months to 6 months but there was no effect of the RUN/MEM treatment on P6 cell survival (one-way ANOVA, F2, 42= 5.192, P=0.0097, Tukey multiple comparisons 2 months vs. Sedentary P =0.0094). The RUN/MEM treatments lead to a 13% loss of P6 BrdU+ cell during the same 4-month period, but this loss is not significantly different from the sedentary controls. [BrdU counting methods can be found in appendix A].

5.4.3 Integration and activity

It has been shown that once adult-born cells fully integrate and become mature they are morphologically, functionally and physiologically the same as developmentally-born cells (Kempermann et al., 2003; Laplagne et al., 2006; Zhao et al., 2006; Stone et al., 2011). While
these two populations become functionally similar once mature, their developmental patterns are quite distinct. Adult-born cells mature at a delayed rate compared to their developmental counterpart, with a slower presentation of spines and dendritic growth which are related to the glutamatergic and afferent connections of these new cells (Overstreet-Wadiche et al., 2006; Zhao et al., 2006). Not only are the afferent connections delayed during the maturation of adult-born cells, so are the efferent connections to the CA3 region when compared to developmentally-born cells (Zhao et al., 2006). Here we show that developmentally-born cell shown an early peak in IEG activity in integrating cells, with the peak in zif268 expression, shifted 1 week earlier compared to previous findings in adult-born cells (Snyder et al., 2009a, See table 5-1). These differences may be related to differences in the local environment between the adult and developing brain. In development, two differences in the background network activities may influence the quicker maturation of these cells: the enhanced GABAergic depolarization, and an environment rich in activity-dependent gene expression (Overstreet-Wadiche et al., 2006; Pedroni et al., 2014; Götz et al., 2016, see section 1.3).

5.4.4 Difference between the developmental and general granule cell populations

Adult-born cells are often compared to the general population of DG neurons, which consists of both developmentally-born and older, mature adult-born cells. The use of this general mature population of DG neurons has led to the belief that there is increased recruitment of adult-born cells into memory circuits (Ramirez-Amaya et al., 2006; Kee et al., 2007). To rectify the issue of a general mature population Stone and colleagues (2011) analyzed the heterogeneous population of the DG by looking at both the developmentally-born and adult-born cells individually and found that the integration rates were equivalent regardless of when the cells were born (Stone et al., 2011). These findings highlight the importance of birth dating your
comparison population, as there is not an increased recruitment of adult-born over developmentally-born cells as earlier studies have suggested (Ramirez-Amaya et al., 2006; Kee et al., 2007). While this study does suggest that once mature adult-born cells are similar to developmentally-born and seem to function and be recruited in a similar fashion (Laplagne et al., 2006; Zhao et al., 2006; Laplagne et al., 2007; Stone et al., 2011), others have found that when a neuron is born affects the type of memory process they are recruited for. Tronel et al (2015) found that adult-born cells are more involved in spatial memory formation, task novelty, and are affected by the prior learning experience. While developmentally-born cells were shown to be recruited when rats had to discriminate between dissimilar contexts (Tronel et al., 2015). This increased recruitment in developmentally-born cells during discrimination of dissimilar contexts is similar to findings by Nakashiba and colleagues (2012) that showed that older (~6wk) granule cells are required for pattern completion which included the ability to compare distinct pairs of experiences, while young adult-born neurons were crucial for contextual discrimination of similar contexts (Nakashiba et al., 2012).
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<tr>
<th><strong>Developmentally-born</strong></th>
<th><strong>Adult-born</strong></th>
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<tbody>
<tr>
<td><strong>Appearance of spines</strong></td>
<td>Starts: 12 days</td>
</tr>
<tr>
<td><strong>Axons at CA3</strong></td>
<td>Reach CA3 before adult-born</td>
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| **Dendrites**            |                | • Delayed dendritic growth  
                           |                | • Similar dendritic complexity between 12 days developmental and 16-day adult |
| **Overall Maturation**   |                | • Delayed maturation  
                           |                | • Lower expression of NeuN at 12 days (47% adult vs. 67% in developmental) |
| **Cellular properties once mature** |                | • Decreased capacitance |
|                          | • Lower IR at maturation  
                           |                          | • Increasing adult-born decreases activity in DG  
                           |                          | • Decreasing adult-born increases activity in developmental  
                           |                          | • Impact via inhibition or synaptic redistribution  
                           |                          | • Show random bursts of deploing activity (GDPs) |
| **Cellular environment** | • Increased GABAergic network activity  
                           | • Increasing adult-born decreases activity in DG  
                           | • Increasing transcription factor activation  
                           | • Decreasing adult-born increases activity in developmental  
                           | • Show random bursts of deploing activity (GDPs)  
                           | • Impact via inhibition or synaptic redistribution  
                           | • Increasing adult-born decreases activity in DG  
                           | • Decreasing adult-born increases activity in developmental  
                           | • Show random bursts of deploing activity (GDPs) |
| **Activation**           | • Peak IEG at 2 weeks  
                           | • No IEG before 2 weeks of age  
                           | • Peak IEG at 3 weeks  
                           | • Large cell loss between 1-4 weeks- when cells are immature  
                           | • Cells show stable survival after 4 weeks up to 11 months |
| **Cell survival**        | • Complete/ Stable Early survival  
                           | • Large cell loss between 1-4 weeks- when cells are immature  
                           | • 17% loss between 2-6 months  
                           | • Cells show stable survival after 4 weeks up to 11 months |

*Table 5-1: Updated figure from section 1-3: comparison of developmental and adult populations.*

Newly added information is both bolded and underlined.

5.5 How to increase adult neurogenesis: still an important question

Since the discovery of adult neurogenesis and the finding that these new neurons integrate and contribute significantly to the synaptic circuitry in the DG, (van Praag et al., 2002; Jessberger and Kempermann, 2003; Laplagne et al., 2006; 2007), ways to manipulate these
populations have been studied and extensively used. The most prominent methods to increase rate of adult neurogenesis including environmental enrichment (Kempermann et al., 1997a), exercise (van Praag et al., 1999a; Kennard and Woodruff-Pak, 2012), antidepressant treatment (Malberg et al., 2000), seizures (Jessberger et al., 2007a), NMDA-receptor modulation (Cameron et al., 1995; Maekawa et al., 2009) and learning and memory (Gould et al., 1999). While there has been extensive research done in the area of increasing adult neurogenesis many methodological issues related to the time and effectiveness of treatment, as well as the translatability from rodent to human models still exist.

For the purpose of this thesis, two prominent methods to increase adult neurogenesis were focused on: wheel running and NMDA receptor blockade. Wheel running has been shown to increase proliferation and survival and is a widely used as a method to increase neurogenesis (van Praag et al., 1999b; van Praag, 2008; Baek, 2016; Motta-Teixeira et al., 2016). Wheel running has been shown to not only affect cellular properties but to also cause behavioural changes such as increasing spatial learning and memory on the water maze task (van Praag et al., 2005). Our chosen NMDA receptor antagonist, Memantine (MEM) has been shown to increase the proliferation, creating mature granule neurons that are incorporated into memory circuits (Maekawa et al., 2009; Ishikawa et al., 2014). In terms of learning, MEM treatment is shown to increase spatial memory in relation to the increase in new adult-born cells (Ishikawa et al., 2014). Understanding how to best increase adult-born population over long time intervals was an important extension to the current understanding of these two methods, as well to allow for a direct comparison of sex and treatment effects. Most studies examine neurogenesis regulation over short time periods, ranging from hours to weeks (van Praag et al., 1999b; Adlard et al., 2005; Bolz et al., 2015; Dostes et al., 2016; Nguemeni et al., 2018), often not looking at the
sustained impact on neurogenesis. Since most disorders of hippocampal function are chronic, show deterioration over time, and include an age component, identifying whether neurogenesis can be increased over extended intervals was the goal.

Our findings show a transient like effects for increased neurogenesis which agrees with others, who have found decreases effects over time or who only look at the initial increases in neurogenesis (van Praag et al., 1999a; Naylor et al., 2005; van Praag et al., 2005; Kronenberg et al., 2006; Clark et al., 2010). This findings of transient increases in adult neurogenesis with constant running and single MEM injection after only a 30 days lead us to want to investigate if there was a benefit to compounding the effects of each of these treatments (see Chapter 3).

We next followed one treatment after the other, such as to initiate a second treatment when the initial period of increase begins to decrease after 4 weeks. We found that this combination of treatments was overall effective in increasing neurogenesis, prolonging the effects of each individual treatment, but was most effective in male (see Chapter 3). A possible reasons why our combination of treatments was successful in prolonging increases in adult neurogenesis is that each method promotes proliferation differently (see chapter 3).

5.5.1 Sex difference and direct comparisons

There are clear sex difference in the rates of Alzheimer's disease and depression, and the manifestations of these disorders between males and females (Angst et al., 2002; Gutiérrez-Lobos et al., 2002; Silverstein, 2002; Baum, 2005; Irvine et al., 2012). These robust sex differences in disease are known to be influenced, at least in part, by neuroplasticity, making the importance of studying both sexes in conjunction clear. There is a need to study the effectiveness of treatments on increasing adult neurogenesis in both sexes and studies including both sexes allowing for direct comparison are important to highlight the difference in treatment outcomes
(see section 1.2.2.3). Our findings of sex differences in the treatment regimens and their effects on increasing neurogenesis, highlight not only differences in responsiveness to treatments by sex but also how these new cells are added and integrated into the DG. Females showed increases in neurogenesis with 8 weeks of restricted running wheel access, while males responded best with the combination treatments (see chapter 3). Female rodents are generally more active than males (Lightfoot, 2008), increases in neurogenesis from running are larger in females, and female running distance is correlated with the addition of new cells long-term while this is not the case in long-term running for males (Rhodes et al., 2003; Clark et al., 2008; Nguemeni et al., 2018). The differences in responsiveness to treatments between the sexes may not only be from the differences in preference for females to run but also how these new cells are added and integrated into the DG. There are known sex difference in spatial learning with females showing a preference towards cued strategy, and on standard water maze task, where cued strategy cannot be employed, males outperform females (Williams et al., 1990; Chow et al., 2013). Chamizo and collegues (2016) did find that when given running wheels in an enriched environment males and females performed the same on a task where both a cued and spatial strategy could be used, though females still preferred a cued based strategy (Chamizo et al., 2016). The activation of new neurons by spatial learning is also different between the sexes, though males outperform females on spatial tasks, females not male performance is related to the activation of new neurons, which may have to do either with task difficulty or activation based solely on the level of learning (Chow et al., 2013).

While the positive effects of MEM on hippocampal-dependent learning (Ishikawa et al., 2016) and neuroprotective functions (Maekawa et al., 2009; Liu et al., 2014), have been shown, along with our single injection of MEM increasing BrdU+ cells in both males and females. Our
use of multiple doses of MEM was found to be ineffective, with less impact in our female groups 
(see chapter 3). Sex differences in the neurotoxic effect of NMDA receptor antagonist has been 
shown, with adult female rodents showing neurotoxic reactions even at low doses, while males 
show no or lower reactivity (Fix et al., 1995; Jevtovic-Todorovic et al., 2001). Specifically, in 
female rats, pretreatment with low doses of MEM have been shown to impair 24-hour memory 
retention, while in males retention of a 24-hour memory is enhanced with pretreatment of MEM, 
though there have been findings of MEM increasing learning in females at higher doses 
(Zajaczkowski et al., 2000; Creeley et al., 2006; Zoladz et al., 2006). Our females were virtually 
unresponsive to the neurogenic effects of multiple doses of MEM. These observations 
collectively highlight the need to include both sexes in studies of neurogenic treatments because 
of the outcomes, as both our chosen treatment regiments showed clear sex difference.

5.6 Experimental considerations and limitations

Every data chapter covers the limitations found within that experiment. Here I address the 
experimental consideration and limitations that are considered throughout this thesis.

5.6.1 BrdU

Bromodeoxyuridine (BrdU) is a thymidine analog incorporated into the DNA of dividing 
cells during S-phase of the cell cycle (Hayes and Nowakowski, 2002; Taupin, 2007). BrdU has 
been widely used for birth dating and monitoring cell proliferation, as well as the development of 
the nervous system, and confirmation of neurogenesis in the adult brain, including in humans. It 
is important to note that BrdU is a marker of DNA synthesis, not cell proliferation, and labels 
nuclei of cells are in S-phase but not any other phases of the cell cycle (Hayes and Nowakowski, 
2002; Taupin, 2007). While the use of BrdU comes with many advantages it is not without 
pitfalls and limitations.
Some concerns have been raised about BrdU being a toxic and mutagenic substance, which may disrupt normal proliferation, or maturation processes either cellularly or developmentally (Hayes and Nowakowski, 2002; Taupin, 2007). When administered at high doses, BrdU has been shown to have adverse effects on embryonic and neonatal rats (Nagao et al., 1998; Kolb et al., 1999). Although lower doses, such as ours (BrdU 50 mg/kg intraperitoneal (I.P.) single injection) have no apparent toxic effect on development, while still providing substantial labelling (Miller and Nowakowski, 1988). A higher systemic dose of BrdU is required to optimally label all S-phase cells in the adult brain, this increased doses (+200 mg/kg I.P) does not appear to disrupt cell proliferation, the population of immature neurons, or increase cellular death in vivo (Cameron and McKay, 2001; Hancock et al., 2009). Two important points about the toxicity of BrdU should be mentioned, first BrdU would have less toxicity in the adult brain than in the embryonic and neonatal brain, as the blood-brain barrier is more developed in adult, which develops around P10 in rats (Ribatti et al., 2006). Secondly, using BrdU under in vitro and in vivo conditions is different, with the survival of newborn neurons using similar BrdU doses different under each condition. Under in vivo conditions, BrdU administered to adult rats does not decrease cell proliferation or immature neurons (Hancock et al., 2009), while in vitro studies have reported that BrdU interfered with the survival of newborn neurons (Caldwell et al., 2005).

An important note about our thymidine analogs, CldU and IdU, used in chapter 3, while advantageous in allowing for two populations labelled within one animal these labels do come with some limitations in terms of dosing. These markers are known to show cross-reactivity in terms of antibodies used to detect these antigens; however, lower dosages, equimolar to 50mg/kg BrdU, have been shown not to cross-react (Vega and Peterson, 2005). The use of the lower, non-
saturating, doses in chapter 3 may have caused a reduction in the labelling overall, as Cameron & McKay (2001) showed that at saturating doses (100-300mg/kg) compared to a 50mg/kg dose of BrdU, twice as many cells were labelled and more cells were found at 4 weeks post-injection. The second issue is that these markers are known to label fewer cells than BrdU, even at equimolar doses (Taupin, 2007).

Another limitation of BrdU is the potential for labelling neurons going through DNA repair. As DNA repair involves DNA synthesis and because BrdU is a marker of DNA synthesis, not cell proliferation, it is possible that BrdU immunohistochemistry may not only detect dividing cells but also cells undergoing DNA repair (Cameron and McKay, 2001; Taupin, 2007). BrdU dilution is another factor that must be taken into account when using this thymidine analog. BrdU that is taken up by precursor cells will continue to label daughter cells with each division until it is diluted below the limits of detection. BrdU-labeled daughter cells have been shown to be added for a period of approximately four cell cycles, or 4 days (Cameron and McKay, 2001; Hayes and Nowakowski, 2002; Dayer et al., 2003). A single injection of BrdU will not label all cells born over a 4-day period, but the age of a cell in the dentate gyrus labelled with BrdU cannot be determined more precisely than within a 4-day range (Dayer et al., 2003). Redivision of BrdU$^+$ cells dilute incorporated BrdU levels until the level in the daughter cells is too low to be detected by immunohistochemical methods. Two possibilities occur with the redivision of BrdU cells: one labelled precursor can become two labelled daughter cells, leading to the continued generation of new cells. Or one labelled precursor becomes two unlabeled daughter cells (due to label dilution) decreasing the number of BrdU-labeled cells (Dayer et al., 2003). Both possibilities can occur as some daughter cells will continue to divide, with some of the BrdU-labeled cells are likely to disappear, while others become postmitotic (Cameron and
McKay, 2001; Hayes and Nowakowski, 2002; Dayer et al., 2003). For our studies redivision of BrdU+ cells could give the false impression that P6-labeled neurons are stable between 1-8 weeks after injection and infrequent/delayed division between 2-6 months could lead to BrdU dilution below the detection threshold, giving the appearance of cell loss. However, these alternative explanations are unlikely, but a possible limitation when working with BrdU which requires the use of secondary immunohistochemical markers (see chapter 2 for further explanation).

While the dilution of the BrdU-label is known to occur within 4 cell cycles, another issue is label retention by infrequently-dividing stem cells. Studies have shown BrdU-retaining cells after long survival interval, with cells showing strong labelling with little punctate pattern, supporting the possibility that a subpopulation of hippocampal progenitors divides infrequently from early development into adulthood (Angevine, 1965; Mathews et al., 2010). Early postnatal or embryonic proliferating cells which dividing infrequently have been shown to constitute a considerable fraction of adult-dividing cells in the DG (Mathews et al., 2010). In our developmental cells (P6) only 5% of our 8-week-old cells were found to be non-neuronal (NeuN−), and PCNA expression from 1 week onwards was negligible, indicating that revision of our BrdU+ cell is a small possibility, but unlikely (see chapter 2). Even if this entire population of NeuN-/BrdU+ cells consisted of slowly-dividing stem cells that diluted their BrdU between 2-6 months, this cannot account for the observed 17% drop in developmentally-born neurons. Though, as a single BrdU injection labels 100,000+ cells (see chapter 2), understanding the percentage of this population consisting of infrequently dividing cells that divide again through adulthood is an important factor to take into account.
A final issue with the use of BrdU is that a single pulse will not yield an estimate of the true total population. BrdU is cleared from the adult dentate gyrus within 2 hours (Cameron and McKay, 2001; Hayes and Nowakowski, 2002) and because progenitor cells in the dentate gyrus have been determined to have a cell cycle time of ~25 h in adults (Cameron and McKay, 2001)[though there appears to be a progressive lengthening of the cell cycle throughout adulthood, as during the early postnatal period the cell cycle is ~16 hour (Nowakowski et al., 1989; Cameron and McKay, 2001)], only a portion of the entire population of cycling cells is labelled. To solve this problem and label the total proliferating population at any on time would require multiple injections, maintaining BrdU availability over at least a full cell cycle, thought the fact that BrdU is passed on to daughter cells, adds the complication of labelling the whole proliferating population (Taupin, 2007; Hanson et al., 2011).

5.6.2 IEGs for activity makers

Here to look at the recruitment of both developmentally-born and all granule neurons (DAPI+) immediate early genes (IEGs) were utilized. Changes in IEG expression are correlated with neuronal firing and IEGs are shown to be up-regulated when a neuron fires an action potential, indicating that IEGs are a good proxy for neuronal activity (Sheng and Greenberg, 1990; Guzowski et al., 1999; Chawla et al., 2005; Guzowski et al., 2005; Kovács, 2008; Minatohara et al., 2015). Though IEG expression data appear to agree with the single unit findings it should be noted that the IEG data only indicate cell expression at some point during the session (Jung and McNaughton, 1993; Chawla et al., 2005; Schmidt et al., 2012). Another issue with the use of IEGs is the finding that immature (<2-week old) adult-born cells do not express IEG markers and until early maturation at 4 weeks is reached suboptimal recruitment, displayed by IEGs, of these newly added adult-born cells is reported (Kee et al., 2007; Snyder et
al., 2009a; Stone et al., 2011; Huckleberry et al., 2015). This would mean that with our manipulations to increase adult neurogenesis, we would be unable to measure the activity and recruitment of these newly added cells during the last block of treatment (last 2-4 weeks), to see if while we do see a decrease in both developmentally-born and total DG activity, it may be these younger newly added adult-born cells that maybe activate, but unlabeled by IEGs.

5.6.3 Behavioural or cognitive testing

Our present work did not include any behavioural or cognitive testing since learning experiences can alter neurogenesis and cellular death (Kempermann et al., 1997b; Gould et al., 1999; van Praag et al., 1999a; Fabricius et al., 2008; Oomen et al., 2011) and our primary goal was to understand the relationship between the populations within the DG. Behavioural testing could have affected our interpretations about manipulations of adult neurogenesis on the granule cell populations. With this in mind, our lack of behavioural or cognitive testing does limit the interpretations of our findings. First, our finding of the delayed death of developmentally-born DG neurons suggests that death of mature functional DG neurons may be a part of normal development/ageing. This would be in line with findings that some memories may transform or be forgotten over time (Mumby et al., 1999; Kitamura et al., 2009; Kitamura and Inokuchi, 2014), and a combination of survival and death of cells within the DG could enabling detailed memory for important events while minimizing overreliance on obsolete information (Mumby et al., 1999; Richards and Frankland, 2017). Secondly, after manipulations of adult neurogenesis, we found that activity within the DG was altered, thought our use of exposure to a novel environment limits our interpretations of these findings. While novel environmental exposure does increase cellular activity (Chawla et al., 2005; Satvat et al., 2011; Schmidt et al., 2012), behavioural/cognitive testing allows for more detailed information about how each population is
affected by a manipulation in terms of function as well as how the recruitment to a more advanced task would affect the behavioural and cognitive ability.

Even without the use of behavioural or cognitive testing our findings are relevant for a number of psychiatric disorders. The restricted window of zif268 expression found in developmentally-born cells may render some neurons vulnerable to neurodevelopmental insults to the hippocampus (Alberini and Travaglia, 2017). The survival of developmentally-born DG neurons is also relevant for a number of disorders that are associated with structural changes in the hippocampus, and indicate that the developmental neuronal population may be at risk for insult. Our findings of decreased activity in the DG with increased neurogenesis may relate to the possibility that the addition of new neurons could lead to an alternation in the aberrant levels of activity seen in these neurological disorders. As many neurological diseases, including depression (Campbell et al., 2004; Milne et al., 2012), Alzheimer’s disease (Schmitt, 2005; Gorman, 2008), and MCI (Kircher et al., 2007) are associated with increased activity in the hippocampus, which may lead to the neuronal loss and reduced hippocampal volume seen in these diseases (McKinnon et al., 2009; Yassa et al., 2010; Boldrini et al., 2013). The use of behavioural manipulations would allow for future work to translate our findings to behavioural deficits found in human disorders by looking at the functional properties of both the developmental and adult population and how these two interact during cognitive challenge.

5.7 Future Directions

5.7.1 As adult neurogenesis naturally decreases does the survival of developmentally-born cells increase?

Adult neurogenesis is known to substantially decline during ageing, beginning at 6 months of age (Kuhn et al., 1996). By measuring the changing rates of adult neurogenesis and
the loss of developmentally-born cells in adulthood, the homeostatic balance between these populations may be observed. Both developmentally-born (P6) and adult-born (1-12 month) cells can be analyzed using multiple mitotic labels allowing for within-subjects analysis. By looking at the natural balance of cellular addition and loss between these two populations over time one could visualize the change in the rate of loss of the developmental cell population in relation to the rate of addition of the adult-born cells. Animals would receive behavioural testing on a novel object placement (place recognition) paradigm, a hippocampal-dependent memory task (Abbott et al., 2016). This would allow for the induction of activity-dependent IEG expression (Kesner et al., 2014) (as in our previous studies), as well as allow for the functionality and integration of both the developmental and adult neurons to be accessed. This behavioural testing will also allow for any cognitive changes in performance during ageing to be evaluated in relation to cellular populations. We would expect that as animals age, adult neurogenesis will naturally decline, which will correlate with a lower loss of developmentally-born cells as compared to when animals were younger. Also the pattern of IEG activity is of interest, as studies have shown that both developmental and adult-born cells show similar activation patterns during spatial tasks (Stone et al., 2011). It will be interesting to know if this pattern of recruitment changes with age and the natural decline in adult neurogenesis, as previous work has been done in young animals and the balance between the two populations was not taken into account previously.

5.7.2 Examining cellular survival at other developmental stages

It is important to understand if the loss of developmentally-born cells found between 2-6 months of age is specific to postnatal day 6, or is standardized over the developmental period. As postnatal day 6 was chosen to label neurons at the peak of DG development (Schlessinger et al., 1975), other important windows relevant to hippocampal development must be chosen, Prenatal
day 19 and postnatal day 21 to evaluate if this loss is occurring throughout development. The chosen prenatal time point correlates with the beginning of the dentate migration and the start of when the tertiary dentate matrix can be seen, both important milestones in DG formation as well as the end of the CA1 and CA3 formation (Altman and Bayer, 1990a). While P21 is chosen as the postnatal time point because the hippocampus has been shown to come online around this time window, and animals at this age demonstrate adult-like learning patterns (Clelland et al., 2009). Cell death and the long-term survival of cells born during development would be processed the same as in Cahill et al (2017).

5.7.3 Does activity in developmentally-born cells over time reflect turnover of hippocampal memory?

It is well established that some memories undergo consolidation and can be supported by extra-hippocampal structures over time. We propose that the turnover of developmentally-born neurons in the DG circuit either by functional or cellular replacement by adult-born neurons provides a mechanism by which memories can be cleared from the hippocampus. Future studies could look to examine developmentally-born cell activity after the retrieval of recent memories, which still requires the hippocampus, or remote memories, which does not always require the hippocampus. This could be tested through the employment of the contextual fear conditioning, which only temporarily requires the hippocampus (Frankland et al., 2004), and the spatial water maze task, where the hippocampus plays a more permanent role (Mumby et al., 1999; Clark et al., 2005). This study would allow for a more functional look at the homeostatic balance within the hippocampus allowing for a clear understanding of the transient or permanent functions of DG neurons, as well as the role developmentally-born cells play in memory. Developmentally-born (P6) cells would be labelled and at 2 months of age, when these cells are known to start to
die off, rats would then be subjected to contextual fear conditioning or a standard spatial water maze task. Tests would occur either recently (next day) or remotely (6 months of age), and cellular activity in the developmentally-born and total DG neurons would be analyzed. At the recent time point, it is expected that there will be large numbers of activated developmentally-born cells after both the tests. Conversely, at the remote time point, there will be reduced activation of developmentally-born cells during the context fear memory test, which consolidates outside of the hippocampus; however, not the water maze test, which always remains hippocampal-dependent.

5.8 Relevance to human mental health

In humans, reduced hippocampal volume is typically interpreted as a sign of damage and is observed in a number of disorders including depression (McKinnon et al., 2009), mild cognitive impairment (MCI) (Yassa et al., 2010), and Alzheimer’s disease (AD) (Jack et al., 2000). While the mechanisms underlying hippocampal atrophy are multifaceted, changes in adult neurogenesis could contribute to structural damage as well as recovery (Schoenfeld et al., 2017). Our findings of the loss of developmentally-born DG neurons during adulthood highlights the importance of including cellular age and investigating older developmentally-born neurons in pre-clinical models to understand which populations are being affected in the large-scale structural changes in the hippocampus, and sometimes the DG in particular seen (see chapter 2).

External manipulations known to increase adult neurogenesis in rodents have been shown in adult humans to increased hippocampal volume and improvements in cognition in healthy individuals (Pereira et al., 2007; Erickson et al., 2011; Killgore et al., 2013). There are promising results for these treatments restoring hippocampal functions and improvements in hippocampal structure in mental health disorders such as depression (Babyak et al., 2000; Malberg et al.,
2000; Czéh et al., 2001; Sheline et al., 2003; Cunha et al., 2013; Amidfar et al., 2017; Kerling et al., 2017), MCI, and AD (Abbott et al., 2004; Hamer and Chida, 2008; Parachikova et al., 2008; Nichol et al., 2009; Thomas and Grossberg, 2009; Di Santo et al., 2013; Ishikawa et al., 2014; Sun et al., 2015, see section 1.4). Antidepressant treatment has been shown to restores dentate gyrus granule cell number in depressed patients (Boldrini et al., 2013; Mahar et al., 2017), while physical exercise has been shown to increases hippocampal volume in individuals with mild cognitive impairment (Brinke et al., 2015). While both antidepressants and physical exercise are known for their ability to increase adult neurogenesis (van Praag et al., 1999b; Malberg et al., 2000), our findings indicate that changes in the developmentally-born cell population could also contribute to changes in total granule cell number. The developmental population may be more at risk for loss and these neuroprotective treatments may help to protect this population, or newly born adult neurons may act to replace this lost population, though more work is needed.

Developmentally-born neurons appear to be fundamentally different from (even relatively old) adult-born neurons. Adult-born neurons appear to display more experience-dependent plasticity (Snyder et al., 2009c; Tronel et al., 2010; Lemaire et al., 2012; Tronel et al., 2015), as well as respond and have distinct functions in contextual encoding (Nakashiba et al., 2012; Danielson et al., 2016). It then becomes the question of whether it is, in fact, desirable to rescue developmentally-born neurons or if replacing them with new neurons may, in fact, be beneficial, particularly if they are less plastic and could have formed maladaptive associations. First, we must understand if developmentally-born neurons are being replaced by adult neurogenesis. If adult neurogenesis is replacing developmentally-born cells, then damage to dentate cells can be repaired with the addition of new adult-born cells. On the other hand, if adult neurogenesis is simply adding to the already established developmentally-born cells, adding
more adult-born cells will not fix impairments. If this is the case then strategies to protect the developmentally-born cell populations are important, as additional adult-born cells would not alleviate the dysfunction within the hippocampus.

Increased activity within the hippocampus has been associated with many neurological diseases, including depression (Campbell et al., 2004; Milne et al., 2012), AD (Schmitt, 2005; Gorman, 2008), and MCI (Kircher et al., 2007). This increased activity may lead to excitotoxic cell death (Lipton and Chen, 2004; Johnson and Kotermanski, 2006), which could possibly account for (some) of the neuronal loss and reduced hippocampal volume seen in these diseases (McKinnon et al., 2009; Yassa et al., 2010; Boldrini et al., 2013). Finding, including ours, that increasing adult neurogenesis leads to a decrease in activity in the DG (Ikrar et al., 2013; Drew et al., 2016; Adlaf et al., 2017), indicate that it is possible that the addition of new neurons could reduce the aberrant levels of activity seen in these neurological disorders (see chapter 4). It is important to understand which populations are most highly affected when neurogenesis is manipulated. Knowing which populations are either lost during disease and if new adult-born cells can replace the function of these populations to restore function, or simply inhibit the function of overactive developmentally-born neurons is an important insight into treatments that alter new neurons.
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Appendices

Appendix A

Methods for total BrdU counts Figure 5-1

Treatments: In both experiments, only male rats were used. They were injected with the thymidine analog BrdU (50 mg/kg, intraperitoneal; Sigma, B500205, St. Louis, MO, USA) at P6, to label neurons born at the peak of granule cell birth (Schlessinger et al., 1975). At 2 months of age, treatments to manipulate adult neurogenesis began (see below) and continued until rats were 6 months of age, a group of animals were sacrificed at 2 months of age to look at pretreatment number of developmentally-born numbers.

Constant running wheel access: To assessed the long-term effects of continuously housing male rats with access to a running wheel rats were pair housed with constant access to running wheels for 4 months (N=8 per group) (see Fig 3-2, chapter 3).

Alternating RUN/MEM: Animals were administered 4 X 4-week blocks of treatment, treatment blocks consisted of MEM blocks consisting of 4 weekly MEM injections (35 mg/kg each) and RUN blocks consisted of rats being placed individually in running wheel cages for 4 hours on weekdays (N=15-16 per group) (see Fig 4-1, chapter 4).

Microscopy and sampling. Constant running group: Quantification of total DAB-stained BrdU\(^+\) cells was performed under brightfield microscopy using stereological principles. A 1 in 12 series of sections spanning the entire dentate gyrus was examined with a 40x objective and an Olympus CX41 microscope. All BrdU\(^+\) cells located within the granule cell layer were counted in each section and counts were multiplied by 12 to estimate the total number of BrdU\(^+\) cells per DG (bilaterally)(Fig. 5A, constant running group). Alternating RUN/MEM: Quantification of
total DAB-stained BrdU$^+$ cells was performed in a 1 in 12 series of sections spanning the entire dentate gyrus using stereological principles. All stereological analyses were performed by a single investigator using a computer-assisted stereology system consisting of a Olympus CX41 light microscope with a motorized stage and StereoInvestigator software (MicroBrightField Bioscience, Williston, VT). Sections were outlined at 2× magnification and counts were performed at 40× magnification using a counting frame (70 × 70 µm), disector height (8 µm), dual vertical guard zone height (4 µm), sampling grid (220 × 220 µm), while maintaining an acceptably low coefficient of error (Fig 5B).