The role of the dentate gyrus and adult neurogenesis in hippocampal-basal ganglia associated behaviour

by

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TABLE OF CONTENTS

	Page
Declaration	vi
Acknowledgments	vii
List of Tables	viii
List of Figures	ix
Abstract	xii

CHAPTER 1 **Introduction and outline of thesis**

General introduction	1
Neuroanatomical organisation of the dentate gyrus	1
The dentate gyrus: a role in cognition and behaviour	3
Hippocampal-basal ganglia interactions	4
The physiology of neurogenesis	4
Neurogenesis: A role in cognition and behaviour	7
Neurogenesis and stress	9
Outline of the thesis	10

CHAPTER 2 **Effects of learning and chronic unpredictable stress on adult neurogenesis**

Abstract 11

Introduction 12

Materials and methods 14

Results 23

Discussion 35

CHAPTER 3 **The role of the dentate gyrus in hippocampal and hippocampal-basal ganglia dependent behaviour**

Abstract 39

Introduction 40

Materials and methods 42

Results 48

Discussion 61

CHAPTER 4 **Concluding remarks**

Introduction 65

Stress effects on neurogenesis and cognition 65

Surgical lesion effect on cognition 67

Recommendations for future work 68

CONCLUSIONS 71

REFERENCES 73

APPENDIX A	Ethics approval	83
APPENDIX B	Autoshaping software protocol	85
APPENDIX C	Stimulus response learning software protocol	91
APPENDIX D	Delay discounting probe trial software protocol	97
APPENDIX E	Simple discrimination learning software protocol	101
APPENDIX F	Immunohistochemistry protocol	107
APPENDIX G	Example of the pattern separation problems used in the lesion experiment	113
APPENDIX H	Cresyl violet staining protocol	115

DECLARATION

This thesis contains no material published elsewhere, from any other thesis or any other person's work, except where due reference is made, and has not been submitted for the award of any other degree or diploma in any other tertiary institution.

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LIST OF TABLES

Page

Table 2.1 List of experimental groups and their tasks. 14

Table 2.2 Randomisation of daily chronic stress regime applied over a 21 day period for each rat. 17

Table 2.3 List of the methamphetamine dose and administration regime. 22

Table 2.4 Summary table of significant results from chronic unpredictable stress. 33

Table 3.1 List of experimental groups and their tasks. 43

Table 3.2 Summary table of significant results from neurosurgical lesions of the hippocampus. 59

LIST OF FIGURES

		Page
Figure 1.1	Anatomical representation of the hippocampal formation in the rat compared to human.	2
Figure 1.2	Schematic of an anterior coronal section on of the hippocampus and schematic representation of the sub-regions comprising the hippocampus, consisting of the dentate gyrus made up of the dorsal and rostral blades.	5
Figure 1.3	Illustration of the unilateral circuitry of the hippocampus and the neurogenic region of the dentate gyrus and the migratory pathway of the newly generated cells.	6
Figure 2.1	Social defeat cage and divider used during the social interaction.	18
Figure 2.2	The physical restraint apparatus used during the chronic stress protocol.	19
Figure 2.3	Illustration of the images presented during the simple discrimination task.	20
Figure 2.4	Illustration of the images presented during the stimulus-response task.	21
Figure 2.5	Learning mediated BrdU+ labelled cell survival in the dentate gyrus.	24
Figure 2.6	Comparision of observed and predicted cell survival.	25

Figure 2.7	Stimulus-response: Task acquisition. Mean correct response per session block for both experimental groups across acquisition for the 27 days.	26
Figure 2.8	Impulsivity task: Large reward preference acquisition. Mean responses per session block for large reward key for both experimental groups across acquisition for the 14 days.	28
Figure 2.9	Impulsivity probe trials: Mean response per session for large reward key for both experimental groups across acquisition.	29
Figure 2.10	Effect of dose of methamphetamine on distance travelled in the locomotor activity.	29
Figure 2.11	Simple discrimination task acquisition. Mean correct response per session block for both experimental groups across acquisition for the 14 days.	31
Figure 3.1	Photograph depicting the elevated plus maze within the experimental testing room.	47
Figure 3.2	Illustration of the "Transfer" criteria used to measure transfer latency.	47
Figure 3.3	Representative microphotographs of sham and hippocampal lesion showing deformation and cellular thinning of the dentate gyrus as well as extensive cell loss in CA1-CA3 regions in the lesioned animal.	49
Figure 3.4	Simple discrimination: task acquisition. Mean correct response per session block for both lesion and sham groups across 14 days of testing.	50

Figure 3.5	Delayed non-matching to place small pattern separation task: Task acquisition. Mean correct arm choice per session block for both lesion and sham groups across 12 days of testing.	51
Figure 3.6	Delayed non-matching to place large pattern separation task: Task acquisition. Mean correct arm choice per session block for both lesion and sham groups across 12 days of testing.	52
Figure 3.7	Reference memory deficits in lesioned animals.	53
Figure 3.8	Stimulus-response: Task acquisition. Mean correct response per session block for both lesion and sham groups across the 27 days of testing.	54
Figure 3.9	T-maze task acquisition.	55
Figure 3.10	Impulsivity task: Large reward preference acquisition. Mean correct response per session block for both experimental groups across acquisition for the 14 days.	56
Figure 3.11	Impulsivity probe trials: Mean response per session for large reward key for control and lesion groups across acquisition.	57
Figure 3.12	Effect of dose of methamphetamine on distance travelled in the locomotor activity assay.	58
Figure F.1	Illustration of signal amplification by avidin-biotin complex formation.	110

ABSTRACT

The ability of the brain to continually generate new neurons throughout life is one of the most intensely researched areas of modern neuroscience. While great advancements in understanding the biochemical mechanisms of adult neurogenesis have been made, there remain significant obstacles and gaps in connecting neurogenesis with behavioural and cognitive processes such as learning and memory. The purpose of the thesis was to examine by review and laboratory experimentation the role of the dentate gyrus and of adult neurogenesis within the hippocampus in the performance of cognitive tasks dependent on the hippocampal formation and hippocampal-basal ganglia interactions. Advancement in understanding the role of neurogenesis in these processes may assist in improving treatments for common brain injury and cognitive diseases that affect this region of the brain.

Mild chronic stress reduced the acquisition rate of a stimulus-response task (p=0.043), but facilitated the acquisition of a discrimination between a small and a large reward (p=0.027). In locomotor activity assays, chronic stress did not shift the dose-response to methamphetamine. Analysis of 2,5-bromodeoxyuridine incorporation showed that, overall, chronic mild stress did not effect survival of neuronal progenitors . However, learning of the tasks had a positive influence on cell survival in stressed animals (p=0.038). Microinjections of colchicine produced significant lesions of the dentate gyrus and surrounding CA1-CA3 and neocortex. Damage to these regions impaired hippocampal-dependent reference memory (p=0.054) while preserving hippocampal independent simple discrimination learning. In a delay discounting procedure, the lesions did not induce impulsive-like behaviour when delay associated with a large reward was introduced. The experiments uphold a current theory that learning acts as a buffer to mitigate the negative effects of stress on neurogenesis.

CHAPTER 1

Introduction and outline of the thesis

GENERAL INTRODUCTION

The ability of the brain to continually generate new neurons throughout life is one of the most intensely researched areas of modern neuroscience. While great advancements in understanding the biochemical mechanisms of adult neurogenesis have been made, there remain significant gaps connecting this phenomenon with higher order functions, such as learning and memory. The dentate gyrus of the hippocampus has been identified as one of two major neurogenic regions, along with the subventricular zone of the olfactory bulbs, which sustain adult neurogenesis (Gage 2002; Ehninger and Kempermann 2008; Mongiat and Schinder 2011). It has been suggested that since neurogenesis occurs within the hippocampus (an area that has long been associated with learning and memory), new neurons created in this area may contribute to the function of this structure (Eichenbaum 2000; Fortin et al., 2002; Frankland and Bontempi, 2005). However, the answer as to *why* this astounding ability occurs in only two localised areas and *how* it may contribute to brain function remains to be elucidated. This thesis seeks to further understand the functional role of neurogenesis in hippocampal activity and its relationship between hippocampal-basal ganglia interactions. The main focus will be the role of dentate gyrus and adult neurogenesis in mediating hippocampal-basal ganglia behaviour, specifically habit learning, impulsivity and psychostimulant-induced motor activity as these forms of behaviour maybe indirectly influenced by systems-level interactions between the basal ganglia and the hippocampal systems.

NEUROANATOMICAL ORGANISATION OF THE DENTATE GYRUS

The dentate gyrus is a sub-region of a larger anatomical structure known as the hippocampal formation. Figure 1.1 illustrates the anatomical differences of the hippocampus between the rat and human. Despite notable differences between the two species similarities and generalisations can be drawn. The dentate gyrus is comprised

primarily of granular neurons, and receives the main afferent projections from higher cortical regions such as the entorhinal cortex (Lie et al., 2004). The dentate gyrus projects almost exclusively to the CA3 sub-region of the hippocampus via the mossy-fibre projection system.

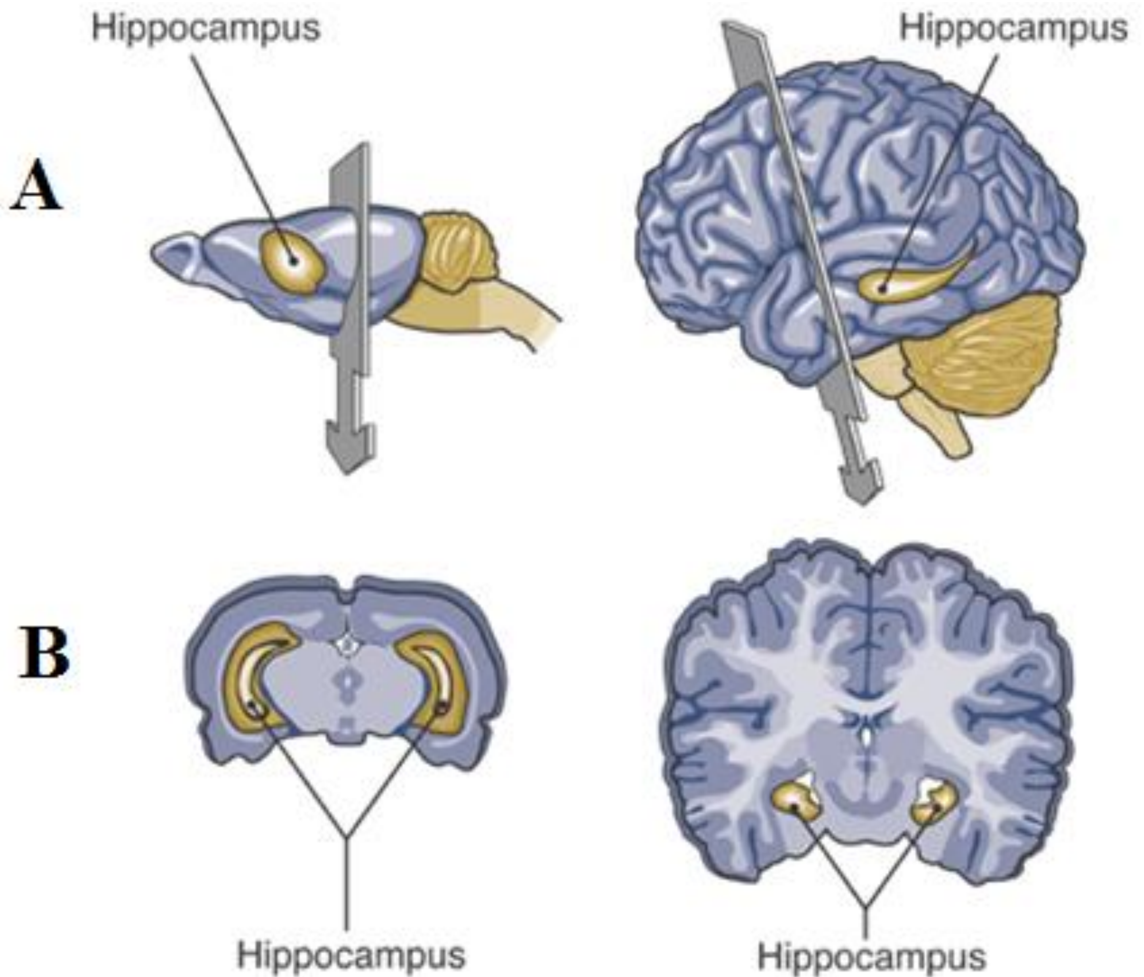

Figure 1.1.

Anatomical representation of the hippocampal formation (A), coronal section (B) in the rat (left) compared to human (right), illustrating the hippocampus region (brown). In both species this structure sits below the surface of the neocortex and is believed to share some similar functions. Adapted from Hiller-Sturmhöfel and Swartzwelder (2005).

The CA3 subregion projects to the CA1 region, which in turn projects to higher cortical regions (Lie et al., 2004). This circuitry has been suggested to underlie the unique functionality of the hippocampus (Tonegawa and McHugh, 2008).

THE DENTATE GYRUS: A ROLE IN COGNITION AND BEHAVIOUR

The hippocampus has been shown to be vital for the processing of spatial discrimination. In particular, more recently the hippocampus has been proposed to be involved in a special subset of spatial discriminations, referred to as 'structural' discriminations (Aggleton and Pearce 2001). A configural task can be thought of as a simple discrimination task, whereby the subject is required to distinguish one stimulus from another. A structural task is similar, in that the subject must discriminate one stimulus from another, yet is different in that the stimuli are comprised of the same elements, and only differ in their structural make-up (Aggleton et al., 2007). An example of a structural task is the presentation of two stimuli, 'AB' and 'BA', where both reward and non-reward stimuli consist of the same elements A and B but differ in the spatial order in which they are presented (Aggleton et al., 2007). Sanderson et al. (2006) conducted a series of experiments which showed that specific lesions of the hippocampus in rats impaired performance of structural learning tasks but not configural learning. This prompted the notion that the hippocampus was important for learning the structure of visual arrays (Sanderson et al., 2006). However, as previous studies have involved lesioning the entire hippocampal system, the unique contribution of the hippocampal sub-regions (dentate gyrus and CA regions) is not clear. Furthermore, the involvement of adult hippocampal neurogenesis in structural learning remains to be established.

The hippocampus has also been associated with learning and memory. Specifically, it has been thought to be responsible for the formation and retrieval of declarative memories (Aggleton et al., 2007). However, the exact mechanisms underlying this aspect of hippocampal functionality has remained elusive. Moreover, the specific sub-regions of the hippocampus, CA1, CA3 and the dentate gyrus have been implicated in rapid acquisition of contextual memory (Lee and Kesner, 2004). Furthermore, the dentate gyrus has been proposed to mediate pattern separation (Leutgeb et al., 2007). Clelland et al. (2009) demonstrated that adult neurogenesis, within the dentate gyrus of mice was involved in pattern separation. However, conversely this did not affect performance of a pairwise associative learning task, a task known to be dependent upon the hippocampus (Clelland et al., 2009). These findings indicate that dentate gyrus and dentate gyrus derived-neurogenesis are involved in certain aspects of hippocampal dependent tasks but not others.

HIPPOCAMPAL-BASAL GANGLIA INTERACTIONS

The role of the basal ganglia in motor behaviour and disorders affecting movement such as parkinson's and huntington disease has been well established (Obeso et al., 2000; Ring and Serra-Mestres, 2001; Obeso et al., 2008). However, this region of the brain has also been implicated in aspects of learning and memory, such as, stimulus-response learning (Packard and Knowlton, 2002). Stimulus-response learning or 'habit-learning' is a form of association learning whereby the subject learns to elicit a particular response, in the presence of a given stimulus. This type of learning has been shown to be significantly impaired in rats with basal ganglia lesions, while paradoxically, lesions of the hippocampus improved habit-learning (Packard and Knowlton, 2002). These findings suggest the presence of an interaction between the hippocampal and basal ganglia regions during the acquisition of stimulus-response associations.

Furthermore, the hippocampus is known to interact with other regions of the brain. For example, the hippocampus and basolateral amygdala have been associated with memory consolidation (Roozendaal et al., 2004). It remains to be determined whether there is a unique contribution from a specific subregion of the hippocampus to this interaction. Evidence by Roozendaal et al. (2003) demonstrated that the effects of glucocorticoids on retrieval of long-term spatial memory depend on the hippocampus. Therefore, one of the aims of this thesis is to explore the effects of lesions of the dentate gyrus on habit-learning, as this may be indirectly influenced by a systems-level interaction between the basal ganglia and the hippocampal systems in adult rats.

THE PHYSIOLOGY OF NEUROGENESIS

The cellular progression from birth to functional integration of adult derived neural stem cells has been extensively researched (Lledo et al., 2006; Alvarez-Buylla and Lim, 2004; Conover and Notti, 2008; Balu and Lucki, 2009; Luikart et al., 2011). Neural stem-like cells proliferate in the sub-granular zone of the dentate gyrus (brown region of Figure 1.2). These precursor cells then differentiate into a specialised cell types (neuron, astrocyte, or oligodendrocyte), most commonly the neuronal type. During differentiation, immature cells migrate into the granule cell layer of the dentate gyrus (brown region of Figure 1.2). In the case of neurons, when the precursor cell matures into a granule neuron,

it becomes integrated into the dentate gyrus granule cell layer, establishing synaptic connections with mossy cell fibres from the entorhinal cortex as well extending projections into the CA3 region (Figure 1.3, Lie et al., 2004). Once these synaptic connections are formed the cells are deemed as functionally integrated into the pre-existing hippocampal circuitry. Figure 1.3 illustrates the process of proliferation, migration and integration of adult derived cells.

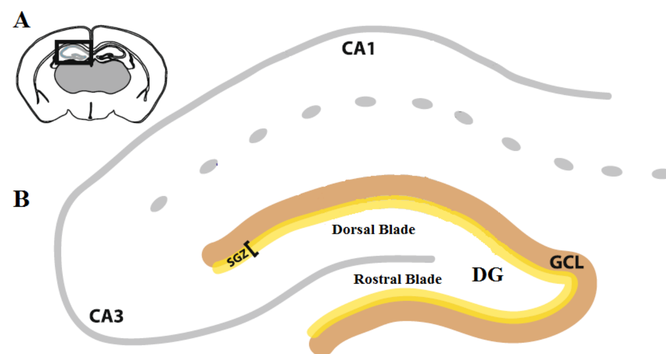

Figure 1.2.

Schematic of an anterior coronal section on of the rat hippocampus (A) and schematic representation of the sub-regions comprising the rat hippocampus (B), consisting of the dentate gyrus (DG) made up of the dorsal and rostral blades, CA3 and CA1. The yellow region depicts the subgranular zone of the DG. The brown region depicts the granular cell layer of the DG. Adapted from Masiulis et al. (2011).

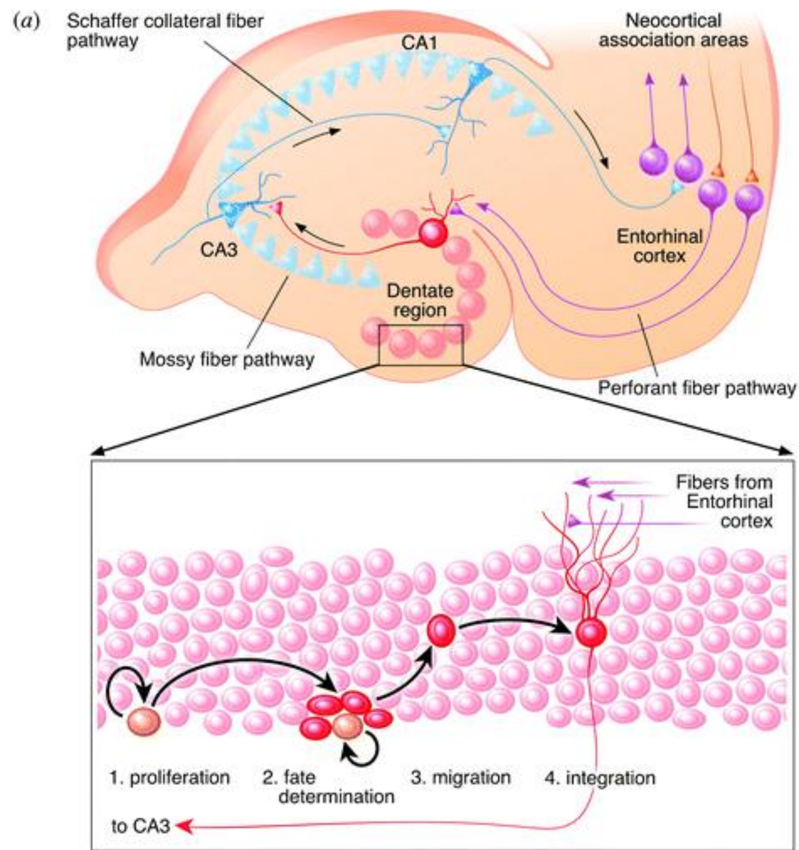

Figure 1.3.

Illustration of the circuitry of the human hippocampus (upper image) and the neurogenic region of the dentate gyrus and the migratory pathway of the adult generated cells (lower image). 1-2. Proliferation and fate determination: Stem-like cells in the subgranular zone give rise to immature neurons. 3. Migration: Immature neurons migrate into the granule cell layer of the dentate gyrus. 4. Integration: Immature neurons mature into new granule neurons and become functionally incorporated into the existing hippocampal circuitry, receiving afferent projections from the entorhinal cortex and extending efferent projections into the CA3 region. Adapted from Lie et al. (2004)

The microenvironment of the neurogenic region has been implicated in the regulation of proliferation, as well as determination of the cell type (neuronal or glial cell, Song et al., 2002). The specific mechanisms which govern this interaction between the cell and the extracellular microenvironment remain largely unknown. Despite this however, some advancement has been made. For instance, the Wnt protein pathway has been proposed to mediate such an interaction (Lie et al., 2005). The Wnt proteins have long been known for their involvement in embryonic neurogenesis, as well as other developmental processes. Recently these proteins have been implicated in mediating the rate of neuronal proliferation within the dentate gyrus in adulthood (Kleber and Sommer, 2004). Adult

hippocampal stem cells have been shown to express receptors and signalling components for Wnt proteins (Moon, 2004). Building on this premise, Lie et al. (2005) identified that expression of the Wnt3 protein mediated neurogenesis, and that over expression of Wnt3 increased neural progenitors within the hippocampal neural niche. Conversely, blockade of the Wnt3 protein led to a decrease in stem cell proliferation (Lie et al., 2005). Together these findings reveal that the Wnt signalling pathway plays a key role in regulating adult hippocampal neurogenesis, and sheds light on the mechanisms by which the microenvironment of the subgranular zone and hippocampal neural niche affect adult neurogenesis and, by extension, neuronal circuitry within the hippocampal region. Other factors that regulate neurogenesis, such as growth factors, hormones and neurotransmitters will also be important, but are beyond the scope of this study.

NEUROGENESIS: A ROLE IN COGNITION AND BEHAVIOUR

Until the functional role of neurogenesis is understood, efforts to connect abnormal hippocampal function with cognitive deficits maybe incomplete. Previous studies have shown recall of recently acquired memory is initially dependent upon the hippocampus, but this dependency decreases as the memory is consolidated in other cortical regions such as the neocortex (Dudai, 2004; Frankland and Bontempi, 2005). Kitamura et al. (2009) extended this line of research by showing that adult neurogenesis modulates the hippocampus-dependent period of associative fear memory. These authors demonstrated that a decrease in neurogenesis was accompanied by prolonged hippocampal dependency of an associative fear memory. Conversely, it was shown that rats allowed to freely exercise on a running wheel, exhibited a significant increase in neurogenesis and a decreased hippocampal dependency of the aforementioned fear memory trace (Kitamura et al., 2009). Together these findings indicate that neurogenesis is one possible mechanism modulating the process by which memory is consolidated from the hippocampus to other cortical regions. These findings suggest that continual integration of new neurons from neurogenesis disrupts existing hippocampal circuitry, and that this disruption leads to the clearance of recent trace memories that are initially dependent upon the hippocampus (Feng et al., 2001; Lledo et al., 2006). Neurogenesis may account for this mechanism of *how* memory traces are consolidated from hippocampal to other cortical areas. However, exactly how this disruption contributes to consolidation remains to be determined.

There is further evidence linking adult hippocampal neurogenesis with cognitive function. Hernandez-Rabaza et al. (2007; 2008) carried out a series of experiments involving lesions of the dentate gyrus and showed a number of specific impairments including deficits in working memory, reference memory and contextual learning. These authors went on to show that irradiated animals (focalised fractionated irradiation ablates hippocampal neurogenesis) were impaired in forming coherent contextual representations in a fear conditioning task (Hernandez-Rabaza et al., 2008). This is an example of a task which is affected by both lesions of the dentate gyrus and decreases in adult hippocampal neurogenesis. Further investigation is needed to tease out the specific contribution and involvement of neurogenesis and the dentate gyrus to this behaviour

Hippocampal neurogenesis has also been associated with behavioural flexibility. In a study by Garthe et al. (2009) it was revealed that mice treated with temozolomide (a DNA-alkylating agent), showed an 80% decrease in hippocampal neurogenesis. Moreover, rats treated with temozolomide failed to employ adaptive search strategies when locating an escape platform within the Morris water maze (MWM). Furthermore, it was proposed that a lack of newborn granule hippocampal cells prevented the rats from adapting to a reversal of platform placement when the cues used to signal the platform placement had changed (Garthe et al., 2009). These findings demonstrate that impaired adult hippocampal neurogenesis may induce cognitive rigidity manifested as an inability to modify previously learnt behavioural strategies. Moreover, Ferragud et al. (2010) revealed that mice who displayed submissive behaviour following chronic intermittent exposure to a social stressor exhibited a greater tendency to engage in habit-based behaviour (Ferragud et al., 2010). Coupled with the finding of reduced hippocampal neurogenesis in submissive mice, it was suggested by Ferragud et al. (2010) that impaired neurogenesis promotes invariable, rigid behavioural patterns, including habits.

Most recently adult neurogenesis has been linked with emotional memory (Kirby et al., 2011). Distorted emotional memory is characteristic of several psychological disorders such as anxiety, depression and post-traumatic stress disorder. Kirby et al. (2011) demonstrated that lesions of the basolateral amygdala suppressed hippocampal neurogenesis, while conversely, lesions of the central nucleus of the amygdala produced no effect on neural proliferation (Kirby et al., 2011). These findings extend the notion that newly created neurons within the dentate gyrus contribute to emotional memory circuits

and provide a possible mechanism by which emotional memories become distorted in the diseased brain (e.g. Sahay and Hen, 2007).

One of the greatest challenges in establishing a link between neurogenesis and cognition is the development of behavioural assays which accurately probe this question (Clelland et al., 2009). Previous researchers have attempted to investigate this link by utilising various paradigms such as the Morris water maze, radial arm maze, and T-maze (Aggleton et al., 2007; Clelland et al., 2009; Ferragud et al., 2010). While these studies have yielded insights into the functional role of neurogenesis, improved behavioural tasks are required that are more sensitive to disruptions in neurogenesis, in order to better understand the specific contribution of these newly generated adult cells. One aim of this thesis is to develop behavioural tasks utilising the Bussey-Saksida touchscreen system which are sensitive to dentate gyrus lesions and disruptions in neurogenesis.

NEUROGENESIS AND STRESS

The rate of neuronal proliferation does not remain constant throughout life. Several environmental factors have been shown to influence neurogenesis, eliciting both down- and up-regulating effects; variables associated with down-regulation or a decrease in proliferation generally include environmental stressors, such as illicit drugs and alcohol consumption, as well as chronic stress and aging (Lledo et al., 2006). In addition focal fractionated radiation has been shown to ablate neurogenesis, and has been extensively used as a research tool. Variables which have been shown to up-regulate, or increase neurogenesis in rats are voluntary exercise and environment enrichment (Lledo et al., 2006). Chronic stress has been shown to negativity affect neurogenesis. However, to what extent stress-induced neurogenesis impairment has on cognition remains to be better understood. Therefore, a further aim of the current project is to explore the effects of chronic stress on adult neurogenesis and its impact on cognitive functioning in rats.

OUTLINE OF THE THESIS

This thesis explores the functional role of the dentate gyrus and neurogenesis in hippocampal activity and its relationship between hippocampal-basal ganglia interactions. By doing so, it attempts to bridge some gaps connecting adult neurogenesis with cognition. Neuroscience experimental techniques were applied to laboratory-reared rats. Behavioural tasks were performed in Bussey-Saksida touchscreen operant chambers, T-maze, a radial arm maze, an elevated plus maze and an open field. Some of the tasks administered are rat homologues of human neuropsychological tasks, and this helps to connect animal research with clinical application, further helping to bridge the species divide. Neurosurgical manipulations involved selective neurotoxic lesions of the dentate gyrus and other manipulations derived from chronic intermittent exposure to stress. Within this framework, this thesis was aimed at studying the role of the dentate gyrus, in general, and of adult neurogenesis in the hippocampus, in particular, in several interrelated cognitive tasks. These tasks are focused on two basic dimensions, hippocampal-basal ganglia interactions and hippocampal function.

The thesis includes two experimental chapters. Chapter 2 comprises a study of the effects of unpredictable chronic stress on adult neurogenesis and on performance involving hippocampal-basal ganglia associated function as well as hippocampal independent functioning. Chapter 3 assesses the role of the dentate gyrus in similar tasks that comparisons could be made between both types of manipulations. Chapter 4 briefly summarises all two experimental chapters and proposes potential new investigations needed to further advance these studies. The final chapter of conclusions completes the thesis. An application for Ethics approval was made by the author and subsequent approval obtained to conduct this research at the University of Canterbury, New Zealand under the supervision of Dr. Juan Canales (Appendix A).

CHAPTER 2

Effects of learning and chronic unpredictable stress on adult neurogenesis

ABSTRACT

The brain continually produces new neurons into adulthood. These adult derived progenitor cells migrate from the subgranular zone of the dentate gyrus and become functionally integrated into existing hippocampal circuitry. Once integrated, the functional contribution of these cells remains controversial. This chapter investigates the effect of chronic mild stress on adult neurogenesis and hippocampal-basal ganglia associated behaviour. Mild chronic stress slow the acquisition of a stimulus-response task ($p=0.043$), while conversely, stress facilitated acquisition of an operant task requiring discrimination between large and small reward ($p=0.027$). In locomotor activity assays, chronic stress did not shift the dose-response to methamphetamine, indicating that the primary affect of the stress affected some forms of hippocampal-basal ganglia behaviour while preserving others. Analysis of 2,5-bromodeoxyuridine incorporation showed that, overall, chronic mild stress did not affect survival of neuronal progenitors. However, learning of the tasks had a positive influence on cell survival in stressed animals ($p=0.038$). The experiments uphold a current theory that learning acts as a buffer to mitigate the negative effects of stress on neurogenesis.

INTRODUCTION

The subgranular zone of the dentate gyrus produces between 5,000-10,000 progenitor cells (PC) per day in the adult rat (Cameron and McKay, 2001). This daily production does not remain constant throughout life; instead factors such as exercise, environmental enrichment and dietary intake have been shown to increase adult neurogenesis (Kempermann and Gage, 1997; van Praag at al., 1999; Park and Lee, 2011). On the contrary, illicit drug and alcohol consumption has been shown to impair PC proliferation and survival (Eisch et al., 2000; Hernandez-Rabaza et al., 2006). Furthermore, binge ethanol consumption has been shown to decrease hippocampal neurogenesis, while paradoxically, moderate ethanol consumption has been shown to increase hippocampal PC proliferation (Nixon and Crews, 2002; Aberg et al., 2005).

Interestingly, stress has also been shown to have a similar duel effect, both increasing and decreasing adult neurogenesis. Chronic *predictable* stress has been shown to increase adult neurogenesis within the dentate gyrus of rats. Parihar et al. (2011) showed predictable mild chronic stress was associated with enhanced hippocampal neurogenesis and a decline in depressive-like and anxiety-like behaviour in adult male rats. It was further demonstrated that exposure to predictable chronic stress improved hippocampal-dependent memory function (Parihar et al., 2011). While conversely, chronic *unpredictable* stress has been linked to a decrease in hippocampal PC proliferation. In a series of experiments conducted by Dias-Ferreira et al. (2009) rats exposed to a 21 day chronic unpredictable stress regime exhibited a significant decline in PC proliferation within the dentate gyrus. The authors went on to show stressed rats also performed worse in decision-making tasks. Together these findings suggest the predictability of the stress plays an import role in the nature of its impact on behaviour and cellular functioning.

While decreasing the rate of PC production has been associated with a decline in specific behavioural task performance, the effects of an increase in cell production is not as clear. It is well established that exercise in rats (such as time logged on a running wheel) produces an increase in cell proliferation, up to a 100% increase (van Praag et al., 1999; Shors, 2009). However, simply more PC does not necessitate improved cognitive performance (Rhodes et al., 2003). In a study conducted by Curlik and Shors (2011) it was shown that rats who exercised exhibited increases in adult neurogenesis, however, this excess of new cells were lost if not integrated into hippocampal circuitry. It was shown by

rats that learnt a fear conditioned response had significantly greater newly born neurons incorporated into the dentate gyrus sub-region of the hippocampal formation. This suggests the integration of PC within the hippocampus is mediated by hippocampal-dependent learning (Curlik and Shors, 2011). These findings are important, as they demonstrate a potential functional role of adult neurogenesis within the hippocampus. However, the involvement of neurogenesis in hippocampal-basal ganglia behaviour remains to be determined. One aim, therefore, of this chapter was to investigate the potential role of dentate gyrus derived PC in hippocampal-basal ganglia dependent behaviour, assessed by a stimulus-response impulsivity task and stimulant-induced locomotor activity.

Hippocampal neurogenesis has also been associated with behavioural flexibility (Garthe et al., 2009). It has been further implicated that impaired adult hippocampal neurogenesis may induce cognitive rigidity manifested as an inability to modify previously learnt behavioural strategies. Furthermore, chronic stress has been shown to enhance habit-based behaviour, coupled with the knowledge that stress reduces hippocampal neurogenesis (Ferragud et al., 2010). These two lines of research provide additional support of the implication of neurogenesis in habit-based learning. All these findings suggest impaired neurogenesis promotes invariable and rigid behavioural patterns, including habits (Ferragud et al., 2010). Because these cognitive deficits have been observed in several neurological disorders (e.g. Olley et al., 2007; Marazziti et al., 2010; Poletti et al., 2012) the role of neurogenesis in cognitive deficits is strong.

Hippocampal-dependent learning is impaired by stress and infusion of stress hormones (Dias-Ferreira et al., 2009; McGaugh and Roozendaal, 2002). Conversely, it is known that chronic mild unpredictable stress has been shown to suppress hippocampal neurogenesis (Surget et al., 2011). The implementation of both physical and psychological stressors, coupled with exposure on an unpredictable schedule is designed to mimic the variability of stressors encountered in daily life, and is a well established research protocol (Dias-Ferreira et al., 2009). Therefore, the aim of this chapter was to explore the effects of unpredictable chronic stress on adult neurogenesis and its impact on hippocampal-basal ganglia dependent function.

MATERIALS AND METHODS

Experimental design

Neurogenesis was manipulated by using a chronic mild unpredictable stress protocol (see details later). A total of twenty-eight (28) male PVGc-hooded rats were obtained from the colony within the Animal Facility of the Psychology Department of the University of Canterbury. A simple experimental design comprising one treatment and one untreated control group was applied in a randomised layout. The treatment was a period of unpredictable physiological stress aimed to examine various functional parameters of dentate gyrus derived neurogensis. A selection of specific cognitive and locomotion tasks were applied to the treated and control groups. These tasks represent two types of brain function (1) non-hippocampal dependent tasks and (2) hippocampal-basal ganglia dependent tasks (Table 2.1).

Table 2.1.

List of experimental groups and their tasks (total number of rates N=28). Rats performing the hippocampal-basal ganglia dependent tasks were divided into a further 2 groups with a 3:4 ratio (3 rats / 4 rats (due to the odd number of rats within the condition)). These two groups were counterbalanced with a reversal of the task in order to eliminate any potential bias for responding to any one side of the touch-screen.

	Non-hippocampal dependent tasks	**Hippocampal-basal ganglia dependent tasks**
CONTROL	**n=7**	**n=7**
	Simple Discrimination task	Stimulus-Response Task
		Delay discounting (Impulsivity)
		Locomotor Assay
STRESS	**n=7**	**n=7**
	Simple Discrimination task	Stimulus-Response Task
		Delay discounting (Impulsivity)
		Locomotor Assay

The hippocampal-basal ganglia dependent task group preformed the stimulus-response, impulsivity and locomotor assay. The non-hippocampal dependent task group preformed the simple discrimination task, additional configural and structural discrimination tasks were planned for this group, however the rats were unable to transition from simple discrimination to configural discrimination tasks (see Discussion).

General Animal Housing Procedure

Laboratory rats were housed in standard housing conditions of 4 rats per cage (opaque plastic cage; 50 cm long by 30 cm wide by 23 cm high). The rats were kept on a 12 h light-dark cycle. Initially water and rat chow was available *ad libitum* prior to food deprivation. Before commencing the stress procedure, the rats were matched in a pairwise fashion based on pre-training performance and assigned to either the stress or control group. Once the rats were assigned a group, they were re-caged such that, only stressed animals were housed together (housing stress and non-stressed animals together may indirectly influence the behaviour of the non-stressed animal). No behavioural testing occurred during the 21 day stress period.

Experimental Procedure

Behaviour shaping protocol

The rats were placed on food deprivation during training so as to increase motivation to seek out a food reward; deprivation was to 90% of initial free feeding body weight, with food rationed accordingly. Rats were weighed no less than three times a week to ensure they did not fall below 90%. Training involved shaping the rats to nose poke the touchscreen for a food reward. During the first session the rat was left in the chamber for 30 mins, with all lights turned off and the food reward tray filled with five pellets for the rat to eat. The criterion for moving to the next stage of pre-training was for the rat to have found and eaten the pellets in 30 min. If this was not achieved, 30 min sessions were repeated until all pellets had been eaten. For the next phase of shaping, rats were hand-shaped to receive a food pellet reward when they reared up on a shelf in front of the touchscreen, while nose poking the top. The criterion for moving to the final stage of

training was for the rat to receive five pellets in 15 minutes. Sessions were repeated once a day until this was achieved.

During the final phase of shaping, rats were required to nose-poke an image on the touchscreen (a white square) to obtain a food reward. In order to elicit a food reward, the rat must touch one of the two stimuli displayed on the screen. Delivery of the reward was signalled by illumination of the food tray and a tone. An entry to collect the food turned off the tray light and began a timeout of 10 s (to allow the rat to eat the food pellet). After the timeout period, the stimuli were displayed again. The criterion for completing the final stage of training was for each rat to complete 30 trials in 30 mins.

Once the rats had demonstrated consistent nose-poke responses (three consecutive sessions of 30 trials in 30 min), the unpredictable stress procedure began. Consistent nose poking was achieved after approximately 21 days of pre-training. This is noted to be substantially longer compared to other published training time frames (Bussey-Saksida 2009; Bussey-Saksida 2010; Lafayette Instruments Company) where consistency was achieved after about 7 days.

Neurogenesis Manipulation (Stress Procedure)

Once the rats had learned to nose poke, they were given *ad libitum* access to food, and received two injections of 2,5-bromodeoxyuridine (BrdU, 200 mg/kg, i.p.) once a day for two days, in order to label proliferating cells in the dentate gyrus. On the third day the stress procedure began. The chronic mild unpredictable stress protocol was based on Dias-Ferreira et al. (2009) which employs three types of stressors: social defeat, forced swimming and physical restraint. Stressed and non-stressed rats were weighed no less than three times a week as a measure of their overall health. Rats assigned to the stress group were exposed once a day to one of three stressors: social defeat, forced swimming or physical restraint. Stressors were randomly distributed throughout a 21 day period and arbitrarily scheduled within the rat’s 12 hr dark cycle (0800 hrs-2000 hrs). Each cage (housing 4 rats) experienced the same stressor each day. Table 2.2 shows the stress regime over the 21 day period.

Table 2.2.

Randomisation of daily chronic stress regime applied over a 21 day period for each rat (ID). S= forced swim, D = social defeat, R = physical restraint. Rats were housed in 4 per cage, each exposed to a different stressor each day with the 4 rats in each cage experiencing the same stressor each day). NOTE: During day 17 a major earthquake occurred, restricting access to the research laboratory over several days. Due to restricted access each rat experienced the three stressors as follows; physical restraint (8) times, forced swim (8) times and social defeat (5) times this was considered sufficient for the experiment.

	Stress Day																				
Rat ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
C4R3BK	S	D	R	S	D	D	R	R	D	R	S	R	R	D	R	S	S	S	R	S	S
C1R4P	S	D	R	S	D	D	R	R	D	R	S	R	R	D	R	S	S	S	R	S	S
C7R2N	S	D	R	S	D	D	R	R	D	R	S	R	R	D	R	S	S	S	R	S	S
C6R4R	S	D	R	S	D	D	R	R	D	R	S	R	R	D	R	S	S	S	R	S	S
C6R2R	R	R	D	S	R	R	D	S	S	D	S	D	S	S	D	R	R	S	S	R	R
C4R1BK	R	R	D	S	R	R	D	S	S	D	S	D	S	S	D	R	R	S	S	R	R
C7R4N	S	R	R	D	S	S	R	R	D	S	D	D	S	D	R	S	R	S	S	R	R
C2R1Y	R	D	R	S	R	D	S	S	D	D	R	S	R	S	D	S	S	R	R	S	R
C2R2Y	R	D	R	S	R	D	S	S	D	D	R	S	R	S	D	S	S	R	R	S	R
C6R1R	R	D	R	S	R	D	S	S	D	D	R	S	R	S	D	S	S	R	R	S	R
C4R4BK	R	D	R	S	R	D	S	S	D	D	R	S	R	S	D	S	S	R	R	S	R
C8R4G	S	R	D	R	R	R	D	R	S	S	D	D	S	D	S	R	R	S	S	R	S
C7R1N	S	R	D	R	R	R	D	R	S	S	D	D	S	D	S	R	R	S	S	R	S
C2R4Y	R	R	R	D	S	R	D	D	R	S	S	D	R	D	S	S	R	S	S	R	S

Social defeat

The social defeat stress was based on the resident-intruder paradigm. The experimental male (the "intruder", a young adult PVGc male) was placed inside a resident Wistar rat home cage (opaque plastic cage length 62 cm, width 40 cm and height 22 cm) with a resident Wistar rat male approximately 6 months old . The animals were allowed to interact for a maximum of 10 min, immediately afterwards, the intruder was physically separated from the resident by a wire mesh divider within the resident cage for a further 60 min (Figure 2.1). To avoid individual differences in intensity of defeat, each social defeat

session was conducted with a resident randomly chosen from the group of four Wistar residents.

Figure 2.1.

Social defeat cage and divider used during the social interaction.

<u>Forced swim</u>

Animals were placed inside a cylinder container (diameter 32 cm and height 59 cm) filled with 21 ± 1ºC water for 10 min. Temperature was monitored after each rat to maintain consistency, with water changed after every four animals.

<u>Physical Restraint:</u>

The rats were immobilised inside a clear Perspex rectangular container (length 20 cm, height 8 cm and width 4.5 cm). The restraint containers were sized for rats (280-350 g) and had holes for breathing. The ends were adjustable with a metal rod, to adjust the space available for the rat, which had to be kept to a minimum during restraint (Figure 2.2).

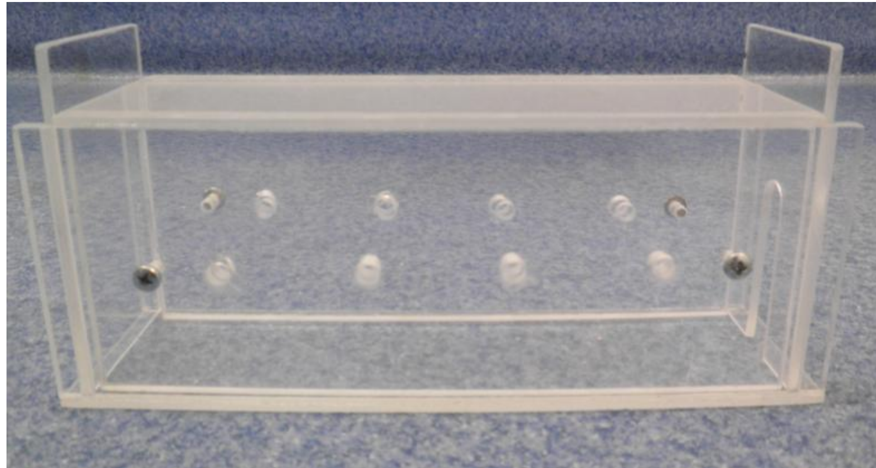

Figure 2.2.

The physical restraint apparatus used during the chronic stress protocol.

Behavioural Tasks

Once the stress procedure was completed, the behavioural tasks commenced. Table 2.1 depicts the assignment of behavioural tasks to animal groups. Appendices B to E describe the software protocol in detail.

Non-hippocampal dependent tasks

Simple Discrimination Task (Bussey-Saksida Chamber)

The rats were presented with two images (a cross and an oval) to assess discrimination. One image (the cross) was always reinforced with a food reward (Precision Pellets) and conversely the oval never received reinforcement. The images were counter balanced throughout the trials to control for any positioning-response-bias. Figure 2.3 illustrates the stimulus used during the simple discrimination task.

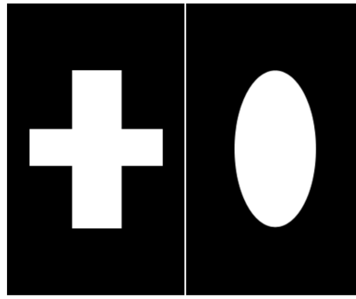

Figure 2.3.

Example of the cross and oval stimuli used for the simple discrimination task. During this task the cross was always reinforced with a food pellet, and conversely the circle always elicited a 30 sec time-out.

Hippocampal-basal ganglia dependent tasks

Stimulus-response learning (Bussey-Saksida Chambers)

Habit learning was tested by a stimulus-response task. This task involved visuomotor conditional learning. Rats were placed inside Bussey-Saksida chambers. Once the task began the rats were presented a series of images, paired with a high or low pitch sound, and were required to learn a rule of the type if shape '+' with high pitch is presented, respond to the left location; and if shape '0' with low pitch is presented, respond to the right location (Figure 2.4). Rats had to nose-poke the correct location to obtain a food reward (Precision pellets). Incorrect responses turned the house light on and started a time-out of 20 s. Food delivery was accompanied by illumination of the tray light and a tone. An entry to collect the food turned off the tray light and began a timeout of 20 s (to allow the rat to eat the food pellet). After the timeout period another image was displayed. The rats had to complete 30 trials in 30 min.

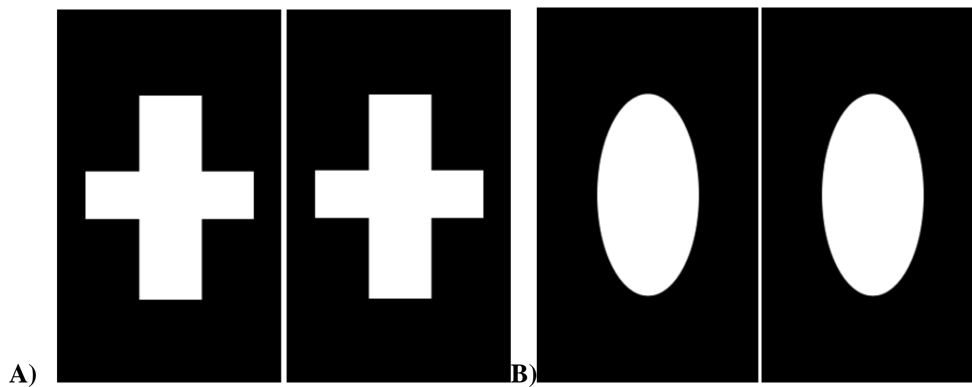

Figure 2.4.

Illustration of the two image sets used during the stimulus response task. A) When two crosses were presented, the left image was reinforced. B) When two ovals were presented the right image was reinforced.

Impulsivity Task (Bussey-Saksida Chambers)

Impulsivity was tested using a delay discounting task. The rats were presented with two images (white squares). The left image was rewarded with one pellet delivered immediately, and the right image rewarded with three pellets delivered immediately. Training continued until the animal reached learning criteria of 90% preference for the large (3-pellet) reward. Once this acquisition occurred, the delay-discounting probe trials began. Probe trials involved discounting the large reward by introducing a time-delay. The following times were used as delays; 3, 10, 30 and 100 sec. Probe trials were counterbalanced across both groups.

Locomotor assays

Once the impulsivity task was completed the animals were placed individually into a black perspex open field (60 cm x 49 cm x 35 cm) and monitored with a video tracking system and image analysis software (Viewpoint 2.5, Champagne au Mont D'Or, France). The rats received two days of habituation to the open field for 90 min sessions each day in a drug-free state. On the third day (test day), rats received a five min pre-habituation to the open field prior to receiving saline or methamphetamine (0.1, 0.3 or 1 mg/kg i.p.). Treatments were administered in a counterbalanced fashion over four sessions (Table 2.3). Locomotor activity was then recorded for 90 min.

Table 2.3.

List of the methamphetamine dose and administration regime. Dosages were counterbalanced over 4 days, with each animal exposed to all dose conditions during this time. Both lesion and sham control animals were randomly assigned a Condition No. (1-7).

Condition No.	Session 1	Session 2	Session 3	Session 4
1	Saline	1 mg/ml	0.3 mg/ml	0.1 mg/ml
2	0.1 mg/ml	Saline	1 mg/ml	0.3 mg/ml
3	0.3 mg/ml	0.1 mg/ml	Saline	1 mg/ml
4	1 mg/ml	0.3 mg/ml	0.1 mg/ml	Saline
5	Saline	1 mg/ml	0.3 mg/ml	0.1 mg/ml
6	0.1 mg/ml	Saline	1 mg/ml	0.3 mg/ml
7	0.3 mg/ml	0.1 mg/ml	Saline	1 mg/ml

Immunohistochemistry and adrenal gland removal

Before introducing the stress treatments, rats were injected with 2,5-bromodeoxyuridine (BrdU, 200 mg/kg, i.p.) in order to label proliferating cells in the dentate gyrus to examine long-term survival. On completion of the behavioural assays rats were deeply anesthetised with sodium pentobarbital and the adrenal glands removed and weighted, rats were then traPCardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer. The brains were then removed, post-fixed in the same fixative solution for 24 hours and transferred to 20% glycerol solution for cryoprotection. Coronal sections (30 um) were collected of the dorsal hippocampus with a sliding microtome. BrdU labelling was carried out on free-floating sections using the Avidin-Biotin Complex (ABC) Method, a standard Immunohistochemistry staining technique. Free-floating sections were washed in a solution of phosphate buffered saline with 0.5% Triton (PBS-Tx) for 5 mins x 3. Endogenous peroxidase was then blocked with a solution of 10% methanol 3% H_2O_2 in PSB 0.1M. DNA denaturation was achieved by incubating the sections in HCL 1N (hydrochloric acid 1 Normal) at 4°C for 10 mins, followed by incubation in HCL 1N at room temp for 10 mins, followed by incubation in HCL 2N at 40°C for 20 mins. Sections were then washed in buffered borate 0.1M pH 8.5 for 15 mins. Sections were then washed in PBS-Tx 0.1M with 5% NGS (Normal Goat Serum). Sections were then incubated with the primary antibody anti-BrdU (1:150; Vector Laboratories) for 2 nights at 4°C. Sections were washed in PBS-Tx then incubated with the secondary anti-body (1:400; biotinylated Goat anti-mouse; Vector Laboratories), followed by amplification with avidin-biotin complex. Cells were visualised with the solution of 3,3'-diaminobenzidine (DAB) (Sigma

Laboratories). BrdU+ cells were manually counted in the dentate gyrus with 40x magnification (Zeiss microscope). The total number of cells were then summed and divided by the number of sections counter for each animal, then expressed as a number of BrdU+ (cells/sections). Full immunohistochemistry procedural details are provided in Appendix F.

Statistical analyses

Data was processed using the statistical package Statview (SAS Institute Inc.) primarily using ANOVA for repeated measures and one-way ANOVA for single events and linear regression (Microsoft Excel). Where treatment differences were marginal ($0.05 < p < 0.20$) and obvious non-normality of residuals were present classical transformations were applied. An arcsine transformation was applied to percentage data and a natural logarithm transformation applied to cell counts and growth data to test for differences. Back-transformed means were subsequently reported where applied. Other measures of error including standards errors of the mean (SEM) were used with all graphical output.

Multiple linear regression was also used to test for significance of learning and stress and their interaction on neural cell survival by the centering method of Howell (2010). This method avoids multicollinearity between the main effects and interaction terms. Such an approach is more robust and universal than ANOVA.

RESULTS

Immunohistochemistry: Effects of stress

Nine animals were excluded from the analysis because of insufficient cell staining that could not confirm BrdU+ cell numbers. A one-way ANOVA revealed no significant difference in BrdU positive cells between stressed and control animals (mean 7.4 cells/section) $F(1,17) = 0.01$, $p = 0.909$. However stressed animals did exhibit a 3-fold increase in variance than the control animals (Control 6.2, Stress 20.2 $(\text{cells/section})^2$). This finding indicates the stress procedure produced non-specific effects on cell survival, in the dentate gyrus.

Immunohistochemistry: Effects of learning

Animals in both hippocampal-basal ganglia tasks and non-hippocampal dependent task groups were pooled and re-grouped into either learning (≥70% learning) or no learning categories (<70% learning). A one-way ANOVA revealed a difference in BrdU+ labelled cells between animals that learnt (8.1 cells/section) either the simple discrimination or stimulus-response task compared to those who did not learn (4.9 cells/section), $F(1,17) = 3.42$, $p = 0.082$ (Table 2.4; Figure 2.5).

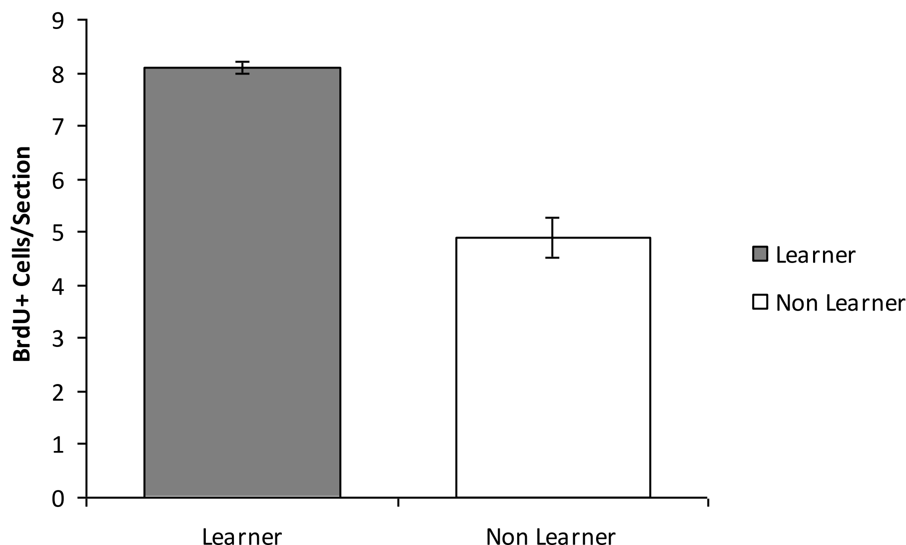

Figure 2.5.

Learning mediated BrdU+ cell survival in the dentate gyrus. Significant difference, p=0.082.

Due to the large difference in variance between the stressed and non-stressed groups and non-normally distributed residuals a general linear regression model was fitted using Excel (Microsoft Corporation). The learning percentage data was transformed by an angular transformation (arcsine) and the cell/section data by natural logarithms. A multiple regression model of the form $Y = a + bX1 + cX2 + dX1X2$ was fitted where a, b, c and d are coefficients, Y is the natural logarithm of cells/section, X1 is 0 for the unstressed group and 1 for stressed group and X2 is the arcsine of the percentage learning (degrees) and X1X2 is the centered interaction term (Howell, 2010). The fit showed an overall marginal significance of stress and learning (p=0.109) similar to the categorical analysis above (Figure 2.5), with insignificant main effects of stress and learning but an

important interaction of stress with learning (p=0.038) (Figure 2.6). The fitted coefficients and standard errors of coefficients (in brackets) are:

Constant	a = 1.41 (0.39), p=0.003
Stress	b = -0.06 (0.21), p=0.775
Learning	c = 0.009 (0.006), p=0.160
Stress*Learning	d = 0.029 (0.013), p=0.038

These findings indicate that learning had a positive influence on cell survival in stressed animals but no effect on unstressed animals.

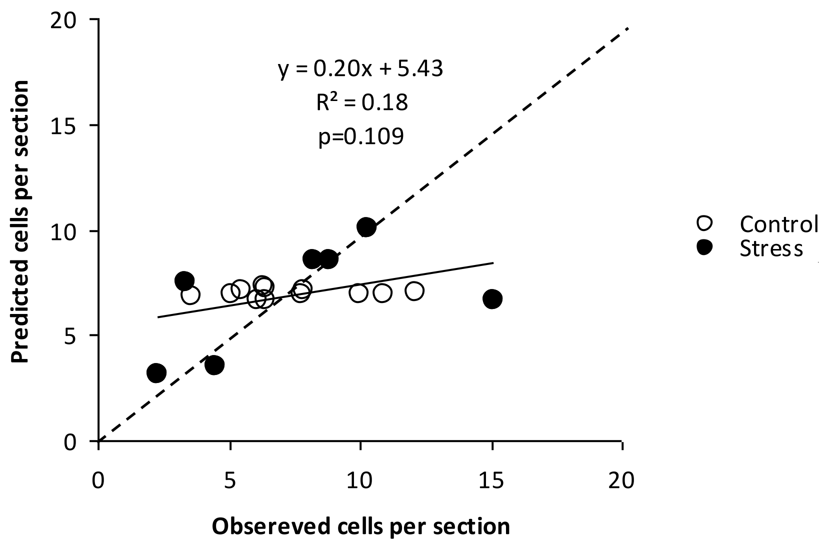

Figure 2.6.

Comparison of the overall observed and predicted cell survival (p=0.109) showing a significant interaction between stress and learning (p=0.038). The dashed line indicates the 1:1 ideal relationship.

While there is large variance the effects of learning are clearly seen and points to the need to design new experiments to articulate the role of stress in affecting interacting functions involving the hippocampus and dentate gyrus.

Hippocampal-basal ganglia dependent tasks

Stimulus-response: Task Acquisition

A repeated measure ANOVA revealed a significant difference in correct responses over time (blocked trials), $F(8, 320) = 48.92$, $p < 0.0001$, indicating both Stress and Control groups acquired the task. It was further shown that a significant difference existed between the two experimental groups, $F(1,40) = 4.35$, $p = 0.043$ (Figure 2.7). Taken together, these findings indicate that the chronic mild stress regime was sufficient to produce a 5.6% deficit in task acquisition (mean 56.9 vs. 60.2%, respectively).

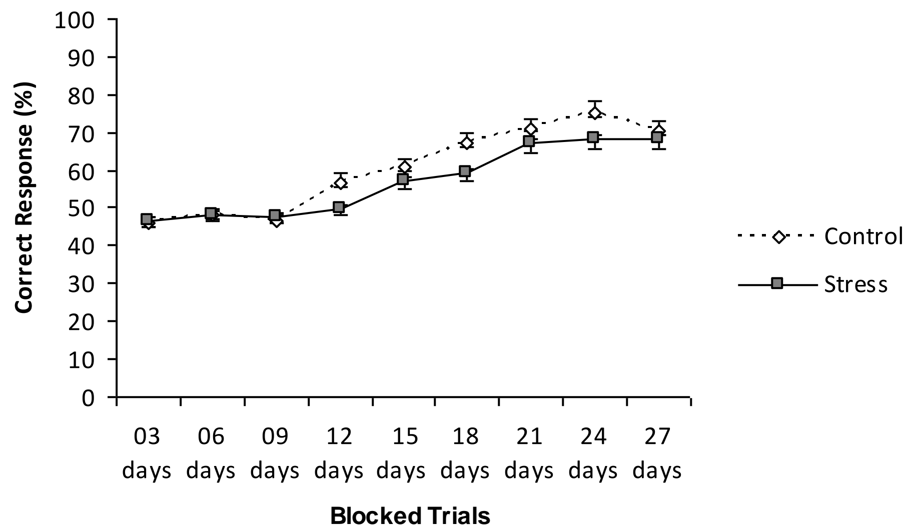

Figure 2.7.

Stimulus-response: Task acquisition. Mean correct response (%) per session block (±SEM) for both experimental groups across acquisition for the 27 days. Trials were blocked into 3 days per block (90 trials). Both Stress and Control groups showed a significant difference in correct response over blocked trials p= 0.0001. A significant difference between groups control (mean 56.9%) and stress (mean 60.2%) was seen (p=0.043).

Stimulus-response: Response latency

A repeated measure ANOVA revealed a significant difference in response latency over time, providing further support that both groups learnt the task, $F(8, 320) = 8.29$, $p < 0.0001$. Response latency was measured as the time taken from when the images were displayed in the touched screen to when the animal nose-poked to illicit a response

(measured in sec) and is considered a measure of learning. No significant difference was observed between stressed and control animals (mean 4.8 sec), $F(1, 40) = 0.83$, $p = 0.367$.

Stimulus-response: Left-right response bias

A one-way ANOVA revealed no significant difference in responses bias on day 1 of testing, $F(1, 12) = 1.29$, $p = 0.278$, indicating both Stress and Control groups had no bias for either left or right response key. It was further shown that on the final day of testing no significant preference for either response key had occurred between the two experimental groups, $F(1, 12) = 0.14$, $p = 0.719$. Taken together, these findings indicate both experimental groups had no bias in selecting left or right response keys. These findings complement the conclusion that over the 27 days, both groups learnt the response task.

Impulsivity task: Large preference acquisition

A repeated measure ANOVA revealed a significant difference preference for the immediate large over blocked trials, $F(6, 156) = 16.74$, $p < 0.0001$, indicating both Stress and Control groups acquired the task. It was further shown that a significant difference existed between the two experimental groups with the unstressed group (mean 74.1%) taking longer to achieve the same proficiency as the stressed group (mean 83.9%), $F(1,26) = 5.49$, $p = 0.027$, as shown in Figure 2.8. Taken together, these findings indicate the chronic mild stress regime was sufficient to produce a facilitatory effect of approximately 13.3% on reward preference acquisition.

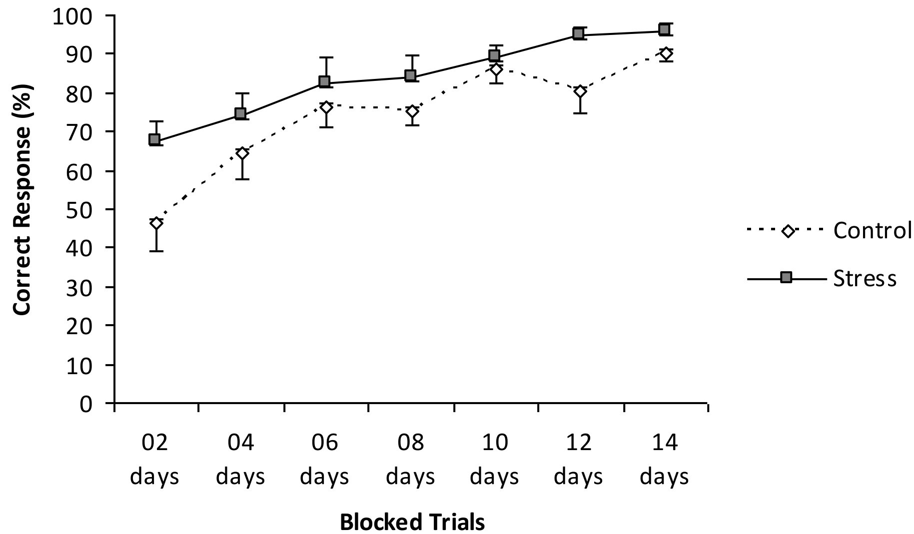

Figure 2.8.

Impulsivity task: Large reward preference acquisition. Mean responses (%) per session block for large reward key (±SEM) for both experimental groups across acquisition for the 14 days. Trials were blocked into two days per block (60 trials). Both Stress and Control groups showed a significant difference in correct response over blocked trials, $p<0.0001$. Moreover, stress was significantly different from the control group, $p=0.027$.

Impulsivity Task: Delay Discounting Probe Trials

A repeated measures ANOVA revealed there was no significant difference between the stress and control groups during the delay-discounting probes; $F(1, 12) = 0.001$, $p = 0.974$ (Figure 2.9). However, a decrease in large reward preference was observed in stress animals across all probe delays (3 sec delay 19.78%, 10 sec delay 23.06%, 30 sec delay 15.19% and 100 sec 20.53%) compared to control animals (3 sec delay 1.48%, 10 sec delay 2.87%, 30 sec delay 1.49% and 100 sec delay 9.22%), as shown in Figure 2.9. These results suggest a trend toward delay-discounting reward contingency sensitivity in stressed animals that is exhibited by the greater percentage decrease from the large reward preference. However, this may be a reflection of the large reward preference already established (Figure 2.8). Moreover, due to the lack of statistical significance it is not possible to confirm this trend. This data set maybe confounded by the probe trial testing occurring 49 days post stress exposure, and further exacerbated by limited sample size (N=14).

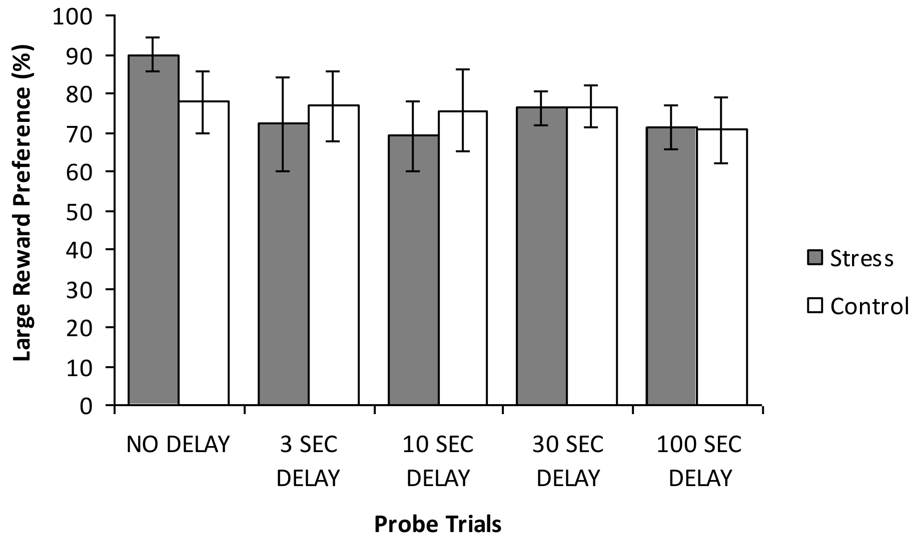

Figure 2.9.

Impulsivity probe trials: Mean response (%) per session for large reward key (±SEM) for both experimental groups across acquisition. No significant difference between experimental groups across probe trials, p=0.974.

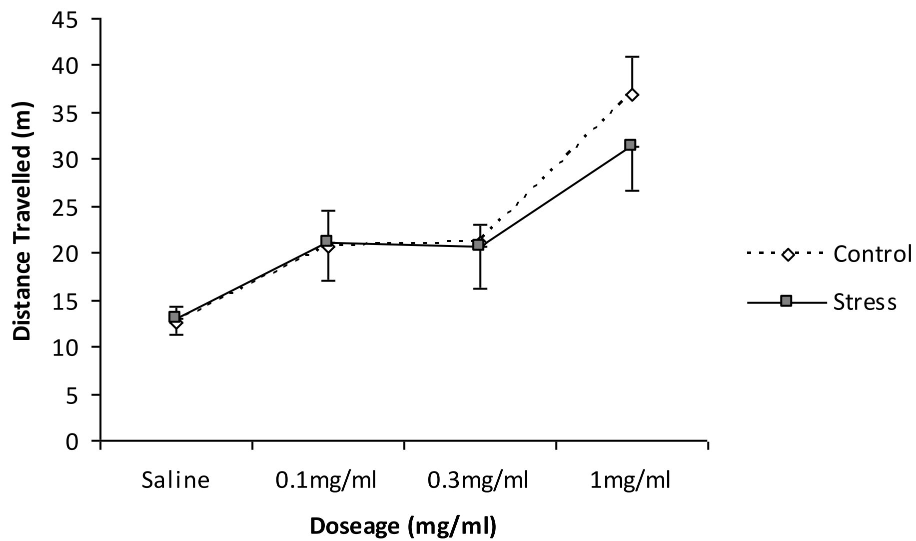

Figure 2.10.

Effect of dose of methamphetamine on distance travelled in the locomotor activity showing that a dose of 0.3 mg/ml and 1 mg/ml signifcantly increased distance travelled, $p<0.0001$. While stress did not produce sensetivity to methamphetamine-induced locomotion, p=0.714.

Locomotor Activity Assay

A repeated measures ANOVA revealed a significant difference between dose of methamphetamine on locomotor activity, $F(3, 36) = 16.52$, $p < 0.0001$ (Figure 2.10). A highly significant interaction was observed between methamphetamine dose and Time, $F(24, 288) = 4.92$, $p < 0.0001$. Despite no significant overall difference between the stress and control groups, $F(1, 12) = 0.14$, $p = 0.714$ (Table 2.5 and Figure 2.10).

Non-hippocampal dependent tasks

Simple Discrimination: Task acquisition

A repeated measure ANOVA revealed a significant difference in correct responses over blocked trials, $F(6, 156) = 27.65$, $p < 0.0001$, indicating both Stress and Control groups acquired the task. However, it was further shown that no significant difference was observed between the experimental groups, $F(1, 26) = 1.31$, $p = 0.263$ (Figure 2.11). Moreover, a significant interaction was observed between stress and control group over time, $F(6, 156) = 3.22$, $p = 0.005$. Despite this interaction, that is considered not important, these findings indicate the chronic mild stress regime was not sufficient to produce any deficit in acquisition of a simple discrimination task.

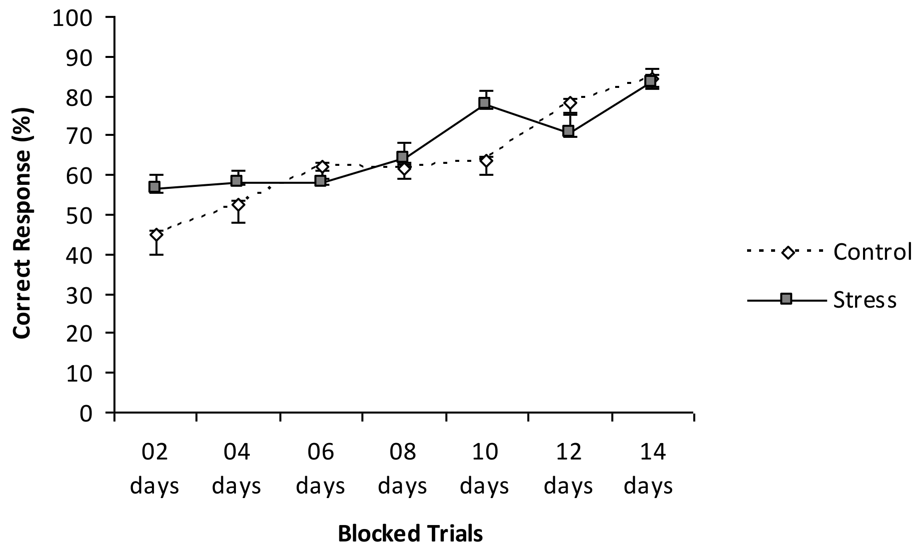

Figure 2.11.

Simple discrimination task acquisition. Mean correct response (%) per session block (±SEM) for both experimental groups across acquisition for the 14 days. Trials were blocked into two days per block (90 trials). A significant difference was seen in both experimental groups over time, $p<0.0001$. No significant difference between the Stress and Control groups was observed, $p=0.263$.

Simple Discrimination: Response Latency

A repeated measure ANOVA revealed a significant difference in response latency over time, providing further support that both groups learnt the task; $F(6, 144) = 13.22$, $p < 0.0001$. Despite no significant difference between stress and control groups, $F(1,24) = 0.47$, $p = 0.830$. Response latency was measured as the time taken from when the images was displayed in the touchscreen to when the animal nose-poked to illicit a response (measured in sec) and is considered a measure of learning. One animal from the control group were excluded from response latency analysis as its response times exceeded 10 min, significantly skewing the data.

Adrenal Gland Size

At the time of sacrifice the animal was deeply anesthetised and the adrenal gland was removed and weighted. A 22% increase in the right adrenal gland was observed in stress animals (mean 367.6 mg) compared to control animals (219.5 mg). While only a 5.7%

increase was observed in the left adrenal gland. However, a One-way ANOVA revealed this was not significant, $F(1, 26) = 3.20$, $p = 0.085$ (Table 2.4). Due to the marginal significance, initial data was transformed by natural logarithms to provide a slight increase in significance, $p = 0.074$. However, despite the improvement, the conclusions remain unchanged. This difference could be confounded by delay measurement of the adrenal gland 58 days post exposure from chronic mild stress, and exacerbated by limited sample size (N=28).

Animal Size: Tibia Length and Animal Weight

The adrenal gland size may also reflect total body mass so the left tibia was removed and measured after perfusion. There was no significant difference between tibia length across stress and control groups $F(1, 54) = 0.35$, $p = 0.557$. In addition, there was no significant difference between body mass across stress and control groups. Taken together, these findings indicate both experimental groups were similar in mass. Therefore any observed difference in adrenal gland maybe attributable to the chronic stress procedure.

Table 2.4.

Summary of key results showing the various tasks, their dependent variable of the control and stressed groups (means) together with their standard errors and degree of significance of time and main effects and interaction (INT) of main effect with time. The probability of significance is not listed if it was not tested and is shown in bold if considered significant. [A] Main effects reclassified from stressed to learnt (≥70% learning) and non-learnt (<70% learning). [B] Main effects of stress and learning in a multiple regression analysis (see text).

Class	Treatment	Control (SE)	Stressed (SE)	Time (p)	Main (p)	INT (p)	Figure
BrdU+ cells	Stress	7.3 (0.73) c/s	7.5 (1.7) c/s		P=0.909		No Figure
BrdU+ cells	[A]Learning	[A]8.1 (0.12) c/s	[A]4.9 (0.39) c/s		[A]**P=0.082**		Fig 2.5
Multiple regression of transformed data	[B] Stress*Learning	(see text for multiple regression equation coefficients)		[B] Stress P=0.775	[B] Learning P=0.160	[B] **P=0.038**	Fig 2.6
Adrenal gland		219.5 (15.9) mg	267.6 (21.6) mg		**P=0.085**		No Figure
			Hippocampal-basal ganglia dependent tasks				
Stimulus-response: Task acquisition	Stress	60.2 (1.10) %	56.9 (0.94) %	**P=0.0001**	**P=0.043**		Fig 2.7
Stimulus-response: Response latency	Stress	5.05 (0.30) s	4.56 (0.38) s	**P<0.0001**	P=0.367		No Figure
Left Right preference (Preference for left)	Stress	Day1: 66.6 (6.7) % Day27: 54.2 (4.7) %	Day1: 79.4 (8. 6) % Day27: 50 (9.2) %		P=0.278 P=0.719		No Figure No Figure
Impulsivity large reward preference	Stress	74.1 (2.3) %	83.9 (2.0)%	**P<0.0001**	**P=0.027**		Fig 2.8

Hippocampal-basal ganglia Dependent tasks Continued

Class	Treatment	Control (SE)	Stressed (SE)	Time (p)	Main (p)	INT (p)	Figure
Impulsivity task: Delay discounting probe trials	Stress	0 s: 77.8 (8.0) % 3 s: 76.7 (9.1)% 10 s: 75.6 (10.4) % 30 s: 76.7 (5.2) % 100 s: 70.7 (8.4)%	0 s: 89.9 (4.3)% 3 s: 72.1 (12.1) % 10 s: 69.1 (8.9) % 30 s: 76.2 (4.3) % 100 s: 71.4 (5.6) %		P=0.974		Fig 2.9
Locomotor activity assay	Stress	0 mg: 12582 (1632) cm 0.1 mg: 20684 (3887) cm 0.3 mg: 21084 (1984) cm 1 mg: 36830 (4187) cm	0 mg: 13047 (1701) cm 0.1 mg: 21218 (4139) cm 0.3 mg: 20588 (4351) cm 1 mg: 31378 (4661) cm	**P<0.0001**	P=0.714	**P<0.0001**	Fig 2.10

Non-hippocampal dependent tasks

Class	Treatment	Control (SE)	Stressed (SE)	Time (p)	Main (p)	INT (p)	Figure
Simple discrimination: Task acquisition	Stress	63.9 (1.8) %	67.1 (1.6) %	**P<0.0001**	P=0.263	**P=0.005**	Fig 2.11
Simple discrimination: response latency	Stress	4.17 (0.39) s	13.79 (8.27) s	**P<0.0001**	P=0.830		No Figure

DISCUSSION

This study has shown that mild chronic unpredictable stress did not reduced BrdU+ labelled cells 58 days post-stress exposure in adult rats. In addition, learning-mediated cell survival was enhanced within the dentate gyrus following stress (Table 2.4 and Figure 2.6). This suggests an interaction between stress and learning-mediated cell survival. These findings are in accordance with previous results which demonstrate enhanced learning following stress, and learning promoting neural stem cell survival (Curlik and Shors, 2011). Given the delayed time from stress exposure to BrdU+ quantification, biological/homeostatic mechanisms may have contributed to biological equilibrium of any proliferation and/or integration disruption induced or attributed to chronic stress. Overall, these findings suggest that any disruption to cell survival induced by stress is transient and does not produce chronic or static effects on neurogenesis, as supported by previous literature.

These results show the importance of learning in mediating adult derived neural stem cell survival in the hippocampus as shown in previous studies (Shors et al., 2002). More specifically, this study has confirmed previous findings of Curlik and Shors, 2011 demonstrating that learning does not necessarily have to be dependent on the hippocampus to promote cell survival within the hippocampal formation, as evident by hippocampal-basal ganglia dependent learning and non-hippocampal dependent learning producing a similar affect in this study. As such, this study has highlighted that hippocampal-basal ganglia dependent learning promotes neural stem cell survival in the dentate gyrus of adult rats. Furthermore, that hippocampal independent learning facilitates cell survival within the dentate gyrus. Therefore confirming previous work that learning in general promotes neural stem cell survival within the hippocampus in adulthood (Leuner et al., 2006; Shors, 2008).

Mild chronic stress also inhibited stimulus-response learning by about 5%. While conversely, facilitating acquisition of reward contingency by at least 13%. These findings highlight the multidimensional and complex effects of stress on cognition and suggest stress inhibits some aspects of cognition, while improving others. While further still producing no significant effect on others e.g. simple discrimination. Such a response is not unexpected and supports earlier findings.

It is known that cells within the hippocampus are sensitive to changes in adrenal steroids. It has been demonstrated that administration of corticosterone decreases adult neurogenesis, while conversely adrenalectomy produces a marked increase in cell proliferation (Cameron and Gould, 1994). It is clear that stress plays a significant role in mediating the cellular population of the dentate gyrus. While this study has not supported a simplistic effect of stress in adult neurogenesis, such an involvement has been documented (Schoenfeld and Gould, 2012). It is likely that there are many factors that confound the linking of neurogenesis with stress. Such factors include body mass and nutritional intake. In this study the rats were kept at 90% of free feed weight to avoid dietary restriction induced reduction effect on neurogenesis (Park and Lee, 2011). Further, adrenalectomy analysis indicated a 22% increase in gland size in stressed animals; however statistical analysis revealed this difference was not highly significant (p=0.082). Chronic stress is known to induce adrenal hyperplasia and hypertrophy within the adrenal gland (Ulrich-Lai et al., 2006). The findings presented here may be confounded by a long interval from the end of stress to post-mortem analysis of 58 days, thereby potentially allowing time for biological equilibrium of adrenal gland size. This interpretation is supported by previous findings which indicate an initial increase in adrenal hypertrophy followed by cellular equilibrium after 14 days following unilateral adrenalectomy (Pellegrino et al., 1963). The experiments show that learning acts as a buffer to mitigate the negative effects of stress on neurogenesis. But without prior exposure to stress, learning did not enhance cell survival (Figure 2.6).

The series of experiments provided important steps into the experimental techniques of neuroscience. One experimental observation provided evidence that the rats had no initial or final bias in selecting the left or right-hand image on the touch screen. This was important to exclude a potential source of unexplained variance in subsequent tasks. There are many other potential confounding factors that may have affected the results. For example, different training protocols have been associated with both positive and negative neurogenesis (Olariu et al., 2005). It is not possible to say categorically that the nature (predictable or unpredictable) of the training might be an explanation for the apparent contradictory response.

A dose response to psycho-stimulant induced locomotor activity was observed across both groups indicating that the primary affect of the stress did not produce an increase in

basal line locomotion, nor sensitivity to methamphetamine-induced locomotor activity. Together, the findings of impaired stimulus-response and improved reward acquisition indicate chronic stress affects similar neural systems differently.

The findings here open up new lines of enquiry to explore if training tasks can be developed to increase neurogenesis and integration of new cells into the hippocampus in ways to combat declines associated with stress and decreased learning. Is it possible to prevent the decline in cell integration with a pre-emptive training programme or indeed increase cellular integration? What is clear from this study is that distinction needs to me made between the types of stress and learning. That is, what neurological systems are involved (distinction has been made here regarding hippocampal-basal ganglia systems), and how does activation of these systems related to learning, and subsequent effects on hippocampal neurogenesis and how can this be reliably tested and repeated by others? Clearly, new experiments are needed to help answer these questions.

Overall, the experiment confirms earlier experimental work and theory that learning mediates cell survival with respect to cellular integration into the hippocampus in rats. While this study has confirmed the current theory it could be expanded by applying more specific and beneficial physical exercise and cogitative tests aimed at isolating only dentate gyrus functions. Future experiments involving hippocampal and dentate gyrus function should also consider including other behavioural tests that are not dependent on the hippocampus function to act as secondary controls (see Chapter 3).

CHAPTER 3

The role of the dentate gyrus in hippocampal and hippocampal-basal ganglia dependent behaviour

ABSTRACT

This chapter investigates the role of the dentate gyrus in the performance of cognitive tasks dependent upon the hippocampus and hippocampal-basal ganglia interactions. Microinjections of colchicine produced significant lesions of the dentate gyrus and surrounding CA1-CA3 and neocortex. Damage to these regions impaired hippocampal-dependent reference memory ($p=0.054$) while preserving hippocampal independent simple discrimination learning. In a delay discounting procedure, the lesions did not induce impulsive-like behaviour when delay associated with a large reward was introduced. In addition, there was no significant effect on stimulant-induced locomotor activity. These findings support the role of the hippocampus in reference memory but question the implication of the hippocampus in the regulation of impulse control. Further research is required to fully understand the neural mechanisms of hippocampal-basal ganglia interactions and the resulting contributions to behaviour and cognition.

INTRODUCTION

The hippocampus has long been associated with learning and memory. This region of the brain is also vital for processing of spatial discrimination, pattern separation and structural discrimination (Aggletion et al., 2007; Leutgeb et al., 2007). Furthermore, disruption of the hippocampal formation has been shown to significantly affect behaviour associated with reference memory. Many studies have documented the involvement of the hippocampus in reference memory, using a variety of tasks (Sharma and Kulkarni, 1992; Eichenbaum, 1999). However, the specific involvement of the specific sub-regions of the hippocampus remains to be clearly established.

Previous findings suggest the presence of an interactive mechanism between the hippocampus and the basal ganglia in mediating various forms of behaviour, such as habit learning (Atallah et al., 2004). The role of the basal ganglia in motor behaviour and disorders affecting movement is well established (Obeso et al., 2000; Ring and Serra-Mestres, 2001; Obeso et al., 2008). In parallel, there is some evidence documenting the ability of the hippocampus to influence behaviour that predominantly depends on the integrity of the basal ganglia. For example, the finding that ablation or lesion of the hippocampal formation induces an increase in locomotor activity, both spontaneous and stimulant-induced (Teitelbaum and Milner, 1963; Cassel et al., 1998), suggests an interaction between hippocampus and basal ganglia. Furthermore, the basal ganglia has also been implicated in certain aspects of learning and memory (Packard and Knowlton, 2002). Stimulus-response learning, a form of habit-based associative learning is impaired in rats with lesions of the basal ganglia (Featherstone and McDonald, 2004). Astonishingly, stimulus-response learning has been shown to be significantly altered in rats with striatal lesions, while, lesions of the hippocampus have been shown to improve habit-learning (Packard and Knowlton, 2002). These findings provide evidence of an interaction between the hippocampus and basal ganglia in stimulus-response learning and locomotor activity. However, the exact nature of the mechanism underlying this interaction remains to be determined.

The basal ganglia have also been implicated in impulsivity. The tendency to act on one's impulse as opposed to thought is a symptom present in several neuropsychiatric disorders, such as attention deficit hyperactivity disorder (ADHD), bipolar disorder,

schizophrenia and addiction disorders (Barkely, 1997; Verdejo-Garcia et al., 2008). Furthermore, it has been proposed that impulsivity is a behavioural manifestation of hippocampal impairment, or abnormality (Mariano et al., 2009). However, it is yet to be determined whether selective lesions of the dentate gyrus are sufficient to promote impulsive-like behaviours.

The involvement of the hippocampus in locomotor activity has been well established (Teitelbaum and Milner, 1963; Wilkinson et al., 1993). Ablation of the hippocampus has been shown to increase stimulant-induced locomotor activity (Wilkinson et al., 1993). It has been proposed that the effect of the hippocampus on locomotor activity is mediated by DA release (Wilkinson et al., 1993). Furthermore, serotonin (5-HT) has been implicated in locomotor behaviour since a decrease in serotonin production within the median raphe nucleus has been shown to correlate with decreases in locomotor activity (Takahashi et al., 2000). By showing that microdialysis infusion of serotonin or a monoamine oxidase inhibitor produced locomotor hyperactivity. Takahashi et al. (2000) demonstrated the involvement of hippocampal serotonin in locomotor activity. It was proposed hippocampal efferent neurons receiving projections from the median raphe nucleus were responsible for mediating this effect (Takahashi et al., 2000).

Locomotor activity is significantly influenced by the basal ganglia and hippocampus. It is yet to be shown whether a similar influence on locomotor activity can be made by removal of the hippocampal sub-region known as the dentate gyrus or cornu ammonis regions (CA1-CA3). Therefore the aim of this chapter was to investigate the effects of selective dentate gyrus lesions on habit-learning, impulsivity and psycho-stimulant induced locomotor activity. All of these responses are associated with hippocampal-basal ganglia interactions. In addition to reference memory and pattern separation, general behaviour that is primarily dependent upon the hippocampus and these and one non-hippocampal function were examined to assist interpretation.

MATERIALS AND METHODS

Experimental design

A total of twenty-five (N=25) male PVGc-hooded rats were obtained from the colony within the Animal Facility of the Psychology Department of the University of Canterbury. A simple experimental design comprising one treatment and an untreated (sham) control with animals assigned in a randomised layout. The treatment was a surgical lesion targeted at the dentate gyrus to study its effect on various functional parameters associated with hippocampal and hippocampal-basal ganglia activity. A selection of specific cognitive and locomotor tasks was applied to the treated and control groups. These tasks represented three types of brain function (1) a non-hippocampal-dependent task, (2) a hippocampal-dependent task and (3) a hippocampal-basal ganglia dependent task. Table 3.1 summarises the tasks experienced by each group.

Non-hippocampal-dependent tasks

One non-hippocampal-dependent task was designed to ensure that the rats retained non-hippocampal function in order to assist in interpretation of results. This was a simple discrimination task.

Hippocampal-dependent tasks

The hippocampal-dependent tasks comprised two tests of a pattern separation and a reference memory task.

Hippocampal-basal ganglia dependent tasks

The hippocampal-basal ganglia dependent task group performed a stimulus-response-habit learning, delayed discounting-impulsivity task and a locomotion task. Additional configural and structural discrimination tasks were planned; however the rats were unable to transition from simple discrimination to configural discrimination tasks (see Discussion).

Table 3.1.

List of experimental groups and their tasks (total number of rates N=25). Rats performing the hippocampal-basal ganglia dependent tasks were divided into a further 2 groups with a 3:4 ratio (3 rats / 4 rats (due to the odd number of rats within the condition)). These two groups were counterbalanced with a reversal of the task in order to eliminate any potential bias for responding to any one side of the touchscreen.

	Hippocampal-basal ganglia dependent tasks	**Non-hippocampal dependent and hippocampal dependent tasks**
CONTROL	**(n=5)**	**(n=7)**
	Simple discrimination: B-S Chambers	Habit learning: T-maze
	Pattern separation: Radial arm maze	Stimulus-response: B-S Chambers
	Reference memory: Plus maze	Impulsivity task: B-S Chambers
		Locomotor assay: Open Field
		Reference memory: Plus Maze
LESION	**(n=6)**	**(n=7)**
	Simple discrimination: B-S Chambers	Habit learning: T-maze
	Pattern separation: Radial arm maze	Stimulus-response: B-S Chambers
	Reference memory: Plus maze	Impulsivity task: B-S Chambers
		Locomotor assay: Open field
		Reference memory: Plus maze

General Animal Housing Procedure

All rats were housed in standard housing conditions of 4 rats per cage (opaque plastic cage; 50 cm long by 30 cm wide by 23 cm high). Rats were kept on a 12 h light-dark cycle. Initially water and rat chow were available *ad libitum* prior to food deprivation. Following surgery all rats were housed individually for a recovery period of 3 weeks, after which they were returned to their original housing conditions.

Experimental Procedure

Animals were trained to nose-poke a touchscreen to elicit a food reward, as described in Chapter 2. Once the rats had demonstrated consistent nose-poke responses (3 consecutive sessions of 30 trials in 30 min), rats were then assigned to the treatment (lesion) and control groups. Rats were matched in a pairwise fashion based on pre-training performance and assigned to either the lesion or control group. Once rats were assigned a group, the surgery procedure was commenced.

Lesion Surgery

Surgery was based on the protocol of Hernandez-Rabaza et al. (2008). One beforehand, the rats were intraperitoneally administered 50 mg/kg of carprofen to induce analgesia, extending the treatment where necessary if any sign of distress was apparent in the animal. Male PVGc rats (25) were anaesthetised with Avertin (a mixture of 2,2,2-tribromoethanol and tertiary amyl alcohol) injected at a dose 2 ml per 100 g and then mounted on a stereotaxic apparatus on a flat skull position. The skull was then exposed and an inverted V-shaped hole was drilled bilaterally at the level of the dorsal hippocampus. A stainless steel needle (31G) was mounted on the stereotaxic arm and connected with polyethylene tubing to a microsyringe driven by a precision pump (Harvard apparatus). The rats received a total of 10 injections (0.2 µl each) of the neurotoxic solution, five into each hemisphere. Colchicine (Sigma, UK) was dissolved in sterile saline (0.9%) and injected at a concentration of 4 µg/µl. The needle was lowered into the brain at the following stereotaxic coordinates: AP -2.3, -3.1, -3.8, -4.5, -5.3; ML 0.9, 1.1, -1.8, 2.4, 3.4; DV 3.5, 3.3, -2.9, -2.8, 3.1 (from brain surface, Paxinos and Watson, 2004). Sham lesions were produced by injecting sterile saline solution (0.2 µl each) at the same stereotaxic coordinates. The needle was kept in place for two minutes to reduce flow back. Neotopic H solution (containing local anaesthesia and antibiotic properties) was placed at the surgery site. The rats were then placed in individual cages and allowed to recover from surgery for 4 weeks before tests began. During the third week of recovery (21 days after the surgery) rats were subjected to the food deprivation regime (exactly as above) and weight was maintained at 90% of their post-surgery weight (i.e., the weight taken on the day that the second deprivation regime begins). Behavioural testing commenced four weeks after the surgery.

Following surgery, animals were tested on a variety of behavioural tasks. The stimulus response task, impulsivity task, and simple discrimination tasks were conducted within the Bussey-Saksida touch-screen chamber, and stimulant induced locomotor activity in the open field as detailed in Chapter 2. Animals were also assessed on pattern separation, habiting learning and reference memory as detailed below.

Pattern Separation Task: Radial Arm Maze

Pattern separation was based on Clelland et al. (2009) protocol and was tested using a delayed non-matching to sample (DNMP) task within a 8-arm radial arm maze (35-cm-wide wooden hub (painted black) with 8 aluminium arms 65 cm long by 8.5 cm wide, 3 cm high borders. Clear perspex guillotine doors, were controlled by an overhead pulley system. All testing occurred within the animal's dark cycle (8 am to 8 pm). All animals received three days of habituation prior to experimental testing. On the first day of habituation rats were allowed to freely explore the maze with all arms open. On the second and third day of habituation all maze arms were baited with two chocolate favoured Precision pellets, and the rats were required to enter a minimum of four arms and eat the pellets placed in each food-well of the arm in order to complete habituation. On day four, experimental trials began. Pattern separation was measured by the rat's ability to separate sample (old) arms from correct (new) arms. Rats received four trials per day for 12 consecutive days. During testing, rats received one sample (forced trial), followed by three choice trials. The rats were returned to the holding cage between each choice trial, and all other rats from the home cage were tested before the first rat began the second choice trial, so as to maximise the inter-trial interval. During the forced trial only the start arm and forced arm were open. Once the rat ate the food at the end of the forced arm, the guillotine door was closed for 30 sec. During the choice trials, the old (forced) arm and new (correct) arm were open, and the rat was required to avoid the old arm, and enter a new (correct) arm to obtain the food reward. Animals were tested on two sets of pattern separation problems, a spatially small separation and a spatially large separation. Correct and old arms varied in distance in each small and large problem set (small and large, see Appendix G for examples).

Habit-Learning (Elevated T-Maze)

The habit learning task was conducted in an elevated T-maze. The maze was made of wood, with a runway 67 cm long and 10 cm wide, with two 40 cm long side arms of equal width (10 cm). The walls of the maze were two cm high, with a guillotine door used to open the start section, as well as two additional wooden blocks used to block access to each arm. The habit learning task consisted of locating two food pellets positioned invariably at either end of a T-maze arm. The reinforced arm was counterbalanced across both groups of rats. The rats received three habituation sessions, lasting three minutes, during this time the rat was allowed to freely explore the T-maze. During the habituation sessions 5 food pellets were placed at the end of both maze arms. On the fourth day Rats received 10 trials per day until 5 consecutive correct choices were made on the same day. During the probe trial, the rat was placed 180 degrees keeping the position of the rewarded arm fixed, so was to probe the choice strategy of the rat. The direction of the animal's choice of arms was used to assess the learning strategy of the animal, as this would reveal a strategy using either allocentric or egocentric cues when responding in this task.

Reference Memory Task (Elevated Plus Maze)

The elevated plus maze was used to investigate reference memory, as described by Sharma and Kulkarni (1992). The plus maze comprised two arms with clear perspex walls (50 x 10 x 25 cm). Two identical arms crossed the clear arms consisting of black wooded walls of the same dimensions. These two sets of arms were connected with a central square (16 x 16 cm). Altogether the maze was attached to a 1 m high wooden stand (Figure 3.1) Prior to testing rats were counterbalanced and randomly assigned a start arm (either the left or right open arm (clear perspex). On the first day of testing, the rat was placed at the end of one of the open arms facing outward away from the central square so as to reduce the tendency to begin blindly running once placed in the maze. The time taken for the rat to move from the open arm and enter a closed arm was recorded manually with a stop watch. Entering a closed arm was defined as the back legs passing from the centre square into the arm (Figure 3.2). This time is referred to as the initial transfer latency (ITL). Once the ITL was recorded the animal was allowed to explore the rest of the maze for an additional 30 s and then returned to its home cage. After 24 h the rat was placed in the same starting

location as previously and the transfer latency was recorded again. This time it was referred to as the retention transfer latency (RTL).

Figure 3.1.

Elevated plus maze within the experimental testing room.

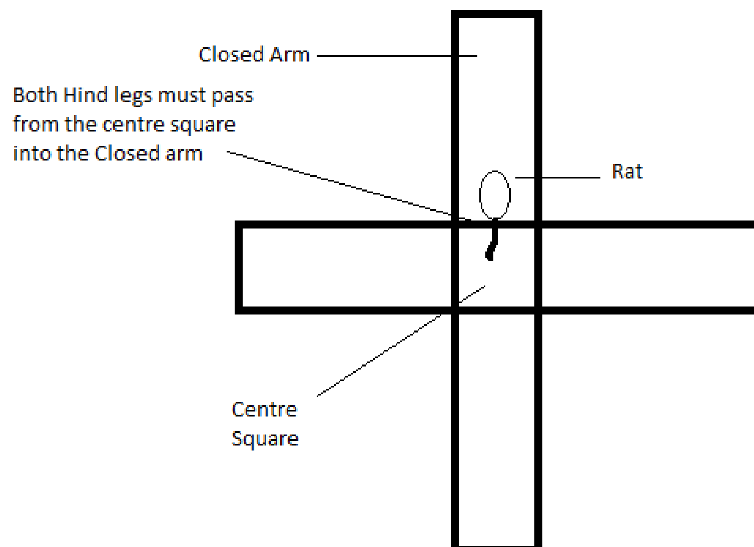

Figure 3.2.

Illustration of the “Transfer” criteria used to measure transfer latency.

Histology

On completion of the behavioural tasks, rats were perfused with 4% paraformaldehyde in 0.1M phosphate buffer. The brains were then removed, post-fixed in the same fixative solution for 24 h, then transferred to Phosphate buffer with 20% glycerol and 0.05% sodium azide solution for cryoprotection. Free-floating coronal sections were then cut through the dorsal hippocampus on a cryostat and stained with cresyl violet (Appendix H). Microphotographs of 10x magnification were taken of representative sections to confirm the location and extent of lesions.

Statistical analyses

Data was processed using the statistical package Statview (SAS Institute Inc.) primarily using repeated measures ANOVAs and the associated graphic tools including standard errors of the mean (SEM).

RESULTS

Histology

Lesions of the dentate gyrus were not selective. Near complete lesions of the entire hippocampus were observed in lesioned animals. In addition, damage to overlying cortical areas was also observed. Surprisingly, one lesioned animal appeared to have no structural damage to the dentate gyrus or surrounding CA1-CA3 or cortical areas. Subsequent testing of this animal revealed that its behaviour was abnormal (exhibiting similar deficit behaviour to other lesioned animals) and it was therefore removed from further analysis. Figure 3.3 shows example representative microphotographs of sham and lesioned animals recorded during the experiments.

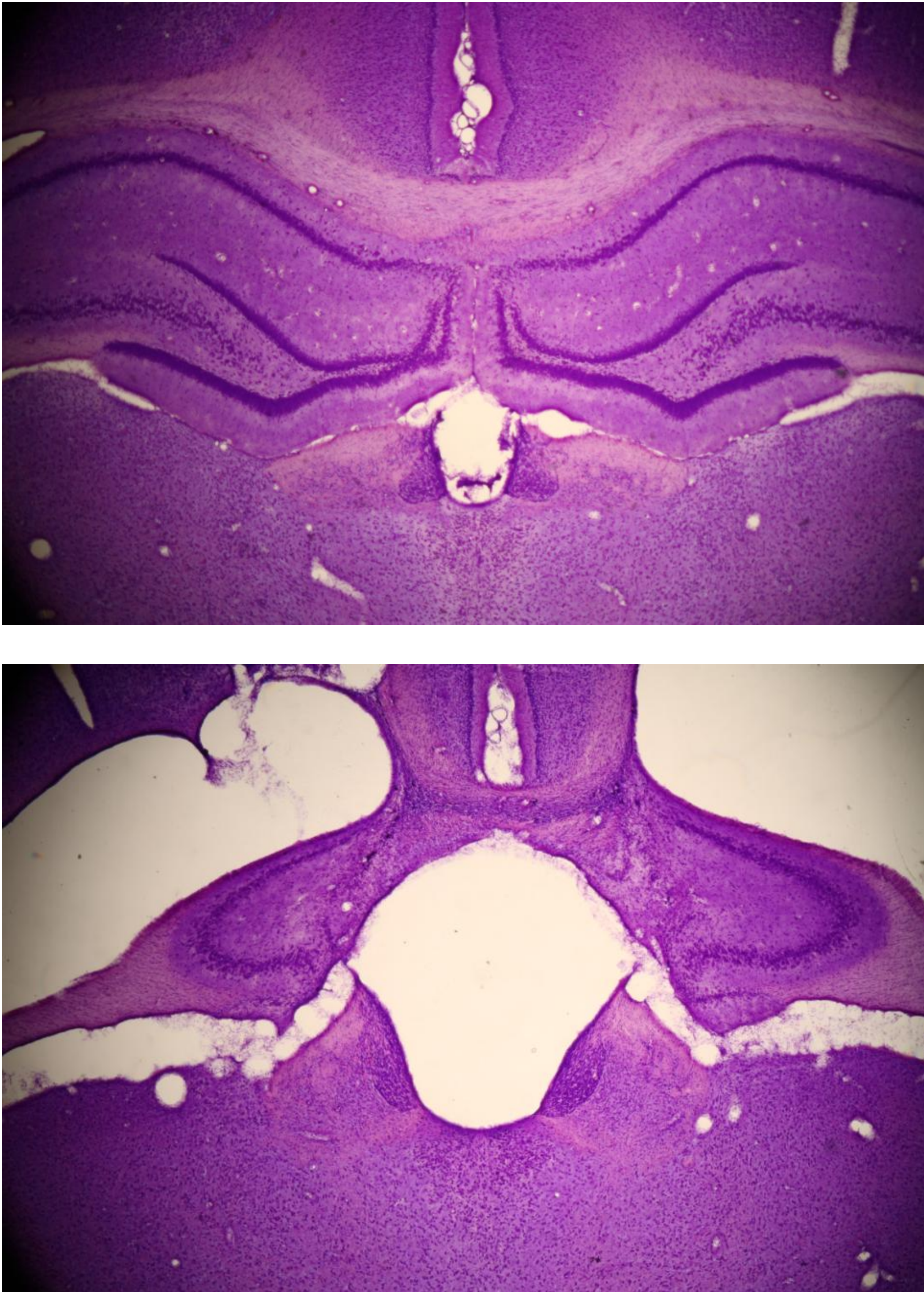

Figure 3.3.

Representative microphotographs of sham (top) and hippocampal lesion (bottom). Showing deformation and cellular thinning of the dentate gyrus as well as extensive cell loss in CA1-CA3 regions in lesioned animals (bottom). These coronal sections are located at approximately -3.14 from bregma.

Non-hippocampal-dependent tasks

Simple Discrimination: Bussey-Saksida Chambers:

Figure 3.4 shows the acquisition of the simple discrimination task by both control and lesioned animal groups. A repeated measure ANOVA revealed a significant difference of percentage correct responses over blocked trials, $F(6, 120) = 9.03$, $p < 0.0001$, indicating both lesion and control groups learnt the simple discrimination, despite no significant difference between the two groups (mean 72.2%), $F(1,20) = 1.48$, $p = 0.238$. In addition no significant interaction was observed between the experimental group and blocked trials, $F(6, 120) = 1.42$, $p = 0.212$. These findings suggest a lesion of the dentate gyrus and surrounding hippocampus is not sufficient to impair acquisition of a simple discrimination task. This supports previous findings as simple discrimination behaviour has been shown to be independent from the hippocampal formation.

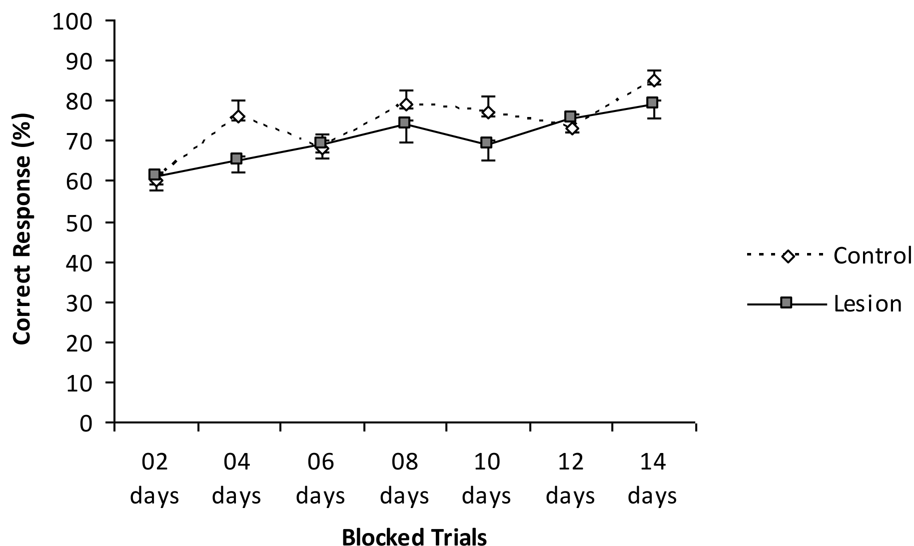

Figure 3.4.

Simple discrimination: Task acquisition. Mean correct response (%) per session block (±SEM) for both lesion and sham groups across 14 days of testing. Trials were blocked into 2 days per block (60 trials). Both Lesion and Control groups showed a significant increase in correct response over blocked trials, p<0.0001.

Hippocampal-dependent tasks

Small pattern separation

Figure 3.5 shows the acquisition of the delayed non-matching to place small pattern separation task by both sham control and lesioned animal groups. However a repeated measure ANOVA revealed no significant difference in correct arm choice over blocked trials, $F(3, 93) = 0.82$, $p = 0.484$, indicating neither lesion or sham control animals were unable to learn the small-separation task. In addition, no significant difference was observed between the two groups (mean 50.4%), $F(1, 31) = 0.05$, $p = 0.822$ (Figure 3.5). As the sham control group was unable to discriminate between small spatial separations, it is not possible to infer the effect of the lesion surgery on this task. The reasons are unclear why the rats in the control group could not learn the task (see Discussion).

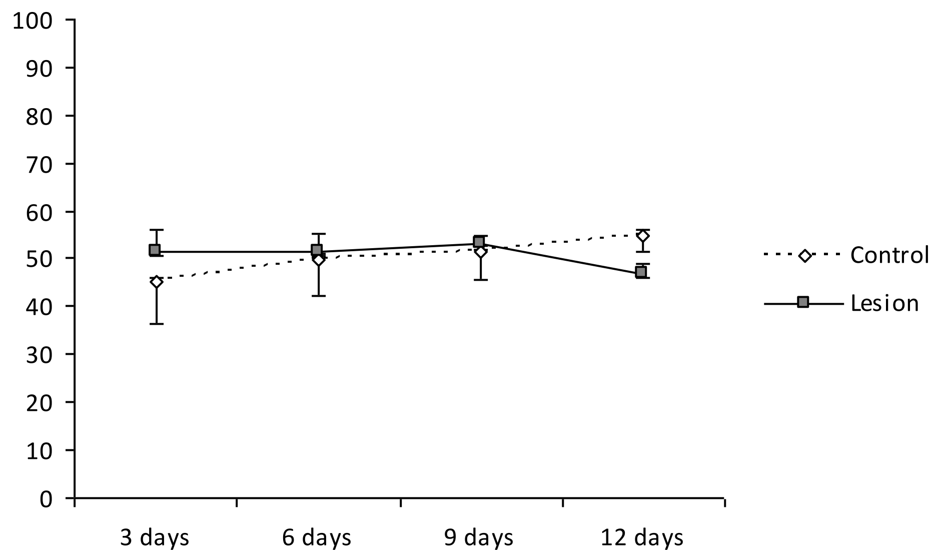

Figure 3.5.

Delayed non-matching to place small pattern separation task: Task acquisition. Mean correct arm choice (%) per session block (±SEM) for both lesion and sham groups across 12 days of testing. Trials were blocked into 3 days per block (12 trials). Both Lesion and Control groups were unable to learn to separation task.

Large pattern separation

Figure 3.6 shows the acquisition of the delayed non-matching to place large pattern separation task by both sham control and lesioned animal groups. A repeated measure ANOVA revealed no significant difference in correct arm choice over blocked trials, $F(3, 93) = 0.60$, $p = 0.616$, indicating neither lesion nor sham control animals were unable to learn the large-separation task. Further, no significant difference was observed between the two groups (mean 50.7%), $F(1, 31) = 0.70$, $p = 0.411$ (Figure 3.6). As the sham control group was unable to discriminate between large spatial separations, it is not possible to infer the affect of the lesion surgery on this task. The reasons are unclear why the rats the control group could not learn the task (see Discussion).

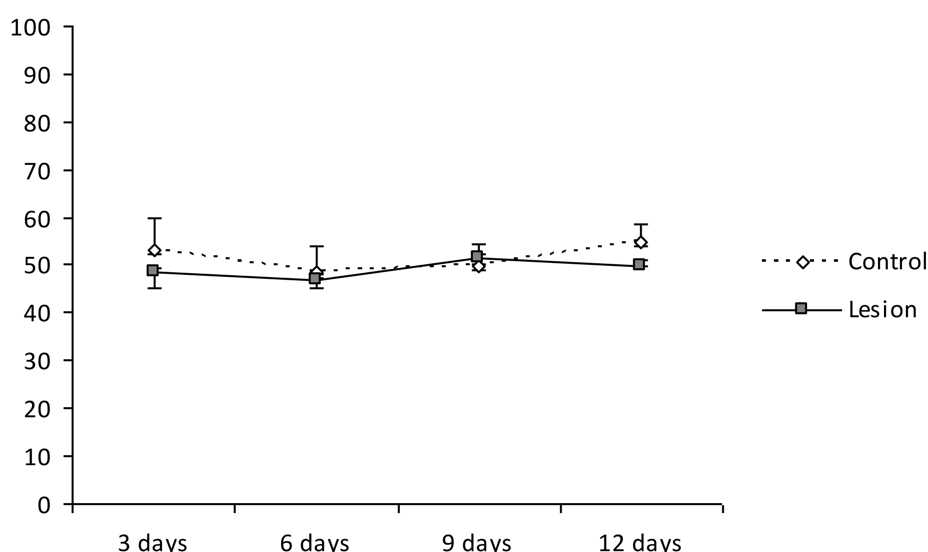

Figure 3.6.

Delayed non-matching to place large pattern separation task: Task acquisition. Mean correct arm choice (%) per session block (±SEM) for both lesion and sham groups across 12 days of testing. Trials were blocked into 3 days per block (12 trials). Both Lesion and Control groups were unable to learn to separation task.

Reference memory task: Elevated plus maze

Figure 3.7 shows the initial transfer latency and retention trial latency of both sham control and lesion animal groups. A repeated measure ANOVA revealed a significant interaction between the experimental groups and the retention trial latency (RTL), $F(1,22) = 4.16$, $p = 0.054$ (Figure 3.7) indicating that control animals significantly decreased

transfer latency after 24 hrs retention, while lesioned animals performed in the same way as the ITL (Figure 3.7). There was no overall difference between experimental groups (mean 18.7 sec), $F(1,22) = 0.002$, $p = 0.961$. These findings indicate that lesions of the dentate gyrus and surrounding hippocampus produced a deficit in reference memory measured in the elevated plus maze.

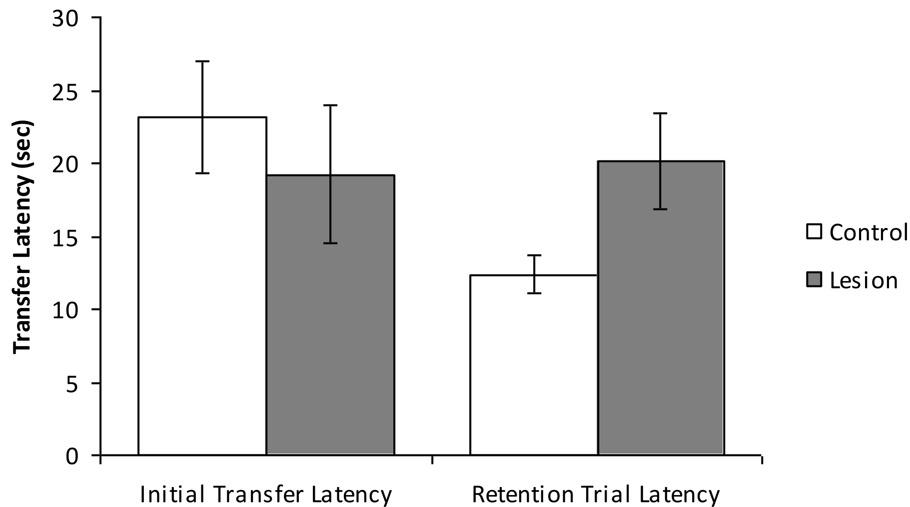

Figure 3.7.

Comparison of reference memory deficits showing the significant interaction between lesioned and control animals (p=0.054).

Hippocampal-basal ganglia dependent tasks

Stimulus response learning: Bussey-Saksida Chambers:

Figure 3.8 shows the acquisition of the stimulus-response task by both control and lesioned animal groups. A repeated measure ANOVA revealed a significant effect for percentage of correct responses over blocked trials, $F(8, 296) = 39.18$, $p < 0.0001$, indicating that both lesion and control groups learnt the stimulus-response task, despite no significant difference between the two groups (mean 60.5%), $F(1,37) = 0.66$, $p = 0.420$. However a significant interaction was observed between experimental group and blocked trials, $F(8, 296) = 2.45$, $p = 0.014$. This may indicate that the lesioned animals had more difficultly learning over time but that lesions of the dentate gyrus and surrounding CA1-CA3 regions is not sufficient to facilitate acquisition of a stimulus-response learning task.

Conversely, this data also shows that the lesions do not produce a serious deficit in stimulus-response learning.

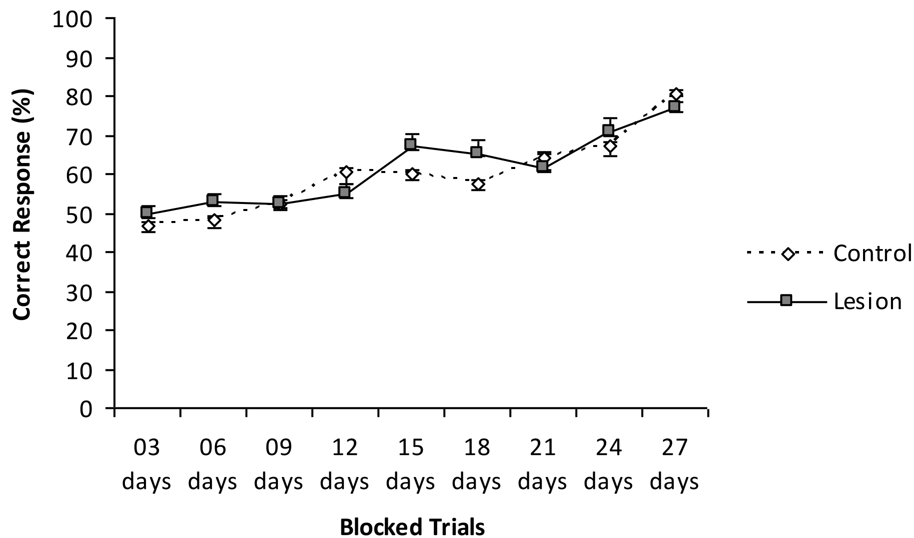

Figure 3.8.

Stimulus-response: Task acquisition. Mean correct response (%) per session block (±SEM) for both lesion and sham groups across the 27 days of testing. Trials were blocked into 3 days per block (90 trials). Both Lesion and Control groups showed a significant increase in correct response over blocked trials, $p<0.0001$.

Habit Based learning: T-Maze

A one-way ANOVA revealed no significant difference in acquisition rate of the habit learning task (mean 12.9 trials), $F(1, 11) = 2.21$, $p = 0.165$, despite a 43.3% increase in trials to acquire the task (Figure 3.9). Due to the large increase in time to acquire the task this potentially may be significant when $p < 0.1$ with a larger sample size (>N=14) because increasing sample size may decrease the residual error. No significant difference in learning strategy (allocentric or egocentric) between the experimental groups was observed, $F(1,11) = 0.15$, $p = 0.707$. These findings indicate that the brain lesions applied did not produce a deficit in acquisition of habit-based learning, and did not affect the type of learning strategy used during acquisition.

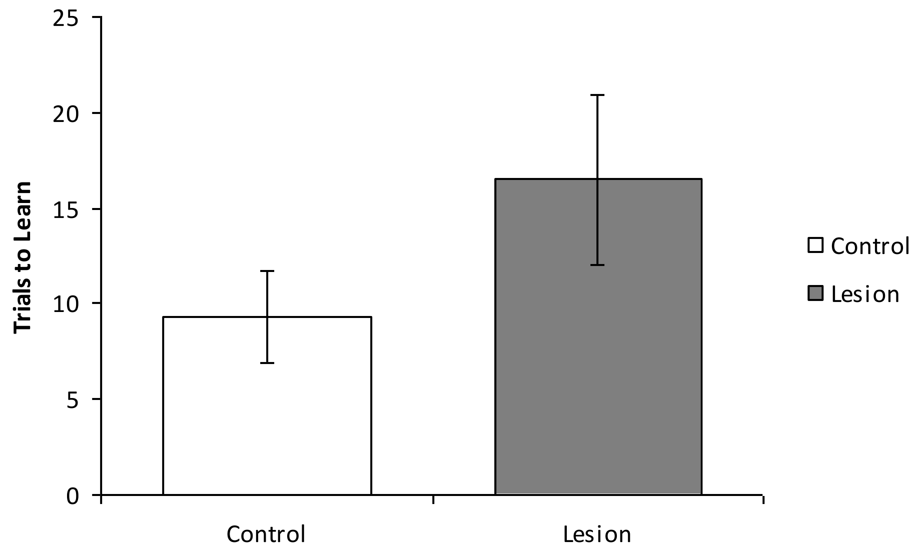

Figure 3.9.

T-maze task acquisition showing large standard errors between the groups with no significant difference between the groups, $p = 0.165$.

Impulsivity task: Large reward preference acquisition

A repeated measure ANOVA revealed a significant difference in preference for the immediate large reward over blocked trials, $F(6, 144) = 9.34$, $p < 0.0001$, indicating both Lesion and Control groups acquired the task. However, no significant difference was observed between the two experimental groups (mean 72.8%), $F(1, 24) = 2.23$, $p = 0.149$, as shown in Figure 3.10. Due to the marginal significance, the initial percentage data was transformed using arcsine transformation, however no significant decrease in p-value was observed. Taken together, these findings indicate that lesions of the dentate gyrus and CA1-CA3 do not produce a deficit in reward preference acquisition.

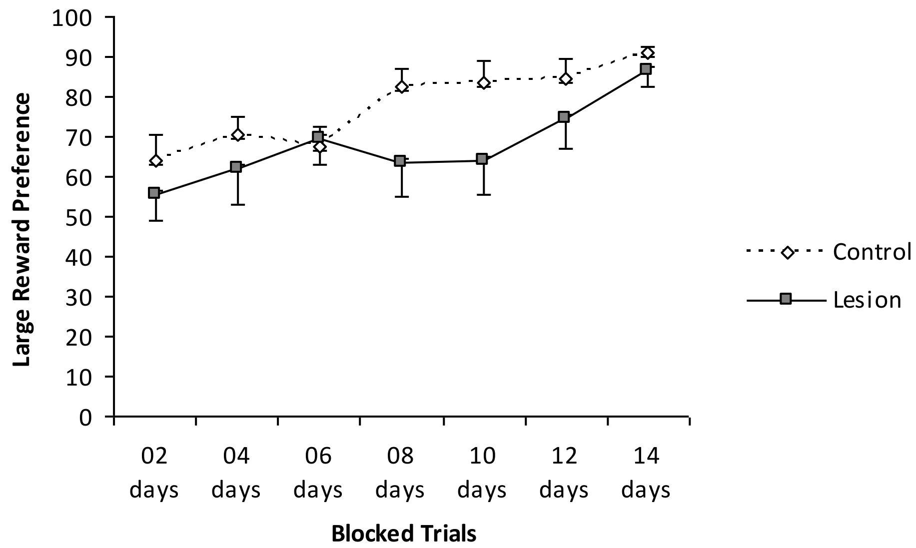

Figure 3.10.

Impulsivity task: large reward preference acquisition. Mean correct response (%) per session block (±SEM) for both experimental groups across acquisition for the 14 days. Trials were blocked into 2 days per block (60 trials). Both Lesion and Control groups showed a significant difference in correct response over time (block trials), $p<0.0001$. However, there were no significant difference overall between the lesion and control groups, $p=0.149$.

Impulsivity task: Delay discounting probe trials

A repeated measures ANOVA revealed a significant difference between the probe trials, $F(5, 55) = 5.02$, $p = 0.015$. Despite no significant difference between the experimental groups, $F(1, 11) = 0.10$, $p = 0.760$, (Figure 3.11). There were similar decreases in reward preference in lesion animals across all probe delays (3 sec delay 24.2%, 10 sec delay 16.9%, 30 sec delay 40.2%, 100 sec delay 41.4% and 300 sec delay 35.4%) compared to control animals (3 sec delay 21.9%, 10 sec delay 24%, 30 sec delay 36.4%, and 100 sec delay 30.7% and 300 sec delay 48.5%) (Figure 3.11). The results suggest lesion surgery did not enhance or attenuate the natural decrease in preference for the large reward that occurs with increased delay.

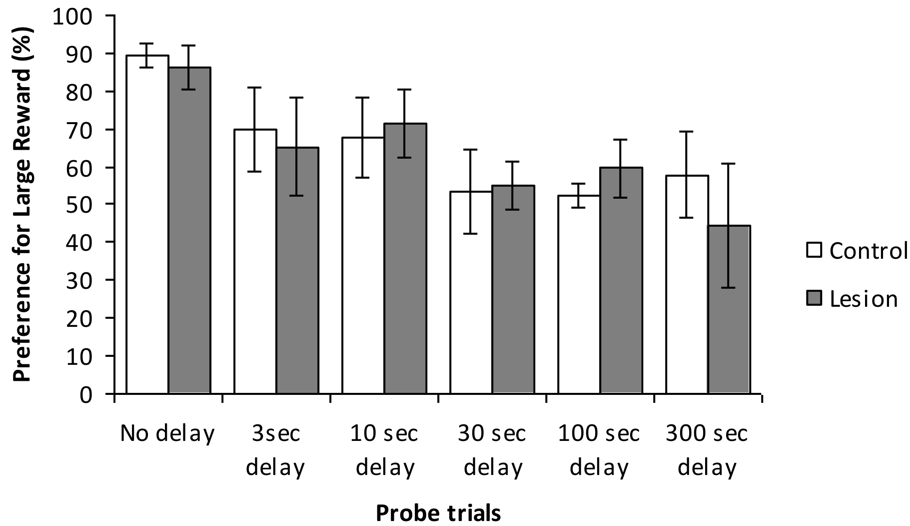

Figure 3.11.

Impulsivity probe trials: Mean response (%) per session for large reward key (±SEM) for control and lesion groups across acquisition. A significant difference in large preference during delay-discounting, $p=0.015$.

<u>Stimulant-Induced Locomotor Assay:</u>

A repeated measure ANOVA revealed a significant effect of methamphetamine dose on locomotor activity, $F(3, 33) = 14.98$, $p < 0.0001$ (Figure 3.12). In addition a highly significant interaction was observed between methamphetamine dose and time, $F(24, 264) = 3.38$, $p < 0.0001$. Despite no significant difference between the lesioned and control animals, $F(1, 11) = 0.59$, $p = 0.459$. These results show a dose response of methamphetamine on locomotor activity, while lesions of the dentate gyrus and CA1-CA3 did not affect baseline locomotor activity, nor stimulant-induced locomotor activity.

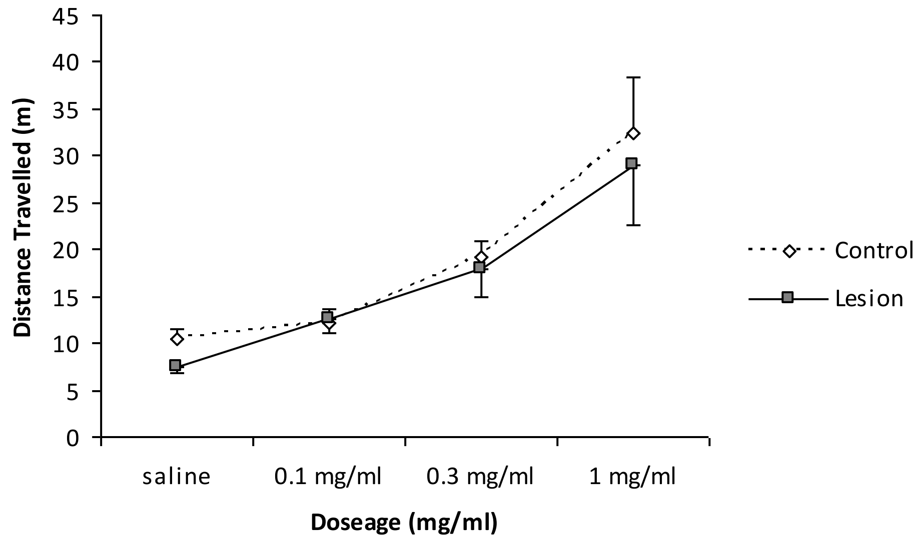

Figure 3.12.

Effect of dose of methamphetamine on distance travelled in the locomotor activity assy. A dose of 1 mg/ml signifcantly increased adulation, $p<0.0001$.

Table 3.2.

Summary of key results showing the various tasks, their dependent variable of the control and lesioned groups together with their standard errors and degree of significance of time and main effects and interaction of main effect with time. The probability of significance is not listed if it was not tested.

			Non-hippocampal dependent tasks				
Class	Treatment	Control (SE)	Lesioned (SE)	Time (p)	Main (p)	INT (p)	Figure
Simple discrimination: Task acquisition	Surgery	74.1 (1.5) %	70.2 (1.7) %	**P<0.0001**	P=0.238	P=0.212	Fig 3.4

			Hippocampal Dependent tasks				
Class	Treatment	Control	Lesioned	Time (p)	Main (p)	INT (p)	Figure
Pattern separation (small)	Surgery	50.4 (3.3) %	50.3 (1.6) %	P=0.484	P=0.822		Fig 3.5
Pattern separation (large)	Surgery	51.7 (2.5) %	49.7 (1.0) %	P=0.616	P=0.411		Fig 3.6
Reference memory	Surgery	ITL: 23.2 (3.8) s	ITL: 19.2 (4.7) s				
		RTL: 12.4 (1.3) s	RTL: 20.1 (3.3) s		P=0.961	**P=0.054**	Fig 3.7

Hippocampal-basal ganglia dependent tasks

Class	Treatment	Control (SE)	Lesioned (SE)	Time (p)	Main (p)	INT (p)	Figure
Stimulus-response: Task acquisition	Surgery	59.68 (0.99) %	61.3 (1.7) %	**P=0.0001**	P=0.420	**P=0.014**	Fig 3.8
Habit learning T-maze	Surgery	9.3 (2.4)	16.5 (4.5)		P=0.165		Fig 3.9
Impulsivity large reward preference	Surgery	77.7 (1.98) %	67.9 (2.93) %	**P<0.0001**	P=0.149		Fig 3.10
Impulsivity task: delay discounting probe trials	Surgery	0 s: 89.2 (3.3) %	0 s: 86.1 (5.9) %				
		3 s: 69.6 (11.1) %	3 s: 65.3 (13.1) %	**P=0.015**	P=0.760		Fig 3.11
		10 s: 67.8 (10.5) %	10 s: 71.5 (8.9) %				
		30 s: 53.4 (11.1) %	30 s: 54.8 (6.3) %				
		100 s: 52.3 (3.3) %	100 s: 59.7 (7.7) %				
		300 s: 57.7 (11.3) %	300 s: 44.4 (16.3) %				
Locomotor activity assay	Surgery	0 mg: 10355 (1139) cm	0 mg: 7463 (727) cm				
		0.1 mg: 12065 (1537) cm	0.1 mg: 12506 (1499) cm	**P<0.0001**	P=0.459	**P<0.0001**	Fig 3.12
		0.3 mg: 19231 (1621) cm	0.3 mg: 18004 (2987) cm			**dose/time***	
		1 mg: 32345 (6044) cm	1 mg: 29015 (6354) cm				

DISCUSSION

This study has shown that, in rats, microinjections of the neurotoxin colchicine produced significant damage of the dentate gyrus and surrounding CA1-CA3 regions and neocortex. The damage was severe in most animals destroying most of the hippocampus (Figure 3.6). It was evident that the lesion surgery was not specific to the dentate gyrus. Despite the greater damage attained the study confirms the importance of the hippocampus in cognitive function and provides a way forward to successfully achieve more precise surgical outcomes.

While lesions of the dentate gyrus were observed the degree of lesion expression was not well controlled. The control group that also receive a sham surgery with no neurotoxin recovered well postoperatively and did not appear to suffer any complications of surgery and exhibited normal behaviour throughout testing. It will be important in future experiments to consider a broader dose range of colchicine in separate experiments to establish a more reliable methodology. Only one quantity (10 microinjections of 0.2 µl) was applied in these experiments based on the experiments of Hernandez-Rabaza et al. (2008). According to previous work, this dose range is conservative. Furthermore, the extensive damage observed is consistent with high dose infusions of colchicine (Hernandez-Rabaza et al., 2008). Two possibilities explain the observed damage. The first involving human error, where dosage calculation errors may have resulted in an increased concentration of colchicine and therefore an increase dosage was infused. This is however unlikely, as dose calculations were checked before the neurotoxin was made. In addition, colchicine dosages were made on the day of surgery for the specific number of surgeries that day. It is still possible however, that a calculation/weighting error was made originally, and carried over each day into all colchicine dose makings. The second possibility is a heightened sensitivity of the PVGc strain to colchicine. This is believed to be more likely as the damage observed is consistent with a high dosage of colchicine. Moreover, previous unpublished work in this Laboratory has shown methamphetamine toxicity in PVGc rats with moderate doses that are typically nontoxic with other strains.

It was not possible to fully test a suite of hippocampal-dependent tasks because simple transition from simple discrimination to configural discrimination could not be attained. It is possible that the task was too complex; however previous studies have used similar tests with learning taking approximately 3-4 days. The testing here was abandoned after 24 days of testing. In addition, both control and lesioned animals were unable to learn a pattern separation task as measured by a delay non-matching to sample paradigm. Previous work has reported learning after 15 days of testing (Clelland et al., 2009). This test was abandoned after 24 days of testing. Despite slow learning both lesion and sham populations did learn a simple discrimination task increasingly over a period of 14 days. This shows that lesioned animals were able to acquire the simple discrimination task despite significant damage to the hippocampus. This supports previous findings that simple discrimination learning relies on other cortical regions, independent of hippocampal functioning. There is also the possibility that the rat base population has some inbred deficiencies like retardation. Fourteen days is considered extremely long to learn a simple discrimination task with typical leaning periods reported in the range of 3-4 days (90 trials) (Bussey et al., 2008). To avoid this problem in the future, the experiment could be conducted across a selection of laboratories with diverse genetic populations.

Despite the learning difficulties, the results did show a significant interaction between the lesion and sham control groups in the reference memory task. The control animals had significantly shorter time to transfer from the exposed arm to the closed arm. These observations indicate that the applied lesions produced a deficit in reference memory measured in the elevated plus maze. This effect on memory is an important finding of this study corroborating earlier work implicating the hippocampus involvement in reference memory, as well as highlighting where future work should continue to focus.

Despite significant damage to the dentate gyrus and surrounding hippocampal regions, the lesion group had no great performance difference compared to the sham group in a wide range of tasks (e.g. habit learning and locomotor activity). There were, however, some interesting interactions attributed to loss of the hippocampal region where both control and lesion animals learnt the stimulus-response task in the touchscreen chambers (Table 3.2). These findings suggest lesions of the dentate gyrus and surrounding hippocampus is not sufficient to facilitate acquisition of a stimulus-response learning task in the touchscreen environment. Conversely, this data also shows significant damage of the

hippocampus does not produce a deficit in stimulus-response learning. Furthermore, a 43% increase in trials to learn a habit learning task in a T-maze environment was shown in lesioned animals compared to controls. While this finding was not statistically significant, it does highlight the importance of different testing environments on measuring similar behaviours (touchscreen Vs T-maze).

Despite significant damage to the dentate gyrus and surrounding hippocampus, lesioned animals were able to acquire a preference for a large food reward, similar to control animals (Table 3.2). This coincides with previous experimental findings (Chapter 1). Furthermore, a curious observation was the similar decrease in reward preference of lesioned animals during delay discounting to control animals. Lesioned animals exhibited similar variability to the reward contingency, and appeared to display similar patterns of response behaviour as controls. This suggests that lesions of the hippocampus and overlying cortex do not produce a deficit in reward preference, nor does it enhance or attenuate the natural decrease in preference for the large reward that occurs with increased delay. These findings are opposed to previous results demonstrating impairment of delay-discounting tasks following hippocampal lesions, and therefore questioning the implication of the hippocampus in the regulation of impulse control (Mariano et al., 2009). In addition, as the surgical lesions were not localised to the dentate gyrus the specific contribution of this area to impulsivity and impulsive-like behaviour could not be established.

While lesioned animals showed a greater positive deviation in distance travelled (m) during the high dose of methamphetamine compare to baseline distance (saline), as opposed to control animals (Figure 3.12). This difference in deviation appears to be a reflection of the treatment group deviation in the initial baseline measure (saline) rather than the effect of surgical manipulation of the dentate gyrus and subsequent hippocampus. The study clearly showed significant non-hippocampal effects such as the dose-response relationship between methamphetamine and locomotor activity. Furthermore, it was not shown that the lesions of the dentate gyrus and surrounding hippocampus produced an effect on locomotion. Lesions of the hippocampus are known to affect stimulant-induced locomotor activity. However the present results to do not support such findings (Wilkinson et al., 1993).

Overall, the experiments maintain the status quo in indentifying the dentate gyrus sub-region of the hippocampus and surrounding CA1-CA3 regions as being significantly involved in memory functions. More refined experiments are required to better understand the individual roles of specific subregions of the hippocampus in behaviour and cognitive function.

CHAPTER 4

Concluding remarks

INTRODUCTION

This thesis has explored the role of the dentate gyrus and adult neurogenesis in hippocampal dependent and hippocampal-basal ganglia dependent cognitive tasks. Two primary lines of experimentation were conducted. This first was to explore the effects of unpredictable stress on neurogenesis and cognitive tasks (Chapter 2) and the second to examine the effects of surgical lesions on similar cognitive tasks (Chapter 3). Despite significant challenges during this period of study (e.g. Canterbury earthquake), and serious learning difficulties of the rat population and rather damaging brain lesions applied the results still advance the science of understanding cognitive function and brain structures. There is a long way to go in establishing proven theories because of the natural difficulties of studying the brain in any animal. Nevertheless, it is possible to advance ideas and new experiments to pursue.

STRESS EFFECTS ON NEUROGENSIS AND COGNITION

The first experiments (Chapter 2) uphold a current theory that learning mediates cell survival in the dentate gyrus. Learning of the tasks had a positive influence on cell survival in stressed animals. Stress was also shown to inhibit stimulus-response learning, while conversely facilitating reward preference. Two forms of cognition dependent upon hippocampal-basal ganglia interactions. These findings highlight the complex nature of stress-brain function interactions. These findings open up new lines of enquiry to explore if training tasks can be developed to increase neurogenesis and integration of new cells into the hippocampus in ways to combat declines in cognitive performance associated with unpredictable stress. The question was raised "is it possible to prevent the decline in cell integration with a pre-emptive training programme or indeed increase cellular integration?" (Chapter 2). What is clear from this study is that distinction needs to be made with the type of stress – predictable or unpredictable as well as types of learning and

the neural systems involved. The very nature of the complexity of this work means that large facilities and a larger population of animals will be needed. The experiments did suffer from low degrees of freedom primarily from low sample sizes for which double or triple numbers (N>50) should be considered in future experiments addressing similar issues. Indeed, use of multiple populations from different laboratories might be needed to remove suspicion of potentially inbred rats with impaired cognitive function.

The need to classify stress in different ways might help attain predictable results and develop beneficial programmes tailored to facilitate or attenuate integration of new cells into the hippocampus, both would have research and clinical applications. This study has shown that after chronic stress learning enhanced cell survival and appears to improve performance on specific cognitive tasks (Chapter 2). Alternative stress regimes and classification, such as standardised measures/definitions of acute/chronic stress, or regimes comprised of different stressors, may yield differing affects on learning and neuronal integration, thereby providing further insight into the effects of stress on brain function. Such classifications might help develop more consistent testing regimes and this should be a consideration for future work. One of the limitations in studies involving stress is to be sure a stress procedure is eliciting a stress response and is measured. Such issues were addressed here by measuring the differences in the size of the adrenal gland but this was not strongly statistically significant (22% difference, $p = 0.082$, Chapter 2). But other options are available such as corticosteroid assays before and after stress exposure and measuring behavioural patterns that are associated with anxiety such as time spent in the open arm compared to closed arm of the elevated plus maze or a light-dark box.

Overall, the experiment confirms earlier experimental work and theory that unpredictable stress is associated with both decrease and increase in behaviour task performance, as well as highlighting the involvement of learning in mediating cell survival within the dentate gyrus, as well as addressing key methodological issues to improve future experimentation in this field. The software protocols established in this thesis will help to develop more sensitivity behavioural tasks targeted at probing neurogenesis for future experiments (Appendices B to E). While this study has confirmed previous findings it could be expanded by applying more specific and beneficial physical exercise and cognitive tests aimed at isolating only dentate gyrus functions. Future experiments involving hippocampal (CA1-CA3) and dentate gyrus function should also consider

including other behavioural tests that are not dependent on the hippocampus function to act as secondary controls (see Chapter 3).

SURGICAL LESION EFFECT ON COGNITION

Successful surgery produced significant lesions of the dentate gyrus and surrounding CA1-CA3 hippocampal regions. Damage to these hippocampal regions impaired hippocampal dependent reference memory, while preserving hippocampal independent simple discrimination learning.

One problem encountered in this work was that it was not possible to fully test a suite of hippocampal dependent tasks because transition from simple discrimination testing to configural discrimination testing could not be attained. Reasons have been suggested including the possibility that rats were not suitable for such tests potentially because of genetic learning deficiencies. This reason is attractive because the tasks that normally take 3-4 days to learn could not be learned in 24 days. Nevertheless, the rats did learn sufficient tasks (e.g. reference memory, stimulus-response learning and delayed-discounting tasks; Chapter 3) that enabled assessments of potential interactions within the hippocampus to be made. Indeed, observations of the number of cells integrated into the granular cell layer of the dentate gyrus of the rats in the stress experiment (Chapter 2) suggest that the degree of neural proliferation and integration is similar to previous reports. Coupled with demonstrated task acquisition (Chapters 2 and 3) it was shown that neurological processing was sufficient to conclude that any baseline cognitive impairment did not compromise the overall experiments to the extent that assessment of the role of the dentate gyrus within the hippocampus is invalid. The work shows that brain trauma, like what was applied here in rats, in the hippocampus can have significant behavioural effects. This is also likely in humans and clinical treatments might involve cognitive exercise designed to integrate new cells into the dentate gyrus (Chapter 2).

In conclusion, the work confirms earlier work showing the important role of the hippocampus in reference memory but not impulsive-like behaviours. Areas to advance this field of research include the development of more accurate surgical techniques and more targeted hippocampal sub-regions specific dependent tasks.

RECOMMENDATIONS FOR FUTURE WORK

Increased size of experimental unit

The interaction between stress and brain-behaviour function is inherently complex. The experiments presented here suffered from large variance and low degrees of freedom primarily from low sample sizes which produced difficulty delineating specific effects of stressors on brain-behaviour functioning, at both the behavioural and cellular levels. Future experiments should consider double or triple sample size in order to increase sensitivity to measure target interactions.

Design new stress protocols and experiments

Predictable and unpredictable stress has been shown to exhibit differing effects on neurobehavioural functionality. It is important to make sure the stresses being imposed are producing the planned effects on stress response (both individually and as a whole) when introducing multiple stressors as part of a lager stress regime.

Previous studies have shown experiencing stress with a home-cage match animal or odor-impregnated objects reverse stress-decreased neurogenesis in mice (Cherng et al., 2010). Future stress protocols should consider limiting stress exposure to multiple animals at a time, as the presence of home-cage animals may produce non-specific effects on neurogenesis, thereby introducing statistical noise to data interpretation.

Wnt signalling has also been shown to mediate neurogenesis (Lie et al., 2005). Future experiments might be designed to investigate the Wnt protein as a possible mechanism (and research tool) by which stress effects adult hippocampal neurogenesis.

More targeted behavioural tasks

One of the greatest challenges in investigating neurogenesis and cognition in general is the development of behavioural assays which accurately probe the desired behaviour/cognitive function (Clelland et al., 2009). Previous researchers have attempted to investigate neurogenesis utilising various paradigms such as the Morris water maze,

radial arm maze, and T-maze (Aggleton at al., 2007; Clelland et al., 2009; Ferragud et al., 2010). While the thesis has yielded insights into the functional role of neurogenesis, the specific contribution of newly generated adult neurons to hippocampal activity remains unclear. Future experiments should consider the implementation of touchscreen environments (Chapter 2), as they offer enhancing capability to investigate behaviour and cognition associated with complex visual arrays or temporal parameters.

Lesion other sub-regions of the hippocampus

Furthermore, as the hippocampus is comprised of sub-regions (CA1-CA3 and dentate gyrus) future experiments should consider the involvement of individual sub-regions to hippocampal performance, as well as, and potentially more importantly, how each specific sub-region activity is orchestrated together to produce hippocampal function. Is there a miss-match between individual performance and group performance? Is the whole of the hippocampus function greater than the sum of its parts?

To date, the primary means of neurogenesis manipulation have focused on inhibiting neurogenesis within the region known to produce it i.e. subgranular zone of the dentate gyrus. Future work should consider the development of technologies and protocols which will allow for manipulation of individual dorsal/ventral blades of the dentate gyrus to probe for differences in functioning attributable to these highly localised areas.

Cross genetic strain experiments

Future work should consider incorporating varying strains of animals to elucidate the involvement of genetic variability in behaviour and cognition, as well as to remove suspicion of potentially inbred rats. This could be achieved by establishing collaborative experiments with other laboratories to coordinate multi-strain experiments without the cost or logistics of housing additional or multiple stains of laboratory animals.

Strengthen links with human experiments

An ultimate aim is to understand the human brain. An avenue for future research should develop technologies which will allow for in vivo quantification of neural stem cells within the human brain. Previous work has demonstrated proof of concept based on this premise utilising existing MRI technology (Manganas et al., 2007). Future work should aim to build upon this work, as well as the development of cheaper and less cumbersome investigative tools (e.g imaging software). Introducing more cost effective research tools would allow smaller laboratories to contribute to an area of scientific inquiry normally exclusive to large research organisations and hospitals.

CONCLUSIONS

The review and experimental work presented here highlight the importance of the dentate gyrus region of the brain and adult neurogenesis in hippocampal dependent and hippocampal-basal ganglia dependent cognitive tasks. Despite significant setbacks in the experimental programme because of a catastrophic earthquake in the early part of this research; clear linkages were shown between cognitive function and neurogenesis in rats. The work confirms earlier work on the effects of stress and brain lesions on cognitive performance and as well as learning on neurogenesis and new lines of enquiry have been highlighted requiring more investigation. These include developing a larger range of stress and hippocampal dependent and hippocampal-basal ganglia dependent cognitive tasks. The development of more accurate surgical techniques and more sensitive behavioural tasks should focus ongoing research in this field.

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APPENDIX A

Ethics Approval.

ANIMAL ETHICS COMMITTEE

Secretary, Lynda Griffioen
Email: animal-ethics@canterbury.ac.nz

AEC Ref: 2011/12R

2 September 2011

Dr Juan Canales & James O'Leary
Department of Psychology
UNIVERSITY OF CANTERBURY

Dear Juan and James

I am pleased to inform you that the Animal Ethics Committee (AEC) has approved your application entitled: "Hippocampal-basal gangalia interactions in the making of structural and stimulus-response associations"

Approval has been granted:
Approval has been granted:
(a) for the use of the following:
64 RAT-PVG – Male
4 Wistar – Female
4 Wistar – Male
(b) for your research project to be undertaken from 2 September 2011 to 31 May 2012. If you require an extension of this period please contact the AEC Secretary.

As part of AEC's new Code of Ethical Conduct all applicants receiving approval to work on animals are required to provide a final report at the completion of their project. The purpose is to provide the AEC with a record of your use of animals and what was achieved by your research project. We are very much interested in your findings and to learn what you have achieved. Following the completion date indicated above you are asked to provide this report using the new Final Report form which is available at the AEC web site (https://intranet.canterbury.ac.nz/research/ethics.shtml).

On an annual basis the University is legally required to provide to MAF statistical data on all animal manipulations undertaken in a calendar year. To assist us in collating this information you are also required to complete and return to the AEC Secretary the attached MAF Animal Manipulation Statistical form 30 days after the completion of this project, or once every three years, which ever comes first. If no animals have been manipulated in your project please provide a "Nil" return. Please also find enclosed a copy of the Animal Welfare (Records and Statistics) Regulations 1999 for your information, together with a list of Animal Type Codes and brief guideline notes for your assistance.

Yours sincerely

pp

Associate Professor Jim Briskie
Chair
University of Canterbury Animal Ethics Committee

University of Canterbury Private Bag 4800, Christchurch 8140, New Zealand. www.canterbury.ac.nz

APPENDIX B

The software schedule used for shaping animals to touch the screen for a food pellet reward in the Bussey-Saksida chambers.

ABET II - Schedule

Schedule Name: Autoshaping UNBIAS

Schedule Description: First habituation with images presented

Environment Link: Environment 1

Name: Chamber1

Usage: 16 inputs/30 outputs

Location: 1

Installed: Yes

Inputs		Outputs		Outputs with Intensity	
Line	Description/Label	Line	Description/Label	Line(s)	Description/Label
1	Tray #1	1	HouseLight #1		
2	BIRBeam #1	2	TrayLight #1		
3	FIRBeam #1	3	Feeder #1		
4	RightFIRBeam #1	4	Sound_On #1		
5	FeederFault #1	5	White_Noise_Off #1		
6	FeederReport #1	6	Sound #1		
		7	Sound #2		
		8	Sound #3		

Conditions and Actions:

Group	Name	Goto	Condition	Action
1	Set Blank Images	4	When _Schedule_Timer ≥ 0	Background.DisplayCurrentImageAtLocation (1) Background.DisplayCurrentImageAtLocation (2) Background.DisplayCurrentImageAtLocation (3) Turn on White_Noise_Off #1
1	Sound 2	1	When A2Sound2 = 1	Turn on Sound #2
4	Start ITI	5	When _Schedule_Timer ≥ 0	Start ITI_Timer
↳	Starts the ITI			
5	No Increment Trial Coun	6	When ITI_Timer ≥ ITI And First_Trial = True	No Action
↳	First time when _TrialCounter=0 ignore, increment thereafter			
5	Increment Trial Counter	6	When ITI_Timer ≥ ITI And First_Trial = False	Increment _Trial_Counter
↳	First time when _TrialCounter=0 ignore, increment thereafter			
5	Position 1 Screen Touch	5	When Background.GridTouch = Position_1	Increment Position_1_Touches_during_ITI
↳	Counts touches to the left screen window during ITI			

Conditions and Actions (continued):

Group	Name	Goto	Condition	Action
5	Position 2 Screen Touch	5	When Background.GridTouch = Position_2	Increment Position_2_touches_during_ITI
5	Position 3 Screen Touch	5	When Background.GridTouch = Position_3	Increment Position_3_touches_during_ITI
6	Display Image	7	When _Schedule_Timer ≥ 0	Images.DisplayCurrentImageAtLocation(2) Training_Images[1].DisplayAtLocation(1) Training_Images[1].DisplayAtLocation(3) Turn off TrayLight #1 Start Image_Timer GetNextImage Training_Images GetNextImage Images
7	Set correct response key	9	When Images["Image 1"].ImageLocation = Position_2	Correct_Grid_Position = Grid_Position_1.Value Increment Image_1_counter GetNextValue Grid_Position_1
7	Set correct response key	9	When Images["Image 2"].ImageLocation = Position_2	Correct_Grid_Position = Grid_Position_3.Value Increment Image_2_Counter GetNextValue Grid_Position_3
9	Correct Image Touched	11	When Training_Images.GridTouch = Correct_Grid_Position	Background[1].DisplayAtLocation(1) Background[1].DisplayAtLocation(2) Background[1].DisplayAtLocation(3) Pulse Sound_On #1 for Tone_Duration ms. Pulse Feeder #1 2 times, time = 50 ms., inter-pulse time = 200ms. Turn on TrayLight #1 Increment Correct_Counter First_Trial = False
↳	Rewards a touch to the image			
11	Reward Collected Start I	1	When Tray #1 = Activated	Turn off TrayLight #1
11	Schedule End Protocol	12	When _Trial_Counter = 30	No Action
12	End of Schedule	-		

Variables:

ABET II - Schedule

Variables:

Name	Type	Value
_Schedule_Timer	Timer	0.000
_Trial_Counter	Integer	0
_Trial_Timer	Timer	0.000
A2Sound2	Integer	1
Correct_Counter	Integer	0
Correct_Grid_Position	Integer	0
Feeder_Pulse_Time	Integer	100
First_Trial	Boolean	True
Image_1_counter	Integer	0
Image_2_Counter	Integer	0
Image_Time	Integer	30
Image_Timer	Timer	0.000
Incorrect_Grid_Position	Integer	0
ITI	Integer	20
ITI_Timer	Timer	0.000
Position_1	Integer	1
Position_1_Touches_during_ITI	Integer	0
Position_2	Integer	2
Position_2_touches_during_ITI	Integer	0
Position_3	Integer	3
Position_3_touches_during_ITI	Integer	0
Timeout_timer	Timer	0.000
Tone_Duration	Integer	1000

ABET II - Schedule

Lists:

Background

Type: Image

Mode: Random Equal Number

Grid Layout: Custom

Background Color: Black

Excluded Locations: N/A

Values: There is 1 image in this list, named "Image 1". Image previews are not available on reports.

Grid_Position_1

Type: Integer

Mode: Random Equal Number

Values: 1, 1, 1, 1, 1

Grid_Position_3

Type: Integer

Mode: Random Equal Number

Values: 3, 3, 3, 3, 3

Images

Type: Image

Mode: Random Equal Number

Grid Layout: Custom

Background Color: Black

Excluded Locations: N/A

Values: There are 2 images in this list. Image previews are not available on reports.
[1]: 'Image 1"
[2]: 'Image 2"

ABET II - Schedule

Lists:

Training_Images

Type: Image

Mode: Random Equal Number

Grid Layout: Custom

Background Color: Black

Excluded Locations: N/A

Values: There is 1 image in this list, named "Image 1". Image previews are not available on reports.

APPENDIX C

The software schedule used for stimulus-response learning in the Bussey-Saksida chambers.

ABET II - Schedule

Schedule Name: Stimulus Response Task (Standard)

Schedule Description: Stimulus Response Task (Standard). The animal is presented with 1 of 2 image pairs, paired with a sound que (high pitch or low pitch). The sound signals the rewarded image (high pitch = left image rewarded, low pitch = right image rewarded).

Environment Link: Environment 1

Name: Chamber1

Usage: 16 inputs/30 outputs

Location: 1

Installed: Yes

Inputs		Outputs		Outputs with Intensity	
Line	Description/Label	Line	Description/Label	Line(s)	Description/Label
1	Tray #1	1	HouseLight #1		
2	BIRBeam #1	2	TrayLight #1		
3	FIRBeam #1	3	Feeder #1		
4	RightFIRBeam #1	4	Sound_On #1		
5	FeederFault #1	5	White_Noise_Off #1		
6	FeederReport #1	6	Sound #1		
		7	Sound #2		
		8	Sound #3		

Conditions and Actions:

Group	Name	Goto	Condition	Action
1	Set Blank Images	4	When _Schedule_Timer ≥ 0	Background.DisplayCurrentImageAtLocation (1) Background.DisplayCurrentImageAtLocation (2) Background.DisplayCurrentImageAtLocation (3) Turn on White_Noise_Off #1
1	Sound 1	1	When A1Sound1 = 1	Turn on Sound #1
1	Sound 2	1	When A2Sound2 = 1	Turn on Sound #2
1	Sound 3	1	When A3Sound3 = 1	Turn on Sound #3
4	Start ITI	5	When _Schedule_Timer ≥ 0	Start ITI_Timer
↳	Starts the ITI			
5	No Increment Trial Coun	6	When ITI_Timer ≥ ITI And First_Trial = True	No Action
↳	First time when _TrialCounter=0 ignore, increment thereafter			
5	Increment Trial Counter	6	When ITI_Timer ≥ ITI And First_Trial = False	Increment _Trial_Counter

Conditions and Actions (continued):

Group	Name	Goto	Condition	Action
↳	First time when _TrialCounter=0 ignore, increment thereafter			
6	Display Center Image	7	When _Schedule_Timer ≥ 0.000	Images.DisplayCurrentImageAtLocation(2) GetNextImage Images
7	Display Image	8	When Images["Image 1"].ImageLocation = Position_2	Response_Keys[Image 1].DisplayAtLocation (1) Response_Keys[Image 2].DisplayAtLocation (3) Turn on Sound #1 Turn off TrayLight #1 Turn off Sound #2 Turn off Sound #3 GetNextImage Response_Keys Pulse Sound_On #1 for 1000 ms.
7	Display image 2	8	When Images["Image 2"].ImageLocation = Position_2	Response_Keys[Image 3].DisplayAtLocation (1) Response_Keys[Image 4].DisplayAtLocation (3) Turn on Sound #3 Turn off TrayLight #1 Turn off Sound #1 Turn off Sound #2 Pulse Sound_On #1 for 1000 ms.
8	Set correct response key	9	When Images["Image 1"].ImageLocation = Position_2	Correct_Grid_Position = Grid_Position_1.Value GetNextValue Grid_Position_1
8	Set correct response key	9	When Images["Image 2"].ImageLocation = Position_2	Correct_Grid_Position = Grid_Position_3.Value GetNextValue Grid_Position_3
9	Incorrect right	10	When Correct_Grid_Position = Position_1	Incorrect_Grid_Position = Position_3
9	Incorrect left	10	When Correct_Grid_Position = Position_3	Incorrect_Grid_Position = Position_1

ABET II - Schedule

Conditions and Actions (continued):

Group	Name	Goto	Condition	Action
10	Correct Image Touched	12	When Response_Keys.GridTouch = Correct_Grid_Position	Background[1].DisplayAtLocation(1) Background[1].DisplayAtLocation(2) Background[1].DisplayAtLocation(3) Turn on Sound #2 Turn on TrayLight #1 Turn off Sound #1 Turn off Sound #3 Pulse Sound_On #1 for 1000 ms. Pulse Feeder #1 2 times, time = 50 ms., inter-pulse time = 400ms. Increment Correct_Counter First_Trial = False
↳	Rewards a touch to the image			
10	Incorrect Image Touch	11	When Response_Keys.GridTouch = Incorrect_Grid_Position	Background.DisplayCurrentImageAtLocation (1) Background.DisplayCurrentImageAtLocation (2) Background.DisplayCurrentImageAtLocation (3) Increment Incorrect_Counter Turn on HouseLight #1 Start Timeout_timer
11	Incorrect Timeout	1	When Timeout_timer ≥ 20.000	Turn off HouseLight #1
11	Schedule End 2	15	When _Trial_Counter = 30	No Action
12	Reward Collected Start I	1	When Tray #1 = Activated	Turn off TrayLight #1
12	Schedule End Protocol	15	When _Trial_Counter = 30	No Action
15	End of Schedule	-		

ABET II - Schedule

Variables:

Name	Type	Value
_Schedule_Timer	Timer	0.000
_Trial_Counter	Integer	0
_Trial_Timer	Timer	0.000
A1Sound1	Integer	0
A2Sound2	Integer	1
A3Sound3	Integer	0
Correct_Counter	Integer	0
Correct_Grid_Position	Integer	0
Delay_timer	Timer	0.000
Feeder_Pulse_Time	Integer	100
First_Trial	Boolean	True
Image_Time	Integer	30
Image_Timer	Timer	0.000
Images_Displayed	Integer	0
Incorrect_Counter	Integer	0
Incorrect_Grid_Position	Integer	0
ITI	Integer	10
ITI_Timer	Timer	0.000
Position_1	Integer	1
Position_2	Integer	2
Position_3	Integer	3
Timeout_timer	Timer	0.000
Tone_Duration	Integer	1000

ABET II - Schedule

Lists:

Background

Type: Image

Mode: Random Equal Number

Grid Layout: Custom

Background Color: Black

Excluded Locations: N/A

Values: There is 1 image in this list, named "Image 1". Image previews are not available on reports.

Grid_Position_1

Type: Integer

Mode: Random Equal Number

Values: 1, 1, 1, 1, 1

Grid_Position_3

Type: Integer

Mode: Random Equal Number

Values: 3, 3, 3, 3, 3

Images

Type: Image

Mode: Random Equal Number

Grid Layout: Custom

Background Color: Black

Excluded Locations: N/A

Values: There are 2 images in this list. Image previews are not available on reports.
[1]: 'Image 1"
[2]: 'Image 2"

ABET II - Schedule

Lists:

Response_Keys

Type: Image

Mode: Random Equal Number

Grid Layout: Custom

Background Color: Black

Excluded Locations: N/A

Values: There are 4 images in this list. Image previews are not available on reports.
[1]: 'Image 1"
[2]: 'Image 2"
[3]: 'Image 3"
[4]: 'Image 4"

APPENDIX D

The software schedule used for the delay discounting probe conditions in the Bussey-Saksida chambers.

ABET II - Schedule

Schedule Name: Delay Discounting Task (Impulsivity) 10sec delay condition

Schedule Description: Delay Discounting Task used to measure Impulsivity. The animal is present with 2 images, one reinforced with a small instant reward, the other a large but delayed reward. This protocol has a 10sec delay discount, additional delay protocols were made by simply changing (increase/decrease) the delay time.

Environment Link: Environment 1
Name: Chamber1
Usage: 16 inputs/30 outputs
Location: 1
Installed: Yes

Inputs		Outputs		Outputs with Intensity	
Line	Description/Label	Line	Description/Label	Line(s)	Description/Label
1	Tray #1	1	HouseLight #1		
2	BIRBeam #1	2	TrayLight #1		
3	FIRBeam #1	3	Feeder #1		
4	RightFIRBeam #1	4	Sound_On #1		
5	FeederFault #1	5	White_Noise_Off #1		
6	FeederReport #1	6	Sound #1		
		7	Sound #2		
		8	Sound #3		

Conditions and Actions:

Group	Name	Goto	Condition	Action
1	Set Blank Images	3	When _Schedule_Timer ≥ 0	Background.DisplayCurrentImageAtLocation (1) Background.DisplayCurrentImageAtLocation (2) Turn on White_Noise_Off #1
1	Sound 2	1	When A2Sound2 = 1	Turn on Sound #2
3	Start ITI	5	When _Schedule_Timer ≥ 0	Start ITI_Timer
↳	Starts the ITI			
5	No Increment Trial Coun	6	When ITI_Timer ≥ ITI And First_Trial = True	No Action
↳	First time when _TrialCounter=0 ignore, increment thereafter			
5	Increment Trial Counter	6	When ITI_Timer ≥ ITI And First_Trial = False	Increment _Trial_Counter
↳	First time when _TrialCounter=0 ignore, increment thereafter			
5	Position 1 Screen Touch	5	When Background.GridTouch = Position_1	Increment Position_1_Touches_during_ITI
↳	Counts touches to the left screen window during ITI			

ABET II - Schedule

Conditions and Actions (continued):

Group	Name	Goto	Condition	Action
5	Position 2 Screen Touch	5	When Background.GridTouch = Position_2	Increment Position_2_touches_during_ITI
6	Display Image	7	When _Schedule_Timer ≥ 0	Large_Stimulus[1].DisplayAtLocation(1) Training_Images[1].DisplayAtLocation(2) Turn off TrayLight #1 GetNextImage Training_Images
7	Image Touched left	8	When Large_Stimulus.GridTouch = Position_1	Background[1].DisplayAtLocation(1) Background[1].DisplayAtLocation(2) Pulse Sound_On #1 for Tone_Duration ms. Turn on TrayLight #1 Increment Correct_Counter First_Trial = False Increment LEFT_TOUCH Start Delay_timer
↳	Rewards a touch to the image			
7	Image Touched right	9	When Training_Images.GridTouch = Position_2	Background[1].DisplayAtLocation(1) Background[1].DisplayAtLocation(2) Pulse Sound_On #1 for Tone_Duration ms. Pulse Feeder #1 for 1000 ms. Turn on TrayLight #1 Increment Correct_Counter First_Trial = False Increment RIGHT_TOUCH
8	Delay	9	When Delay_timer ≥ 10	Pulse Feeder #1 4 times, time = 50 ms., inter-pulse time = 400ms.
9	Reward Collected Start I	1	When Tray #1 = Activated	Turn off TrayLight #1
9	Schedule End Protocol	10	When _Trial_Counter = 24	No Action
10	End of Schedule	-		

ABET II - Schedule

Variables:

Name	Type	Value
_Schedule_Timer	Timer	0.000
_Trial_Counter	Integer	0
_Trial_Timer	Timer	0.000
A2Sound2	Integer	1
Correct_Counter	Integer	0
Delay_timer	Timer	0.000
Feeder_Pulse_Time	Integer	100
First_Trial	Boolean	True
Image_Time	Integer	30
Image_Timer	Timer	0.000
ITI	Integer	10
ITI_Timer	Timer	0.000
LEFT_TOUCH	Integer	0
Position_1	Integer	1
Position_1_Touches_during_ITI	Integer	0
Position_2	Integer	2
Position_2_touches_during_ITI	Integer	0
Position_3	Integer	3
Position_3_touches_during_ITI	Integer	0
RIGHT_TOUCH	Integer	0
Tone_Duration	Integer	1000

Lists:

Background

Type: Image

Mode: Random Equal Number

Grid Layout: Custom

Background Color: Black

Excluded Locations: N/A

Values: There is 1 image in this list, named "Image 1". Image previews are not available on reports.

Large_Stimulus

Type: Image

Mode: Sequencial

Grid Layout: Custom

Background Color: Black

Excluded Locations: N/A

Values: There is 1 image in this list, named "Image 1". Image previews are not available on reports.

Training_Images

Type: Image

Mode: Sequencial

Grid Layout: Custom

Background Color: Black

Excluded Locations: N/A

Values: There is 1 image in this list, named "Image 1". Image previews are not available on reports.

APPENDIX E

The software schedule used for simple discrimination learning in the Bussey-Saksida chambers.

ABET II - Schedule

Schedule Name: Simple discrimination task

Schedule Description: Simple discrimination task. The animal is presented with one image pair (cross & oval). One image is ALWAYS rewarded (the cross). The incorrect image (oval) produces a time-out if touched.

Environment Link: Environment 1

Name: Chamber1

Usage: 16 inputs/30 outputs

Location: 1

Installed: Yes

Inputs		Outputs		Outputs with Intensity	
Line	Description/Label	Line	Description/Label	Line(s)	Description/Label
1	Tray #1	1	HouseLight #1		
2	BIRBeam #1	2	TrayLight #1		
3	FIRBeam #1	3	Feeder #1		
4	RightFIRBeam #1	4	Sound_On #1		
5	FeederFault #1	5	White_Noise_Off #1		
6	FeederReport #1	6	Sound #1		
		7	Sound #2		
		8	Sound #3		

Conditions and Actions:

Group	Name	Goto	Condition	Action
1	Set Blank Images	4	When _Schedule_Timer ≥ 0	Background.DisplayCurrentImageAtLocation (1) Background.DisplayCurrentImageAtLocation (2) Background.DisplayCurrentImageAtLocation (3) Turn on White_Noise_Off #1
1	Sound 2	1	When A2Sound2 = 1	Turn on Sound #2
4	Start ITI	5	When _Schedule_Timer ≥ 0	Start ITI_Timer
↳	Starts the ITI			
5	No Increment Trial Coun	6	When ITI_Timer ≥ ITI And First_Trial = True	No Action
↳	First time when _TrialCounter=0 ignore, increment thereafter			
5	Increment Trial Counter	6	When ITI_Timer ≥ ITI And First_Trial = False	Increment _Trial_Counter
↳	First time when _TrialCounter=0 ignore, increment thereafter			

ABET II - Schedule

Conditions and Actions (continued):

Group	Name	Goto	Condition	Action
10	Incorrect Image Touch	11	When Response_Keys.GridTouch = Incorrect_Grid_Position	Background.DisplayCurrentImageAtLocation (1) Background.DisplayCurrentImageAtLocation (2) Background.DisplayCurrentImageAtLocation (3) Increment Incorrect_Counter Turn on HouseLight #1 Start Timeout_timer
11	Incorrect Timeout	1	When Timeout_timer ≥ 20.000	Turn off HouseLight #1
11	Schedule End 2	15	When _Trial_Counter = 30	No Action
12	Reward Collected Start I	1	When Tray #1 = Activated	Turn off TrayLight #1
12	Schedule End Protocol	15	When _Trial_Counter = 30	No Action
15	End of Schedule	-		

ABET II - Schedule

Variables:

Name	Type	Value
_Schedule_Timer	Timer	0.000
_Trial_Counter	Integer	0
_Trial_Timer	Timer	0.000
A2Sound2	Integer	1
Correct_Counter	Integer	0
Correct_Grid_Position	Integer	0
Delay_timer	Timer	0.000
Feeder_Pulse_Time	Integer	100
First_Trial	Boolean	True
Image_Time	Integer	30
Image_Timer	Timer	0.000
Images_Displayed	Integer	0
Incorrect_Counter	Integer	0
Incorrect_Grid_Position	Integer	0
ITI	Integer	10
ITI_Timer	Timer	0.000
Position_1	Integer	1
Position_2	Integer	2
Position_3	Integer	3
Timeout_timer	Timer	0.000
Tone_Duration	Integer	1000

ABET II - Schedule

Lists:

Background

Type: Image

Mode: Random Equal Number

Grid Layout: Custom

Background Color: Black

Excluded Locations: N/A

Values: There is 1 image in this list, named "Image 1". Image previews are not available on reports.

Grid_Position_1

Type: Integer

Mode: Random Equal Number

Values: 1, 1, 1, 1, 1

Grid_Position_3

Type: Integer

Mode: Random Equal Number

Values: 3, 3, 3, 3, 3

Images

Type: Image

Mode: Random Equal Number

Grid Layout: Custom

Background Color: Black

Excluded Locations: N/A

Values: There are 2 images in this list. Image previews are not available on reports.
[1]: 'Image 1"
[2]: 'Image 2"

Lists:

Response_Keys

Type: Image

Mode: Random Equal Number

Grid Layout: Custom

Background Color: Black

Excluded Locations: N/A

Values: There are 4 images in this list. Image previews are not available on reports.
[1]: 'Image 1"
[2]: 'Image 2"
[3]: 'Image 3"
[4]: 'Image 4"

APPENDIX F

IMMUNOCYTOCHEMISTRY BRDU PROTOCOL

1. **Wash samples in PBS-Tx 0.5% for 5 min, repeat 3 times on orbital agitated.**

   Note: The use of Triton (Tx) increases the permeability of the cell membranes, this promotes anti-gen retrieval of the BrdU complex (incorporated into the cell DNA).

2. **Block endogenous Peroxidase: Wash samples in solution of 10% methanol 3% H_2O_2 in PBS 0.1M and Place on orbital agitated for 10 min.**

   Note: endogenous peroxidase exits throughout the body. If the endogenous peroxidase is not blocked, using the Horse-radish peroxidase (HRP)-antibody may result in, non-specific background staining of the endogenous peroxidase and not the biotinylated ABC complex (***Step 11and13***). This non-specific background staining is significantly reduced by pre-treatment of the cells/tissues with hydrogen peroxide (Ramos-Vara, 2005).

3. **Wash samples in PBS-Tx 0.5% for 5 min on the orbital agitated. Repeat 3 times.**

   Note: This step is used to wash away excess solution from step 3. This is to control for any uncontrolled chemical reactions between residual solutions from previous steps (inter-step contamination). PBS-Tx washes are used throughout the protocol for this purpose.

4. **Incubate samples in a solution of HCL 1N (1 normal hydrochloric acid) at 4^oC for 10 min without orbital agitation (i.e. in a fridge).**

   Then - Incubate samples in a solution of HCL 2N (2 normal hydrochloric acid) at room temperature for 10 min without orbital agitation.

Then - Incubate in solution of HCL 2N (2 normal hydrochloric acid) at 40°C for 20 min without orbital agitation (i.e. in an oven).

Note: 1N or 2N refers to 1normal or 2normal (1 molar/2 molar – Therefore the weight of 1 mole of HCl = 36.5 g and the weight of 2 moles of HCl = 73 g).

Immunostaining of the labelled cells requires a step to denature DNA, allowing the antibody access to the BrdU complex from within the DNA. Hydrogen chloride is used as a denaturing agent to break open the DNA structure of the labelled cells.

5. **Wash samples in a solution of buffered borate 0.1M PH: 8.5 for 15 min on orbital agitated.**

   Note: 3.8 g of Boric acid in 100 ml of distilled water (add sodium hydroxide 1M to increase pH until 8.5).

6. **Wash samples in Normal Goat Serum (NGS) 5% in PBS-Tx 0.1M for 45 min on Orbital agitator.**

   Note: Normal Goat Serum is used to reduce nonspecific binding of the primary and secondary antibodies to reaction surfaces of non-targeted cells (i.e. non-BrdU+ cells). Goat serum is chosen as the secondary antibody was produced in goat. Blocking the reaction of the antibodies to non-Brdu labelled cells helps to maximise the signal-to-noise ratio (Normal Goat Serum (10%), KPL, Gaithersburg USA).

7. **Primary Anti-body: Anti-BrdU: Incubate sample in a solution of Anti-BrdU 1/250 in PBS-Tx with NGS 1% for 2 days (2 overnights) at 4°C (i.e. in a fridge) with orbital agitation.**

   Note: The primary anti-body binds to the BrdU antigen incorporated into the target cells DNA.

8. **Wash samples in PBS-Tx 0.5% for 5 min on the orbital agitated. Repeat 3 times.**

9. **Secondary Antibody: Wash samples in solution of Goat anti-mouse 1/400 in PBS-Tx with 1% NGS with orbital agitation for 1 hour (cover to reduce light exposure).**

   Note: Reconstitute antibody by adding 1mm distilled water. After 30 min prepare ABC solution, this will provide enough time for the Avidin (A) and Biotin (B) molecules to bind together to form the Avidin Biotin Complex (ABC). See *Step 11.*

10. **Wash samples in PBS-Tx 0.5% for 5 min on the orbital agitated. Repeat 3 times.**

11. **Avidin Biotin Complex (ABC): Wash samples in a solution of 10 ul/ml** Avidin and 10 ul/ml of Biotin and 1% NGS in PBS-Tx for 1hour with orbital agitation. Cover to reduce exposure to light.

    Note: Avidin is a glycoprotein with a high affinity for Biotin (Vitamin B7). Avidin has four binding sites for biotin (1 Avidin molecule binds with four biotin molecules). Avidin-Biotin Complex is used for signal amplification of the antibody-antigen complex (Key, 2006). Using avidin-biotin systems for protein detection allows amplification of the original protein signal to improve detection of proteins expressed at low levels. This is achieved by forming large avidin-biotin complexes with the secondary biotinylated antibody (Figure F.1).

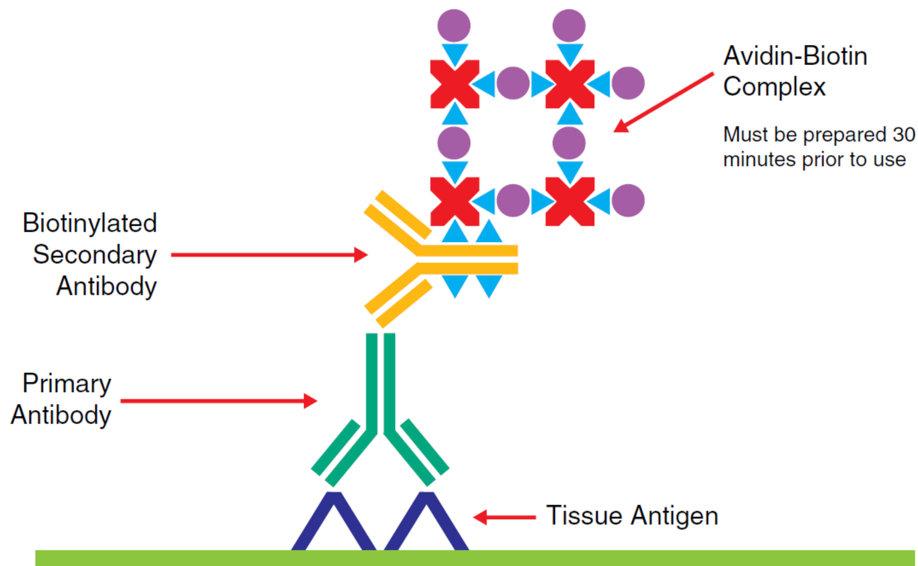


Figure F.1.

Illustration of signal amplification by avidin-biotin complex formation. Adapted from Key (2006).

12. Wash samples in PBS 0.1M for 5 min with orbital agitation.

13. Wash samples in solution of diaminobenzidine tetrahydrochloride (DAB) 5 mg/ 10 ml and H_2O_2 12 mg/10 ml in PBS 0.1M.

Note: DAB (33,3'-diaminobenzidine tetrahydrochloride; MW = 214.1) is oxidized in the presence of peroxidase and hydrogen peroxide producing a brown, alcohol-insoluble precipitate at the site of enzymatic activity i.e. the Avidin-biotinylated complex (Instructions DAB Substrate; 1-4, Thermo Scientific, Rockford, IL, USA).

14. Wash samples in PB 0.1M for 5 min with orbital agitation. Repeat 3 times.

Note: The final wash is used to stop the DAB-Peroxidase reaction.

15. Mount Samples on Subbed Microscope Slides. Leave to try for 24 hr.

16. Dehydration and Differentiation: Place Mounted Microscope slides in a bath of;

70% ETHANOL for 2 min, then
95% ETHANOL for 2 min, then
95% ETHANOL/ACID for 40 sec, then
100% ETHANOL for 2 min, then
100% ETHANOL for 2 min, then

17. Cleaning: Place in Bath of Xylene for 5 min, Repeat.

18. Mount Cover slips onto slides with mountant (DPX) in fume cupboard.

References

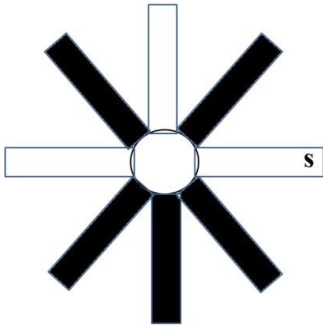
Key M. (2006). Immunohistochemical staining methods, Fourth Edition. 183p.

Ramos-Vara J. (2005). Technical aspects of Immunohistochemistry. Veterinary Pathology, 42, 405-426.

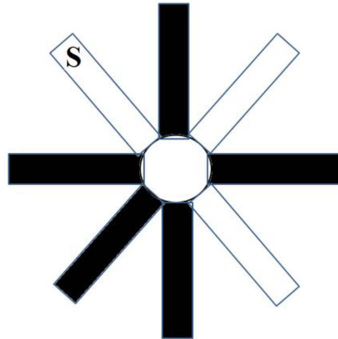
APPENDIX G

Example of the pattern separation problems used in the lesion experiment (Chapter 3) with starting arms (S), open arms (white) and closed arms (black) indicated.

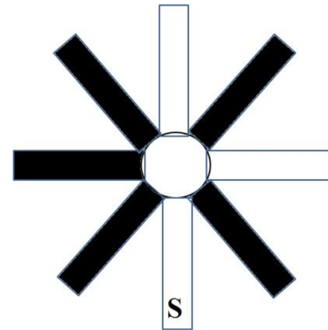
Small Separation 1A



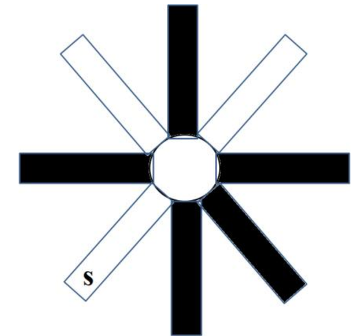
Separation 1B



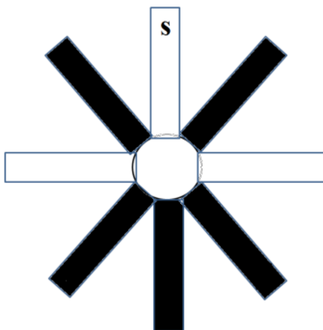
Separation 1C



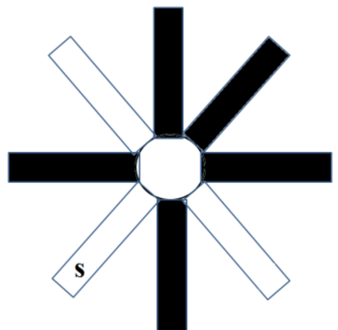
Separation 1D



Large Separation 2A



Separation 2B



APPENDIX H

Cresyl violet staining protocol. A strict protocol was followed to ensure consistent colour stain across all brains.

Process	Steps	Solution	Time	Comments
Delipidisation (defatting)	1	70% ETHANOL	10 dips	70% ethanol = (736 ml of 96% Ethanol + 264 ml distilled water)
	2	95% ETHANOL	10 dips	
	3	100% ETHANOL	10 dips	Change 100% Ethanol after 4 trays
	4*	100% ETHANOL	5 min	Each dip must cover slides – no less than 250 ml in each staining dish
	5	95% ETHANOL	10 dips	
	6	70% ETHANOL	5 min	
Hydration	7	Water (Distilled)	1 min (change every time)	Change distilled water after each tray
Stain	8*	0.5% cresyl violet solution	12 min	Filter before use – only use 4 times – keep old solution separate to new stock (DO NOT MIX TOGETHER). Record number of uses on bottle.
Rinsing	9	Water (Distilled)	2 min (change every time)	Change Distilled water after each tray
	10	Water (Distilled)	2 min (change every time)	Change Distilled water after each tray
Dehydration and differentiation	11	70% ETHANOL	2 min	
	12	95% ETHANOL	2 min	
	13*	95% ETHANOL/ACID	45 sec (depending on freshness/number of uses of Cresyl Violet	95% Acid/Alcohol = 400 ml of 95% Ethanol + 1ml (using insulin syringe) of Glacial Acetic Acid.
	14	100% ETHANOL	2 min	
	15*	100% ETHANOL	2 min	
Clearing	16	Xylene	5 min	
	17	Xylene	5 min	
Mount	18	On Subbed Slide with Depex		

*Change after 4th tray of slides.