

**ADRENALECTOMY-INDUCED NEURONAL DEGENERATION:  
DEVELOPMENT OF A NOVEL ANIMAL MODEL OF COGNITIVE  
DYSFUNCTION AND NEUROGENIC TREATMENT STRATEGIES**

**SIMON SPANSWICK**

**M.Sc. Psychology and Neuroscience, University of Lethbridge, 2005**

A Thesis

Submitted to the School of Graduate Studies  
of the University of Lethbridge  
in Partial Fulfilment of the  
Requirements for the Degree

**DOCTOR OF PHILOSOPHY**

Department of Neuroscience  
University of Lethbridge  
LETHBRIDGE, ALBERTA, CANADA

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## **Dedication**

For Grandma,

## **Abstract**

Long-term adrenalectomy (ADX) results in a specific loss of dentate gyrus granule cells in the hippocampus of adult rats, occurring over a period of weeks to months. This loss of granule cells results in cognitive deficits in a number of tasks that depend on intact hippocampal function. The gradual nature of ADX-induced cell death and the ensuing deficits in cognition are similar to those experienced by patient populations suffering from a variety of pathological conditions. Here we present an animal model by which we use ADX to produce a loss of granule cells within the hippocampus of rats. We also provide experimental evidence for a treatment strategy by which the lost granule cells may be replaced, with the goal of functional recovery in mind.

## **Acknowledgements**

There is a long list of people that deserve recognition for their efforts during the course of my Ph.D. work. First and foremost I would like to thank Dr. Robert Sutherland for his continued support and guidance throughout my graduate studies, I am a better researcher for it. A number of members of the Sutherland lab have also contributed greatly to the completion of this work. I would specifically like to thank Jonathan Epp, Hugo Lehmann, Neale Melvin, Fraser Sparks, and last but not least, Melinda Wang for their help. My sincerest thanks also goes to Doug Bray for all his technical expertise, your collaborative ability and patience are greatly appreciated.

I would also like to take the time to recognize the members of my Ph.D. supervisory committee, thank you to Dr. Robert McDonald, Dr. Olga Kovalchuk, Dr. Carolyn Harley, and Dr. Deborah Saucier. Thank you to the publishers of Hippocampus, Learning and Memory, and the Journal of Neuroscience Methods for their kind permission to reprint a number of previously published figures.

It behooves me to thank all those friends and family that stood behind me during this endeavour. Without your support none of this would have been possible. A special thank you goes to Lindsay Akins, with you by my side I am a better person.

## Table Of Contents

<b>Approval/Signature Page</b> .....	<b>p. ii</b>
<b>Dedication</b> .....	<b>p. iii</b>
<b>Abstract</b> .....	<b>p. iv</b>
<b>Acknowledgements</b> .....	<b>p. v</b>
 <b>CHAPTER ONE</b>	
<b>Adrenalectomy-Induced Neuronal Degeneration: Development Of A Novel Animal Model Of Cognitive Dysfunction And Neurogenic Treatment Strategies</b> .....	
	<b>p. 1</b>
Hippocampus: Structure And Function.....	p. 3
Hippocampus And Memory.....	p. 5
The Dentate Gyrus.....	p. 9
Neurogenesis In The Adult Hippocampus.....	p. 13
Animal Models Of Cognitive Impairment.....	p. 24
 <b>CHAPTER TWO</b>	
<b>Adrenalectomy-Induced Cell Loss In The Dentate Gyrus Of The Rat</b> p. 31	
<b>Experiment One: Time-Course Of Adrenalectomy-Induced Cell Death In The Dentate Gyrus</b> .....	
	p. 33
Methods.....	p. 33
Results.....	p. 36
Discussion.....	p. 37
<b>Experiment Two: Quantification Of The Dentate Gyrus Granule Cell Layer After Chronic Adrenalectomy</b> .....	
	p. 40
Methods.....	p. 40
Results.....	p. 43
Discussion.....	p. 44
<b>General Discussion</b> .....	
	p. 47
 <b>CHAPTER THREE</b>	
<b>Loss Of Function After Long-Term Adrenalectomy: Electrophysiological And Behavioural Indices</b> .....	
	<b>p. 51</b>
<b>Experiment One: Electrophysiological Properties Of The Damaged Dentate Gyrus After Adrenalectomy In Rats</b> .....	
	p. 54
Methods.....	p. 54
Results.....	p. 58
Discussion.....	p. 60
<b>Experiment Two: Spatial Deficits In The Morris Water Task After Chronic Adrenalectomy In Rats</b> .....	
	p. 63
Methods.....	p. 65
Results.....	p. 67
Discussion.....	p. 70

<b>Experiment Three: Object-Context Specific Memory Deficits Associated With Loss Of Hippocampal Granule Cells After Adrenalectomy.....</b>	<b>p. 72</b>
Methods.....	p. 72
Results.....	p. 75
Discussion.....	p. 77
<b>General Discussion.....</b>	<b>p. 82</b>

**CHAPTER FOUR**

<b>Continuing Neurogenesis In The Adrenalectomized Brain.....</b>	<b>p. 85</b>
<b>Experiment One: Time-Course Of Adrenalectomy-Induced Increase In Proliferation In The Adult Dentate Gyrus Of The Rat..</b>	<b>p. 87</b>
Methods.....	p. 87
Results.....	p. 89
Discussion.....	p. 96
<b>Experiment Two: Combined Environmental Enrichment And Exercise Increases Neurogenesis In The Damaged Dentate Gyrus</b>	<b>p. 100</b>
Methods.....	p. 100
Results.....	p. 104
Discussion.....	p. 106
<b>Experiment Three: A Novel Method For Reliable Nuclear Antibody Detection In Tissue With High Levels Of Pathology-Induced Autofluorescence.....</b>	<b>p. 110</b>
Methods.....	p. 112
Results.....	p. 115
Discussion.....	p. 116
<b>Experiment Four: Survival Of Adult-Born Neurons In The Dentate Gyrus After Adrenalectomy.....</b>	<b>p. 119</b>
Methods.....	p. 119
Results.....	p. 122
Discussion.....	p. 122
<b>General Discussion.....</b>	<b>p. 124</b>

**CHAPTER FIVE**

<b>A Novel Animal Model Of Cognitive Dysfunction And Neurogenic Treatment Strategies.....</b>	<b>p. 127</b>
---	---------------

<b>REFERENCES.....</b>	<b>p. 149</b>
------------------------	---------------

<b>APPENDIX ONE.....</b>	<b>p. 172</b>
--------------------------	---------------

**FIGURES**..... **p. 175**

## List Of Figures

**Figure 1.** (A) Bilateral adrenalectomy (ADX) resulted in a significant decrease in circulating CORT across all post-surgical time-points. (B) The dentate gyrus of a control rat labeled for the cell death marker Fluoro-Jade B. No cells positive for Fluoro-Jade B were evident in the hippocampal granule cell layer of any of the control animals. (C) Fluoro-Jade B positive cells in the superior, lateral blade of the dentate gyrus in an ADX rat three days after surgery (indicated by arrows)..... p. 175

**Figure 2.** Representative Fluoro-Jade B labeled sections from ADX rats at 2 weeks (A), 4 weeks (B), 8 weeks (C), and 23 weeks (D) after surgery. Fluoro-Jade B labeling was evident throughout the entire granule cell layer of the dentate gyrus at all of the above time-points and was most concentrated in the superior, lateral blade. Fluoro-Jade B labeling had decreased substantially by 23 weeks post-surgery but was still apparent throughout the granule cell layer..... p. 176

**Figure 3.** (A) Complete removal of the adrenal glands (ADX) resulted in a significant decrease in CORT. A subset of ADX rats (INC-ADX) did not differ from controls, suggesting that the ADX was incomplete. (B) ADX also resulted in a significantly reduced weight gain over the 10-week degeneration period. Those rats that were deemed INC-ADX did not differ significantly from controls in terms of weight gain..... p. 177

**Figure 4.** (A) As determined by the Cavalieri method, ADX rats had a significantly smaller dentate gyrus granule cell layer volume compared with both controls and incomplete ADX rats. (B) Further analysis with the optical fractionator showed that ADX resulted in a significant loss of granule cells. (C-E) Representative DAPI-labeled sections taken from a control (C), incomplete ADX (D), and an ADX rat (E) ADX-induced degeneration was most prevalent in the superior blade of the dentate gyrus (arrows)..... p. 178

**Figure 5.** Time-course of CORT levels, taken at 4 and 20 hours after oral administration of 1 mg of corticosterone in ADX rats. This method and amount of CORT was successful in producing a diurnal rhythm in ADX rats..... p. 179

**Figure 6.** (A) Chronic ADX resulted in a significantly attenuated fEPSP slope relative to control rats, despite ADX rats receiving replacement CORT at the time of data collection. (B) A tendency for ADX rats to have a lower population spike amplitude compared to intact controls was apparent..... p. 180

**Figure 7.** Representative DAPI-labeled sections showing electrode

placement in the dorsal dentate gyrus of a control (A) and an ADX rat (B). Red circles highlight the approximate placement of the tip of the electrode..... p. 181

**Figure 8.** Performance in the Morris water task, pre- and post-chronic treatment with CORT. (A) Prior to daily administration of CORT for six weeks, ADX rats displayed a deficit in novel platform locations as measured by latency to locate the hidden platform. (B) Post-treatment analysis revealed a deficit in the ability of ADX rats to locate both novel and familiar platform locations. Asterisks indicate statistical significance p. 182

**Figure 9.** Examples of the object discrimination tasks. (A) Novel object preference. (B) Novel place preference. (C) Object/context mismatch task..... p. 183

**Figure 10.** Performance of control and ADX rats was similar in novel object preference (A) and novel place preference (B); both groups discriminated the novel object or place at greater than chance levels. (C) ADX animals were significantly impaired relative to controls and did not discriminate above chance levels during the novel object/context mismatch paradigm. (Dashed lines) Chance, (\*) significant difference..... p. 184

**Figure 11.** (A) Total number of Ki67 positive cells quantified in the dentate gyrus granule cell layer of controls and ADX rats at 24 hours, 1 week, 2 weeks, and 4 weeks after ADX surgery. ADX rats had significantly more Ki67 positive cells than controls at 24 hours after surgery, a tendency for ADX rats to have more Ki67 positive cells than controls was apparent at the 1 week time-point. No significant group differences were evident at the 2 or 4-week time-points. (B) A replication of the time-course using improved immunohistochemical and quantification techniques revealed a significant difference in the total Ki67 cell number estimate at 1 week but not 4 weeks after ADX surgery. Asterisks indicate a significant difference..... p. 185

**Figure 12.** (A) Chronic-ADX significantly reduced the volume of the dentate gyrus granule cell layer in rats housed in the home-cage. A combination of six weeks of enriched housing and wheel running significantly increased the volume of the granule cell layer in ADX rats, compared to their home-cage counterparts. (B) The alternating treatment of enrichment and wheel running significantly increased the number of doublecortin (DCX) positive cells in the dentate gyrus of ADX and control rats. Asterisks denote significance..... p. 186

**Figure 13.** Regardless of ADX, the combined enrichment and exercise treatment significantly increased neurogenesis as indexed by doublecortin (DCX). Representative pictures of DCX positive cells in

the dentate gyrus of (A) home-cage controls, (B) controls exposed to wheel running and enrichment, (C) ADX rats housed in the home-cage, and (D) ADX rats exposed to wheel running and enrichment..... p. 187

**Figure 14.** Representative pictures of Control (A-C), ADX (D-F), and Stroke (G-I) rats. Nuclear labeling was apparent in all tissue sections and was only visible under the DAPI filter (A,D,G). Autofluorescence was minimal in control rats and was not readily apparent under either the FITC (B) or the TRITC (C) filters. ADX resulted in high levels of autofluorescence in both the dorsal and ventral blades of the dentate gyrus and was observed under both FITC (E) and TRITC (F) filters. Similarly, autofluorescence was high in the peri-infarct area of the stroke tissue and was apparent under both filters (H,I)..... p. 188

**Figure 15.** Confocal images of Control (A,B), ADX (C,D), and Stroke (E,F) tissue. Arrows indicate minimal amounts of autofluorescence in Control tissue (B) and a significant amount in ADX and Stroke tissue (C-F). Confocal analysis revealed that autofluorescence sometimes surrounds, but never co-localizes with the DAPI or Hoechst (data not shown) signal ..... p. 189

**Figure 16.** (A) Control tissue labeled with Ki67, areas indicated with an arrow are shown at a higher magnification (20X) in the insert. (B) Unlabeled ADX tissue displaying typical levels of autofluorescence. Inserts show areas marked by arrows at a higher magnification. Autofluorescence associated with damage looks strikingly similar to the Ki67 signal observed in the control. (C) Confocal image showing co-localization of Ki67 (red) and DAPI in control tissue at high magnification (indicated by arrows) ..... p. 190

**Figure 17.** BrdU/NeuN co-localization (indicated by downward arrows) in the dentate gyrus granule cell layer of control and ADX rats at two and eight weeks after administration of BrdU. (A) A BrdU/NeuN positive cell in the granule cell layer of a control rat at the two-week survival time-point. (B) BrdU/NeuN positive cell in an ADX rat 2 weeks after injection, further co-localization with the nuclear marker DAPI avoided false positives as a result of increased levels of autofluorescence. (C) Co-localization of BrdU/NeuN at the 8-week time-point in a control rat. (D) BrdU/NeuN/DAPI co-localization in an ADX rat at the 8-week survival time-point. Smaller, upward facing arrows highlight areas of high autofluorescence as determined by a lack of co-localization with the DAPI signal..... p. 191

## List Of Abbreviations

ADX.....	Adrenalectomy
ANOVA.....	Analysis of variance
BOLD.....	Blood oxygenation level dependent
BrdU.....	5-bromo-2-deoxyuridine
CCAC.....	Canadian Council on Animal Care
CORT.....	Corticosterone
DAPI.....	4', 6-diamidino-2-phenylindole
DCX.....	Doublecortin
ET-1.....	Endothelin-1
fEPSP.....	Field excitatory post-synaptic potential
fMRI.....	Functional magnetic resonance imaging
GFAP.....	Glial fibrillary acidic protein
MWT.....	Morris water task
NMDA.....	N-methyl-D-aspartate
PBS.....	Phosphate buffered saline
PFA.....	Paraformaldehyde
PSA-NCAM....	Poly-Sialated Neural Cell Adhesion Molecule
SGZ.....	Subgranular zone
TUNEL.....	Terminal dUTP nick end labeling

## **Chapter One**

### **Adrenalectomy-Induced Neuronal Degeneration: Development Of A Novel Animal Model Of Cognitive Dysfunction And Neurogenic Treatment Strategies**

The use of animal models to attempt to mimic important aspects of pathologies associated with disorders of the central nervous system is pervasive throughout the field of neuroscience. Afflictions as varied as Alzheimer's disease, stress, depression, traumatic brain injury, sleep disturbances, and autism have been the focus of intense investigation. Even within a single pathological condition there exists a wide diversity of models, each attempting to delineate a specific cause and/or potential treatment strategy for the condition in question.

Animal models concerned with deficits of function and recovery of cognitive abilities have frequently focused on the hippocampus, potentially due to the susceptibility of the hippocampus to a great variety of pathologies. This is evident in a number of models attempting to characterize deficits in behaviour associated with Alzheimer's disease (Götz & Ittner, 2008), stress (Duman & Monteggia, 2006), stroke (DeVries et al., 2001), and traumatic brain injury (Fujimoto et al., 2004), to mention only a few. The following thesis describes a novel animal model of hippocampal neuronal loss that results in cognitive impairments in the adult rat. We also provide experimental evidence for a treatment strategy by which the lost neurons may be replaced, with the goal of functional recovery in mind.

Specifically, we describe an experimental manipulation that produces a gradual and targeted loss of granule cells in the dentate gyrus subfield of the hippocampus of adult rats. We utilize histological and stereological quantification techniques to determine the time-course and extent of this cell loss. Next we assess the effects of this cell loss on the electrophysiological properties of the dentate gyrus and characterize its ensuing impact on behaviour. A potential treatment strategy is described via the manipulation of ongoing adult dentate gyrus neurogenesis. We show that neurogenesis continues in the adult dentate gyrus, despite a significant loss of granule cells. Furthermore, we demonstrate that neurogenesis in rats with dentate gyrus granule cell loss can be up-regulated in a manner similar to normal adult animals. This up-regulation of adult neurogenesis results in a significant re-population of the dentate gyrus granule cell layer. We offer the up-regulation of adult neurogenesis as a treatment strategy, such that the re-population of the granule cell layer may result in behavioural recovery.

It is perhaps best to begin with a description of “normal” hippocampal structure and function and then briefly consider some animal models that attempt to contribute to our understanding of hippocampal pathologies that result in disturbances of cognition. The following sections will discuss the basic structure of the hippocampus and some of the evidence for its role in certain memory processes. The focus of discussion will then shift to the dentate gyrus subfield of the hippocampus, specifically examining evidence for its role in behaviour and the phenomenon of ongoing adult neurogenesis.

## **Hippocampus: Structure And Function**

The hippocampus is a relatively simply organized, three layered structure situated in the medial temporal lobe. One way that the hippocampus has been viewed is as a tri-synaptic pathway (Rudy, 2008). Inputs from polymodal cortical association areas into the hippocampus arrive indirectly through connections in the perirhinal and parahippocampal cortices (Amaral & Lavenex, 2007). The latter two cortical regions project to the entorhinal cortex, layer II of which innervates the dentate gyrus subfield via the perforant path (synapse one). Dentate gyrus projections terminate in the CA3 subfield via the mossy fiber pathway (synapse two), which in turn projects via the Schaffer collaterals to the CA1 subfield (synapse three). Neurons in the CA1 region project to the subiculum which outputs back to the entorhinal cortex and a number of other subcortical areas (Lavenex & Amaral, 2000), these regions then project to numerous neocortical structures.

The foregoing description indicates that the tri-synaptic pathway is a small part of a larger, more complex network involving the hippocampus. For example, projections from entorhinal cortex also terminate in areas CA3 and CA1 of hippocampus (Amaral & Lavenex, 2007). Perforant path projections from layer II of the entorhinal cortex innervate the molecular layer of CA3, much like in the dentate gyrus (synapse one of the tri-synaptic pathway). The entorhinal cortex also projects to CA1, although in this case it is layer III cells that innervate the molecular layer. The complex nature of the connections involved in hippocampal pathways has caused some to refer to it as the “medial temporal hippocampal system”, also including the entorhinal, perirhinal and parahippocampal cortices (Rudy, 2008).

By the time information reaches the hippocampus it is highly processed. The perirhinal and parahippocampal cortices have been suggested as the initial stages in which multimodal information arriving from numerous cortical sites is integrated (Lavenex & Amaral, 2000). Integrated information is then directed to the entorhinal cortex in which further processing is performed and then transmitted into the dentate gyrus, as described above. Given its relative position in terms of information processing the hippocampus receives convergent information from a large number of brain areas (Rudy, 2008). This information is processed within hippocampal circuitry in a strictly unidirectional manner (Amaral & Lavenex, 2007) and outputs back to the cortex and subcortical regions (Eichenbaum, 2002). Thus, one can think of the hippocampus as sitting atop a hierarchical recurrent processing loop.

Given the nature of the information the hippocampus receives and its output of that information back to neocortical sites, the question arises, what is it the hippocampus doing? This seemingly simple question has been the topic of heated debate for some years. Brenda Milner and Wilder Penfield first presented information on the effects of hippocampal damage on memory in humans at the 1955 Transactions of the American Neurological Association (Milner & Penfield, 1955). They describe two individuals (patient P.B. and F.C.) each of whom had undergone medial temporal lobe resection in an attempt to control epileptic seizures. Curiously, both of the individuals presented with an unexpected loss of recent memory, extending for approximately four years prior to

surgery (Milner, 2005). This initial report was followed by what is now considered to be one of the seminal papers in memory research (Scoville & Milner, 1957).

### **Hippocampus And Memory**

Scoville and Milner's (1957) initial description of the effect of lesions limited to the medial temporal lobe in humans suggested that medial temporal lobe structures, especially the hippocampus, play a crucial role in the storage and recall of certain memories. Further experimentation suggested that the hippocampus is critical for what have been termed "declarative memories" (Cohen & Squire, 1980). Typically declarative memories are thought to contain episodic (event knowledge) and/or semantic (factual knowledge) information (Milner et al., 1998). Interestingly, after lesions limited to the medial temporal lobe, learning of nondeclarative (procedural elements, priming, practice effects) memories is left relatively intact (Milner et al., 1998; Eichenbaum, 1999).

Recent evidence suggests that there is a wider range of involvement for the hippocampus in memory than previously thought (Sutherland et al., 2001; Driscoll et al., 2005; Epp et al., 2007). There is evidence to suggest that the hippocampus supports a myriad of functions, including, but not limited to, pattern separation (Marr, 1971; Leutgeb et al., 2007), spatial representations (O'Keefe & Nadel, 1978; Muller, 1996), path integration (McNaughton et al., 1996; Kubie & Fenton, 2008), and stress response (Sapolsky, 1996).

Even *within* the field of memory research the role of the hippocampus is the focus of great debate (Anagostaras et al., 2001; Isaacson, 2002, Sutherland et al., 2010).

Perhaps one of the more hotly contested topics relates to the involvement of the hippocampus in memory processes over the long-term. In their now famous work with patient H.M., Scoville and Milner (1957) discovered a period of amnesia for recent (extending up to approximately three years), but not remote events prior to bilateral hippocampal damage. In addition, they noted an inability of H.M. to form new episodic memories after the surgical intervention.

In their summary Scoville and Milner (1957) make several points that are worth reiterating. The first is that bilateral removal of the medial temporal lobe produces a loss of recent memory as long as the damage includes at least the anterior portion of the hippocampus. This loss of memory is proportional to the degree of damage within the hippocampal formation (unilateral lesions seem to be insufficient to cause long lasting memory deficits). Removal of the medial temporal lobe produces both anterograde and retrograde amnesia but leaves older memories (including technical skills) intact. Finally, this memory loss is dissociated from general intelligence and perceptual abilities as they are left intact in the amnesiacs in this study.

A more recent examination of the lesion site in patient H.M. by Corkin et al. (1997) confirms Scoville's original surgical report indicating that much of the medial temporal lobe had indeed been removed, although to a lesser degree than was originally postulated. Specifically, much of posterior hippocampus was left intact, although at the

time of imaging the remaining tissue was extremely atrophied (Corkin et al., 1997). Since H.M.'s death on December 2<sup>nd</sup> of 2008, efforts have been made in an attempt to better reconstruct and analyze the site of lesion and its effects on surrounding structure (<http://thebrainobservatory.ucsd.edu/hmblog/>).

The most widely discussed account of the findings of Scoville and Milner (1957) is now commonly referred to as the “standard model of consolidation” (see Squire et al., 2004 for review). Simply put, the standard model asserts that the medial temporal lobe (specifically the hippocampus) plays a time-limited role in declarative memory. As declarative memories become older they depend less upon the hippocampus itself, gradually becoming dependent upon other neocortical structures. A prediction is made such that lesions limited to the hippocampus should interfere with recent, but not remote declarative memories (Squire et al., 1984).

Studies involving rats (Kim & Fanselow, 1992; Anagnostaras et al., 1999) and human subjects (Rempel-Clower et al., 1996; Bayley et al., 2006) offer some support for the standard model. Several lines of evidence suggest that recent, but not remote memories are lost after lesions focused on the hippocampus. Two of the more widely cited papers by Kim and Fanselow (1992) and Anagnostaras et al. (1999) show that lesions limited to the dorsal hippocampus in rats produce amnesia for contextual fear if they are made one day, but not 28 or 50 days after learning. Both papers conclude that this demonstrates the time-limited nature of the hippocampus in contextual fear memory. The interpretation of such findings is marred by the fact that damage to the hippocampus

is limited to the dorsal region (in both experiments produced by two, bilateral electrolytic lesions), potentially leaving the majority of the hippocampal formation intact. This makes claims about the role of the entire hippocampus in memory difficult to decipher at best.

A more thorough review of experimental work with rats reveals a large number of exceptions that the standard model of consolidation cannot account for (Nadel & Moscovitch, 1997; Sutherland et al., 2010). There are now a number of reports showing that lesions of the hippocampus result in deficits in memory, independent of time, in both humans (Cipolotti et al., 2001; Steinvorth et al., 2005) and rats (Sutherland et al., 2001; Broadbent et al., 2006; Epp et al., 2007; Sutherland et al., 2008). Epp et al. (2007) show that amnesia for two-choice picture discriminations is equal at recent (one-day) and remote (60-day) time-points, suggesting a role for the hippocampus in at least some picture discriminations regardless of the age of the memory. Furthermore, they show a significant positive correlation between lesion size and postoperative performance, suggesting that incomplete lesions of the hippocampus may be insufficient to produce ensuing memory disturbances.

Despite differences in theoretical approaches in regards to the hippocampus, there is general agreement that it is critically involved in certain forms of memory, including but not limited to, memory for space (Morris et al., 1982; Sutherland et al., 1982, 1983; Astur et al., 2002), and context (Kim & Fanslow, 1992; O'Brien et al., 2006; Sutherland et al., 2008). A number of papers have chosen instead to focus on the specific contribution of the different subfields within the hippocampus to memory processing and

storage. There is evidence to suggest that different subfields may provide something unique to these processes (Leutgeb et al., 2007; Hunsaker & Kesner, 2008; Farovik et al., 2010). For the purposes of this discussion the focus will reside on the dentate gyrus subfield of the hippocampus.

### **The Dentate Gyrus**

Recent attention has been directed to the dentate gyrus subfield of the hippocampus. The dentate gyrus has been labeled by some, the “gateway to the hippocampus” (Westbrook, 2000, it is however, important to remember that significant hippocampal input is also provided via the temporo-amonic pathway). Input to the dentate gyrus originates from the entorhinal cortex via the perforant path. Specifically, layer II neurons in the entorhinal cortex project to dendrites in the molecular layer of the dentate gyrus. Projections from entorhinal cortex may be further divided into lateral and medial pathways, which terminate in the superficial and middle third of the molecular layer within the dentate respectively (Amaral & Lavenex, 2007).

The principal cell field within the dentate gyrus is the granule cell layer. In the rat, the granule cell layer is composed of approximately 2 million granule cells (West et al., 1991; Spanswick et al., 2010). New granule cells are born throughout the life of an adult mammal (discussed below; Kempermann et al., 2004) and likely contribute to dentate gyrus function (van Praag, 2002). Possibly because of the high levels of inhibition, firing rate of cells in the dentate gyrus is low compared to other brain regions, even when compared to other hippocampal subfields (Barnes et al., 1990; McClelland & Goddard,

1996). Congruent with electrophysiological data, behavioural activation of immediate early genes in the dentate involves a very small proportion of cells compared to surrounding structures (Chawla et al., 2005).

The dentate gyrus contributes to spatial tasks (Sutherland et al., 1983; Islam et al., 1995; Xavier et al., 1999; Spanswick et al., 2007). Deficits in the fixed hidden platform (Islam et al., 1995), moving hidden platform (Spanswick et al., 2007), and working memory (Xavier et al., 1999) versions of the Morris water task have been reported in animals with dentate gyrus damage. Xavier et al. (1999) used different versions of the Morris water task to assess reference and working memory in rats with colchicine-induced lesions of the dentate gyrus. With repeated training, rats with dentate gyrus lesions showed marked improvement in the reference memory task (a single, fixed platform location). Xavier et al. (1999) assessed working memory by moving the platform to a new location each day. Control rats quickly learned the new location of the platform on each day of training, whereas rats with dentate gyrus damage showed no improvement over the course of the experiment. Xavier et al. (1999) suggest that the working memory deficits in rats with dentate gyrus lesions are due to a lack of flexibility in regards to place information. In the reference memory task (single platform location) limited flexibility is required to successfully locate the platform on subsequent trials. In contrast, the working memory task (novel platform everyday) requires rats to acquire new information on a daily basis, thus requiring flexible updating of spatial information.

An alternate explanation is that the working memory version of the Morris water

task contains a high degree of overlap from one day to the next. Despite differences in platform location a number of items remain similar from one day to the next (the pool, distal cues, lighting conditions, transport to and from the room, etc.). A given rat is required to differentiate between episodes in the testing context to successfully locate the hidden platform. Computationally speaking, interference as a result of overlapping inputs (highly similar environmental cues across days) needs to be reduced to successfully perform the working memory task employed by Xavier et al. (1999).

Given low probability of firing rate and immediate early gene activity in cells within the dentate gyrus (Barnes et al., 1990; McClelland & Goddard, 1996; Chawla et al., 2005), it has been proposed as a candidate structure to reduce interference between overlapping inputs (Treves & Rolls, 1994; McClelland & Goddard, 1996; Norman & O'Reilly, 2003). This ability to create very different representations despite inputs that share common features is termed pattern separation. Computational theories of the role of the dentate gyrus in pattern separation find some experimental support from performance in spatial (Kesner et al., 2004; Chawla et al., 2005; Leutgeb et al., 2007), contextual (Spanswick et al., 2010) and other discrimination tasks (Bakker et al., 2008).

Leutgeb et al. (2007) recorded from multiple single granule cells in the dentate gyrus of the freely moving rat to determine their potential role in pattern separation. Most of the cells are place cells that fire preferentially when the rat is in a specific location within the environment (O'Keefe & Dostrovsky, 1971; Muller et al., 1987; Moser et al., 2008). In the Leutgeb et al. (2007) experiment, rats were placed in a square environment

and allowed to explore for 10 minutes, every 10 minutes thereafter the environment was altered, such that after 60 minutes the environment was circular in shape. The activity of place cells in the dentate gyrus and CA3 was recorded in each phase of the morph from square to circle. Leutgeb et al. (2007) note that minor changes to the environment in which the rat was tested (a single step in the change from square to circle) produced pronounced changes in the spatial map within the dentate gyrus while only altering CA3 place cell activity minimally. In contrast, large changes in the environment produced distinct populations of place cell maps in CA3, the dentate responded to much smaller changes in which overlap between “morphed” environments was extremely high.

Evidence from fMRI studies involving human participants is consistent with computational theory and findings from rat research (Kirwan & Stark, 2007; Bakker et al., 2008). Bakker et al. (2008) measured brain activity during a pattern separation task in human subjects. People were shown picture sets, such that some of the pictures were exact copies of previously encountered images, completely novel pictures, or slightly altered versions of a previous picture. During trials in which altered versions of a previous picture were shown (high degree of overlap, yet different), activity was higher in the dentate gyrus/CA3 region (as determined by increase in BOLD signal) than when novel, or previously encountered pictures were shown. At face value these results compliment findings from rat research, confirming a role for the dentate gyrus in the pattern separation process. However one should interpret findings from human pattern separation fMRI studies with some skepticism, as the authors themselves acknowledge that a myriad of factors aside from pattern separation have been shown to influence

hippocampal activity (Bakker et al., 2008). Importantly, Bakker et al. (2008) recognize that the results from their study depend on changes in blood oxygen level, noting that if overall activity in the dentate gyrus remained similar throughout the experiment the detection of pattern separation becomes difficult. The idea that overall activity in dentate gyrus remains stable (although individual neuron firing rates may differ) regardless of alterations made to incoming inputs is more consistent with data collected from experiments involving rats (Leutgeb et al., 2005).

In addition to its proposed role in pattern separation, the dentate gyrus is one of only two brain regions to contain a stem cell niche that continues to add new neurons in adulthood (Kempermann, 2006). The phenomenon of ongoing neurogenesis in the adult brain has been well documented and is currently a focus of intense interest within the neuroscience domain. A search on ISI Web of Science utilizing the terms “dentate gyrus neurogenesis” reveals nearly 400 research papers published on the topic in 2009 alone.

### **Neurogenesis In The Adult Hippocampus**

The discovery of adult-born neurons in the dentate gyrus by Joseph Altman in 1963 served to intensify investigation into this structure. His initial finding has enjoyed several “re-discoveries” since. In 1977, Kaplan and Hinds reported the presence of new neurons in the visual cortex, olfactory bulb and hippocampus of adult rats. A few years later, Goldman and Nottebohm (1983) discovered seasonally dependent volume changes in the high vocal centre of songbirds and noted the presence of new neurons within the same region. Gould et al. (1992) confirmed neurogenesis in the dentate gyrus of adult rats

and later reported on the modulation of neurogenesis in the same region via adrenal steroids (Cameron & Gould, 1994). It should be noted however that the phenomenon of neurogenesis in the adult brain was not met with entirely open arms. A number of skeptics clung to the doctrine of no new neurons after development (Rakic, 1985; Kaplan, 2001). Nonetheless, the available data are entirely consistent with the idea that the dentate gyrus continues to produce new neurons throughout adult life and this phenomenon is now generally accepted.

It is recognized that there are two very active neurogenic zones within the adult brain. Both the olfactory bulb and the dentate gyrus continue to incorporate new neurons throughout adulthood (Kempermann, 2006). Several other areas within the adult brain have been suggested to be neurogenic (Gould et al., 1999), but there is some doubt as to the validity of the phenomenon (Nowakowski & Hayes, 2000). Recent investigation suggests that the neurogenesis in the neocortex is relatively sparse, but may contribute significantly to neocortical function (Cameron & Dayer, 2008).

The process of the birth, migration, survival, and integration of new neurons in the adult brain is now a well-described phenomenon, both in the dentate gyrus (Kempermann et al., 2004a) and the subventricular zone (Alvarez-Bulleya & Garcia-Verdugo, 2002). The focus of this discussion will be the process and modulation of adult neurogenesis within the dentate gyrus. It should be made clear that the term neurogenesis refers not to a single thing but to a process consisting of several phases (as mentioned above). It is for sheer simplicity's sake that we refer to the steps of cell birth, migration,

survival and integration into an existing network under the umbrella term of neurogenesis.

Adult hippocampal neurogenesis occurs within the subgranular zone (SGZ) of the dentate gyrus. The SGZ is typically defined as a two to three cell thick layer located immediately below the granule cell layer of the dentate gyrus (Kempermann, 2006). There is some debate as to whether the SGZ contains stem cells in the strictest definition (Seaberg & van der Kooy, 2002) and for this reason the cells responsible for the production of new neurons in the adult brain are typically referred to as progenitor or precursor cells (Kempermann et al., 2004a). The SGZ is a highly vascularized structure and progenitor cells tend to cluster around the blood vessels within the region (Palmer et al., 2000), typically possessing vascular end feet (Filippov et al., 2003). SGZ progenitors express glial fibrillary acidic protein (GFAP), are positive for the intermediate filament protein nestin (Kempermann et al., 2004a), but are negative for the astrocytic marker S100beta (Filippov et al., 2003). Furthermore, progenitor cells are morphologically distinct from mature astrocytes (Garcia et al., 2004) but curiously have similar electrophysiological properties, displaying passive voltage currents (Filippov et al., 2003).

Progenitor cells in the SGZ undergo asymmetric division, producing a transiently amplifying progenitor cell as described by Kempermann et al. (2004a). These cells divide rapidly, massively expanding the pool of potential neurons. During this stage, cells shift from a Nestin-positive, GFAP-negative, highly proliferative state, to a more neuronal

phenotype, during which they express the microtubule-associated protein doublecortin (Francis et al., 1999; Rao & Shetty, 2004). Doublecortin positive cells migrate radially into the granule cell layer where they begin to establish network connections, transiently expressing the calcium binding protein calretinin (Brandt et al., 2003). These cells extend mossy-fiber axons into CA3, eventually become fully integrated into the granule cell layer (Carlén et al., 2002; van Praag et al., 2002; Song et al., 2005). Fully integrated granule cells switch from the brief expression of calretinin to the neuronal specific protein NeuN and the calcium binding protein calbindin (Brandt et al., 2003).

Congruent with histological (Kempermann et al., 2004a) and electrophysiological (van Praag et al., 2002) evidence of functional integration, immature neurons in the adult dentate gyrus display activation of immediate early genes (Jessberger & Kempermann, 2003; Smith et al., 2006; Snyder et al., 2009). Jessberger and Kempermann (2003) report that six-week-old adult-born granule cells (as determined by BrdU/NeuN co-localization) display c-fos activity as a result of Morris water task acquisition. Furthermore, the same proportion of six-week old and mature granule cells was activated by the behavioural task. To determine a time-course of immediate early gene expression, Jessberger and Kempermann (2003) induced seizure activity in groups of mice that had been injected with BrdU at differing time points. They discovered an increase in the proportion of new cells showing immediate early gene activity as a function of time. Immediate early gene activity was non-existent in one and 15 day old cells, with approximately 50 percent of 25 day-old cells expressing c-fos. By 35 days of age no difference in expression of c-fos between new and mature granule cells was noted.

Conversely, others have reported an increase in behavioural activation of immature granule cells (Ramirez-Amaya et al., 2006; Kee et al., 2007). Using a triple labeling technique, Ramirez-Amaya et al. (2006) showed co-localization of BrdU/NeuN and the immediate early gene Arc in immature (five-month old) neurons after spatial exploration. Adult born neurons expressed Arc in a greater proportion than the surrounding mature granule cells, a phenomenon that is indicative of an enhanced plasticity in this population.

Some have suggested different behavioural paradigms and prior experience may account for differences in the number of immature cells expressing immediate early genes (Snyder et al., 2009). Tashiro et al. (2007) report that prior experience in an enriched environment increases the activation of new neurons (indicated by the immediate early genes c-fos and Zif268) when rats are re-exposed to the same, but not a different, environment.

It is likely that the time points and methods utilized to quantify neurogenesis and immediate early gene activation will influence the numbers of new neurons showing immediate early gene activation. The above studies assess immediate early gene expression at differing time points, ranging from 25 days (Jessberger & Kempermann, 2003) to five months (Ramirez-Amaya et al., 2006). It is plausible that if other mechanisms of plasticity vary according to the age of adult born granule cells (van Praag et al., 2002) so too will the expression of immediate early genes. Recent evidence shows

that learning episodes activate more adult-born neurons in rats than mice (Snyder et al., 2009). This suggests that a number of other factors (genetics, environment, diet, etc.) can also influence immediate early gene expression in adult born neurons.

Cameron and McKay (2001) estimate that approximately 9000 new cells are generated each day in the adult dentate gyrus. Despite the high levels of apoptosis amongst newborn neurons (Biebl et al., 2000), a great number of new neurons are constantly being integrated into the granule cell layer throughout the life of an animal. A report by Bayer et al. (1982) finds a significant increase in the number of hippocampal granule cells during the lifespan of rats. Bayer et al. (1982) estimate that the number of granule cells in the right hippocampus of Wistar rats increased from approximately 0.9 million cells at one month to roughly 1.3 million granule cells at one year in age.

In addition to describing in great detail the process of birth, survival, and integration of new neurons in the adult hippocampus, numerous modulatory factors have been identified. These range from interesting, potentially useful treatment strategies (pharmacological factors, stress, exercise, environmental enrichment, and learning), to the truly bizarre (tooth removal, soothing music, and cosmic radiation).

Given the susceptibility of the hippocampus to stress (McEwen, 1994; Sapolsky, 1996) it is not surprising that the first report of the modulation of adult neurogenesis was related to stress hormones. In 1994 Cameron and Gould reported that acute administration of corticosterone to adult rats significantly decreased the number of

progenitor cells in the dentate gyrus as determined by [<sup>3</sup>H]thymidine labeling. Not only did they note the down-regulation of neurogenesis as a result of increased corticosterone, Cameron and Gould (1994) reported an up-regulation of neurogenesis as a result of removing corticosterone via adrenalectomy. Many studies have followed, reporting on the down-regulation of neurogenesis as a result of a variety of different stressors, including: psychosocial stress (Gould et al., 1997), fox odour (Tanapat et al., 2001), restraint stress (Pham et al., 2003), and inescapable shock (Malberg & Duman, 2003). All of the aforementioned studies report a significant decrease in proliferation, suggesting a common mechanism of different types of stress.

Modulation of neurogenesis by pharmacological factors has been described, with particular attention paid to antidepressants (Malberg et al., 2000; Kodama et al., 2004; Huang & Herbert, 2006). More specifically, chronic treatment with antidepressant drugs increases the proliferation, but not survival, of new neurons as indicated by BrdU labeling (Malberg et al., 2000). Using a reporter mouse line, Encinas et al. (2006) showed that fluoxetine specifically targets what they call “Amplifying Neural Progenitors” (also called Type-2 or putative progenitor cells, Kempermann et al., 2004a) acting to increase the number of symmetric divisions. It is unclear as to the exact mechanisms by which fluoxetine and other selective serotonin reuptake inhibitors up-regulate neurogenesis. However, a report by Santarelli et al. (2003) showed that in a 5-HT<sub>1A</sub> receptor knockout mouse the neurogenic effect of fluoxetine was eliminated. The chronic administration (varying greatly from 15 days to four weeks) of fluoxetine and the ensuing increase of adult hippocampal neurogenesis has been taken by some as a link between fluoxetine’s

neurogenic qualities and its role in the alleviation of depression (Malberg et al., 2000; Santarelli et al., 2003; Encinas et al., 2006).

Environmental enrichment and exercise (specifically wheel running) have been employed as non-invasive methods to increase neurogenesis. Kempermann et al. (1997) were the first to report an increase in proliferation and survival of new neurons in the hippocampus of adult mice housed in enriched conditions. Briefly, mice were housed in either standard experimental housing conditions (four per cage) or in a larger enriched environment (12 per cage) containing a variety of toys and access to running wheels for 40 days. Both proliferation (BrdU+ cells one day after injection) and survival (BrdU+ cells four weeks after injection) were increased in enriched animals relative to home-cage controls. Using a similar methodology, Nilsson et al. (1999) reported increased survival of newborn neurons in adult rats. Curiously, there was no net effect on proliferation in direct contrast to what had been previously reported by Kempermann et al. (1997). A crucial difference between the two experiments (apart from species) was the presence of running wheels in the enrichment condition. The effects of wheel running and enrichment it seems are dissociable. In a review of the data on the effects of wheel running and environmental enrichment on neurogenesis, Olson et al. (2006) report that proliferation and survival of neurons are increased respectively. The mechanisms behind this dissociation are still not clearly understood. Olson et al. (2006) suggest that changes in blood flow and the resulting increase in delivery of growth factors such as FGF-2 may be responsible for the increase of cell proliferation. In regards to survival, stimulation of incoming inputs via novelty/complexity in the enriched environment may suffice to

encourage long-term survival of adult born neurons (Kempermann, 2006).

Given the sheer number of new neurons added to the dentate gyrus throughout the life of an adult animal (Cameron & McKay, 2001) researchers have speculated that newborn neurons in the adult brain may contribute something functional to the operation of the hippocampal network. Much research has focused on the potential role of new neurons in the adult brain (Kempermann et al., 2004b; Doetsch & Hen, 2005; Aimone et al., 2006, Wiskott et al., 2006).

Aimone et al. (2006) propose an interesting role for new neurons in the encoding of time in the formation of new memories. They note that given the sparse level of encoding present in the dentate gyrus, it is a structure suited for disambiguation of overlapping inputs (pattern separation). The role of the dentate gyrus in pattern separation is by no means a novel concept (Marr, 1971; McClelland et al., 1995; Rolls, 1996). Much research supports the involvement of the dentate gyrus in pattern separation, computationally (Marr 1971; McClelland et al., 1995), behaviorally (Gilbert et al., 1998; Gilbert et al., 2001), as well as at the cellular level (Chawla et al., 2005; Leutgeb et al., 2007).

Noting that an inhibition of neurogenesis does not produce behavioural effects similar to lesions of the dentate gyrus (but see Jessberger et al., 2009), Aimone et al. (2006) propose that it is solely mature granule cells that participate in the orthogonalization of incoming inputs, thus providing a mechanism by which events

sharing common features can be discriminated. Aimone et al. (2006) reason that immature adult born neurons, prior to becoming indistinguishable from their mature counterparts, may contribute something unique to memory formation in the form of temporal information.

As immature neurons in the adult dentate gyrus are more excitable than mature granule cells (van Praag et al., 2002), Aimone et al. (2006) state that this population is likely to have an opposing effect on orthogonalization of incoming inputs. They theorize that activity in the dentate gyrus should become less sparse, as the population of immature cells will respond to a wider range of incoming inputs, thereby increasing the overlap between events. Since the immature population of adult-born neurons changes as a function of time (new cells continually are born, mature and integrate into the existing network) those events that are temporally distal should activate an immature network that is more distinct from its previous state than events that are closer in time. Thus, overlap in events occurring closer in time will be greater than those spaced further apart. This provides a mechanism by which networks of cells can determine the temporal relatedness of events. A testable hypothesis is evident, mature granule cells contribute to pattern separation via sparse encoding and the immature population of granule cells provides temporal information.

In seemingly direct contrast to the theory proposed by Aimone et al. (2006), Wiskott et al. (2006) suggest that the enhanced plasticity experienced by immature granule cells serves to *increase* the pattern separation abilities of the dentate gyrus.

Briefly, Wiskott et al. (2006) argue that new (and thus more plastic) neurons are suited to encoding aspects of novel environments whereas mature granule cells remain “fixed”, adapted to environments previously experienced. Adult neurogenesis constantly provides the dentate gyrus with a pool of immature neurons that are able to encode novel information. When the situation demands, these neurons are activated and eventually incorporated into the existing network, those that are not, undergo apoptosis. This fits with evidence of transient amplification of progenitor cells (Kempermann et al., 2004a) and high rates of apoptosis amongst the pool of adult born neurons in the dentate gyrus (Biebl et al., 2000).

Recent evidence supports the idea that new neurons are indeed involved in the pattern separation process (Clelland et al., 2009; Creer et al., 2010). Creer et al. (2010) for example, show that mice housed with a running wheel, and thus higher levels of neurogenesis (as determined by 5-bromo-2-deoxyuridine (BrdU) administration), performed better on a spatial pattern separation task than their standard-housed cohorts. Interestingly, when running did not result in increased levels of neurogenesis (as in aged mice) the increase in ability to pattern separate did not occur. Prior research has demonstrated that reductions in hippocampal neurogenesis result in a decreased ability to successfully pattern separate (Clelland et al., 2009). Taken together, these studies suggest a functional role for new neurons in the pattern separation process.

As mentioned above, the structure (and to a lesser degree the function) of the normal hippocampus is well described. Much work has also been done examining the

effect of injury and disease pathology on hippocampal function. Although typically not the sole target of pathological states, the hippocampus is often involved (Walker et al., 2007). Hippocampal pathology has been associated with numerous conditions, including but not limited to, stress (Sapolsky, 1996; Duman & Monteggia, 2006), stroke (DeVries et al., 2001), epilepsy (Sloviter, 2007), Alzheimer's disease (SantaCruz et al., 2005; Götze & Ittner, 2008), sleep disorders (Meerlo et al., 2008), and traumatic brain injury (Fujimoto et al., 2004). Even within a given pathological condition there exists a wide range of models, each of which attempts to delineate a specific cause and potential treatment strategy for the condition under investigation.

### **Animal Models Of Cognitive Impairment**

Animal models that attempt to elucidate cognitive impairment usually include an assessment of hippocampal pathology. This is not surprising given the association between dysfunctions in hippocampal operation and a number of pathological states. Alterations in hippocampal electrophysiology (Pavlidis et al., 1993; Karst & Joëls, 2001), neurogenesis (Jin et al., 2004; Jessberger et al., 2005), cell death (Sloviter et al., 1989; Hicks et al., 1993), immediate early gene expression (Rosi et al., 2005; Hansson & Fuxe, 2008), and ultimately behaviour (McEwen & Sapolsky, 1995; Palop et al., 2003; Fujimoto et al., 2004) have all been reported as a result of a variety of disease states or traumatic insults.

Alterations in adult neurogenesis have been noted as a result of a number of pathological conditions (Jin et al., 2004; Jessberger et al., 2005; Kaneko & Sawamoto,

2009; Kuge et al., 2009). Seizure activity induced by injections of kainic acid has been shown to up-regulate the division of doublecortin positive cells in the dentate gyrus in mice (Jessberger et al., 2005), resulting in a pool of ectopic immature neurons that potentially may contribute to epileptic pathology. A similar up-regulation of immunohistochemical markers of immature neurons has been observed in postmortem tissue obtained from humans suffering from Alzheimer's disease (Jin et al., 2004). Increases in doublecortin, PSA-NCAM, TUC-4, and NeuroD, all of which are proteins expressed in newly born neurons, are found in the hippocampus of brains with confirmed Alzheimer's disease pathology.

A significant loss of neurons, as well as accumulation of  $\beta$ -amyloid and tau, resulting in neurofibrillary tangles, are all associated with cognitive deficits as a result of Alzheimer's disease (Näslund et al., 2000; Riley et al., 2005; Götz & Ittner, 2008). Degeneration of cells in both the hippocampus and entorhinal cortex has been implicated in the pathology of Alzheimer's disease (Walker et al., 2007). In humans, Alzheimer's-induced atrophy of the hippocampus is positively correlated with deficits in several memory tasks (Deweert et al., 1995; Köhler et al., 1998; de Toledo-Morrell et al., 2000; Chupin et al., 2009). Using magnetic resonance imaging, de Toledo-Morrell and colleagues (2000) show that the volume of the left and right hippocampi predict performance on a verbal and spatial recall task, respectively, in individuals with a clinical diagnosis of Alzheimer's disease.

A number of animal studies mirror the behavioural deficits observed in the human patient population, reporting cognitive impairments as a result of Alzheimer's pathology (Janus et al., 2000; Palop et al., 2003; SantaCruz et al., 2005). Animal models of Alzheimer's disease often involve experimentally induced increases in the protein tau (resulting in neurofibrillary tangles, SantaCruz et al., 2005), or amyloid- $\beta$  (Palop et al., 2003), two pathological hallmarks of Alzheimer's disease also found in the human patient population (Näslund et al., 2000; Riley et al., 2005). The majority of animal models utilize performance in the hidden platform version of the Morris water task as an index of hippocampal function. This version of the Morris water task requires the subject to locate a hidden platform in a swimming pool through the use of distal cues present in the testing room. The ability of rodents (Morris et al., 1982; Sutherland et al., 1982,1983) and humans (Astur et al., 2002) to perform this task successfully is severely diminished as a result of damage limited to the hippocampus. Palop et al. (2003) and SantaCruz et al. (2005) show that mice with Alzheimer's brain pathology (neurofibrillary tangles and amyloid- $\beta$ , respectively) experience difficulties in the Morris water task, a finding that is similar to spatial deficits experienced by human Alzheimer's patients (de Toledo-Morell et al., 2000; Laczó et al., 2010).

Medial temporal lobe cell loss however, is not a unique symptom of Alzheimer's disease, as it has been described in other forms of dementia. Frontotemporal lobar degeneration, dementia with Lewy bodies and vascular dementia all involve a gradual, yet significant loss of cells specifically within the hippocampus (Walker et al., 2007). There are a number of other non-dementia related pathological states that also involve a

loss of hippocampal neurons, including alterations in stress hormones (Sloviter et al., 1989; Uno et al., 1989), traumatic brain injury (Hicks et al., 1993), epilepsy (Meldrum, 2002), and ischemia (Hartman et al., 2005).

Traumatic brain injury produces two related, yet dissociable processes, immediate damage to neurons and a delayed onset of cell death within the hippocampus (Hicks et al., 1993; Yamaki et al., 1998; Kokiko & Hamm, 2007). Immediately following traumatic brain injury, oxidative stress, changes in cerebral blood flow, altered metabolism, massive release of neurotransmitters, and ensuing neuronal depolarization all contribute to rapid cellular death (Fujimoto et al., 2004; Kokiko & Hamm, 2007; Ansari et al., 2008). Neuronal death within the hippocampus (typically observed in CA3) occurs at later time points after the initial insult (Hicks et al., 1993; Yamaki et al., 1998; Huh & Raghpathi, 2007).

The loss of hippocampal neurons after traumatic brain injury has been associated with cognitive deficits in a number of hippocampal dependent tasks (Hicks et al., 1993; Fox et al., 1998; Browne et al., 2004; Thompson et al., 2006; Lu et al., 2007). Thompson et al. (2006) report deficits in the acquisition of a single platform location in the Morris water task four weeks after traumatic brain injury in rats. Additionally, rats with traumatic brain injury were impaired in the acquisition of a new platform location and the subsequent probe trial. Using a controlled cortical impact model, Lu and colleagues (2007) also demonstrate that 31-35 days after traumatic brain injury, rats are impaired relative to controls in their ability to learn the location of a single platform location.

Furthermore, Lu et al. (2007) report a significant and continuing loss of cells in the CA3 region of hippocampus.

Delayed neuronal loss in the hippocampus is observed after global ischemia in rats (Pulsinelli et al., 1982; Hartman et al., 2005) and has also been reported in monkeys (Tabuchi et al., 1992; Yamashima et al., 2007), as well as humans (Petito et al., 1987), albeit with a truncated timeline relative to cell loss associated with the dementias and traumatic brain injury. Hippocampal cell loss after global ischemia is usually observed within the CA1 subfield and is typically thought to have ceased by seven days after the initial ischemic insult (Yamashima et al., 2007).

Behavioural assessment of rats subjected to global ischemic events is typically conducted soon after recovery from surgery (Hartman et al., 2005; Langdon et al., 2008). For example, in a study by Hartman et al. (2005) rats received a transient (10-minute) occlusion of the carotid artery and one week later they were tested them in a number of versions of the Morris water task. Hartman et al. (2005) report deficits in a “hard” place-learning, but not in a cued, version of the Morris water task. Importantly, those rats that performed poorly in place learning had a significant loss of CA1 neurons.

In a recent study, Langdon et al. (2008) showed that neuronal loss in the CA1 region of hippocampus of rats was evident as late as 270 days after global ischemia (as determined by the histological labeling of activated microglia), suggesting a much longer period of cellular degeneration than previously suggested. Using a battery of behavioral

tasks, Langdon et al. (2008) show persistent memory deficits in the radial arm maze, the Morris water task, and the t-maze in rats showing long-term loss of CA1 neurons.

A common phenomenon shared by the pathological states discussed above is the gradual and specific loss of cells within the hippocampus. This loss of neurons is associated with cognitive deficits in tasks that require intact hippocampal function. Here we describe a novel animal model that uses a unique combination of granule cell induced death and treatment strategies that manipulate the phenomenon of ongoing neurogenesis within the adult hippocampus, in an attempt to replace lost granule cells and induce functional recovery. Using adrenalectomy (ADX) we are able to selectively induce a loss of hippocampal granule cells that occurs over a number of weeks or months. As mentioned above, the gradual loss of hippocampal neurons is a defining symptom of a number of pathological states. Our animal model allows us to assess the effects of this cell loss on the physiological function of the hippocampus and ultimately on the cognitive abilities of the freely moving animal. Furthermore, we are able to assess the effectiveness of treatment strategies aimed at replacing lost hippocampal neurons.

In chapter 2 we describe ADX-induced granule cell loss utilizing the cell death marker, Fluoro-Jade B, in combination with stereological estimates of granule cell layer volume and total number estimates of granule cells. We show that cell death after ADX continues for at least 23 weeks. Stereological estimates of the total number of granule cells show that by ten weeks after ADX approximately 50 percent of the granule cells have died.

Chapter 3 describes the ensuing loss of function at the electrophysiological and behavioural levels. We provide evidence from two tasks that have been shown to depend on intact hippocampal function that the loss of granule cells results in disturbances of behaviour. Furthermore we demonstrate that the behavioural deficits we observe are due to loss of granule cells and not loss of corticosterone due to ADX *per se*.

Chapter 4 shows that adult neurogenesis continues within the damaged dentate gyrus, replicating earlier findings showing that removal of corticosterone via ADX increases proliferation in the dentate gyrus. We report a novel finding that this associated increase in neurogenesis is only a transient phenomenon, eventually returning to levels similar to normal animals. Finally, we show that the neurogenic niche within the damaged dentate gyrus can respond appropriately to modulatory factors. Using techniques that have been shown to up-regulate neurogenesis in the normal rat we increase neurogenesis in the ADX model. This increase in neurogenesis results in a significant repopulation of the dentate gyrus granule cell layer, providing us with a potential treatment strategy. The ultimate goal of which is to replace lost granule cells and restore normal cognitive function.

## **Chapter Two**

### **Adrenalectomy-Induced Cell Loss In The Dentate Gyrus Of The Rat**

The loss of granule cells in the dentate gyrus resulting from bilateral adrenalectomy (ADX) was first reported by Robert Sloviter and colleagues in 1989. They observed a selective loss of dentate gyrus granule cells in the hippocampus, three to four months after complete, bilateral removal of the adrenal glands from adult rats. Using immunohistochemical and electrophysiological techniques, Sloviter et al. (1989) found that other hippocampal subfields were relatively unaffected by ADX, demonstrating the specificity of cell loss after ADX. Importantly, rats that received corticosterone (CORT) replacement immediately after ADX did not display cell loss from the granule cell layer. This suggests that lack of CORT may trigger the degeneration of granule cells in the dentate gyrus.

Research by Gould et al. (1990) confirmed the specificity of ADX-induced cell loss, showing the presence of pyknotic cells in the hippocampal granule cell layer (but not CA1 or CA3) of rats, three days after ADX. Subsequent investigation by Woolley et al. (1991) demonstrated that specific activation of type 1 adrenal steroid receptors via aldosterone is also sufficient to prevent the loss of dentate gyrus granule cells associated with ADX, providing identification of an initial trigger of the mechanism by which post-ADX granule cell death occurs. Woolley et al. (1991) also confirmed the specificity of cell death in the rat hippocampus, noting a significant increase in pyknotic cells in the granule cell layer of the dentate gyrus seven days after ADX. Using electron microscopy,

Sloviter et al. (1993) provide further evidence for apoptotic cell death in the dentate gyrus granule cell layer as a result of ADX. They report a number of morphological changes in dentate granule cells that are characteristic of apoptosis (condensed nuclear chromatin, compaction of cytoplasm, blebbing). Other markers of cell death have been employed to characterize granule cell loss after ADX, including, but not limited to, silver impregnation (Jaarsma et al., 1992; Park et al., 2002), caspase-9 (Andrés et al., 2006), and terminal dUTP nick end labeling (TUNEL, Greiner et al., 2001).

The majority of studies on ADX-induced neural degeneration focus on cell death measures at a single time-point, relatively soon after ADX (typically within the first week), however degeneration in the dentate gyrus continues for weeks if not months after removal of the adrenal glands (Jaarsma et al., 1992). This ADX-induced loss of cells in the hippocampus is similar to that experienced by individuals suffering from a variety of dementias (Walker et al., 2007), traumatic brain injury (Hick et al., 1993), and stroke (Petito et al., 1987). As of yet no one has systematically examined the ongoing loss of granule cells in the hippocampus as a result of ADX.

Fluoro-Jade B has been suggested to be an efficient and reliable method by which to label degenerating neurons (Schmued & Hopkins, 2000). Numerous studies have employed Fluoro-Jade B to assess neuronal death after insult (Pitkänen et al., 2002; Lee & Agoston, 2009; Moon et al., 2009), however this method of cell death detection has not been applied to the ADX model. Here we adrenalectomize rats and utilizing the cell death marker Fluoro-Jade B (Schmued & Hopkins, 2000) at a number of time-points, ranging

from days to weeks after surgery, attempt to measure a time-course of cell death in the granule cell layer.

## **Experiment One**

### **Time-course Of Adrenalectomy-Induced Cell Death In The Dentate Gyrus**

#### Methods

Male Long-Evans rats (n = 101) were obtained from the University of Lethbridge breeding colony. At the onset of the experiment the rats were approximately 60 days of age. Rats were housed two per cage in a 12-hour light/dark cycle (lights on at 07:30) and received ad libitum access to food and water for the duration of the study. All experimental procedures were conducted in accordance with the University of Lethbridge and Canadian Council on Animal Care (CCAC) animal welfare committee guidelines.

#### Surgery

Rats underwent either ADX or corresponding control surgery. Immediately prior to surgery, rats were injected with buprenorphine (0.05 mg/kg, i.p.) to minimize pain and discomfort. Anesthesia was induced using a 4% concentration of isoflurane (oxygen at 1.5 litres per minute). A surgical plane was maintained at a 2% concentration of isoflurane for the remainder of the surgical procedure. Upon induction, the flanks were shaved and swabbed three times with Hibitane® (4% chlorhexidine gluconate) followed by a single swab of alcohol (70% isopropyl). ADX involved making an approximately 4 cm long incision through the skin in each flank using a number 11 scalpel. Muscle was then blunt dissected and held open with a surgical retractor. The adrenal glands were

removed using organ forceps and the muscle wall of the peritoneal cavity was sutured closed with Vicryl (3-0 coated, Ethicon Inc.). Skin was sutured closed using the same procedure. Control surgery was performed in a similar fashion without the removal of the adrenal glands. Immediately following surgery, all rats were injected intramuscularly with penicillin (0.2 ml, 300,000 IU). All ADX rats were provided 0.9 % saline in lieu of drinking water for the remainder of the experiment.

### Histology

In order to determine a time-course of ADX-induced cell death, rats were euthanized and perfused for histological examination at varying time points after ADX (24 hours, 2-6 days, 1 week, 2 weeks, 4 weeks, 8 weeks, and 23 weeks). At each time point, rats were injected with a lethal dose of sodium pentobarbital (500 mg/kg, i.p.) and perfused transcardially with approximately 200 ml of phosphate buffered saline (0.1M PBS) followed by an equal volume of a 4% paraformaldehyde (PFA) solution. Brains were extracted and stored in the same 4% PFA solution for 24 hours. The brains were then transferred to a 30% sucrose, 0.1M PBS solution until they had sunk and were ready to be sectioned on a freezing sliding microtome (American Optical, model #860; Buffalo, NY, USA). Sections were taken employing a sampling fraction of 1/5 and were cut at a thickness of 40  $\mu$ m.

### Corticosterone Analysis

To assess the effectiveness of ADX, approximately 2.0 ml of trunk blood were collected at the time of perfusion. Blood samples were kept on ice until they were

centrifuged at 10,000 rpm for eight minutes. The separated serum was removed and stored at -80 °C until the samples were assayed. To determine the level of circulating corticosterone a Coat-A-Count ® Corticosterone radioimmunoassay was performed according to kit instructions.

### Cell Death

To determine the time-course of cell death after ADX, a single series of tissue was labeled with the cell death marker Fluoro-Jade B (Schmued & Hopkins, 2000). Tissue sections were mounted on slides and allowed to dehydrate for 24 hours. Slides were then dipped in a solution of 0.06% potassium permanganate in distilled H<sub>2</sub>O for 15 minutes on a lab rotator. Tissue sections were then washed in distilled H<sub>2</sub>O for two minutes, after which they were incubated in a solution containing a 0.0004% solution of Fluoro-Jade B for 30 minutes. After a series of washes in distilled water (one minute each, for a total of three) slides were air-dried, placed in HemoDe for one minute and then coverslipped with Permount® (FisherScientific).

### Analysis

Analysis of Fluoro-Jade B was performed using a Zeiss Axioskop 2 Motplus epifluorescent scope through a FITC filter. Tissue was observed at varying magnifications, employing Plan Neofluor 20x/0.5 and 40x/0.75 objectives. Two sections containing dorsal dentate gyrus were examined in each rat (approximately -3.14 mm and -4.16 mm posterior to bregma, Paxinos & Watson, 1998) for the presence of Fluoro-Jade B positive cells.

## Results

All statistical analyses were conducted employing an alpha level of 0.05, group means are reported as plus/minus the standard error of the mean. Five ADX rats had CORT levels similar to controls, preventing hippocampal granule cell death. These rats were removed from the study and not included in any further analysis. ADX surgery resulted in significantly lower CORT levels,  $F(1,62) = 229.39$ ,  $p < 0.001$  (Figure 1A). On average, control rats had  $293.82 \pm 12.19$  ng/ml of circulating CORT, with ADX rats having a mean CORT level of  $12.87 \pm 13.98$  ng/ml. A significant effect of post-surgical time was present,  $F(6,62) = 3.09$ ,  $p = 0.01$ . Mean CORT levels were  $201.47 \pm 23.31$ ,  $83.29 \pm 23.31$ ,  $136.05 \pm 28.55$ ,  $129.35 \pm 26.07$ ,  $191.362 \pm 22.81$ ,  $171.22 \pm 23.93$ , and  $160.69 \pm 25.51$  ng/ml at the 24-hour, 1-week, 2-week, 4-week, 6-week, 8-week, and 23-week time-points respectively. A significant surgery by time interaction was also evident,  $F(6,62) = 2.88$ ,  $p = 0.02$ . Low levels of CORT were evident in ADX rats across all time-points, ranging from  $6.41 \pm 32.97$  ng/ml at 24 hours post-surgery,  $8.91 \pm 32.97$  ng/ml 1 week after surgery,  $11.66 \pm 46.63$  ng/ml at 2 weeks,  $11.15 \pm 40.38$  ng/ml at the 4-week time-point,  $21.42 \pm 32.26$  ng/ml at 6 weeks,  $16.29 \pm 35.34$  ng/ml at 8 weeks, to  $14.28 \pm 39.51$  ng/ml at 23 weeks after ADX surgery. Control rats showed the highest levels of CORT at the 24-hour time-point ( $396.53 \pm 32.97$  ng/ml), which decreased over the following weeks ( $157.67 \pm 32.97$ ,  $260.44 \pm 32.97$ ,  $247.54 \pm 32.97$ ,  $361.30 \pm 32.26$ ,  $326.15 \pm 32.26$ ,  $307.10 \pm 32.26$  ng/ml at 1, 2, 4, 6, 8, and 23 weeks after surgery).

## Fluoro-Jade B

Control rats did not display any Fluoro-Jade B positive cells at any of the post-surgical time-points. Fluoro-Jade B labeling was evident in ADX rats by three days after surgery (Figure 1B-C). The number of Fluoro-Jade B positive cells present in the dentate gyrus granule cell layer of ADX rats increased steadily from three to seven days. At three days after ADX, cells positive for Fluoro-Jade B were located in the lateral edge of the granule cell layer in the superior blade of the dentate gyrus. At the two-week time point following surgery, Fluoro-Jade B labeling was evident throughout the entire granule cell layer of ADX rats (Figure 2A), but was still greatest in the lateral edge of the superior blade. This remained to be the case at both the four and eight week time points (Figure 2B-C). By 23 weeks after ADX, the number of Fluoro-Jade B positive cells had decreased dramatically, although some labeling was still visible throughout the granule cell layer of ADX rats (Figure 2D).

## Discussion

ADX surgery significantly reduced CORT levels compared to control rats. Unusually high levels of CORT were evident in control rats at the 24-hour time point after surgery, likely due to the stress of the surgical procedure itself. This is evident in the time by surgical condition interaction. Importantly, a main effect of surgery was evident, showing that ADX surgery successfully reduced CORT levels across all time-points.

In control rats Fluoro-Jade B positive cells were non-existent at all time-points after surgery, confirming previous findings (Schmued et al., 1997). By three days after

surgery, a small number of Fluoro-Jade B positive cells were evident in ADX brain tissue. At this time-point, Fluoro-Jade B positive cells were concentrated in the lateral edge of the superior blade of the dentate gyrus. This is congruent with the time-course (Gould et al., 1990; Jaarsma et al., 1992) and pattern of cell loss observed after ADX, with the greatest loss of cells typically occurring in the same region (Sloviter et al., 1995; Roozental et al., 1998). Conrad and Roy (1995) suggest that the superior blade of the dentate gyrus is more susceptible to cell loss after ADX and our finding of initial granule cell loss at three days after ADX lends support to this idea.

Using injections of the thymidine analog BrdU at postnatal day nine to determine the age of post-natal born granule cells, Cameron and Gould (1996) determined that older granule cells in the dentate gyrus are more susceptible to cell death after ADX. Cameron and Gould (1996) injected rats with BrdU on postnatal day six and again just prior to ADX surgery. Those BrdU positive cells expressing mature phenotypes were more likely to also show phenotypes characteristic of apoptosis. Recently labeled cells (those undergoing cell division just prior to ADX) were less likely to display characteristics of cell death. These findings suggest that granule cell age is at least one factor that can influence the fate of a given granule cell after ADX. Given that dentate gyrus granule cell layer development begins with the superior blade and progresses to the inferior blade in an outside-inside pattern (Schlessinger et al., 1975; Kempermann, 2006), older granule cells are more likely to reside in the areas typically associated with observed apoptotic profiles a few days after ADX.

Taken together, the pattern of cell loss that we observe, as well as the observations of others (Conrad & Roy, 1995; Sloviter et al., 1995; Roozental et al., 1998) three days after ADX suggest that the older granule cells of the dentate gyrus are more susceptible to cell death as a result of ADX. This fits with evidence from studies showing that dentate gyrus development begins with the lateral, superior blade (Schlessinger et al., 1975; Kempermann, 2006), as the first signs of ADX-induced cell loss occur in this area. Additionally, BrdU studies (Cameron & Gould, 1996) showing that BrdU positive cells expressing mature phenotypes are more likely to present with an apoptotic profile after ADX than their immature counterparts bolster this idea.

Beginning at two weeks, and continuing to eight weeks after ADX more Fluoro-Jade B labeling was evident, eventually appearing throughout the entirety of the dentate gyrus granule cell layer. By 23 weeks, Fluoro-Jade B labeling had decreased, but was still evident, clearly showing that cell death was still occurring at this point. To our knowledge no studies have examined the occurrence of ADX-induced cell death up to twenty-three weeks or beyond.

Here we are successfully able to determine the onset of cell death after ADX using the histological marker Fluoro-Jade B, supporting prior research on the phenomenon (Gould et al., 1990; Jaarsma et al., 1992). We also confirm the pattern of cell loss reported by others (Conrad & Roy, 1995; Sloviter et al., 1995; Roozental et al., 1998) at early time-points (three to seven days) after ADX. Our observations show a large pool of granule cells undergoing ADX-induced cell death throughout the granule

cell layer of the dentate gyrus at longer time-points (two to eight weeks). This loss of granule cells in the dentate gyrus continues until at least 23 weeks after ADX, a finding that has not been reported before.

In an attempt to further characterize hippocampal granule cell loss as a result of ADX, we assessed granule cell layer volume and estimated the number of remaining granule cells using the Cavalieri volume estimator and optical fractionator techniques (West et al., 1978, 1991; Mouton, 2002; Schmitz & Hof, 2005) after 10 weeks of dentate gyrus granule cell layer degeneration in ADX rats.

## **Experiment Two**

### **Quantification Of The Dentate Gyrus Granule Cell Layer After Chronic**

#### **Adrenalectomy**

##### **Methods**

Fifty-two male Long-Evans rats were obtained from the University of Lethbridge breeding colony. All rats were housed in pairs in a 12-hour light/dark cycle and had ad libitum access to food and water during the extent of the experiment. At the start of the experiment rats were approximately 50 days of age. All experimental procedures were conducting according to the guidelines of the Animal Welfare Committee at the University of Lethbridge and the CCAC.

## Surgery

As discussed above (p. 33), rats received ADX or control surgery. Post-surgically, all ADX rats received 0.9% saline in lieu of drinking water. Rats remained in their home-cages for a period of 10 weeks to allow for sufficient loss of DG granule cells. During the degeneration period rats were weighed on a weekly basis.

## Corticosterone Analysis

Six-weeks after ADX surgery, blood samples were collected from the tail vein. Rats were anesthetized using isoflurane (induction at 4%, surgical plane at 2%), once at a stable anesthetic plane the tail was swabbed with alcohol and approximately 1.0 ml of blood was collected using a Heparin (Sandoz Canada Inc.) coated 23 gauge needle. Blood samples remained on ice until they were centrifuged at 8,000 rpm for 10 minutes. Serum was drawn off and kept at -80 °C until samples were assayed. Analysis of samples was done utilizing a corticosterone EIA kit (Cayman Chemical).

## Histology

Rats were perfused employing the same method as discussed in Experiment 1. Brains were post-fixed in 4% PFA for 24 hours and then transferred into a 30% sucrose, 0.1M PBS solution until they were ready for sectioning. Tissue sections were cut using a frozen sliding microtome at a sampling fraction of 1/12.

To measure the extent of dentate gyrus granule cell loss as a result of ADX a single series of tissue was labeled with 4', 6-diamidino-2-phenylindole (DAPI, Sigma).

Free-floating sections were incubated in a 1:1000 dilution of DAPI in 0.1 M PBS for 25 minutes after which they were washed two times for seven minutes each time in 0.1 M PBS. Tissue was then immediately mounted and cover-slipped utilizing an anti-fade mounting medium. Slides were stored in the dark at 4°C until tissue analysis was performed.

#### Granule Cell Layer Volume

Cavalieri volume estimates were performed utilizing a Zeiss Axio Imager.M1 microscope in concert with Stereo Investigator software (version 8.21.2, MicroBrightField). Quantification of brain tissue took place under a Plan Neofluor 10x/0.3 lens. A pilot study determined that a grid size with an area per point of 100  $\mu\text{m}^2$  would allow for a sufficient number of contact points between the grid and damaged dentate gyrus in ADX rats. The same grid size was used to assess volume of control animals. Coefficients of Error were consistently below 0.06 across all groups.

#### Granule Cell Number Estimates

A subset of control (n = 7), and ADX (n = 5) tissue was chosen by picking those animals that most closely represented the mean volume in each group. A second series of tissue in this subset was labeled with DAPI (as above) and total cell number estimates in the dentate gyrus granule cell layer were performed utilizing the Optical Fractionator technique in Stereo Investigator (MicroBrightField). Estimates of total cell number were done using a Plan Apochromat 100x/1.4 oil immersion objective. A pilot study

determined that a 180 x 180  $\mu\text{m}$  sampling grid with a 12 x 12  $\mu\text{m}$  counting frame and 20  $\mu\text{m}$  dissector zone was sufficient and yielded Coefficients of Error below 0.09 for all rats.

## Results

An alpha level of 0.05 was used for statistical analysis and all means are reported as plus/minus standard error of the mean. ADX significantly reduced CORT levels compared to control rats,  $F = 6.39$ ,  $p < 0.01$  (Figure 3A). A subset of ADX rats had CORT levels similar to controls, most likely a result of incomplete ADX. This resulted in three experimental groups: intact controls ( $n = 18$ ), ADX rats ( $n = 20$ ), and incomplete ADX rats ( $n = 14$ ). A one-way ANOVA revealed a significant difference between the three groups in regard to weight gain over the 10-week degeneration period,  $F(2,49) = 7.37$ ,  $p < 0.01$  (Figure 3B). Further Bonferroni *post-hoc* analysis showed that weight gain did not differ significantly between control and incomplete ADX rats ( $p = 1.00$ ). However, both groups gained significantly more weight than ADX rats (control vs. ADX,  $p < 0.05$ , incomplete ADX vs. ADX,  $p < 0.05$ ). Weight change over 10 weeks was  $203.06 \pm 22.63$  grams for controls,  $165.86 \pm 14.14$  grams for incomplete ADX rats, and  $109.70 \pm 17.14$  grams for ADX rats.

### Granule Cell Layer Volume

A one-way ANOVA ( $F(2,49) = 18.52$ ,  $p < 0.001$ ) revealed a significantly lower dentate granule cell layer volume in ADX rats compared to both controls (Bonferroni *post-hoc*,  $p < 0.001$ ), and incomplete ADX animals ( $p = < 0.001$ ). No significant volume difference was noted between incomplete ADX and controls ( $p = 1.00$ ). Dentate granule

cell layer volumes were as follows: controls  $2.93 \pm 0.06 \text{ mm}^3$ , incomplete ADX  $2.96 \pm 0.10 \text{ mm}^3$  and ADX  $2.13 \pm 0.14 \text{ mm}^3$  (Figure 4A). Mean Coefficients of Error were  $0.046 \pm 0.002$  for Controls,  $0.046 \pm 0.004$  for incomplete ADX, and  $0.057 \pm 0.003$  for ADX rats.

### Granule Cell Number Estimates

Analysis of the optical fractionator data revealed a significant difference between control and ADX rats in terms of total number of cells in the dentate gyrus granule cell layer,  $F(1,10) = 34.84$ ,  $p < 0.001$ . On average, controls had  $1.89 \pm 0.11$  million cells, with ADX rats having a mean of  $0.98 \pm 0.10$  million cells (Figure 4B). Coefficients of Error were  $0.06 \pm 0.01$  and  $0.09 \pm 0.01$  for controls and ADX rats respectively.

### Correlations

There was a significant correlation between weight gain and granule cell layer volume,  $r(52) = 0.518$ ,  $p < 0.01$ . In the subset of rats that the optical fractionator was performed a significant correlation between dentate gyrus granule cell layer volume and total cell number,  $r(12) = 0.904$ ,  $p < 0.01$ , was apparent.

## Discussion

Removal of the adrenal glands resulted in a significant decrease in the amount of circulating CORT. A subset of rats that had undergone ADX surgery had CORT levels similar to intact controls, suggesting that the ADX surgery was incomplete. This is further supported by increased weight gain and larger dentate gyrus volume measures

than other ADX rats. Those ADX rats that had CORT levels similar to controls gained similar amounts of weight and did not differ from controls in terms of dentate gyrus volume.

After 10 weeks of ADX, approximately 25 percent of the volume of the granule cell layer had been lost. These findings are congruent with previous studies showing granule cell loss in the dentate gyrus after long-term ADX (Sloviter et al., 1989, Armstrong et al., 1993; Roozental et al., 1998; Spanswick et al., 2007). Typically, measures of dentate gyrus damage after ADX involve approximation of differences in area (Sloviter et al., 1989; Roozental et al., 1998), using a few representative sections from dorsal dentate gyrus.

Here we apply unbiased stereological techniques to provide a reliable estimate of dentate gyrus granule cell layer volume after ADX. These methods allow for more reliable comparisons, both within and across studies than compared to assumption based methodologies. Our estimate of a 25 percent loss of granule cell layer volume after ten-weeks of ADX is smaller than that reported by Conrad and Roy (1993), who find a 45 percent reduction after 22 weeks. Clearly the time-course accounts for the differences between these studies. We also report a significant loss (roughly 50 percent) of granule cells as determined by the optical fractionator technique. Sousa et al. (1997) find a similar percentage of lost neurons after 120 days of ADX, a much longer time-point. The loss of granule cells after ADX may not be a linear phenomenon, as initially after ADX cell loss is restricted to the lateral edge of the superior blade. Several weeks after ADX, cell loss is

evident throughout the extent of the granule cell layer, but is diminished by 23 weeks.

This shows a rapid increase of cell loss in response to ADX, reaching an asymptote for a number of weeks, followed by a noticeable decline at longer time-points.

A second possibility is that between-study strain differences may account for variation in hippocampal granule cell loss. In the current study we use the Long Evans rat, whereas Sousa et al. (1997) employ Wistar rats. Strain differences in rodents have been reported in spatial behaviour (Harker & Whishaw, 2002), dentate gyrus electrophysiology (Diana et al., 1994), hippocampal cell number (Wimer & Wimer, 1989), and dentate neurogenesis (Perfilieva et al., 2001; Kempermann & Gage, 2002). It stands to reason that granule cell responses to removal of CORT may also be influenced by strain.

The Cavalieri technique for volume estimation allows for a relatively quick and easy estimate of ADX-induced damage in the dentate gyrus. However, the “gold standard” of analysis (Saper, 1996; West & Coleman, 1996) is an estimate of the total number of remaining cells after ADX. The optical fractionator revealed that 10 weeks of ADX-induced cell death resulted in the loss of approximately 50 percent of dentate gyrus granule cells. Not surprisingly, there was a significant correlation between volume and total granule cell number in the dentate gyrus, suggesting that the decrease in volume after ADX is at least in part due to the loss of granule cells.

Despite observing a 50 percent reduction in the total number of granule cells remaining after 10 weeks of ADX, we report a much lower (25 percent) decrease in the volume of the dentate gyrus granule cell layer. This suggests that the density of the remaining granule cell layer is much lower in ADX rats than controls. Prior reports have described an increase in the density of pyknotic cells throughout the granule cell layer of ADX rats (Woolley et al., 1991), the idea that the density of surviving granule cells is decreased in the hippocampus of long-term ADX rats is congruent with this report. Furthermore, our finding is in agreement with that of Sousa et al. (1997) who report approximately a 50 percent loss of granule cells but only a third loss of granule cell layer volume after 120 days of ADX in Wistar rats.

The amount of time required to perform the optical fractionator (approximately five hours per animal) versus the Cavalieri technique (approximately 25 minutes per animal), and the significant correlation between volume estimates and total cell number, justifies our use of the Cavalieri technique as a standard method to estimate granule cell layer depletion after long-term ADX.

### **General Discussion**

Here we replicate previous research showing that bilateral removal of the adrenal glands in the adult rat produces a gradual and specific loss of cells in the granule cell layer of the dentate gyrus (Sloviter et al., 1989; Gould et al., 1990; Jaarsma et al., 1992). A novel aspect of our work is that using the cell death marker Fluoro-Jade B we show that cell death after ADX is apparent by three days after surgery and continues for a

period of at least 23 weeks. The initial time of onset and location of cell death after ADX as determined by Fluoro-Jade B is similar to those using alternate indicators of cell loss (Jaarsma et al., 1992). Furthermore, the initial site of cell loss as a result of ADX in our study, as well as others (Jaarsma et al., 1992) is congruent with findings showing that mature granule cells are more susceptible to cell death after complete removal of circulating CORT (Cameron & Gould, 1996).

Although Fluoro-Jade B has been utilized in a number of studies as an indicator of neuronal death after insult (Schmued & Hopkins, 2000; Pitkänen et al., 2002; Liu et al., 2009) its specificity for neuronal death has been questioned. Several studies have indicated that Fluoro-Jade B labeling may also occur in astrocytes (Columbo & Puissant, 2002; Anderson et al., 2003) and microglia (Damjanac et al., 2007). In addition, the processing involved in Fluoro-Jade B labeling is slightly destructive, often resulting in relatively poor quality tissue. Thus, a number of tissue sections are lost, or are not of high enough quality to include in analysis. Given these problems we decided to utilize other indicators of granule cell layer degeneration in addition to Fluoro-Jade B. Specifically we utilized two stereological techniques in an attempt to better quantify cell loss after ADX.

Using the Cavalieri technique we show that the volume of the dentate gyrus granule cell layer is significantly decreased after 10 weeks of ADX. This finding is by no means novel, as a number of reports of a depleted granule cell layer after ADX are available (Sloviter et al., 1989; Armstrong et al., 1993; Roozental et al., 1998). However, estimations of dentate gyrus damage after ADX have usually involved an approximation

of area (Sloviter et al., 1989; Roozental et al., 1998) relying on a minimal number of “representative” sections from each rat, a method that is less than optimal for biological structures (Mouton, 2000). The Cavalieri volume estimator proves an efficient and unbiased method by which the volume of the dentate gyrus after ADX can be estimated. As such, more reliable comparisons can be made between, and within experiments.

Although the Cavalieri technique provides an accurate and reliable measure of volume of the dentate gyrus granule cell layer, the ultimate concern may be the number of granule cells lost after ADX. To quantify the extent of cell loss after ADX we employed the optical fractionator technique to estimate the number of granule cells within the hippocampus of a subset of control and ADX rats. We show that 10 weeks after ADX there is approximately half of the number of granule cells remaining in the hippocampus of rats. A significant and strong correlation between volume and total cell number estimates was evident. Therefore, we utilize the Cavalieri technique as the standard method by which to calculate granule cell layer degeneration after ADX.

We provide evidence from two lines of research demonstrating the degeneration of the granule cell layer after chronic ADX. Using the histological marker of cell death Fluoro-Jade B, we show that ADX-induced granule cell loss continues in the adult dentate gyrus for a period of at least 23 weeks. Confirming this finding with unbiased stereological techniques, we report a significant loss of dentate gyrus granule cells as determined by estimation of volume and total cell number. A question that arises as a result of this phenomenon is what are the functional implications of this significant loss

of dentate gyrus granule cells? In the following chapter we assess the effects of granule cell loss on the electrophysiological properties and behavioural output of the dentate gyrus in the rat.

## **Chapter Three**

### **Loss Of Function After Long-Term Adrenalectomy: Electrophysiological And Behavioural Indices**

Long-term ADX produces a gradual loss of granule cells that is specific to the dentate gyrus subfield of the hippocampus (Chapter 2; Sloviter et al., 1989). A number of studies have shown spatial learning and memory deficits in animals with damage focused on the dentate gyrus (Sutherland et al., 1983; Islam et al., 1995; Xavier et al., 1999; Kesner et al., 2004). The gradual loss of hippocampal neurons as a result of other brain pathologies is also associated with loss of related cognitive function in both rats (Palop et al., 2003; Thompson et al., 2006) and humans (de Toledo-Morell et al., 2000; Laczó et al., 2010). Here we conduct three experiments in an attempt to assess the functional consequences of long-term ADX-induced granule cell death. Specifically, we assess the effects of granule cell loss on the electrophysiological properties and behavioural contributions of the dentate gyrus.

In their initial report of hippocampal granule cell loss three to four months after ADX in the rat, Sloviter et al. (1989) measured evoked potentials in CA1 and the dentate gyrus of control and ADX rats in response to afferent stimulation. Sloviter et al. (1989) noted that despite a relatively normal electrophysiological response in the CA1 subfield of ADX rats there was a complete absence of evoked potentials in the granule cell layer of the dentate gyrus. A subset of ADX rats that were administered replacement CORT immediately after surgery and then daily until testing did not differ from normal animals,

showing that replacement CORT prevents the ensuing loss of granule cells after ADX and the associated alterations in dentate gyrus electrophysiology.

With the exception of the report by Sloviter et al. (1989), much like post-ADX reports of cell death (Gould et al., 1990; Park et al., 2002), electrophysiological studies have been performed at time-points very soon after surgery. A number of experiments have reported alterations in granule cell electrophysiology, usually ranging from 3 (Stienstra et al., 1998; Wossink et al., 2001; Krugers et al., 2007) to 10 days (Margineanu et al., 1994) after ADX. The short time course of these experiments means that little is known about the effects of chronic ADX and the associated gradual loss of hippocampal granule cells on the electrophysiological properties of the dentate gyrus.

Using *in vivo* recording techniques, Krugers et al. (2007) found that three days after ADX, the maximal field excitatory post-synaptic potential (fEPSP) was significantly reduced in the dentate gyrus of ADX rats compared to intact controls. Synaptic potentiation as a result of theta-burst stimulation was also significantly muted as a result of ADX. Both of these effects were prevented by replacing CORT in the drinking water of ADX rats, prompting Krugers et al. (2007) to state that loss of granule cells after ADX is not necessary for alterations in dentate gyrus electrophysiology. This claim is supported by studies showing changes in dentate gyrus electrophysiology as a result of chronic increases in CORT levels in the absence of notable hippocampal granule cell death (Karst & Joëls, 2003). Conversely, even at these acute time-points post-ADX the removal of CORT has already initiated the apoptotic cascade in a number of granule cells

(Gould et al., 1990; Jaarsma et al., 1992). These cells are not likely to be making a normal functional contribution to dentate gyrus electrophysiology.

Margineanu and colleagues (1994) showed that 10 days after ADX, rats have lower population spike amplitudes, require a higher current intensity to produce a normal response, and have slowed neuronal responses compared to controls. These deficits are not present in rats 24 hours after ADX, despite no detectable circulating CORT.

Margineanu et al. (1994) suggest that although circulating CORT levels and mineralcorticoid receptor availability reach stable levels by 24 hours after surgery it takes at least two weeks for maximal glucocorticoid receptor availability, perhaps underlying the delayed time-course of electrophysiological changes due to ADX.

By 10 days after ADX a significant number of hippocampal granule cells have died or are currently undergoing apoptosis (see Chapter 2, Experiment 1; Gould et al., 1990). ADX-induced granule cell death is first observed at approximately three days post-surgery (Chapter 2, Experiment 1; Gould et al., 1990; Jaarsma et al., 1992). At 24 hours post-surgery, although CORT levels have decreased dramatically, granule cell death is not yet apparent. This suggests that the loss of granule cells is likely to contribute significantly to the electrophysiological alterations after ADX, despite the fact that loss of CORT also contributes.

Alterations in CORT and loss of granule cells due to ADX are two related, yet potentially dissociable processes, each likely exerts an effect on the electrophysiological

properties of the dentate gyrus. With the exception of a single study (Sloviter et al., 1989) all of the aforementioned experiments have examined the electrophysiological properties of the dentate gyrus soon after ADX surgery in rats with or without replacement CORT. No long-term studies have been conducted to assess the effects of loss of granule cells on the electrophysiological properties of the dentate gyrus after chronic ADX.

Here we describe the effects of granule cell loss as a result of chronic (10-week) ADX on granule cell layer electrophysiology. Rats underwent ADX and after 10 weeks of degeneration were administered daily CORT. Input/output (I/O) curves were performed on awake, freely behaving control and ADX rats and population spike amplitude and fEPSPs were recorded.

## **Experiment One**

### **Electrophysiological Properties Of The Damaged Dentate Gyrus After Adrenalectomy In Rats**

#### Methods

A number of rats ( $n = 8$ ) were utilized from the University of Lethbridge breeding colony. All housing conditions were identical to those previously discussed (p. 33). Experimental procedures were performed in accordance with the University of Lethbridge Animal Care Committee and CCAC guidelines.

### Surgery

All surgical procedures for ADX were performed in the exact manner described before (p. 33). Rats underwent ADX surgery at approximately 50 days of age and were returned to their home-cages for 10 weeks prior to electrophysiological recording. ADX rats received 0.9% saline in lieu of drinking water for the duration of the experiment.

### Hormone Replacement

For one week prior to, and during electrophysiological implantation and recording, replacement CORT was provided via oral administration. CORT (1.0 mg daily, Sigma) was suspended in sesame oil and delivered orally on a cookie. Control rats were given sesame oil vehicle on a cookie without the CORT suspension. CORT was administered approximately three hours prior to lights out in the colony room (19:30).

To determine the effectiveness of our CORT replacement strategy, a separate group of rats (n = 5) were placed on CORT replacement one-week after ADX surgery. After seven days of CORT replacement (using the strategy described above) blood was collected at 4 and 20-hour intervals after CORT administration in two separate groups of rats. Blood samples were centrifuged at 10,000 rpm for eight min and plasma was collected and stored at -80°C until analysis. CORT levels were determined using a Coat-A-Count® Corticosterone radioimmunoassay and were performed according to the kit directions.

## Electrode Implantation

One week after CORT replacement began, control and ADX rats were implanted with stimulating and recording electrodes. Rats were administered buprenorphine (0.05 mg/kg, i.p.) immediately prior to the procedure. The rat's head was shaved and the animal was placed in a stereotaxic setup (KOPF® instruments). The scalp was swabbed three times with Hibitane®, followed by a single swab of alcohol (70% isopropyl). After anesthesia was induced using a 4% concentration of isoflurane (oxygen at 1.5 litres per minute) a stable surgical plane was maintained at approximately 2%. A midline incision of the scalp was made using a number 10 scalpel, after which the periosteum was incised and then blunt-dissected away from the skull. Bregma was used as a landmark to determine drill sites for stimulating and recording electrode placement. Holes were drilled in the skull at 8.1 mm posterior and 4.3 mm lateral to Bregma for stimulating electrodes as well as at 3.2 mm posterior and 1.7 mm lateral to Bregma for recording electrodes. The stimulating and recording electrodes were then lowered to approximately 3.0 and 3.5mm below Bregma into the perforant path and granule cell layer of the dentate gyrus respectively. The exact location of the electrodes was adjusted so as to optimize the recorded response in the granule cell layer to perforant path stimulation. A reference electrode and two ground screws were also implanted during the procedure to allow for differential recording. Leads from the electrodes were soldered to amphenol connectors, which in turn were placed into a 9-pin connection plug (Molino & McIntyre, 1972), which was then fastened to the skull using dental acrylic. Upon completion of electrode implantation, rats recovered for 24 hours before they were returned to their homecages where they remained until electrophysiological recording began.

### Electrophysiology

Prior to electrophysiological recording, rats were pre-exposed to the recording context once a day, for two days, for a period of 20 minutes each time. The recording context consisted of a plexiglass box approximately 30 cm x 45 cm in size with standard housing bedding on the floor. On the second day, the connecting plug on the head of the rat was connected to a wire harness from the commutator in an attempt to further acclimate them to the procedure. I/O curves were performed on day three, using six stimulus intensities (50, 100, 200, 300, 400, and 500  $\mu$ A). Each stimulus intensity was repeated 10 times at an interval of 0.05 Hz. The fEPSP slope and population spike amplitude was averaged at each stimulus intensity to provide a curve for each rat. All electrophysiological data was collected and analyzed utilizing SciWorks software (DataWave® Technologies).

### Histology

Rats were perfused according to the same protocol discussed before (p. 34). Brains were post-fixed in 4% PFA for 24 hours and then transferred into a 30% sucrose solution until ready for sectioning. 40  $\mu$ m thick tissue sections were cut using a frozen sliding microtome at a sampling fraction of 1/12.

A single series of tissue was labeled with DAPI (Sigma) to determine electrode location. Free-floating sections were incubated in a 1:1000 dilution of DAPI in 0.1 M PBS for 25 minutes after which they were washed twice for seven minutes each time in

0.1 M PBS. The sections were then mounted and cover-slipped utilizing an anti-fade mounting medium. Sections were observed with a Zeiss Axioskop 2 Motplus epifluorescent scope through the DAPI filter.

## Results

A single control and two ADX rats were removed from the study due to an inability to optimize the electrophysiological response, the animals in question were not included in any further statistical analysis. As before, all means are reported as plus/minus the standard error of the mean and an *alpha* level of 0.05 was employed for all statistical tests.

### Hormone Replacement

Analysis of blood plasma with radioimmunoassay revealed that the CORT replacement strategy that was utilized produced a mean level of  $222.36 \pm 9.22$  ng/ml of CORT in ADX rats four hours after oral administration. At the 20-hour time-point CORT levels had decreased drastically to  $21.44 \pm 4.11$  ng/ml producing a diurnal rhythm in ADX rats on CORT replacement (Figure 5).

### Electrophysiology

A repeated measures analysis of variance revealed a significant difference between control and ADX rats in fEPSP slope,  $F(1,3) = 19.02$ ,  $p < 0.05$  (Figure 6A). Group means for fEPSP slope were  $469.37 \pm 34.93$  for control rats and  $228.52 \pm 42.78$  for ADX rats. Field EPSP slope steepened significantly as stimulation intensity increased,

$F(5) = 8.54$ ,  $p < 0.05$ . At a stimulation intensity of 50  $\mu\text{A}$  the mean fEPSP slope was  $107.35 \pm 53.229$ , increasing at 100  $\mu\text{A}$  ( $205.26 \pm 40.68$ ), 200  $\mu\text{A}$  ( $326.39 \pm 22.15$ ), 300  $\mu\text{A}$  ( $443.44 \pm 43.35$ ), 400  $\mu\text{A}$  ( $479.14 \pm 68.31$ ), and 500  $\mu\text{A}$  ( $532.07 \pm 96.57$ ).

There was a tendency for control and ADX rats to differ in regards to population spike amplitude,  $F(1,3) = 9.14$ ,  $p = 0.057$  (Figure 6B). Control rats had a mean population spike amplitude of  $2200.55 \pm 643.70$ , with the mean population spike amplitude of  $876.47 \pm 788.37$  in the ADX group. A significant within-subject effect,  $F(5) = 8.60$ ,  $p < 0.05$  showed that population spike amplitude increased as a function of increasing stimulation. Population spike amplitude was  $495.26 \pm 498.60$ ,  $886.88 \pm 533.00$ ,  $1669.64 \pm 491.99$ ,  $2049.03 \pm 577.25$ ,  $2063.97 \pm 600.37$ , and  $2066.28 \pm 598.43$  at stimulation intensities of 50, 100, 200, 300, 400, and 500  $\mu\text{A}$ , respectively.

The ratio of population spike amplitude to fEPSP slope was calculated for each rat at each stimulus intensity. A repeated measures analysis of variance revealed no significant differences between ADX and control rats ( $p = 0.601$ ).

### Electrode Location

Electrode placement was assessed in all control and ADX rats included in the study. DAPI labeled sections showed electrode tracks ending in the granule cell layer of the dentate gyrus of control and ADX rats (Figure 7). A noticeable degeneration of the granule cell layer was apparent in all ADX rats included in the study.

## Discussion

Here we show an attenuation of the electrophysiological response of the dentate gyrus in chronic ADX rats. Specifically we report a significantly lower fEPSP slope and a tendency for a decreased population spike in ADX rats after 10 weeks of granule cell layer degeneration when compared to intact controls. Importantly, all ADX rats were receiving replacement CORT at the time of data collection.

To determine if the CORT replacement strategy was effective, blood samples were obtained from two groups of ADX rats, 4 and 20 hours after oral administration of CORT. The data suggest that a diurnal rhythm of CORT was present in ADX rats that is similar to intact controls (Atkinson et al., 2006; Huang and Herbert, 2006). The deficits we observe in electrophysiology are therefore likely due to depletion of the granule cell layer and not complete removal of circulating CORT *per se*.

Replacement of CORT immediately after surgery prevents alterations in electrophysiology within the dentate gyrus of ADX rats (Stienstra et al., 1998). A similar effect of CORT replacement occurs in regard to apoptosis, as CORT replacement also prevents ADX-induced cell death in the dentate gyrus (Sloviter et al., 1989). Curiously, changes in dentate gyrus electrophysiology (24 hours post-ADX) appear prior to notable cell death (three days post-ADX), suggesting that loss of granule cells is not a necessary criteria for ADX-induced disturbances in dentate gyrus electrophysiology (Stienstra et al., 1998).

It is likely that the processes of CORT removal and granule cell loss are two related, yet dissociable phenomena. Both low (Stienstra et al., 1998) and high (Karst & Joëls, 2003) levels of CORT have been shown to influence hippocampal granule cell physiology. Decreases in fEPSP slope have been observed just 24 hours after ADX in rats (Stienstra et al., 1998), despite an absence of apoptotic profiles in the granule cell layer of the dentate gyrus. Karst and Joëls (2003) report increases in dentate gyrus granule cell excitability in response to administration of CORT in rats that have undergone three weeks of chronic stress. These findings suggest that alterations in CORT can disrupt normal dentate gyrus granule cell function in the absence of significant granule cell death. Our concern here was not the modulatory effects of CORT on granule cell function, but rather the effect that a specific, gradual loss of granule cells has on the field potentials recorded in the dentate gyrus.

The gradual loss of granule cells as a result of chronic ADX produces disturbances in dentate gyrus function. Sloviter et al. (1989) report changes in dentate gyrus electrophysiology as a result of long-term ADX but due to lack of replacement CORT are not able to dissociate the effects of CORT disruption from loss of hippocampal granule cells. Our CORT replacement strategy affords us the opportunity to offer the loss of dentate gyrus granule cells as an explanation for the lowered fEPSP slope in ADX rats. The fEPSP slope is associated with the number of granule cells synchronously depolarizing in response to perforant path stimulation. The shallower fEPSP slope evident at several stimulus intensities in our chronic ADX rats fits with the significant loss of dentate gyrus granule cells.

ADX rats differed significantly from controls in regards to fEPSP slope and displayed a tendency to differ in regards to population spike amplitude. Population spike amplitude has been described as a function of the number of neurons (in this case granule cells) synchronously discharging in response to a stimulation event (Anderson et al., 1971). The effects of ADX on population spike have been studied before, Margineanu et al. (1994) report a reduction in population spike by 10 days after ADX, noting a threefold increase in stimulation intensity necessary to induce a standard population spike in ADX rats. Margineanu and colleagues (1994) conclude that loss of granule cells due to ADX (10 days of ADX induces a notable amount of granule cell apoptosis) in combination with alterations to granule cell excitability account for the changes they observe. Our current findings are congruent with previous reports of population spike attenuation as a result of ADX but extend the time-course greatly.

The small group sizes involved in this pilot study (three control and two ADX rats) suggests that the lack of a significant difference between control and ADX rats in regards to population spike amplitude is likely due to high within group variability and low statistical power. A brief examination of the raw data (Figure 6B) shows that the addition of a few more animals is likely to result in a significant between group differences in population spike amplitude.

Despite relatively small groups sizes we report an attenuation of fEPSP slope and population spike amplitude in ADX rats as a result of loss of granule cells in the dentate

gyrus. The gradual loss of granule cells results in measurable electrophysiological dysfunction despite replacement of corticosterone (CORT). In addition to alterations in electrophysiology, impairments in behaviour have been noted in chronic adrenalectomy (ADX) rats.

## **Experiment Two**

### **Spatial Deficits In The Morris Water Task After Chronic Adrenalectomy In Rats**

Behavioural deficits as a result of long-term ADX have been reported in the Morris water task (Armstrong et al., 1993; Conrad & Roy, 1995; McCormick et al., 1997; Roozental et al., 1998; Spanswick et al., 2007) and the open-field (Islam et al., 1995). Roozental et al. (1998) note that long-term ADX produces impairments in both the acquisition and retention of a spatial, but not cued version of the Morris water task. Islam et al. (1995) report similar disturbances in behaviour after ADX, as evidenced by poor performance in 24-hour retention of a hidden platform location in the same task. Interestingly, Islam et al. (1995) also report a significant decrease in rearing in ADX rats in an open field task, suggesting that ADX may adversely affect explorative behaviour.

There has been debate as to whether the deficits experienced by rats with long-term ADX are a result of a loss of CORT or due to depletion of the granule cell layer itself. Conrad and Roy (1995), and McCormick et al. (1997) report that acute CORT replacement is sufficient to alleviate some of the spatial deficits associated with chronic ADX. Specifically, Conrad and Roy (1995) report that in a hidden platform version of the Morris water task employing a single goal location, replacement of CORT for four days

prior to and continuing during behavioural testing is sufficient to improve performance of ADX rats. McCormick et al. (1997) provide similar evidence, reporting that replacement of CORT for 5 or 10 days prior to Morris water task training restores the performance of ADX rats to that of controls.

These findings have prompted some to conclude that the removal of CORT is responsible for the behavioural deficits experienced by ADX rats and not the loss of granule cells *per se* (McCormick et al., 1997). This suggests that the granule cells of the dentate gyrus may not be important for certain spatial tasks, a finding that is at odds with other lesion studies (Sutherland et al., 1983; Xavier et al., 1999; Jeltsch et al., 2001). For example, Xavier et al. (1999) find that colchicine-induced lesions of dentate gyrus produce spatial deficits in both a reference and working memory version of the Morris water task. Jeltsch et al. (2001) find a dose-dependent effect of colchicine induced DG granule cell layer damage on spatial ability in a working memory version of both the Morris water task and the radial arm maze.

In contrast to studies that report amelioration of ADX-induced behavioural deficits via administration of CORT we have shown that chronic administration of CORT after six weeks of ADX does not alleviate spatial deficits in a moving platform version of the Morris water task (Spanswick et al., 2007). Here we describe the effects of long-term ADX on the behaviour of rats in a moving platform version of the Morris water task. In a second experiment we delineate the behavioural deficits associated with ADX-induced granule cell loss in three versions of an object recognition task (novel object,

object/place, and object/context mismatch). We show that dentate gyrus granule cell loss produces deficits that are specific to detecting a mismatch between object and context. These findings add support to the idea that hippocampus and specifically the dentate gyrus is crucial in situations in which discrimination based on context is important. We also provide further evidence that the behavioural deficits associated with ADX are not a result of loss of CORT *per se*.

### Methods

Sixty-two male Long-Evans rats were obtained from the University of Lethbridge breeding colony. Rats were housed in a 12-hour light/dark cycle, two to three animals per cage and received ad libitum access to food and water throughout the experiment. At the beginning of the experiment rats were approximately 60 days of age. All procedures were conducted according to CCAC and University of Lethbridge Animal Welfare committee guidelines.

### Surgery

General surgical procedures were similar to those previously described (p. 33). Rats received post-surgical injections of buprenorphine (0.05 mg/kg i.p.) and remained in their homecages for six weeks to allow for sufficient degeneration of the granule cell layer. ADX rats received 0.9% saline in lieu of drinking water for the remainder of the experiment.

### Morris Water Task

After six weeks of granule cell degeneration, but before CORT replacement, ADX and control rats were trained to locate a hidden platform utilizing distal cues present in the testing room. Water temperature in the Morris water task was maintained at approximately 21°C and skim milk powder was added to render the water opaque. Each rat was given 60 seconds to locate the hidden platform, upon which they remained on the platform for approximately 8 seconds before they were returned to their holding cage. Any rat that failed to locate the platform within the allotted time was removed from the pool and returned to their holding cage until the next trial. Latency to locate the hidden platform was recorded for each trial. Using a novel/familiar platform location paradigm we were able to assess both memory acquisition and 24-hour retention. As we moved the platform location every second day, odd days indicate acquisition of a novel platform location and even days represent retention of a previously acquired platform location. Rats were given eight trials per session, starting twice from each of the four cardinal compass points in a pseudo-random order. A single session was run each day for 12 consecutive days (six platform locations) to establish baseline performance prior to CORT administration. After treatment with CORT, rats were tested on three new platform locations utilized the methods as described above.

### Hormone Replacement

CORT was administered after the six-week degeneration period and the baseline measure in the Morris Water Task. Replacement CORT was administered to all ADX rats (1 mg daily, Sigma) in the same manner as described above (p. 55). Hormone

replacement was carried out for six weeks before rats were retested in the Morris water task. Prior to retesting, CORT was delivered approximately three hours prior to lights out. Administration of CORT continued for the duration of the experiment and was given one hour after behavioural testing.

### Histology

Upon completion of the study rats were perfused according to the previously discussed protocol (p. 34). Brains were sectioned using a frozen sliding microtome at a thickness of 40 microns, employing a section-sampling fraction of 1/5. Completeness of ADX was determined by weight gain (p. 44) and granule cell layer thickness.

## Results

All means are reported as plus/minus standard error of the mean and an alpha level of 0.05 was used for all statistical analyses.

### Pre-treatment Performance

To determine if ADX and control rats differed in regards to latency to locate the hidden platform during pretreatment training in novel locations in the Morris water task a repeated-measures analysis of variance (ANOVA) was performed. Statistical analysis revealed a significant main effect of group over six novel platform locations in the Morris water task,  $F(1,39) = 5.08$ ,  $p = 0.03$ . Group means indicate that ADX rats took significantly longer than controls to locate the hidden platform ( $14.70 \pm 0.45$  seconds and  $11.90 \pm 0.67$  seconds, respectively) during novel platform locations (Figure 8A). The

repeated-measures analysis revealed a significant within-subject effect of day,  $F(5) = 55.75$ ,  $p < 0.001$  with both groups decreasing their mean latencies from the first novel platform location to the last. Mean latencies for the first novel platform location were  $23.23 \pm 2.96$  seconds for controls and  $33.34 \pm 1.68$  seconds for ADX rats. By the sixth novel platform location, latencies had decreased to  $7.37 \pm 0.98$  seconds and  $8.23 \pm 0.56$  seconds for controls and ADX rats respectively. There was also a significant within-subject effect of trial,  $F(7) = 60.48$ ,  $p < 0.001$ , with latency to locate the novel platform decreasing from Trial 1 to Trial 8 in both groups. Latency to locate the novel platform for Trial 1 was  $27.02 \pm 2.50$  seconds for controls and  $29.04 \pm 1.42$  seconds for ADX rats. By the eighth trial, latency to locate the platform had dropped to  $5.25 \pm 1.14$  seconds and  $8.60 \pm 0.64$  seconds for controls and ADX rats respectively.

A second repeated-measures ANOVA revealed no significant between group differences for familiar platform locations prior to CORT replacement,  $F(1.39) = 2.60$ ,  $p = 0.115$  (Figure 8A). There was a significant within-subject effect of day,  $F(5) = 19.37$ ,  $p < 0.001$  suggesting that both groups had improved with exposure to the familiar platform locations. For the first platform location, control rats had a mean latency of  $11.25 \pm 2.31$  seconds, with ADX animals taking an average of  $14.61 \pm 1.31$  seconds to locate the familiar platform. By platform location six, mean latencies had decreased to  $4.25 \pm 0.65$  seconds for controls and  $5.12 \pm 0.37$  seconds for ADX rats. There was also a significant within-subject effect of trial in familiar platform locations  $F(7) = 24.63$ ,  $p < 0.001$ . Mean latency to locate the familiar platform dropped from Trial 1 ( $13.41 \pm 2.15$  seconds for

controls and  $17.95 \pm 1.22$  seconds for ADX rats) to Trial 8 ( $6.76 \pm 0.51$  seconds for controls and  $5.00 \pm 0.89$  seconds for ADX rats) in both groups.

### Post-treatment Performance

After six weeks of CORT replacement, rats were again run in the Morris water task. A repeated-measures ANOVA revealed a significant difference between control and ADX rats to locate a novel, hidden platform,  $F(1,39)$ ,  $p = 0.022$ . As with pre-treatment performance, ADX rats took significantly longer than controls to locate the platform. Group means were  $4.93 \pm 0.59$  seconds for controls and  $6.54 \pm 0.33$  seconds for ADX rats (Figure 8B). There was no significant effect of day,  $F(5) = 1.105$ ,  $p = 0.336$ , suggesting asymptotic performance had been achieved by both control and ADX rats on novel platform locations. As with pre-treatment performance there was a significant within-subject effect of trial in latency to locate the novel platform,  $F(7) = 37.10$ ,  $p < 0.001$ . Both control and ADX rats lowered their latencies from Trial 1 ( $11.74 \pm 2.98$  seconds for controls and  $19.66 \pm 1.70$  seconds for ADX rats) to Trial 8 ( $3.05 \pm 0.41$  seconds for controls and  $3.74 \pm 0.23$  seconds for ADX rats).

Control and ADX rats differed significantly in their ability to locate a familiar, hidden platform location,  $F(1,39) = 8.95$ ,  $p = 0.005$ . Control rats took on average  $3.14 \pm 0.27$  seconds to locate the familiar platform location, with ADX rats requiring a mean of  $4.07 \pm 0.15$  seconds (Figure 8B). As with novel platform locations there was no significant effect of day for familiar platform locations,  $F(5) = 0.223$ ,  $p = 0.801$ , again implying asymptotic latencies in both groups. A significant within-subject effect of trial

was evident,  $F(7) = 5.99$ ,  $p < 0.001$ , with both control and ADX groups lowering their latency to locate the familiar platform from Trial 1 (controls  $4.15 \pm 1.07$  seconds, ADX  $6.06 \pm 0.61$  seconds) to Trial 8 (controls  $2.54 \pm 0.32$  seconds, ADX  $3.45 \pm 0.18$  seconds).

A one-way ANOVA was run to determine if between-group variability in swim speed could account for the significant differences in latency to locate the hidden platform. Analysis revealed no significant difference between control and ADX rats,  $F(1,39) = 2.819$ ,  $p = 0.101$ .

## Discussion

Control rats were able to successfully learn and remember several hidden platform locations in a moving platform version of the Morris water task. This is evident in the decrease in latency to locate the platform within each session as well as across several sessions. After six weeks of granule cell layer degeneration and prior to CORT replacement ADX rats performed significantly worse than controls during novel platform locations in the Morris water task. The behavioural deficits we observe in the Morris water task in ADX rats prior to CORT replacement are similar to those spatial deficits previously reported as a result of long-term ADX (Armstrong et al., 1993; Islam et al., 1995). These findings are also in agreement with studies using colchicine as a method to damage the dentate gyrus granule cell layer (Sutherland et al., 1983; Xavier et al., 1999; Jeltsch et al., 2001). Our results add support to a body of evidence that suggests the dentate gyrus plays an important role in spatial navigation in the rat.

After six weeks of CORT replacement, behavioural testing revealed a deficit in ADX rats not only in novel platform locations but also in familiar locations. Thus, the loss of CORT *per se* is not sufficient to account for the spatial deficits in ADX rats. Additionally the results are not explained by a difference in swim speed between control or ADX rats. The spatial deficits in ADX rats in the Morris water task do not disappear as a result of chronic CORT replacement.

Our findings differ from those of McCormick et al. (1997) who report that replacement of CORT was sufficient to reverse the behavioural deficits associated with ADX in the Morris water task. Accordingly, the results of McCormick et al. (1997) suggest that hippocampal granule cells are not important for normal spatial learning. A possible explanation for the difference between this and the McCormick et al. (1997) study is the version of the Morris water task employed to assess spatial behaviour. Within task manipulations have been shown to influence the involvement of the hippocampus (Dudchenko et al., 2000; Rudy et al., 2002). The simple manipulation of employing multiple platform pairings in the current study compared to a single pair in the McCormick et al. (1997) study may serve to alter hippocampal involvement.

The main finding from this study supports the idea that the granule cells of the hippocampus are critical for normal spatial leaning. Furthermore, we provide evidence that the spatial learning deficits associated with long-term ADX are a result of granule cell loss and are not explained by a loss of CORT alone. To further examine this phenomenon we tested rats in a battery of object discrimination tasks. Specifically, we

evaluated performance of ADX rats on CORT replacement in a novel object, object/place and object/context mismatch task.

### **Experiment Three**

#### **Object-Context Specific Memory Deficits Associated With Loss Of Hippocampal Granule Cells After Adrenalectomy**

##### Methods

Fifty-two male Long-Evans rats were obtained from the University of Lethbridge breeding colony. All rats were housed in pairs in a 12-hour light/dark cycle and had ad libitum access to food and water during the extent of the experiment. At the start of the experiment rats were approximately 50 days of age. As before all experimental procedures were conducting according to the guidelines of the Animal Welfare Committee at the University of Lethbridge and the CCAC.

##### Surgery

Adrenalectomy was performed as has been described before (p. 33). All ADX rats received 0.9% saline in lieu of drinking water for the extent of the study. Six weeks after ADX surgery, blood samples were collected and analyzed as described previously (p. 34).

##### Behaviour

Starting at 10 weeks, for one week prior to, and during behavioral testing, rats received a daily oral administration of replacement CORT (p. 55). After one week of

CORT replacement rats were evaluated in one of three object exploration tasks. Rats were initially pre-exposed to the discrimination context (devoid of any objects) once a day for 10 minutes, for a total of two days prior to behavioral testing. For novel object recognition and object/place discrimination the context consisted of a white, square plastic box approximately 60 cm by 60 cm in size with standard housing bedding on the floor. Prior to each exposure the walls of the context were wiped down with a cleaning solution (Clinicide, Bimeda-MTC). On the third day behavior was assessed. In the novel object recognition task, rats were placed in the context and allowed to explore two identical objects for five minutes. Rats were then removed from the context for a period of 10 minutes, during which the context and objects were cleaned and one of the objects was replaced with an object the rat had not previously encountered (Figure 9A). After the 10-minute delay period, rats were re-introduced to the context for three minutes and allowed to investigate the objects. The object/place task was run in a similar fashion, except that one of the identical pair of objects was moved relative to its previous location (Figure 9B). An investigation ratio was calculated for both the novel object and the object/place tasks by dividing the time spent investigating the novel object/place by the total time spent investigating both objects.

The object/context mismatch was performed in a similar fashion to that as originally described by Mumby et al. (2002). Pre-exposure for the object/context mismatch involved exposing rats to two different contexts for 10 minutes each, one immediately after the other, each day, for a total of two days. During the pre-exposure period the contexts were devoid of any objects. Context A was a white, square plastic box

60 x 60 cm in size, housed in a brightly lit room devoid of other cues. Context B was a large black, circular bin 60 cm in diameter and was housed in a cue rich, dimly light room. Both contexts had standard bedding on the floor and the walls of both were wiped clean prior to each exposure.

On test day each context housed a unique pair of identical objects and rats were given five minutes to explore each context, one immediately after the other. After exposure to both contexts and a five-minute delay period (during which both contexts and objects were cleaned), rats were re-exposed to one of them, this time with one object from each (Figure 9C). As with the other discrimination tasks, rats were allowed to explore for three minutes. As above, an investigation ratio was calculated by determining the time spent investigating the novel/mismatched object and dividing it by the total time spent investigating both.

### Histology

Upon completion of behavioural testing rats were perfused using the same method as has been discussed earlier (p. 34). Once ready to cut, brains were sectioned on a frozen sliding microtome employing a section-sampling fraction of 1/12 at a thickness of 40 microns. To determine the effect of ADX on granule cell layer volume a single series was labeled with DAPI utilizing the same technique as previously mentioned (p. 41). Completeness of ADX was determined using CORT analysis (p. 34) and hippocampal granule cell layer volume (p. 42).

## Results

An alpha level of 0.05 was used for statistical analysis and all means are reported as plus/minus standard error of the mean. Analysis of CORT levels, dentate gyrus volume and granule cell number have been reported in chapter 2 (p. 43). Given that incomplete ADX does not reduce CORT levels enough to produce a loss of dentate granule cells (Sloviter et al., 1989) and incomplete ADX animals have been shown to act similarly to controls in various tests of memory (McCormick et al., 1997), incomplete ADX animals were grouped with control animals for the purposes of this study.

The combination of incomplete ADX and control rats was further supported by Cavalieri volume estimates, with a one-way ANOVA ( $F(2,49) = 18.52, p < 0.001$ ) revealing a significantly lower dentate granule cell layer volume in ADX rats compared to both controls (Bonferroni post-hoc,  $p < 0.001$ ), and incomplete ADX animals ( $p = < 0.001$ ). No significant volume difference was noted between incomplete ADX and controls ( $p = 1.00$ ). Dentate granule cell layer volumes were as follows: controls  $2.93 \pm 0.06 \text{ mm}^3$ , incomplete ADX  $2.96 \pm 0.10 \text{ mm}^3$ , and ADX  $2.13 \pm 0.14 \text{ mm}^3$  (Figure 4A, Chapter 2). Mean Coefficients of Error were  $0.046 \pm 0.002$  for Controls,  $0.046 \pm 0.004$  for incomplete ADX, and  $0.057 \pm 0.003$  for ADX rats. The pattern of granule cell loss in ADX rats was similar to previous reports (Conrad and Roy 1995; Roozendaal et al. 1998; Spanswick et al. 2007) with the dorsal blade of the dentate gyrus displaying the highest level of degeneration. The dentate gyrus granule cell layer of incomplete ADX rats was indistinguishable from that of controls (Figure 4A, Chapter 2).

## Behavior

Statistical analysis revealed no significant difference between control and ADX rats in regards to time spent investigating the identical object pair during the trial phase of novel object recognition,  $F(1,16) = 0.214$ ,  $p = 0.65$ . Control rats spent  $62.27 \pm 8.16$  seconds investigating the object pair with ADX rats investigating the objects for  $67.49 \pm 7.41$  seconds. The mean investigation ratio for control rats during the test phase of novel object recognition was  $0.70 \pm 0.04$ , while ADX rats had an average investigation ratio of  $0.70 \pm 0.03$  (Figure 10A). As determined by a one sample t-test, investigation ratios for controls and ADX during novel object recognition differed significantly from chance (0.5),  $t(8) = 4.39$ ,  $p < 0.01$  and  $t(7) = 6.41$ ,  $p < 0.01$  respectively. A one-way ANOVA revealed no significant difference between investigation ratios for controls and ADX rats  $F(1,16) = 0.003$ ,  $p = 0.957$ .

Time spent investigating the object pair during the trial phase of object/place recognition did not differ significantly between groups,  $F(1,17) = 1.041$ ,  $p = 0.322$ , with control and ADX rats investigating the objects an average of  $71.37 \pm 6.13$  and  $81.09 \pm 7.33$  seconds respectively. The mean investigation ratio for control rats during the test phase of object/place recognition was  $0.59 \pm 0.04$  and as determined by a one-sample t-test differed significantly from chance,  $t(10) = 2.39$ ,  $p < 0.05$ . ADX rats had a mean investigation ratio of  $0.55 \pm 0.02$  which also differed significantly from chance levels,  $t(7) = 2.464$ ,  $p < 0.05$ . The investigation ratio for controls and ADX rats did not differ significantly from one another,  $F(1,17) = 0.618$ ,  $p = 0.443$  (Figure 10B).

Time spent investigating the object pairs did not differ significantly between control and ADX rats during trial phase one,  $F(1,14) = 0.49$ ,  $p = 0.495$ , or trial phase two,  $F(1,14) = 1.079$ ,  $p = 0.316$ . Trial phase one investigation times were  $91.58 \pm 10.94$  seconds for controls and  $103.83 \pm 13.30$  seconds for ADX rats. Control rats investigated the object pair for  $83.90 \pm 8.25$  seconds during trial phase two, with ADX rats investigating the objects for  $100.30 \pm 15.23$  seconds. During the context/object mismatch task, control rats spent significantly more time investigating the novel context/object pairing with a mean investigation ratio of  $0.68 \pm 0.04$  which, as determined by a one sample t-test, differed significantly from chance levels,  $t(8) = 4.573$ ,  $p < 0.05$ . ADX rats did not investigate the novel pairing at levels greater than chance (mean =  $0.54 \pm 0.03$ ),  $t(6) = 1.15$ ,  $p = 0.295$ . Analysis of variance revealed a significant difference between the investigation ratios of control and ADX rats, with controls investigating the object out of context more than ADX rats,  $F(1,14) = 7.29$ ,  $p < 0.05$  (Figure 10C). A significant correlation between investigation of the out of context object and granule cell layer volume was also apparent,  $r(16) = 0.499$ ,  $p < 0.05$ .

## Discussion

Control animals were able to successfully discriminate between a previously encountered and novel object, between objects that were in familiar vs. new locations, and between objects that were in expected vs. unexpected contexts. This is evident in their preferential exploration of the novel object, displaced object, or out-of-context objects. Importantly, animals with dentate granule cell loss were similar to controls in both the novel object recognition task and the displaced object task. Only during the

object/context mismatch task did animals with dentate granule cell loss differ significantly from controls and fail to discriminate between the out-of-context vs. in-context objects. As control and ADX rats investigated the objects during the trial phase of exploration similarly, the memory deficits we observe are not due to a lack of object investigation.

It is important to note that at the time of behavioral testing (as well as one week prior) our ADX animals were on replacement CORT. Experiment one demonstrates that the method of CORT we utilize is sufficient to mimic a relatively normal diurnal rhythm in ADX rats. As mentioned above, there is debate surrounding behavioral deficits as a result of chronic ADX as both removal of circulating CORT and degeneration of the granule cell layer may be responsible for observed deficits in ADX animals. Several studies have indicated that replacement of CORT is sufficient to at least partially reverse the behavioral deficits associated with ADX (Conrad & Roy, 1995; McCormick et al., 1997). Our current findings, as well the results of experiment 2, and those using colchicine as a method to ablate the dentate gyrus (Sutherland et al., 1983; Xavier et al., 1999; Jeltsch et al., 2001) do not support this conclusion. Conversely, small lesions limited to the CA3 region of the hippocampus have been shown to elevate circulating CORT levels and induce deficits in spatial tasks that are subsequently reversed by the synthesis inhibitor metyrapone (Roozendaal et al. 2001). This has been taken as evidence that increases in circulating CORT are responsible for spatial deficits as a result of damage to the hippocampus.

As previously discussed (p. 71), a potential explanation for the disparity between the reports of ADX-induced deficits may be the sensitivity of the task used to assess behavior. Here we provide evidence of another behavioral task that is highly sensitive to the loss of granule cells as a result of ADX. Given the significant correlation between granule cell number and behavioral performance in the object/context mismatch task the loss of CORT alone is not sufficient to account for these deficits.

The current findings suggest that the granule cells of the dentate gyrus are critical when information about context is important for making a discrimination. An alternative explanation is that the object/context shift task was simply more difficult than both the object place and novel object recognition tasks and thus engaged the hippocampus more than the other discrimination tasks. Using a delay versus trace conditioning paradigm Beylin et al. (2001) find that the hippocampus may become engaged simply as a function of task difficulty. In contrast to the results found by Beylin et al. (2001), Agster et al. (2002) note that rats with lesions limited to the hippocampus do not differ from controls in difficult tasks that place heavy demands on memory. Several others have reported similar findings, suggesting that task difficulty may not activate the hippocampus preferentially (Bunsey & Eichenbaum, 1996; Dudchenko et al., 2000).

The current results, specifically the decrease in ability of control rats to make object/place discriminations when compared to either novel object recognition or in the object/context mismatch task (Figure 10), could potentially be explained as due to an increase in task difficulty. It has been previously reported, task difficulty preferentially

engages the hippocampus (Beylin et al. 2001) one would expect rats with dentate gyrus granule cell loss to show deficits relative to controls. This is not what we observe. Rather, rats with granule cell loss discriminate at similar levels as controls when an object is displaced from its original position, suggesting that task difficulty is not preferentially engaging the hippocampus. Instead, a deficit is only observed in granule cell depleted rats when discrimination in controls is relatively high, further supporting the idea that task difficulty is not responsible for the deficit we observe.

An alternative explanation for the relatively poor performance of both control and ADX rats in the object/place discrimination task (~ 60%) may be the method employed to assess place discrimination. Although the room in which the rats were tested contained a number of contextual cues, the box itself was devoid of any. In our object/place discrimination task we relocated one object from one corner to another, perhaps inadvertently increasing difficulty due to the symmetrical nature of the box. Despite control and ADX rats both discriminating at significantly greater than chance levels the discrimination levels were lower than our other tasks, potentially obscuring a deficit.

Here we show that rats with a significant loss of granule cells in the dentate gyrus fail to discriminate between a novel and previously encountered object/context pair but are unimpaired in both novel object and object/place discriminations. In this study we use a relatively short retention interval (5-10 min), similar to those previously employed for the object context mismatch task (Mumby et al. 2002; O'Brien et al. 2006). Despite the short retention, the behavior of ADX rats was impaired specifically in the object/context

mismatch task. Prior research has demonstrated that larger disruptions of normal dentate gyrus function can also impair novel object recognition (Lee et al. 2005; Jessberger et al. 2009) as well as spatial behavior (Sutherland et al. 1983; Xavier et al. 1999; Gilbert et al. 2001). A possible explanation for this disparity is that discriminations based upon context may typically require a higher degree of computation, perhaps due to an increase of complexity in information load, resulting in a greater sensitivity to dentate gyrus granule cell layer disruption. Thus, a longer degeneration period in ADX animals (resulting in greater granule cell loss) may eventually produce deficits in novel object and object/place discriminations. It is worthy of note that Lee et al. (2005) report behavioral deficits after colchicine infusions into dorsal dentate gyrus (resulting in a 97% loss of granule cells in that region) in a novel object recognition paradigm. Lee and colleagues utilized a configuration of five objects, whereas in the current study we employ only two, suggesting that information load may indeed explain the inconsistency.

An alternate possibility is that animals with dentate gyrus granule cell loss are more susceptible to interference during the object/context mismatch task. Hippocampal granule cells may contribute to segregating two similar object exploration memories in an attempt to reduce interference due to overlap, a process sometimes referred to as pattern separation (McClelland and Goddard 1996; Norman and O'Reilly 2003; Leutgeb et al. 2007). Multiple theories have hypothesized the hippocampus as a structure that might serve to reduce interference (Marr 1971; Shapiro and Olton 1994, McClelland et al. 1995; Rolls, 1996). Given its sparse level of activity (Barnes et al. 1990; Chawla et al. 2005), particular attention has been paid to the dentate gyrus as a potential mediator of pattern

separation (Gilbert et al. 2001; Lee et al. 2004; Leutgeb et al 2007; Clelland et al. 2009). A failure in pattern separation may provide an explanation for the deficits we observe during the object/context mismatch task in our ADX rats.

Our finding that the selective loss of dentate gyrus granule cells produces a deficit in object memory only when the rat must remember the context in which the object had been previously encountered adds to a converging body of evidence that suggests the hippocampus is essential in storing and retrieving certain types of context memories (Rudy, 2009). At face value the results indicate that the hippocampal granule cells contribute to segregating two similar memories of object exploration, a process sometimes referred to as pattern separation (McClelland & Goddard, 1996; Norman & O'Reilly, 2003, Leutgeb et al., 2007). Specifically, we show that hippocampal granule cells are necessary for supporting associations between objects and contexts. Furthermore we find that adrenalectomy produces behavioral deficits in rats despite CORT replacement, clearly showing that loss of CORT is not responsible alone for the behavioral deficits associated with chronic adrenalectomy.

## **General Discussion**

In the current chapter we performed electrophysiological and behavioural assays to assess the function of the damaged dentate gyrus. The results from this set of experiments show that long-term ADX produces loss of function at the electrophysiological and behavioural level. Preliminary data from experiment 1 demonstrate that at the very least, field excitatory post-synaptic potentials are muted in

chronic ADX animals. These alterations in the electrophysiological properties of the dentate gyrus were apparent despite ADX rats being administered replacement CORT. The loss of hippocampal granule cells as a result of long-term ADX results in measurable electrophysiological deficits in the awake, freely moving rat.

Long-term ADX produces behavioural deficits in two tasks that are not ameliorated by replacement CORT. ADX rats on CORT replacement are impaired relative to controls in a moving platform version of the Morris water task. This is evident in the increased latency of long-term ADX rats to locate both a novel and familiar hidden platform location. Behavioural impairments were also apparent in the context/object mismatch task, during which ADX rats did not investigate the out of context object at greater than chance levels. These findings add to a body of evidence that suggests that hippocampal granule cells are critical for spatial navigation (Sutherland et al. 1983; Xavier et al. 1999; Gilbert et al. 2001) and situations in which information about context is important (Leutgeb et al., 2007; McHugh et al., 2007).

The object/context mismatch task provides a sensitive index to measure a behaviour that is dependent upon the integrity of the hippocampal granule cell layer. This is evident in the positive correlation between granule cell layer volume and discrimination ability in rats. Such a method allows us to not only reliably assess behavioural deficits as a result of granule cell layer damage but also provides us the opportunity to examine potential treatment strategies. One such strategy is to repopulate

the damaged layer with adult-born granule cells that are able to support functional recovery.

## Chapter Four

### Continuing Neurogenesis In The Adrenalectomized Brain

It is well established that the birth of new neurons in the brain continues throughout all of adulthood (Alvarez-Bulleya & Garcio-Verdugo, 2002; Kempermann et al., 2004). Numerous modulators of the neurogenic process have been discovered, ranging from the administration of pharmacological agents (Malberg et al., 2000; Santarelli et al., 2003), growth factors (Kuhn et al., 1997), exercise (Kempermann et al., 1997; Olson et al., 2006), environmental enrichment (Kempermann et al., 1997; Brown et al., 2003), to stress and stress hormones (Gould et al., 1992; Cameron & Gould, 1994; Tanapat et al., 2001), to name just a few.

One of the first descriptions of neurogenic modulation in the adult was provided by Gould and colleagues in 1992. They measured an increase in proliferation in the adult dentate gyrus after ADX. Gould et al. (1992) adrenalectomized rats and six days later administered a single dose of tritiated thymidine ( $^3\text{H}$ -thymidine). They noted a significant (almost three-fold) increase in the density of  $^3\text{H}$ -thymidine labeled cells in ADX rats compared to controls. Further studies have confirmed this phenomenon, finding increases in proliferation within the dentate gyrus at time-points ranging from six to twelve days post-ADX (Cameron & Gould, 1994; Montaron et al., 1999; Wong & Herbert, 2005). Unfortunately all of the data regarding increases in proliferation after ADX in the adult have been collected at acute time-points (Gould et al., 1992; Cameron & Gould, 1994; Montaron et al., 1999; Wong & Herbert, 2005).

In an important set of experiments, Brunson et al. (2005) adrenalectomized rats at postnatal day 10 and then supplemented them with low levels of CORT (10 mg/L in the drinking solution) for the remainder of the study. They report no significant increase in proliferation (as determined by injections of BrdU 24 hours prior to perfusion) at either three months or twelve months of age. Furthermore, they replicate the acute increase in neurogenesis in the hippocampus after ADX in adult rats (Gould et al., 1992; Cameron & Gould, 1994; Montaron et al., 1999; Wong & Herbert, 2005). Brunson et al. (2005) note that when CORT replacement was ceased in postnatal day 10 ADX rats, no such increase was evident. The results from this study suggest that the relationship between CORT and proliferation in the adult dentate gyrus is not as simple as was first proposed. Although lower levels of CORT are associated with increased neurogenesis at acute time-points, these findings suggest that compensatory mechanisms (discussed below) exist that return neurogenesis to a baseline level.

Other regulators of adult neurogenesis have been shown to produce only transient effects. For example, Naylor et al. (2005) show that short (nine-day) but not long (24-day) periods of wheel running induce an increase in dentate gyrus cell proliferation in adult mice. A similar phenomenon has been reported in an enriched environment (Kempermann & Gage, 1999). A number of studies have demonstrated that the selective serotonin reuptake inhibitor fluoxetine, administered daily for between one and four weeks results in increases in neurogenesis within the adult hippocampus (Malberg et al., 2000; Malberg & Duman, 2003; Santarelli et al., 2003; Huang & Herbert, 2006), specifically targeting a class of early neural progenitors (Encinas et al., 2006). We have

recently reported that chronic, daily administration (six-week) of fluoxetine did not result in a permanent increase in cell proliferation in the adult dentate gyrus (Spanswick et al., 2007).

In the current experiment we were interested in determining whether a transient increase in cell proliferation is associated with ADX. We adrenalectomized rats and quantified levels of proliferation at several time-points (ranging from 24 hours to four weeks) after ADX using the proliferative marker Ki67 (Scholzen & Gerdes, 2000; Amrein et al., 2004).

## **Experiment One**

### **Time-Course Of Adrenalectomy-Induced Increase In Proliferation In The Adult Dentate Gyrus Of The Rat**

#### Methods

Forty-six rats were taken from the breeding colony at the University of Lethbridge. Rats were housed as has been discussed before (p. 33). All procedures followed the guidelines set by the University of Lethbridge and CCAC animal welfare committees. At the beginning of the experiment rats were approximately 60 days of age.

#### Surgery

All surgical procedures were identical to those discussed before (p. 33). Rats underwent either bilateral adrenalectomy or corresponding control surgery. ADX rats

were provided with 0.9% saline in lieu of drinking water for the duration of the experiment.

### Corticosterone Analysis

Trunk blood was collected at the time of perfusion to determine the effectiveness of ADX surgery. Blood samples were processed and analyzed as described previously (p. 34) using a Coat-A-Count ® Corticosterone radioimmunoassay performed according to kit instructions.

### Histology

Rats were sacrificed at four time-points after surgery (24 hours, 1 week, 2 weeks, and 4 weeks). At each time-point after ADX, rats were perfused utilizing the method described previously (p. 34). Brains were sectioned at a thickness of 40 µm on a freezing sliding microtome at a section-sampling fraction of 1/5. A single series of sections was processed for the endogenous cell cycle marker Ki67.

### Ki67 Immunohistochemistry

To reduce endogenous peroxidase, tissue sections were incubated into 0.3% H<sub>2</sub>O<sub>2</sub> for 25 minutes. Tissue sections were then washed in 0.1M PBS three times for seven minutes each wash. Brain tissue was then transferred to a primary antibody solution containing a 1:1000 dilution of rabbit anti-Ki67 antibody (Novacastra) in 0.1M PBS, 0.5% Triton-X (Sigma) and 1% normal goat serum. Sections were incubated at room temperature in the primary solution for approximately 16 hours and then washed three

times in 0.1M PBS. The tissue was transferred to the secondary solution containing a 1:1000 dilution of anti-rabbit biotinylated antibody (Vector Laboratories Inc.) in 0.1M PBS and was incubated at room temperature for one hour and then washed three times in 0.1M PBS. An ABC kit (Vector Laboratories Inc.) was prepared according to kit instructions and tissue was incubated in this solution at room temperature for 45 minutes and then washed three times in 0.1M PBS. Visualization of the Ki67 antigen was produced using a DAB kit (Vector Laboratories Inc.). Sections were mounted onto glass slides, allowed to dry and dehydrated in increasing concentrations of ethanol and cover slipped with Permount® (Fisher Scientific).

### Quantification

Ki67 positive cells were quantified in the granule cell layer and the subgranular zone of the dentate gyrus. A cell was defined as positive for Ki67 if the signal was dissociable from background (greater than) and localized to the nucleus. All cells positive for Ki67 were counted in a single series of tissue (1/5 section sampling fraction) and summed. Cell counts were performed using a Nikon E-600 microscope matched to a 100X oil immersion objective.

### Results

An alpha level of 0.05 was utilized for statistical analysis, all means are reported as plus/minus the standard error of the mean. Three rats were removed from the study due to incomplete ADX as indicated by significantly higher CORT levels than their successfully adrenalectomized cohorts ( $p < 0.001$ ) and were not included in any further

statistical analysis. ADX significantly reduced CORT levels,  $F(1,35) = 101.55$ ,  $p < 0.01$ . Mean CORT levels for control rats was  $265.55 \pm 16.49$  ng/ml with ADX rats having an average of  $9.53 \pm 19.33$  ng/ml. There was also a significant effect of post-surgical time on CORT levels,  $F(3,35) = 4.35$ ,  $p = 0.01$ . Mean CORT levels were  $201.47 \pm 23.31$ ,  $83.29 \pm 23.31$ ,  $136.05 \pm 28.55$ , and  $129.35 \pm 26.07$  ng/ml at the 24-hour, one-week, two-week, and four-week time-points respectively. A significant surgery by time interaction was also apparent  $F(3,35) = 4.57$ ,  $p < 0.01$ . ADX rats had low levels of CORT across all time-points, ranging from  $6.41 \pm 32.97$  ng/ml at 24 hours post-surgery,  $8.91 \pm 32.97$  ng/ml one week after surgery,  $11.66 \pm 46.63$  ng/ml at two weeks, to  $11.15 \pm 40.38$  ng/ml at the four week time-point. Control rats showed high levels of CORT at the 24-hour time-point ( $396.53 \pm 32.97$  ng/ml), which decreased over the following weeks ( $157.67 \pm 32.97$ ,  $260.44 \pm 32.97$ ,  $247.54 \pm 32.97$  ng/ml at one, two, and four weeks, Figure 1A).

### Ki67

A significant main effect of surgery was detected,  $F(1,34) = 8.63$ ,  $p < 0.01$ . ADX rats expressed a mean of  $6730 \pm 473.25$  Ki67 positive cells and controls had a mean of  $4884 \pm 413.56$  cells positive for Ki67. There was also a significant main effect of time,  $F(1,34) = 11.37$ ,  $p < 0.001$ , with mean expression of Ki67 ranging from  $3549.17 \pm 570.76$  cells at 24 hours after surgery,  $8110 \pm 570.76$  cells at one week after surgery,  $6493.33 \pm 699.04$  cells two-weeks post surgery, to  $5075.50 \pm 663.17$  cells four weeks after surgery. There was no significant interaction between surgery and time,  $p = 0.35$  (Figure 11A).

Although further analysis was not required given the lack of a significant interaction term, our a priori prediction that increases in neurogenesis may be transient and the raw data suggested that group means may differ at 24 hours and one week post-ADX, but not at later time-points. To this end, t-tests were performed employing the Bonferroni correction factor resulting in an alpha level of 0.0125. One-tailed t-tests revealed a significant difference between ADX and control rats 24 hours after surgery in regards to the number of Ki67 positive cells  $t(10) = 4.64, p < 0.001$ . The control group had a mean of  $2586.67 \pm 779.00$  cells positive for Ki67 whereas ADX rats expressed a mean of  $4511.67 \pm 653.99$  Ki67 positive cells. At one-week after surgery groups did not differ significantly although there was a tendency for ADX rats to have more Ki67 positive cells than controls,  $t(10) = 1.88, p = 0.045$ . Control rats had a mean of  $6366.67 \pm 1506.71$  cell positive for Ki67, with ADX rats expressing on average  $9853.33 \pm 4284.91$  Ki67 positive cells. Groups did not differ significantly at either the two-week ( $p = 0.075$ ) or the four-week time-points ( $p = 0.36$ ).

A correlation analysis was performed on control and ADX groups. There was a significant inverse relationship between levels of CORT and number of Ki67 positive cells in control rats,  $r(19) = -0.60, p = 0.002$ , that was absent in ADX rats ( $p = 0.67$ ).

## Discussion

As expected, ADX produced a significant drop in CORT levels across all time-points. An interaction between time and surgical condition was evident, accounted for by

the unusually high levels of CORT in controls at 24 hours post-surgery, a factor likely caused by the stress associated with the surgical manipulation.

The results of Experiment 1 confirm previous findings of an increase in cell proliferation soon after ADX (Gould et al., 1992; Cameron & Gould, 1994; Montaron et al., 1999; Wong & Herbert, 2005). Our results show that cell proliferation is enhanced 24 hours after ADX and remains high at one-week post-surgery. Proliferation returns to levels similar to intact controls by two weeks and remains comparable to control levels at four weeks post-ADX. Importantly, this suggests that the ADX-induced increase in proliferation is only transient.

Given the potential inadequacies associated with our immunohistochemical (lack of complete antibody penetration, Melvin and Sutherland, 2009) and quantification techniques (specifically the lack of guard zones; Mouton, 2002) a further cohort of animals was procured and a replication was performed. One week and four weeks after ADX, tissue was analyzed for Ki67 using unbiased stereological techniques (West et al., 1991; Mouton, 2002). These time-points were chosen to represent the ensuing increase (one week) and return to baseline levels (four weeks) after ADX as determined by our prior experiment (p. 90).

## Methods

Rats were obtained from the University of Lethbridge breeding colony (n = 9). All animals were housed in standard conditions and had ad-libitum access to food and water

for the duration of the experiment. All methods were conducted according to the University of Lethbridge and CCAC animal welfare guidelines.

### Surgery

Rats received ADX or corresponding control surgery, post-surgical care was performed in an identical manner, mentioned previously (p. 33). All ADX rats received 0.9% saline in lieu of drinking water for the remainder of the experiment.

### Histology

Rats were perfused at either one or four-weeks post-surgery. Perfusion was performed in the same manner as discussed before (p. 34). Brains were sectioned on a freezing sliding microtome at a thickness of 40  $\mu$ m employing a section-sampling fraction of 1/12. A single series of tissue was processed for Ki67/DAPI.

### Ki67 Immunohistochemistry

Brain tissue was incubated on a lab rotator in a primary solution consisting of a 1:1000 rabbit anti-Ki67 antibody (Novacastra) and 0.3% Triton-X (Sigma) in 0.1M PBS for approximately 24 hours at room temperature. Tissue was then washed three times in 0.1M PBS for seven minutes per wash. A second incubation was performed in a solution of 0.1M PBS containing a 1:500 dilution of Cy3-conjugated donkey Anti-rabbit IgG (Jackson ImmunoResearch), sections remained in secondary for approximately 24 hours (Melvin & Sutherland, 2009). During the last 20 minutes of the incubation a 1:1000 dilution of DAPI (Sigma) was added to the well. Tissue was washed twice and then

mounted on gelatin coated glass slides and coverslipped using an anti-fade fluorescent mounting medium. Mounted slides were stored in the dark at 4 °C until quantification.

### Quantification

All quantification was performed using a Zeiss Axio Imager.M1 microscope matched to a 100x oil immersion objective. Total cell number estimates were performed utilizing the optical fractionator technique (West et al., 1991; Mouton, 2002). An estimate of the total number of Ki67 positive cells was determined using the equation:

$$E(N) = \sum O \times (1/ssf) \times (1/asf) \times (1/tsf)$$

Where E(N) is the estimate of the total number of cells,  $\sum O$  is the sum of the objects counted, ssf is the section sampling fraction, asf is the area sampling fraction, and tsf is the thickness sampling fraction (Mouton, 2002).

Immunohistochemical procedures resulted in a post-processing tissue thickness of 32  $\mu\text{m}$ . A dissector height of 20  $\mu\text{m}$  was employed, allowing for a guard zone of 6  $\mu\text{m}$  on both the top and bottom of the section. Given the relative infrequency of Ki67 positive cells an area-sampling fraction of one was employed, thus all cells on a given section were quantified (within the appropriate dissector height), resulting in the final equation:

$$E(N) = \sum O \times (1/(1/12)) \times (1) \times (1/(20/32))$$

Only cells that were positive for Ki67 and located within the subgranular zone or the granule cell layer of the dentate gyrus were quantified. In cases of high ADX-induced autofluorescence co-localization with DAPI was used to aid in identification (Spanswick et al., 2009). Cells that were visible under the TRITC and DAPI, but not FITC filters were deemed to be Ki67 positive.

## Results

All group means are reported as plus/minus standard error of the mean, an alpha level of 0.05 was used for all statistical analysis.

### Ki67

A two by two ANOVA revealed a significant main effect of surgery on the number of Ki67 positive cells present within the dentate gyrus,  $F(1,5) = 31.03$ ,  $p < 0.01$ . On average, control rats had  $4212.60 \pm 393.54$  cells positive for Ki67, with ADX rats expressing a mean of  $7464.00 \pm 431.10$  Ki67 positive cells. There was also a main effect of time,  $F(1,5) = 24.13$ ,  $p < 0.01$ , with rats in the one-week group expressing a mean of  $7271.80 \pm 431.10$  Ki67 positive cells. At the four-week time point the group mean was  $4404.80 \pm 393.54$  cells positive for Ki67. A significant interaction was apparent,  $F(1,5) = 6.78$ ,  $p < 0.05$ . The control group expressed mean of  $4886.00 \pm 609.67$  and  $3539.20 \pm 497.79$  Ki67 positive cells at one and four weeks respectively. The ADX group had a mean of  $9657.60 \pm 609.67$  cell positive for Ki67 at one week and  $5270.40 \pm 609.67$  Ki67 positive cells at four weeks (Figure 11B). Post-hoc tests revealed a significant difference between the number of Ki67 positive cells in ADX and control groups at the one-week

post-surgical time-point ( $t(2) = -5.91$ ,  $p < 0.05$ ) that was not evident at four weeks after ADX surgery ( $p = 0.125$ ).

## Discussion

Using unbiased stereological techniques we provide additional evidence that ADX produces a transient increase in proliferation as determined by Ki67 expression. We replicate our initial finding (p. 90) and that of others (Gould et al., 1992; Cameron & Gould, 1994; Montaron et al., 1999; Wong & Herbert, 2005) that at acute time-points after ADX (ranging from 6 to 12 days) proliferation within the adult dentate gyrus is significantly increased. We further replicate our finding that proliferation returns to control levels by four weeks.

Brunson et al. (2005) have previously reported levels of proliferation similar to control animals in 3 and 12 month old rats that were adrenalectomized on postnatal day 10. The results from the Brunson et al. (2005) study suggest that the increase in proliferation observed acutely after ADX may only be a brief phenomenon, our findings confirm this idea. We have previously demonstrated that 14 weeks after surgery, expression of Ki67 in ADX rats is similar to intact controls (Spanswick et al., 2007). These findings in combination with the current experiment demonstrate that ADX-induced increases in dentate gyrus proliferation are transient in nature.

The inverse relationship between CORT levels and the number of Ki67 positive cells in the dentate gyrus of control rats suggests that CORT normally inhibits

proliferation. This finding confirms prior reports of the effects of stress hormones on neurogenesis (Gould et al., 1992; Cameron & Gould, 1994; Mirescu & Gould, 2006).

The current findings fit with a larger body of evidence that indicates that many of the ways by which proliferation is up-regulated in the adult dentate gyrus only do so in a transient fashion. Similar results have been reported in an enriched environment (Kempermann & Gage, 1999), wheel running (Naylor et al., 2005; Kronenberg et al., 2006; Snyder et al., 2009), after administration of fluoxetine (Spanswick et al., 2007), and as a result of brain damage (Park & Enikolopov, 2010). The similarity in outcome with such diverse approaches suggests the presence of very important regulatory mechanisms that function to return levels of proliferation to a “normal” baseline in response to some perturbation.

A potential explanation for the return of enhanced proliferation to baseline levels is that differences in the expression of corticosterone receptors (specifically mineralocorticoid and glucocorticoid receptors) may underlie the up and down-regulation of neurogenesis in the adult brain (Kempermann, 2006). The mineralocorticoid (type 2) receptor is only expressed in mature cells in the dentate gyrus, whereas the glucocorticoid (type 1) receptor is expressed in both precursor and mature granule cells (Kempermann, 2006). Thus, it is possible that the effects of stress hormones on proliferation may be mediated through the glucocorticoid receptor itself. In agreement, Naylor et al. (2005) note that 24 days of wheel running not only fails to increase proliferation, but also increases the size of the adrenal glands relative to short-term (nine-day) running,

suggesting an increase in a stress-related response. Given the current results and those of Brunson et al. (2005) showing that the effects of adrenalectomy on proliferation seem to be transient, it is likely that the glucocorticoid receptor is working in concert with other regulatory factors. N-methyl-D-aspartate (NMDA) receptor activity has been shown to modulate the effects of corticosterone on neurogenesis, such that if the receptor is blocked the ensuing decrease in proliferation in response to corticosterone does not occur (Cameron et al., 1998).

The increase in proliferation of progenitor cells within the dentate gyrus in response to fluoxetine has also been shown to depend on corticosterone (Huang & Herbert, 2006; Alahmed & Herbert, 2008). By dampening the diurnal rhythm of CORT in rats via the implantation of a slow release CORT pellet, Huang & Herbert (2006) prevented the fluoxetine-induced increase in proliferation that is typically observed. In addition, they note that increased serotonin release (due to 8-OHDPAT, a 5-HT<sub>1A</sub> receptor agonist) is attenuated as a result of the dampened corticosterone rhythm. This finding suggests that corticosterone is playing a modulatory role in the function of 5-HT<sub>1A</sub> receptors.

Of importance for this thesis is the fact that the stem cell niche remains viable in the damaged dentate gyrus. Despite a significant loss of granule cells after ADX, proliferation (Experiment 1) and neural migration (Spanswick et al., 2007) continue at levels similar to intact controls. Furthermore, this seems to be unaffected by CORT replacement status. Here we show that ADX rats without replacement CORT, exhibit

levels of Ki67 similar to controls at two and four weeks after ADX. Previously we have demonstrated that ADX rats on replacement CORT for a period of eight weeks display levels of proliferation and neural migration in the dentate gyrus that is akin to controls (Spanswick et al., 2007). Taken together, these findings show that the birth of new neurons continues in the dentate gyrus despite significant damage to the granule cell layer.

Neurogenesis within the normal adult dentate gyrus can be modulated by a myriad of factors (Kempermann, 2006). Increases (Kuhn et al., 1997; Kempermann et al., 1998; Malberg et al., 2000) and decreases (Parent et al., 1999; Tanapat et al., 2001; Bizon & Gallagher, 2003) of neurogenesis are commonly reported as a result of numerous stimuli. Given our results showing that neurogenesis continues in the damaged dentate gyrus we want to determine if the dentate gyrus progenitor cell niche in the long-term ADX rat could be modulated by factors that have been demonstrated to up-regulate neurogenesis in the normal animal. Specifically we are interested in determining if neurogenesis could be up-regulated via exercise (Kempermann et al., 1997; Olson et al., 2006) and environmental enrichment (Nilson et al., 1999; Kempermann, 2006) within the damaged dentate gyrus in an attempt to replace granule cells that had been lost as a result of ADX.

Indirect evidence from our earlier work (Spanswick et al., 2007) suggests that this may be the case. We found no effect of eight weeks of daily fluoxetine administration on expression of Ki67. This finding differs from other reports describing an increase in neurogenesis as a result of chronic administration of fluoxetine (Malberg et al., 2000;

Kodama et al., 2004). Eight weeks of administration greatly exceeds the duration used in previous studies. Despite a lack of a significant difference in neurogenesis between ADX and controls, there was a trend for ADX rats that were treated with fluoxetine to have a thicker dentate than those that were untreated, despite a lack of a significant difference in neurogenesis between ADX and controls after eight weeks of treatment. This suggests that fluoxetine may have induced a transient increase in neurogenesis, resulting in a thicker dentate gyrus granule cell layer.

In the following experiment we utilize a combination of exercise and environmental enrichment in an attempt to up-regulate neurogenesis in the ADX brain. We assess the levels of doublecortin present in the dentate gyrus of ADX rats and utilize Cavalieri volume estimates to determine the effect of increasing neurogenesis on the damaged dentate gyrus granule cell layer.

## **Experiment Two**

### **Combined Environmental Enrichment And Exercise Increases Neurogenesis In The Damaged Dentate Gyrus**

#### **Methods**

A number of rats ( $n = 46$ ) were obtained from the University of Lethbridge breeding colony. All rats were housed as previously discussed (p. 33) and all experimental procedures were performed in accordance to University of Lethbridge and CCAC animal welfare guidelines.

### Surgery

All surgical procedures were identical to those discussed before (p. 34). At approximately 60 days of age rats underwent either ADX or corresponding control surgery. ADX rats were provided with 0.9% saline in lieu of drinking water for the duration of the experiment. Rats remained in their home cages for a period of 10 weeks prior to subsequent experimental manipulations. During the 10-week degeneration period rats were weighed each week.

### Hormone Replacement

After the 10-week degeneration period rats began to receive CORT replacement therapy for the remainder of the experiment. As described before (p. 55), rats received an oral dose of CORT each day approximately three hours prior to lights out. Control rats received vehicle without the CORT suspension.

### Enrichment/Wheel Running

For a total of six weeks, rats were placed in 24-hour alternating conditions of group-housed environmental enrichment and individual access to a running wheel. The enriched environment consisted of a circular pool 150 centimeters in diameter and 45 centimeters in height. A wire mesh was placed over the top to prevent escape. The pool was filled with a variety of toys, different lengths of polyvinyl tubing and different substrates in an attempt to encourage exploration. Rats were group housed (n = 6) in the enriched environment in order to induce social interaction beyond that experienced in the home cage. Toys were rearranged daily and exchanged on a weekly basis to maintain the

novelty of the environment. Rats were individually transferred to wheel running cages (Mini Mitter, Bend, OR, USA) each of which contained a counter in order to monitor running behavior. All rats had ad libitum access to food and water for the duration of the enrichment/wheel running condition. As a control, a group of ADX rats and a group of control surgery rats were left in their home-cages for the duration of the experiment.

### Histology

Upon termination of the study rats were perfused according to our standard protocol (p. 34). All brains were sectioned employing a 1/12 section sampling fraction at a thickness of 40  $\mu\text{m}$ . A single series of tissue was labeled with DAPI (p. 41) for volumetric quantification of the granule cell layer within the dentate gyrus.

### Immunohistochemistry

A series of tissue was labeled with doublecortin (DCX). Free floating sections were incubated for 24 hours on a lab rotator in a primary solution containing a 1:1000 dilution of goat doublecortin antibody (Santa Cruz Biotechnology) in 0.1M PBS and 0.3% Triton-X (Sigma). Sections were rinsed three times in 0.1M PBS and then incubated in a biotinylated rabbit anti-goat secondary (1:1000 in 0.1M PBS, Vector Labs) for a further 24 hours. Sections were then rinsed again in 0.1M PBS and transferred to a 1:500 tertiary solution of Streptavidin-conjugated Alexa 568 (Molecular Probes) in 0.1M PBS for one hour. As with DAPI, sections were mounted on 1% gelatin covered glass slides and coverslipped with an anti-fade mounting medium.

## Quantification

The volume of the dentate gyrus granule cell layer was determined using the Cavalieri method (Chapter 2, Experiment 2). Images of DAPI labeled sections from a single series of tissue were taken using a Zeiss Axioskop 2 MotPlus epifluorescent scope attached to a QImaging Retiga CCD camera (Burnaby, British Columbia, Canada). Images were then analyzed using ImageJ software (<http://rsb.info.nih.gov/ij/>) in which a sampling grid with an area per point of 0.02 square millimeters was created and randomly thrown over each image. The total number of points in contact with the granule cell layer in each section was counted. The number of points per section was multiplied by the area associated with each point, the section thickness and then the section sampling fraction. These numbers were summed to provide the total estimated volume of the granule cell layer within the dentate gyrus. To ensure that volume estimation using ImageJ software produced similar results to the method previously reported in chapter 2 (p. 43) utilizing MicroBrightField software, a control experiment was performed (Appendix 1).

A subset of rats from each control group (home-cage  $n = 8$ , enrichment  $n = 6$ ) were analyzed for DCX, all ADX animals were included in the analysis. DCX was quantified using a Zeiss Axioskop 2 MotPlus epifluorescent scope using a Plan Neofluor 20x/0.5 objective through a TRITC filter. If necessary, a 40x/0.75 objective was used to further delineate cell bodies. Given the relative frequency of cells expressing DCX, we quantified every second section within a single series in a single hemisphere (resulting in a section sampling fraction of 1/24). The total number of DCX positive cells was summed across all sections. Only those cells exhibiting a cytoplasmic DCX positive

signal in the granule cell layer or sub-granular zone were counted. DCX positive cells were counted as they came into focus in an attempt to reduce double counts.

## Results

An alpha level of 0.05 was employed for all statistical analyses and group means are reported as plus/minus standard error of the mean. ADX resulted in a significant decrease in CORT levels,  $F(1,29) = 71.32$ ,  $p < 0.01$ . Mean CORT levels were  $373.37 (\pm 26.43)$  ng/ml and  $18.169 (\pm 32.717)$  ng/ml for controls and ADX rats respectively. There was no significant effect of housing condition (home-cage vs. enrichment),  $p = 0.58$  and no significant surgery by housing interaction,  $p = 0.56$ . A number of ADX rats ( $n = 13$ ) displayed CORT levels that were significantly greater than their ADX cohorts ( $p < 0.001$ ), indicating incomplete ADX. These rats were removed from the study and were not included in the final data analysis.

### Enrichment/Wheel Running

All rats given access to a running wheel ran throughout the alternating enrichment/wheel running paradigm. There was no effect of ADX surgery ( $p = 0.93$ ) on the amount of running throughout the experiment. On average, control rats ran  $568.76 \pm 138.77$  revolutions per day and ADX rats ran  $589.31 \pm 169.95$  revolutions. There was also no significant effect of day ( $p = 0.23$ ) or day by surgery interaction ( $p = 0.64$ ).

### Granule Cell Layer Volume

As determined by the Cavalieri method, ADX resulted in a significant decrease in dentate gyrus granule cell layer volume,  $F(1,21) = 32.11$ ,  $p < 0.01$ . Control rats had a mean dentate gyrus granule cell layer volume of  $2.89 (\pm 0.08)$  cubic millimeters and ADX animals had a mean volume of  $2.12 (\pm 0.11)$  cubic millimeters. There was a tendency for a difference between housing conditions (home-cage vs. enrichment),  $F(1,21) = 3.79$ ,  $p = 0.065$ , with home-cage rats having a mean volume of  $2.37 (\pm 0.10)$  cubic millimeters and enriched environment animals having a mean volume of  $2.64 (\pm 0.09)$  cubic millimeters. A tendency for a surgery by housing interaction was apparent,  $F(1,21) = 3.55$ ,  $p = 0.073$ , with control animals in the home-cage and enriched environment having mean volumes of  $2.89 (\pm 0.12)$  and  $2.90 (\pm 0.11)$  cubic millimeters respectively. ADX animals had a mean volume of  $1.86 (\pm 0.16)$  cubic millimeters in the home-cage and  $2.38 (\pm 0.15)$  cubic millimeters in the enriched environment (Figure 12A).

To determine the exact nature of the differences in granule cell layer volume, pairwise comparisons were performed. A significant difference between control rats ( $2.89 \pm 0.12$  cubic millimeters) and ADX rats ( $1.86 \pm 0.16$  cubic millimeters) housed in their home-cages was apparent,  $F(1,21) = 25.55$ ,  $p < 0.001$ . Further pairwise comparisons revealed a significant volumetric difference between ADX animals that remained in the home-cage ( $1.86 \pm 0.16$  cubic millimeters) versus those kept in the enriched environment ( $2.38 \pm 0.15$  cubic millimeters),  $F(1,21) = 5.74$ ,  $p = 0.026$ . A significant difference was also apparent between control and ADX rats within the enriched environment ( $2.90 \pm$

0.11 and  $2.38 \pm 0.15$  cubic millimeters respectively),  $F(1,21) = 8.09$ ,  $p = 0.01$ , but no significant difference was evident between control animals kept in the home-cage and those housed in the enriched environment,  $p = 0.96$ .

### Doublecortin

Doublecortin positive cells were present in all groups and were typically located in the sub-granular zone or inner third of the granule cell layer (Figures 12B, 13A-D). ANOVA revealed a significant main effect of housing condition (home-cage vs. enrichment),  $F(1,23) = 8.24$ ,  $p < 0.01$ . Mean number of doublecortin positive cells was  $308.80 (\pm 27.54)$  in the home-cage rats compared to  $427.25 (\pm 30.72)$  in the enrichment group. There was no significant effect of ADX ( $p = 0.12$ ) or surgery by housing condition interaction ( $p = 0.71$ ).

## Discussion

Ten weeks of ADX was effective in significantly reducing the volume of the dentate gyrus granule cell layer. A combination of six weeks of environmental enrichment and wheel running increased the volume of the granule cell layer in ADX rats compared to those that remained in the home-cage. Hence, enhancing neurogenesis was successful in at least partially repopulating a damaged dentate gyrus in adult animals.

Using the Cavalieri method we discovered that approximately 30 percent of the dentate gyrus volume was lost after 10 weeks of ADX. This finding is consistent with others that have reported a loss of granule cells after long-term ADX (Sloviter et al.,

1989, Armstrong et al., 1993; Roozental et al., 1998), as well as our previous reports (p. 43; Spanswick et al., 2007). Despite the significant depletion of granule cells in the dentate gyrus caused by ADX, hippocampal neurogenesis continued, suggesting that the sub-granular zone remains viable after long-term ADX. Cells positive for DCX, an endogenous marker of neuroblasts, were found in both ADX groups (home-cage and enrichment).

Increases in neurogenesis have been reported soon after ADX (Yehuda et al., 1989; Cameron & Gould, 1994). Specifically, Cameron and Gould (1994) and Yehuda et al. (1989) employed tritiated thymidine to show an increase in proliferation at seven and fourteen days respectively, after bilateral removal of the adrenal glands. Here we found comparable levels of DCX expression between home-cage rats that received 10 weeks of ADX followed by six weeks of CORT replacement and their respective home-cage control group. We confirm our previous finding (p. 90; Spanswick et al., 2007), as well as those reported by others (Brunson et al., 2005), that early stages of neurogenesis seem to return to control levels after a transient period of up-regulation after ADX.

Regardless of adrenal status, a significantly greater number of DCX positive cells were found in the rats that received the enriched environment and wheel running. This is consistent with extensive evidence suggesting that neurogenesis can be increased by enriched environmental housing (Kempermann et al., 1997; Auvergne et al., 2002; Brown et al., 2003) and exercise (van Praag et al., 1999; Rhodes et al., 2003). It is believed that the effects of exercise and environmental enrichment are dissociable, such

that exercise specifically targets the proliferation of new cells. In contrast, the effects of enrichment are limited to their survival (Olson et al., 2006; Tashiro et al., 2007). The effects of exercise and enrichment on adult neurogenesis extended to the rats that received 10 weeks of ADX followed by six weeks of CORT replacement.

A combination of exercise and enrichment was used in the current study in an attempt to target the early (proliferation) and late (survival) stages of neurogenesis, maximizing the chances of re-populating the damaged dentate gyrus. The significantly higher levels of DCX in rats that received the enrichment/exercise treatment show that earlier stages of neurogenesis had indeed been up-regulated. The increase in volume of the dentate gyrus in ADX rats that experienced enrichment and exercise suggests that the survival of adult born granule cells was also increased.

Taken at face value our results differ from Naylor et al. (2005) who report a transient increase in proliferation as a result of voluntary wheel running at nine, but not 24 days in rats. Kempermann and Gage (1999) also demonstrated that short-term but not long-term exposure to an enriched environment increased proliferation in mice, suggesting a similar transient phenomenon. Here we show that after six weeks of combined enrichment/wheel running treatment levels of DCX are still significantly increased relative to controls. Differences in the method used to stimulate an increase in neurogenesis may provide an explanation.

In their comparison of the effects of short-term (nine-day) versus long-term (24-day) running on proliferation, Naylor et al. (2005) provided access to running wheels on consecutive days. Similarly, Kempermann and Gage (1999) included running wheels in their enriched environment allowing uninterrupted access on consecutive days. In the current study our rats were given access to running wheels every second day, alternated with environmental enrichment. By interleaving enrichment with exercise we may have extended the duration of the associated increase in proliferation. An alternate possibility is that switching between wheel running and a relatively novel, enriched environment every 24 hours may maintain a degree of novelty that has been suggested as responsible for increases in neurogenesis (Kempermann & Gage, 1999).

Here we report that after long-term ADX the sub-granular zone in the dentate gyrus remains a viable substrate for adult neurogenesis. New neurons continue to proliferate at a rate that is similar to intact animals. Furthermore, we provide evidence that neurogenesis can be manipulated in the damaged dentate gyrus. This up-regulation of neurogenesis is associated with an increase in dentate gyrus granule cell layer volume, suggesting that repopulation of the granule cell layer has occurred.

To provide more direct evidence of the survival of new neurons in the dentate gyrus of ADX rats a follow-up experiment was designed. Here we examine both early and later phases of survival of adult born neurons. Specifically we looked for the presence of NeuN, a protein only expressed in mature neurons (Kempermann, 2006), in adult born granule cells in the ADX animal. Rats were injected with the thymidine

analogue BrdU and two or eight weeks later perfused. Using confocal microscopy, BrdU labeling and its co-localization with the neuronal specific marker NeuN was assessed. Unfortunately, given the high levels of ADX-induced autofluorescence in the granule cell layer of the dentate gyrus assessment of BrdU/NeuN positive cells proved difficult. Following is a description of a published study designed to minimize the potential confound of autofluorescence in the damaged brain (Spanswick et al., 2009).

### **Experiment Three**

#### **A Novel Method For Reliable Nuclear Antibody Detection In Tissue With High Levels Of Pathology-Induced Autofluorescence**

An unfortunate consequence and potential problem in studying neurogenesis in the damaged brain is the autofluorescence that accompanies it. A number of studies report increased levels of autofluorescence as a result of brain damage (Brooke et al., 1996; Chung et al., 1997; Liu et al., 2002) and even aging (Brizzee et al., 1974; Amenta et al., 1988). Interestingly, the reliable manner in which damaged tissue autofluoresces has been suggested as a valuable method to dissociate between damaged and normal brain tissue (Chung et al., 1997; Bottiroli et al., 1998).

A clear dissociation between intended and autofluorescent signal is critical when quantifying brain phenomena. The co-localization of BrdU and NeuN for example, becomes very difficult in the damaged brain as a result of particles that fluoresce under a wide range of wavelengths of laser light. Thus, the difficulty in discriminating between

true BrdU labeled neurons and damage-induced fluorescence may artificially inflate numbers and distribution of neurogenesis after brain damage.

A variety of techniques have been put forth in an attempt to minimize or eliminate autofluorescence. Treatment of tissue with cupric sulfate or sudan black (Schnell et al., 1999) has been shown to reduce lipofuscin related autofluorescence. As well, treatment with sodium borohydride has proven useful in the reduction of glutaraldehyde-induced autofluorescence (Tagliaferro et al., 1997) and has also been applied to DNA microarrays (Raghavachari et al., 2003). The aforementioned methods all have disadvantages, one of which is the reduction of *all* fluorescent labeling.

Procedures such as sudan black and cupric sulfate have been documented to reduce intended immunofluorescent signal in addition to autofluorescence (Schnell et al., 1999), even at low concentrations. This requires a difficult decision on the part of the observer. Given that the signal to noise ratio may have been only slightly altered, or not at all, these techniques do not necessarily resolve the issue.

In direct contrast to those methods that attempt to reduce spurious signal, techniques that serve to increase intended fluorescent signal are available. Signal amplification via tyramide has been suggested as a method to overcome some of the issues surrounding autofluorescence (McKay et al., 1997). Unfortunately the autofluorescence associated with damage remains. While the intended signal is indeed

increased, as with methods such as sudan black a difficult decision is still required on the part of the observer.

What is clear is that no method can satisfactorily eliminate autofluorescence associated with brain damage. Despite a reduction in background signal as a result of several techniques (e.g., sudan black, cupric sulfate), or an increase in intended signal (via tyramide amplification), autofluorescent particles still remain, potentially influencing further analysis of tissue sections. A method to help dissociate real from spurious signal is therefore required if fluorescent techniques are to be used in the damaged brain.

Here we show using confocal microscopy that damage-induced autofluorescence does not co-localize with the nuclear specific markers DAPI or Hoescht. Co-localization of nuclear specific antibodies (e.g., Ki67, BrdU, NeuN) with these nuclear specific markers will therefore suffice to eliminate the potential confound of autofluorescence in the damaged brain.

#### Methods

Adult, male Long-Evans rats (n = 8) were obtained from the University of Lethbridge breeding colony, all housing procedures were as previously described (p. 33). All experimental procedures were done in accordance with the University of Lethbridge and CCAC animal welfare guidelines.

## Surgery

At approximately 60 days of age, rats either received adrenalectomy or corresponding control surgery according to the methods discussed previously (p. 33). Rats remained in their home-cages for 10 weeks to ensure a significant loss of granule cells in the dentate gyrus.

A separate group of rats received a stroke in the hippocampus. Stroke was induced by microinjecting endothelin-1 (ET-1, Sigma-Aldrich) at two sites in each hemisphere. Holes were drilled through the skull employing Bregma as a landmark. Sites were located as follows: 3.5 and 5.3 millimeters posterior to Bregma,  $\pm 2.4$  and  $\pm 5.3$  millimeters medial/lateral at each site respectively. Depths were determined using Bregma and were -3 and -7 millimeters at each site respectively. ET-1 was infused at a concentration of 3.8 pmole in 0.1M PBS at a flow rate of 0.05  $\mu$ l per minute for three minutes at the anterior site and six minutes at the posterior site. Injection cannulae were left in place after infusion for an additional five minutes to allow for diffusion of ET-1 from the tip. Post-surgical care included the administration of buprenorphine HCl (0.05 mg/kg) as an analgesic.

## Histology

Rats were perfused according to the previously discussed protocol (p. 34). Brains were sectioned on a frozen sliding microtome at a thickness of 40  $\mu$ m. ADX and control brains were sectioned employing a 1/12 section sampling fraction and stroke brains were sectioned utilizing a 1/5 sampling fraction.

### Immunohistochemistry

A single series of tissue was labeled with DAPI utilizing the same set of procedures described elsewhere (p. 41). A separate series of tissue was labeled with Hoescht. Sections were incubated in a 1:8000 solution of Hoescht in 0.1M PBS for three minutes at room temperature on a lab rotator. As with DAPI, sections were washed twice for seven minutes and then mounted on slides and coverslipped.

A third series of control tissue was processed for Ki67/DAPI. Free-floating sections were incubated in a primary solution containing rabbit anti-Ki67 (Novacastra) at a dilution of 1:1000 in 0.1M PBS, 0.3% Triton-X for 24 hours on a lab rotator at room temperature. Sections were then washed three times (seven minutes per wash) in 0.1M PBS. Tissue was then incubated in a secondary solution containing a 1:500 dilution of anti-rabbit Cy3 (Jackson ImmunoResearch) for 24 hours as above. A 1:1000 dilution of DAPI was added to the secondary solution during the last 25 minutes of the incubation period. Tissue was then washed twice in 0.1M PBS for seven minutes each time and then mounted and coverslipped using a fluorescent mounting medium.

### Tissue Analysis

Tissue sections were analyzed using a Zeiss Axioskop 2 Motplus epifluorescent microscope to determine if DAPI/Hoescht labeling was effective and if autofluorescence in ADX and stroke tissue was noticeable at lower magnification. Tissue was observed at various levels of magnification using Plan Neofluor 10x/0.3, 20x/0.5, and 40x/0.75 lenses

through DAPI, FITC, and TRITC filters. Given that ADX and stroke sections were only stained with DAPI or Hoescht any signal present using the FITC or TRITC filters was considered erroneous and deemed autofluorescent. Those sections that were deemed autofluorescent were then further scrutinized using confocal microscopy. Ki67/DAPI labeled sections were analyzed using the same microscope and levels of magnification.

### Confocal Microscopy

Confocal microscopy was performed using a Nikon C1+ confocal system (Nikon Eclipse TE 2000-u microscope). Tissue was scanned under a 60x/1.2 Plan apo lens at a 2-4x digital zoom using 408, 488, and 543 nm wavelength lasers. Z-stacks were taken from several sites in the dentate gyrus granule cell layer of two control rats to determine baseline levels of autofluorescence. Corresponding z-stacks were also taken from two ADX rats that displayed high levels of autofluorescence as well as the peri-infarct region from two stroke rats. A step size of 0.5  $\mu\text{m}$  was utilized for all z-stacks and typical z depth for a stack was approximately 25  $\mu\text{m}$ .

## Results

Control tissue displayed little to no autofluorescence when examined using low magnification epifluorescent microscopy (Figure 14A-C). DAPI/Hoescht labeling in control tissue was consistent across sections and was not visible under the FITC or TRITC filters. ADX-induced brain damage caused a significant amount of autofluorescence under both the FITC and TRITC filters in the areas associated with cell loss (Figure 14D-F). This was also the case with ET-1 tissue (Fig 14G-I), especially in

the peri-infarct region. As with controls, DAPI/Hoescht labeling was apparent in both ADX and ET-1 tissue.

Autofluorescence appeared in two specific forms: a general increase in background levels, and punctate glowing particles that are easily dissociable from background (despite the increase) in both the ADX and ET-1 tissue sections. Further scrutiny with confocal microscopy confirmed low levels of autofluorescence in control animals, specifically in the dentate gyrus granule cell layer. As mentioned before, high levels of autofluorescence were observed in both ADX and ET-1 tissue. Analysis of the z-stacks taken from ADX and stroke tissue revealed a perfect co-localization of the autofluorescent signal collected from the 488 and 548 nm lasers, resulting in a gold/yellow appearance. Importantly, this autofluorescence never co-localized with the DAPI/Hoescht signal, strongly suggesting that it is not nuclear in nature (Figure 15).

Ki67 labeling was apparent in control tissue (Figure 16A) and at lower levels of magnification looked similar to autofluorescent signal (Figure 16B). However, confocal microscopy revealed a co-localization of Ki67 and DAPI (Figure 16C) that is dissociable from autofluorescence.

## Discussion

Autofluorescent signals were minimal in control tissue, implying that our perfusion technique is alone not responsible for high levels of autofluorescence. Low levels of autofluorescence were present in some control tissue as viewed under FITC and

TRITC filters. Given the age of the rats at perfusion (approximately six months) and the nature of the autofluorescence (peri-nuclear particles) this may be the lipofuscin-like signal that has been previously reported (Schnell et al., 1999). One would therefore expect this to vary as a function of age. Unpublished data from our lab suggests that lipofuscin-like autofluorescence is absent in younger control rats using the same perfusion protocol, furthering the suggestion that PFA is not the cause of autofluorescence *per se* and that it is likely age-related.

Nuclear labeling was consistent in control tissue and was specific to the DAPI filter in our epifluorescent microscope. When autofluorescence was observed in control tissue it was only apparent under the FITC and TRITC filters. Investigation with confocal microscopy revealed a perfect co-localization of autofluorescent signal when excited by the 488 and 548 nm lasers resulting in a gold/yellow like appearance. Importantly, the signal associated with autofluorescence never co-localized with either the nuclear marker DAPI or Hoescht using confocal microscopy at higher levels of magnification (60x), another observation that is consistent with the idea that this is an accumulation of lipofuscin in the cytoplasm of the cells in question (Schnell et al., 1999).

Tissue from animals receiving either ADX or ET-1 displayed high levels of autofluorescence when observed using epifluorescent and confocal microscopy. Nuclear labeling with DAPI/Hoescht was similar to controls in both ADX and ET-1 tissue, such that the signal was evident under only the DAPI filter and was consistent across tissue sections. As ADX involved no direct manipulation of brain tissue, processes related to

cell death itself seem sufficient to produce high levels of autofluorescence. This is further supported by the fact that levels of background and punctate autofluorescence were highest in regions subjected to cell death associated with ADX, specifically the granule cell layer of the dentate gyrus. Background levels and punctate autofluorescence in ET-1 tissue were highest in the peri-infarct region, although some autofluorescent particles were occasionally noted in the granule cell layer of the dentate, a considerable distance from the infarct. These findings confirm previous reports that apoptosis itself is sufficient to induce autofluorescence (Levitt et al., 2006).

At low levels of magnification (10-40x) autofluorescence was strikingly similar to the typical pattern and colour of BrdU/NeuN co-localization that is often reported (Sharp et al., 2002; Kempermann et al., 2003; Urrea et al., 2007). This is apparent specifically in damaged tissue. Thus, analysis of co-localized nuclear markers in the damaged brain could potentially result in overestimating the number of multiple labeled cells as a result of high levels of autofluorescence. This is evidenced by the similarity between autofluorescent particles in ADX animals and Ki67 signal observed in controls. Utilizing confocal microscopy one can differentiate between intended nuclear labeling and spurious autofluorescent signal.

Given that the nuclear markers DAPI or Hoescht do not co-localize with autofluorescent signal in control or brain damaged tissue it is possible to dissociate one from the other. This becomes extremely useful under circumstances in which spurious fluorescent signal increases in the brain, whether it is a result of aging (Schnell et al.,

1999; Neumann & Gabel, 2002) or cell death (Levitt et al., 2006). Any signal that co-localizes with DAPI or Hoescht is by nature not autofluorescent. Methods that utilize nuclear markers (e.g. Ki67, BrdU, NeuN) are still viable in the aged/damaged brain as long as it is demonstrated that they co-localize with a nuclear counter stain. Furthermore, techniques that require co-localization of specific markers such as BrdU/NeuN can reliably be performed in the damaged/aged brain as long as DAPI/Hoescht counterstaining is present.

Our results from Experiment 3 demonstrate that enrichment/exercise induced up-regulation of neurogenesis results in volumetric increases in the damaged dentate gyrus. We are also able to successfully identify BrdU/NeuN positive neurons in the damaged brain (Experiment 3) via co-localization with the nuclear specific marker DAPI. Thus, a pilot study was designed to determine if immunohistochemical measures of adult born neuronal survival could be performed in the damaged dentate gyrus.

## **Experiment Four**

### **Survival Of Adult-Born Neurons In The Dentate Gyrus After Adrenalectomy**

#### **Methods**

Twenty-two rats were taken from the breeding colony at the University of Lethbridge. Housing conditions were as described elsewhere (p. 33). All experiments were conducted according to the guidelines set forth by the University of Lethbridge animal welfare committee and the CCAC.

### Surgery

At 60 days of age rat underwent ADX or corresponding control surgery as described in detail elsewhere (p. 33). Rats remained in their home-cages for 10 weeks to allow for sufficient loss of granule cells.

### Hormone Replacement

After the 10-week degeneration period rats were placed on replacement CORT (1 mg daily, as described on p. 55). Rats were administered CORT for seven days before any other experimental manipulations were made and remained on CORT until the cessation of the experiment.

### BrdU Administration

Rats received two pulses of BrdU (Sigma), at 10-hour intervals, via intraperitoneal injection. BrdU was dissolved in a sterile-filtered 0.1M PBS solution at a concentration of 50 milligrams per milliliter. The solution was warmed immediately prior to administration to aid the incorporation of BrdU. BrdU was injected at 150 milligrams per kilogram, resulting in an injection amount of 1.5 milliliters for a 500 gram rat.

### Histology

Either two or eight weeks after administration of BrdU, rats were perfused in the same method as described before (p. 34). Tissue sections were taken using a frozen sliding microtome at a thickness of 40  $\mu\text{m}$ , employing a section sampling fraction of 1/12.

### Immunohistochemistry

A single series of tissue was processed for BrdU/NeuN/DAPI. Tissue was incubated in 2M HCl at room temperature for one hour, after which several PBS washes were performed over a period of 90 minutes. Brain tissue was then incubated for 24 hours on a lab rotator at room temperature in a primary solution containing 0.1M PBS, 0.3% Triton-X, a 1:200 dilution of rat anti-BrdU (AbD serotec), and a 1:2000 dilution of mouse anti-NeuN (Chemicon). After incubation for 24 hours, tissue was washed three times in 0.1M PBS for a duration of seven minutes per wash. Sections were then transferred to a secondary solution of 0.1M PBS containing a 1:500 dilution of goat anti-rat biotinylated IgG (Chemicon) and a 1:250 dilution of goat anti-mouse Alexa Fluor 488 (Invitrogen) and were incubated for a further 24 hours. After incubation in the secondary solution, tissue was washed three times in 0.1M PBS and incubated for one hour in a 0.1M PBS solution containing a 1:500 dilution of streptavidin Alexa Fluor 568 (Invitrogen). A 1:1000 dilution of DAPI was added in the last 25 minutes of the tertiary incubation. Upon completion of the tertiary incubation, tissue was washed three times in 0.1M PBS, mounted on glass slides and coverslipped using an anti-fade mounting medium.

### Confocal Microscopy

A Nikon C1+ confocal system (Nikon Eclipse TE 2000-u microscope) was employed for all confocal analysis. Tissue was scanned under a 60x/1.2 Plan apo lens using 408, 488, and 543 nm wavelength lasers. Z-stacks were taken from several sites in the dentate gyrus granule cell layer in control and ADX rats, in both the two and eight

week survival groups. A step size of 0.5  $\mu\text{m}$  was utilized for all z-stacks and typical z depth for a stack was approximately 25  $\mu\text{m}$ . A cell was considered positive for BrdU/NeuN if a nuclear signal from both the 488 nm and 543 nm lasers co-localized in the x, y, and z axes in control rats. Given the increase in autofluorescence associated with damage (Experiment 4) a cell was only considered BrdU/NeuN positive in ADX rats if co-localization with the DAPI (408 nm) laser was also apparent.

## Results

BrdU/NeuN positive cells were present in the granule cell layer of control rats at both two and eight weeks after injections of BrdU (Figure 16A-B). ADX rats on CORT replacement also displayed co-localization of BrdU/NeuN in combination with DAPI (Figure 16C-D).

## Discussion

Here we employ the thymidine analog BrdU to assess the survival of adult born neurons at early (two-week) and late (eight-week) time-points in the damaged dentate gyrus. Utilizing confocal microscopy, we report BrdU/NeuN positive cells in the dentate gyrus of control rats at two and eight weeks after injection with BrdU. This finding fits with a vast body of evidence showing the maturation of adult-born neurons in the dentate gyrus of rats (van Praag et al., 2002; Kempermann et al., 2004).

Rats with a damaged dentate gyrus as a result of chronic (10-week) ADX also displayed BrdU/NeuN positive cells. Given the increase in autofluorescence we further

co-localized BrdU/NeuN with the nuclear marker DAPI, resulting in a triple label (BrdU/NeuN/DAPI) that allowed us to differentiate intended from spurious signal (Spanswick et al., 2009). The evidence suggests that after ADX, adult born neurons survive for a period of at least eight weeks. This finding supports our indirect evidence showing a thicker granule cell layer in ADX rats after treatment with fluoxetine (Spanswick et al., 2007) as well as increases in dentate gyrus granule cell layer volume after a combination of exercise and enrichment (Experiment 2).

We specifically chose two and eight weeks post-BrdU injection in an attempt to encapsulate early and later survival times after the proliferation of cells in the adult dentate gyrus. Our early time-point (two weeks) falls after the peak of NeuN expression in adult-born neurons (approximately three days, Kempermann et al., 2004) after which a selection between recruitment and elimination occurs (Biebl et al., 2000). This stage of cell development is typically associated with a more stable survival rate (Kempermann et al., 2003), suggesting that the remaining cells are more likely to become incorporated into the hippocampal network. Our late (eight-week) time-point falls after the range of time required for adult-born granule cells to become indistinguishable from their surrounding counterparts (Kempermann et al., 2004). By this stage adult-born neurons are functionally integrated into the surrounding circuitry, displaying similar electrophysiological properties (van Praag et al., 2002) and immediate early gene expression (Jessberger & Kempermann, 2003) to mature granule cells.

Here we show that adult born neurons survive in the damaged dentate gyrus for at least eight weeks. This augments our findings that factors that serve to increase neurogenesis in the normal animal result in an increase in the number of granule cells in the damaged dentate gyrus. Together, these results suggest that it may be possible to employ methods to repopulate a depleted granule cell layer in the adult animal.

### **General Discussion**

The current chapter shows that neurogenesis continues in the adult dentate gyrus after brain damage induced by long-term ADX. This suggests that despite a significant loss of granule cells, the subgranular zone remains a viable substrate for the birth of new neurons in the adult animal. Indices of proliferation such as Ki67 and DCX, as well as those of survival (BrdU/NeuN) are present at comparable levels to those in the “normal” brain. We replicate previous findings showing that ADX itself is capable of up-regulating the proliferative stages of neurogenesis (Gould et al., 1992; Cameron & Gould, 1994; Montaron et al., 1999; Wong & Herbert, 2005), but note that these effects are ephemeral in nature.

The discovery that the increase in proliferation after ADX is transient is novel, yet fits with a larger body of evidence that reports similar effects as a result of a number of other modulatory factors (Kempermann & Gage, 1999; Brunson et al., 2005; Naylor et al., 2005; Spanswick et al., 2007). Importantly, we show that after a brief increase in proliferation after ADX, adult neurogenesis returns to a stable baseline. This indicates the presence of compensatory mechanisms, the exact machinery of which remains somewhat

of a mystery. Interestingly, corticosterone has been implicated in other accounts of transient bursts in neurogenesis (Naylor et al., 2005) as well as a mechanism for antidepressant-based increases in proliferation (Huang & Herbert, 2006; Alahmed & Herbert, 2008). A potential explanation is that the glucocorticoid receptor itself is acting in concert with other receptor systems, as has been reported in regards to the 5-HT<sub>1A</sub> receptor (Huang & Herbert, 2006), as well as the NMDA receptor (Cameron et al., 1998). Changes in the expression of these other receptor systems (up/down-regulation) after neurogenic stimulation may account for the eventual return to baseline levels.

The current data demonstrate that factors that modify the neurogenic process within the normal brain are able to do so after long-term ADX. Fluoxetine (Malberg et al., 2000; Malberg & Duman, 2003; Santarelli et al., 2003; Huang & Herbert, 2006), exercise (Naylor et al., 2005; Olson et al., 2006; Tashiro et al., 2007), and exposure to enriched environments (Kempermann et al., 1997; Kempermann & Gage, 1999; Olson et al., 2006) have all been demonstrated to increase neurogenesis within the brain. These effects extend to the ADX rat (Spanswick et al., 2007; Experiment 2). Utilizing a unique combination of exercise and enrichment we were able to significantly increase the number of DCX positive cells present in the damaged dentate gyrus.

We also present immunohistochemical (Experiment 4) and volumetric evidence (Experiment 2) that these adult-born neurons survive in the damaged dentate gyrus. Despite ADX increasing autofluorescence in the damaged dentate gyrus we are able to co-localize the nuclear marker DAPI with BrdU/NeuN providing a reliable method by

which to examine long-term survival of adult-born neurons after brain damage (Spanswick et al., 2009). Immunohistochemical evidence in combination with unbiased quantification of dentate gyrus granule cell layer volume allows us to assert that long-term survival of adult-born neurons occurs within the damaged dentate gyrus.

A critical point is that the treatments we utilize to stimulate neurogenesis in ADX rats result in an increase in the volume of the dentate gyrus granule cell layer, a measure that is significantly correlated with granule cell number (p. 44). This demonstrates that we are able to partially repopulate a damaged dentate gyrus and suggests that it may be possible to repair a damaged dentate gyrus, the ultimate goal of which is functional recovery.

## **Chapter Five**

### **A Novel Animal Model Of Cognitive Dysfunction And Neurogenic Treatment Strategies**

Here we describe a novel animal model by which we can selectively induce a gradual loss of granule cells in the hippocampus of the adult rat. The degeneration occurs over a period of weeks to months. The loss of granule cells results in persistent deficits of cognition that are similar to those experienced by individuals suffering from a number of pathological and disease states. We highlight potential treatment strategies that target the endogenous neural stem cell niche within the adult hippocampus, resulting in an up-regulation of neurogenesis. This increase in the birth of neurons within the adult rat is sufficient to at least partially re-populate a damaged dentate gyrus. Utilizing a variety of immunohistochemical, electrophysiological, stereological, and behavioural methods we can assess both the loss and potential recovery of function in the adult animal.

Using the histological marker Fluoro-Jade B we are able to determine the onset and time-course of continuing cell death in the hippocampus as a result of ADX. Our data confirm the limited number of prior reports of ADX-induced granule cell death (Gould et al., 1990; Woolley et al., 1991; Andrés et al., 2006), but extend the time-course to a period beyond five months, something that has not been reported before. Our findings demonstrate that hippocampal granule cell loss associated with ADX begins as soon as three days after removal of CORT and continues over a period of weeks to months in the adult animal.

We show that the loss of granule cells as a result of ADX is initially localized to the superior, lateral blade of the dentate gyrus. This is congruent with the observations of others (Conrad & Roy, 1995; Sloviter et al., 1995) and fits with the idea that older granule cells are more susceptible to ADX-induced cell death (Cameron & Gould, 1996). Studies using BrdU to assess the age of cells displaying apoptotic profiles after ADX (Cameron & Gould, 1996) and the development of the dentate gyrus (Schlessinger et al., 1975; Kempermann, 2006) show that more mature granule cells are the first to die as a result of CORT removal. At more distant time-points after ADX the absolute number and extent of cell death throughout the granule cell layer increases. This continuing loss of hippocampal granule cells in the adult rat as a result of ADX is a nice feature of this model that resembles slow neurodegenerative processes of neuronal loss experienced by individuals suffering from various forms of dementia (Walker et al., 2007), delayed hippocampal neuronal loss associated with stroke (Petito et al., 1987), as well as traumatic brain injury (Hicks et al., 1993). The relatively slow loss of neurons as a result of ADX is also congruent with other neurodegenerative disorders including, but not limited to Huntington's (Kumar et al., 2010) and Parkinson's disease (Lees et al., 2009).

By employing an unbiased stereological approach we are able to quantify the extent of hippocampal granule cell loss in the ADX brain. Using indices of volume and estimates of total cell number we confirm the conclusions of studies using histological cell death makers. Specifically, using the Cavalieri method we show that long-term (10-week) ADX results in an approximate 25 percent decrease in the volume of the dentate gyrus granule cell layer. Further analysis with the optical fractionator technique reveals a

50 percent decrease in the number of cells within the granule cell layer of chronic ADX rats compared to intact controls. Our results resemble those of prior research demonstrating a much smaller dentate gyrus granule cell layer after chronic ADX (Sloviter et al., 1989; Roozental et al., 1998). Importantly, the improvement of quantification techniques beyond that of the “representative section” allows us to make more reliable and accurate comparisons, both within and between experiments.

A pilot study assessing the electrophysiological properties of the damaged dentate gyrus determined that a loss of granule cells due to chronic ADX resulted in a muted electrophysiological response. Both fEPSP slope and population spike amplitude were reduced in ADX rats on CORT replacement after ten weeks of granule cell degeneration, relative to controls. These measures are a function of the number of granule cells synchronously participating in granule cell layer function and show a significantly attenuated response, a finding that is congruent with our histological analysis and our stereological quantification of the granule cell layer in the chronic ADX rat. The observed 25 percent decrease in the volume of the dentate gyrus and approximate 50 percent loss of granule cells suggests a lower density of granule cells remaining in the dentate gyrus of ADX rats compared to controls. This decrease in dentate gyrus granule cell layer density fits with the decrease in fEPSP slope and population spike amplitude, as fewer granule cells are near enough the recording electrode to contribute to the measured electrophysiological response.

A debate exists within the scientific literature, as two possibilities may explain the deficits associated with long-term ADX. The first is that complete removal of circulating CORT produces electrophysiological and learning/memory disturbances, as alterations in CORT have been reported to influence both (Conrad & Roy, 1995; McCormick et al., 1997; Stienstra et al., 1998; Karst & Joëls, 2003; Krugers et al., 2007). The second is that the loss of granule cells within the dentate gyrus is responsible for disturbances of electrophysiology and cognition and not loss of CORT *per se*, a theory that is in agreement with a number of lesion studies (Sutherland et al., 1983; Xavier et al., 1999; Jeltsch et al., 2001).

At acute time-points after ADX (prior to measurable cell death) alterations in granule cell physiology have been reported (Stienstra et al., 1998). Changes in granule cell physiology have also been reported as a result of chronic increases in CORT via stress paradigms (Karst & Joëls, 2003). Both of these phenomena occur in the absence of notable granule cell loss, suggesting that modulations in CORT are sufficient to produce alterations in the electrophysiological properties of hippocampal granule cells. On the contrary, the removal of CORT initiates cell death even when measured at short time-points after ADX (Gould et al., 1990; Jaarsma et al., 1992). Thus, it is likely that cells are suffering adverse effects of CORT removal, interfering with their ability to contribute to the function of the dentate gyrus, despite not displaying measurable characteristics of cell death. Alterations in the dendritic morphology of granule cells have been shown soon after ADX (Gould et al., 1990; Wossink et al., 2001). Gould et al. (1990) report a significant decrease in the cell body area and the number of dendritic branch points of

granule cells by seven days after ADX, despite a relatively “normal” appearance, suggesting a reduced ability of these cells to participate in normal granule cell layer function.

Due to the fact that our ADX rats were receiving replacement CORT during the course of our experiment we can assert that the alterations in electrophysiology were due to a loss of granule cells and not from a removal of circulating CORT alone. To confirm the effectiveness of our oral CORT replacement strategy we assayed CORT levels in a subset of rats at two time-points (4 and 20 hours) after administration. CORT levels in our ADX rats on hormone replacement were similar to intact controls (Experiment 1, Chapter 3; Spanswick et al., 2007), suggesting that our method of replacement is effective at inducing a normal diurnal rhythm. This latter point is critical in that it allows us to dissociate the effects of CORT removal from granule cell loss in chronic ADX rats. We utilized the same, proven method of CORT replacement in ADX animals to determine the effect of granule cell loss associated with chronic ADX on behaviour.

The loss of granule cells due to long-term ADX resulted in cognitive deficits in two tasks that have been shown to depend on intact hippocampal function. Using a moving platform version of the Morris water task we demonstrated that rats with dentate gyrus damage performed poorly relative to controls, replicating prior research that suggests damage focused on the dentate gyrus (including ADX) produces deficits in spatial tasks (Sutherland et al., 1983; Xavier et al., 1999; Armstrong et al., 1993; McCormick et al., 1997). We show that ADX rats take longer to locate a hidden platform

within the Morris water task, regardless of whether it was in a familiar or novel location. This significant increase in time to find the platform is not explained by differences in swim speed between ADX and intact control animals. During our investigation, all of our ADX rats received replacement CORT and still presented with spatial deficits in the Morris water task. This finding adds to a body of evidence that suggests a loss of granule cells is sufficient to produce cognitive deficits. Thus, the behavioural deficits we observe in chronic ADX animals cannot be explained by removal of CORT alone.

Our finding the ADX animals on CORT replacement suffer cognitive deficits in the Morris water task is divergent from reports showing the amelioration of spatial deficits in long-term ADX rats by acute administration of CORT (Conrad & Roy, 1995; McCormick et al., 1997). McCormick et al. (1997) show that acute (5 or 10 day) replacement of CORT in chronic ADX rats is sufficient to restore behaviour to that of controls in a version of the Morris water task, despite a significant decrease in the area of the dentate gyrus granule cell layer in ADX rats. This report not only differs from our finding that CORT replacement does not ameliorate the effects of ADX on spatial behaviour, but also those that use alternate methods to lesion the dentate gyrus granule cell layer (Sutherland et al., 1983; Xavier et al., 1999; Jeltsch et al., 2001), as well as cell recording experiments that suggest hippocampal granule cells are involved in spatial information processing (Leutgeb et al., 2007; Moser et al., 2008).

Methodological differences between our experiment and that conducted by McCormick and colleagues (1997) may in part account for the disparate results.

McCormick et al. (1997) employ only two goal locations in the Morris water task. Other reports of similar effects of CORT on spatial behaviour in long-term ADX rats also employ relatively simple analyses. For example, Conrad & Roy (1995) employ a single hidden platform location, using only two of the four available cardinal compass start locations. Utilizing multiple platform pairings in the Morris water task, we show that CORT replacement does not improve the behaviour of chronic ADX rats. Our multiple platform method allows us to assess both acquisition and retention performance across multiple goal locations, providing us with a greater ability to potentially detect relatively small performance deficits as a result of hippocampal granule cell loss. The use of the four cardinal compass start locations in our experiment versus the two employed by Conrad & Roy (1995) requires rats to locate the goal platform from a larger number of distinct start-points, providing greater opportunity to assess spatial ability. Hippocampal involvement can be influenced by within task manipulations (Dudchenko et al., 2000; Rudy et al., 2002), suggesting that the simple manipulation of increasing the number of platform locations may also account for the differing results. Additionally, our report of spatial disturbances in rats that have granule cell loss is more consistent with the current understanding of hippocampal function, offering a more parsimonious explanation for the data.

Others have demonstrated that a threshold of damage is required before reliable deficits can be observed in tasks that have shown to depend on intact hippocampal function (Moser et al., 1993; Epp et al., 2008; Sutherland et al., 2008; Lehmann et al., 2010). By varying the extent of hippocampal lesion size, Epp et al. (2008) showed a

significant inverse correlation between hippocampal damage and performance of rats in a visual discrimination task. Similar reports exist in contextual fear conditioning (Sutherland et al., 2008) and spatial navigation (Epp et al., 2008). The amount of damage produced as a result of lesion, in combination with the sensitivity of the task used to assay hippocampal (in our case specifically dentate gyrus) function, will likely predict the presence or absence of quantifiable behavioural deficits. We show that six weeks of ADX-induced cell loss is sufficient to produce reliable deficits in a moving platform version of the Morris water task, which as described above is arguably more sensitive than single platform methodologies.

It is reasonable to conclude that there are two related, yet dissociable processes that occur as a result of ADX. The first is the complete removal of CORT, which has been reported as sufficient to alter granule cell physiology at acute time-points (Steinstra et al., 1998; Kruger et al., 2007). The second is the ensuing loss of granule cells, beginning three days after ADX and occurring over a number of months, which also produces physiological changes in the dentate gyrus granule cell layer (Chapter 2, Experiment 1). Our concern is not the effects of alterations in CORT *per se*, but rather the result of a significant and gradual loss of granule cells.

To further understand the behavioural deficits associated with loss of hippocampal granule cells we observed ADX rats in three versions of an object preference task after 10 weeks of granule cell layer depletion. We assessed the ability of ADX rats to discriminate between a novel and a previously experienced object (novel

object preference), an object that had been moved to a new location versus an old location (novel location preference), and a novel versus previously encountered object/context pairing (object/context mismatch task). Prior investigation has shown that rats with damage limited to the hippocampus perform novel object recognition at levels similar to controls, but display deficits when information about context (O'Brien et al., 2006) or place (Mumby et al., 2002) becomes important for the discrimination.

ADX rats did not falter in resolving discriminations based on object novelty or location, performing at levels similar to control rats. Only when ADX rats were required to remember the context in which an object had been previously encountered did they display impairments of cognition. During the object/context mismatch task ADX animals performed significantly worse than controls and failed to discriminate the novel object/context pairing at greater than chance levels. Importantly, as during the previous electrophysiological and behavioural assessments, all ADX rats were on a regimen of replacement CORT using the same method of administration as described above. This is another line of evidence showing that the removal of CORT alone does not explain the observed behavioural deficiencies of chronic ADX rats.

Our results show that the granule cells of the dentate gyrus are necessary for supporting associations between contexts and objects. Furthermore they are congruent with anatomical (Westbrook, 2000; Rudy, 2008) and physiological (Barnes et al., 1990; McClelland & Goddard, 1996; Chawla et al., 2005) evidence, as well as some theoretical standpoints (Marr, 1971; Rudy & Sutherland, 1989; McClelland & Goddard, 1996;

Norman & O'Reilly, 2003) in regards to hippocampal function. Curiously, our ADX rats did not differ from controls during the novel place preference task, a finding that is at odds with several reports of the involvement of the dentate gyrus in spatial information processing (Sutherland et al., 1983; Xavier et al., 1999; Jeltsch et al., 2001; Leutgeb et al., 2007; Moser et al., 2008). This is likely caused by the relative poor performance of controls animals during the place preference task (Figure 10B). Our translocation of one of the objects from one corner to the other (Figure 9B) may have inadvertently increased the difficulty of the task.

The hippocampus receives highly convergent inputs from a number of brain regions (Rudy, 2008). By the time these inputs arrive at the hippocampus they are highly processed and contain multimodal information (Lavenex & Amaral, 2000). The sparse activity within the dentate gyrus granule cell layer (Barnes et al., 1990; McClelland & Goddard, 1996; Chawla et al., 2005) suggests that multimodal information is represented by a relatively small population of granule cells. This small population of granule cells is likely to contain a combination of multimodal stimulus information, an idea that was synthesized by Sutherland and Rudy (1989) in their “configural association theory”.

In agreement with anatomical and physiological properties of the hippocampus, Sutherland and Rudy (1989) suggest that the hippocampus is responsible for producing what they term “configural associations”. Specifically, they suggest that the hippocampus binds together information from elementary stimuli, resulting in unique representations that differ from the elemental stimuli themselves (Sutherland & Rudy, 1989). In a more

recent discussion of configural association theory Rudy and Sutherland (1995) suggest that the hippocampus may serve to highlight differences between configural representations, an idea that is similar to theories of pattern separation (Marr, 1971; McClelland & Goddard, 1996; Rolls, 1996). Pattern separation has been suggested as a process that is mediated by the hippocampus (Marr, 1971; McClelland & Goddard, 1996; Rolls, 1996) with particular attention paid to the dentate gyrus (Treves & Rolls, 1994; McClelland & Goddard, 1996; Norman & O'Reilly, 2003). Our findings showing that rats with damage focused on the dentate gyrus perform poorly in tasks that require an association between context and object fit with configural association theory (Sutherland & Rudy, 1989; Rudy and Sutherland, 1995) as well as theories of pattern separation (Treves & Rolls, 1994; McClelland & Goddard, 1996; Norman & O'Reilly, 2003). Additionally, our experimental findings are congruent with behavioural evidence from studies assessing pattern separation ability and dentate gyrus function (Kesner et al., 2004; Chawla et al., 2005; Leutgeb et al., 2007). Recent evidence suggests that not only is activity in the dentate gyrus sparse, but the same population of hippocampal granule cells respond even in changing environments (Leutgeb et al., 2007; Colgin et al., 2008) Leutgeb and colleagues (2007) show that when the context in which a rat is placed slowly changes from square to circle (and vice versa) the same dentate gyrus granule cells produce place fields regardless of the shape of the context. Despite no change in the absolute population of cells producing place fields, individual granule cells alter their firing rate in concert with alterations to the environment, a phenomenon referred to as rate remapping (Colgin et al., 2008), this has been suggested as a mechanism by which the reduction of interference via pattern separation may occur.

The dominant view is that the dentate gyrus, in some form or another, aids in the reduction of overlap between inputs (Treves & Rolls, 1994; McClelland & Goddard, 1996; Norman & O'Reilly, 2003; Leutgeb & Moser, 2007). An alternate possibility is that the high degree of overlap seen in the population of hippocampal granule cells responding in different environments might instead reduce the orthogonalization of incoming inputs, in a manner similar to what has been proposed by Aimone et al., (2006, discussed in Chapter 1, p. 21). This massive overlap in the population of active granule cells could potentially increase the continuity between events.

As with individuals suffering cognitive deficits as a result of hippocampal cell loss (de Toledo-Morell et al., 2000; Laczó et al., 2010) we show that rats with loss of dentate gyrus granule cells experience deficits in behaviour. These deficits can be quantified reliably in a spatial navigation task as well as a discrimination task based on an association between an object and the context in which it was previously encountered. Importantly, the deficits we observe in rats after long-term ADX are analogous to those experienced by people displaying a loss of hippocampal function (de Toledo-Morell et al., 2000; Astur et al., 2002; Marschner et al., 2008; Pascalis et al., 2009). Spatial deficits as a result of hippocampal atrophy due to Alzheimer's disease (Laczó et al., 2010) and hippocampal damage associated with surgical resection (Astur et al., 2002) have been reported in the virtual Morris water task, the human analogue of the spatial task we employed to assess behaviour in our ADX animals (Chapter 3, Experiment 2). Astur et al. (2002) show that human patients with surgical-induced unilateral hippocampal damage

took significantly longer to locate a hidden platform and spent less time “swimming” in the correct quadrant during a probe trial in the virtual Morris water task than their age-matched cohorts. The disruption of novelty preference by altering the context in which objects are presented has been observed in human patients suffering from hippocampal damage (Pascalis et al., 2009), a finding that is congruent with our data, showing diminished novel object/context association in ADX rats (Chapter 3, Experiment 3). Pascalis and colleagues (2009) show that when the context in which an object is first presented (familiarization) and subsequently re-introduced (test) is changed, patients with hippocampal damage show a lack of object preference, despite intact object recognition when the background context remained constant.

Previous reports show that relatively soon after ADX the proliferation of new neurons is enhanced in the rat dentate gyrus (Gould et al., 1992; Montaron et al., 1999; Wong & Herbert, 2005). This has been suggested to result from a decrease in circulating CORT, as an inverse relationship between the birth of new neurons and CORT levels has been observed in the developing rat (Gould et al., 1992). Replicating the initial finding by Gould et al. (1992) in the adult animal, we show that one week after removal of circulating CORT the number of proliferating cells is increased in the dentate gyrus. We are the first to report however, that at longer time-points after ADX in adult animals, proliferation returns to a level that is similar to intact controls. By two weeks after ADX, proliferation in the dentate gyrus of ADX rats is no longer different from that of controls and remains at control levels at longer (four-week) time-points.

Our finding that ADX only transiently increases adult hippocampal neurogenesis fits with a growing body of evidence showing similar effects of other modulators of the neurogenic process. Transient changes in neurogenesis have been reported in response to environmental enrichment (Kempermann & Gage, 1999), exercise (Naylor et al., 2005), and chronic administration of fluoxetine (Spanswick et al., 2007). This suggests that although the neural stem cell niche within the hippocampus may be modulated, regulatory factors exist that normalize the stem cell niche after stimulation. The mechanisms of such factors are unknown, although some have suggested that decreases in stimulus novelty may influence the response (Kempermann & Gage, 1999).

A plausible explanation is that, as in response to pharmacological agents, alterations in cell physiology occur in response to neurogenic treatments. For example, chronic administration of the antidepressant fluoxetine results in an ensuing down-regulation of postsynaptic 5-HT receptors, which in turn eventually produces an increase in brain-derived neurotrophic factor (BDNF; Meyer & Quenzer, 2005). BDNF has been demonstrated as a requirement for basal levels of adult neurogenesis (Lee et al., 2002) and its infusion into the lateral ventricle has been shown to up-regulate the proliferation of new neurons in the subventricular zone (Pencea et al., 2001). Alterations at the cellular level are likely to influence brain plasticity at a variety of levels, including the birth of new neurons in the adult. Long-term changes in CORT receptor sensitivity or number may account for the eventual return of neurogenesis to baseline levels. Mineralcorticoid receptor availability requires only 24 hours to reach stable levels after removal of CORT via ADX. Conversely, maximal glucocorticoid receptor availability requires at least two

weeks (Margineau et al., 1994), the time at which neurogenesis has returned to control levels after ADX (Chapter 4, Experiment 1). It is plausible that the sensitivity of these receptor subtypes is also altered, perhaps explaining the ensuing de-correlation between CORT levels and rate of neurogenesis.

Our six-week administration of fluoxetine (Spanswick et al., 2007) during which we report no increase in proliferation may represent an entirely different stage of cellular response than those which report an increase at more acute time-points (Malberg et al., 2000). Similar findings have been reported in the electrophysiological properties of the dentate gyrus in response to fluoxetine administration. Stewart and Reid (2000) show that 15 days of fluoxetine is sufficient to produce an increase in the slope of the fEPSP and population spike in the dentate gyrus of adult rats. Conversely, Keith et al. (2007) show no alterations in dentate gyrus electrophysiology after six weeks of daily fluoxetine administration. These findings mirror directly what has been reported in regards to adult neurogenesis and chronic fluoxetine treatment. Specifically, these converging lines of evidence show that the increase in plasticity associated with chronic fluoxetine administration is a transient phenomenon, providing further evidence that regulatory mechanisms exist that return enhanced brain plasticity to baseline levels.

To validate our initial findings we quantified the number of Ki67 positive cells in ADX rats at two post-surgical time-points using improved immunohistochemical (Melvin & Sutherland, 2009) and quantification techniques (Mouton, 2002). Specifically, we employed the optical fractionator technique in an attempt to reliably estimate the number

of Ki67 positive cells present within the dentate gyrus of ADX and control rats. The optical fractionator provides an unbiased estimate of the total number of objects within a given reference space (the dentate gyrus), allowing more reliable across, and between experiment comparisons. Compared to quantification techniques that utilize an insufficient number of representative sections, the optical fractionator is not influenced by the spatial distribution of objects (in this case cells positive for Ki67) across the reference space under investigation (West, 2002). As a result, the optical fractionator provides a more accurate estimate of the actual number of objects under scrutiny.

Modifications to our immunohistochemical technique were necessary to ensure an accurate estimate of the total number of Ki67 positive cells. Melvin and Sutherland (2009) have shown that the standard method of one-hour long secondary antibody incubations is insufficient to penetrate the entire depth of relative thick (40  $\mu\text{m}$ ) tissue sections, resulting in a lack of labeling in the middle of a given section. As the optical fractionator involves sampling from a reference space sandwiched between a top and bottom guard zone, labeling throughout the entire height of the tissue section is critical to ensure proper quantification. As such we replicate our initial report, and those of others (Gould et al., 1992; Montaron et al., 1999; Wong & Herbert, 2005), that proliferation is increased in the dentate gyrus soon after ADX. By four weeks after ADX we show a return of proliferation to baseline levels using unbiased stereological quantification, confirming our prior result, but with a more appropriate technique.

Of note is that even after a significant degeneration of the granule cell layer the stem cell niche within the subgranular zone remains a viable substrate for the birth of new neurons in the adult. We are the first to show that dentate gyrus neurogenesis continues at normal levels after chronic (longer than 10 days) ADX in the adult rat. We are also the first to show that at much longer time-points (six to ten weeks after ADX) that in combination with CORT replacement, methods that increase neurogenesis in the normal animal continue to do so in the damaged dentate gyrus.

As in normal animals, we show that the stem cell niche in the subgranular zone of chronic ADX rats can be modulated. Using a combination of exercise and environmental enrichment we show that early stages of neurogenesis can be up-regulated in the ADX rat. A 24-hour alternating regimen of wheel running and enriched environment exposure resulted in a significant increase in the number of migrating neuroblasts, as indicated by the microtubule associated protein DCX. This effect was similar to that which was observed in our controls, as well as to other reports of exercise-induced alterations in neurogenesis (van Praag et al., 1999; Rhodes et al., 2003). Our observation provides further evidence of an intact neural stem cell niche in the dentate gyrus of ADX rats. This up-regulation of neurogenesis resulted in a significant increase in the volume of the dentate gyrus of ADX rats, relative to their home-cage ADX cohorts. Although at the six-week time-point the volume of the granule cell layer in treated ADX rats was still smaller than controls, the continuing high levels of DCX expression suggest that increasing the length of exposure to the combination of enrichment and exercise may suffice to restore the volume of the dentate gyrus granule cell layer to normal. The significant increase in

dentate gyrus granule cell layer volume in the enriched ADX rats provides indirect evidence that adult born neurons survive in the damaged dentate gyrus.

Taken at face value our finding that the number of DCX positive cells is significantly increased, even after six weeks of wheel running and enrichment, is contrary to the reports of transient increases in neurogenesis as a result of exercise or enrichment (Kempermann & Gage, 1999; Naylor et al., 2005). The alternating method of exercise and enrichment we employed may provide an explanation for the disparate results. Every 24 hours, rats were transferred from a wheel running cage to a novel environment (modified on an almost daily basis). Thus, stimulus novelty remains relatively higher throughout the experiment. This factor may account for the continued increase we observe in adult neurogenesis.

To further confirm long-term survival of adult-born neurons in the damaged dentate gyrus we performed a pilot study. We injected chronic ADX rats receiving CORT replacement with the thymidine analog BrdU and assessed them for the presence of BrdU/NeuN positive cells at two and eight weeks after BrdU administration. At early (two-week) and late survival (eight-week) time-points, cells positive for the neuronal marker NeuN in combination with BrdU were evident in the granule cell layer of chronic ADX rats, showing cells born at the time of BrdU administration had adopted a neuronal fate and remained in the granule cell layer.

By two weeks after exit from the cell cycle the number of surviving adult born neurons in the hippocampus remains highly stable (Kempermann, 2006), even when measured at time-points as long as 11 months after injection with BrdU (Kempermann et al., 2003). Our eight-week time-point falls well after the period by which newly generated neurons become active participants in granule cell layer function (van Praag et al., 2002) and respond to behavioural stimulation (Jessberger & Kempermann, 2003). As adult neurogenesis results in the functional integration of new cells to an existing network (van Praag et al., 2002), a potential treatment strategy becomes available. Inducing an increase in the number of new neurons born in the damaged dentate gyrus offers the possibility of replacing lost hippocampal granule cells with functionally integrated adult-born neurons.

Preliminary evidence from our laboratory suggests that increasing adult neurogenesis in an attempt to repopulate a damaged dentate gyrus is a viable approach (Lai et al., 2007). After 10 weeks of granule cell layer degeneration due to ADX, rats were placed on CORT and administered a cocktail of growth factors (FGF-2 and sonic hedgehog) via an osmotic mini-pump for six-weeks in an attempt to increase neurogenesis within the hippocampus. Both FGF-2 (Kuhn et al., 1997; Rai et al., 2007) and sonic hedgehog (Ahn & Joyner, 2005; Palma et al., 2005) have been shown to increase neurogenesis in normal animals, specifically targeting early stages of the neurogenic process. During administration of growth factors, ADX rats were group-housed in an enriched environment, which has been shown to increase survival of adult born neurons in normal animals (Kempermann et al., 1997; Olson et al., 2006). ADX rats

that received the neurogenic treatments presented with a greater hippocampal granule cell layer volume compared to home-cage housed ADX rats given vehicle. Furthermore, the volume of the hippocampal granule cell layer in treated ADX rats did not differ from that of controls, indicating, at least by this measure, a complete re-growth of the previously degenerated hippocampal subfield. Importantly, those ADX rats that had an increased dentate gyrus granule cell layer volume as a result of treatment with an enriched environment, FGF-2, and sonic hedgehog performed significantly better than their ADX cohorts receiving vehicle and did not differ from controls in a contextual discrimination task. This initial finding suggests a functional re-population of the dentate gyrus granule cell layer in the adult animal, something that has not been reported before. Although more work is required to confirm the results, this initial finding suggests that functional recovery in the adult animal is a distinct possibility.

### **General Conclusions**

The described animal model produces a specific and gradual degeneration of neurons within the adult hippocampus. The gradual loss of hippocampal neurons is a hallmark symptom of a number of pathological states, including traumatic brain injury (Hicks et al., 1993; Yamaki et al., 1998; Huh & Raghupathi, 2007), stroke (Petito et al., 1987; Langdon et al., 2008), and a number of dementias (de Toledo-Morrell et al., 2000; Walker et al., 2007). Similar to patients that suffer hippocampal atrophy due to prolonged cell loss we show that rats with a long-term loss of hippocampal granule cells suffer from cognitive deficits in tasks that have been shown to depend upon intact hippocampal function.

We provide a method by which one form of intrinsic plasticity of the adult brain can be modulated in an attempt to induce functional recovery. Here we employ behavioural and pharmacological techniques that increase the birth and survival of new neurons in the hippocampus. We show that re-population of the damaged dentate gyrus via adult-born neurons is a viable approach to induce functional recovery.

Treatment strategies employing transplantation of exogenous stem cells have proven to be relatively ineffective in alleviating function deficits (Shetty & Turner, 1996; Jeltsch et al., 2003; Turner & Shetty, 2003). A number of issues result from such methods, including the long-term survival of grafted cells and the inability to determine conclusively whether or not grafted cells functionally integrate into an existing neural network (Lindvall & Hagell, 2002). Here we manipulate a naturally occurring system that continually results in the functional integration of new neurons into an existing network within the adult animal.

The functional integration of new neurons in the adult animal provides a treatment strategy by which an increase in the number of new cells results in a re-population of previously damaged brain region. The effectiveness of treatment methods designed to induce re-population of the granule cell layer and induce functional recovery can be assessed using the available tools and techniques discussed in the preceding chapters. Importantly, this demonstration of the loss of function associated with brain damage and the development of treatment strategies that result in functional recovery provides a

model by which the required conditions for replacing neural circuitry in other brain regions may be explored.

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## Appendix One

In an attempt to confirm the accuracy of dentate gyrus granule cell layer volumetric analysis conducted using ImageJ software (<http://rsb.info.nih.gov/ij/>), a comparison was performed between results collected from ImageJ and software designed specifically for stereological analysis (Stereo Investigator, version 8.21.2, MicroBrightField). The volume of the dentate gyrus granule cell layer was calculated using both programs in a single group of rats and then statistically compared.

### Methods

A subset of control rats (n = 8) from Chapter 4, Experiment 2, “Combined Environmental Enrichment And Exercise Increases Neurogenesis In The Damaged Dentate Gyrus” was employed for this experiment. All experimental procedures were performed in accordance with the University of Lethbridge and CCAC animal welfare committee guidelines.

A single series of tissue (1/12 section sampling fraction) was stained with Cresyl Violet according to standard lab protocol. The tissue was analyzed utilizing ImageJ (<http://rsb.info.nih.gov/ij/>) and Stereo Investigator software (version 8.21.2, MicroBrightField) by two experimentally blind, independent raters.

### Image J

Images of Cresyl Violet sections were taken using a Zeiss Axioskop 2 MotPlus epifluorescent scope attached to a QImaging Retiga CCD camera (Burnaby, British

Columbia, Canada) using a Plan Neofluor 10x/0.3 objective. Images were then imported into ImageJ software (<http://rsb.info.nih.gov/ij/>) after which a sampling grid was randomly thrown over each image. An area per point of 0.02 square millimeters was determined as an efficient and reliable size for the purposes of this study. The total number of contact points between the granule cell layer and the grid was quantified for each section. The number of contact points per section was multiplied by the area associated with each point (0.02 square millimeters), the section cut thickness (40 microns), and the section sampling fraction (1/12). These numbers were then summed to provide an estimated total volume of the dentate gyrus granule cell layer (as described in Mouton, 2002).

### Stereo Investigator

The same Cresyl Violet sections that were analyzed via ImageJ were quantified by an independent rater using Stereo Investigator software (version 8.21.2, MicroBrightField). A Plan Neofluor 10x/0.3 lens was employed for all quantification. As previously mentioned (p. 42), a pilot study revealed that a grid with an area per point of  $100 \mu\text{m}^2$  was sufficient to provide enough contact points between the grid and granule cell layer in the dentate gyrus. Volume analysis was performed according to standard protocol provided by MicroBrightField.

### Results

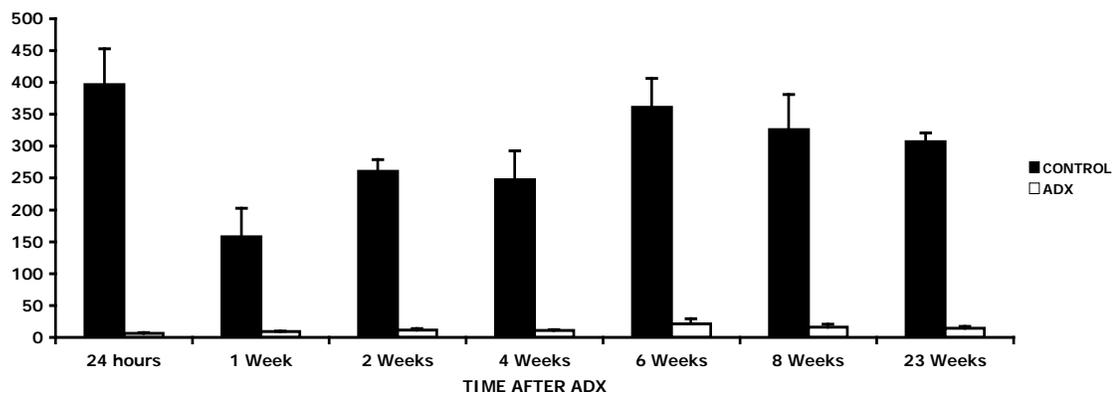
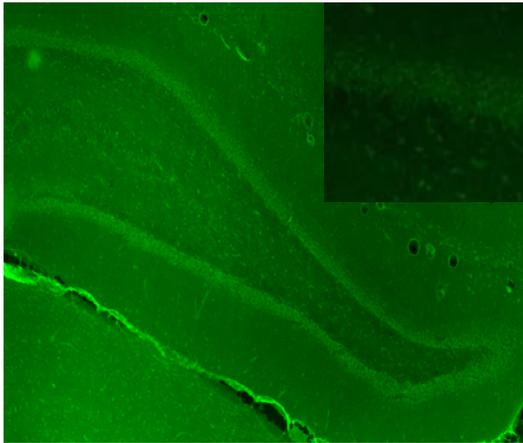
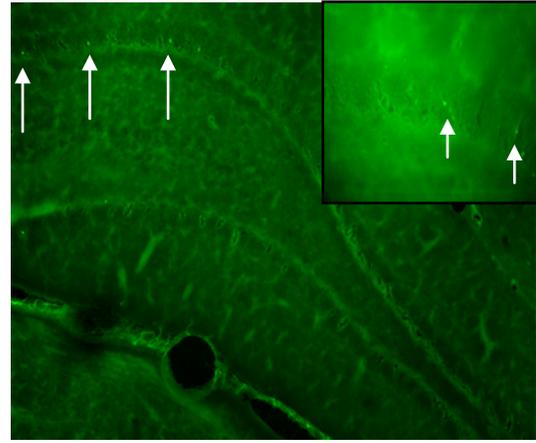
A one-way analysis of variance determined that there was no significant difference between mean group volumes,  $F(1,15) = 1.86$ ,  $p = 0.19$ . As analyzed by

ImageJ software the mean volume of the dentate gyrus granule cell layer was  $2.85 \pm 0.09$  cubic millimeters. Analysis by Stereo Investigator software revealed a group mean of  $3.09 \pm 0.16$  cubic millimeters. Furthermore, a significant positive correlation between the volumes as determined by analysis in ImageJ and Stereo Investigator was evident  $r(8) = 0.842$ ,  $p < 0.01$ .

## Discussion

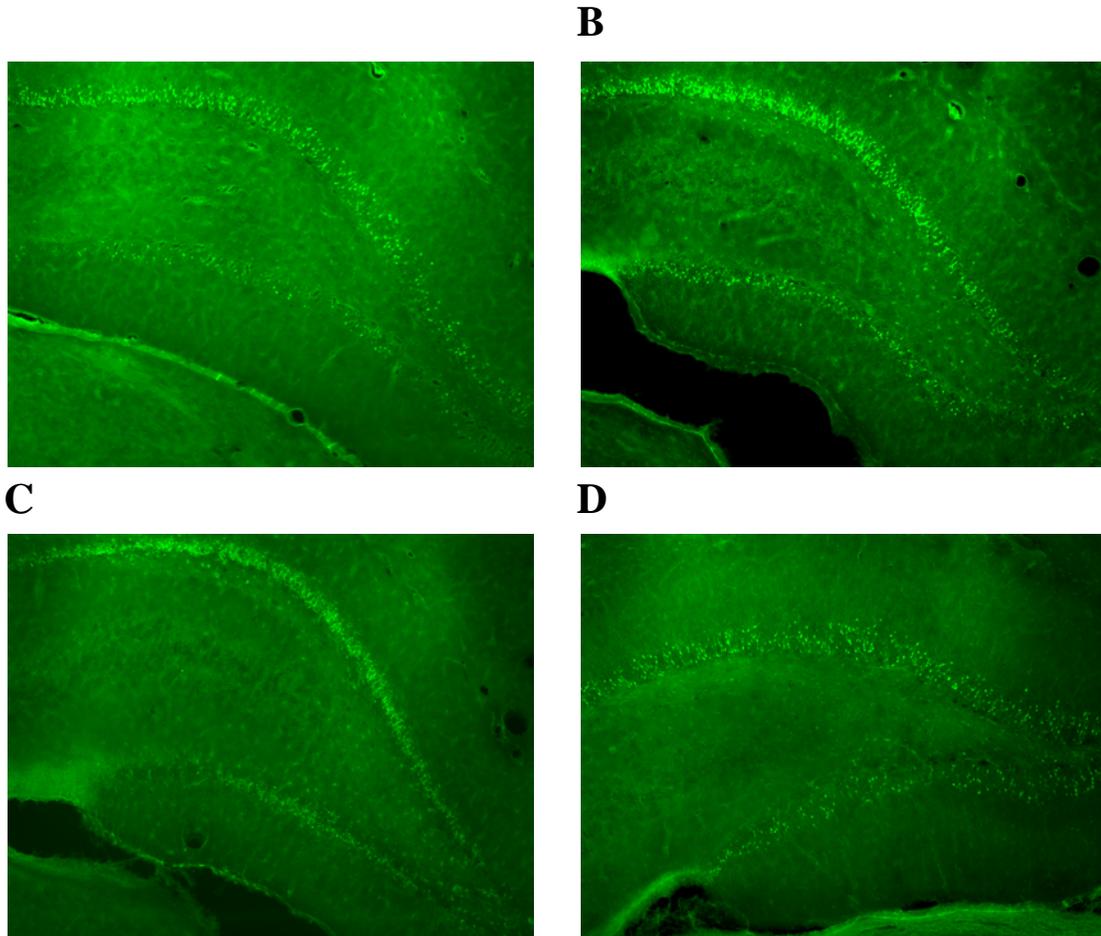
Here we show that volume estimates conducted using ImageJ do not differ significantly from those produced by Stereo Investigator, a dedicated stereological software package. We also report a significant, positive correlation between the two computer programs in regards to estimates of volume. This confirms that volume estimation using ImageJ is a viable alternative to software designed specifically for stereological analysis. Regardless of the program employed to assess volume, the Cavalieri method remains a viable technique by which to estimate total volume of a given structure.

The relatively high cost of software packages designed specifically to perform stereological analysis may be prohibitive for some investigators. Here we show that using the free software package ImageJ, estimates of total volume can be achieved in a manner similar to more expensive alternatives.

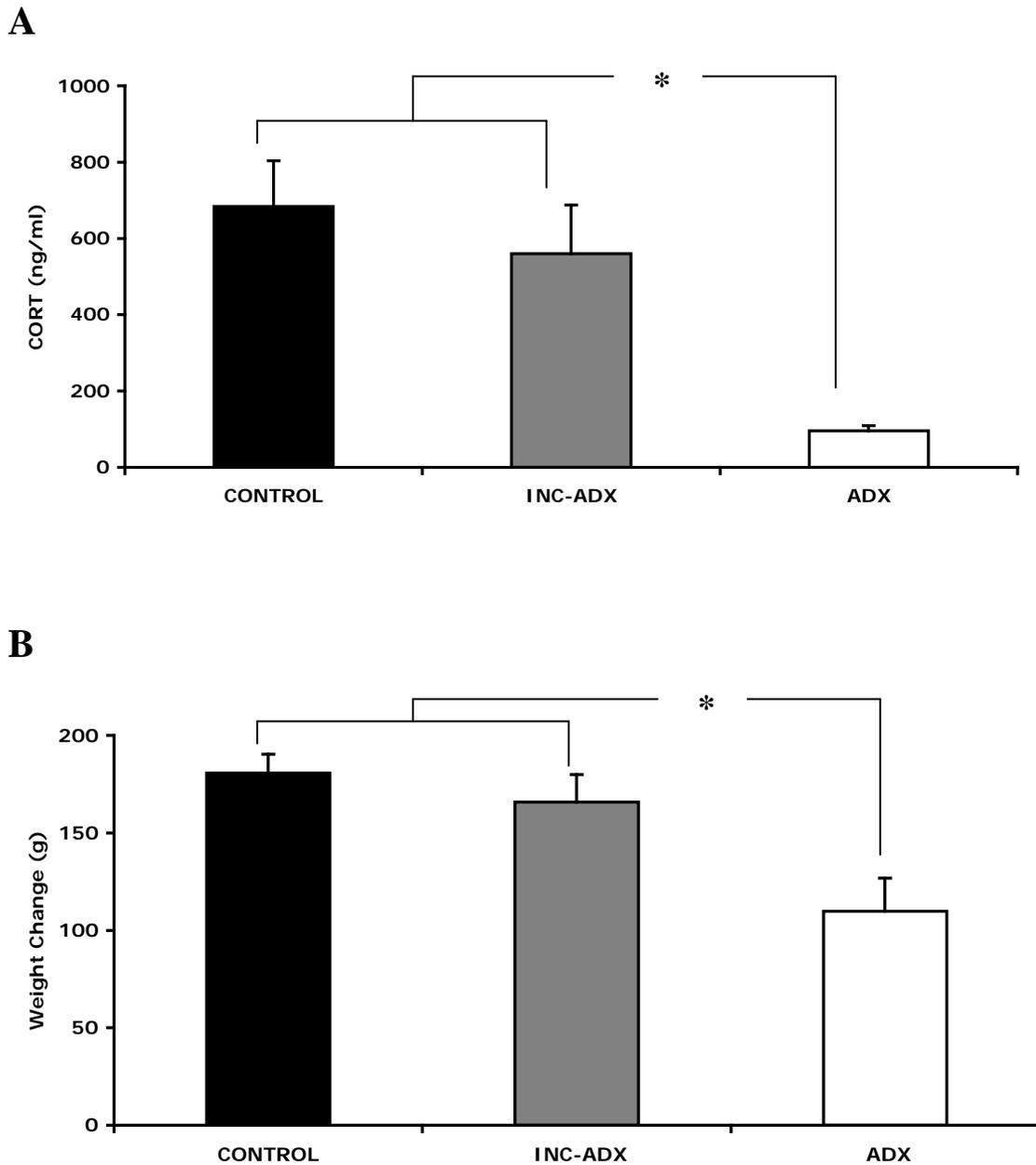
**A****B****C**

**Figure 1.** (A) Bilateral adrenalectomy (ADX) resulted in a significant decrease in circulating CORT across all post-surgical time-points. (B) The dentate gyrus of a control rat labeled for the cell death marker Fluoro-Jade B. No cells positive for Fluoro-Jade B were evident in the hippocampal granule cell layer of any of the control animals. (C) Fluoro-Jade B positive cells in the superior, lateral blade of the dentate gyrus in an ADX rat three days after surgery (indicated by arrows).

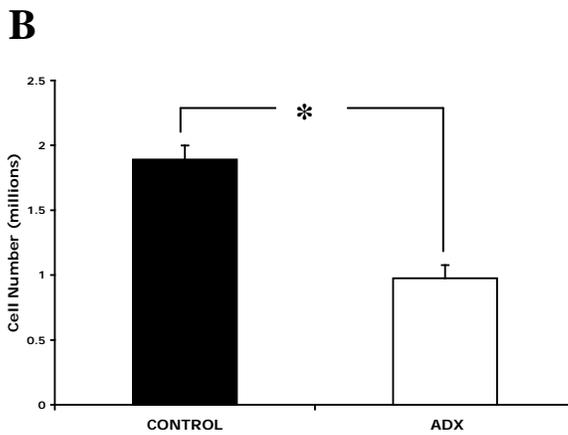
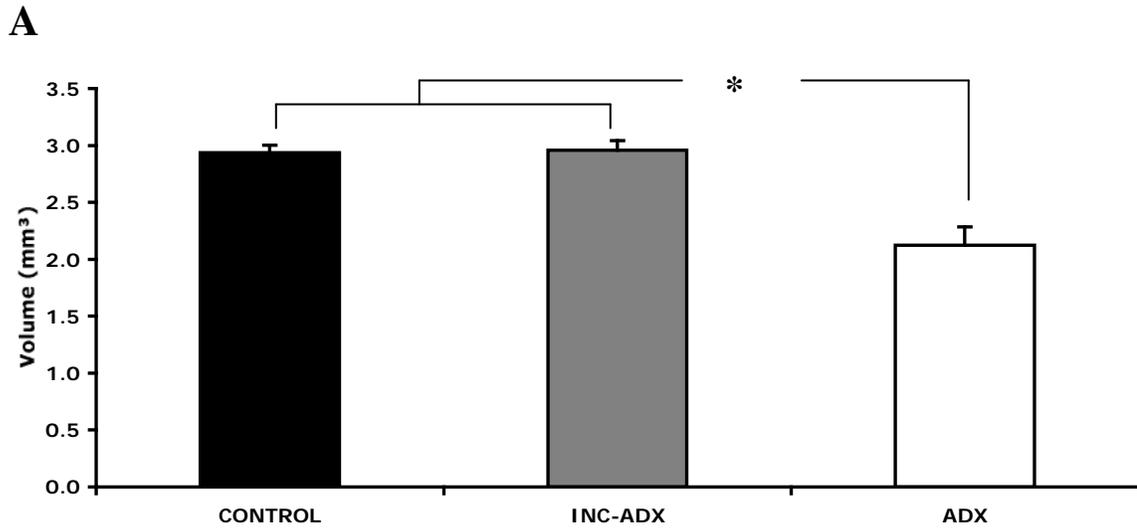
**A**



**Figure 2.** Representative Fluoro-Jade B labeled sections from ADX rats at 2 weeks (A), 4 weeks (B), 8 weeks (C), and 23 weeks (D) after surgery. Fluoro-Jade B labeling was evident throughout the entire granule cell layer of the dentate gyrus at all of the above time-points and was most concentrated in the superior, lateral blade. Fluoro-Jade B labeling had decreased substantially by 23 weeks post-surgery but was still apparent throughout the granule cell layer.



**Figure 3.** (A) Complete removal of the adrenal glands (ADX) resulted in a significant decrease in CORT. A subset of ADX rats (INC-ADX) did not differ from controls, suggesting that the ADX was incomplete. (B) ADX also resulted in a significantly reduced weight gain over the 10-week degeneration period. Those rats that were deemed INC-ADX did not differ significantly from controls in terms of weight gain. Adapted from Spanswick et al., 2010 (re-printed with permission).



**C**

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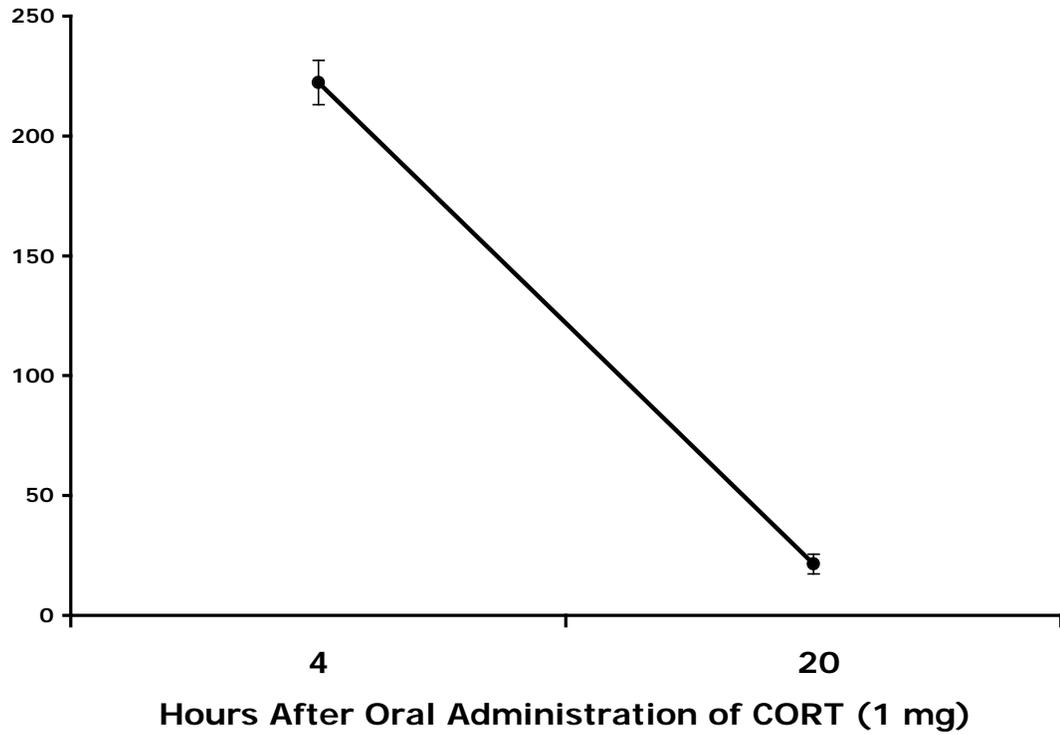
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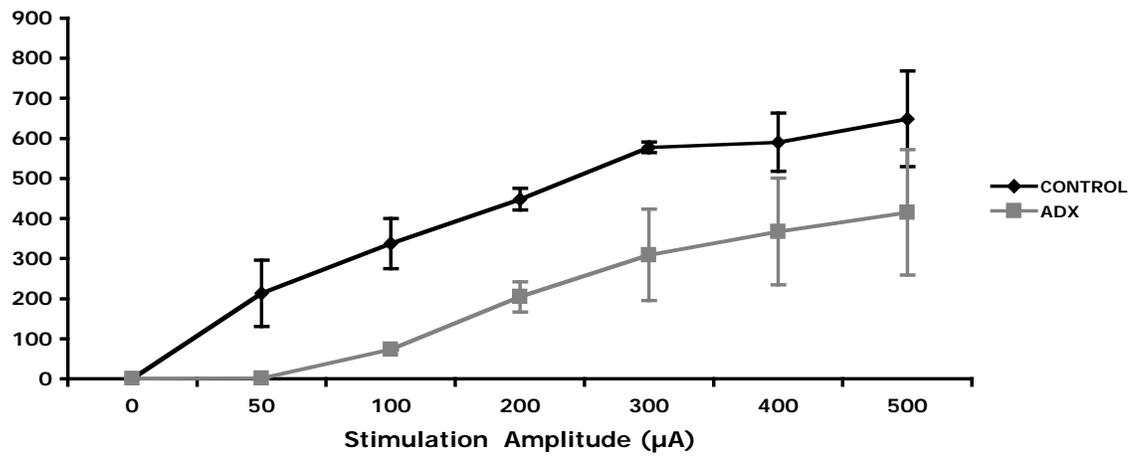
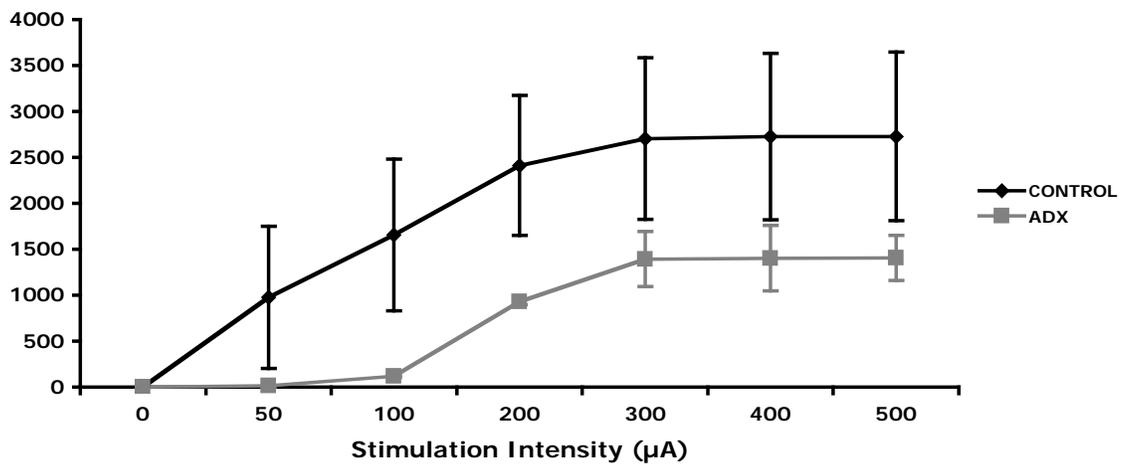
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**Figure 4.** (A) As determined by the Cavalieri method, ADX rats had a significantly smaller dentate gyrus granule cell layer volume compared with both controls and incomplete ADX rats. (B) Further analysis with the optical fractionator showed that ADX resulted in a significant loss of granule cells. (C-E) Representative DAPI-labeled sections taken from a control (C), incomplete ADX (D), and an ADX rat (E) ADX-induced degeneration was most prevalent in the superior blade of the dentate gyrus (arrows). Adapted from Spanswick et al., 2010 (re-printed with permission).



**Figure 5.** Time-course of CORT levels, taken at 4 and 20 hours after oral administration of 1 mg of corticosterone in ADX rats. This method and amount of CORT was successful in producing a diurnal rhythm in ADX rats (Spanswick et al., 2007, re-printed with permission).

**A****B**

**Figure 6.** (A) Chronic ADX resulted in a significantly attenuated fEPSP slope relative to control rats, despite ADX rats receiving replacement CORT at the time of data collection. (B) A tendency for ADX rats to have a lower population spike amplitude compared to intact controls was apparent.

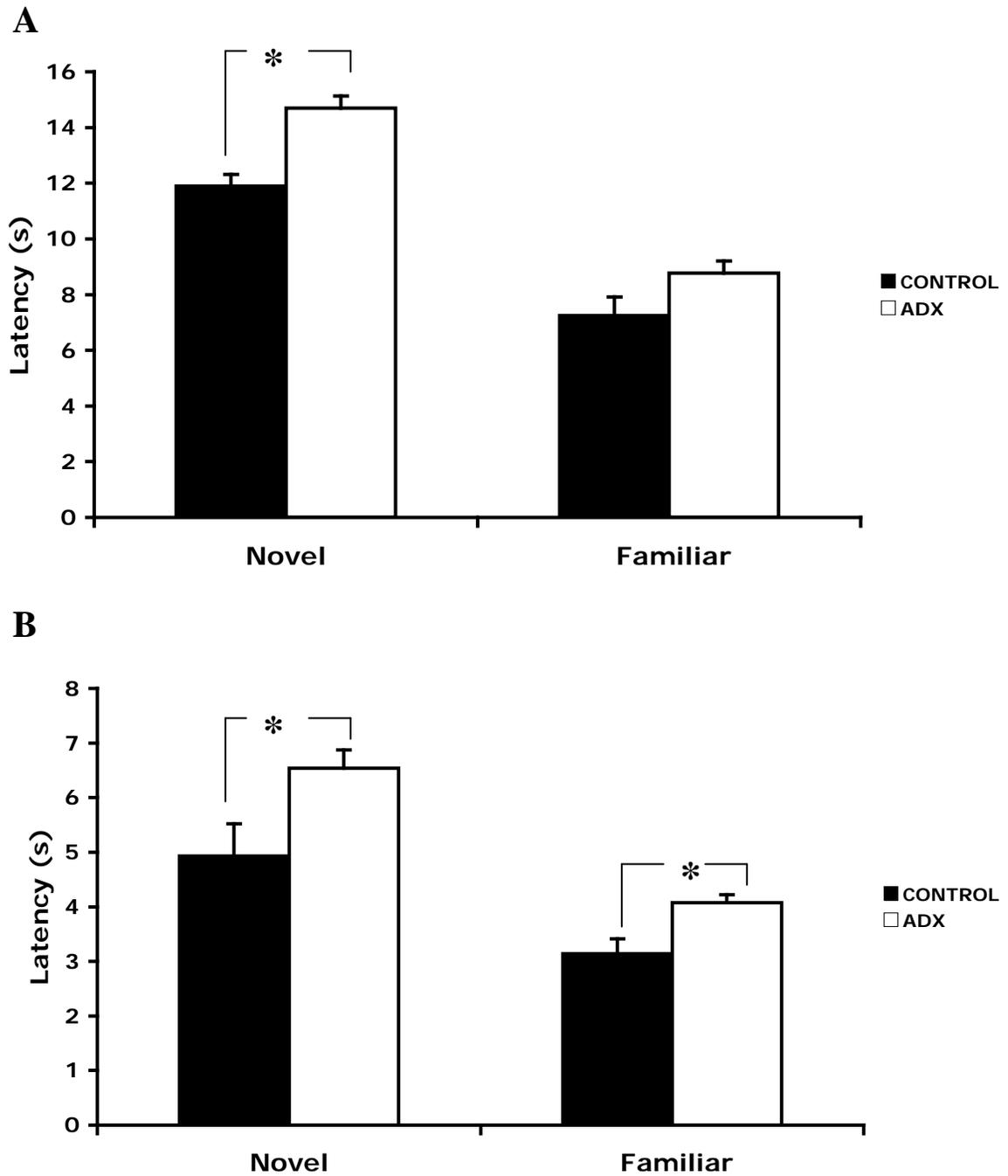
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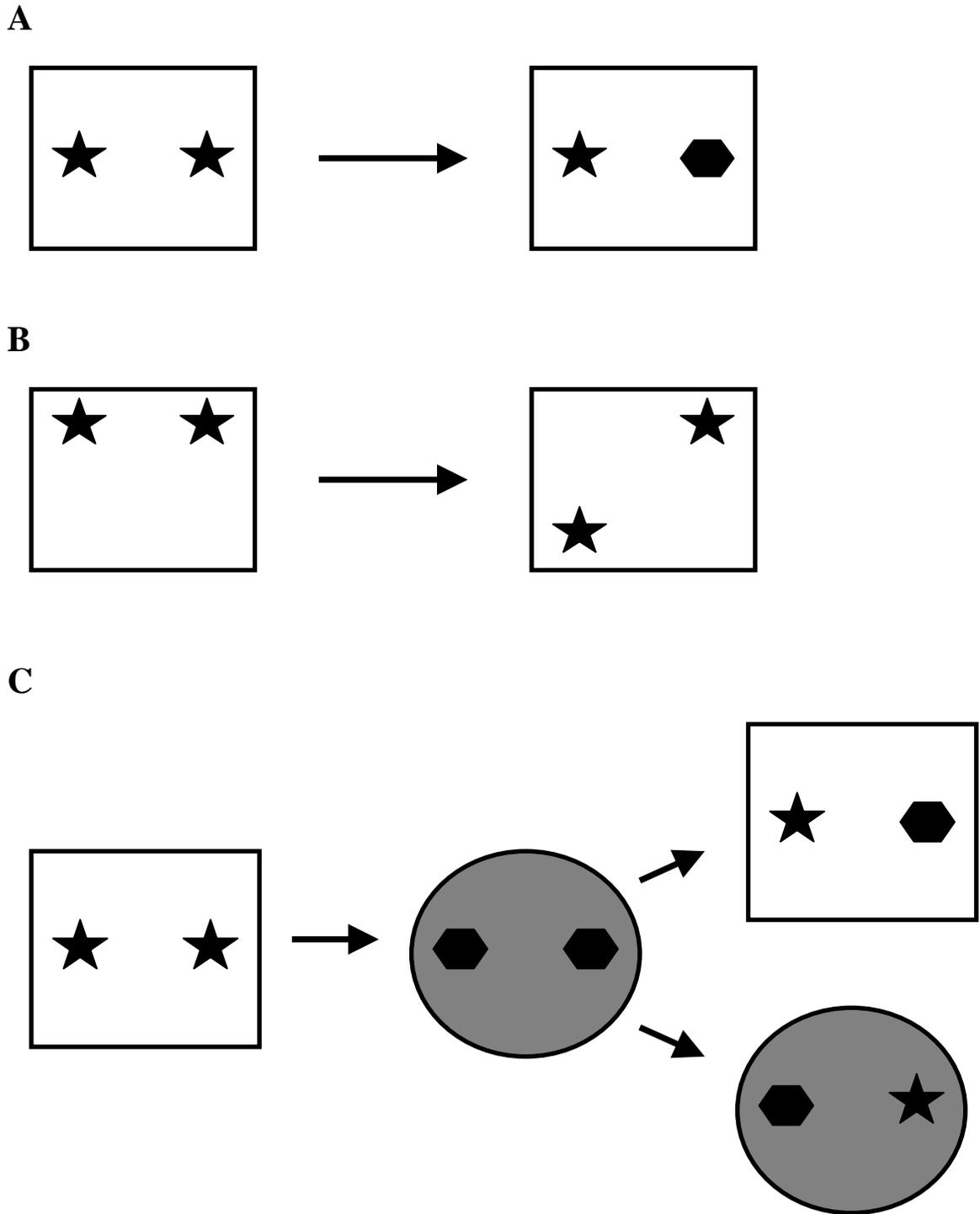
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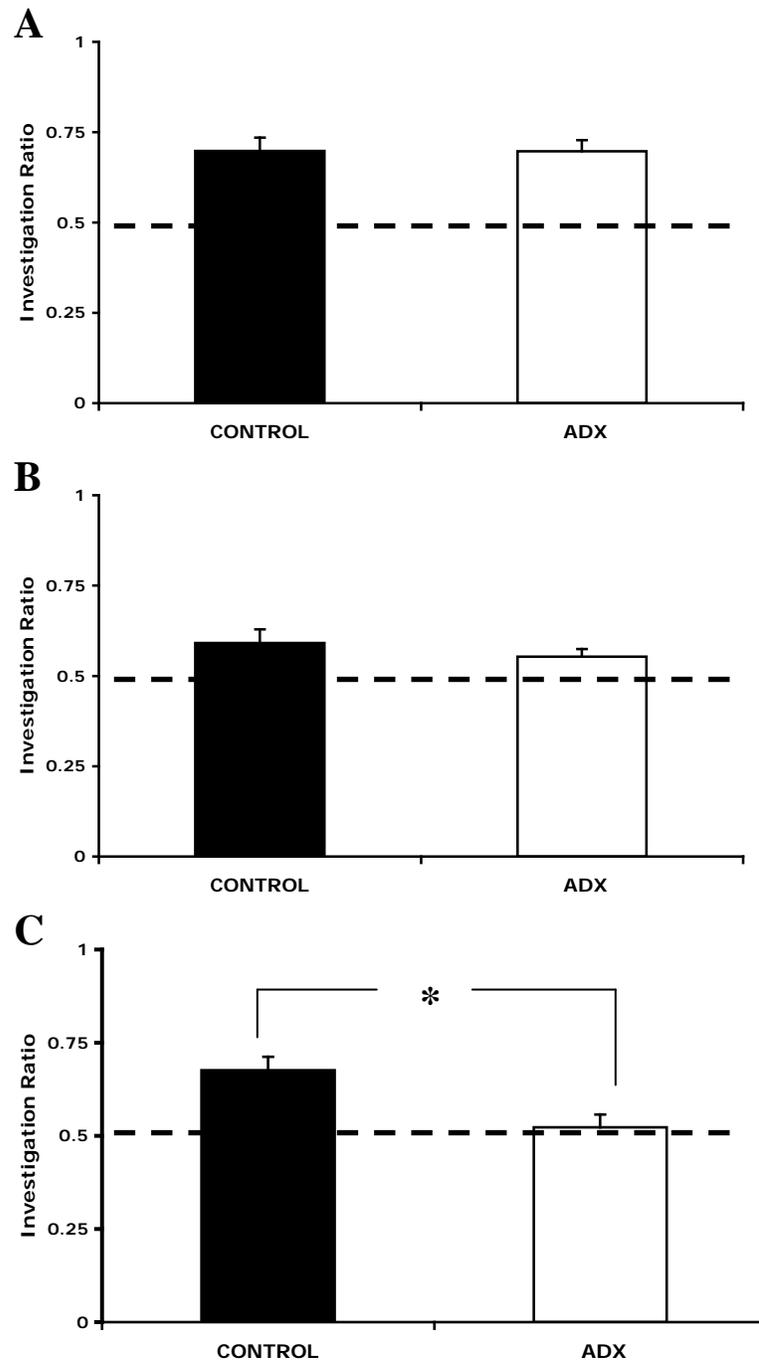
**Figure 7.** Representative DAPI-labeled sections showing electrode placement in the dorsal dentate gyrus of a control (A) and an ADX rat (B). Red circles highlight the approximate placement of the tip of the electrode.



**Figure 8.** Performance in the Morris water task, pre- and post-chronic treatment with CORT. (A) Prior to daily administration of CORT for six weeks, ADX rats displayed a deficit in novel platform locations as measured by latency to locate the hidden platform. (B) Post-treatment analysis revealed a deficit in the ability of ADX rats to locate both novel and familiar platform locations. Asterisks indicate statistical significance (Adapted from Spanswick et al., 2007, re-printed with permission).

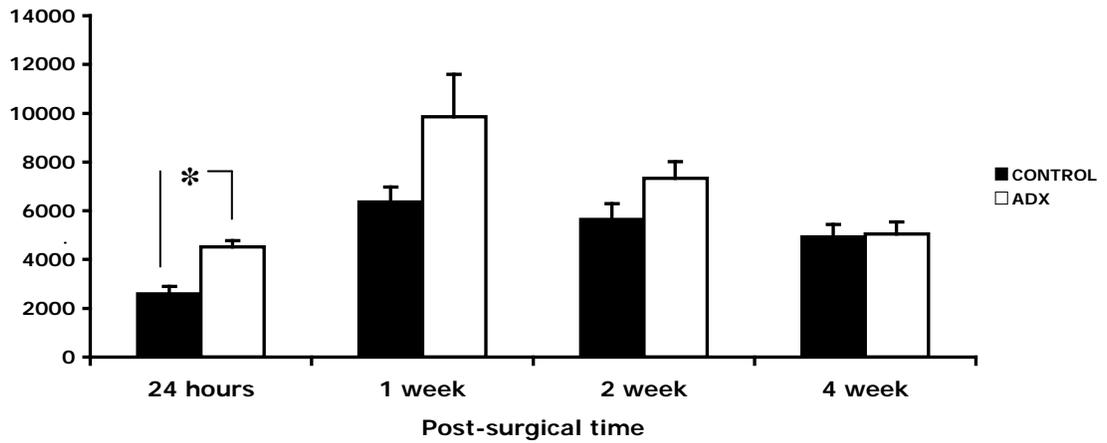


**Figure 9.** Examples of the object discrimination tasks. (A) Novel object preference. (B) Novel place preference. (C) Object/context mismatch task (Spanswick et al., 2010, reprinted with permission).

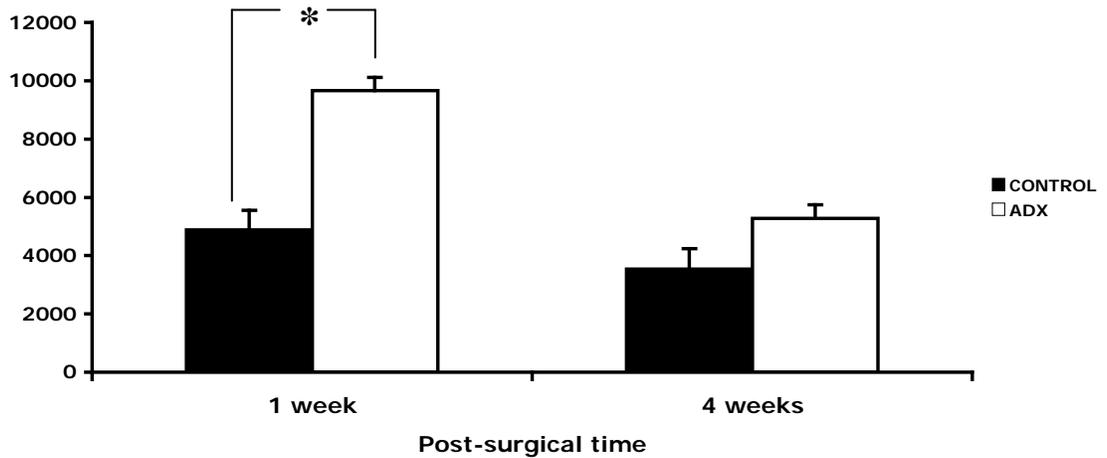


**Figure 10.** Performance of control and ADX rats was similar in novel object preference (A) and novel place preference (B); both groups discriminated the novel object or place at greater than chance levels. (C) ADX animals were significantly impaired relative to controls and did discriminate above chance levels during the novel object/context mismatch paradigm. (Dashed lines) Chance, Asterisks denote significant difference (Adapted from Spanswick et al., 2010, re-printed with permission).

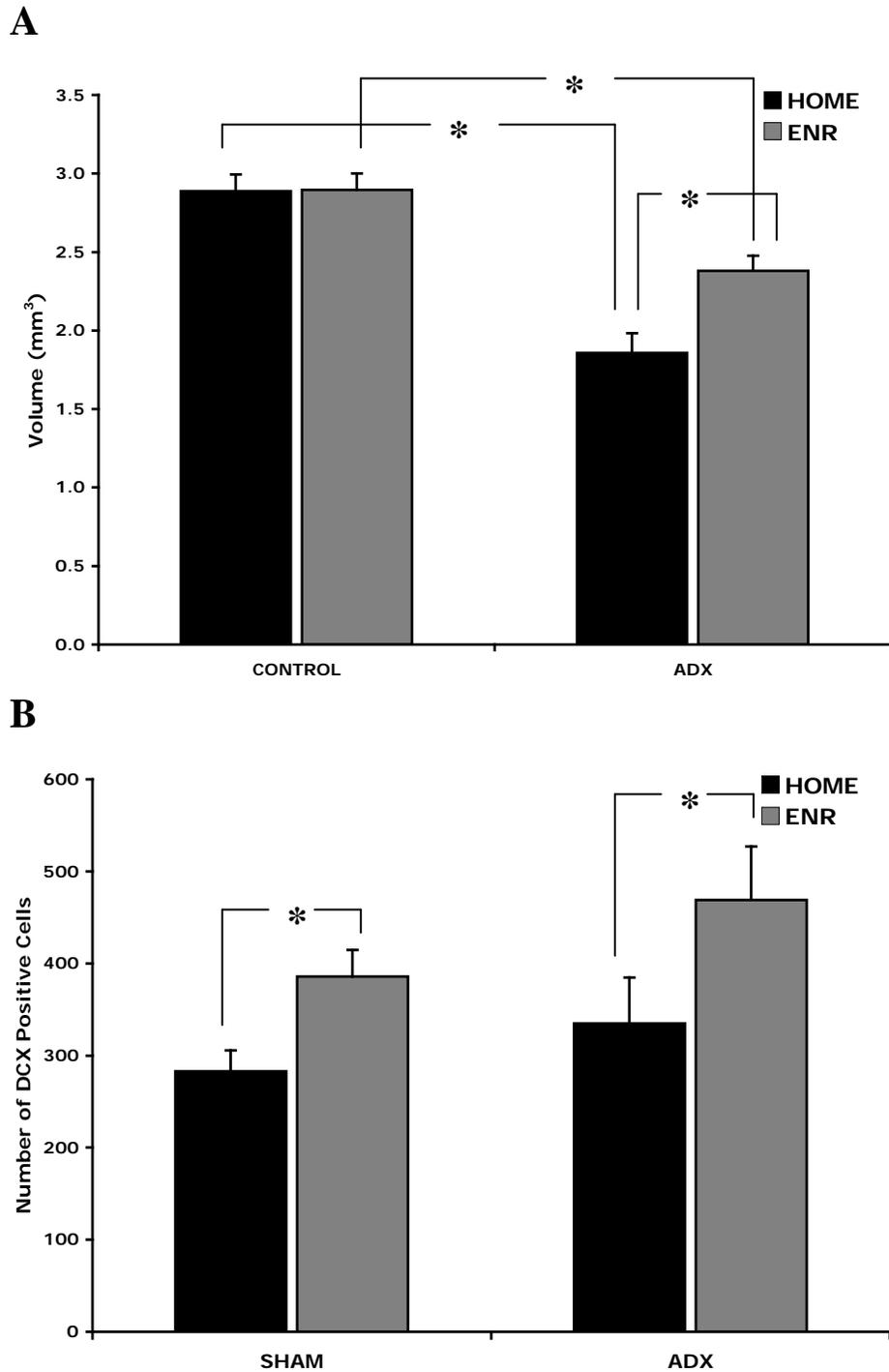
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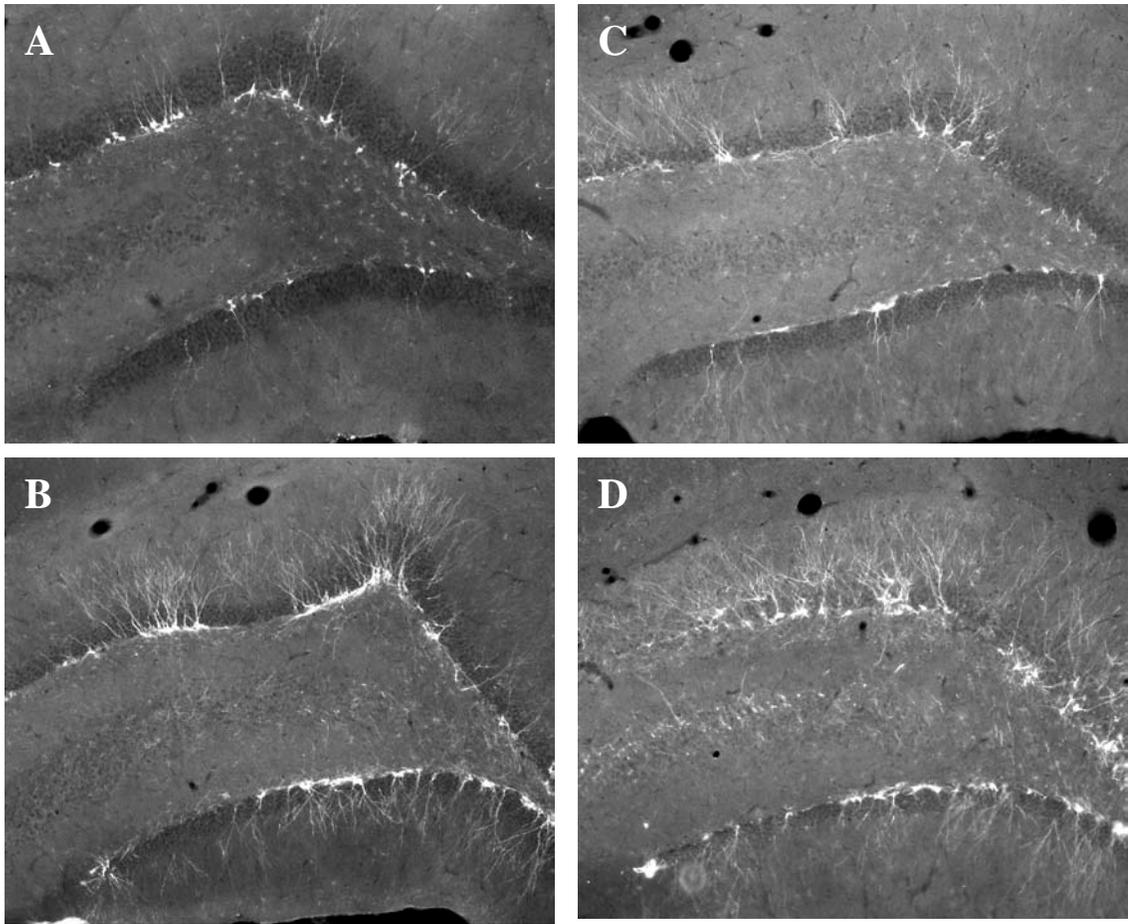
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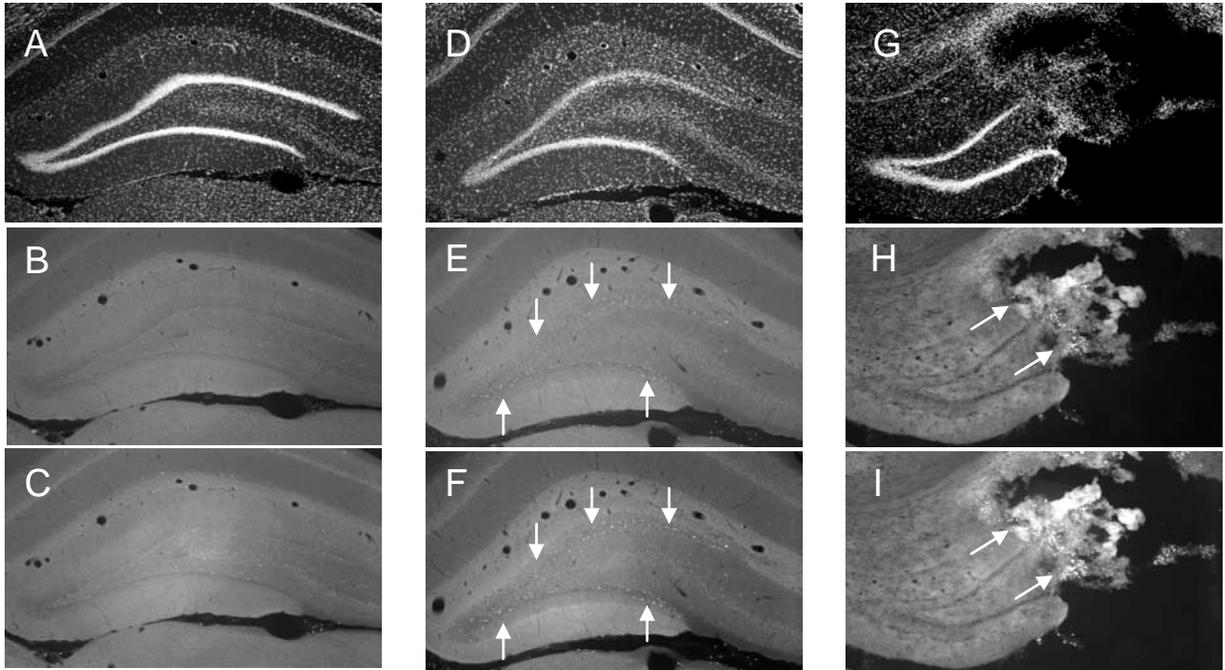
**Figure 11.** (A) Total number of Ki67 positive cells quantified in the dentate gyrus granule cell layer of controls and ADX rats at 24 hours, 1 week, 2 weeks, and 4 weeks after ADX surgery. ADX rats had significantly more Ki67 positive cells than controls at 24 hours after surgery, a tendency for ADX rats to have more Ki67 positive cells than controls was apparent at the 1 week time-point. No significant group differences were evident at the 2 or 4-week time-points. (B) A replication of the time-course using improved immunohistochemical and quantification techniques revealed a significant difference in the total Ki67 cell number estimate at 1 week but not 4 weeks after ADX surgery. Asterisks indicate a significant difference.



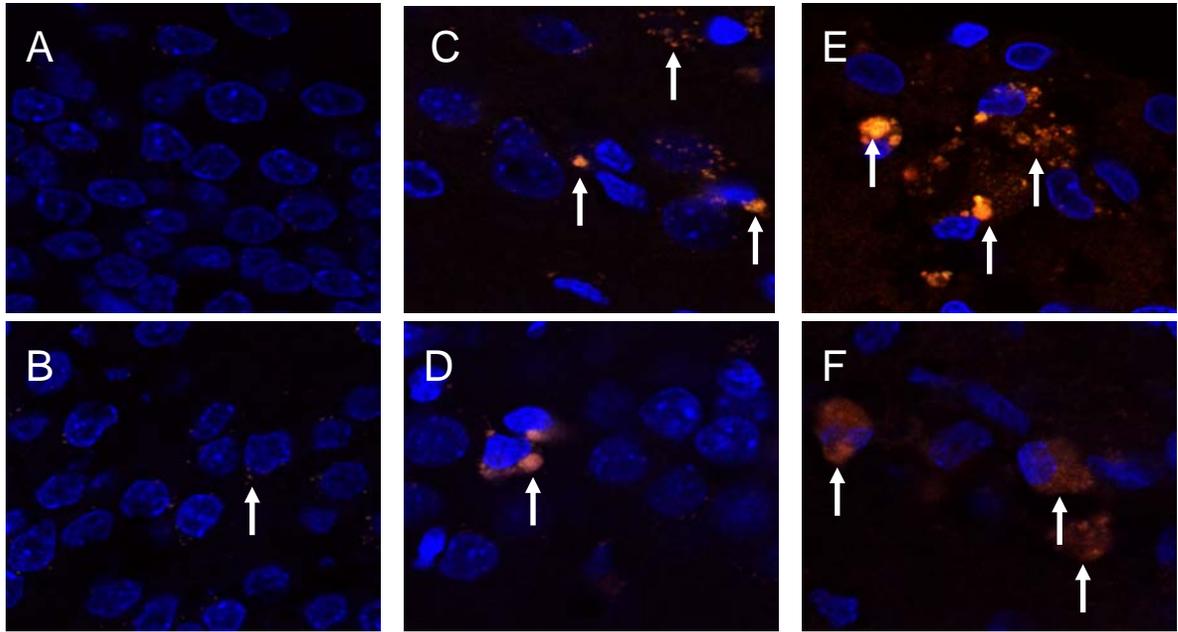
**Figure 12.** (A) Chronic-ADX significantly reduced the volume of the dentate gyrus granule cell layer in rats housed in the home-cage. A combination of six weeks of enriched housing and wheel running significantly increased the volume of the granule cell layer in ADX rats, compared to their home-cage counterparts. (B) The alternating treatment of enrichment and wheel running significantly increased the number of doublecortin (DCX) positive cells in the dentate gyrus of ADX and control rats. Asterisks denote significance.



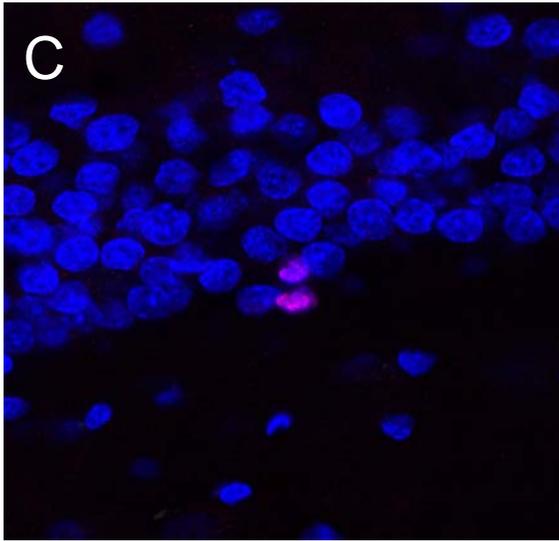
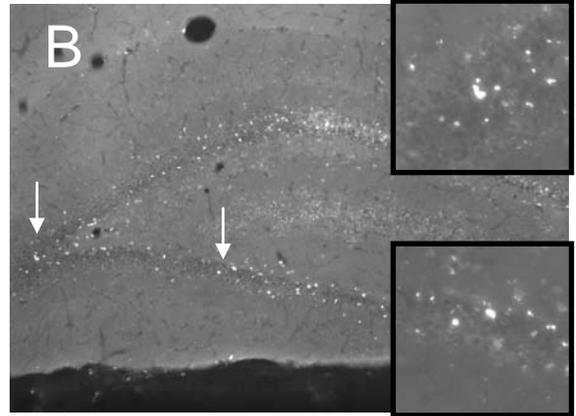
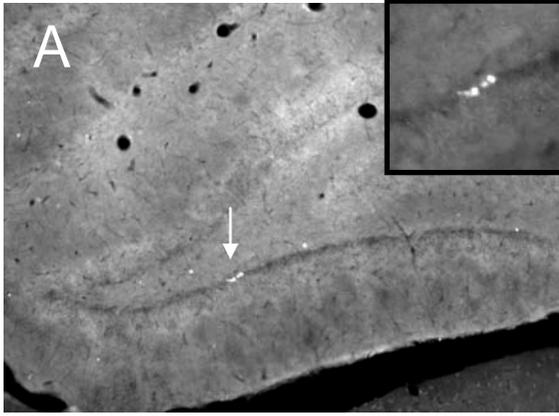
**Figure 13.** Regardless of ADX, the combined enrichment and exercise treatment significantly increased neurogenesis as indexed by doublecortin (DCX). Representative pictures of DCX positive cells in the dentate gyrus of (A) home-cage controls, (B) controls exposed to wheel running and enrichment, (C) ADX rats housed in the home-cage, and (D) ADX rats exposed to wheel running and enrichment.



**Figure 14.** Representative pictures of Control (A-C), ADX (D-F), and Stroke (G-I) rats. Nuclear labeling was apparent in all tissue sections and was only visible under the DAPI filter (A,D,G). Autofluorescence was minimal in control rats and was not readily apparent under either the FITC (B) or the TRITC (C) filters. ADX resulted in high levels of autofluorescence in both the dorsal and ventral blades of the dentate gyrus and was observed under both FITC (E) and TRITC (F) filters. Similarly, autofluorescence was high in the peri-infarct area of the stroke tissue and was apparent under both filters (H,I). From Spanswick et al., 2009 (re-printed with permission).



**Figure 15.** Confocal images of Control (A,B), ADX (C,D), and Stroke (E,F) tissue. Arrows indicate minimal amounts of autofluorescence in Control tissue (B) and a significant amount in ADX and Stroke tissue (C-F). Confocal analysis revealed that autofluorescence sometimes surrounds, but never co-localizes with the DAPI or Hoechst (data not shown) signal. From Spanswick et al., 2009 (re-printed with permission).



**Figure 16.** (A) Control tissue labeled with Ki67, areas indicated with an arrow are shown at a higher magnification (20X) in the insert. (B) Unlabeled ADX tissue displaying typical levels of autofluorescence. Inserts show areas marked by arrows at a higher magnification. Autofluorescence associated with damage looks strikingly similar to the Ki67 signal observed in the control. (C) Confocal image showing co-localization of Ki67 (red) and DAPI in control tissue at high magnification (indicated by arrows). From Spanswick et al., 2009 (re-printed with permission).

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**Figure 17.** BrdU/NeuN co-localization (indicated by downward arrows) in the dentate gyrus granule cell layer of control and ADX rats at two and eight weeks after administration of BrdU. (A) A BrdU/NeuN positive cell in the granule cell layer of a control rat at the two-week survival time-point. (B) BrdU/NeuN positive cell in an ADX rat 2 weeks after injection, further co-localization with the nuclear marker DAPI avoided false positives as a result of increased levels of autofluorescence. (C) Co-localization of BrdU/NeuN at the 8-week time-point in a control rat. (D) BrdU/NeuN/DAPI co-localization in an ADX rat at the 8-week survival time-point. Smaller, upward facing arrows highlight areas of high autofluorescence as determined by a lack of co-localization with the DAPI signal.