Disambiguating the Similar: Investigating Pattern Separation in Medial Temporal Lobe Structures Using Rodent Models

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Declaration

The work in this dissertation was carried out between October 2011 and April 2015 at the Department of Psychology, University of Cambridge, under the supervision of Dr. Lisa M. Saksida and Professor Timothy J. Bussey. This dissertation is the result of my own work and collaborations are specified in the text.

This dissertation is not substantially the same, in whole or in part, as any that I have submitted, or is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution, except as specified in the text.

I have made every attempt to reference properly for any idea or finding that is not my own.

The length of this dissertation is less than 60,000 words.

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Publications

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Bekinschtein, P., Kent, B. A., Oomen, C., Clemenson, D., Gage, F., Saksida, L. M., & Bussey, T. J. (2013). Consolidating unique memories: BDNF in the dentate gyrus is required for spatial pattern separation. *Cell Reports,* 5, 1-10.

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Summary

This dissertation investigates the mechanisms underlying pattern separation, using rodent models and behavioural tasks that assess the use of representations for similar stimuli. Pattern separation is a theoretical mechanism involving the transformation of inputs into output representations that are less correlated to each other. Because of this orthogonalizing process, similar experiences are stored as discrete non-overlapping representations. Studying pattern separation emphasizes the important but often overlooked fact that successful memory involves more than just remembering events over a period of time, but also differentiating between similar memories.

Through a series of experiments this dissertation adds support to the literature that the dentate gyrus (DG) subregion of the hippocampus is important for pattern separation when encoding spatial and contextual inputs. Using the Spontaneous Location Recognition (SLR) task it is shown the brain-derived neurotrophic factor (BDNF) can improve performance by acting via N-methyl-D-aspartate (NMDA) glutamate receptors in the DG and adult-born hippocampal neurons. Manipulating the level of neurogenesis by inhibiting Wnt signalling or by administering acyl-ghrelin systemically is shown to impair and enhance performance on SLR, respectively. Using a novel exposure paradigm in combination with SLR, it is demonstrated for the first time that the relationship between pattern separation and neurogenesis may be reciprocal, such that inhibiting neurogenesis impairs pattern separation, enhancing neurogenesis improves pattern separation, and performing pattern separation enhances the production or survival of adult-born hippocampal neurons. Finally, it is shown that the TgTau^{P301L} mouse model of dementia exhibits spatial and object recognition memory impairments once aged, recapitulating a dementia-like phenotype. Understanding the mechanisms that contribute to effective pattern separation may help elucidate the processes underlying the memory impairment experienced by AD patients.

This dissertation concludes with a critical discussion about whether pattern separation can be studied using behavioural paradigms.

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Abbreviations

Aβ	β-amyloid
ABC	avidin-biotin enzyme complex
AD	Alzheimer's disease
AP5	D-2-amino-5-phosphonopentanoic acid
APOE	apolipoprotein E
APV	D-2-amino-5-phosphonopentanoate
BDNF	brain-derived neurotrophic factor
BrdU	5′-Bromo-2-deoxyuridine
Ca2+	calcium ions
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CR	caloric restriction
CREB	cyclic AMP response element-binding protein
CRE	cAMP response elements
CSF	cerebrospinal fluid
D2	discrimination ratio
DAB	diaminobenzidine
DCX	doublecortin
DG	dentate gyrus
DMSO	dimethyl sulfoxide
ERK	extracellular signal-regulated protein kinase
fMRI	functional Magnetic Resonance Imaging
FTD	frontotemporal dementia
FTD-17	frontotemporal dementia with Parkinsonism linked to chromosome 17
GFP	green fluorescent protein
GHSR	growth hormone secretagogue receptor; ghrelin receptor
H_2O_2	hydrogen peroxide
HCL	hydrochloric acid
ICV	intracerebroventricular
IGF-1	insulin-like growth factor-1
IP	intraperitoneal injection
ITI	inter-trial interval
LD	Location Discrimination
LR	Location Recognition
LTP	long-term potentiation
M	mean
MAPK	mitogen-activated protein kinases
MCI	Mild Cognitive Impairment
MWM	Morris water maze
mRNA	messenger ribonucleic acid
NAD	aldehyde dehydrogenase
NBF	neutral buffered formalin
NDS	normal donkey serum
NFT	neurofibrillary tangle
NGS	normal goat serum
NMDA	*N*-methyl-*D*-aspartate
OR	Object Recognition
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with 0.1% triton

PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
R-H	Representational-Hierarchical Perspective
SAL	saline
SEM	standard error of the mean
SIRT1	NAD-dependent protein deacetylase sirtuin-1
SLR	Spontaneous Location Recognition
TB	Tris buffer
Tg	transgenic
Tg+	transgenic
Tg-	non-transgenic littermate control
TgTau^{P301L}	TgTau(P301L)23027
TMZ	temozolomide
TrkB	tropomyosin receptor kinase B
TUNL	Trial-unique Nonmatching-to-Location
VEH	vehicle

Chapter 1: Introduction

The aim of my dissertation is to investigate pattern separation processes in medial temporal lobe structures, with specific focus on plasticity-related mechanisms in the hippocampus. Pattern separation is a theoretical computational mechanism involving the transformation of similar inputs into outputs that are less correlated with each other. In the brain, pattern separation is hypothesized to create distinct ensemble neural responses from overlapping input. By transforming similar experiences into discrete representations, pattern separation is postulated to increase the likelihood of accurate memory encoding and subsequent retrieval, which is fundamental to successful episodic memory. The following chapter will provide an introduction to pattern separation by reviewing the early computational literature where an orthogonalization process occurring in neural networks was first proposed. This will be followed by a brief summary of experimental evidence suggesting that pattern separation is occurring in the hippocampus and then an introduction to the Representational-Hierarchical perspective to describe pattern separation as a process that is not localized to the hippocampus but rather a ubiquitous mechanism throughout the brain. This chapter will conclude with an overview of the experiments described in this dissertation, which tested rodent models on behavioural paradigms that were designed to allow for the parametric manipulation of task parameters in order to evaluate pattern separation mechanisms.

1.1. Hippocampus

The field of memory research has been particularly hippocampus-centric since the infamous case of Henry Molaison, referred to as “Patient H.M.” Following bilateral medial temporal lobe resections, performed to treat intractable epilepsy, H.M. suffered from persistent anterograde amnesia (Scoville & Milner, 1957). Prior to H.M.'s surgery the locus of memory in the brain was largely a mystery, thus the drastic effects of removing portions of the temporal lobe were unexpected. H.M.’s memory impairment was particularly specific to the memory for new experiences, as he demonstrated an inability to form memories for day-to-day events. It was Scoville and Milner’s (1957) seminal article describing H.M.'s condition that began the intense study of the role of the hippocampus in memory formation, which was originally thought to be the critical

region of H.M.'s damage. Specifically, this case stimulated study into the role of the hippocampus for *episodic memory* (Tulving, 1972), defined as memory for autobiographical episodes or personal events that include temporal-spatial components.

Much of the work described throughout this dissertation focuses on processes occurring in the hippocampus when forming episodic memories, and specifically examines how similarity between stimuli affects memory encoding and retrieval. It is hypothesized that when stimuli are similar, such as when identical landmarks are placed in close proximity to each other, the input reaching the hippocampus is overlapping. When stimuli are similar but distinct, the hippocampus engages a process - referred to as *pattern separation* in the computational literature - to amplify differences in the representations. By separating the overlapping inputs during processing, it reduces interference amongst stored memories. A failure in pattern separation is thought to, at least in part, underlie amnesia associated with hippocampal damage, such as in Alzheimer's disease (AD).

Before discussing the specific mnemonic functions of the hippocampus, the following section will very briefly outline a few important neuroanatomical features of this brain region. Although the anatomy of the hippocampus is not the focus of this dissertation, some of its unique anatomical properties are thought to enable a pattern separation process to occur when encoding episodic memories.

1.1.1. Neuroanatomy of the hippocampus

The cytoarchitecture of the hippocampus was first described in detail by the definitive work of Ramón y Cajal (1911) and has been reviewed more recently by several others (e.g., Amaral & Witter, 1989; van Strien, Cappaert, & Witter, 2009; Amaral, Scharfman, & Lavenex, 2007).

The hippocampus is a large structure located within the medial temporal lobe of the forebrain, consisting of distinct and interconnected subregions, such as the dentate gyrus (DG), *cornu ammonis* regions (e.g., CA1 and CA3), and subiculum. The dorsal portion of the hippocampus extends behind the septum and the ventral portion extends to more

temporal regions of the brain. Figure 1.1 and 1.2 show horizontal and coronal sections of the rat hippocampus.

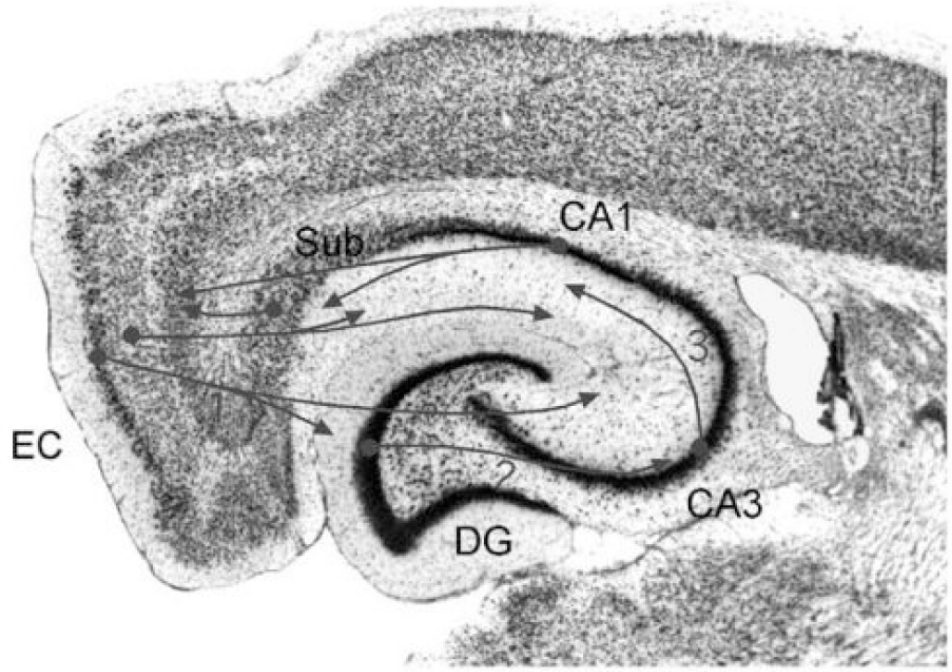

Figure 1.1. Nissl-stained horizontal section of the hippocampus. Figure is reproduced from Amaral, Scharfman, & Lavenex (2007; with permission from Elsevier). The entorhinal cortex (EC), dentate gyrus (DG), CA3, CA1, and subiculum (Sub) are labelled. Arrows identify key projections: (1) perforant path, (2) mossy fibres, and (3) Schaffer collaterals.

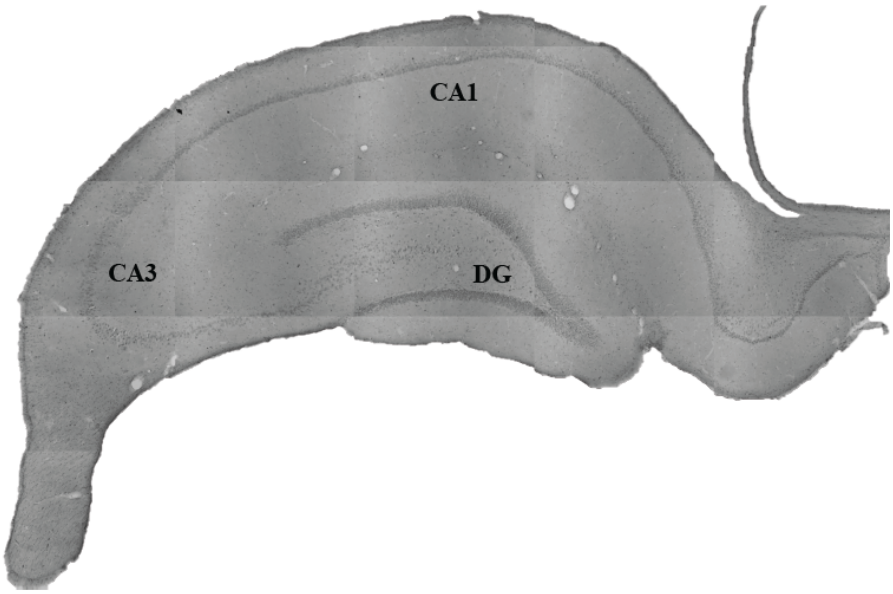

Figure 1.2. Hematoxylin-stained coronal section of the hippocampus. The DG, CA3, and CA1 subregions are labelled. This is an anterior section from a rat in Experiment 1 of Chapter 5.

The synaptic organization of the hippocampus is often described as a trisynaptic loop, referring to the DG, CA3, and CA1 synaptic connections, collectively. Each of these subregions has a distinctive pattern of afferent and efferent connections (Swanson & Cowan, 1977). The circuit begins with the major input coming from the entorhinal cortex, which provides polymodal cortical sensory information to the hippocampus via a unidirectional perforant path. Although the DG is often described as the first input region of the hippocampus, the entorhinal cortex also directly innervates pyramidal cells in CA3 and CA1. The perforant path to the DG originates mainly in layer II of the lateral entorhinal cortex and medial entorhinal cortex, whereas CA1 and the subiculum receive axons projecting from layer III (Steward & Scoville, 1976). After the perforant path reaches the DG, the trisynaptic pathway continues with granule cells in the DG projecting to pyramidal neurons in CA3 via the mossy fibre pathway. This connection between the DG and CA3 subregions is where pattern separation is postulated to occur and there is also strong evidence for this as well, which will be discussed in later sections. From the mossy fibre pathway, the trisynaptic circuit then continues with CA3 projecting to CA1 via Schaffer collaterals. For a detailed review of the trisynpatic circuitry see van Strien, Cappaert, and Witter (2009).

In simplified circuit diagrams, the hippocampal formation is often discussed as having connections that are predominantly unidirectional; however, the hippocampal circuitry also propagates signals through important back projections (Swanson et al., 1978; Swanson et al., 1981). For example, key anatomical features of CA3 include pyramidal cells that project back to the DG as well as recurrent collaterals within CA3, such that CA3 pyramidal cells actually receive the majority of their input from other CA3 pyramidal cells (Hjorth-Simonsen, 1973; Li et al., 1994; Laurberg, 1979; Amaral & Witter, 1989; Ishizuka et al., 1990; Wittner et al., 2007). There are also back projections from CA1 to CA3 (Laurberge, 1979; Amaral et al., 1991; Swanson et al., 1981) and from the subiculum to CA1 (Kohler, 1985; Finch et al., 1983).

Much of the work described in this dissertation focuses on the DG region of the hippocampus. The neuroanatomy of the DG is described in detail by Amaral, Scharfman, and Lavenex (2007). Briefly, the DG is a trilaminar structure with distinct layers identified as the granule cell layer, polymorph cell layer, and molecular layer. Throughout these layers there are three key types of neurons: granule cells, dentate

pyramidal basket cells, and mossy cells. The granule cell layer is thought of as the main cell layer and contains densely packed granule cells (Seress & Pokorny, 1981). In the rat there is an estimated one million DG granule cells (Boss et al., 1985; West et al., 1991), which represents a large anatomical divergence from the estimated 200,000 neurons in the entorhinal cortex that project to the granule cells (Amaral et al., 1990). Granule cells are the principal cells of the mossy fibre pathway to CA3, and interestingly exhibit uniquely sparse activity patterns, such that granule cell action potential rates are relatively low and only 1 - 2% of the total population is active at a given time (Chawla et al., 2005; Alme et al., 2010). The divergence from the entorhinal cortex to the DG and this sparse activity are hypothesized to be features that enable pattern separation. The DG molecular layer contains the dendrites of the granule cells, fibres of the perforant path that originate in the entorhinal cortex, and a small number of interneurons. The polymorphic cell layer contains several cell types, including mossy fibre cells.

As will be discussed in more detail in subsequent chapters, a distinctive feature of the DG is that unlike most other brain regions, the DG continues to generate new principal neurons that become functionally integrated into the neural circuitry throughout adulthood. The only other region in the mammalian brain where this also occurs is in the subventricular zone, from which cells migrate to the olfactory bulbs. Evidence suggests, that the creation of these hyper-plastic cells may contribute to hippocampal-dependent memory by enabling pattern separation. The specific role of hippocampal neurogenesis in pattern separation will be the focus of Chapters 3 to 5.

Finally, to transition over to a discussion of hippocampal mnemonic functions, a final feature of the hippocampus that should be mentioned is place cells. Place cells are predominantly found in CA1 and CA3 subregions of the hippocampus and are defined as pyramidal neurons that are tuned to distinct spatial locations (O'Keefe, 1976; Kjelstrup et al., 2008). Place cells encode specific place fields and were first discovered by O'Keefe and Dostronvsky in 1971, which resulted in O'Keefe being awarded the 2014 Nobel Prize in Physiology or Medicine (O'Keefe & Dostronvsky, 1971; O'Keefe, 1976). These cells were identified using single unit recording, revealing that certain cells displayed maximal unit firing only when a rat was in a specific position in the maze (O'Keefe, 1976). This was a landmark discovery that solidified the hippocampus

as being vital for spatial processes and stimulated decades of research into the spatial memory function of the hippocampus.

1.1.2. Prominent psychological theories of hippocampal memory

The discovery of hippocampal place cells and the aforementioned case study of H.M., inspired several theories about the role of the hippocampus in spatial and episodic memory (e.g., O'Keefe & Nadel, 1978; Squire & Zola-Morgan, 1991). Because Chapters 2 to 5 will describe experiments designed to elucidate hippocampal-dependent spatial memory processes, this section will briefly mention a few of the most prominent psychological theories of general hippocampal function that have guided much of the background research that inspired my specific hypotheses.

A groundbreaking book written by John O'Keefe and Lynn Nadel (1978) proposed that the hippocampus is responsible for maintaining a cognitive map. Their work established a spatial theory of hippocampal function that followed from the landmark studies by Edward Tolman (1948), who first introduced this psychological concept of a cognitive map. O'Keefe and Nadel suggested that the hippocampal cognitive map has two distinct systems that guide spatial learning and memory: the 'taxon' system, which uses egocentric spatial cues (i.e., informs the route), and the 'locale' system for allocentric spatial encoding (i.e., informs the map). Place cells were hypothesized to be the basic functional unit of this system. Extensions of the spatial map were proposed to be fundamental to episodic memory and much of the evidence in support of their theory came from early experiments in rodents.

Some of the earliest studies were conducted by David Olton, who designed radial arm mazes for assessing spatial learning and memory. Using the radial mazes, Olton was able to demonstrate a place-learning deficit in hippocampal-lesioned rats. Using a four-arm maze, Olton (1972) showed that after hippocampal lesions, performance of the rats was dramatically impaired, as they could no longer utilize information about the location of a water reward. The research group then replicated this finding by testing hippocampal-lesioned rats on an eight-arm radial maze, using food as the reward, and found that the lesioned rats were similarly impaired at locating the arm containing the food (Olton, Walker, & Gage, 1978). These studies helped solidify the hippocampus as

important for spatial memory and the radial arm maze remains a commonly used method for evaluating spatial memory in rodents today.

Another highly influential model for hippocampal function was proposed by Squire and Zola-Morgan (1991). Their model was largely influenced by the work done by Scoville and Milner (1957) on patients, including H.M., who had bilateral medial temporal lobe removal. Their work was also influenced by Mortimer Mishkin (1982) who had developed an animal model of human amnesia in the monkey by lesioning the hippocampus and amygdala. Squire and Zola-Morgan (1991) offered a model of the medial temporal lobe memory system that placed the hippocampus in a central position, working together with adjacent regions such as the entorhinal cortex, perirhinal cortex, and parahippocampal cortices. They proposed that these structures were responsible for governing long-term memories. According to the model, long-term memory was divided into declarative and nondeclarative memory. Declarative memory was split further into memories for facts and events (i.e., episodic memory). Nondeclarative memory was split into skills and habits, priming, simple classical conditioning, and nonassociative learning. Long-term potentiation (LTP), a type of plasticity in which high-frequency stimulation causes a persistent increase in synaptic strength and will be discussed in more detail in Chapter 2, was proposed as a possible mechanism for rapidly forming conjunctions between unrelated events.

Much of the evidence for this model came from non-human primates and human patients suffering from medial-temporal lobe amnesia. For example, Zola-Morgan, Squire, and Amaral (1986) conducted a thorough histological examination on patient R.B. who after suffering an ischemic stroke, experienced anterograde amnesia without other noticeable forms of cognitive impairment. The histological analysis revealed that the damage was limited to the CA1 subregion of the hippocampus. The same research group then replicated these findings in monkeys with bilateral damage specifically to the hippocampus (Zola-Morgan, Squire, & Amaral, 1989). The medial temporal lobe memory system quickly became a dominant theory in the field of memory research. A fundamental principle was that acquiring new memories was distinct and separable from other cognitive abilities, such as perception, which will be discussed in contrast to the Representational-Hierarchical theory in a later section of this chapter.

These theories proposed by O'Keefe and Nadel (1978) and Squire and Zola-Morgan (1991) are two of the most prominent psychological theories of hippocampal function and established the hippocampus as important for the interconnected mechanisms of spatial processing and episodic memory formation. However, research examining the specific mnemonic deficits experienced by patients with amnesia has helped to further illuminate hippocampal function.

Importantly, failure in episodic memory does not always reflect forgetting an event over time, but can also result from confusing distinct events. For example, some evidence suggests that even though patients diagnosed with AD exhibit profound memory deficits, they do not necessarily have accelerated rates of forgetting, which has traditionally been considered a hallmark of the disease (Christensen et al., 1998; Money et al., 1992). This is partly because memories of our everyday lives often include similar routines and environments, which makes episodic memory particularly vulnerable to interference (Tulving, 1972).

Surprisingly, recognition that successful memory requires accurately differentiating between similar representations is often neglected by the majority of memory research, which - as in the theory proposed by Squire and Zola-Morgan (1991) described above - emphasizes dichotomies such as short-term and long-term memory, implicit and explicit memory, and semantic and episodic memory. Most theories focus on the different types of memory and neglect underlying components that may transcend the categories, such as how similarity of inputs can interfere with mnemonic processes. However, overcoming interference is essential for accurate memory and may also be a contributing factor to the cognitive deficits experienced by patients suffering from amnesia and dementia caused by hippocampal damage.

1.1.3. Interference theory of amnesia

The interference theory of amnestic syndrome was proposed by Warrington and Weiskrantz (1970; 1978) to explain the surprising finding that amnestic patients could demonstrate good verbal retention under certain conditions. The researchers found that the method by which memory in amnestic patients was evaluated is a crucial factor in the degree of mnemonic deficit they exhibit. This followed from the paradoxical finding

that when evaluating memory in patients with amnesia, providing partial information, such as fragmented letters or the initial letters of a word, was a more effective retrieval strategy than showing the patient a whole word and asking them to respond "yes" or "no" to whether it was a target word seen in the previous list of fragmented words (Warrington & Weiskrantz, 1970). The experiments demonstrated that retrieval by partial information reduced false-positive responses, possibly due to the fact that the number of alternatives that would match the fragmented words was more limited. The authors concluded that long-term memory could be demonstrated in amnestic patients when the method of retrieval minimized interference. In contrast, the method of learning did not differentially affect retention in amnestic patients, suggesting that it is inappropriate to characterize amnesia as a failure of consolidation, which is often thought (Warrington & Weiskrantz, 1970). These findings are similar to those from studies of dementia patients, who show differences in memory performance depending on whether a task involves "free recall" or "cued recall," such that patients can more often successfully recall using the cued strategy (Christensen et al., 1998). This has been interpreted as evidence that the memory has been stored (i.e., consolidated) but that the disease pathology is disrupting retrieval; however, poor encoding can also result in retrieval deficits so it is difficult to disentangle these processes.

There is some evidence that increased vulnerability to interference is an early manifestation of disease in patients with dementia and the vulnerability gets worse with disease progression (Lowenstein et al., 2004; 2007). Loewenstein and colleagues (2004) showed that after controlling for overall memory impairment, AD patients and patients diagnosed with Mild Cognitive Impairment (MCI) were more affected by proactive and retroactive semantic interference than age-matched controls, when asked to recall common household objects that had been previously presented. Interference was manipulated by presenting new but semantically related objects at different time points. This increased vulnerability to interference has been linked to false recognition memory in a mouse model of AD (Romberg et al., 2012), and complements the higher rates of false memories exhibited by MCI and AD patients (Budson et al., 2001; Yeung et al., 2013). These specific memory impairments associated with dementia will be discussed further in Chapter 6 when I describe how a specific deficit in pattern separation may underlie the heightened susceptibility to interference, and how this can result in false memories in AD (Budson et al., 2001).

1.2. History of pattern separation

The following section will describe how the concept of pattern separation - as it is currently used in memory research - evolved. This is important because whether or not behavioural research, such as the research presented in this dissertation, studies *pattern separation*, is a topic of debate (Santoro, 2013). Some argue that behavioural approaches are only studying *discrimination* and that the term pattern separation should be reserved for use in computational models. By exploring the computational literature that first proposed pattern separation as an important process for hippocampal-dependent memory, it helps clarify our operational definition of the term and explain why behavioural data support the existence of this postulated computation. Although it is true that behavioural tasks used to study pattern separation do involve discrimination, the following chapters will provide evidence that qualitatively different mechanisms are engaged when discriminating between similar versus dissimilar stimuli, suggesting unique processes are occurring. This behavioural research does not study pattern separation directly, but rather provides evidence in keeping with the process of pattern separation that was proposed by early computational models. Chapter 7 will discuss this debate in more detail and argue that it is appropriate and beneficial to share terminology across levels of analysis.

1.2.1. Early computational models of the hippocampal memory system

In parallel to the psychological theories of hippocampal memory, described above, and experimental data emphasizing the hippocampus as critical for spatial memory processes, computational modellers also developed formal and quantitative descriptions of how the hippocampal memory system functions.

The seminal work of David Marr (Marr, 1971) was pioneering in developing a mathematical model of the hippocampal memory system, consisting roughly of the CA 1 - 3 fields, DG, entorhinal cortex, and adjacent structures. He designed a simple model to satisfy some of the biological constraints known at the time, in an attempt to describe the hippocampus as a memorizing device. Taking a general systems level view, Marr provided a computational model to explain how the hippocampal system processes memory temporarily. According to his model, an initial event evokes a pattern of

activity across unrelated cells and an autoassociation process then quickly couples the neuronal population that has been active together and stores the pattern as temporary comprehensive representations. These representations are gradually moved to permanent storage in the cortex. The model proposed the hippocampus as a region in which simple representations of an event or episode were formed and stored as particular patterns of activity, and provided the first theoretical basis for how the hippocampus might form memory traces.

Marr's model also proposed a pattern completion process that allowed subsequent activity patterns to retrieve the original memory trace. Using pattern completion, memory recall was aided by 'subevents,' which were identified as events that elicited a fraction of the original pattern of activity and could subsequently be used to enable recall of the whole previous event. Even though incomplete, a subevent can activate the whole of the event because elements of a neuronal population become coupled together during storage through the autoassociation process. Thus, the whole of the event can be correctly reconstructed when activated by a subevent. This is an important feature of the model because it allows the system to identify continuity between similar events and enables recall even after some synapses in the network are damaged or no longer active.

Marr's model proposed that the hippocampus was a temporary store for these representations, prior to being transferred and re-stored in the cerebral cortex, which Marr postulated occurred during sleep and other situations when reactivation might occur (Marr, 1970). The transient storage in the hippocampus was thought to make for a more efficient system by filtering information to be coded as a permanent memory in the cortex. Although Marr's model bears a resemblance to the hippocampus (e.g., multilayer, topographic ordering, and a feedback loop), the model lacked important details, such as key projections (e.g., direct projections from entorhinal cortex to CA1 and CA3), making it an extremely influential but incomplete model.

1.2.2. Challenges for early computational models of the hippocampal memory system

Following Marr's groundbreaking work, subsequent models continued to emphasize the role of the hippocampus in memory formation for an episode or event by storing unique

combinations of elements associated together by temporal contiguity. However, early computational models of hippocampal memory had three prominent challenges; the models needed to (1) allow incomplete or partial information to enable accurate recall, (2) have storage mechanisms that would not overload the system's capacity, and (3) retain detail and precision during encoding.

Marr's initial model provided a solution for the first challenge by outlining the autoassociation and pattern completion mechanisms by which subevents could enable recall of an integrated memory store; however, such detailed encoding of subevents could result in overloading the capacity of the system being modelled (i.e., the hippocampus). Thus, Marr's model neglects the second challenge: as the memory system reaches saturation, interference causes retrieval errors. Marr's model also neglects the third challenge, which is described by O'Reilly and McClelland (1994), as a trade off between accurate *pattern completion* and *pattern separation.*

To deal with the second challenge, early computational models of associative memory attempted to estimate the memory capacity of the system. Memory capacity was defined as the maximum number of patterns that could be stored effectively (i.e., enabling accurate recall). The maximum number of events a memory system can store depends upon the size of each input event and the number of cells used for each representation. Hopfield (1982) demonstrated by computer simulation that saturation of the memory system could cause the degradation of stored information and increase the probability that memories would become irretrievable. Similar to Marr, Hopfield's network model utilized Hebb's associative learning rule, in which memories are formed by strengthening the connections between cells that are simultaneously activated.

One solution for increasing the memory capacity of a system is to use sparse encoding. The direct storage of associations between firing cells that was suggested by Marr's model places heavy demands on the neural network. Revised theoretical models of memory storage have since demonstrated that as encoding becomes sparser, the capacity of the associative memory system becomes larger (Amari, 1989). This increase in capacity results in more patterns being effectively stored, and thus increases the number of memories that can be retrieved.

However, while incorporating sparse encoding into the model increases the memory capacity, the risk with sparse encoding is that representations of similar input patterns become more correlated. The problem is that as patterns become correlated, memories for similar events are more likely to become confused or merge (Hopfield, 1982). This problem emphasizes that a successful memory system requires the ability to both store a vast amount of information and enable accurate recall.

Although these models described associative memory systems in general, and were not specifically focused on the hippocampus, the same principles apply when formulating a realistic model of the hippocampal network. What the computational modellers needed was a mechanism that allowed for sparse encoding, without losing the precise detail necessary to keep similar memories separate. Thus, drawing from these models of associative memory, it was concluded that any model of the hippocampal memory system required sparse coding to increase memory capacity and a mechanism for encoding similar inputs in a way to enable successful recall. Although not included in Marr's (1971) model of the hippocampal memory system, Marr's (1969) model of learning in the cerebellum, introduced the concept of *pattern separation* that was later adopted by hippocampal models to provide the necessary mechanism.

1.2.3. Pattern separation incorporated into the hippocampal memory system

According to Marr, granule cells in the cerebellum act as pattern separators, serving to orthogonalize overlapping inputs (1969). This process amplifies the discrepancies between similar patterns, translating overlapping activity of mossy fibres into activation of parallel fibres that overlap proportionately much less. In Marr's model of the cerebellum, mossy fibres communicate afferent input events to the cerebellar cortex where they are then stored as *codon representations*. Codon representations created an economical method of storage, much like the sparse coding in models mentioned above. According to Marr's model of the cerebellum system, similar inputs would have markedly less similar codons because of the pattern separation process. Following Marr (1969) and the subsequent work by Torioka (1979) and Gibson and colleagues (1991), who also developed models of neural systems that made an explicit consideration of

input pattern overlap as an independent variable, pattern separation was introduced into models of the hippocampal memory system.

The pivotal work by O'Reilly and McClelland (1994) provided the complementary-learning-systems model, which proposed the hippocampus as a dual memory system similar to the model initially proposed by Marr (1971). The complementary-learning-systems model suggested that the hippocampus was responsible for encoding contents of specific episodes by binding together temporally coincident events before the information was transferred to the cortex as more stable representations of the world.

O'Reilly and McClelland (1994) articulated one of the key challenges for models of memory as a trade off between pattern separation and pattern completion. Pattern separation occurs at the time of storage and allows similar representations to be distinct. It is a process that enables a network to reduce overlap between similar input patterns, prior to being stored, as a way to reduce the probability of interference during memory recall. In contrast, pattern completion occurs at the time of recall and allows overlapping input patterns to trigger recall of an existing memory, instead of creating a distinct new one. It enables a network to retrieve stored output patterns when input patterns are only partial or degraded. The trade-off refers to the observation that maximal pattern separation of different episodes is necessary to avoid confusion; whereas, maximal pattern completion in the recurrent autoassociative system depends on how close the probe pattern is to the stored memory, and thus relies on similarity.

O'Reilly and McClelland (1994) argue that the unique anatomical and physiological properties of the hippocampus may minimize this trade-off, which makes the hippocampus ideally suited for memory encoding. For example, inhibitory interneurons that form local feedback circuits may serve to regulate activity (McNaughton & Morris, 1987). Additionally, the unique properties of the DG, in particular, and its projections ideally support the trade off. For example, even though the DG has four to six times as many excitatory neurons as other hippocampal subregions, the DG displays an unusually low level of activity compared to other regions (Squire et al., 1989). Furthermore, the mossy fibre pathway from DG to CA3 is a sparse and topographic projection and the projections from the entorhinal cortex are strictly feedforward to DG and CA3 without any direct feedback from the hippocampus to the entorhinal cortex

(McNaughton & Nadel, 1990). Thus, O'Reilly and McClelland (1994) provide a compelling model for the complementary roles of pattern separation and pattern completion in the hippocampal memory system that is supported by its anatomical and physiological properties.

Around the same time, other researchers were also developing computational models of the hippocampal memory system to deal with the trade off between pattern separation and pattern completion. McNaughton and Morris (1987) outlined a simple neuronal model of hippocampal circuitry that explicitly incorporated a role for hippocampal plasticity. Their model suggested that LTP - referred to as long-term enhancement - was fundamentally involved in memory formation. Another computational model for hippocampal memory function that incorporated pattern separation was proposed by Rolls (1987), and developed further by Treves and Rolls (1994). Their model suggested that arbitrary associations are formed by single neurons in the hippocampus. According to their model, there is a preprocessing network in the DG that separates overlapping patterns prior to sending the representations to CA3, which then operates as an autoassociative memory system. There are more similarities than differences between these simple neuronal models, which all propose an important contribution of pattern separation to hippocampal memory.

More recently, computational models have suggested that hippocampal neurogenesis plays a unique role in pattern separation, although these models have drawn mixed conclusions about the specific role of the new cells (Becker, 2005; Becker et al., 2009; Weisz & Argibay, 2009; Aimone et al., 2009). For example, Aimone and colleagues (2009) describe a computational model in which immature granule cells are more active than the overall granule cell population. According to their model, immature neurons decrease pattern separation when inputs are dissimilar, referred to as a 'pattern integration' effect. The authors also suggest that neurogenesis in the hippocampus provides a temporal code for memories (Aimone et al., 2009), as the relative enhanced plasticity of immature neurons only occurs during a limited time-window and can thus provide a unique sub-population linked to a memory trace in time. In contrast, Becker (2005, 2009) proposes a model in which neurogenesis acts to reduce interference by enhancing pattern separation mechanisms, and Aimone and colleagues (2006) and Becker and Wojtowicz (2007) propose models in which the plasticity of young neurons

yields different functional populations, potentially improving pattern separation. However, according to Weisz and Argibay's (2009) model, neurogenesis is associated with less pattern separation; thus, both Aimone and colleagues (2009) and Weisz and Argibay (2009) demonstrate that the heterogeneity introduced by new neurons can attenuate pattern separation.

The exact function of adult-born neurons in the hippocampus has yet to be agreed upon. The functional role of neurogenesis is debated in both the computational and experimental literature; however, there is strong evidence that the new cells play an important role in pattern separation. This will be discussed in further detail throughout Chapters 3 to 5.

1.3. Experimental data in support of pattern separation

Stemming from these computational models of pattern separation as an important mechanism in the hippocampal memory system, experimental evidence from electrophysiology, immediate early-gene, behaviour, and human imaging studies have accumulated in support of pattern separation in the hippocampus playing a role in episodic memory formation. Although much of the research discussed throughout this dissertation focuses on pattern separation occurring in the hippocampus, as a mechanism to promote accurate spatial memory, the process of keeping similar representations distinct is likely important for most brain regions and cognitive processes. The following section will briefly describe the experimental evidence for pattern separation occurring in the hippocampus and will follow with a discussion of pattern separation in other regions of the brain.

1.3.1. Electrophysiology evidence of pattern separation

Electrophysiology studies have accumulated some of the strongest evidence for pattern separation in the hippocampus and are consistent with the predictions from the computational models discussed above. For example, an early *in vivo* study monitoring freely moving rats performing an eight-arm radial maze task, demonstrated that granule cells exhibit sparse coding (Jung & McNaughton, 1993). Subsequently, an *in vivo* patch-clamp study provided evidence that single mossy fibres connecting a DG granule

cell and CA3 pyramidal cell can act as "conditional detonators" such that fast and repetitive firing of the presynaptic granule cell is powerful enough to fire a downstream CA3 neuron (Henze et al., 2002). This provides possible evidence for what McNaughton and Morris (1987) refer to as the *detonator potential* of the mossy fibres. A detonator is a powerful synapse that consistently causes depolarization and firing of the post-synaptic neuron (McNaughton & Morris, 1987).

Further evidence for pattern separation occurring in the hippocampus comes from electrophysiology studies measuring the ability of neurons to disambiguate small differences in cortical input patterns. Using a behavioural paradigm that gradually morphed enclosures, changing from a circle to a square, or vice versa, Wills and colleagues (2005) recorded from hippocampal place cells in CA1 and found that the rate of remapping was abrupt, even though the sensory input was changed incrementally. The authors concluded that because highly similar environmental input created dramatically different representations, these results provided evidence for pattern separation and an autoassociation network that coordinates a large number of place cells.

Some of the most convincing experimental evidence that this pattern separation process occurs in the DG subregion comes from Leutgeb and colleagues (2007) who provided the first evidence for a neuronal substrate of pattern separation in the DG by demonstrating that minimal changes in the shape of an environment can substantially alter correlated activity patterns among place-modulated granule cells. Similar to the study above, they trained rats to run in square and circular enclosures with flexible walls. The flexibility of the walls enabled the experimenters to transform the enclosures through a series of five intermediate stages, which changed the enclosure from square to circular, or vice versa. These physical changes were intended to progressively alter the sensory input patterns. By measuring population activity in the DG during this manipulation, it was revealed that DG activity was highly sensitive to small changes in the environment. Specifically, the activity patterns of the DG showed that the smallest differences produced a decrease in the correlation of the population, which was not observed in CA3. This study also confirmed sparse firing in the DG granule cell population because the number of active cells was lower in the DG than CA3, while the DG and CA3 regions showed similar peak firing rates.

More recently, Neunuebel and Knierim (2014) provided further direct neurophysiological evidence for the existence of a pattern separation process in the DG. Their study confirmed hallmark predictions from computational models (e.g., McNaughton & Morris, 1987; Rolls, 1987; Treves & Rolls, 1994) that the DG performs pattern separation and CA3 performs pattern completion. By measuring both input and output representations they were able to for the first time explicitly test whether outputs are more similar or less similar than the inputs (i.e., pattern completion or pattern separation, respectively). In their study, rats were trained to run around a track with local and global cues placed near the testing arena. The experimenters systematically rotated the cues to cause graded changes in sensory input. While manipulating the cues, the researchers simultaneously recorded single-unit activity from the DG and CA3. The results provided direct evidence that the DG sends degraded input to CA3, but that CA3 produces an output pattern that more closely reflects the originally stored representations, suggesting pattern separation and pattern completion mechanisms in the DG and CA3, respectively.

1.3.2. Behavioural evidence of pattern separation in the hippocampus

Complementing this electrophysiology evidence, findings from behavioural studies also support a pattern separation mechanism in the DG. Although behavioural paradigms are unable to measure the computational process of pattern separation directly, our lab and others have developed tasks in which similarity of to-be-remembered stimuli is varied parametrically, to assess the use of separated representations. The goal of these behavioural studies is to demonstrate the functional relevance of pattern separation, as described by computational models, to cognition and behaviour.

A variety of modified tasks have been used to study pattern separation such as delayed-match-to-sample spatial maze (Gilbert, Kesner, & DeCoteau, 1998; Gilbert, Kesner, & Lee, 2001), contextual fear conditioning (McHugh et al., 2007; Sahay et al., 2011a; Nakashiba et al., 2012; Tronel et al., 2012), novel context exploration (Hunsaker, Rosenberg, & Kesner, 2008), radial arm maze (Clelland et al., 2009), object-place association (Lee & Sollivan, 2010), touchscreen Location Discrimination (LD) (McTighe et al., 2009; Clelland et al., 2009; Creer et al., 2010), and Spontaneous Location Recognition (SLR) (Hunsaker, Rosenberg, & Kesner, 2008; Bekinschtein et

al., 2013; 2014; Kent et al., 2015). In humans, a combination of functional Magnetic Resonance Imaging (fMRI) and a novel object detection task has been used to study pattern separation (Kirwan & Stark, 2007; Bakker et al., 2008; Lacy et al., 2011). The assumption underlying these tasks is that the representations formed after effective pattern separation will be useful when there is a high demand on resolving the confusability of inputs, such as when performing tasks requiring discrimination of similar contexts, locations, or whole episodes (Oomen et al., 2014).

Experiments employing behavioural tasks have produced strong evidence suggesting that pattern separation occurs in the hippocampus (McDonald & White, 1995; Frankland et al., 1998; Gilbert et al., 1998; McTighe et al., 2009) and specifically the DG subregion (Gilbert, Kesner, & Lee, 2001; Hunsaker, Rosenberg, & Kesner, 2008; McHugh et al., 2007; Bakker et al., 2008; Lee & Solivan, 2010). In particular, plasticity-related mechanisms in the DG appear important for pattern separation (McHugh et al., 2007; Bekinschtein et al., 2013), as well as adult-born hippocampal neurons (Clelland et al., 2009; Creer et al., 2010; Sahay et al., 2011; Nakashiba et al., 2012; Tronel et al., 2012; Kheirbek et al., 2012; Bekinschtein et al., 2014; Kent et al., 2015). The role of hippocampal plasticity and adult neurogenesis will be the focus of Chapters 2 to 5, which will describe the behavioural evidence in further detail.

To my knowledge the first behavioural evidence of a separation-dependent effect resulting from hippocampal damage was provided by McDonald and White (1995). In their study, rats were trained on an eight-arm radial maze and were then evaluated on their ability to discriminate between adjacent or widely separated arms of the maze. Hippocampus-lesioned rats were impaired on this place-learning task when differentiating between adjacent arms. In the adjacent arm condition, the cues identifying the locations to be discriminated were ambiguous because of the high amount of overlap between the cues, making it more difficult to distinguish than the other condition. Using another behavioural task, Frankland and colleagues (1998) provided evidence that the dorsal hippocampus, specifically, is the essential hippocampal region needed to discriminate between similar contexts. They demonstrated this by using a modified version of the contextual fear conditioning paradigm. Mice with electrolytic dorsal hippocampus lesions showed roughly equal levels of freezing in two similar chambers, even though they were only ever shocked in

one of the chambers. In contrast, the sham-lesioned mice froze more in the shock-context than in the no-shock context. Although these first two studies did not explicitly aim to study the process of pattern separation, the data lend support for a pattern separation mechanism occurring in the hippocampus. Because these paradigms (i.e., radial arm maze and contextual fear conditioning) allow for the manipulation of similarity between contextual stimuli, they have since been used in studies that have the main objective of studying pattern separation. It is hypothesized that by varying the similarity of the stimuli to be encoded, that these tasks change the load on pattern separation.

The first group to explicitly study pattern separation behaviourally was Gilbert and colleagues (1998). They developed a behavioural paradigm that allowed the similarity of distal cues to be systematically varied. The task was run in a dry maze with baited food wells and the rat was tasked with discriminating between a baited well (i.e., correct location) and an unbaited well (i.e., incorrect location), which were both marked with identical objects. The experimenters then systematically varied the distance between the marked wells. The performance of the rats with complete hippocampal lesions was linearly associated with separation, which was not the case for the controls. As distance between the correct and incorrect locations increased, performance of the lesioned rats improved. The authors concluded that the function of the hippocampus was to preserve the uniqueness of a memory representation by separating incoming spatial information. A follow-up study then demonstrated that rats with selective DG lesions, but not CA1 lesions, showed the same separation-dependent deficits on this task (Gilbert, Kesner, & Lee, 2001), confirming an important role of the DG.

To investigate this DG-dependent process further, Hunsaker, Rosenberg, and Kesner, (2008) developed a spontaneous task, which did not require training and took advantage of a rat's innate preference for novelty. The experimenters demonstrated that DG-lesioned rats explored objects that had been moved to a novel location (i.e., closer together than placement during the sample phase) and a novel environment (i.e., changed from a circular arena to a square arena) less than the CA3-lesioned rats explored the novel location and environment. It was concluded that the lower levels of exploration exhibited by the DG-lesioned rats suggested that the DG was critical for

spatial pattern separation for both the detection of metric changes in the distance between objects and in environmental geometry.

Along with these tasks that require hand testing, our lab group previously developed an automated touchscreen-based spatial discrimination task, referred to as LD, that allowed for the locations of stimuli to be systematically varied (McTighe et al., 2009). Using LD, the experimenters demonstrated that rats with dorsal hippocampal lesions were impaired when discriminating between similar locations on the screen. Performance of the sham-lesioned animals was unaffected by spatial separation. This replicated the separation-dependent impairment demonstrated by the hand testing tasks described above.

Pattern separation has also been studied in humans (e.g., Bakker et al., 2008; Lacy et al., 2011; Azab et al., 2014). Bakker and colleagues (2008) developed a test of pattern separation to be used in combination with fMRI. This study used semantically related targets and lures to investigate mnemonic interference. Participants viewed a series of pictures of everyday objects and were then asked to determine if an object was new, a repetition of a previously seen object, or a different version of a previously seen object (i.e., lure). Presenting a lure elicited increases in blood flow in DG/CA3 regions that looked similar to the pattern elicited by a novel stimulus, and not the pattern elicited by a repeated stimulus. This pattern suggested that activity limited to the DG/CA3 regions showed a strong bias towards pattern separation and not pattern completion, which was seen in other regions. Lacy and colleagues (2011) had a similar design but parametrically rotated objects in the series of pictures to explicitly control target-lure similarity. The fMRI activity provided evidence of pattern separation in the DG/CA3 regions, such that the activity was highly sensitive to small changes in input. These studies provide indirect evidence that pattern separation may occur in the DG/CA3 regions of the human brain as well.

This section has briefly outlined some of the behavioural evidence for pattern separation occurring in the hippocampus, and specifically the DG. However, as mentioned previously, the hippocampus retains a unique form of plasticity throughout adulthood that enables the production of functionally integrated new neurons. This process is referred to as hippocampal adult neurogenesis. Some computational models suggest that

adult born DG granule cells play an important role in pattern separation (Aimone & Gage, 2011) and there is growing evidence in support of this claim from behavioural experiments (Clelland et al., 2009; Creer et al., 2010; Sahay et al., 2011a; Nakashiba et al., 2012; Tronel et al., 2012; Bekinschtein et al., 2014; Kent et al., 2015). For example, the first experimental evidence was provided by Clelland and colleagues (2009), who demonstrated using two different methodologies, that reducing hippocampal neurogenesis impaired performance on a delayed non-matching to sample eight-arm radial maze and the LD touchscreen task.

Further support for the important role of hippocampal neurogenesis for pattern separation comes from contextual fear conditioning experiments using a modified paradigm to assess pattern separation (Sahay et al., 2011a; Tronel et al., 2012; Nakashiba et al., 2012). The modified paradigm requires the subject to differentiate between shock and no-shock contexts, which vary in similarity. In this paradigm, a less distinct pair of contexts would share an identical metal grid floor, but still have unique odours, ceilings, and lighting, whereas the distinct pair would vary on all parameters (Sahay et al., 2011a). There is evidence that rodents with reduced neurogenesis are impaired when having to discriminate between the similar contexts but not dissimilar contexts (Sahay et al., 2011a; Tronel et al., 2012; Nakashiba et al., 2012). A caveat when analyzing data from experiments using this methodology is that although similarity is manipulated, it is not parametrically altered like it is in other behavioural paradigms (e.g., SLR and LD). The behavioural evidence and various methodologies will be discussed in more detail throughout the following chapters. The remainder of this chapter will turn to pattern separation outside of the DG to emphasize that although pattern separation occurs in the DG, it is not a unique function of the DG.

1.4. Pattern separation outside the dentate gyrus

As described above, it is well established that the hippocampus is important for encoding episodic memories, which involves the storage and recollection of unique events. Pattern separation is hypothesized to be an essential component of this process by allowing for the storage of non-confusable representations of these episodes.

Together, the aforementioned studies provide evidence that a pattern separation process occurs in the DG and experimental support for the predictions of the early computational models. DG granule cells, because of their low firing rates (Jung & McNaughton, 1993) and sparse connectivity with CA3 pyramidal cells (Amaral et al., 1990), appear to be particularly adapted to maintain and transmit the orthogonalized information. There is so much accumulating evidence from electrophysiological and behavioural studies in support of the role of the DG for pattern separation, that it is often wrongly assumed that pattern separation only happens in the DG. The following chapters offer further evidence in support of pattern separation occurring in the DG; however, it is important to acknowledge that pattern separation occurs outside the DG as well. The wider literature suggests that the ability of neural networks to encode two relatively similar inputs as distinct representations may be a fundamental property of neural networks in general, and not a unique function of the DG. The ability of neural networks to produce outputs that are less correlated than their inputs is exhibited by other regions. In fact, in Marr's (1969) seminal work, he initially discussed pattern separation as a process occurring in the cerebellum, which was years prior to anybody suggesting that it may also be a process involved in the hippocampal memory system.

1.4.1. Representational-Hierarchical model

Work done by Bussey and Saksida (2002, 2005, 2007), provides a theoretical framework and experimental evidence for pattern separation occurring throughout the ventral visual stream and medial temporal lobe. Although the term pattern separation is not engrained in the theory, the process that enables the accurate discrimination between similar stimuli is analogous.

Bussey and Saksida (2002) proposed a neural network model referred to as the "Perceptual-Mnemonic/ Feature-Conjunction" model**,** which has since been modified to the "Representational-Hierarchical" (R-H) theory of temporal lobe function. This view provides a continuous, hierarchical account of cognitive processing focused mainly on the organisation of representations throughout the ventral visual-perirhinal-hippocampal processing stream.

The R-H theory has been instantiated in a connectionist model, simulating lesions by removing the corresponding component of the model (Bussey & Saksida, 2002; Bussey et al., 2002; 2003; Cowell et al., 2006). Deficits arising from lesions are assumed to be due to the loss of conjunctive representations at a specific level of representation. The connectionist model formalized assumptions of the R-H theory and generated a large number of predictions that have since been successfully tested (McTighe et al., 2010; Barense et al., 2012a; Barense et al., 2005; Lee et al., 2005; Graham et al., 2006; Bartko et al., 2010; Tyler et al., 2004; Newsome et al., 2012; Yeung et al., 2013).

1.4.2. Representational-Hierarchical model versus modular view

The R-H model uses a hierarchical continuum instead of a modular framework, which was once the prevailing view in the field (e.g., Squire & Zola-Morgan, 1991). The model is in agreement with others who have also questioned the modular approach in favor of continuous accounts of temporal lobe function (e.g., Palmeri & Tarr, 2008; Palmeri & Gauthier, 2004; Gaffan, 2002; Nadel & Peterson, 2013). Instead of trying to map psychological constructs onto anatomical modules in the brain the R-H model suggests that the focus be on the functions of brain regions in terms of what computations they perform and the representations they contain. According to the R-H model, regions along the ventral visual stream use the same neural and cognitive mechanisms, and the same processing algorithms are assumed in each component of the model. In contrast, the modular perspective is that the brain is divided into discontinuous modules, each defined according to a particular psychological function with different functional and neuronal algorithms.

Proponents of the modular view of cognitive processes assert that specific cognitive functions such as memory and perception are isolated to specific brain regions (Hampton, 2005; Squire et al., 2004). As discussed in a previous section, a prevalent modular theory is that the perirhinal cortex and hippocampus are part of the medial temporal lobe memory system that form a functionally homogenous structure governing declarative memory (Squire & Zola-Morgan, 1991). Evidence against this unitary memory system comes from studies using monkeys, rodents, and humans that suggest an anatomical distinction between systems underlying spatial and nonspatial memory, where the perirhinal cortex and hippocampus operate independently of one another

(reviewed by Brown & Aggleton, 2001). Specifically, the perirhinal cortex appears important for visual object recognition, whereas the hippocampus plays a more dominant role in spatial processing (Bird & Burgess, 2008; Bussey et al., 2000; Ennaceur, Neave, & Aggleton, 1996). This dissociation of the memory function provides strong evidence against the idea of a unitary medial temporal lobe memory system (Squire & Zola-Morgan, 1991), and supports the notion that functional differences result from the representations held in each brain region (i.e., contextual/spatial versus object).

In contrast to the modular view, the R-H theory questions the anatomical modularization on the basis of psychological notions, and argues that brain regions needed for specific tasks are those that contain the necessary representations (Bussey & Saksida, 2005). According to the R-H perspective, behavioural tasks can be designed in such a way to enable researchers to assess the use of specific levels of representation for cognition and behaviour. For example, differentiating between recognition memory for a spatial location versus recognition memory for an object, varies the dependency on representations at the level of space and the level of the object, and thus the hippocampus and perirhinal cortex, respectively.

1.4.3. Representation-Hierarchical perspective of pattern separation

The R-H account of cognitive organization expands throughout the ventral visual stream-perirhinal-entorhinal-hippocampal continuum and supposes that each brain region within the hierarchy can perform pattern separation for the level of stimulus complexity that it represents. Whereas a modular approach would aim to find the pattern separation locus in the brain (i.e., the DG), the R-H approach suggests that the specific locus depends on the level of processing at which pattern separation is occurring. For example, the perirhinal cortex is responsible for encoding highly similar objects as distinct and by the time these separated object representations reach the hippocampus, episodes may be further separated into distinct representations defined by space and time.

Behavioural experiments used to evaluate pattern separation have the underlying assumption that representations formed after effective pattern separation are helpful

when performing behavioural tasks when there are overlapping inputs, such as when discriminating between similar locations or objects. According to the R-H view, the level of stimulus complexity determines which region performs pattern separation rather than being a fixed functional role of a specific brain structure. The model's only assumption is that differences in complexity of stimulus representations in specific brain regions, account for functional differences. For example, the level of stimulus complexity required to solve the tasks (i.e., changes in spatial location and environmental contextual cues) determines whether performance relies on the DG/hippocampus performing pattern separation.

The R-H model suggests that the perirhinal cortex is responsible for complex visual discrimination between stimuli that have a high degree of 'feature ambiguity' because the perirhinal cortex holds the most complex conjunctive representations in the ventral visual stream, or the 'gestalt' of a distinct object whole. In contrast, the hippocampus contains complex, multimodal representations of multiple objects and their visuo-spatial relationship. The particular function of the perirhinal cortex and hippocampus in perceptual discrimination of objects and objects in space is to keep separate distinct representations. In other words, the perirhinal cortex and hippocampus are important for object and spatial pattern separation, respectively.

By resolving ambiguity between overlapping inputs from the level below in the hierarchy, each step uses a pattern separation mechanism. Each processing step, within the ventral-visual stream, involves increasingly complex configural representations, which eventually include elements of time and space, thought to be represented in the hippocampus. Thus, the hippocampus is most useful for recognition memory of stimulus material that involves rich representational content, such as space, time, and context of an event. This view is in obvious conflict with views in which brain regions are specialized for particular cognitive functions, such as memory in the medial temporal lobe (Squire, 1992), perception in the inferotemporal cortex (Schacter & Tulving, 1994), or object-context associations in entorhinal cortex (Wilson et al., 2013a,b).

1.4.4. Evidence of pattern separation in the perirhinal cortex

Evidence of a pattern separation process occurring in the perirhinal cortex comes from a series of experiments that were motivated by the R-H model. In one study, rhesus monkeys with perirhinal cortex lesions exhibited performance on a visual discrimination task that was dependent upon feature ambiguity, or rather was similarity-dependent. The lesioned monkeys were unimpaired on a discrimination task of grey scale clipart when feature ambiguity was low, mildly impaired when feature ambiguity was at an intermediate level, and severely impaired when feature ambiguity was high (Bussey, Saksida, & Murray, 2002; 2003). In the follow-up study, monkeys with perirhinal lesions had trouble with the acquisition and performance of single-pair discriminations between grey-scale pictures that were made perceptually difficult by blending stimuli together. The blending process was used to increase 'feature ambiguity,' and thus similarity. In contrast, the same monkeys with perirhinal lesions showed no impairment when discriminating between perceptually easier, low feature ambiguity pictures (Bussey, Saksida, & Murray, 2003).

Moreover, just as the perceptually easy, low feature ambiguity discriminations were spared following perirhinal lesions, performance on perceptually difficult visual discriminations based on size and colour were also unaffected by the lesions. Because discriminations of size and colour were challenging but did not have overlapping features, it provides evidence for a selective role in discriminating feature ambiguity rather than another aspect of visual perception. This work extends the concept of pattern separation from the spatial domain in the hippocampus-dependent tasks to the object domain in perirhinal-dependent tasks.

Similarly in rats, perirhinal damage does not affect discrimination of stimuli with few overlapping features; however, perirhinal lesions do impair a rat's ability to discriminate between complex compound stimuli that contain overlapping features (Eacott, Machin, & Gaffan, 2001). These findings are consistent with the monkey data described above. To further investigate this pattern separation process in the perirhinal cortex, Bartko and colleagues (2007a,b) found that rats with perirhinal lesions had a selective deficit in object recognition and discrimination tasks using similar objects with high levels of feature ambiguity. It is this function of discriminating feature ambiguity

for objects, that is thought to be analogous to performing pattern separation, but at the level of objects (Cowell, 2012; Gilbert & Kesner, 2003).

1.4.5. Pattern separation in other regions of the brain

Although the strongest experimental evidence is for pattern separation occurring in the DG and perirhinal cortex, there is some evidence of pattern separation throughout the ventral visual stream and other streams such as the dorsal visual stream and cortical auditory processing stream, which are thought to be organized in a similar hierarchy with increasingly complex representations (reviewed by Goodale, 2011 and Rauscheker & Scott, 2009, respectively). It is possible that a pattern separation mechanism reduces ambiguity between the stages of processing, such that higher-level representations resolve ambiguity from lower-level ones.

Evidence from electrophysiology studies has also revealed that different stages of the ventral visual stream are responsible for resolving ambiguity and conjunctive coding (Logothetis & Sheinberg, 1996; Sheinberg & Logothetis, 1997). For example, single-unit activity in the ventral visual stream of monkeys, shown a series of images including some that are ambiguous as a result of presenting incongruous images to each eye, has revealed that a portion of neurons activate exclusively when the target images are presented, whereas other cells are active only when ambiguous images are presented (Logothetis & Sheinberg, 1996; Sheinberg & Logothetis, 1997). These cells that become active when resolving ambiguity when presented with overlapping input may be engaging in a pattern separation process.

Furthermore, there is evidence in monkeys and humans that neurons in the ventral visual pathway show sparse and selective activity for specific individuals, landmarks, and objects (e.g., Desimone et al., 1984; Logothetis & Sheinberg, 1996; Sheinberg & Logothetis, 1997; Baker, Behrmann, & Olson, 2002; Quiroga et al., 2005), confirming that neurons throughout the visual stream exhibit pattern-selective responses. For example, electrophysiology studies have revealed that many neurons in the inferotemporal cortex, which is the final stage of the ventral visual stream, respond selectively to complex visual objects, such as faces (Desimone et al., 1984). Similarly, using single-cell recording inferotemporal cortex of monkeys, Baker, Behrmann, and

Olson (2002) identified neuronal responses that were highly selective for individual parts and for the whole of the previously learned images. The pattern of activity reflected conjunctive encoding, such that the summed response to individual features of the stimulus were less than the response to the conjuctions of those features. Responses could not be modelled as the sum of independent responses to the parts, but rather the firing rate depended nonlinearly on the conjunction of elements to represent the whole.

In humans, Quiroga and colleagues (2005) also confirmed highly specific complex visual representations throughout the medial temporal lobe. They recorded from eight patients and found cells with very low baseline activity and sparse, explicit, and invariant encoding of visual percepts. Interestingly, this study showed that different views of the same individuals, landmarks, animals, or objects selectively activated a subset of the cells (Quiroga et al., 2005). The sparse, highly selective activation may represent an electrophysiological signature of pattern separation, and suggests that these cells may be important for the transformation of complex visual input into distinct representations. The findings support the existence of "whole-preferring" conjunctive representations and suggest that new visual representations are established in the cortex during visual discrimination learning. This process has also been referred to as 'unitization,' in which stimulus elements are treated as a single entity after being fused into unitary representations, creating complex conjunct representations of object or contents of a scene (Graf & Schacter, 1989).

Using a similar paradigm and fMRI imaging, Motley and Kirwan (2012) provide evidence of pattern separation in the ventral visual stream that is amplified in the hippocampus. The experimenters parametrically manipulated the similarity of images by using photographs of objects rotated in 5-degree intervals. This was done in order to examine the effect of similarity on the response function of the medial temporal lobe. The participants were asked to identify the image as new, repeated, or rotated. Regions were defined as performing pattern separation if there was a marked difference between the repeat and small-rotation conditions in activity. The activity resembling pattern separation (i.e., best fit curve was a power function with decreasing slope) was observed throughout the ventral stream, but to a lesser degree than in the hippocampus.

This process of remapping of inputs to facilitate subsequent information processing and memory formation is not isolated to the ventral visual stream or medial temporal lobe. The dorsal visual stream is an independent anatomical pathway that is thought to extract visuomotor information to influence object recognition for manipulable objects, such as tools (Almeida et al., 2010). Single-cell recording in monkeys and fMRI studies in humans reveal selective activation of the posterior parietal and ventral premotor cortices by images of specific categories of objects (Chao & Martin, 2000; Murata et al., 2000). For example, Murata and colleagues (2000) recorded single-unit activity in the dorsal visual stream and found neurons that were selective for shape, size, and orientation of simple geometric objects. The selective responding to particular stimuli is analogous to that demonstrated throughout the ventral visual stream.

Pattern separation processes have also been studied in other regions as well, such as the CA1 region of the hippocampus, amygdala (AM), and olfactory bulbs (OB). Gilbert and colleagues (2001) evaluated what they referred to as temporal pattern separation in the CA1 region. In their study, rats were allowed to visit each arm of an eight-arm radial maze in a randomly determined sequence. This was followed by a test phase in which the rats were presented with two arms and were required to choose the arm that had been presented earlier during the sample sequence. The two arms presented during the test phase varied in temporal separation, such that the number of arms visited between the two test arms were varied systematically. The findings revealed that CA1 lesions selectively impaired temporal pattern separation, and not spatial pattern separation. As predicted, DG lesions had the opposite effect.

There is also limited evidence that a pattern separation process occurs in the amygdala, based on reward value (Gilbert & Kesner, 2002). Gilbert and Kesner (2002) compared hippocampal or amygdala-lesioned rats with controls, using an anticipatory contrast paradigm in which the experimenters monitored a rat's intake of two sucrose drinking water solutions that had different percentages of sucrose. The anticipatory contrast effect is when a rat suppresses intake of a less preferred substance (e.g., lower sucrose concentration), after learning that a more preferred substance will follow (e.g., higher sucrose concentration). The findings revealed that the amygdala-lesioned rats were impaired and did not show the anticipatory suppression when offered a 2 % and then a 16 % sucrose solution. The low consumption ratio suggested that the amygdala-lesioned

rats were unable to discriminate between the solutions. In contrast, the hippocampal-lesioned rats performed similarly to the control group. Importantly, the amygdala-lesioned group performed normally on the other conditions and the researchers demonstrated that this was not due to a perceptual deficit, concluding that this was a deficit in mnemonic pattern separation for reward value.

Because of the growing interest in neurogenesis and particularly the role of adult-born hippocampal neurons for pattern separation in the DG (discussed in detail in Chapters 2 to 5), there has also been some attention to the role of adult-born cells in the olfactory bulbs. Like the hippocampal system, the olfactory system processes complex spatiotemporal patterns, but these inputs are evoked from scents. It is possible that spatial information is so complex that it requires hyperplastic, hyper-responsive cells, which are not necessary for other types of pattern processing. Molecularly similar odorants can evoke highly overlapping patterns within the olfactory bulb, which may require a process of pattern separation for high acuity odour discrimination. Wilson and colleagues (Barnes et al., 2008; Wilson, 2009) used analysis of ensemble single-unit activity and found that ensembles of rat olfactory bulb neurons decorrelate similar inputs. This process of pattern separation may help enable natural odours, which are derived from odorant mixtures, to be perceived as configural objects (Jinks & Laing, 2001; Kay et al., 2003). How adult neurogenesis contributes to the olfactory bulb network is still unclear, but it may play a critical role in pattern separation for olfactory acuity and difficult discriminations (Mandairon et al., 2006; Moreno et al., 2009; Valley et al., 2009; Breton-Provencher et al., 2009; for reviews see Wilson, 2009; Sahay et al., 2011b and Breton-Provencher et al., 2012).

1.5. Conclusion

To summarize, pattern separation was first proposed in computational models as a mechanism for transforming overlapping inputs into separate and distinct representations. This process was postulated to enable accurate memory retrieval. There has since been a growing body of evidence from electrophysiological and behavioural studies that suggest that pattern separation occurs in the DG to discriminate between similar spatial locations and environmental contexts. Before progressing to the following chapters, which add further experimental evidence in support of pattern

separation occurring in the DG, it was important to acknowledge that pattern separation is not a unique function to the DG, but rather may occur throughout the brain. The R-H model provides a framework for understanding pattern separation throughout the ventral visual stream, as a mechanism for encoding ambiguous stimuli as distinct wholes. This is not to claim that the same neural mechanisms that are important for pattern separation in the DG are necessarily ubiquitous for pattern separation in other areas. It is possible that spatial information is so complex that it requires the hyperplastic and hyper-responsive adult-born cells in the DG, which are not necessary for other types of pattern processing.

1.6. General overview

There is substantial evidence that the pattern separation process first proposed by computational models, occurs throughout the brain. In particular, the DG region of the hippocampus has a well-established role in performing a pattern separation function when encoding spatial and contextual inputs. Similarly, the perirhinal cortex performs a pattern separation function when processing objects.

The following chapters will describe experiments that have been designed to further elucidate the mechanisms underlying pattern separation. Table 1.1 provides a list of the chapter descriptions. First, I will describe the development of the Spontaneous Location Recognition (SLR) task and evidence for a role of brain-derived neurotrophic factor (BDNF) and N-methyl-D-aspartate (NMDA) receptors in the DG for pattern separation (Chapter 2). Second, I will describe the role of adult-born hippocampal neurons in pattern separation by describing an experiment in which neurogenesis was inhibited (Chapter 3) and an experiment in which neurogenesis was increased (Chapter 4). Third, I will provide evidence that the process of pattern separation may have a reciprocal relationship with neurogenesis, such that neurogenesis may be increased after repeated pattern separation, and this can improve subsequent performance on a spatial memory task (Chapter 5). Finally, I will conclude by describing a longitudinal experiment that provided a cognitive phenotype of a mouse model of dementia, to specifically explore how tau pathology in the frontotemporal cortex interferes with cognition (Chapter 6).

Although Chapter 6 does not explore the mechanisms underlying pattern separation directly, a deficit in pattern separation is hypothesized to underlie some of the cognitive impairments experienced by patients with dementia (Yassa et al., 2010; Ally et al., 2013; Wesnes et al., 2014). For example, fMRI scans combined with cognitive tasks have revealed that age-related deficits of pattern separation are more pronounced in patients diagnosed with Mild Cognitive Impairment (MCI) or AD (Yassa et al., 2010; Ally et al., 2013). There is also a correlation between cerebrospinal fluid concentration of amyloid β42 and the ability to make difficult visual discriminations but not easier discriminations (Wesnes et al., 2014). Because an important step in developing effective therapeutics for dementia is establishing a valid animal model of disease, the aim of the experiments described in Chapter 6 was to validate the TgTau^{P301L} mouse as a model of tauopathy.

Table 1.1. Outline of chapters

Chapter 1	**General Introduction:** History and definition of pattern separation
Chapter 2	**Plasticity and Pattern Separation**: Evidence for a role of BDNF and NMDA receptors
Chapter 3	**Neurogenesis and Pattern Separation**: Evidence for a role of hippocampal neurogenesis
Chapter 4	**Ghrelin and Neurogenesis:** Evidence that ghrelin administration can increase neurogenesis and improve pattern separation
Chapter 5	**Reciprocal Relationship of Pattern Separation and Neurogenesis:** Evidence that performing pattern separation increases neurogenesis and improves subsequent pattern separation
Chapter 6	**Tau Pathology and Cognition:** Evidence that the TgTau^{P301L} model of dementia shows memory impairments at advanced age
Chapter 7	**General Discussion:** The pattern separation debate

Chapter 2: Pattern separation is mediated by brain-derived neurotrophic factor (BDNF) and N-methyl-D-aspartate (NMDA) glutamate receptors in the dentate gyrus of rats

Pattern separation is a theorized process that orthogonalizes inputs to keep memories distinct and resistant to confusion. The aim of this chapter is to examine the role of plasticity-related mechanisms in the dentate gyrus (DG) region of the hippocampus during pattern separation. This chapter provides the necessary foundation for the work presented in Chapters 3 to 5 by describing how the Spontaneous Location Recognition (SLR) task was validated for evaluating pattern separation, and the evidence that pattern separation occurs during the encoding and/or consolidation phase of memory processing when encountering similar spatial locations or contexts. Experimental evidence is provided showing that brain-derived neurotrophic factor (BDNF) and N-methyl-D-aspartate (NMDA) glutamate receptors in the DG are necessary when encoding similar spatial locations. The work described was conducted in collaboration with Dr. Pedro Bekinschtein, who was a postdoctoral researcher in our lab during the first year of my PhD, and the data were previously included in my First Year Report (Kent, 2012).

2.1. Introduction

The hippocampus supports several mnemonic processes critical for memory encoding and retrieval. Among these processes is pattern separation in the spatial domain, a mechanism suggested to reduce interference among similar memory representations by encoding a location or context as distinct from another similar location or context. These orthogonal neural representations for similar hippocampal input are essential for effective episodic memory.

As discussed in Chapter 1, pattern separation was a concept first proposed by computational models of neural circuits as a mechanism for transforming similar inputs into distinct representations (O'Reilly & McClelland, 1994). Convergent lines of evidence from electrophysiology (Leutgeb et al., 2007; Neunuebel & Knierim, 2014), immediate-early genes (Vazdarjanova & Guzowsi, 2004; Kubik et al., 2007), behaviour (Gilbert, Kesner, & Lee, 2001; Hunsaker, Rosenberg, & Kesner, 2008; McHugh et al., 2007; Lee & Solivan, 2010), and human brain imaging studies (Bakker et al., 2008; Lacy et al., 2011), suggest that a pattern separation process occurs in the dentate gyrus (DG) region of the hippocampus.

The DG seems particular necessary for separating inputs of similar locations and contexts, referred to as *spatial pattern separation.* We sometimes use this specific terminology to differentiate DG-dependent processes from pattern separation occurring in other regions of the brain, such as the perirhinal cortex. For example, previous work from our lab has suggested that pattern separation in the perirhinal cortex is necessary for discriminating highly similar objects (e.g., Bartko et al., 2007a.b), which is sometimes referred to as *object pattern separation.* Although the same computational mechanism may be occurring during pattern separation in all regions, it is possible that there are variations in the specific cellular processes involved when separating inputs at different levels of representation (e.g., *spatial* versus *object*).

When the experiments presented in this chapter were conducted (beginning October 2011), the DG had already been identified as a location where pattern separation occurred; however, the specific neural mechanisms underlying this process were largely unknown. Additionally, there was substantial evidence for the importance of plasticity-related mechanisms underlying memory formation in general, suggesting that hippocampal plasticity in the DG may be important for pattern separation.

2.1.1. Plasticity mechanisms underlying memory

The dominant experimental model for cellular mechanisms underlying hippocampal learning and memory is a phenomenon referred to as long-term potentiation (LTP). LTP was the first demonstration of an experimentally-induced lasting neurophysiological alteration in the mammalian brain and provided compelling

evidence in support of the seminal ideas of Cajal (1894) and Hebb (1949), who had suggested that associative memories are stored as changes in the strength of synaptic connections. This postulated process was later renamed, synaptic plasticity, by Konorski (1948).

LTP is defined as long lasting enhancement in synaptic strength following brief high-frequency stimulation and was first demonstrated in the DG (Lomo, 1966; Bliss & Lomo, 1973; Bliss & Gardner-Medwin, 1973). Bliss and Lomo (1973) provided initial evidence in rabbits that repetitive tetanic stimulation of the perforant path from the enthorinal cortex to the DG elicited a stable increase in the magnitude of the post-synaptic response at monosynaptic junctions. In other words, the granule cells were subsequently more effectively activated by stimulation after the repetitive stimulation. This increase in synaptic efficacy resulting from the repetitive stimulation was shown to last up to 10 hours in anesthetized rabbits (Bliss & Lomo, 1973) and up to 16 weeks in unanesthetized rabbits (Bliss & Gardner-Medwin, 1973). Although initially observed in the hippocampus, there is *in vivo* and *in vitro* evidence of LTP in several brain regions (reviewed by Teyler & DiScenna, 1987).

The first evidence that LTP occurred in conjunction with behavioural learning was from multiunit studies that demonstrated a learning-dependent increase in the firing of CA1 pyramidal neurons during classical conditioning in rabbits (Berger et al., 1976; Berger & Thompson, 1978). This initial evidence was followed by Disterhoft and colleagues' (1986) study, which demonstrated that the neuronal changes induced *in vivo* during conditioning could be preserved and studied *in vitro*. Being able to study these changes *in vitro* enabled the experimenters to examine the specific conditioning-induced biophysical alteration of CA1 pyramidal cells.

Since these influential experiments, the role of LTP in hippocampal memory processes has been a topic of intense study and debate (for reviews see Luscher & Malenka, 2012 and Nicoll & Roche, 2013). Although there are mechanistically distinct forms of LTP occurring at different types of synapses (e.g., mossy fibre versus Schaffer collateral synapses), it is largely accepted that N-methyl-D-aspartate (NMDA)-type glutamate receptors are central to at least one form of hippocampal LTP (Collingridge, Kehl, & McLennan, 1983).

2.1.2. N-methyl-D-aspartate (NMDA) receptors

The NMDA ionotropic glutamate receptor is a membrane-inserted protein complex, which is uniquely both voltage and ligand gated (Laube, Kuhse, & Betz, 1998; Ghafari et al., 2012). The NMDA receptor is highly calcium permeant and activated by the combination of depolarization of the postsynaptic membrane and simultaneous binding of the co-agonist glycine and glutamate to the NR1 and NR2 subunits, respectively (Johnson & Ascher, 1987). Once active, NMDA receptors produce a strong post-synaptic calcium (Ca^{2+}) influx, which can then activate a wide range of signalling pathways [e.g., Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC), protein kinase A (PKA), and mitogen-activated protein kinases (MAPK)] and induce LTP (reviewed by Waltereit & Weller, 2003).

Morris and colleagues (1986) were the first to directly block LTP while assessing hippocampal-dependent memory. The researchers used a pharmacological approach to block LTP, which consisted of an intracerebroventricular (ICV) infusion of an NMDA antagonist (D-2-amino-5-phosphonopentanoic acid; APV or AP5). The rats infused with the NMDA antagonist, at concentrations shown to block LTP in the DG *in vivo,* demonstrated impaired acquisition on the Morris water maze (MWM) task, while sparing visual discrimination learning (Morris et al., 1986; Morris, 1989; Davis, Butcher, & Morris, 1992). Since this first demonstration, strong evidence has accumulated in support of the importance of NMDA receptors for hippocampal LTP and memory (see Morris, 2013 for a review).

However, subsequent studies have shown that DG NMDA receptors are not always essential for spatial learning and memory, and suggest that NMDA receptors have a subtler role than first presumed (e.g., Saucier et al., 1995). For example, rats treated with NMDA antagonists were not impaired on the MWM task when training occurred in a maze located in a distinct testing room (Bannerman et al., 1995). Furthermore, mice genetically engineered to have selective ablation of the GluN1 subunit of NMDA receptors in DG granule cells and CA1 pyramidal cells showed normal spatial reference memory but impaired spatial discrimination on the MWM (Bannerman et al., 2012).

Bannerman and colleagues (2014) review these most recent findings and propose that instead of NMDA receptors in the DG contributing to spatial memory in general, DG NMDA receptors are important only when needing to resolve conflict or inhibit previously learned behavioural responses (Bannerman et al., 2012; Taylor et al., 2014). In other words, DG NMDA receptors are necessary when there are ambiguous or overlapping representations, which could be consistent with evidence suggesting a specific role of DG NMDA receptors in pattern separation (e.g., McHugh et al., 2007).

To directly investigate the involvement of DG NMDA receptors in pattern separation, McHugh and colleagues (2007) developed a transgenic mouse line that lacked the NR1 NMDA receptor specifically in DG granule cells. These transgenic mice were then tested on a modified contextual fear conditioning task, in which the mice had to differentiate between similar shock and no-shock contexts. DG-NR1 knockout and fNR1 littermates showed no detectable differences in performance on the MWM and both groups were able to acquire and retain contextual fear memories. However, to evaluate the contextual specificity of the fear memories, the researchers used a less distinct pair of contexts that shared an identical metal grid floor, but had unique odours, ceilings, and lighting. The results revealed that although both groups initially showed generalized freezing in both chambers, after 12 days of visiting the two chambers daily, the control mice learned to discriminate, whereas the DG-NR1 mice were still impaired. Even after 12 days, the DG-NR1 mice continued to exhibit more freezing in the no-shock chamber, compared to the littermate control group. The researchers also found evidence for NR1-mediated shaping of CA3 encoding, because CA3 pyramidal cells exhibited less context-specific modulation of firing rate in the transgenic mice than in the control mice. Thus, disrupting DG plasticity via a selective knockout of the NR1 subunit of the NMDA receptor impaired the mice's ability to distinguish between similar contexts. It was concluded that this impairment reflected a disruption in pattern separation; however, because this paradigm did not parametrically alter similarity, it is difficult to draw conclusions about pattern separation.

2.1.3. Brain-derived neurotrophic factor (BDNF)

Another important mechanism mediating hippocampal LTP and memory is a small dimeric secretory protein called brain-derived neurotrophic factor (BDNF). BDNF

belongs to the family of neurotrophins, which are molecular signals that allow the nervous system to remain in a plastic state. BDNF acts through high affinity binding with the transmembrane receptor, tropomyosin kinase B (TrkB; Klein et al., 1991). TrkB receptors are located throughout the central nervous system with a high density of receptors in the hippocampus and forebrain (Lapchak et al., 1993; Fryer et al., 1996). Ligand binding to TrkB results in autophosphorylation of tyrosine residues. The autophosphorylation of intracellular tyrosine residues then create docking sites for second messengers and activate various intracellular signalling cascades, such as Ras-mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) and phosphoinositide 3-kinase (PI3K) pathways (reviewed by Numakawa et al., 2010). These BDNF-activated intracellular signalling cascades then activate the transcription factor, cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), which directly regulates gene transcription by binding to cAMP response elements (CRE) sequences in DNA (Finkbeiner et al., 1997).

Importantly, BDNF and the BDNF-induced intracellular signalling pathways have been shown to play an important role in hippocampal synaptic plasticity and memory. For example, NMDA receptor activation (Gwag & Springer, 1993) and training on the hippocampal-dependent MWM (Kesslak et al., 1998) have been shown to increase BDNF mRNA expression in the hippocampus. Similarly, BDNF elicits a dose-dependent increase in the phosphorylation of NMDA receptor subunit 1 in the hippocampus (Suen et al., 1997). Furthermore, Ma and colleagues (1998) showed that infusing BDNF antisense oligonucleotide directly into the DG, prior to memory consolidation, impaired performance on an inhibitory avoidance memory task and inhibited LTP in rats (Ma et al., 1998).

Since these early studies, it has been extensively shown that BDNF is an important molecule for learning and memory processes (reviewed by Bekinschtein et al., 2014), such that BDNF is both necessary and sufficient for the expression of LTP in the hippocampus (Pang et al., 2004) and long-term hippocampal-dependent memory (Bekinschtein et al., 2007).

To evaluate whether BDNF plays a specific role in spatial pattern separation, our lab developed a new behavioural paradigm, the Spontaneous Location Recognition (SLR)

task (Bekinschtein et al., 2013). SLR has several benefits in its design, such as being a spontaneous task that does not require training, using identical choice phases in every condition to allow for direct comparisons, manipulating similarity during encoding, and allowing the experimenter to discriminate between the different stages of memory processing. The task design and rationale will be described in more detail in the following sections and the pros and cons of the paradigm will be discussed again in Chapter 7.

2.1.4. Spontaneous Location Recognition (SLR) task development

SLR is a modified version of an established object-location recognition behavioural paradigm (Ennaceur et al., 1997; Warburton et al., 2000). In the traditional version of the task, rats are exposed to two identical objects during a sample phase. Following a variable delay, one of the objects is displaced to a novel location within the arena. Time spent exploring objects in the novel and familiar locations is analysed and used to infer memory. Several behavioural paradigms have shown that rats have a preference towards novelty and consistently spend more time exploring a novel object or an object in a novel location. SLR takes advantage of this natural preference towards novelty and thus does not require training. Our modified version enables control over the similarity of spatial landmarks during the critical encoding phase, and thus varies the load on pattern separation. Figure 2.1 illustrates the SLR task design.

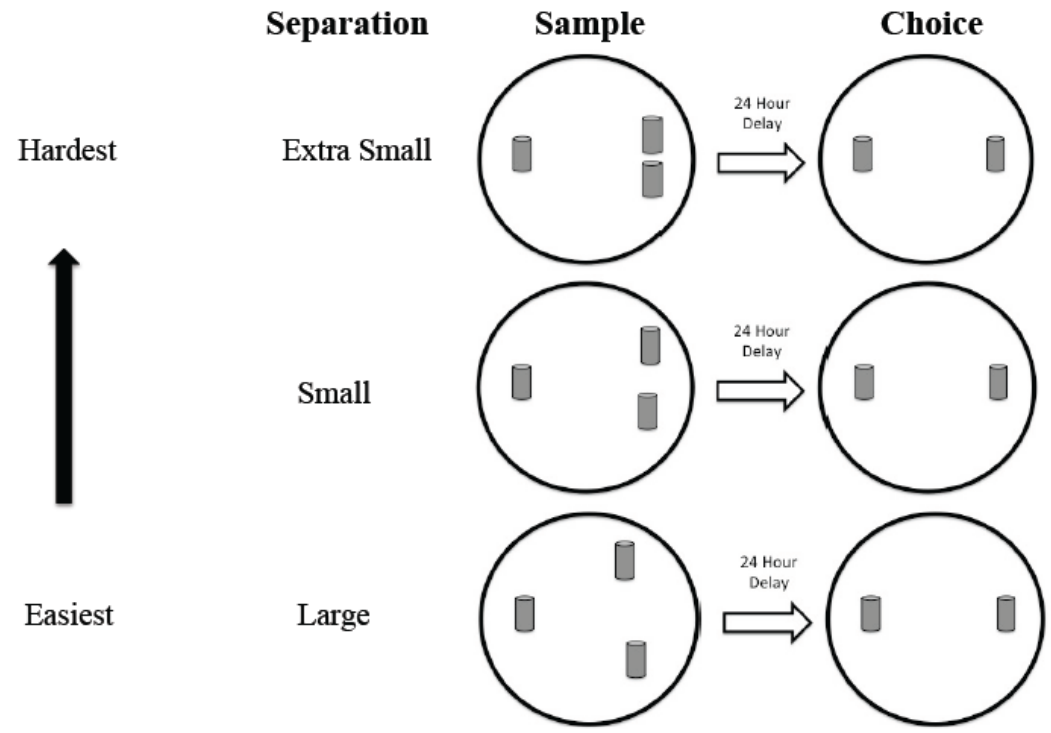

Figure 2.1. Diagram of the SLR task. There are three conditions: extra small separation (proximate objects are 4 spaces apart; top), small separation (proximate objects are 5 spaces apart; middle), and large separation (all objects are 12 spaces apart; bottom). During the sample phase, rats are exposed to three identical objects for 10 min. Twenty-four hours later the rats are exposed to two identical objects, but one of the objects is displaced to a novel location that is in between where two of the sample objects had been previously presented. The most difficult version of the task, in which naive rats consistently do not show a preference for the novel location, is the extra small condition. The moderately difficult condition, in which the naive rats consistently show a preference for the novel location but performance is disrupted when DG processes are inhibited, is the small condition. The easiest version of the task, in which naive rats consistently show a preference for the novel location is the large condition, and performance on this condition is not disrupted when DG processes are inhibited.

During the sample phase of SLR, rats are exposed to three identical objects, two are close together and the third is further apart, in an isosceles triangle arrangement. The distance between the two closest objects is varied to modify the load on pattern separation. During the choice phase, rats are presented with only two objects, one in its original location and one in a novel location, which is between the positions of the two closest objects previously explored during the sample phase.

If the rat recognizes that one of the objects has moved, then it should spend more time with the object in the novel location than with the object in the familiar location (Warburton et al., 2000). The closer the two objects are during the sample phase, the

more difficult it is to separate them as two distinct representations. If representations of the two closest locations are not sufficiently separated, then the novel location presented during the choice phase will activate the same representations in memory and will not be distinguishable.

Bekinschtein and colleagues (2013) conducted a series of experiments using the SLR task to demonstrate an important role of BDNF for spatial pattern separation. The initial experiment demonstrated that the expression of BDNF in the DG is spontaneously increased after rats are exposed to landmarks delineating similar spatial locations (objects separated by a 50° angle, equivalent to 5 spaces apart in the maze), but not dissimilar spatial locations (objects separated by 120° angle, equivalent to 12 spaces apart in the maze). This finding suggested that rats engage a BDNF-associated process of pattern separation in the DG when forming two ambiguous spatial representations (Bekinschtein et al., 2013). Importantly, this separation-dependent pattern of BDNF activation was not seen in the CA1 region of the hippocampus.

Bekinschtein and colleagues (2013) followed up this initial observation by combining intracranial infusions of exogenous BDNF or a BDNF-blocking antibody at different stages of the SLR task. The results revealed that disrupting BDNF at time points before or immediately after the sample phase, but not prior to the choice phase, negatively affected performance on the small separation condition. This pattern of results suggested that spatial representations go through a time-restricted consolidation phase in the DG after spatial landmarks are encoded. Similarly, infusing exogenous BDNF immediately after the sample phase improved performance on the SLR task. These experiments provided the first evidence of a BDNF-dependent pattern separation process occurring during the consolidation, and perhaps the encoding, but not the retrieval stage of memory processing.

Importantly, in all of these experiments, only performance on the small separation condition was affected by the manipulations. In contrast, performance on the large separation condition was unaffected by BDNF-blocking antibodies at any time point. These separation-dependent effects suggest that SLR is a valid task for examining the mechanisms underlying spatial pattern separation and provided the framework for the experiments described in this chapter, as well as in Chapters 3 to 5.

2.1.5. Overview

The following experiment was designed to evaluate whether BDNF enhancement of pattern separation required NMDA receptor activation. Post-sample BDNF infusions into the DG were combined with pre-sample saline or NMDA antagonist (AP5) DG infusions. AP5 is a competitive antagonist of the NMDA-type glutamate receptor that competes with glutamate, thus reducing the activity of the receptors. The results of this experiment showed that pre-sample infusion of AP5 into the DG blocked the beneficial effect of BDNF on performance. This suggests that NMDA receptor activation is part of the mechanism involved in BDNF-dependent pattern separation.

2.2. Methods

2.2.1. Subjects

All procedures were in strict compliance with the guidelines of the University of Cambridge and United Kingdom Animals (Scientific Procedures) Act 1986 and the Amendment Regulations 2012. Eight male Lister Hooded rats (250 – 300 g; Harlan, Olac, Bicester, UK) were housed in groups of two or four on a 12 h light cycle (lights on 19:00 - 07:00). For comfort, the cages contained a cardboard tube that could be used for sleeping. All rats were provided with *ad libitum* access to water and food, except during behavioural testing when food was restricted to 17 g per day for each animal. Rats were handled for 2 consecutive days prior to surgery and for at least another 5 days prior to the start of behavioural testing. All procedures were performed during the dark phase of the light cycle. One rat was removed from the experiment because of a loose headcap and cannula.

2.2.2. Surgery

All rats were implanted bilaterally with 22-gauge indwelling guide cannula targeting the DG (PlasticsOne^{TM}). Rats were deeply anesthetized with an intraperitoneal (IP) injection of ketamine (Ketalar, 90 mg/kg) and xylazine (Rompun, 6.7 mg/kg) and then placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) with the

incisor bar set at - 3.2 mm. Coordinates were - 3.9 mm, ± 1.9 mm, - 3.8 mm (a/p, m/l, d/v from bregma; Paxinos and Watson, 1998). Cannulae were secured using dental cement (A-M Systems) and four jeweler screws. After the cannulae were secured, the rats were sutured. Obturators, which had an outer diameter of 0.36 mm and were cut to sit flush with the tip of the guide cannula, were inserted into the guides and remained there except during infusions. A screw-on dust cap kept the obturators in place. Animals were then housed in pairs and allowed to recover for at least 7 days before behavioural testing.

2.2.3. Infusions

A mock infusion handling session to habituate the rats to the infusion procedure was conducted the day prior to the first sample phase. The following day, rats received bilateral infusions of either recombinant human BDNF (rhBDNF; 0.25 µg/ 0.5 µl/side; Bioscience, Cambridge, UK), competitive NMDA antagonist AP5 (D(-)-2-amino-5-phosphonopentanoic acid, 5 µg/ 0.5 µl/side; Ascent Scientific), or physiological saline (0.5 µl/side; 0.9 % sodium chloride, pH 7.0). As illustrated by Figure 2.2, all infusions took place 15 min prior to sample or 5 min post-sample. Seven rats were run on 3 trials of the extra small separation condition and the order of treatment was counterbalanced.

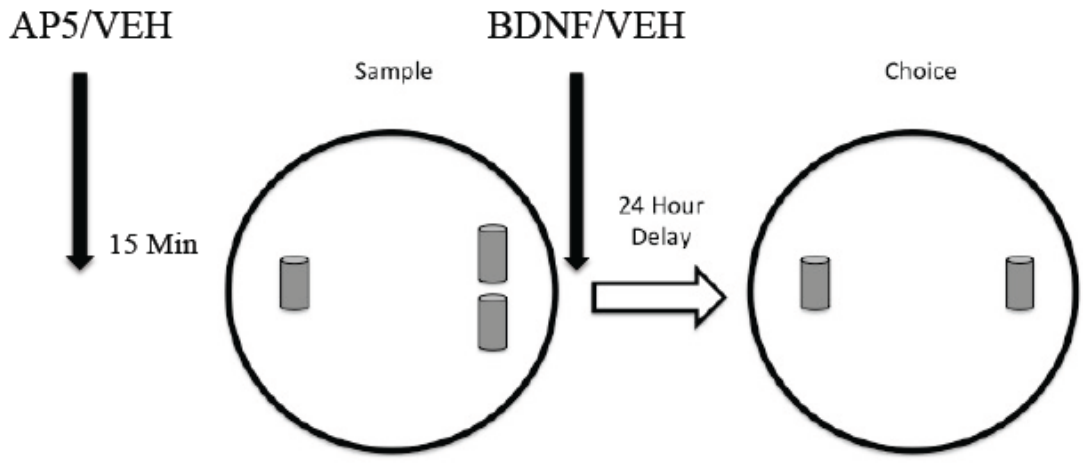

Figure 2.2. Schematic showing the timing of infusions. The testing protocol used the extra small condition of the SLR task. Saline (VEH) or AP5 was infused into the DG 15 min prior to sample, combined with a post-sample saline or BDNF infusion into the DG 5 min after the sample phase.

All infusions took place in a preparation room, under dark lighting conditions, which was separate from the location of behavioural testing. Animals were restrained gently by the experimenter throughout the infusion process. The dust cap and obturators were removed and a 28-gauge injector, which was cut to extend 1 mm beyond the tip of the guide cannula, was inserted. Infusers were constructed by connecting polyethylene tubing (0.38 mm inside diameter, A-M Systems) to the injector cannula. The tubing was then connected to a 5 μl Hamilton syringe. Bilateral infusions were performed simultaneously with a Harvard Apparatus precision syringe pump, with an injection rate of 0.25 μl/min. There was a 1 min rest period before removing the infusers. There were at least 3 days allowed for washout between repeated infusions.

2.2.4. Apparatus

Behavioural testing was conducted in a black plastic circular arena (90 cm diameter x 45 cm high) covered with sawdust bedding and situated in the middle of a dim lit room. Figures 2.3 and 2.4 provide illustrations of the testing room and apparatus. The same testing room and apparatus were used in all SLR experiments discussed in Chapters 2 to 5. The maze was labelled with a white pen to equally divide it into 36 positions, each separated by a 10^{o} angle. The labels were hidden by bedding, which was the same material used as bedding in the home cages. The bedding in the testing arena was mixed up in between each trial to minimize the effects of any remaining scent cues. The testing room had three proximal spatial cues and distal standard furniture. The position of the furniture and each cue was outlined with grey duct tape to ensure that there was the same positioning of cues for each trial. Objects used for testing were tall cylinder containers ~ 20 cm in height (i.e., soda cans, glass beer bottles, ceramic water bottles, and plastic dinosaur eggs). To prevent the rats from moving the objects during exploration, Blu-tack ^{TM} was used to secure the objects in place. Objects were wiped down with 50 % ethanol solution between sessions. A digital camera (Sony ^{TM}) recorded the testing sessions.

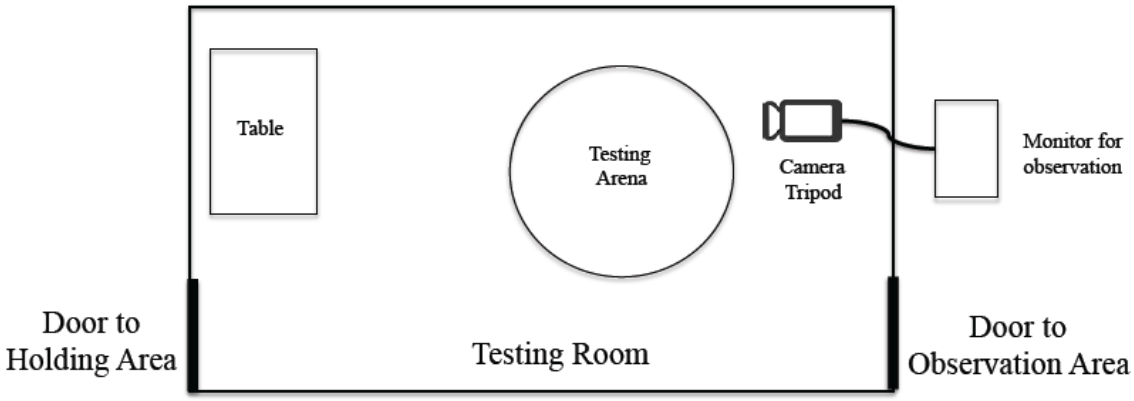

Figure 2.3. Illustration of the testing room. Testing took place in a room situated between the holding area, where the intracranial infusions took place, and the observation area, where the experimenter could monitor the behavioural testing via a video camera connected to a computer monitor.

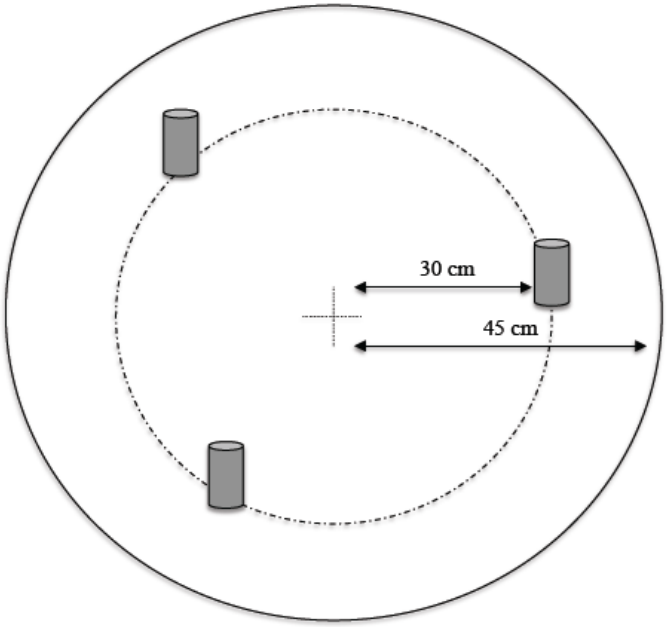

Figure 2.4. Schematic of the testing arena. The circular arena had a diameter of 90 cm and the objects were placed 30 cm from the centre and 15 cm from the outer edge. The dotted circular line was equally divided into 36 placements.

2.2.5. Behavioural procedures

On testing days, rats were held in a holding room under dark conditions, which was adjacent to the testing room. All rats were habituated in 5 consecutive daily sessions.

During habituation, rats were allowed to explore the empty circular arena for 10 min. Testing began 24 h after the fifth habituation session. Seven rats were run on 3 trials of the extra small separation condition.

Each trial consisted of two phases. During the sample phase, three identical objects (A1, A2, and A3) were placed 15 cm from the edge of the open field and 30 cm from the centre. The exact positioning of the objects was chosen pseudorandomly and counterbalanced between groups and within conditions. In the extra small separation condition, two of the objects (A2 and A3) were separated by a 40° angle (i.e., 4 spaces apart in the maze) and the third (A1) by 160° angle (i.e., 16 spaces apart in the maze). The extra small separation condition was used to avoid any ceiling effects because control subjects only perform at chance levels (Bekinschtein et al., 2013). This is useful for assessing enhancements because it had previously been shown that rats infused with recombinant BDNF into the DG showed a preference for the novel location in the extra small separation condition, whereas the control rats did not (Bekinschtein et al., 2013). Rats were allowed to explore the arena and objects for 10 min during the sample phase. At the end of the sample phase, rats were removed from the arena and placed back into their home cage.

After a 24 h delay, each rat was placed back into the arena for the choice phase. During the choice phase, rats were presented with 2 new identical copies (A4 and A5) of the objects previously used during the sample phase. A4 was placed in the previous position of A1 (i.e., familiar location). A5 was placed in between the sample placements of A2 and A3 (i.e., novel location). The choice phase allowed the animals to explore the chamber and objects for 5 min before being returned to their home cage.

2.2.6. Histology

Once behavioural testing was complete, rats were anaesthetized by IP injection with 2 ml of Euthatal (Rhone Merieux, Harlow, Essex, UK) and perfused transcardially with phosphate buffered saline (PBS), followed by 10 % neutral buffered formalin (NBF). The brains were removed and post-fixed in NBF for at least 24 h, followed by immersion in a 30 % sucrose solution for at least 48 h. The brains were cut into 60 μm

sections. Every fifth section was mounted on a gelatin-coated glass slide and placement of the cannulae were verified using a light microscope.

2.2.7. Data collection

In both the sample and choice phases, exploration of an object was defined as a rat directing its nose to an object at a distance of 2 cm or less. Sitting on the object or digging at the base of the object was not considered exploratory behaviour. For the sample phase, exploration was recorded using three stopwatches. For the choice phase, exploration was scored using a computer program written in Visual Basic 6.0 (Microsoft Corp., USA). The program had two keys corresponding to the novel and familiar objects. Exploration was recorded by pressing the appropriate keys at the onset and offset of a bout of exploration. Scoring was done blinded to the treatment condition.

2.2.8. Data analysis

Sample data were analyzed by converting exploration time for each of the three objects into a percentage of the total exploration time and using a one-way analysis of variance (ANOVA) to ensure the three sample objects were being explored equally. A paired two-tailed Student's *t*-test was run to ensure that total sample exploration was equal between pre-sample treatment conditions. Results from the choice phases were expressed as discrimination ratios (D2), calculated as time spent exploring the object in the novel location minus the time spent exploring the object in the familiar location divided by total exploration time.

$$\boldsymbol{D2} = \frac{novel\ location\ exploration - \ familiar\ location\ exploration(s)}{novel\ location\ exploration + \ familiar\ location\ exploration\ (s)}$$

Group mean D2 scores were analyzed with repeated measures ANOVA, followed by post-hoc Student's *t* paired contrasts using the Bonferonni correction. Results are shown as means and ± standard errors of the mean (SEM). All statistical analyses were conducted using SPSS version 22 and Microsoft Excel version 14.4.5. Statistical significance was set at $p < 0.05$, unless running a post-hoc comparison. To control for Familywise error, the level of statistical significance (i.e., α) for post-hoc comparisons

was calculated using the Bonferonni correction, which was calculated as 0.05 divided by the number of statistical comparisons.

2.3. Results

2.3.1. NMDA receptors in DG are necessary for BDNF-dependent enhancement of pattern separation

To test whether BDNF enhancement of pattern separation (previously reported by Bekinschtein et al., 2013) is dependent on glutamate NMDA receptor activation in the DG, we infused a competitive NMDA antagonist AP5 into the DG 15 min before the sample phase and predicted that this pre-sample infusion would block the enhancement effect of post-sample BDNF infusions in the extra small SLR condition. A repeated measures ANOVA followed by post-hoc comparisons revealed that infusions of AP5 abolished the BDNF enhancement of pattern separation in the extra small separation SLR condition ($p < 0.001$, $F(2,12) = 35.319$). The D2 scores for each group are shown in Figure 2.5. There was a statistically significant difference between the D2 scores when the rats received pre- and post-sample saline infusions and when the rats received pre-sample saline and post-sample BDNF ($p < 0.0001$). There was also a statistically significant difference when the rats received pre-sample saline and post-sample BDNF and when the rats received pre-sample AP5 and post-sample BDNF ($p < 0.0001$). There was no difference between the D2 scores when the rats received pre- and post-sample saline and when they received pre-sample AP5 and post-sample BDNF. Thus, the pre-sample AP5 infusions blocked the effect of post-sample BDNF.

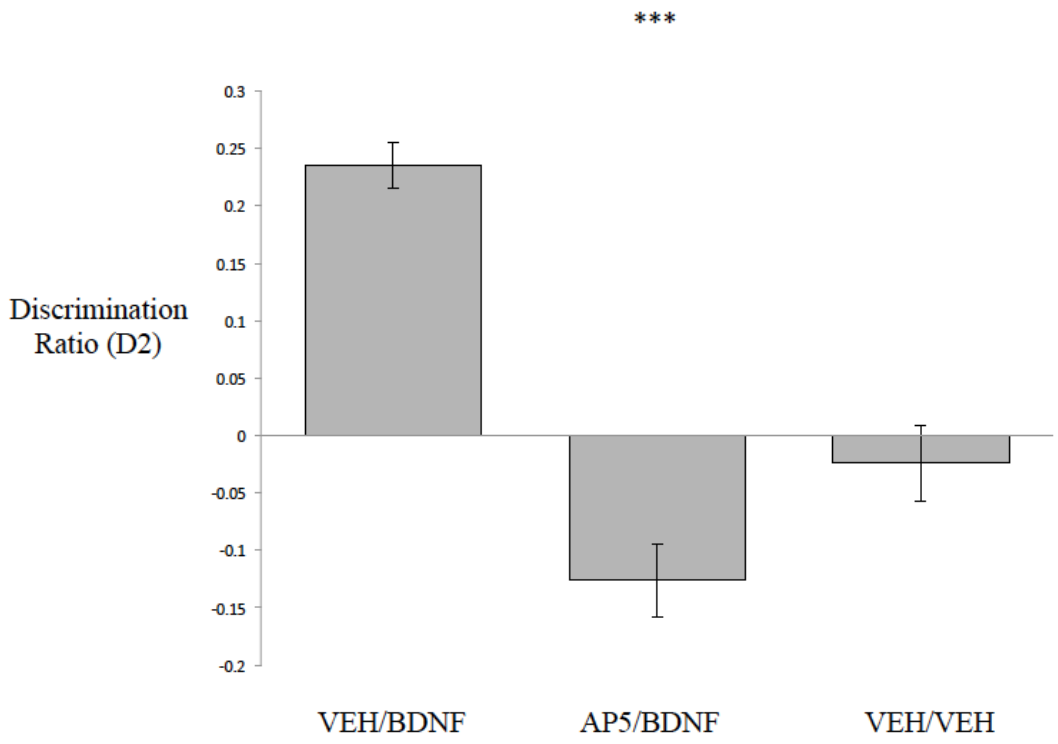

Figure 2.5. Discrimination ratios for the three conditions. The conditions were: (1) saline pre-sample and BDNF post-sample (Veh/BDNF), (2) AP5 pre-sample and BDNF post-sample (AP5/BDNF), and (3) saline pre- and post-sample (VEH/VEH). Pre-sample AP5 infusions blocked the beneficial effect of post-sample BDNF infusions. Positive D2 values reflect a preference for the novel location. *** $p < 0.001$ n = 7. Data are expressed as the mean ± SEM.

As shown in Figure 2.6, rats spent equal amounts of time exploring during the sample phases, regardless of the pre-sample treatment ($p = 0.60$). Similarly, as shown in Figure 2.7, there was also no difference in the proportion of time spent exploring each of the specific locations during the sample phase. Rats explored all three locations equally with no effect of treatment ($p = 0.44$) or location ($p = 0.19$) on exploration during the sample phase. This analysis of sample exploration confirmed that the pre-sample infusions were not having a nonspecific effect on willingness to explore.

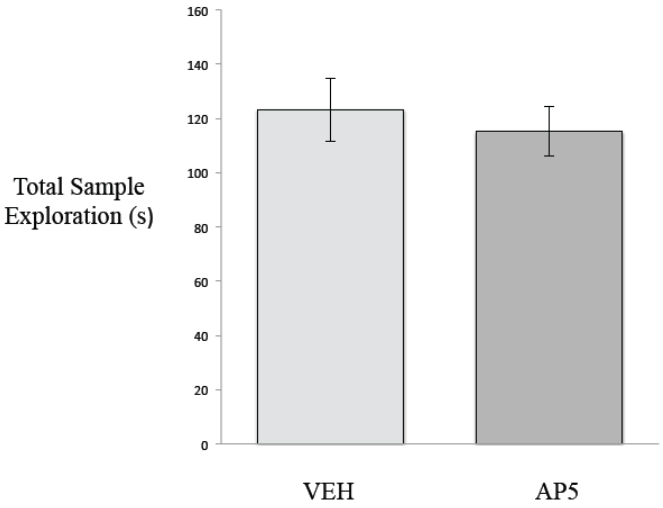

Figure 2.6. Total exploration of the objects during the sample phase. The y-axis represents the total time spent exploring during the sample phase. Pre-sample injections of saline (VEH) or AP5 had no effect on the mean exploratory behaviour during the sample phase. Data are expressed as the mean ± SEM.

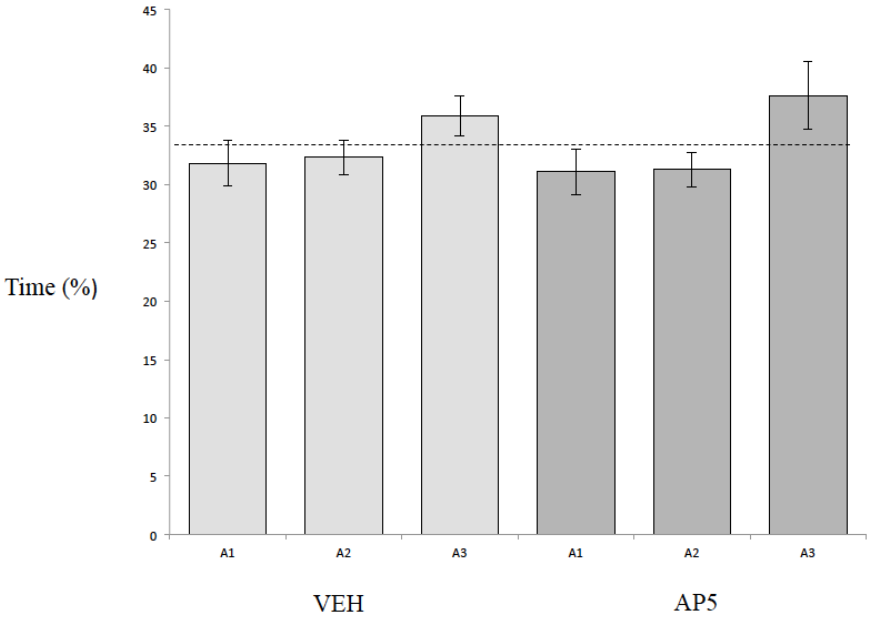

Figure 2.7. Percentage of time spent exploring each of the objects during the sample phase. Rats explored all three locations equally (A1, A2, A3) after saline (VEH) or AP5 infusions. The dotted line represents chance-level preference (33 %). Data are expressed as the mean ± SEM.

2.4. Discussion

Previous work using the SLR task provided compelling evidence that BDNF in the DG

is critically involved in the molecular mechanisms underlying pattern separation (Bekinschtein et al., 2013). The experiment presented in this chapter was designed to examine whether the BDNF enhancement of pattern separation (previously reported by Bekinschtein et al., 2013) required NMDA activation. Rats received combined pre-sample and post-sample infusions and were tested on the extra small separation condition of SLR. The results revealed that pre-sample DG infusions of the competitive NMDA receptor antagonist, AP5, completely blocked the beneficial effect of post-sample BDNF infusions on SLR performance. Only rats that received saline pre-sample infusions combined with post-sample BDNF infusions showed a preference for the novel location during the choice phase. These results suggest that NMDA receptor activation is part of the mechanism involved in BDNF-dependent pattern separation.

These findings are consistent with evidence demonstrating that DG NMDA receptors are required for discriminating between similar contexts (McHugh et al., 2007), and fit with the wider literature suggesting that NMDA receptors are a candidate through which BDNF-related plasticity mechanisms are mediated. Not only are BDNF and TrkB localized at glutamatergic synapses (Drake et al., 1999), but BDNF-induced plasticity and memory have been linked to NMDA glutamate receptor activation in the hippocampus (Suen et al., 1997; Mizuno et al., 2003).

In isolated hippocampal cell cultures, BDNF selectively increases tyrosine phosphorylation of the NMDA receptor subunits NR1, NR2A, and NR2B (Suen et al., 1997; Lin et al., 1998; Caldiera et al., 2007), and this regulation of the phosphorylation of NMDA receptors enhances NMDA receptor activity (Levine et al., 1998). Furthermore, an NMDA receptor antagonist (MK-801) has been shown to block this BDNF enhancement of synaptic transmission (Levine et al., 1998). In addition to these rapid effects on activity, BDNF increases the translation of NMDA mRNA, which upregulates glutamatergic activity and supports NMDA receptor delivery to the plasma membrane (Calderia et al., 2007).

The exact mechanism by which BDNF acts through NMDA receptors is unclear. TrkB receptors are located in axons, nerve terminals, and dendritic spines of glutamatergic pyramidal and granule cells in the hippocampus (Drake et al., 1999) and are present in one-third of glutamatergic synapses in the hippocampus (Pereira et al., 2006). There is

also some evidence that TrkB receptor activation can modulate glutamatergic pathways both pre and post-synaptically (Drake et al., 1999), so the specific process by which BDNF acts on NMDA receptors is not known.

One hypothesis is that the key molecule liking BDNF and NMDA receptors is tyrosine kinase Fyn. Fyn is a downstream neurotrophin in the BDNF/TrkB signalling pathway (Iwasaki et al., 1998) that binds to NMDA receptors (Takagi et al., 1999), and mediates Nr2B phosphorylation at tyr-1472 (Nakazawa et al., 2001). Together this suggests that Fyn may be the direct link between TrkB and NMDA receptor phosphorylation (Mizuno et al., 2003). Further work is needed to elucidate the exact mechanism by which BDNF acts through NMDA receptors in the DG to regulate pattern separation, but the results described in this chapter are consistent with the existing literature.

The rationale behind the SLR task is when objects are closer together it is more challenging to form representations that are distinct and resistant to confusion. This explains why the extra small separation condition is the most difficult for the rats. If representations are not sufficiently separated, then presenting the new intermediate location will activate the same representation in memory of the sample location and thus will not be distinguishable or perceived as novel. Because we have shown that DG manipulations impair performance only in the case where spatial representations need to be separated (i.e., the small and extra small separation conditions), there is strong evidence that SLR is a suitable and reliable task for studying pattern separation (Bekinschtein et al., 2013).

The nature of the SLR task provides several advantages over other tasks used to study pattern separation. The single trial nature, ability to manipulate similarity in a parametric way, identical choice phases in every condition, and not requiring training or rewards are all desirable qualities. However, as with other spontaneous tasks paired with pharmacological manipulations, one limitation is the possibility that the treatments changed non-mnemonic performance variables, such as the animal's motivation to explore an environment or their preference for novelty. Because previous studies demonstrated that the effects of DG manipulations are dependent upon the SLR condition, it is unlikely that these changes in motivation account for the observed differences in discrimination ratios (Bekinschtein et al., 2013). There is no reason to

believe that DG manipulations differentially affect non-mnemonic processes in the small separation condition more than in the large separation condition.

Another potential weakness in the design is the possibility that objects closer together are learned differently than objects further apart. It is possible that animals rely less on distal cues in the small and extra small separation condition, than the large separation condition, and instead use proximal cues. However, this is unlikely to explain our findings because the hippocampus is more often associated with processing distal (allocentric) cues and to a lesser extent, proximal cues (reviewed by Burgess, 2008), such that hippocampal lesions in rodents impair allocentric spatial learning to a greater extent (e.g., Morris et al., 1990; Save & Poucet, 2000).

In summary, Bekinschtein and colleagues (2013) developed the SLR task and identified BDNF as a molecule that is critical for encoding and/or consolidation of similar representations in the DG. The experiment described in this chapter began to explore the critical molecular events underlying the mnemonic process of pattern separation and identified NMDA receptors as a necessary target for BDNF-enhancement. The following three chapters will build on this work to elucidate further how hippocampal plasticity is involved in spatial pattern separation using the SLR task.

Chapter 3: Brain-derived neurotrophic factor (BDNF) interacts with adult-born cells in the dentate gyrus during pattern separation

Hippocampal neurogenesis is a unique form of neural plasticity that results in the generation of new neurons in the dentate gyrus (DG), and persists throughout adulthood. The exact function of these new cells is not yet known; however, there is growing evidence that adult hippocampal neurogenesis makes a distinct contribution to learning and memory, and specifically to pattern separation. Pattern separation is a process for orthogonalizing overlapping input that enables similar events to be stored as distinct representations. The aim of the experiments presented in this chapter was to help elucidate the precise role of adult-born hippocampal neurons and associated molecules. A lentiviral approach was used to specifically block neurogenesis in the DG of rats, by inhibiting Wnt signalling. The results showed that rats with reduced neurogenesis were impaired on the Spontaneous Location Recognition (SLR) task and performance was not rescued by infusion of exogenous brain-derived neurotrophic factor (BDNF). These experiments provide a conceptual advance in our knowledge regarding pattern separation, by revealing that BDNF is one of the upstream signals that affect the plasticity of adult-born young neurons. This work builds on the experiments described in Chapter 2, which demonstrated an important role of hippocampal plasticity-related mechanisms in pattern separation. The experiments described in this chapter were conducted in collaboration with Dr. Pedro Bekinschtein, as well as with Professor Fred Gage and Dr. Dane Clemenson at the Salk Institute in San Deigo, USA. These data have been previously published in Bekinschtein & Kent et al. (2014) as well as in my First Year Report (Kent, 2012).

3.1. Introduction

As described in Chapter 2, we modified a spatial memory paradigm to evaluate pattern separation, referred to as the Spontaneous Location Recognition (SLR) task

(Bekinschtein et al., 2013). Using SLR we demonstrated that N-methyl-D-aspartate (NMDA) receptors in the dentate gyrus (DG) are necessary for BDNF to have an enhancing effect on performance. Having identified the importance of plasticity-related mechanisms in the DG for pattern separation, the next step was to investigate the cell type involved. Because there is some evidence that adult-born neurons are important for pattern separation (e.g., Clelland et al., 2009), the experiments described in this chapter examined the specific role of hippocampal neurogenesis in performance on SLR.

3.1.1. Adult hippocampal neurogenesis

Neural precursor cells are unique in that they are able to self-renew and give rise to neuronal and glial lineages. In the adult mammalian brain, the vast majority of neuronal precursor cells undergo terminal differentiation and are no longer able to divide; however, there is substantial evidence that two regions of the postnatal mammalian brain, the subgranular zone and subventricular zone, maintain a unique form of plasticity that enables the continual production of new neurons throughout adulthood (Altman, 1962; see Aimone et al., 2014 for a review). This process, referred to as adult neurogenesis, produces new cells that during their differentiation into mature neurons possess unique biological properties and eventually become functionally integrated into neural circuits (Carlen et al., 2002; van Praag et al., 2002).

Adult hippocampal neurogenesis in mammals was first discovered by Joseph Altman (1962), who wrote a letter to *Science* describing evidence of proliferation of neurons in the adult rat. This finding challenged the prevailing dogma at the time that the adult mammalian brain was 'post mitotic' with limited regenerative capacity. Altman (1962; 1963) used autoradiography to show that after adult rats were injected with the radioactive nucleoside, thymidine-H^3, glial cells and potentially neurons in the granule cell layer of the DG were labelled, which suggested new cells had been formed. Neurogenesis was suspected because the thymidine-H^3 solution injected was known to label proliferating cells by becoming incorporated into new strands of chromosomal DNA during the synthesis phase (S phase) of mitotic cell division. This finding has been continuously replicated and it has since been shown that a substantial portion of these new cells are neurons (Cameron et al., 1993).

The DG subregion of the hippocampus is characterized by sparse and important projections to CA3 pyramidal cells, through the mossy fibre pathway. The total number of granule cells in the DG of an adult rat is estimated to be 630,000 (Schlessinger et al., 1975), and progenitor cells in the subgranular zone of the DG generate approximately 5000 new neurons each day in 9 - 10 week old adult rats (Gould et al., 1999). These neurons, produced along the border between the granule cell layer and hilus, are initially innervated by septal neurons and mature granule cells and receive feedback from CA3 pyramidal neurons **(**Vivar & van Praag, 2013). By one month of age, these new neurons are innervated by cells in the peririhinal cortex and lateral entorhinal cortex (Vivar & van Praag, 2013), grow axons onto target cells in CA3 (Toni et al., 2008; Sun et al., 2013) and CA2 pyramidal neurons (Llorens-Martin et al., 2015), evoke stable action potentials (Gu et al., 2012), and show enhanced synaptic plasticity that is maintained until 7 - 8 weeks of age (Lemaire et al., 2012). For example, Marin-Burgin and colleagues (2012) demonstrated that 4-week old granule cells require less input strength to reach the action potential threshold, and are more likely to be active *in vivo*.

In addition to unique electrophysiological properties, adult born neurons retain a long-term capacity to alter the shape of their dendritic tree in response to experiences, such as spatial learning (Lemaire et al, 2012). The survival and death of these immature neurons, as well as the enhanced plasticity, appears to be input-dependent through NMDA receptors (Tashiro et al., 2006; Ge et al., 2007) and is affected by experience, including hippocampal dependent learning (Gould et al., 1999; Dobrossy et al., 2003; Dupret et al., 2007; Tashiro et al., 2007; Epp et al., 2007). For example, a groundbreaking study by Gould and colleagues (1999) demonstrated that training on the hippocampal-dependent trace eyeblink conditioning associative learning task doubled the number of adult-born neurons in the DG.

Not only does experience affect neurogenesis, but despite the small number of new neurons that survive, several studies indicate that these new cells make distinct contributions to learning and memory (Shors et al., 2001, 2002; Bruel-Jungerman et al., 2005; reviewed by Koehl & Abrous, 2011 and Oomen et al., 2014). For example, in rodents, down-regulating DG neurogenesis can impair performance on tasks such as the Morris water maze (MWM; e.g., Dupret et al., 2008), trace fear conditioning (e.g., Shors et al., 2002), nonmatching-to-sample (e.g., Winocur et al., 2006), radial-arm maze

(e.g., Clelland et al., 2009), contextual fear conditioning (e.g., Pan et al., 2012), and olfactory discrimination (e.g., Luu et al., 2012). In contrast, upregulating DG neurogenesis has been shown to provide cognitive enhancing effects on similar hippocampus-dependent tasks (e.g., Nilsson et al., 1999; Sahay et al., 2011a; Marlatt et al., 2012). In particular, there is growing evidence from behavioural experiments that adult-born neurons contribute to DG function by facilitating pattern separation (Clelland et al., 2009; Creer et al., 2010; Sahay et al., 2011a; Nakashiba et al., 2012; Tronel et al., 2012; Kheirbek et al., 2012; Bekinschtein et al., 2014; Kent et al., 2015).

3.1.2. Neurogenesis and pattern separation

The first study to provide experimental support for an important role of adult hippocampal neurogenesis for pattern separation was Clelland and colleagues (2009). Their experiment used a delayed non-matching to sample eight-arm radial maze and demonstrated that after undergoing low dose x-irradiation to focally ablate neurogenesis in the hippocampus, mice were selectively impaired when differentiating between adjacent arms (i.e., small separation) but not arms that were further apart (i.e., large separation). The mice were then tested on two hippocampal-dependent touchscreen tasks. The first task evaluated object-in-place learning (i.e., Paired Associates Learning, PAL) and the second was a two-choice spatial discrimination task (i.e., Location Discrimination, LD). The LD task was designed to specifically evaluate spatial pattern separation by controlling the distance between two stimuli presented on the touchscreen (McTighe et al., 2009; Oomen et al., 2013). The irradiated mice showed normal performance on PAL but impaired performance on LD that suggested a selective impairment for fine spatial discriminations, and thus pattern separation. The deficit on the radial arm maze was replicated in mice that had reduced neurogenesis following DG injections of a lentivirus expressing a dominant negative Wnt (dnWnt) protein. Mice with reduced neurogenesis were impaired on the radial arm maze when the correct arm was adjacent to the previously baited arm.

In slight contrast, Swan and colleagues (2014) tested a transgenic mouse model with knocked-down DG neurogenesis, and found that the transgenic mice were impaired on LD but only after reversal. It is unclear why these two knockdown models differed on LD performance but the authors suggest that it could have resulted from the differences

in design and analysis, such that a time-dependent effect and specific impairment in reversal went unnoticed by Clelland and colleagues (2009). Differences in performance could also result from the different methods used to reduce neurogenesis.

Complementing these knockdown studies, Creer and colleagues (2010) demonstrated that voluntary running in young mice was associated with increased neurogenesis in the DG and improved performance on the touchscreen LD task. Hippocampal neurogenesis and task performance were positively correlated in the young mice; however, the running-dependent enhancement in neurogenesis and improvement in performance was not seen in aged mice that exhibited low basal cell genesis. The authors concluded that the increase in neurogenesis was necessary for the running-induced improvement in pattern separation in the young mice.

Further support for the important role of neurogenesis for pattern separation comes from a series of studies employing a contextual fear conditioning paradigm that was modified to assess pattern separation (Sahay et al., 2011a; Tronel et al., 2012; Nakashiba et al., 2012). Sahay and colleagues (2011a) developed a transgenic mouse that enabled the experimenters to inducibly augment the survival of adult-born neurons in the DG. The transgenic mice with increased neurogenesis demonstrated improved performance on a contextual fear conditioning task that used two similar contexts. Mice with increased neurogenesis showed higher levels of discrimination between the two contexts, suggesting that increased neurogenesis improved the ability to differentiate between potentially overlapping contextual representations. In contrast, increasing neurogenesis did not affect performance on Open Field, Light-Dark, Elevated-Plus Maze, or Novelty Suppressed feeding tests. This study also provided support for the findings from Clelland and colleagues' (2009) study because mice lacking adult-born granule neurons were impaired in their ability to distinguish similar contexts in this contextual fear conditioning paradigm.

These findings were replicated by Tronel and colleagues (2012) who developed a double transgenic mouse with impaired DG neurogenesis and tested them on a similar contextual fear conditioning paradigm, which was previously developed by McHugh and colleagues (2007). In this task similar contexts were presented over repeated trials, to evaluate whether the mouse was able to disambiguate the similar contexts. Once

again, the mice with reduced neurogenesis were impaired when discriminating the similar contexts.

Using this same task, Nakashiba and colleagues (2012) tested a triple-transgenic DG-TeTX mouse, which specifically inhibited older granule cells and left younger granule cells largely intact. The aim of this study was to differentiate the function of young and old granule cells during pattern separation. The transgenic mice exhibited enhanced or normal performance when the output of the older granule cells was inhibited. These mice were then treated with irradiation to ablate young adult-born DG granule cells. Without young granule cells, the mice performed worse than control mice when discriminating similar contexts. The authors concluded that the young granule cells but not the older granule cells were required for pattern separation.

3.1.3. Overview

Because of the accumulating evidence from experiments using the radial arm maze, LD touchscreen paradigm, and contextual fear conditioning tasks, demonstrating that adult hippocampal neurogenesis plays an important role in pattern separation, the following experiments were designed to evaluate the role of adult neurogenesis for performance on the SLR task. Because SLR, like LD, parametrically manipulates the similarity of stimuli, these tasks are superior to the fear conditioning paradigms when evaluating pattern separation.

In Experiment 1, a lentiviral vector (LV) injection was used to specifically reduce neurogenesis in the DG of rats, by inhibiting Wnt signalling, which is a principal regulator of adult hippocampal neurogenesis (Lie et al., 2005; Jessberger et al., 2009). Wnt proteins are derived from astrocytes and are important for neural development and differentiation, acting as a key regulator of cell lineage decisions (reviewed by Kleber & Sommer, 2004). Wnt protein signals through a receptor complex composed of members of the Frizzled (Fz) and low-density lipoprotein receptor-related protein (Nrp) families, and activate intracellular signalling pathways including the β-catenin/TCF pathway, which is referred to as the canonical Wnt pathway (Brantjes et al., 2002).

Lie and colleagues (2005) demonstrated that adult hippocampal progenitor cells express receptors and signalling components for Wnt proteins, and that the Wnt β-catenin pathway is active in the subgranule zone of the DG. Using a self-inactivating LV with mutant Wnt1 protein (LV-dnWnt), Lie and colleagues (2005) were able to block Wnt signalling *in vivo,* and demonstrate a marked reduction in bromodeoxyuridine (BrdU)–positive and doublecortin (DCX)-positive neurons, which are commonly used as markers of neurogenesis. This method has since been used successfully to reduce neurogenesis in rats and mice (Clelland et al., 2009; Jessberger et al., 2009).

A benefit of this methodology is that stereotaxic injections specifically target the DG and minimize involvement of extra-DG brain regions. The results of Experiment 1 showed that rats injected with LV-dnWnt had diminished neurogenesis and exhibited a separation-dependent impairment on the SLR task.

In Experiment 2, the same cohort of rats was cannulated and received infusions of recombinant BDNF directly into the DG. The study was designed to rescue performance on the SLR task. The results revealed that rats with reduced neurogenesis did not exhibit performance-enhancing effects of BDNF infusions, whereas the control rats did. These findings suggest that BDNF acts on adult-born neurons in the DG to exert its effect on pattern separation.

3.2. Methods

3.2.1. Subjects

All experimentation was conducted in strict compliance with the UK Animals (Scientific Procedures) Act 1986, the guidelines of the University of Cambridge and the Institutional Animal Care and Use Committee at the Stalk Institute for Biological Studies. Eighteen male Long-Evans rats (380 - 410 g at the start of testing; Harlan, San Diego, CA, USA) were provided with *ad libitum* access to water and food, except during behavioural testing when food was restricted to 17 g per day for each animal. Upon arrival to the Combined Animal Facility at the University of Cambridge, rats were housed four per cage and each cage contained a cardboard sleeping tube. Rats were

handled for at least five days prior to the start of behavioural testing. All procedures were performed during the dark phase of the light cycle (lights on 19:00 - 07:00).

3.2.2. Surgery

Prior to arriving at Cambridge, the rats underwent a procedure to decrease DG neurogenesis by inhibiting Wnt signalling, which was conducted by Dr. Dane Clemenson in Professor Fred Gage's lab (Lie et al., 2005; Jessberger et al., 2009). See Jessberger and colleagues (2009) for a detailed description of the procedure. Lentivirus vectors were prepared as previously described (Lie et al., 2005). All viral stocks were diluted to and injected at 1 x 10^9 transducing units (TU)/ml. The rats were deeply anesthetized with a ketamine/ xylazine/ acepromazine mixture and placed into a stereotaxic apparatus. Rats received either a control green fluorescent protein (GFP) virus (LV-GFP) or a dnWnt virus (n = 9/ group). A total of 6 µl of either LV-GFP or LV-dnWnt was injected over 8 bilateral targets in each hemisphere of the DG: (a/p, m/l, d/v from bregma) – 2.4, ±1, -4.1; -3.2, ±1.2, -4.1; -4, ±2, -3.7; -4.8, ±3, 3.8; -5.4, ±3.8 -4; -5.4, ±4.4, -7.2; -6, ±4, -4.2; -6, ±4, -7.4. At each injection site 0.3 - 0.4 µl of virus was injected slowly over 1 min. Once complete, animals were sutured and given a one-time dose of buprenex.

After Experiment 1, all rats underwent another surgery to implant bilateral 22-gauge indwelling guide cannula targeting the DG (PlasticsOne^{TM}). The surgical procedures were the same as those described in Chapter 2. Briefly, rats were anesthetized with an intraperitoneal (IP) injection of ketamine (Ketalar, 90 mg/kg) and xylazine (Rompun, 6.7 mg/kg), and cannulae were aimed at - 3.9mm, ± 1.9mm, - 3.8mm (a/p, m/l, d/v from bregma; Paxinos and Watson, 1998) and secured using dental cement (A-M Systems) and four jeweler screws. Obturators were inserted into the guides and a screw-on dust cap kept the obturators in place. Following surgery, the rats were housed in pairs and allowed to recover for at least 7 days before further behavioural testing.

3.2.3. Infusions

Prior to the first sample phase of Experiment 2, rats were habituated to the infusion procedure with a mock infusion handling session. The following day rats received

bilateral infusions of either recombinant human BDNF (0.25 µg/ 0.5 µl/side; Byoscience, Cambridge, UK), or physiological saline (0.5 µl/side; 0.9 % sodium chloride, pH 7.0), 5 min after sample. All infusions took place in a preparation room separate from the location of behavioural testing. Animals were restrained gently by the experimenter throughout the infusion process. The same infusion protocol was used as described in Chapter 2. A 28-gauge injector, constructed by connecting polyethylene tubing (0.38 mm inside diameter, A-M Systems) to the injector cannula, was connected to a 5 µl Hamilton syringe. Bilateral infusions were performed simultaneously with a Harvard Apparatus precision syringe pump at a rate of 0.25 µl/min. There was a 1 min rest period before removing the infusers and at least 3 days between repeated infusions.

3.2.4. Behavioural procedures

Experiment 1 and 2 used the same apparatus and SLR protocol described in Chapter 2. Briefly, after 5 days of habituation, rats were shown three identical objects as spatial landmarks as part of the sample phase. The exact positioning was dependent upon the condition (i.e., extra small, small, or large separation condition). During the extra small separation condition, the proximal objects were located 4 spaces apart, which was equivalent to a 40° angle from the centre of the maze. During the small separation condition, the proximal objects were located 5 spaces apart, which was equivalent to a 50° angle from the centre of the maze. During the large separation condition, all three objects were located equidistant apart which was equivalent to 12 spaces and 120° angle from the centre. Rats were given 10 min to explore the objects and arena and were then returned to their home cage for 24 h. The rats were then placed back into the arena and shown two identical objects as part of the choice phase. One object was in a *familiar* location and one was placed in a *novel* location, which was located in between where two of the other previous sample objects had been placed. Rats were allowed to explore the two objects and arena for 5 min.

In Experiment 1, I assessed the effects of a lentiviral vector injection on performance of the SLR task. Eighteen rats were run on two trials of SLR: small separation and large separation conditions. Figure 3.1 illustrates these two conditions. The conditions were counterbalanced between rats, such that half received the small separation condition

during trial 1 and the other half received the large separation condition during trial 1, and the opposite for trial 2. There was at least a 48 h rest period between trials.

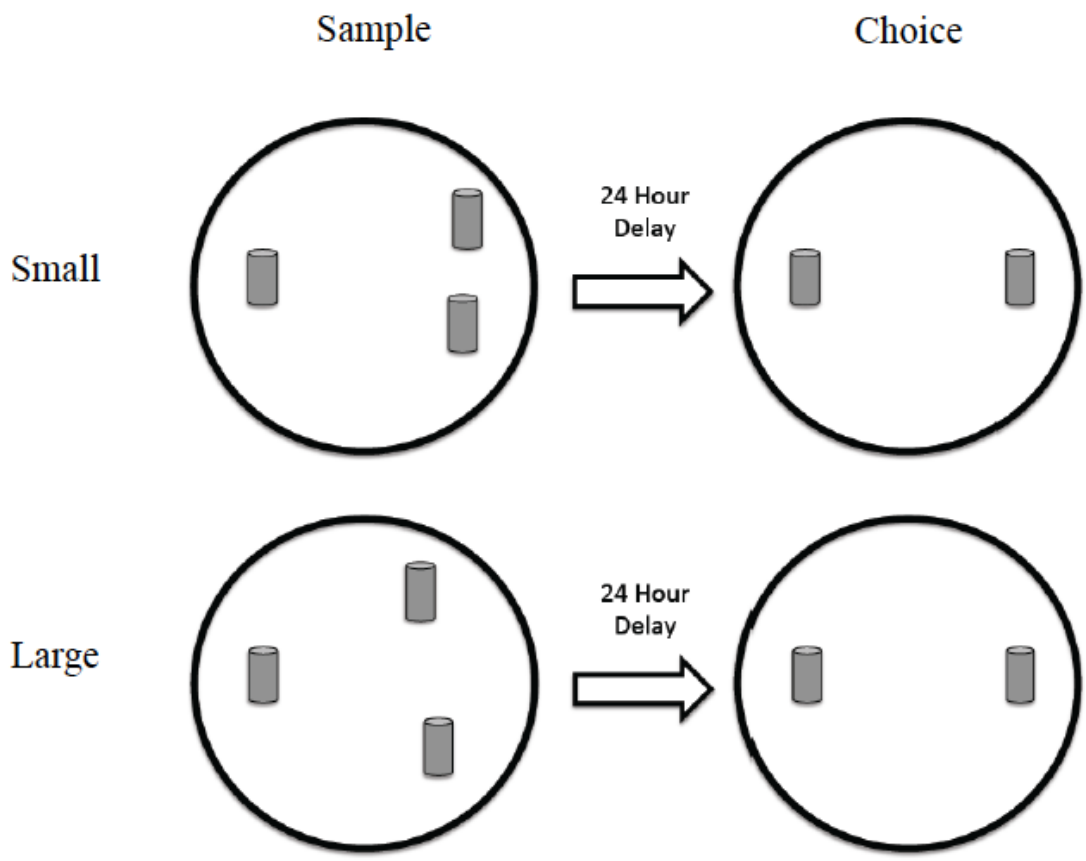

Figure 3.1. Schematic of the small and large SLR conditions used in Experiment 1. Each rat was tested on the small separation condition (top; the proximate objects were placed 5 spaces apart, equivalent to a 50° angle from the centre) and the large separation condition (bottom; objects placed 12 spaces apart, equivalent to 120° angle from the centre) of the SLR task. Rats were shown three objects during the sample phase and then 24 h later were shown two objects during the choice phase. One of the objects presented during the choice phase was in a 'familiar' location and one object was in a 'novel' location. The novel location was directly halfway between the locations of the proximate objects shown during the sample phase.

In Experiment 2, I assessed the effects of a BDNF infusion on performance on the SLR task, and compared rats with attenuated neurogenesis with controls. The same 18 rats were run on two trials of the extra small separation condition with a 72 h rest period between trials. Rats received a BDNF or saline infusion immediately after the sample phase on counterbalanced trials. Figure 3.2 illustrates the extra small SLR condition and the timing of the infusions.

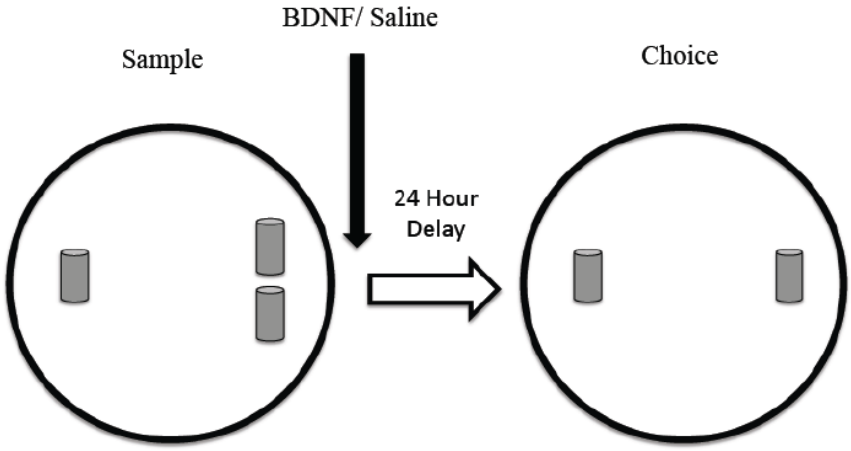

Figure 3.2. Schematic of the extra small SLR condition used in Experiment 2. Each rat was tested using two trials of the extra small separation condition of the SLR task. Two of the three objects presented during the sample phase were placed closer together (4 spaces apart, equivalent to a 40° angle from the centre) than the placement previously used for the small (5 spaces) and large (12 spaces) separation conditions. BDNF or saline was injected into the DG 5 min after the sample phase. Treatment was counterbalanced.

3.2.5. Histology

Once behavioural testing was complete, rats were anaesthetized by IP injection with 2 ml of Euthatal (Rhone Merieux, Harlow, Essex, UK) and perfused transcardially with phosphate buffered saline (PBS), followed by 10 % neutral buffered formalin (NBF). The brains were removed and post-fixed in NBF for at least 24 h, followed by immersion in a 30 % sucrose solution for at least 48 h. The brains were cut in 30 μm sections using a freezing microtome.

3.2.6. Immunohistochemistry

Immunohistochemistry was conducted in collaboration with Dr. Charlotte Oomen who was a postdoctoral researcher in our lab. To assess the level of neurogenesis in animals, brains were prepared for immunohistochemistry for the microtubule-associated protein doublecortin (DCX) as described previously (Oomen et al., 2010). DCX is associated with migration of neuroblasts and used as a marker for immature neurons because it is transiently expressed in proliferating progenitor cells and newly generated neuroblasts

(Brown et al., 2003). DCX immunoreactivity decreases sharply when newly generated cells mature, making it a useful marker for identifying immature neurons.

Sections were incubated in primary antibody (polyclonal goat anti-DCX, Santa Cruz, 1:800) and signal amplification was accomplished by further incubation with biotinylated secondary antibody (horse anti goat, 1:500, Vectorlabs) and avidin-biotin enzyme complex (ABC kit; Elite Vectastain; 1:800). Subsequent chromogen development was performed with diaminobenzidine (DAB; 20 mg/100 ml Tris buffer; TB, 0.01% hydrogen peroxide; H_2O_2). DAB is a chromogen oxidized by H_2O_2 (Hsu & Soban, 1982). The oxidative polymerization of DAB is widely used in histochemistry for identifying biological materials labelled with peroxidase or possessing intrinsic peroxidase activity. DAB staining appears as light brown deposits on the sections.

3.2.7. Data collection

Exploration was defined as previously described in Chapter 2. Only when the rat was directing its nose within 2 cm or less of the object, was it included as exploration. Sitting or digging was not considered exploratory behaviour. The experimenter recorded sample phase exploration using stopwatches and choice phase exploration using a computer program written in Visual Basic 6.0 (Microsoft Corp., USA).

Stereological quantification (StereoInvestigator, Microbrightfield, Germany) was conducted using a stage-controlled brightfield microscope (40x objective). Cells were counted at sites that were selected using systematic random sampling, in every tenth coronal section starting at bregma -2.1 with a total of 6 sections per animal. StereoInvestigator optical fractionator settings for DCX quantification were as follows: grid size 200 x 80 and counting frame 50 x 50, which resulted in an average of 250 markers counted per animal.

3.2.8. Data analysis

For the behavioural analysis, the sample exploration time for each of the three objects was converted into a percentage of the total exploration time and was analysed using a one-way analysis of variance (ANOVA) to ensure the three sample objects were being

explored equally. An unpaired two-tailed Student's *t*-test was also conducted to ensure that total sample exploration was equal between groups. Results from the choice phases were expressed as discrimination ratios (D2), which were calculated as follows:

$$D2 = \frac{novel\ location\ exploration - \ familiar\ location\ exploration(s)}{novel\ location\ exploration + \ familiar\ location\ exploration\ (s)}$$

Group mean D2 scores were analysed with repeated measures ANOVA, followed by *post-hoc* Student's *t* contrasts using the Bonferroni correction.

For the histological analysis, total numbers of DCX-positive cells were quantified by systematic random sampling performed with the Stereo Investigator system (MicroBrightField). DG granule cell layer and molecular layer surface area and volume measurements were performed according to Cavalieri's principle using the Stereo Investigator system (MicroBrightField). Estimates for the total number of DCX-positive cells in the DG were calculated using the random samples and measurements collected. The results from the histological analysis were analysed using Student's *t*-test to compare groups.

All statistical analyses were conducted with SPSS version 22 and Microsoft Excel version 14.4.5. Statistical significance was set at $p < 0.05$, unless running a *post-hoc* comparison. To control for Familywise error, the level of statistical significance (i.e., α) for *post-hoc* comparisons was calculated using the Bonferonni correction, which was calculated as 0.05 divided by the number of statistical comparisons. All data are presented as mean ± standard error of the mean (SEM).

3.3. Results

3.3.1. dnWnt virus injected into the DG reduces hippocampal neurogenesis

We used a lentiviral approach to specifically knock down neurogenesis in the DG of adult rats by inhibiting Wnt signalling, using a dnWnt protein. Levels of neurogenesis

were determined by counting the DCX-positive cells within the DG of LV-GFP (i.e., control) or LV-dnWnt injected rats. LV-dnWnt rats showed a significant decrease in DCX-positive cells compared to LV-GFP rats (p = 0.009, n = 9 per group). Figures 3.3 and 3.4 show the estimated total number of DCX-positive cells and representative images of DCX-stained DG sections from the two groups.

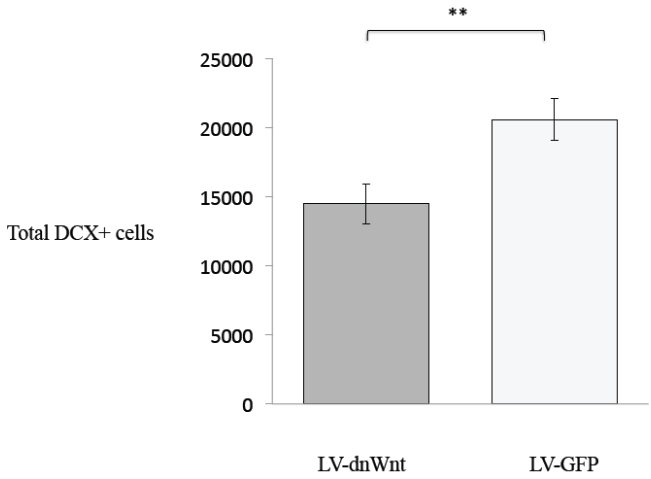

Figure 3.3. Total number of DCX-positive cells in the DG. Rats were treated with a LV-dnWnt (left) or a LV-GFP (right). ** p = 0.009, n = 9 per group. Data are expressed as the mean ± SEM.

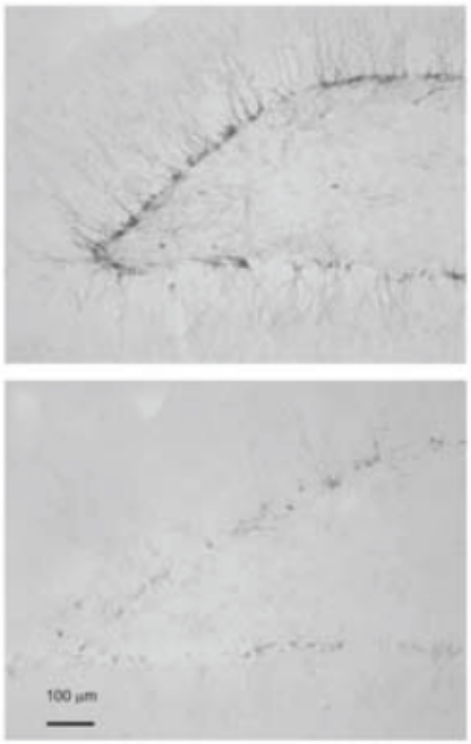

Figure 3.4. Representative images of DCX-stained DG sections. Samples of an LV-GFP-treated rat (top) and an LV-dnWnt-treated rat (bottom) stained with an anti-DCX antibody and DAB.

3.3.2 Reduced neurogenesis impairs spatial pattern separation

To investigate the role of adult-born neurons during pattern separation, we used the SLR task to evaluate the effect of decreasing adult neurogenesis in the DG on memory consolidation when the load on pattern separation was high (i.e., small separation condition) compared to when it was low (i.e., large separation condition). Each rat was tested on both conditions.

A mixed measures ANOVA revealed a significant interaction of treatment and separation (p = 0.012, F (1,17) = 8.23). Figure 3.5 shows the D2 values for each condition. Post-hoc contrasts revealed a significant difference between the LV-dnWnt rats ($p < 0.01$) and control group in the small but not the large separation condition. The LV-dnWnt rats showed a deficit in the small separation condition, but not in the large separation condition, whereas the performance of the control rats did not vary with either configuration.

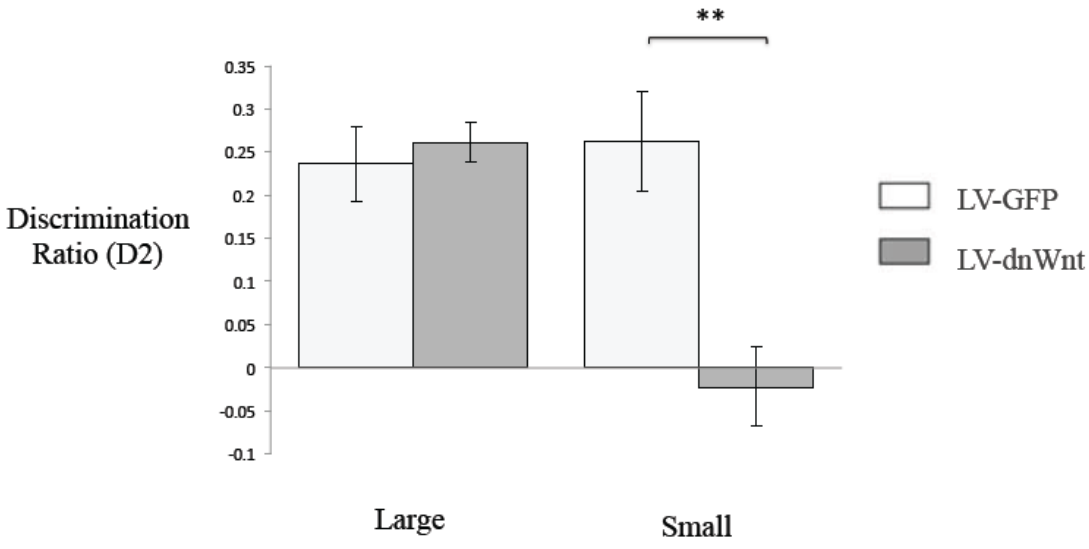

Figure 3.5. Discrimination ratios for LV-GFP and LV-dnWnt rats on the small and large separation conditions of SLR. The LV-GFP and LV-dnWnt rats were evaluated on the large separation (left) and the small separation (right) conditions of the SLR task. Positive D2 values reflect a preference for the novel location. The LV-dnWnt group did not show a preference for the novel location in the small separation condition. ** $p <$ 0.01, n = 9 per group. Data are expressed as the mean ± SEM.

As shown in Figure 3.6, there was no difference between the LV-GFP and LV-dnWnt rats in the total amount of time spent exploring during the sample phase (p = 0.093).

Similarly, as shown in Figure 3.7, both LV-GFP and LV-dnWnt-treated rats spent an equal proportion of time exploring each of the three objects. There was no main effect of treatment (p = 0.50) or location (p = 0.79) on the percentage of time spent exploring each object. This indicates that the differences in discrimination ratios cannot be explained by preferential exploration of the more separated location during the sample phase.

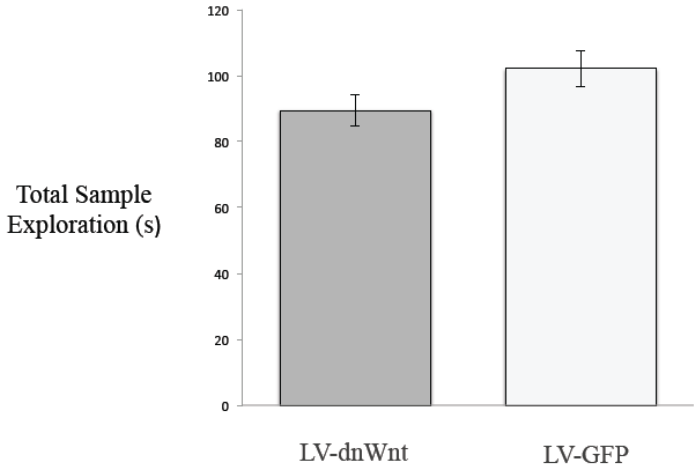

Figure 3.6. Total time spent exploring during the sample phase in Experiment 1. There was no difference between the LV-dnWnt (left) and LV-GFP (right) groups in the total amount of time spent exploring during the sample phase. Data are expressed as the mean ± SEM.

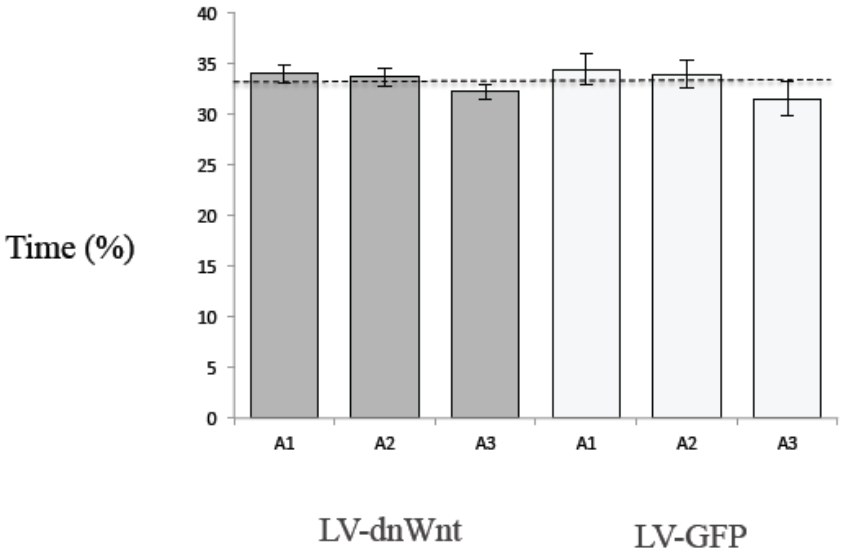

Figure 3.7. Percentage of time spent exploring each of the locations during the sample phase in Experiment 1. The data shown are from the LV-dnWnt (left) and LV-GFP (right) groups during the sample phase of the small separation condition of the SLR task. A1, A2, and A3 represent each of the 3 locations. Data are expressed as the mean ± SEM.

3.3.3. Newborn immature neurons are required for BDNF-dependent pattern separation

To test whether infusing BDNF could rescue the deficit associated with reduced neurogenesis in the LV-dnWnt rats, performance was evaluated on two trials of the extra small separation SLR condition with post-sample BDNF injections into the DG. A two-way repeated measures ANOVA revealed that there was a significant interaction of treatment and drug (p = 0.0077, F (1,17) = 9.26). Post-hoc comparisons showed a significant difference in the effect of the BDNF infusion in LV-GFP rats compared to LV-dnWnt rats ($p < 0.01$). Figure 3.8 shows the D2 values for the extra small separation conditions. BDNF but not saline injections enhanced performance during the choice phase in the LV-GFP group, but this effect was not seen in the LV-dnWnt group.

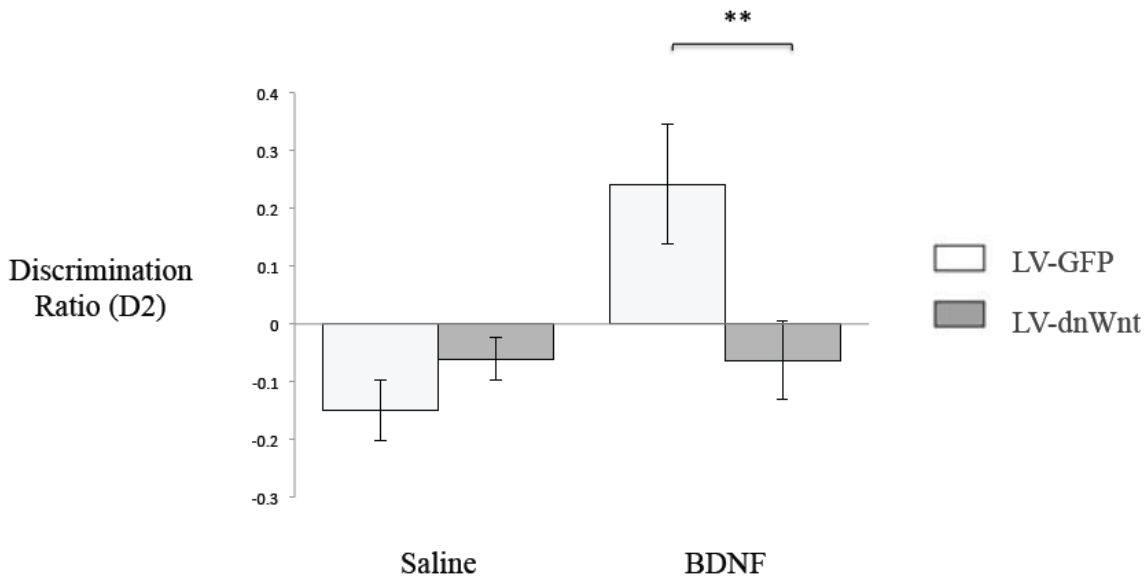

Figure 3.8. Post-sample BDNF but not saline infusions enhanced performance during the choice phase in the control group. The LV-GFP-treated group but not LV-dnWnt-treated group showed a preference for the novel object in the extra small separation condition when treated with post-sample BDNF. Positive D2 values reflect a preference for the novel location. **$p < 0.01$, n = 9 per group. Data are expressed as the mean ± SEM.

As shown in Figure 3.9, there was no difference between the LV-GFP and LV-dnWnt groups in the amount of total time spent exploring during the sample phase (p = 0.60). Similarly, as shown in Figure 3.10, during the sample phase, the LV-GFP and LV-

dnWnt rats spent equal proportion of time exploring each of the three locations. There was no main effect of treatment (p = 1.0) or location (p = .158). This indicates that the differences in the D2 scores cannot be explained by preferential exploration during the sample phase.

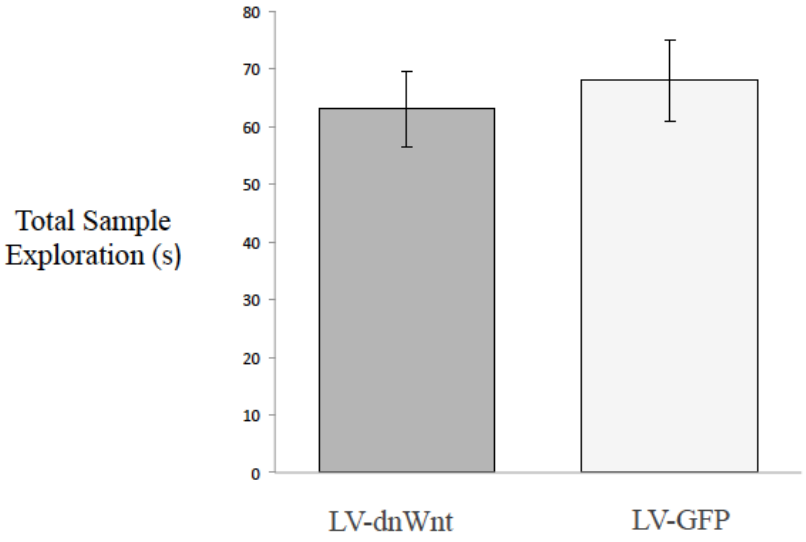

Figure 3.9. Total time exploring during the sample phase in Experiment 2. The LV-dnWnt (left) and LV-GFP (right) groups did not differ in the amount of time spent exploring the sample objects. Data are expressed as the mean ± SEM.

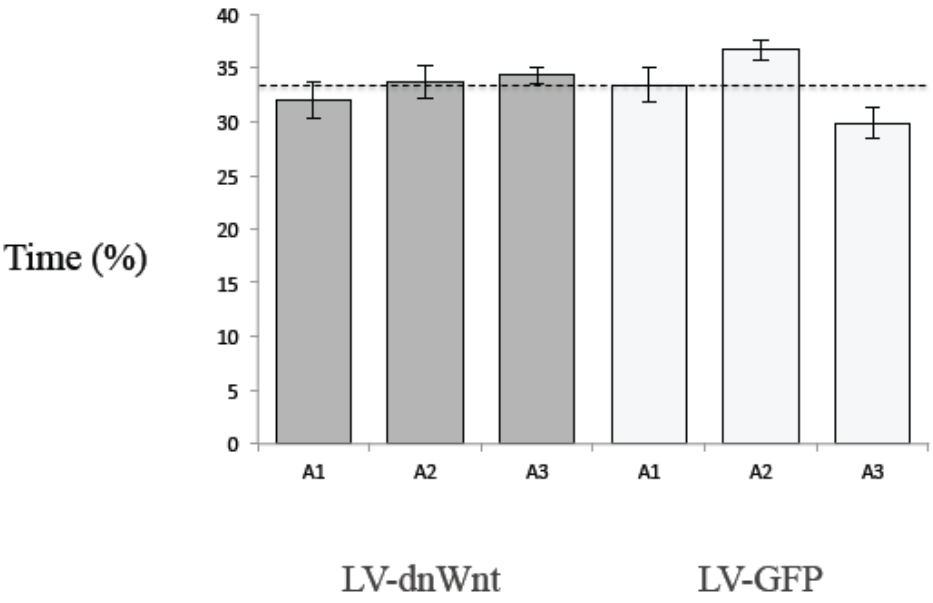

Figure 3.10. Percentage of time spent exploring each of the locations during the sample phase in Experiment 2. Both LV-GFP and LV-dnWnt rats spent equal proportion of time exploring each of the three locations during the sample phase of the extra small separation condition of the SLR task. A1, A2, and A3 represent each of the 3 object locations. Data are expressed as the mean ± SEM.

3.4. Discussion

The experiments described in this chapter investigated the function of hippocampal neurogenesis and further elucidate the mechanisms underlying the actions of BDNF in DG-dependent spatial pattern separation. This builds directly from the experiments presented in Chapter 2 (Bekinschtein et al., 2013). In agreement with previous reports of the important role of neurogenesis for pattern separation (e.g., Clelland et al., 2009; Creer et al., 2010; Sahay et al., 2011a; Nakashiba et al., 2012; Tronel et al., 2012) the results show that DG adult-born neurons are necessary for pattern separation when encoding spatially proximate objects and the BDNF-dependent enhancement.

A lentiviral approach was used to specifically inhibit neurogenesis in the DG of adult male rats by inhibiting Wnt signalling (Lie et al., 2005). Previous studies have validated this method in both rats and mice (Clelland et al., 2009; Jessberger et al., 2009). In the present study, the LV-dnWnt-treated rats, which had inhibited DG neurogenesis, demonstrated impaired recognition when objects were placed in similar spatial locations (i.e., small separation condition) but not dissimilar locations (i.e., large separation condition). This finding is consistent with our hypothesis that attenuating the production of adult-born DG neurons only impairs memory retrieval when spatial representations need to be separated, such as in the small separation condition. The control group of rats that had been injected with LV-GFP, performed equally well when presented with the small or large configurations; thus performance of the control group was independent of the load on pattern separation.

The same rats were then evaluated on the extra small separation condition of the SLR task. The extra small separation condition is used when evaluating enhancement effects because it introduces a higher load on pattern separation by positioning the landmarks closer together. The results showed that infusing recombinant BDNF enhanced performance on the extra small separation condition in the control group, but not in the LV-dnWnt group. Thus, rats with reduced neurogenesis did not exhibit the performance-enhancing effect of BDNF infusions that was exhibited by the control group. This finding indicates that adult-born immature neurons in the DG are required for BDNF action during pattern separation in the SLR task, suggesting that the new neurons are necessary for memory consolidation of similar representations.

The mechanism through which adult-born neurons may enhance DG-dependent pattern separation is not fully understood. The results presented in this chapter suggest that BDNF is one of the upstream signals that affect the plasticity of adult-born neurons during pattern separation. It is possible that the unique physiological properties, such as a relatively low firing threshold and plastic nature of immature adult-born neurons (Schmidt-Hieber et al., 2004; Ge et al., 2007), make these cells more sensitive to BDNF levels present in the hippocampus. Another possibility is that BDNF exerts its effect by upregulating neurogenesis (Scharfman et al., 2005), although this is unlikely to explain the results because the time course of the BDNF requirement for the SLR task (less than 24 hours) and development and incorporation of newborn cells into the circuits (weeks) are very different.

The finding that BDNF may affect the plasticity of adult-born neurons is consistent with previous research. Immunoreactivity for the BDNF receptor, TrkB, is detected during the first week of maturation of proliferating cells in the DG and increases with neuronal maturity (Donovan et al., 2008). Furthermore, ablating the TrkB receptor in progenitor cells has been shown to reduce the growth of dendrites and spines in new neurons, impair LTP, and reduce cell survival (Bergami et al., 2008).

Because BDNF receptors are located on immature neurons, it suggests that the observed effects during pattern separation could be a result of BDNF activating the TrkB receptors on immature neurons. Moreover, because Bekinschtein and colleagues (2013) demonstrated that rats exposed to the small separation condition spontaneously upregulated BDNF, it is possible that the spontaneous expression of BDNF acts on the hyper-plastic new neurons, and together this is the necessary stimulus for memory consolidation of similar spatial representations; as inputs become more similar, more BDNF acting on immature neurons is required to separate the overlapping representation.

Building on the results presented in Chapter 2, which demonstrated that BDNF acts upstream of NMDA receptors in the DG during pattern separation, it is possible that TrkB activation enhances plasticity of the adult-born neurons by interacting with NMDA receptors. It has been shown that BDNF-activated TrkB receptors increases NMDA activation (Levine & Kolb, 2000) and this interaction with BDNF/TrkB

signalling is important for spatial memory (Mizuno et al., 2003). Furthermore, the major excitatory input to DG granule cells is from the entorhinal cortex, transmission depending upon NMDA receptors (Collingridge, 1989), and the survival of adult-born cells is mediated by NMDA activation (Cameron et al., 1995; Tashiro et al., 2006). The critical period for NMDA receptor-dependent survival/death is restricted to the third week after neuronal birth (Tashiro et al., 2006). Together, this suggests that BDNF may be affecting immature neurons via NMDA activation.

There is also some evidence that NMDA receptors on adult-born neurons are important for pattern separation (Kheirbek et al., 2012). Kheirbek and colleagues (2012) demonstrated that transgenic mice with deleted Nr2B-containing NMDA receptors in adult-born granule cells were impaired on a fear conditioning paradigm using similar contexts, which is a commonly used behavioural indictor of a pattern separation deficit. More work is needed to elucidate the exact mechanisms by which BDNF, NMDA receptors, and adult-born neurons interact during pattern separation, but one possibility is that BDNF acts upstream of NMDA receptors that are located on new-born neurons.

More broadly, the mechanism by which adult-born neurons directly support memory processing remains elusive. It is unclear whether adult-born neurons perform the computation underlying pattern separation or just interact with the local network to contribute to the computation. There are several hypotheses as to how the unique electrophysiological properties or the enhanced capacity for structural plasticity of immature neurons may modulate DG network activity. Some suggest that young granule cells act as primary coding units, encoding information during the initial hyper-plastic period (e.g., Becker, 2005; Alme et al., 2010). This is supported by the fact that adult-born neurons are preferentially incorporated in spatial memory networks (Kee et al., 2007; Tashiro et al., 2007). Alternatively, it has been suggested that adult-born neurons control DG function in an indirect manner, by modulating inhibitory tone (see Piatti et al., 2013 for review).

Becker (2005) proposes a model in which neurogenesis acts to reduce interference by directly enhancing pattern separation mechanisms, suggesting that a functional role for neurogenesis is to create distinct memory traces. Becker's simulation shows that neuronal turnover is beneficial when encoding highly confusable items during repeated

learning sessions because the new neurons increase the diversity of available connections. Thus, the plasticity of young neurons yields different functional populations, potentially improving pattern separation.

It should be noted that Groves and colleagues (2013) developed a novel genetic model to knockdown neurogenesis, which ablated 98 % of new neurons in rats. This extensive knockdown had no effect on performance on the radial arm maze, spatial reference memory in the water maze, or contextual or cued fear conditioning. The lack of effect on cued and contextual fear conditioning and the water maze is not surprising, given that similarity was not controlled for. However, the surprising result was that the knockdown did not affect performance on radial arm maze tasks, which were specifically designed to assess pattern separation. The first task used three conditions: single arm, pair arms, and trio arms. The trio arms condition was hypothesized to have the highest load on pattern separation because of the interference created by the adjacent arms in the trio. The single arm was hypothesized to present the lowest load on pattern separation. The researchers also carried out a binary choice, delayed non-matching to sample task using the radial maze, which had been developed by Clelland and colleagues (2009) to evaluate pattern separation in mice. Surprisingly, in both tasks, separation had no effect on performance of the control or knockdown group. In the first radial arm maze task, the most errors were actually made during the single arm condition, which was designed to have the lowest amount of interference and to be the easiest condition.

The conflicting results might be explained by the different methods to reduce neurogenesis. Even though the irradiation used by Clelland and colleagues (2009) and the genetic knockdown used by Groves and colleagues (2013) both resulted in almost complete ablation of new cells in the DG, it is possible that the methods had different unspecified off-target effects that explain the contradictory findings. Another possibility is that the parameters of the tests used by Groves and colleagues (2013) did not vary the load on pattern separation to the same extent as the parameters used by Clelland and colleagues. The eight-arm radial maze used by Clelland and colleagues was a rat-sized radial arm maze (each arm was 76.2 cm long) but used for mice, whereas Groves and colleagues used a twelve-arm radial maze for rats (each arm was 60 cm long). Although the mazes were similar in size, the maze used by Clelland and colleagues is a much

larger relative space for a mouse, than the maze used by Groves and colleagues for a rat. Because of the species variation in size, the difference between arms from a mouse's perspective may appear much larger than the same distance from a rat's perspective. The relative distance between the arms may change the amount of overlap in inputs, and thus the load on pattern separation. Further research is needed to evaluate how maze size and other differences between these studies can affect pattern separation processes.

The Groves and colleagues (2013) study also included a meta-analysis of the adult neurogenesis literature and found no significant effect of ablating adult neurogenesis on spatial memory. If neurogenesis plays a specific role in pattern separation, then the high level of heterogeneity is not surprising given pattern separation was not controlled for in the vast majority of the studies (Bekinschtein et al., 2011).

As a final note, studying the function of hippocampal neurogenesis has potential clinical relevance because neurogenesis has also been identified in humans (Eriksson et al., 1998; Spalding et al., 2013). Eriksson and colleagues (1998) treated cancer patients with BrdU and identified BrdU-immunoreactive cells in the DG granule cell layer, subgranular zone, and subventricular zone. Following this study, Spalding and colleagues (2013) developed a method for identifying neurogenesis in humans by taking advantage of elevated atmospheric ^{14}C levels caused by above ground testing of nuclear bombs during the Cold War (1955-1963). During mitosis ^{14}C is integrated into synthesized genomic DNA with a concentration corresponding to the atmospheric levels at the time. Using this method, Spalding and colleagues (2013) were able to show that neurogenesis occurs in the human hippocampus throughout adulthood, at an estimated rate of 700 new hippocampal neurons each day.

The experiments described in the following two chapters (Chapter 4 and 5) were designed to further elucidate the role of adult hippocampal neurogenesis in spatial pattern separation. Chapter 4 examines the effects of increasing neurogenesis, using a ghrelin-treatment, on SLR performance and then Chapter 5 examines whether it is possible that the process of pattern separation has a reciprocal relationship with neurogenesis, such that engaging pattern separation increases hippocampal neurogenesis. Together, these studies help elucidate the relationship between hippocampal neurogenesis and pattern separation.

Chapter 4: The orexigenic hormone acyl-ghrelin increases adult hippocampal neurogenesis and enhances pattern separation

The gut hormone ghrelin is an orexigenic peptide that is elevated during calorie restriction (CR) and known primarily for stimulating growth hormone release. Recently, there has been accumulating evidence that ghrelin also has important extra-hypothalamic functions, such as enhancing synaptic plasticity and hippocampal neurogenesis. The experiment described in this chapter was designed to evaluate the long-term effects of elevating acyl-ghrelin levels on the number of new adult-born neurons in the dentate gyrus (DG) and performance on the Spontaneous Location Recognition (SLR) task. This builds on the work described in Chapters 2 and 3, which demonstrated that performance on SLR is DG-dependent and sensitive to manipulations of plasticity mechanisms and cell proliferation in the DG. The results presented in this chapter reveal that peripheral treatment of physiological levels of acyl-ghrelin enhances both adult hippocampal neurogenesis and spatial pattern separation. Although not directly tested, these results suggest that the beneficial mnemonic effects of elevated ghrelin levels may result from the increased neurogenesis. This work has been previously published in Kent et al., (2015). The immunohistochemistry and imaging were conducted by Amy L. Beynon and Amanda K. Hornsby in Dr. Jeff Davies's lab at Swansea University.

4.1. Introduction

4.1.1. Ghrelin and caloric restriction

One of the earliest clues that an important relationship exists between metabolic state and cognitive functioning was the benefits following a behavioural intervention referred to as calorie restriction (CR), which reduces daily food intake by approximately 25 %

(reviewed by Fontán-Lozano et al., 2008 and Gillette-Guyonnet & Vellas, 2008). The first demonstration that CR exerts beneficial effects on the brain was by Joseph and colleagues (1983), who showed that CR in rats reduced striatal receptor loss and some of the motor-behavioural deficits associated with ageing. Since this initial study, CR has been repeatedly shown to enhance cognition in rodents (Ingram et al., 1987; Stewart, Mitchell, & Kalant, 1989), to be neuroprotective in animal models of ageing and neurodegenerative disease (Bruce-Keller et al., 1999; Duan et al., 2001; Maswood et al., 2004; Wang, 2005; Youssef et al., 2008; Zhu, Guo, & Mattson, 1999), and to improve memory in humans (Witte et al., 2009). However, the mechanisms underlying the beneficial neuroprotective and cognitive enhancing effects of CR are only beginning to be understood.

The aldehyde dehydrogenase (NAD)-dependent protein deacetylase sirtuin-1 (SIRT1) mediates, at least in part, the cellular effects of CR (Graff et al., 2013; Guarente, 2013) by increasing autophagy and related processes (Bergamini et al., 2003; Morselli et al., 2010). Furthermore, SIRT1 is required for long-term potentiation (LTP) in CA1 hippocampal neurons and activation of the SIRT1 signalling pathway promotes cognition (Gao et al., 2010).

Upstream of SIRT1, the peptide hormone ghrelin is one mechanism by which the cognitive enhancing effects of CR may occur. Whilst predominately known for its growth hormone releasing and orexigenic properties, the list of functions and biological effects produced by the peptide ghrelin continue to be identified. Not only does ghrelin act in the pituitary and hypothalamus to regulate energy homeostasis, appetite, body weight, and adiposity (Castañeda et al., 2010; Chen et al., 2009; Davies et al., 2009; Kojima & Kangawa, 2005; Kojima et al., 1999; Tschöp, Smiley, & Heiman, 2000), but recently the extra-hypothalamic actions of ghrelin, such as pro-cognitive, antidepressant, and neuroprotective properties have also been identified (Andrews, 2011; Asakawa et al., 2001; Frago et al., 2011; Kanehisa et al., 2006; Lutter et al., 2008; Moon et al., 2009; Steiger et al., 2011). Because SIRT1 has been shown to mediate the anti-apoptotic and orexigenic actions of ghrelin (Shimada et al., 2014; Velasquez et al., 2011), it is therefore possible that the circulating levels of ghrelin, which is secreted from the stomach during periods of CR, may link energy balance and cognition through SIRT1.

4.2.2. Ghrelin and memory

Carlini and colleagues (2002) were the first to demonstrate that ghrelin treatment can improve memory retention. The researchers administered the peptide via an intracerebroventricular (ICV) injection in rats and found that the treatment improved memory in a dose-dependent manner, as measured by latency time in a step-down behavioural test. Since that initial study, the beneficial mnemonic effects of intracranial infusions or systemic ghrelin treatment have been repeatedly shown (Atcha et al., 2009; Carlini et al., 2004; 2007; 2008; 2010a; 2010b; Chen et al., 2011; Chen, 2012; Diano et al., 2006; Tóth et al., 2010). Atcha and colleagues (2009) have also replicated the cognitive enhancing effects of ghrelin using two structurally non-peptide ghrelin receptor agonists (Atcha et al., 2009).

Additionally, ghrelin treatments have been shown to affect measures of hippocampal synaptic plasticity (Carlini et al., 2002; 2010b; Chen et al., 2011; Diano et al., 2006) and increase hippocampal cell proliferation and neurogenesis (Li et al., 2013; Moon, et al., 2009; Zhao et al., 2014). A study by Diano and colleagues (2006) demonstrated that ghrelin passively crosses the blood brain barrier and binds directly to neurons in the dentate gyrus (DG), CA1, and CA3 regions of the hippocampus, likely via the only identified ghrelin receptor, the growth hormone secretagogue receptor (GHSR), which is localized in these regions. Their study also showed that the peripheral ghrelin treatment promoted hippocampal LTP generation and increased spine density in the CA1, much like its rapid effects on the synaptic organisation in hypothalamic anorexigenic neurons (Pinto et al., 2004). These changes were accompanied by improvements on the plus-maze, T-maze, and step-down passive avoidance tasks.

As discussed in Chapter 3, hippocampal neurogenesis is a unique form of plasticity that results in the generation of functionally integrated new neurons from progenitor cells in the DG. More recently, it has been shown that systemic ghrelin administration can upregulate neurogenesis (Chen, 2012), and that GHSR null mice have reduced cell proliferation and survival of hippocampal neurogenesis when exposed to social defeat stress (Walker et al., 2014). These findings place ghrelin in a unique position to connect metabolic state with hippocampal neurogenesis and cognition. As most of these previous studies have used supraphysiological doses, the effects of physiological levels of ghrelin are less well understood.

Several studies indicate that adult-born hippocampal cells make distinct contributions to learning and memory and may be particularly important for pattern separation (reviewed by Oomen et al., 2014). For example, in the experiment described in Chapter 3, rats with inhibited DG neurogenesis were impaired on the Spontaneous Location Recognition (SLR) task only in conditions with a high load on pattern separation (Bekinschtein et al., 2014).

4.1.3. Overview

To investigate whether increasing circulating levels of acyl-ghrelin, within the physiological range, could increase DG neurogenesis and lead to lasting effects on neurogenesis-dependent mnemonic processes, rats were given daily injections of either saline or acyl-ghrelin on days 1 to 14, prior to assessing spatial pattern separation using SLR on days 22 to 26. The timing of the injections is illustrated in Figure 4.1. On days 5 to 8 rats also received injections of the thymidine analogue, 5′-Bromo-2-deoxyuridine (BrdU).

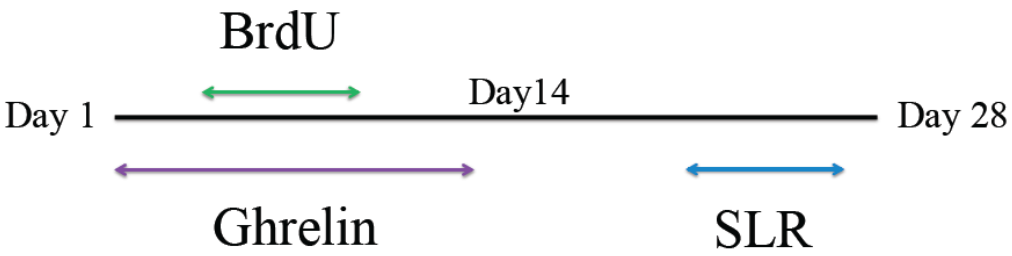

Figure 4.1. Schematic of the timing of the experimental design. Rats were given daily injections of either saline or ghrelin on days 1 - 14 and BrdU on days 5 - 8. Spatial pattern separation was evaluated using SLR on days 22 - 26.

Thymidine is normally incorporated into DNA as a proliferating cell passes through the synthesis phase (S phase) of the cell cycle (Sidman, 1970). BrdU replaces endogenous thymidine during DNA replication and becomes permanently incorporated into the DNA of dividing cells. The effectiveness of BrdU to investigate developmental neurogenesis was first demonstrated by Miller and Nowakowski (1988) and it is now accepted as a robust method for labelling new cells (Ngwenya, Peters, & Rosene, 2005). The development of BrdU largely replaced autoradiography for analysis of neurogenesis.

The results of our study revealed that rats treated with acyl-ghrelin, but not those injected with saline, showed increased numbers of new adult-born neurons and enhanced performance on the SLR task. Because the final injection of acyl-ghrelin was given 8 days before the start of cognitive testing, any observed effects could not be attributed to the exogenous peptide being in circulation during behavioural testing. The results are in keeping with the previous finding that elevating adult hippocampal neurogenesis is sufficient to improve pattern separation (Sahay et al., 2011a).

4.2 Methods

4.2.1. Subjects

All procedures were in strict compliance with the guidelines of the University of Cambridge and Home Office for animal care. Twenty-four male Lister Hooded rats (n = 12/group), 250 – 300 g; Harlan, Olac, Bicester, UK) were housed in groups of four on a 12 h light cycle (lights on 19:00 - 07:00). Each cage contained a cardboard tube. All procedures were performed during the dark phase of the light cycle. All rats were provided with *ad libitum* access to water and food, except during behavioural testing when food was restricted to 16 g per day for each animal to maintain weight at 95 - 100% free-feeding weight. Rats were handled for 2 consecutive days prior to the start of daily injections.

4.2.2. Injections

The acyl-ghrelin peptide (Phoenix Pharmaceuticals, Inc., USA) was dissolved in physiological saline (0.9 % sodium chloride, pH 7.0) at a concentration of 12 μg/ml. This dose of acyl-ghrelin was chosen as it has previously been shown to increase food intake and elevate plasma ghrelin concentrations to similar levels as a 24 h fast in rats (Wren et al., 2001). Intraperitoneal (IP) injections (total dose ~ 0.26 ml; 10 μg/kg) were performed at the same time each day (2 – 3 h after lights off) for 14 consecutive days.

5′-Bromo-2-deoxyuridine (BrdU, Sigma, B5002) was dissolved in physiological saline (0.9 % sodium chloride, pH 7.0) at a concentration of 20 mg/ml. During preparation the solution was heated to 40 – 50 ºC and 1 ml of NaOH (.01 M) was added. BrdU is

incorporated into DNA as a thymidine analogue during the S phase of the cell cycle (Miller & Nowakowski, 1988; Nowakowski, Lewin, & Miller, 1989). IP injections were conducted over 3 consecutive days, on days 5 - 8 of the experiment (each dose ~ 0.8 ml; 50 mg/kg).

4.2.3. Apparatus

Behavioural testing was conducted in the same arena and testing room described in Chapters 2 and 3 (i.e., black plastic circular arena, 90 cm diameter x 45 cm high). The room was dimly lit with intentional spatial cues. Objects used for testing were tall cylinder beverage containers secured with Blu-tack ^{TM}. Objects were cleaned with 50 % ethanol solution between sessions. A digital camera (Sony ^{TM}) recorded the testing sessions.

4.2.4. Behavioural procedures

Details of the SLR task have been previously described in Chapters 2 and 3 (Bekinschtein et al., 2013; 2014). Briefly, unlike other tests of pattern separation, SLR uses a continuous variable as a measure of performance, which yields sufficient data within a single trial to allow manipulations at different stages of memory. Our paradigm enables us to manipulate the similarity of locations at the time of encoding/consolidation, when pattern separation is thought to occur, rather than at retrieval like other tasks used to assess pattern separation (e.g., Clelland et al., 2009; Gilbert et al., 1998).

All rats were habituated over 5 consecutive daily sessions in which they were allowed to explore the empty circular arena for 10 min. Rats were then tested on the small separation condition (proximate objects located 5 spaces apart, which was equivalent to a 50° angle from the centre) and extra small separation condition (proximate objects located 4 spaces apart, which was equivalent to a 40° angle from the centre), and were counterbalanced within groups. Figure 4.2 illustrates the experimental design and SLR task. We used the extra small separation condition to avoid ceiling effects (Bekinschtein et al., 2013; 2014). For both conditions, rats were allowed to explore the arena and objects for 10 min during the sample phase and were then placed back into their home cage for a 24 h delay. During the choice phase, rats were presented with 2 new identical copies of the objects previously used during the sample phase. One of the objects was in

a novel position and one was in a familiar location. The rats were allowed to explore the chamber and objects for 5 min before being returned to their home cage.

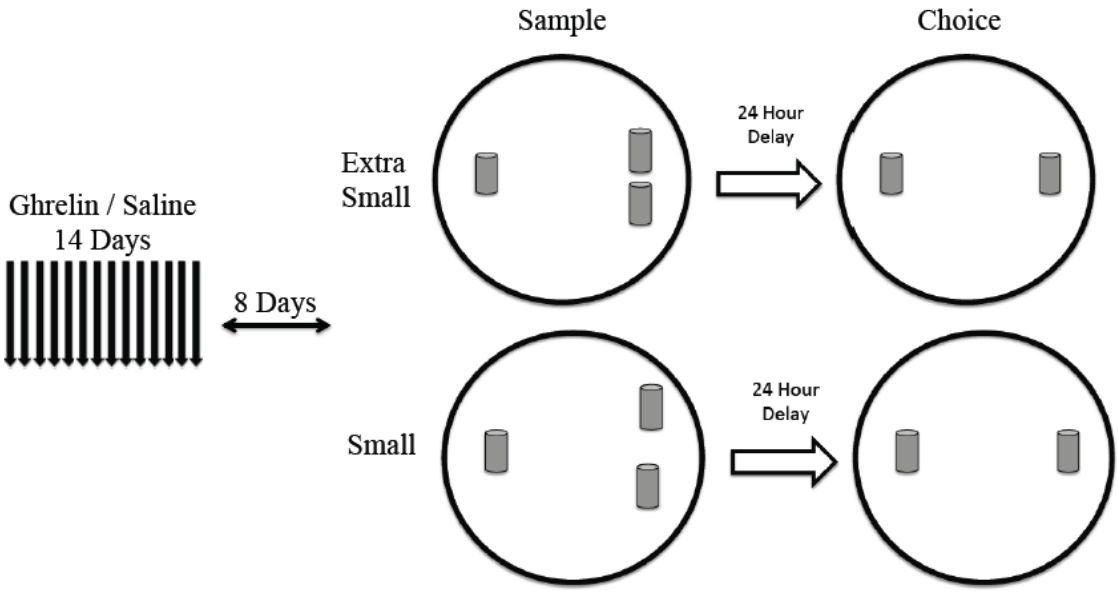

Figure 4.2. Illustration of the experimental design and SLR. After 14 days of daily injections of either ghrelin or saline, rats were given 8 days of rest, which included five daily 10 min sessions of habituation to the testing room and arena. The rats were then tested on two trials of SLR with a 48 h delay between trials. During the sample phase rats were exposed to three identical objects. During the extra small separation condition (top), two of the objects were placed 4 spaces apart (40° angle from the centre). During the small separation condition (bottom), two of the objects were placed 5 spaces apart (50° angle from the centre). In both conditions a third object was placed on the other side of the arena, equidistant from the other two objects. The sample phase was 10 min in duration. There was a 24 h delay between sample phase and choice phase. During the choice phase, rats were exposed to two objects for 5 min, one placed in a novel location and one remaining in a familiar location.

4.2.5. Data collection

In both the sample and choice phases, exploration of an object was defined as described in previous chapters. When the rat was directing its nose within 2 cm or less of the object, it was included as exploration. Sitting on the objects or digging at the base of the objects was not considered exploratory behaviour. The experimenter recorded sample phase exploration using stopwatches. For the choice phase, the experimenter scored exploration using a computer program JWatcher_V1.0, written in Java[TM] (JWatcher, USA). The program had two keys corresponding to the two objects. Exploration was recorded by pressing the appropriate keys at the onset and offset of a bout of

exploration. This was a different program than what was used in the experiments described in Chapters 2 and 3. JWatcher is freely available online and was chosen for its ease of programming.

4.2.6. Histology

Following behavioural testing, rats were anaesthetized by IP injection of Euthatal (2 ml; Rhone Merieux, Harlow, Essex, UK) and perfused transcardially with phosphate buffered saline (PBS), followed by 4 % neutral buffered formalin (NBF). The brains were removed and post-fixed in NBF for at least 24 h, followed by immersion in a 30 % sucrose solution for at least 48 h. Coronal sections (30 μm) were cut along the entire rostro-caudal extent of the hippocampus using a freezing-stage microtome (MicroM, Thermo) and collected (1:12) for free-floating immunohistochemistry.

The following immunohistochemistry protocols were provided by Dr. Jeff Davies and published in Kent et al. (2015).

For the $Brdu^{+}/NeuN^{+}$ staining, sections were washed three times in PBS for 5 min, permeabilised in methanol at – 20 ^{o}C for 2 min and washed in PBS prior to pre-treatment with 2N hydrochloric acid (HCL) for 30 min at 37 ^{o}C. Because BrdU antibodies recognize BrdU in single stranded DNA, immunohistochemistry protocols require that the sections be treated with HCL to break apart the double-stranded DNA into single strands to expose the BrdU. The HCL pre-treatment was followed by washing the sections in 0.1 M borate buffer, pH 8.5, for 10 min. Sections were washed and blocked with 5 % normal goat serum (NGS) in PBS plus 0.1 % triton (PBS-T) for 60 min at room temperature. Sections were incubated overnight at 4 ^{o}C in mouse anti-BrdU (1:50, AbD Serotec), washed as before and incubated in goat anti-mouse AF-568 (1:500, Life Technologies, USA) for 30 min at room temperature in the dark. Sections were washed again prior to a 1 h incubation in mouse anti-NeuN (1:1000, Millipore, USA) diluted in PBS-T. Following another wash the sections were incubated with goat anti-mouse AF-488 (1:500, Life Technologies, USA) in PBS-T for 30 min in the dark. After another wash, sections were mounted onto superfrost^{+} slides (VWR, France) with prolong-gold anti-fade solution (Life Technologies, USA).

For the $BrdU^{+}/Sox2^{+}/S100\beta^{+}$ staining, sections were treated identically to the $BrdU^{+}/NeuN^{+}$ immunohistochemistry described above, with the exception that sections

were first blocked using 5 % normal donkey serum (NDS) in PBS-T for 30 min and subsequently blocked using 5 % NGS in PBS-T for 30 min. Primary antibodies were applied as a cocktail that included rat anti-BrdU (1:400, AbD Serotec), rabbit anti-Sox2 (1:500, Abcam), and mouse anti-S100β (1:1000, Sigma) in PBS-T overnight at 4 ^{o}C. Similarly, secondary antibodies were applied as a cocktail that included donkey anti-rat AF488, donkey anti-rabbit AF568 and goat anti-mouse AF405 (all at 1:500, Life Technologies) in PBS-T for 30 min in the dark. Brain sections were mounted as described above.

For DAB-immunohistochemical analysis of DCX and BrdU labelling, sections were washed in 0.1 M PBS (2 x 10 min) and 0.1 M PBS-T (1 x 10 min). For BrdU-DAB analysis, sections underwent acid treatment and neutralization as described above. Subsequently, endogenous peroxidases were quenched by washing in a PBS plus 1.5 % H_2O_2 solution for 20 min. Sections were washed and incubated in 5 % NDS in PBS-T for 1 h. Sections were incubated overnight at 4 ^{o}C with goat anti-doublecortin (1:200 Santa Cruz Biotechnology, USA) or mouse anti-BrdU (1:200, AbD Serotec) in PBS-T and 2 % NDS solution. Another wash step followed prior to incubation with biotinylated donkey anti-goat (1:400 Vectorlabs, USA) or biotinylated donkey anti-mouse (1:400 Vectorlabs, USA) in PBS-T for 70 min. The sections were washed and incubated in avidin-biotin enzyme complex (ABC; Vectorlabs, USA) solution for 90 min in the dark prior to another two washes in PBS, and incubation with 0.1 M sodium acetate pH 6 for 10 min. Immunoreactivity was developed in Nickel enhanced DAB solution followed by two washes in PBS. Sections were mounted onto superfrost+ slides (VWR, France) and allowed to dry overnight before being de-hydrated and de-lipified in increasing concentrations of ethanol. Finally, sections were incubated for 5 min in histoclear (National Diagnostics, USA) and coverslipped using entellan mounting medium (Merck, USA). Slides were allowed to dry overnight prior to imaging.

4.2.7. Data analysis

For the behavioural analyses, SLR sample data were converted to percentage of the total sample exploration time spent with each of the three objects and analysed using a mixed analysis of variance (ANOVA) to ensure that the three sample objects were being

explored equally. An unpaired two-tailed Student's *t*-test was also conducted to ensure that total exploration was equal between groups. Results from the choice phases were expressed as discrimination ratios (D2), calculated as follows:

$$D2 = \frac{novel\ location\ exploration - \ familiar\ location\ exploration(s)}{novel\ location\ exploration + \ familiar\ location\ exploration\ (s)}$$

Group mean D2 scores were analysed with a repeated measures ANOVA, followed by *post-hoc* contrasts using the Bonferroni correction.

All statistical analyses were conducted with SPSS version 22 and Microsoft Excel version 14.4.5. Statistical significance was set at $p < 0.05$, unless running a *post-hoc* comparison. To control for Familywise error, the level of statistical significance (i.e., α) for *post-hoc* comparisons was calculated using the Bonferonni correction, which was calculated as 0.05 divided by the number of statistical comparisons. All data are presented as mean ± standard error of the mean (SEM).

The histological analyses were conducted by Amy L. Beynon and Amanda K. Hornsby in Dr. Jeff Davies' lab. One-in-twelve series of 30 μm sections (360 μm apart) from each animal was immunohistologically stained (see above) and imaged using a fluorescent microscope (Axioscope, Zeiss) or LSM 710 META upright confocal microscope (Zeiss). $BrdU^+/NeuN^+$ immunoreactive newborn adult neurons were manually counted bilaterally through the z-axis using a 40× objective and throughout the entire rostro-caudal extent of the granule cell layer. Resulting numbers were divided by the number of coronal sections analysed and multiplied by the distance between each section to obtain an estimate of the number of cells per hippocampus (and divided by 2 to obtain the total per DG). For quantification of stem cell self-renewal, one hundred $BrdU^+$ cells were assessed for co-expression with Sox2 and S100β within the subgranule zone of the DG in each brain. The resulting numbers were expressed as a percentage of new stem cells ($BrdU^+/Sox2^+/S100\beta^-$), new astrocytes ($BrdU^+/Sox2^+/S100\beta^+$), or new 'other' cells ($BrdU^+/Sox2^-/S100\beta^-$). DAB-stained sections were imaged using a Nikon 50i microscope (10× objective) and analysed using Image J software. Statistical analyses were carried out using GraphPad Prism 6.0 for Mac. Statistical significance was assessed by unpaired two-tailed Student's *t*-test or one-way ANOVA with Bonferroni's *post-hoc* test unless described otherwise. Pearson correlation and linear regression analysis were used to determine the goodness-of-fit

between number of new adult-born neurons and pattern separation-dependent memory performance.

4.3. Results

4.3.1. Ghrelin treatment improves performance on SLR

To investigate whether ghrelin treatment affects spatial pattern separation, I treated rats with daily injections of either acyl-ghrelin or saline and used the SLR task to evaluate the effects on memory consolidation when the pattern separation load was moderate (i.e., small separation condition) or high (i.e., extra small separation condition). Figure 4.3 shows that both the saline-treated group and the ghrelin-treated group showed a preference for the novel location (i.e., positive D2 score) in the small separation condition, whereas only the ghrelin-treated group showed a preference for the novel location in the extra small separation condition. A two-way repeated measures ANOVA revealed a significant interaction of treatment and separation (p = 0.01, F (1,22) = 8.003).

Post-hoc contrasts revealed a significant effect of separation in the saline-treated group (p < 0.01), but not in the ghrelin-treated group (p = .193). The saline-treated rats showed a preference for the novel location in the small separation condition, but not in the extra small separation condition, whereas the ghrelin-treated rats showed a preference for the novel location in both conditions. There was a significant difference between the saline and ghrelin-treated groups in the extra small separation condition (p = .0001), but no difference between groups in the small separation condition (p = .167).

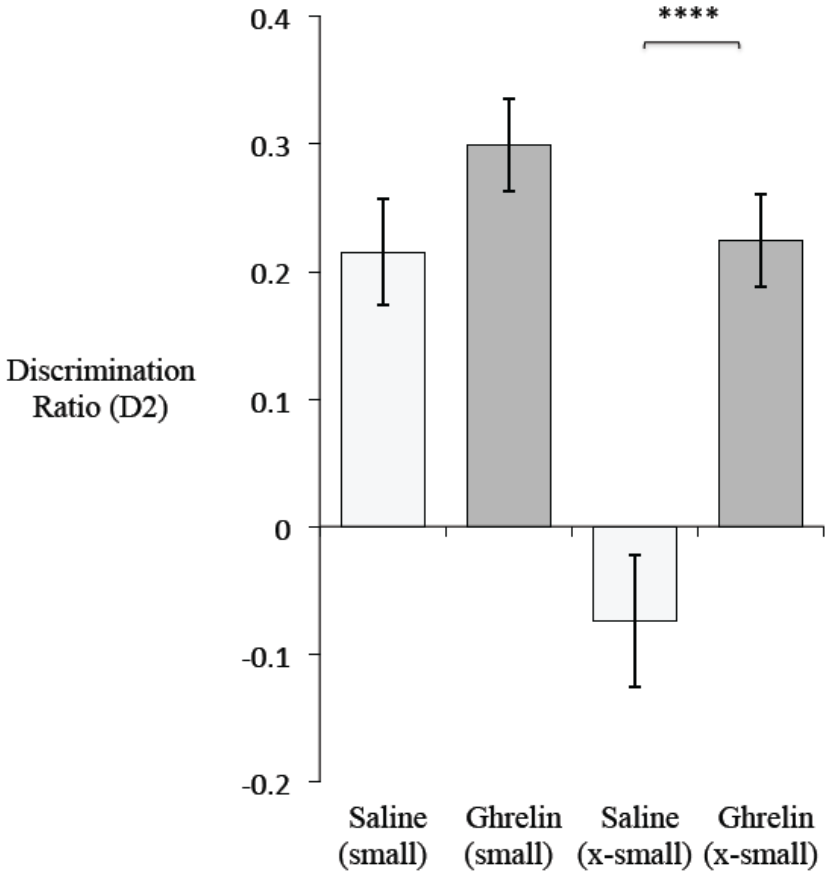

Figure 4.3. Discrimination ratios during the small and extra small conditions of SLR. Rats were treated with saline or ghrelin for two weeks prior to SLR. Each rat was tested on the small and the extra small separation conditions. The y-axis shows the average D2 values, reflecting the preference for the novel location. ****$p < 0.0001$, n = 12 per group. Data are expressed as the mean ± SEM.

As shown in Figure 4.4, there was no difference between groups in the total time spent exploring the objects during the sample phase ($p = 0.38$). Also, as shown in Figure 4.5, both the saline-treated group and the ghrelin-treated group spent equal proportions of time exploring each of the three objects. This indicates that the differences in preference cannot be explained by preferential exploration of the more separated location during the sample phase. There was no main effect of treatment ($p = 0.741$) or condition ($p = 0.818$) on the proportion of time spent exploring each of the sample objects. Similarly, during the choice phase, total time exploring also did not differ between treatment groups ($p = 0.380$) or SLR conditions ($p = 0.301$; data not shown).

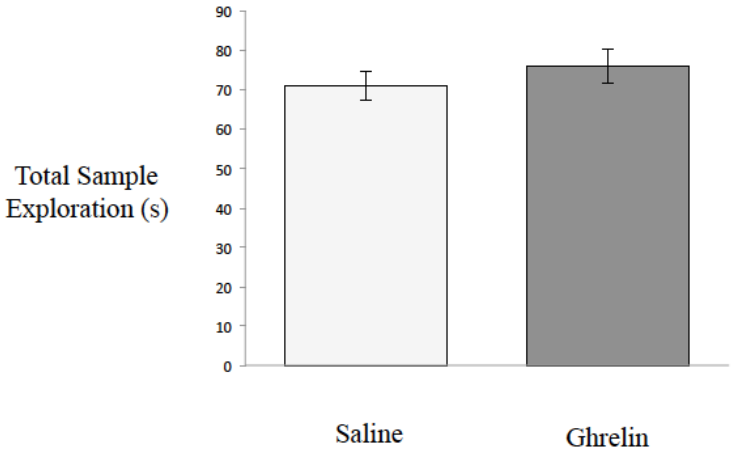

Figure 4.4. Total time spent exploring the objects during the sample phase of Trial 1. There was no difference between the saline-treated group and ghrelin-treated group in total time spent exploring. Data are expressed as the mean ± SEM.

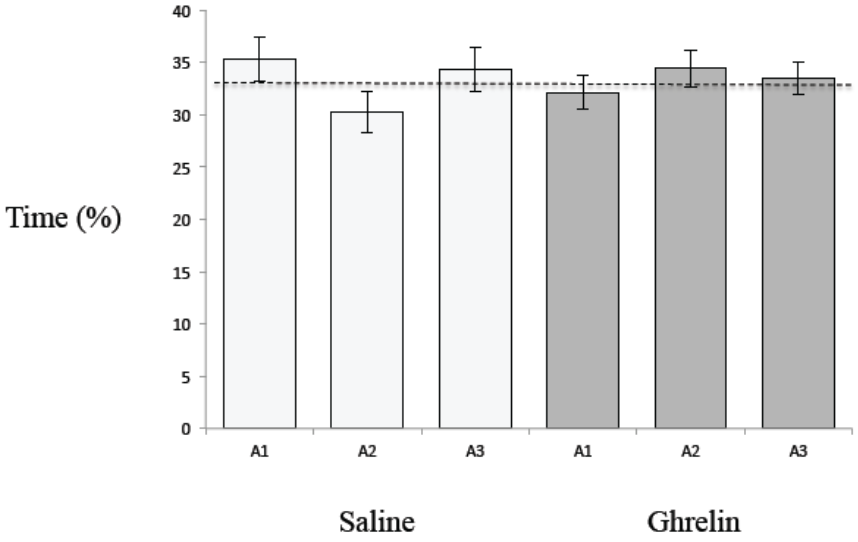

Figure 4.5. Percentage of time spent exploring each of the locations during the sample phases. The dotted line represents chance level (33%). There was no difference between the saline-treated group and ghrelin-treated group in location preference. A1, A2, and A3 represent each of the 3 object locations. Data are expressed as the mean ± SEM.

4.3.2. Ghrelin treatment increases the number of new neurons in the DG of adult rats

To examine whether the daily ghrelin injections increased neurogenesis in the DG, Dr. Jeff Davies performed a BrdU-pulse chase experiment and immunolabelled neurons in the DG were counted. Subsequent analysis showed that acyl-ghrelin treatment

significantly increased (58 %) the total number of new adult-born neurons ($BrdU^+/NeuN^+$) in the DG ($p < 0.001$). Figure 4.6 shows the estimated mean number of $BrdU^+/NeuN^+$ labelled cells in each group. Further analysis revealed that this increase was specific to new neuron formation in the rostral DG ($p < 0.001$; -2.64 mm to -4.56 mm relative to Bregma) rather than the caudal DG (-4.92 mm to -6.48 mm relative to Bregma).

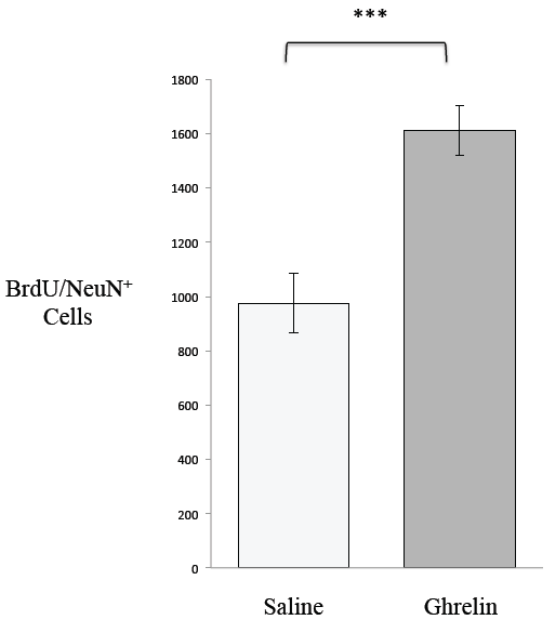

Figure 4.6. Ghrelin treatment increases the total number of new adult-born neurons ($BrdU^+/NeuN^+$) in the DG ($p < 0.001$). The y-axis represents the total estimated number of BrdU-positive neurons in the rostral DG. Data are expressed as the mean ± SEM.

Furthermore, as shown in Figure 4.7, performance on the SLR task was positively correlated with the number of new neurons ($BrdU^+/NeuN^+$) in the rostral DG in the small separation condition (Pearson $r^2 = 0.1663$, $p = 0.0240$) and the extra small separation condition (Pearson $r^2 = 0.1588$, $p = 0.0269$). Higher D2 scores (i.e., stronger preference for the novel location) in both conditions were associated with more neurons in the rostral DG.

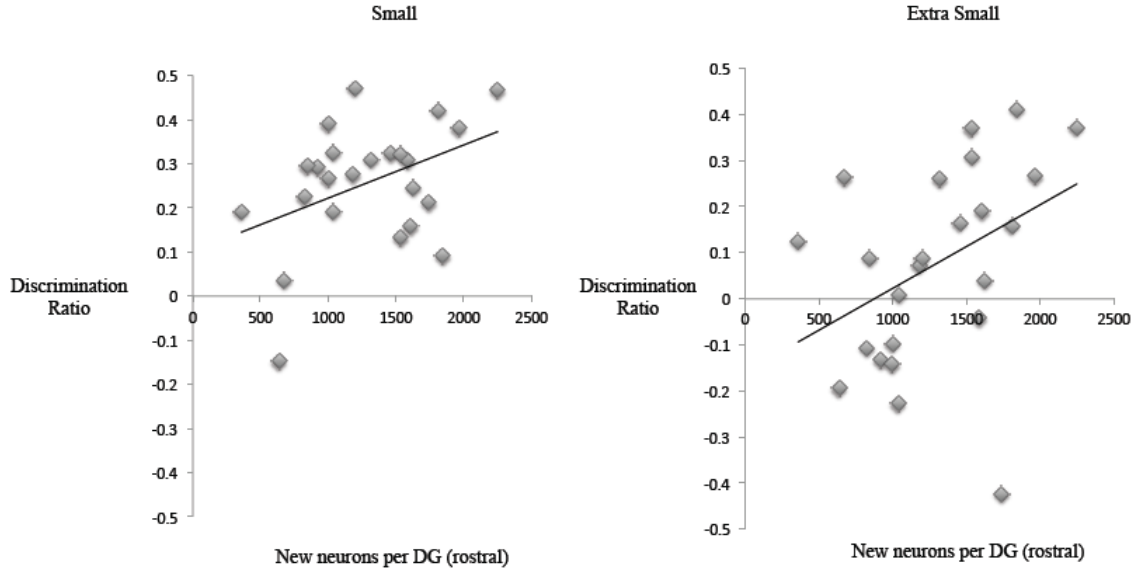

Figure 4.7. Performance on SLR is positively correlated with the number of new DG neurons. The y-axis of the scatterplot represents the preference for the novel location during the choice phase of SLR and the x-axis represents the estimated number of new neurons in the rostral DG. The number of new neurons in the rostral DG was positively correlated with D2 scores in the small separation SLR condition (left; Pearson r^2 = 0.1663, p = 0.0240) and the extra small separation SLR condition (right; Pearson r^2 = 0.1588, p = 0.0269).

Dr. Jeff Davies's analysis showed that there was a 35 % increase in the number of immature neurons (DCX^+) in the DG 14 days after the final acyl-ghrelin injection (p < 0.01). Similarly, analysis of total $BrdU^+$ cell numbers using a DAB-based immunohistochemistry approach revealed a 25 % increase in the DG of acyl-ghrelin-treated rats (p < 0.01), thereby providing further evidence of enhanced neurogenesis. However, acyl-ghrelin did not alter $BrdU^+$ cell number in the hilus (saline, 865.3 ± 79.4 versus ghrelin, 905.7 ± 43.8) or promote the rate of neuronal lineage differentiation in the DG compared to saline control (saline, 71.8 ± 4.5 % versus ghrelin, 74.3 ± 2.7%). Notably, the rate of stem cell self renewal ($BrdU^+/Sox2^+/S100B^-$) and new astrocyte cell formation ($BrdU^+/Sox2^+/S100B^+$) were quantified throughout the rostro-caudal extent of the subgranule zone and showed that acyl-ghrelin did not significantly affect either new stem or new astrocyte cell numbers in the hippocampal niche. Figure 4.8 provides representative images of DCX^+ cells and new neurons co-expressing $NeuN^+$ and $BrdU^+$ in the DG of rats treated with saline or ghrelin.

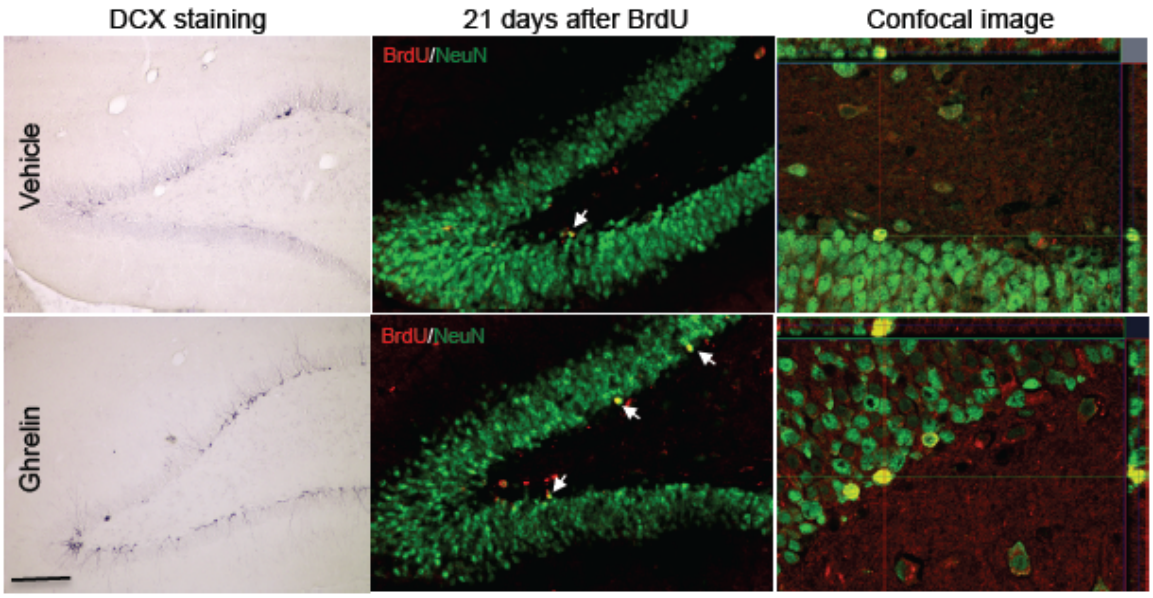

Figure 4.8. Representative images of DCX^+ immature neurons and new adult-born DG neurons (white arrows) co-expressing $NeuN^+$ and $BrdU^+$. Images were provided by Dr. Jeff Davies and published in Kent et al., (2015).

4.4. Discussion

The experiments presented in this chapter were designed to investigate long-term mnemonic effects of increasing adult neurogenesis with daily acyl-ghrelin injections. Using SLR, we evaluated the performance of rats on the small and extra small separation conditions, which vary the load on pattern separation (Bekinschtein et al., 2013; 2014). In support of my hypotheses, the results revealed that peripheral treatment with physiological levels of acyl-ghrelin increased neurogenesis in the DG and also improved spatial pattern separation. The results are in keeping with the finding that elevating adult hippocampal neurogenesis is sufficient to improve pattern separation (Sahay et al., 2011a) and that ghrelin administration can affect spatial cognition (Diano et al., 2006). To my knowledge, this is the first research to look at long-term mnemonic effects of sub-chronic acyl-ghrelin administration, and the first demonstration that acyl-ghrelin enhances spatial pattern separation via a mechanism consistent with elevated adult hippocampal neurogenesis.

In the small separation condition, there was no difference in performance between the saline-treated and ghrelin-treated rats. There was a difference, however, in the extra small separation condition. The extra small separation condition positioned the

landmarks closer together and thus increased the requirement to create less overlapping, unique representations. The results showed specifically that, consistent with previous reports (Bekinschtein et al., 2013; 2014), the saline-treated rats did not show a preference for the novel location in the extra small separation condition, but showed a significant preference for the displaced object in the small separation condition. In contrast, the ghrelin-treated rats showed a preference for the displaced objects in both conditions. Furthermore, histological analysis confirmed that the ghrelin-treated rats had a 58 % increase in the number of new adult-born neurons in the DG, compared to the saline-treated rats.

As described in previous chapters, the rationale behind the SLR task is that when objects are closer together it is more challenging to form representations that are distinct and resistant to confusion, than when objects are further apart. If representations are not sufficiently separated during encoding and/or consolidation, then the presentation of a new intermediate location may activate the same memory representation and thus will not be distinguishable or identified as novel. Because we have shown that DG manipulations impair memory only when similar but distinct spatial representations are formed (i.e., the small and extra small separation conditions, but not the large separation condition), there is strong evidence that SLR is a suitable and reliable task for studying pattern separation (Bekinschtein et al., 2013; 2014).

The nature of the SLR task provides several advantages over other tasks used to study spatial pattern separation. The single trial nature, ability to manipulate similarity in a parametric way, identical choice phases in every condition, and the fact that it does not use rewards are all desirable qualities. However, as with other spontaneous tasks paired with pharmacological manipulations, one limitation is the possibility that the treatments changed non-mnemonic performance variables, such as the animals' motivation to explore an environment or their preference for novelty. Because testing took place 8 to 10 days after the acyl-ghrelin treatment was discontinued, the long-lasting improvements in spatial processing cannot be attributed to the exogenous hormone being in circulation during behavioural testing. Furthermore, acyl-ghrelin did not appear to have an effect on motivation because total exploration times during the sample phase and the test phase did not differ between treatment groups. Thus, it is unlikely that changes in motivation account for the observed differences in discrimination ratios.

Although the exact mechanisms underlying the ghrelin-induced enhancement of pattern separation remains to be determined, our results are in agreement with previous work suggesting an important role of neurogenesis (Bekinschtein et al., 2014; Clelland et al., 2009; Creer et al., 2010; Nakashiba et al., 2012; Sahay et al., 2011a; Tronel et al., 2012). For example, as described in Chapter 3, attenuating neurogenesis in the DG impairs performance on the SLR task (Bekinschtein et al., 2014). Because performance on the SLR task is DG-dependent, and particularly sensitive to manipulations altering plasticity-related factors and neurogenesis, it is reasonable to suggest that the cognitive enhancing effect of the acyl-ghrelin treatment may be a result of increased neurogenesis.

As ghrelin has been shown to cross the blood brain barrier and act on the GHSR (type 1a) in the DG (Diano et al., 2006; Guan et al., 1997), which is the only functional ghrelin receptor characterized (Guan et al., 1997; Howard et al., 1996; Kojima & Kangawa, 2005), it is possible that increasing circulating ghrelin in the present study had direct effects in the DG. However, it is important to recognize that although it is possible that ghrelin acted directly in the hippocampus, the indirect effects of ghrelin cannot be excluded. For example, ghrelin stimulates the production of insulin-like growth factor-1 (IGF-1), which is known to increase neurogenesis (Anderson et al., 2002; Chen, 2012). Additionally, GHSR mRNA is found throughout brain regions other than the hippocampus, such as several hypothalamic nuclei (e.g., arcuate nucleus, ventromedial nucleus, and suprachiasmatic nucleus) and midbrain structures (e.g., ventral tegmental area, substantia nigra, and dorsal raphe nucleus) but the functional relevance is unknown for many of these regions (Zigman et al., 2006). It is possible that the actions of ghrelin outside of the hippocampus are having indirect effects that explain the results.

If ghrelin is acting directly in the DG, a potential mediating mechanism is brain-derived neurotrophic factor (BDNF). There is evidence that BDNF expression is affected by SIRT1-mediated processes in the hippocampus (Zocchi & Sassone-Corsi, 2012). As mentioned previously, the SIRT1 signalling pathway is hypothesized to mediate the cognitive enhancement associated with CR, as well as the anti-apoptotic and orexigenic actions of ghrelin (Shimada et al., 2014; Velasquez et al., 2011). There is evidence that disrupted SIRT1 activity results in a downregulation of BDNF (Gao et al., 2010). For

example, a transgenic mouse model with impaired SIRT1 activity, demonstrated impaired performance on contextual fear conditioning, object recognition, and the MWM, and this impairment was accompanied by reduced BDNF mRNA (Gao et al., 2010). Thus, circulating levels of ghrelin may activate SIRT1, which then affects BDNF expression.

Further research is needed to elucidate the exact pathway by which ghrelin affects pattern separation, but the potential for BDNF to mediate the effect complements the findings described in Chapters 2 and 3. However, the effect of BDNF described in previous chapters was acute, whereas the potential effects of BDNF via the ghrelin treatment would be long lasting. BDNF has been shown to increase neurogenesis (Scharfman et al., 2005), which may provide a mechanism to explain the long-term effects of the ghrelin treatment.

In contrast to our findings, as well as previously published data (Diano et al., 2006; Li et al., 2013), Zhao and colleagues (2014) reported that mice receiving a daily systemic supra-physiological dose (80 μg/kg) of ghrelin for 8 days had no effect on spatial memory, even though there was an increase in hippocampal neurogenesis. In their experiment, spatial memory was assessed via performance on the MWM. This finding is not as surprising as it may appear at first because many previous studies looking at the relationship between an enhancement of neurogenesis and spatial memory have provided mixed results. A meta-analysis conducted by Groves and colleagues (2013), which was previously discussed in Chapter 3, found no significant effect of ablating adult neurogenesis on spatial memory. One hypothesis is that the source of variation contributing to the inconsistent effect is the load on pattern separation in the tasks used to evaluate spatial memory (Bekinschtein et al., 2011). Hippocampal neurogenesis appears to be critically involved in spatial pattern separation and how much the tasks rely on this process could determine the effects manipulating neurogenesis has on performance.

In addition to further elucidating the extra-hypothalamic effects of ghrelin, this research has potential clinical applications. Consistent with aged animal models demonstrating impairments in pattern separation (Burke et al., 2010; 2011; Wilson et al., 2003), healthy older adults also show impaired memory performance and less efficient pattern

separation, compared to younger adults (Toner et al., 2009; Yassa et al., 2011). The pattern of impairment seen in adult humans is similar to that seen in animal models, in that greater dissimilarity is required for elderly participants to successfully encode information as distinct (Yassa et al., 2011).

Furthermore, neurodegenerative disorders often display coexisting metabolic dysfunction, and there are several converging lines of evidence linking metabolic dysfunction with an increased risk of developing Alzheimer's disease (AD) and other dementias (Cai et al., 2012; Kapogiannis & Mattson, 2011; Naderali, Ratcliffe, & Dale, 2009). More specifically, a high fat diet (Handjieva-Darlenska & Boyadjieva, 2009) and obesity (Cummings et al., 2002) are associated with reduced circulating levels of acyl-ghrelin in rats and humans, respectively, and obesity is associated with an increased risk of dementia in humans (Kivipelto et al., 2005; Whitmer et al., 2005; Gorospe & Dave, 2007). Moreover, ghrelin may have potential for preventing or treating neurodegenerative disease, such as AD (reviewed by Gahete et al., 2011 and Kent, 2014). There is a growing literature suggesting that insulin deficiency and insulin resistance act as mediators of AD-type neurodegeneration. This has led some to refer to AD as "type 3 diabetes," a form of diabetes that selectively involves the brain (de la Monet & Wands, 2008; Steen et al., 2005). Because ghrelin has been shown to modulate insulin sensitivity (Chen et al., 2010), as well as several other metabolic and mnemonic effects, ghrelin may be a potential candidate molecule responsible for the relationship between metabolic and cognitive dysfunction. It is possible that disruption of the normal modulation of ghrelin secretion may contribute to the metabolic changes associated with AD.

In summary, the present study investigated the long-term effects of elevating acyl-ghrelin on spatial memory. To the best of my knowledge, this provides the first data demonstrating a previously unknown physiological function for circulating ghrelin, by showing that administering acyl-ghrelin promotes adult hippocampal neurogenesis and pattern separation. This is the first step towards determining whether modulating ghrelin can lead to enhancements in cognition via alterations in neurogenesis.

Chapter 5: Spatial pattern separation upregulates neurogenesis in the dentate gyrus of rats

Adult-born immature neurons in the dentate gyrus (DG) have been previously implicated in spatial pattern separation. As discussed in previous chapters, reducing neurogenesis can cause performance impairments on the Spontaneous Location Recognition (SLR) task when the load on pattern separation is high (Chapter 3). In contrast, increasing neurogenesis is associated with improved performance on the SLR task (Chapter 4). Together, these findings demonstrate that the level of neurogenesis can affect performance on a task requiring pattern separation. What is not yet known is whether the load on pattern separation during learning can affect the level of neurogenesis and subsequent pattern separation. To test this, our lab developed a spontaneous exposure protocol, which varied the placement of objects to change the load on pattern separation. After the exposure protocol, rats were tested on SLR to assess whether repeatedly exposing rats to spatial landmarks that were in similar locations - hypothesized to engage pattern separation - during incidental learning, had any improvements on SLR performance. The results suggest a reciprocal relationship between neurogenesis and pattern separation, such that learning experiences requiring pattern separation can upregulate adult neurogenesis in the DG of rats and this increase of adult-born neurons then enhances performance on subsequent tasks requiring pattern separation. Some of the experiments described in this chapter were conducted in collaboration with Dr. Pedro Bekinschtein.

5.1. Introduction

There is accumulating evidence that adult hippocampal neurogenesis plays an important role in learning and memory, and is particularly important for pattern separation (reviewed by Sahay, Wilson, & Hen, 2011b and Oomen et al., 2014).

To briefly summarize, there is some evidence that down-regulating DG neurogenesis in rodents impairs performance on hippocampal-dependent tasks such as the Morris water maze (MWM; Jessberger et al., 2009), trace fear conditioning (Shors et al., 2002), nonmatching to sample task (Winocur et al., 2006), radial-arm maze (Clelland et al., 2009), and contextual fear conditioning (Winocur et al., 2006; Wojtowics, Askew, & Winocur, 2008; Pan et al., 2012). As discussed in Chapter 3, this neurogenesis-dependent impairment may be due to a specific disruption of pattern separation, and rats with reduced hippocampal neurogenesis exhibit impaired performance on the Spontaneous Location Recognition (SLR) task.

In contrast, some have shown that interventions such as voluntary physical exercise or environmental enrichment, which are known to upregulate DG neurogenesis, improve performance on hippocampal-dependent tasks (Nilsson et al., 1999; Anderson et al., 2000; Rhodes et al., 2003; Cao et al., 2004; Creer et al., 2010; Kim et al., 2010; Marlatt et al., 2012; Mustroph et al., 2012). As examined in Chapter 4, this enhancement may be due to a specific improvement of pattern separation, and rats with increased hippocampal neurogenesis exhibit improved performance on SLR.

5.1.1 Can learning increase neurogenesis?

There is now evidence that this link between neurogenesis and cognition may be reciprocal. Although highly debated, some evidence suggests that learning itself can upregulate the production or survival of newborn neurons in the DG. It has been shown that training in spatial memory tasks (Light et al., 2010; Nokia et al., 2012b) and exposure to cognitively challenging and stimulating environments (e.g., Wainwright et al., 1993; Kempermann, Kuhn, & Gage, 1998; Nilsson et al., 1999) can enhance subsequent learning and memory performance. Evidence suggests that upregulated hippocampal neurogenesis may underlie these cognitive enhancing effects (Gould et al., 1999; Nokia et al., 2012b; Clemenson et al., 2014).

Gould and colleagues (1999) were the first to report that learning a hippocampus-dependent task, the MWM, resulted in a three-fold increase in the survival of neurons born one week before training. This finding was in agreement with evidence that the

survival of adult-born neurons was regulated by excitatory input (Cameron, McEwen, & Gould, 1995) and suggested that the act of learning may affect cell survival.

Since this initial study, other studies have also shown an increase of DG neurogenesis or neuronal survival after MWM training (Ambrogini et al., 2000; Kempermann & Gage, 2002a; Dobrossy et al., 2003; Hairston et al., 2005; Kee et al., 2007; Sisti, Glass, & Shors, 2007; Lemaire et al., 2000). However, several others have found the opposite association and concluded that MWM training causes a reduction in neurogenesis (Ambrogini et al., 2004; Ehninger & Kempermann, 2006; Mohapel et al., 2006; Aztiria et al., 2007), or has no effect on levels of new DG neurons (van Praag et al., 1999; Van der Borght et al., 2005). It is unclear why similar studies have produced such conflicting results but confounds such as stress and exercise, and the inconsistency of setups, protocols, parameters, and age of new cells during training have all been hypothesized as contributing factors (Ehninger & Kempermann, 2006; Epp et al., 2010).

Coinciding with the aforementioned experiments evaluating the link between previous learning and neurogenesis, it was hypothesized that an upregulation of neurogenesis that results from learning may result in improved cognition. To specifically investigate the effect previous learning has on later cognitive functioning, Light and colleagues (2010) trained mice on two distinct eight-arm radial mazes and found that this training was associated with better performance on a subsequent battery of five independent learning tasks. Similarly, Nokia and colleagues (2012b) trained rats on one of two hippocampal-dependent associative learning tasks (i.e., trace and long-delay eyeblink conditioning), and found that this training facilitated performance on the subsequent task when tested one week later. In their study, there was a positive impact on neuronal survival for cells generated after learning, suggesting that the training improved cognitive performance by increasing the number of adult-born neurons in the DG.

In addition to the potential cognitive benefits from training on explicit learning paradigms, rodent studies have also revealed that environmental enrichment can enhance cognitive performance on subsequent learning and memory tests, and increase adult neurogenesis (Nilsson et al., 1999). This offers the possibility that improved cognition as a result of previous cognitive training may be due to the effect of general enrichment and not specifically due to the cognitive training; however, there is some

evidence that it is learning, and not just exposure to training that enhances neurogenesis (Sisti, Glass, & Shors, 2007). This highlights the need for further work to elucidate the mechanisms connecting cognitive training and/or enrichment, neurogenesis, and memory.

Given the debate about whether learning and memory can affect neurogenesis and subsequent cognition, it is possible that the load on pattern separation is one of the critical mechanisms leading to the mixed results (Bekinschtein et al., 2011). Specifically, pattern separation could be the key component of the learning or enrichment experience that engages the DG and increases neurogenesis. To investigate the specific role of pattern separation in the relationship between previous learning, neurogenesis, and cognitive enhancement, our lab developed a spontaneous exposure protocol, which allows the load on spatial pattern separation to be varied. Because specifically evaluating pattern separation when neurogenesis was upregulated and downregulated has helped elucidate the specific function of adult-born neurons in spatial memory, it is possible that evaluating the specific role of pattern separation in learning-induced neurogenesis and cognitive enhancement may hold similar promise for clarifying this relationship further.

The exposure protocol was previously shown to spontaneously increase brain-derived neurotrophic factor (BDNF) in the DG of rats (Bekinschtein et al., 2013) when the spatial landmarks were in similar locations (i.e., small separation condition). Dr. Pedro Bekinschtein (unpublished) was the first to combine the exposure protocol with the SLR task. He compared rats repeatedly exposed to an empty arena (control exposure condition), rats repeatedly exposed to spatial landmarks delineating a small separation (small exposure condition, 5 spaces apart, which is equivalent to a 50° angle from the centre), and rats repeatedly exposed to spatial landmarks delineating a large separation (large exposure condition, 12 spaces apart, which is equivalent to a 120° angle from the centre), and then tested them on the extra small separation condition of SLR (4 spaces apart, which is equivalent to a 40° angle from the centre). Figure 5.1 illustrates the three exposure conditions. The extra small separation SLR condition is used whenever evaluating potential enhancement effects because control subjects perform at chance level (Bekinschtein et al., 2013; 2014). The results of this preliminary study found that rats exposed to the small exposure condition showed a preference for the novel location

when tested on extra small separation SLR, whereas rats exposed to the control or large exposure conditions did not. Figure 5.2 shows the unpublished data from this preliminary experiment conducted by Dr. Pedro Bekinschtein.

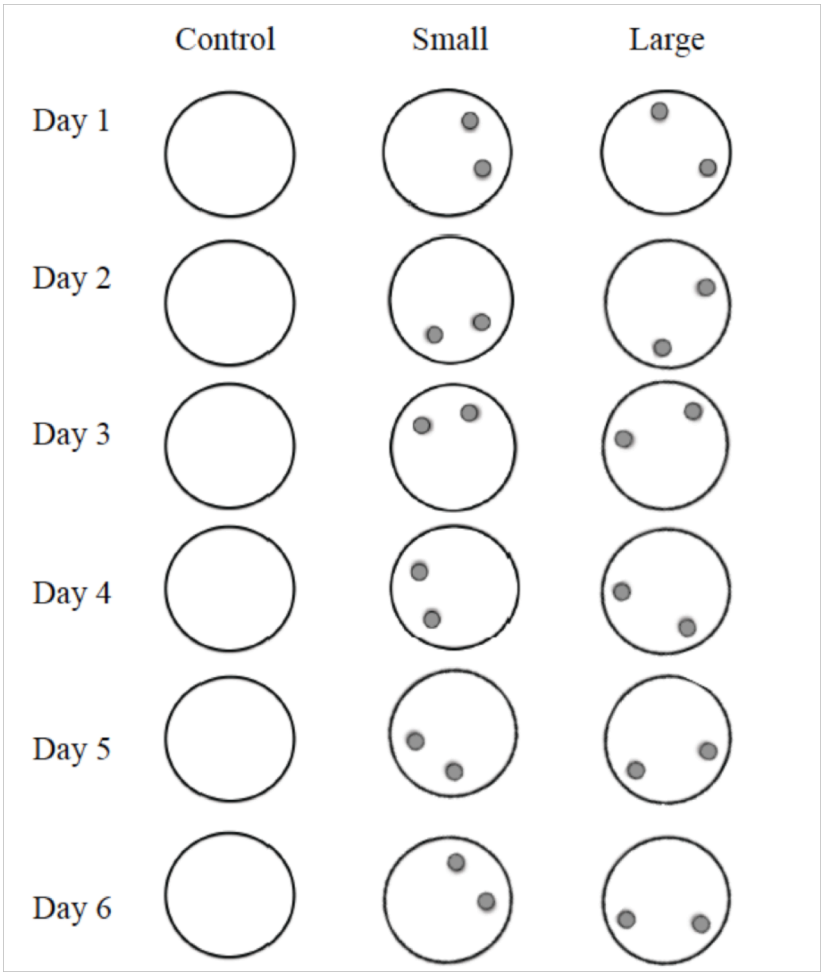

Figure 5.1. Diagram of the exposure protocol. Rats were assigned to the control exposure condition (left; empty arena), small exposure condition (middle; two objects placed 5 spaces apart, equivalent to a 50° angle from the centre), or the large exposure condition (right; two objects placed 12 spaces apart, equivalent to 120° angle from the centre). The placements in the small and large conditions of the exposure paradigm correspond to the locations of the proximate objects in the small separation and large separation conditions of the SLR task, respectively. For six consecutive days, the rats were placed in the arena for 10 min and allowed to explore. The positioning of the objects varied each day and was counterbalanced between rats.

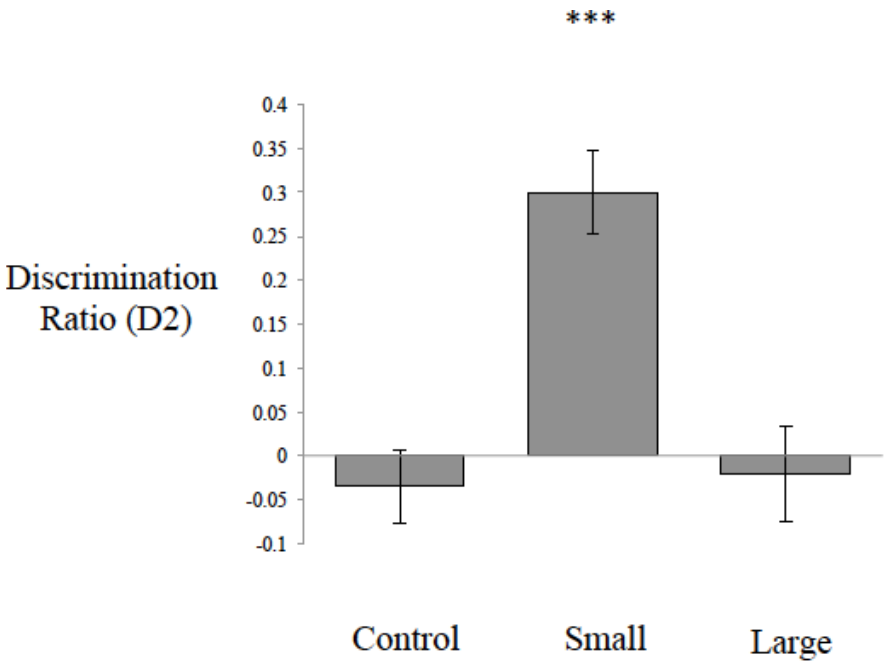

Figure 5.2. Performance on SLR improves after the small exposure protocol. The figure shows unpublished data from Dr. Pedro Bekinschtein. Positive discrimination ratios (D2) reflect a preference towards the novel location. Only the group that was previously in the small exposure condition showed a preference for the novel location during extra small separation SLR. ***$p < 0.001$ (one-way ANOVA $F_{2,21} = 15.268$). Data are expressed as the mean ± SEM.

5.1.2. Overview

Following from this initial study, which suggested that exposure to similar stimuli during implicit learning experiences – thought to engage pattern separation - result in improved performance on tasks requiring pattern separation, three experiments were designed to investigate this relationship further.

In Experiment 1, I investigated the effects of the exposure protocol on hippocampal neurogenesis and found that rats exposed to the small exposure condition had more adult-born neurons than those exposed to the control or large exposure conditions.

In Experiment 2, I investigated whether adult-born neurons were important for the cognitive enhancement associated with the exposure protocol, which was demonstrated in the preliminary work described above. Rats were injected with the dominant negative Wnt lentiviral vector (LV-dnWnt), directly into the DG, to reduce neurogenesis and were then exposed to the small exposure condition. The lentiviral vector (LV) inhibits

Wnt signalling, which is important for neural development and differentiation. This method was used successfully in the experiment described in Chapter 3 to reduce neurogenesis in rats, and has been previously shown to be effective in both rats and mice (Clelland et al., 2009; Jessberger et al., 2009; Bekinschtein & Kent et al., 2014). After the exposure protocol, the rats were tested on the extra small separation condition of SLR, and the results revealed that the knockdown rats were impaired. Rats with reduced neurogenesis did not show a preference for the novel location, even after the exposure treatment.

In Experiment 3, I attempted to replicate these findings using a different method to reduce neurogenesis. Rats were treated with temozolomide (TMZ) and exposed to the small exposure condition, and then tested on the extra small separation condition of SLR. TMZ is a DNA-alkylating agent used as a chemotherapeutic agent to treat central nervous system tumors (Lashkari et al., 2011). It suppresses neurogenesis by attaching a methyl group to DNA residues, which disrupts DNA replication and results in cell death. Treating rats with TMZ has been previously shown to reduce neurogenesis and impair hippocampal dependent learning and memory (Garthe et al., 2009; Nokia et al., 2012a). Furthermore, four weeks of TMZ treatment has been shown to reduce neurogenesis by 40% in mice and impair population-based coding in CA3 for similar but not dissimilar contexts during a contextual fear conditioning task, suggesting a specific impairment in pattern separation (Niibori et al., 2012). This effect was replicated in mice that had a genetically induced reduction of neurogenesis, which suggested that it was the reduction in neurogenesis that caused the deficit in pattern separation. Importantly, TMZ treatment does not affect general health, locomotion, or exploratory behaviour (Garthe et al., 2009).

In agreement with the findings from Experiment 2, the rats treated with TMZ did not show a preference for the novel location on the extra small separation condition of SLR.

5.2. Method

5.2.1. Subjects

Forty-four (24 for Experiment 1 and 20 for Experiment 3) male Lister Hooded rats (250 – 300 g; Harlan, Olac, Bicester, UK) and 24 (Experiment 2) Long-Evans rats (380 - 410 g; Harlan, San Diego, CA, USA) were housed in groups of two to four on a 12 h light cycle (lights on 19:00 - 07:00). Cages were enriched with a cardboard tube. All rats were provided with *ad libitum* access to water and food, except during behavioural testing when food was restricted to 17 g per day for each animal. Rats were handled for two consecutive days prior to any behavioural testing or injections. All procedures were performed during the dark phase of the light cycle. All procedures were in strict compliance with the guidelines of the University of Cambridge, Home Office for animal care, and UK Animals (Scientific Procedures) Act 1986.

5.2.2. Injections and surgery

In Experiment 1, 24 Lister Hooded rats received three consecutive days of intraperitoneal injections (IP) of the thymidine analogue, 5′-Bromo-2-deoxyuridine (BrdU, Sigma-Aldrich, UK), dosed at 60 mg/kg (20 mg/ml, ~ 0.8 ml) in physiological saline (0.9 % sodium chloride, pH 7.0). BrdU replaces endogenous thymidine during DNA replication, becoming permanently incorporated into the DNA of dividing cells. BrdU is widely accepted as a robust method for labelling new neurons (Ngwenya, Peters, & Rosene, 2005). During preparation BrdU was heated to 40 – 50 ºC with 1 ml of NaOH (.01 M) per 100 ml of saline to neutralize the pH. BrdU was injected 1 week prior to the start of the exposure protocol.

In Experiment 2, 24 Long-Evans rats underwent the same procedure outlined in Chapter 3 to decrease neurogenesis in DG by inhibiting Wnt signalling (Lie et al., 2005; Jessberger et al., 2009). See Jessberger and colleagues (2009) for a detailed description of the procedure. Lentiviral vectors (LV) were prepared as previously described (Lie et al., 2005). All viral stocks were diluted to and injected at 1 x 10^9 transducing units (TU)/ml. The rats were deeply anesthetized with a ketamine/ xylazine/ acepromazine

mixture and placed into a stereotaxic apparatus. Rats received either a control green fluorescent protein (GFP) virus or a dnWnt virus (n = 12 /group). A total of 6 µl of either LV-GFP or LV-dnWnt was injected over 8 bilateral targets in each hemisphere of the DG: (a/p, m/l, d/v from bregma) – 2.4, ±1, -4.1; -3.2, ±1.2, -4.1; -4, ±2, -3.7; -4.8, ±3, 3.8; -5.4, ±3.8 -4; -5.4, ±4.4, -7.2; -6, ±4, -4.2; -6, ±4, -7.4. At each injection site 0.3 - 0.4 µl of virus was injected slowly over 1 min. Once complete, animals were sutured and given a one-time dose of buprenex. These surgeries were conducted by Dr. Dane Clemenson in Professor Fred Gage's lab and were approved by the Use Committee at the Stalk Institute for Biological Studies.

In Experiment 3, 20 Lister Hooded rats received three consecutive days of IP injections of TMZ (LKT Laboratories, Inc., USA) dosed at 25 mg/kg (concentration 3.1 mg/ml in physiological saline) or an equivalent volume of physiological saline (0.9 % sodium chloride, pH 7.0) for 4 weeks. I had attempted to increase the concentration of TMZ by using dimethyl sulfoxide (DMSO) but the high percentage (50 %) of DMSO required to dissolve the TMZ caused an adverse reaction in the first 3 rats injected, so I did not continue the injections. These rats were given a week to recover. All rats then started the first set of TMZ injections, using a larger volume and lower concentration of TMZ dissolved in only saline. On the final 3 days of injections, all rats received 3 injections of BrdU (Sigma-Aldrich, UK) dosed at 60 mg/kg (20 mg/ml) in physiological saline (0.9 % sodium chloride, pH 7.0). BrdU injections were conducted at least 2 h after the TMZ or saline injections. During preparation, 100 ml of the BrdU solution was heated to 40 – 50 ºC and 1 ml of NaOH (.01 M) was added to neutralize the pH. Habituation started 24 h after the final injection.

5.2.3. Apparatus

Behavioural testing was conducted in the same black plastic circular arena (90 cm diameter x 45 cm high) used in the experiments described in Chapters 2 to 4. Objects used for the exposure protocol and SLR were tall cylinder containers ~ 20 cm in height and were selected such that they had varying physical attributes of size, reflectance, transparency, and contour (i.e., soda cans, glass beer bottles, ceramic water bottles, plastic flashlights, and plastic dinosaur eggs). To prevent the rats from moving the objects during exploration, Blu-tack™ was used to secure the objects in place. Objects

were wiped down with a 50 % ethanol solution between sessions. A digital camera (Sony ^{TM}) recorded the behavioural testing.

5.2.4. Behavioural procedures

All rats were habituated over 5 consecutive days in which they were allowed to explore the empty circular arena for 10 min. The exposure protocol began 24 h after the fifth habituation session.

In Experiment 1, rats were randomly pre-assigned to one of the three exposure conditions (n = 8 per condition) shown in Figure 5.3: control (no objects), small (2 objects placed 5 spaces apart, equivalent to a 50° angle from the centre), or large (2 objects placed 12 spaces apart, equivalent to a 120° angle from the centre). For six consecutive days, each rat was exposed to their assigned condition for 10 min. Control rats continued as in the habituation trials, with no exposure to objects.

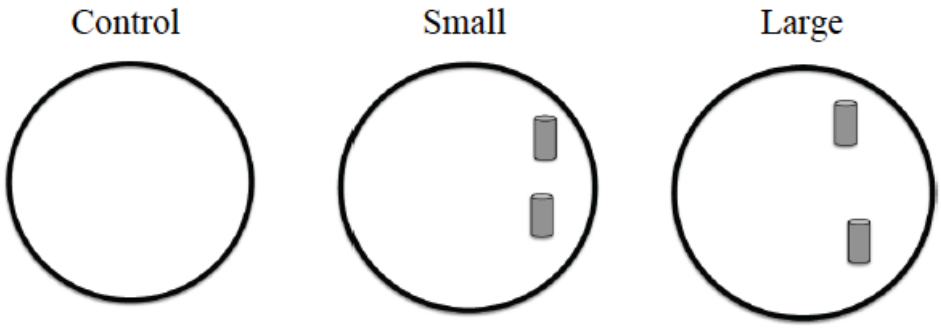

Figure 5.3. Illustration of the three conditions used during the exposure protocol. The control condition (left) was an empty arena. The small condition (middle) had 2 objects placed a "small" distance apart (5 spaces, equivalent to a 50° angle from the centre). The large condition (right) had 2 objects placed a "large" distance apart (12 spaces, equivalent to 120° angle from the centre). The "small" and "large" distances corresponded to the distances used in the small separation and large separation conditions of SLR.

In Experiment 2, the rats injected with the GFP lentiviral vector (LV-GFP) and the rats injected with the dnWnt lentiviral vector (LV-dnWnt) were all exposed to the small exposure condition. The schedule of behavioural testing is shown in Figure 5.4. After 6 days of exposure, the rats were tested on the extra small separation condition of the SLR

task. Figure 5.5 illustrates the extra small separation SLR condition. The task was described previously in Chapters 2 to 4 (Bekinschtein et al., 2013; 2014; Kent et al., 2015).

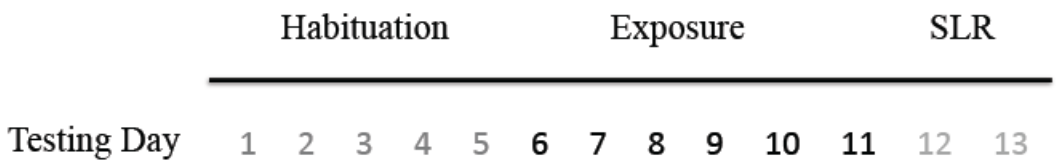

Figure 5.4. Schedule of the behavioural testing used for Experiments 2 & 3. All rats were habituated for 5 days and then went through the exposure protocol for 6 days. This was followed by two days of SLR.

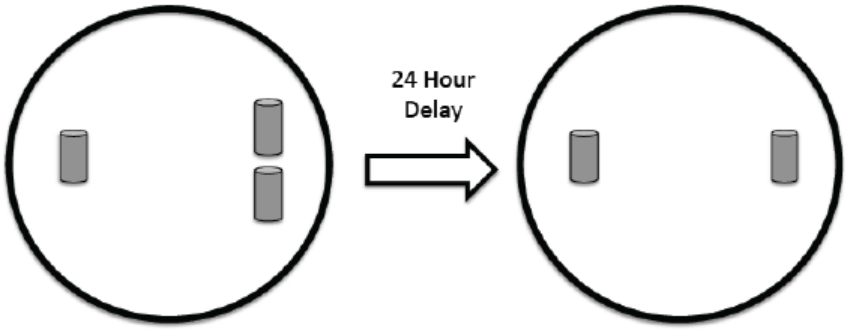

Figure 5.5. Schematic of the extra small SLR condition. During the sample phase (left), rats were shown three objects for 10 min. Two of the objects were placed close together at an "extra small" distance (4 spaces, which was equivalent to a 40° angle from the centre). The rats were then placed back in their home cage for 24 h. During the choice phase (right), the rats were shown two objects. One object was in a novel location and one was in a familiar location.

Briefly, each trial of SLR consisted of two phases. In the sample phase, three identical objects were placed 15 cm from the edge of the open field and 30 cm from the centre. To avoid any ceiling effects, the extra small separation SLR condition was used because the discrimination is so difficult that naïve or vehicle-treated rats only perform at chance levels (Bekinschtein et al., 2014; Kent et al., 2015). In the extra small separation condition, two of the objects are placed only 4 spaces apart, which is equivalent to a 40° angle from the centre. Rats were allowed to explore the arena and objects for 10 min during the sample phase. At the end of the sample phase, rats were removed from the arena and placed back into their home cages. After a 24 h delay, each rat was placed back into the arena for the choice phase. During the choice phase, rats were presented with 2 new identical copies of the objects previously used during the sample phase. One

object was placed in a familiar location and one object was placed in a novel location, which was between the sample placements of the two proximate objects. The choice phase allowed the animals to explore the chamber and objects for 5 min before being returned to their home cage.

In Experiment 3 both groups of rats (i.e., saline-treated and TMZ-treated rats) were exposed to the small exposure condition for 6 days. This was immediately followed by the extra small separation condition of SLR as described above. The same schedule of behavioural testing was used in Experiments 2 and 3, which was illustrated in Figure 5.4.

5.2.5. Histology

After behavioural testing was complete, rats were anaesthetized by IP injection of 2 ml of Euthatal (Rhone Merieux, Harlow, Essex, UK) and perfused transcardially with phosphate buffered saline (PBS), followed by 10 % neutral buffered formalin (NBF). In Experiment 1 the rats were sacrificed 7 days after the last day of the exposure treatment. In Experiments 2 and 3 the rats were sacrificed after completing SLR. The brains were removed and post-fixed in NBF for at least 24 h, followed by immersion in a 30 % sucrose solution for at least 48 h. The brains collected from all of the rats were cut in 30 μm sections using a freezing microtome. Brain sections from Experiment 1 were stained for BrdU, an exogenous thymidine analogue, and doublecortin (DCX), a microtubule-associated protein expressed by immature neurons. Sections from Experiment 2 were stained for DCX. Sections from Experiment 3 were stained for BrdU. Dr. Charlotte Oomen and Dr. Jeff Davies provided the training and protocols used for the immunohistochemistry.

BrdU: Sections from Experiments 1 and 3 were prepared for BrdU immunohistochemistry by blocking endogenous enzymatic activity, using 0.5 % peroxide in phosphate buffer for 20 min, 2 N HCL (37 ^{o}C) for 30 min, and a 0.1 M borate buffer for 10 min, before being incubated in the primary antibody (Experiment 1 used monoclonal murine anti-BrdU, Roche, 1:1000; Experiment 3 used mouse anti-BrdU 1:200, AbD Serotec) at 4 °C overnight. Signal amplification was achieved by incubation with biotinylated secondary antibody (Experiment 1 used sheep anti mouse

GE Healthcare, 1:200; Experiment 3 used biotinylated goat anti-mouse, Vector labs, 1:200) and avidin-biotin enzyme complex (ABC kit, Elite Vectastain, 1:800). Chromagen development was performed with diaminobenzidine (DAB; 20 mg 100 ml^{-1} TB, 0.01 % H_2O_2). Hemotoxylin (Ehrlich, Sigma-Aldrich) was used as a counterstain.

DCX: DCX is used as a marker for immature neurons because it is transiently expressed in proliferating progenitor cells and newly generated neuroblasts (Brown et al., 2003). Sections from Experiments 1 and 2 were prepared as described in Chapter 3 (Oomen et al., 2010). Sections were incubated in primary antibody (polyclonal goat anti-DCX, Santa Cruz, 1:800) and signal amplification was accomplished by further incubation with biotinylated secondary antibody (horse anti goat, Vector labs, 1:500) and ABC (Elite Vectastain; 1:800). Subsequent chromogen development was performed with DAB (20 mg/100 ml Tris buffer; TB, 0.01 % hydrogen peroxide; H_2O_2). DAB is a chromogen that is oxidized by H_2O_2 and appears as light brown deposits on the sections. (Hsu & Soban, 1982).

5.2.6. Data collection

For the behavioural data, exploration of objects was defined as described in Chapters 2 to 4. Sitting on an object or digging at the base of an object was not considered exploratory behaviour. Exploration was measured during the exposure protocol and the sample and choice phases of SLR. For the exposure and SLR sample phase, exploratory behaviour was recorded using stopwatches. For the choice phase of SLR, exploration was scored using the program JWatcher_V1.0, written in Java[TM] (JWatcher, USA). This is the same program used in the experiment described in Chapter 4. The program had two keys corresponding to each object. Exploration was recorded by pressing the appropriate keys at the onset and offset of a bout of exploration.

For the histological data, BrdU-positive cell counts were made blind to the conditions, using a compound light microscope (Leitz LaborLux, 20x objective) and a handheld manual counter. Counting was conducted twice and an average for each DG section was calculated. DCX-positive cell counts were made as described in Chapter 3. Stereological quantification (StereoInvestigator, Microbrightfield, Germany) was conducted using a stage-controlled brightfield microscope (40x objective). Cells were

counted at sites that were selected using systematic random sampling, in every tenth coronal section starting at bregma -2.1 with a total of 6 sections per animal. StereoInvestigator optical fractionator settings for DCX quantification were as follows: grid size 200 x 80 and counting frame 50 x 50, which resulted in an average of 250 markers counted per animal.

5.2.7. Data analysis

Exploration during the exposure protocol and the sample phases of SLR were analysed using a mixed model analysis of variance (ANOVA) or Student's *t*-test to ensure that there was equal exploration between groups.

Results from the choice phases were expressed as discrimination ratios (D2), as follows:

$$D2 = \frac{novel\ location\ exploration - \ familiar\ location\ exploration(s)}{novel\ location\ exploration + \ familiar\ location\ exploration\ (s)}$$

Group mean D2 scores were analyzed with repeated measures ANOVA, followed by *post-hoc* Student's *t* contrasts using the Bonferroni correction.

For the histological analysis, total numbers of DCX-positive cells were quantified by systematic random sampling performed with the Stereo Investigator system (MicroBrightField). DG granule cell layer and molecular layer surface area and volume measurements were performed according to Cavalieri's principle using the Stereo Investigator system (MicroBrightField). Estimates for the total number of DCX-positive cells in the DG, as well as the density of DCX-positive cells in the DG granule cell layer, were calculated using the random samples collected. The results from the histological analysis of Experiment 1 were analysed using a one-way ANOVA, followed by *post-hoc* Student's *t*-test using the Bonferroni correction. The results from the histological analysis for Experiments 2 and 3 were analysed using an unpaired two-tailed Student's *t*-test.

All statistical analyses were conducted with SPSS version 22 and Microsoft Excel version 14.4.5. Statistical significance was set at $p < 0.05$, unless running a post-hoc

comparison. To control for Familywise error, the level of statistical significance (i.e., α) for post-hoc comparisons was calculated using the Bonferonni correction, which was calculated as 0.05 divided by the number of statistical comparisons. All data are presented as mean ± standard error of the mean (SEM).

5.3. Results

5.3.1. Pattern separation during encoding upregulates neurogenesis

To investigate if incidental learning requiring pattern separation upregulates neurogenesis, rats were injected with BrdU 1 week prior to a 6-day exposure protocol. As shown in Figure 5.6, there was no difference in the amount of time spent exploring the objects in the small and large exposure conditions. No objects were presented during the control condition so object exploration could not be measured. As shown in Figure 5.7, there were no statistically significant differences in cells labelled with BrdU ($F_{2,21}$ = 0.607, p = 0.5). Figure 5.8 shows representative images of DG sections labelled with BrdU from the three conditions. In contrast, as shown in Figure 5.9, a one-way ANOVA revealed a statistically significant difference in the overall density of DCX-labelled cells in the DG ($F_{2,21}$ = 5.885; p = 0.009), such that the rats exposed to the small separation exposure condition had a 42 % increase in the density of DCX-positive cells. Post-hoc comparisons revealed no differences between the overall density of DCX-positive cells in the DG of rats from the control and large exposure conditions, but there were significant differences between the rats from the small exposure condition and the control (p = 0.01) and large exposure conditions (p = 0.02). However, as shown in Figure 5.10, there was no statistically significant difference between the total number of estimated DCX-positive cells in the DG (p = 0.20). Figure 5.11 shows representative images of DCX-stained DG sections from the three groups. Although the group difference of BrdU-labelled cells and the total estimated number of DCX-positive cells were not statistically significant, there was the same trend of more cells occurring in the group exposed to the small exposure condition. The small exposure condition had the highest load on pattern separation, suggesting that repeated exposure to spatial landmarks requiring pattern separation increases cell proliferation (i.e., DCX-positive

cells), but may not affect cell survival (i.e., BrdU-labelled cells born 1 week prior to the start of exposure). Although a larger study is needed before drawing this conclusion.

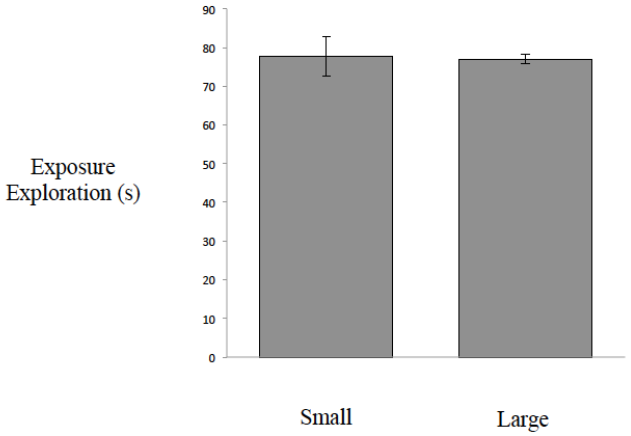

Figure 5.6. Total amount of time spent exploring during the exposure protocol. The y-axis represents the total amount of time spent exploring the objects during a 10 min exposure in the small (left) and large (right) exposure conditions. There was no difference between groups. Data are expressed as the mean ± SEM.

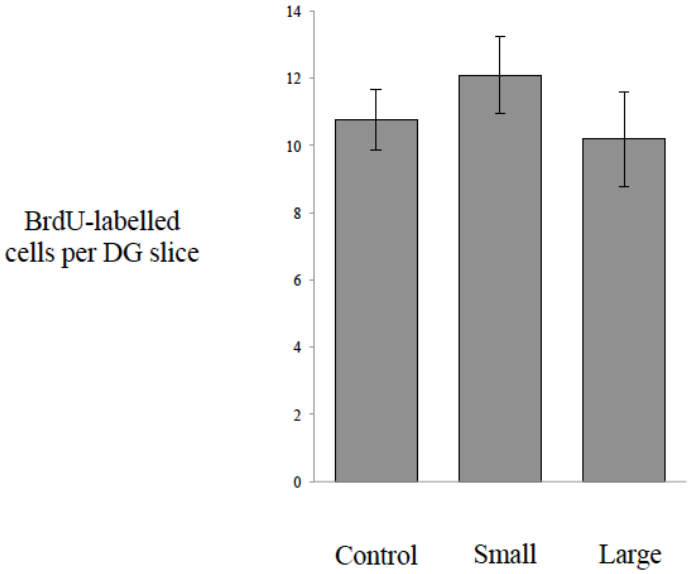

Figure 5.7. BrdU-labelled cells per section of the DG after the exposure protocol. The y-axis shows the mean number of BrdU-labelled cells in a 30 μm section of DG from rats in the control exposure condition (left), small exposure condition (middle), and large exposure condition (right). There was not a statistically significant difference between groups ($p = 0.5$). Data are expressed as the mean ± SEM.

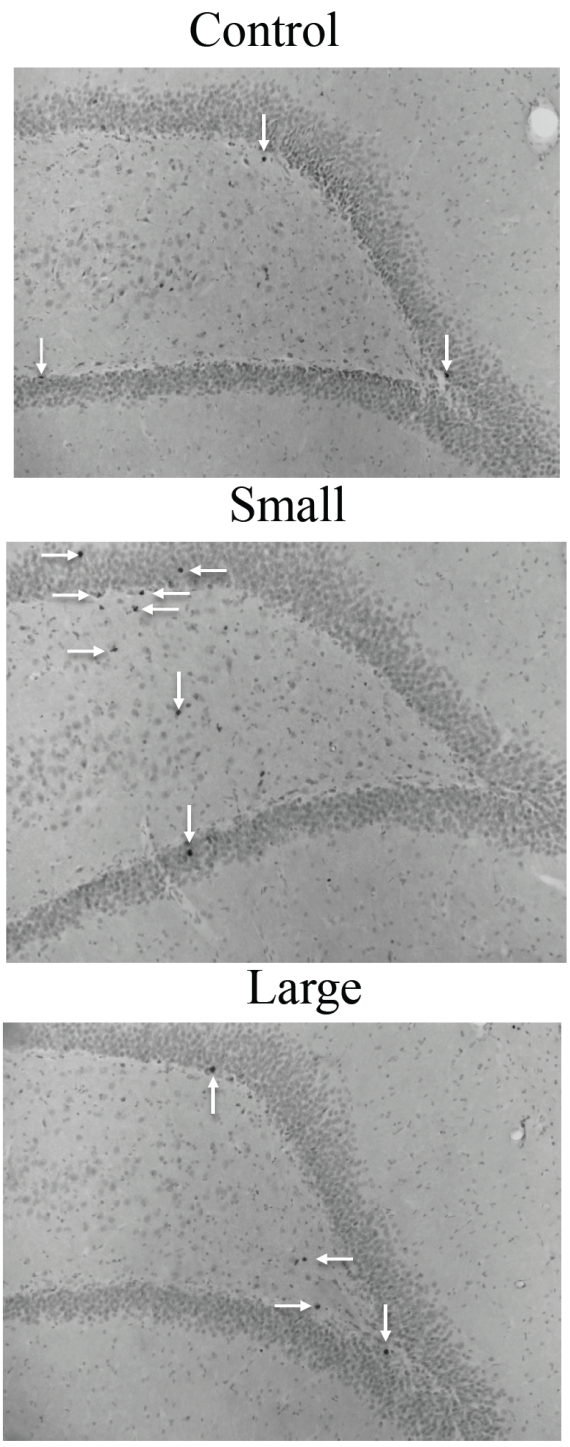

Figure 5.8. Representative images of BrdU-labelled DG sections from Experiment 1. The sections come from rats in the control exposure condition (top), small exposure condition (middle), and large exposure condition (bottom). The white arrows highlight BrdU-positive cells, which appear as dark spots. Images were taken using a digital camera (AxioCam, Zeiss) through the 10x objective on a light microscope (Axio Imager 2, Zeiss).

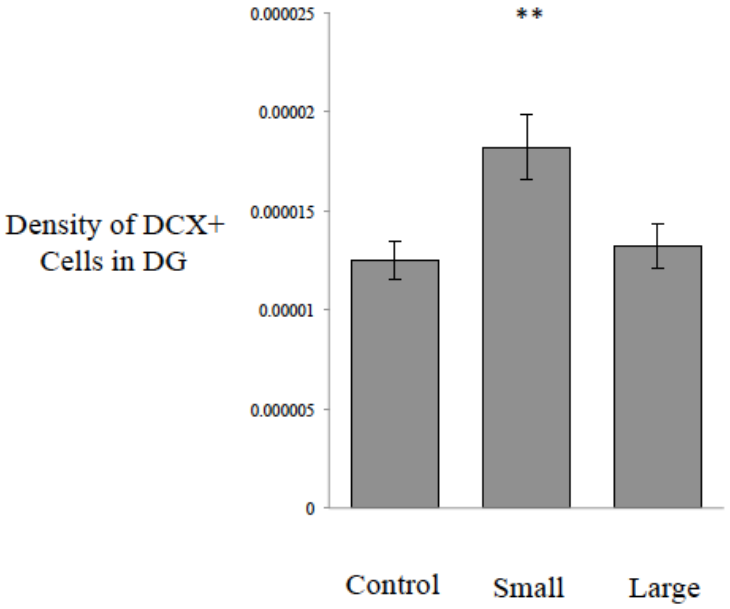

Figure 5.9. Density of DCX-positive cells in the DG after the exposure protocol. The y-axis shows the mean density of DCX-positive cells from the granule layer of rats in the control exposure condition (left), small exposure condition (middle), and large exposure condition (right). Rats in the small exposure condition had a higher density of DCX-positive cells, ** $p = 0.009$. Data are expressed as the mean ± SEM.

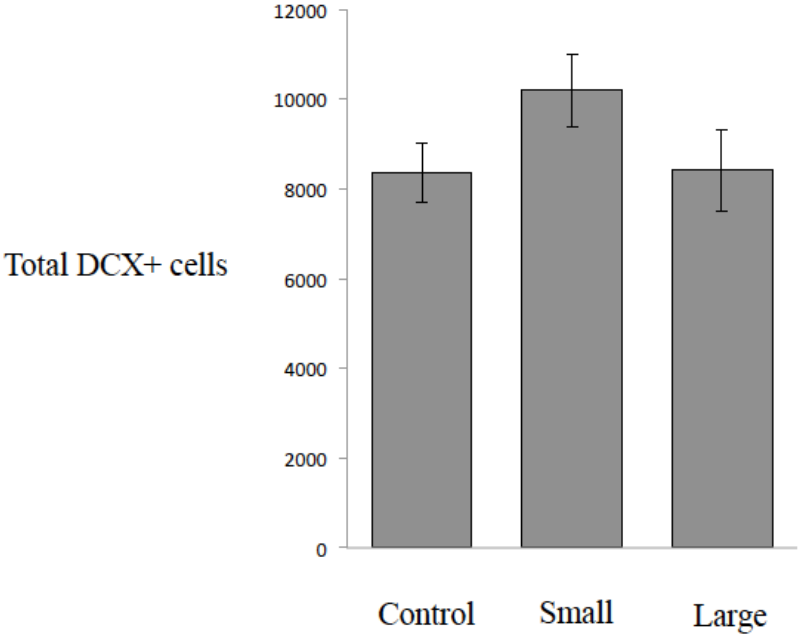

Figure 5.10. Total number of DCX-positive cells in the DG after the exposure protocol. The y-axis shows the total number of DCX-positive cells in the DG from rats in the control exposure condition (left), small exposure condition (middle), and large exposure condition (right). There was no statistically significant difference between the groups ($p = 0.20$). Data are expressed as the mean ± SEM

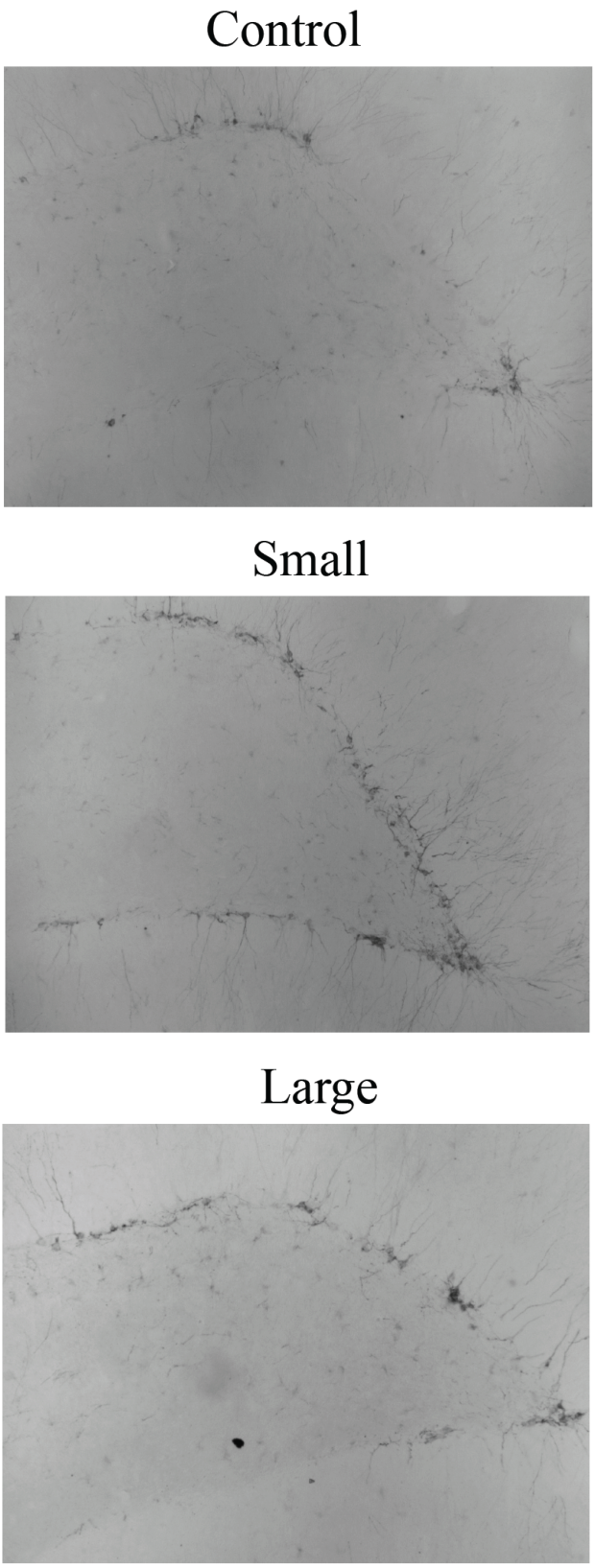

Figure 5.11. Representative images of DCX-stained DG sections from Experiment 1. Samples from the control exposure condition (top), small exposure condition (middle), and large exposure condition (bottom), were stained with an anti-DCX antibody and DAB. Images were taken using a digital camera (AxioCam, Zeiss) through the 10x objective on a light microscope (Axio Imager 2, Zeiss).

5.3.2. Neurogenesis in the DG is necessary for exposure-induced improvement in pattern separation

To investigate the functional significance of the possible upregulation, we used the same lentiviral approach described in Chapter 3 to specifically attenuate DG neurogenesis by inhibiting Wnt signalling. These rats underwent the same 6-day exposure protocol used in Experiment 1. Performance of the LV-dnWnt and LV-GFP groups were then compared on the extra small separation condition of the SLR task. As shown in Figures 5.12 and 5.13, the dnWnt rats had a 30 % reduction in the overall density of DCX-labelled cells in the DG (p = 0.041) and a 15 % reduction in the total number of estimated DCX-labelled cells in the DG (p = 0.025). Figure 5.14 shows representative images of DCX-stained DG sections from the LV-GFP and LV-dnWnt groups.

The results of the SLR task revealed that only the control group, which was injected with LV-GFP, showed a preference for the novel location in the extra small condition (p = 0.016). Figure 5.15 shows the D2 scores for the LV-GFP and LV-dnWnt groups. The group injected with LV-dnWnt that had reduced neurogenesis, did not show a preference for the novel location.

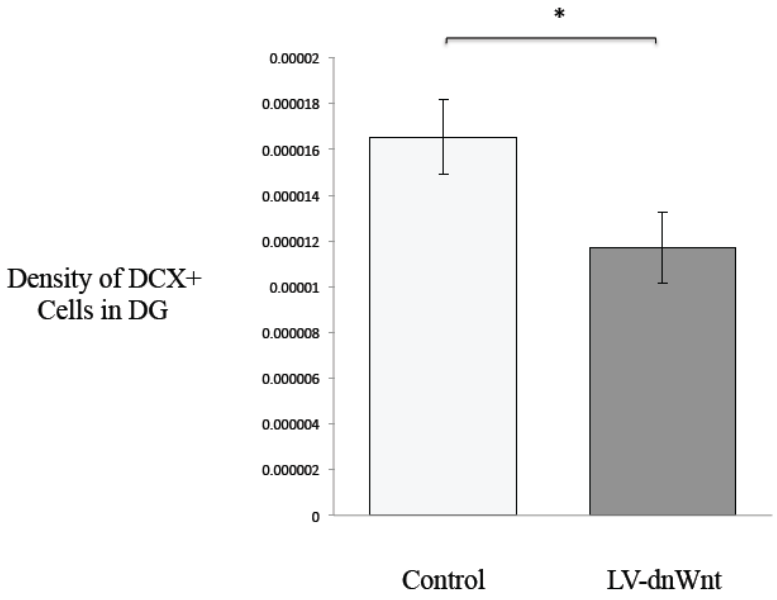

Figure 5.12. Density of DCX-positive cells in the DG of rats from Experiment 2. The y-axis shows the density of DCX-positive cells from DG sections of rats injected with the control LV-GFP (left) and rats injected with LV-dnWnt (right). There was a significant reduction in the density of new cells in the rats injected with the LV-dnWnt (p = 0.041). Data are expressed as the mean ± SEM.

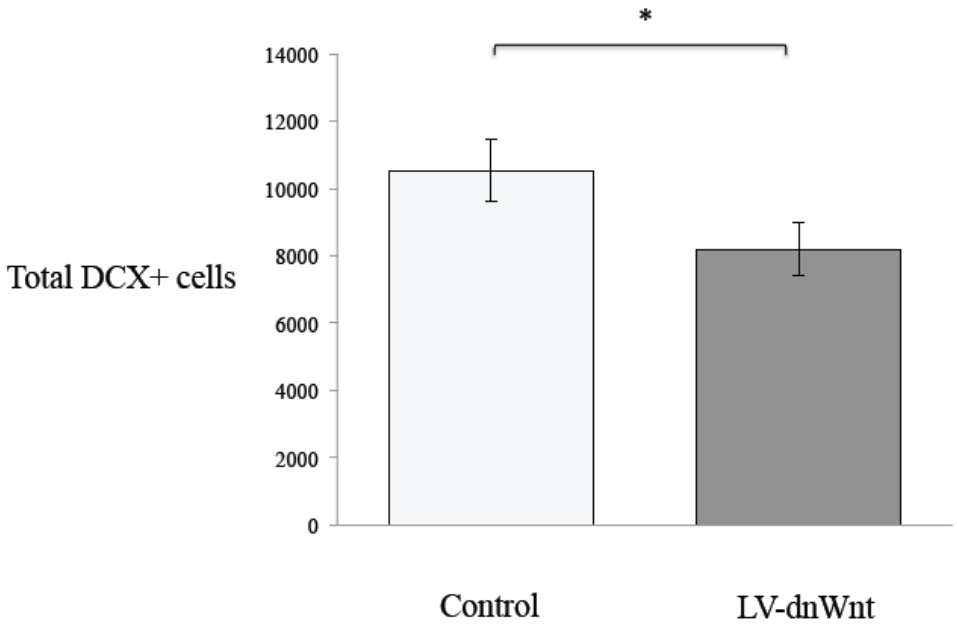

Figure 5.13. Total number of DCX-positive cells in rats from Experiment 2. The y-axis shows the number of DCX-positive cells in the DG of rats injected with the control LV-GFP (left) and rats injected with LV-dnWnt (right). There was a significant decrease in the number of new cells in the rats injected with LV-dnWnt (p = 0.025). Data are expressed as the mean ± SEM.

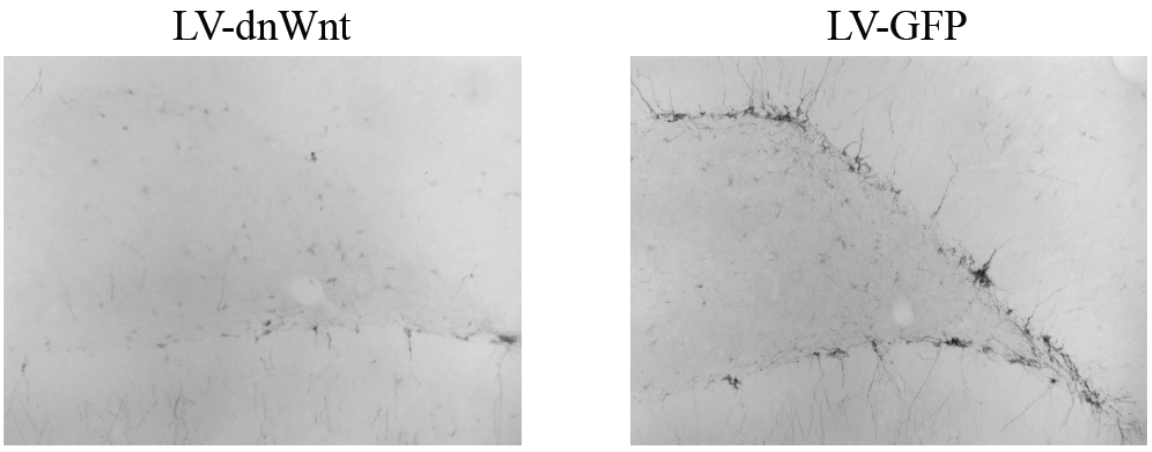

Figure 5.14. Representative images of DCX-stained DG sections from Experiment 2. Sample sections from an LV-dnWnt rat (left) and LV-GFP rat (right) stained with an anti-DCX antibody and DAB. Images were taken using a digital camera (AxioCam, Zeiss) through the 10x objective on a light microscope (Axio Imager 2, Zeiss).

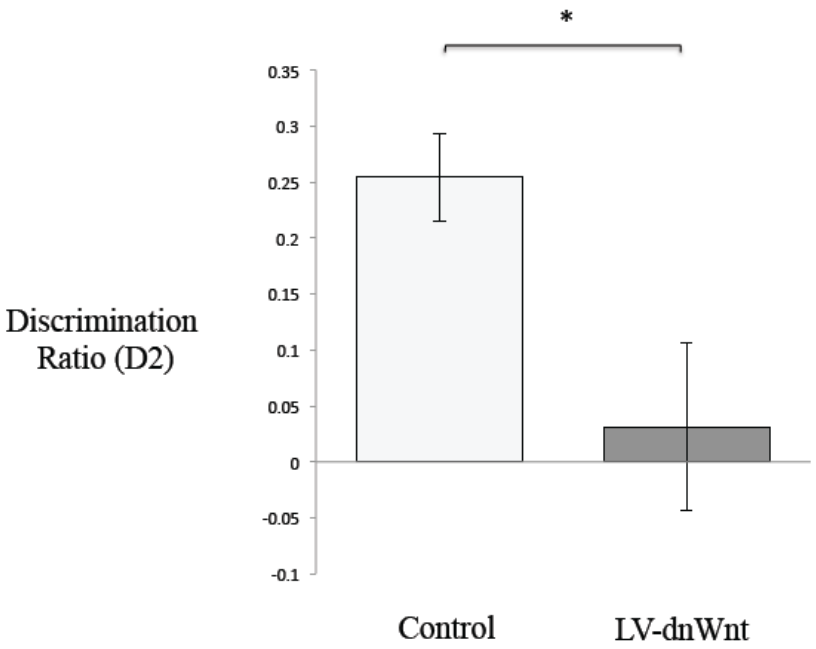

Figure 5.15. D2 scores on extra small SLR in Experiment 2. The y-axis shows the D2 scores of rats injected with LV-GFP (left) and rats injected with LV-dnWnt (right) during the choice phase of the extra small separation condition of the SLR task. Positive D2 scores reflect a preference for the novel location. The control group (LV-GFP) showed a preference for the novel location, but the LV-dnWnt group did not. SLR was conducted after the small exposure protocol. * $p < 0.05$. Data are expressed as the mean ± SEM.

To try and replicate this effect using another method to reduce hippocampal neurogenesis, a new group of rats were treated with TMZ. It should be noted that the final sample size was 8 per group. In the TMZ-treated group, one rat was removed during the first week of injections because it was abnormally sensitive to the needle and was taken out of the study. In the saline-treated group, one rat was removed from the SLR analysis because of an error in object placement. Finally, as a result of excessive jumping out of the maze (> 10 during the 5 min choice phase), two rats (one from each condition) were removed from the analysis of SLR. The removal of subjects reduced the power of this study and the future replication will start with a larger sample size.

Because TMZ had never been used in conjunction with SLR, general levels of exploratory behaviour were analysed to ensure that the treatment did not affect general levels of exploration. Independent samples *t*-tests confirmed that the total amount of exploration during the 6-day exposure protocol was not different between groups (p = 0.20) and the total amount of object exploration during the sample phase of SLR was

not different between groups (p = 0.73). Figure 5.16 shows the total amount of sample exploration for the saline-treated and TMZ-treated groups.

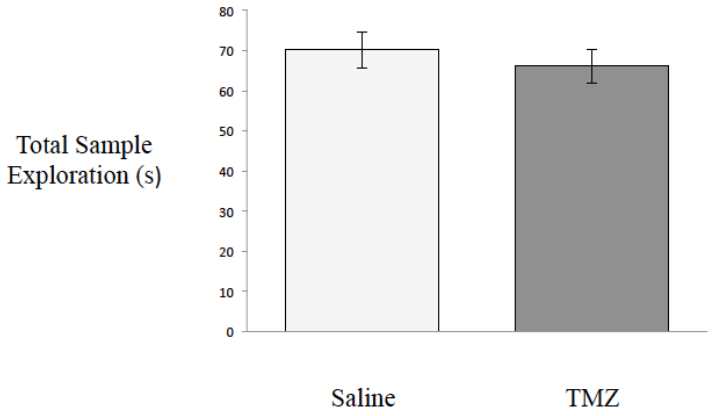

Figure 5.16. Exploration during the sample phase of the extra small condition of SLR in Experiment 3. The y-axis represents the total amount of time spent exploring the objects during the sample phase. There was no difference in the total amount of exploration between the groups. Data are expressed as the mean ± SEM.

As shown in Figure 5.17, during the choice phase of the extra small separation condition of the SLR task, only the saline-treated group showed a preference for the novel location, whereas the TMZ-treated group did not show a preference. However, the difference between groups was not statistically significant (p = 0.09).

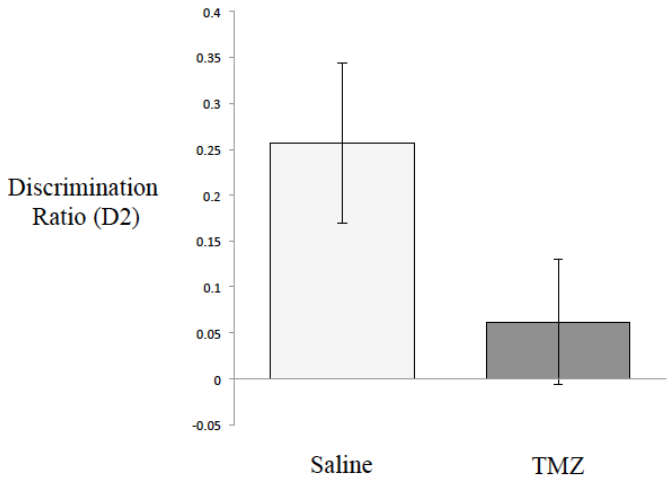

Figure 5.17. D2 scores on the extra small condition of SLR from Experiment 3. The y-axis represents performance during the choice phase of the extra small separation condition of the SLR task. Positive D2 values reflect a preference for the novel location. Only the saline-treated group showed a preference for the novel location, whereas the TMZ–treated group did not show a preference. The difference between groups was not statistically significant (p = 0.09; n = 8 per group). Data are expressed as the mean ± SEM.

Unfortunately, due to an accident in the laboratory, there was a failure in tissue preservation that rendered the tissue unanalyzable. Therefore, histological analysis for Experiment 3 was inconclusive and the level of neurogenesis could not be confirmed.

Together, these results suggest that the exposure protocol with the highest load on pattern separation (i.e., small exposure condition) was associated with enhanced performance on the SLR task in the control groups, but not the dnWnt or TMZ-treated groups that had reduced neurogenesis.

5.4. Discussion

The current study was designed to evaluate whether engaging pattern separation during incidental learning affects production or survival of adult-born neurons in the DG and whether these changes then affect performance on a task that requires pattern separation. The results revealed that rats repeatedly exposed to spatial landmarks that engage pattern separation during encoding had increased DG neurogenesis, as measured by the density of DCX-positive cells in the DG granule layer. The same exposure protocol resulted in improved performance on the SLR task, which requires spatial pattern separation. Importantly, the improvement on SLR following the exposure protocol was not exhibited in rats when hippocampal neurogenesis was inhibited, using two mechanistically different methods. Together, these results suggest that pattern separation can upregulate neurogenesis in the DG, and that this increase can then result in an improved ability to separate overlapping input during encoding. However, larger sample sizes are needed in future replications before drawing these conclusions, particularly because the effect on neurogenesis appears to be small and variable.

This work followed the experiments described in Chapters 2 to 4, demonstrating an important role of BDNF and adult neurogenesis for spatial pattern separation. BDNF is a small dimeric secretory protein with an important role in excitatory transmission, as well as synaptic and structural plasticity in the adult brain (Korte et al., 1995; Kang et al., 1997; Tyler & Pozzo-Miller, 2001; Pang et al., 2004; Bramham & Messaoudi, 2005; Bamji et al., 2006). There is mounting evidence for a role of BDNF in learning and memory (Linnarsson et al., 1997; Mizuno et al., 2000; Tokuyama et al., 2000; Alonso et

al., 2002; Bekinschtein et al., 2007, 2008; Lee, Everitt, & Thomas, 2004), and BDNF has been shown to increase neurogenesis (Scharfman et al., 2005).

Previous work demonstrated that rats exposed to the small exposure condition (objects separated by 5 spaces, equivalent to a 50° angle from the centre), but not the large exposure condition (objects separated by 12 spaces, equivalent to a 120° angle from the centre), exhibit a spontaneous increase of BDNF in the DG (Bekinschtein et al., 2013). This finding suggested that rats engage a BDNF-associated process when presented with two ambiguous spatial representations. Importantly, this separation-dependent pattern of BDNF activation appears to be specific to the DG because it was not seen in other subregions of the hippocampus, such as CA1 (Bekinschtein et al., 2013).

It is possible that the exposure protocol leads to a repeated spontaneous increase of BDNF when the rat is exploring objects in the small exposure condition (Bekinschtein et al., 2013). This increase in BDNF may then act on newborn neurons as shown in Chapter 3 and/or cause the increase in neurogenesis (Scharfman et al., 2005). Further research into the cellular mechanisms is required before drawing conclusions about what is causing both the increase in neurogenesis and improved performance on SLR following the exposure protocol.

One possibility is that the repeated increase in BDNF causes the subsequent improvement in SLR. Because infusing BDNF into the DG has also been show to improve performance on the SLR task, it is possible that this increase leads to the performance enhancement; however, the spontaneous upregulation of BDNF is likely a much smaller quantity than the dose infused in previous experiments (e.g., 0.5 μg infused into the DG) and would not explain why control rats in the extra small separation SLR condition do not experience the same enhancement. Because immature adult-born neurons have enhanced plasticity and are more easily excitable (Ge et al., 2007; Schmidt-Hieber et al., 2004), it is possible that the new cells are more sensitive to BDNF levels present in the DG and regulate the exposure effect. The experiments described in Chapter 3 provide evidence that BDNF acts upon adult-born neurons in the DG during pattern separation because reducing neurogenesis with LV-dnWnt blocked the enhancement associated with BDNF infusions; however, it is possible that different mechanisms are causing the improvements from BDNF infusions and the exposure

protocol, particularly because BDNF is known to have several actions such as regulating long-term potentiation (Lu et al., 2008) and neuronal survival (Alderson et al., 1990).

The observed increase in neurogenesis may also be the cause of improved pattern separation. It has been previously shown that increasing the production of new neurons in the DG, following the introduction of a running wheel, improves performance on an automated touchscreen task of spatial pattern separation in mice (Creer et al., 2010), although the increase in neurogenesis following the introduction of a running wheel is much greater than that from exposure. Similarly, Sahay and colleagues (2011a) genetically augmented the survival of adult-born neurons, and demonstrated that mice with more new neurons had improved performance on a contextual fear conditioning task using similar contexts, which is presumed to require pattern separation. We showed a similar effect in Chapter 4, demonstrating that ghrelin treatment both increased neurogenesis in the DG and improved performance on SLR. Thus, it is possible that the increased neurogenesis associated with the small exposure condition does cause the improved performance on SLR.

Recently, Clemenson and colleagues (2014) reported that mice with increased neurogenesis due to environmental enrichment had an improved ability to discriminate between similar contexts, whereas mice with increased neurogenesis after being provided a running wheel, without further enrichment, did not show this enhancement. This suggests that there may be a fundamental functional difference between neurogenesis induced by different methods (i.e., exercise or environmental enrichment), and its role in pattern separation. These recent findings conflict with evidence that running can improve pattern separation (Creer et al., 2010), but agree with evidence presented in this chapter that the exposure protocol, which is a form of environmental enrichment and implicit learning, can improve pattern separation. The results also serve as a reminder that different mechanisms can upregulate neurogenesis and that the functional implications can vary between these mechanisms. I have tried to corroborate the evidence provided in Chapters 2 to 5 to elucidate the mechanisms underlying performance on SLR but the possibility that parallel processes are affecting performance should not be overlooked.

To summarize, this chapter provides evidence that a simple repeated exposure to similar spatial landmarks during an implicit learning task increases hippocampal neurogenesis and improves subsequent performance on SLR, along with the previously shown spontaneous increase in BDNF (Bekinschtein et al., 2013). Furthermore, there is evidence that the improved performance depends on the increase in neurogenesis. This provides an interesting possibility that the debate surrounding the effects of learning and enrichment on neurogenesis and cognition may be partially clarified by examining the load on pattern separation (Bekinschtein et al., 2011).

Chapter 6: Longitudinal evaluation of TgTau^{P301L} transgenic mice reveals cognitive impairments in old age

An understanding of the mechanisms underlying pattern separation has potential clinical implications for Alzheimer's disease (AD). In humans, there is some evidence of age-related deficits of pattern separation that are more pronounced in patients diagnosed with Mild Cognitive Impairment (MCI) or AD. One of the greatest challenges for finding effective treatments for AD is that we are currently without a valid mouse model that is adequate at reproducing AD phenotypes with predictive validity. To aid in the development of a model, the aim of the experiments described in this chapter was to provide a longitudinal behavioural profile of the TgTau^{P301L} model in multiple cognitive domains, across multiple ages. The P301L is a mutation of the MAPT gene, which encodes tau protein. Tau is the microtubule-associated binding protein implicated in neurodegenerative tauopathies, including frontotemporal dementia (FTD) and AD. These diseases result in the intracellular accumulation of hyperphosphorylated tau in the form of neurofibrillary tangles, the presence of which is associated with cognitive deficits. The findings of the experiments presented in this chapter suggest that the TgTau^{P301L} mouse model recapitulates the relatively extended age of onset of the behavioural symptoms often associated with neurodegenerative diseases. There were no apparent changes in executive function or attention in these animals. However, both object recognition and spatial recognition memory impairments were observed in these mice when aged, which is consistent with a dementia-like phenotype. This study represents the first comprehensive longitudinal analysis of cognition in the TgTau^{P301L} mouse model.

Pattern separation mechanisms were not explictly evaluated for a number of reasons, such as (1) prioritizing the 5-choice serial reaction time test (5-CSRTT) in hopes of detecting early attentional deficits, (2) not having a robust mouse version of the Spontaneous Location Recognition (SLR) task, (3) concern that the touchscreen tasks developed to assess pattern separation would interfere with the 5-CSRTT, and (4) once

a memory impairment was observed when the mice were aged, there was high attrition rate, low sample size, and high variability, which limited the options for testing and led to the prioritization of the histological analysis. However, the modified version of the novel object recognition paradigm that was used did allow for the assessment of vulnerability to interference, which is an indirect method to evaluate pattern separation in the object domain.

The TgTau^{P301L} mice used in these experiments were provided by Professor Paul Fraser at the Tanz Centre for Research in Neurodegenerative Diseases, University of Toronto, Canada.

6.1. Introduction

Alzheimer's disease (AD) threatens to become one of the greatest public health challenges of the 21st century, with an increasing number of ageing people throughout the world at risk. Unfortunately, the aetiology of AD is unknown and currently there are no effective drugs to prevent or cure the memory ailments associated with the pathology (Selkoe, 2011).

AD is the most common form of dementia, which is estimated to be affecting more than 44.4 million people worldwide (Alzheimer's Disease International, 2013). AD was first described by German psychiatrist and neuropathologist Dr. Alois Alzheimer in 1906. Alzheimer had a patient who was suffering from what is referred to as *presenile dementia*, because of her relatively young age of 51. After post-mortem analysis, Alzheimer identified numerous abnormal neural structures throughout her cerebral cortex. The original reports and histological slides from this first patient have since been rediscovered and analysed further by an international team of researchers (Graeber et al., 1997; Graeber, 1999).

Unfortunately, even though it has been over a century since AD was first identified, our understanding of the aetiology remains limited and there are still no effective treatments for this devastating disease. A comprehensive study of all clinical trials for AD treatments in the US found that 99.6 % of trials for potential therapeutics were ineffective and discontinued (Cummings et al., 2014). These failings in developing

effective treatments result in part from our incomplete understanding of the causal mechanisms underlying disease progression and the difficulty in recapitulating AD in animal models, which impedes translation to the clinic.

AD is a progressive neurodegenerative disorder, associated with severe amnesia, personality changes, compromised executive functions, and a variety of other behavioural impairments. The hallmark pathology of AD is the accumulation of misfolded proteins. Specifically, AD is associated with extracellular deposits of insoluble β-amyloid (Aβ) and intraneuronal accumulation of abnormally phosphorylated tau protein. These toxic multimetric complexes were acknowledged in the first reports of Aloysius Alzheimer and are referred to as Aβ *plaques* and tau neurofibrillary *tangles*. Plaques and tangles are associated with neuronal cell death and brain atrophy.

The discovery of the amino acid sequence for the main component of Aβ, isolated from the plaques in AD patients, gave rise to the "amyloid hypothesis" which remains one of the dominant hypotheses in the field (Glenner & Wong, 1984). The strongest genetic risk factor for AD is the apolipoprotein E (*APOE*) gene, linked to chromosome 21 (Heston et al., 1981), which affects Aβ clearance. APOE is a polymorphic gene with 3 common alleles (ε2, ε3, and ε4) that plays a central role in lipid metabolism and transport. The ε4 allele is associated with an increased frequency of late-onset familial AD (Strittmatter et al., 1993) whereas the ε2 allele is associated with a lower frequency of AD, suggesting that it is protective (Corder et al., 1994). Although the amyloid hypothesis of AD may eventually help uncover the causal mechanisms underlying AD and aid in the development of treatments, there is evidence that the other hallmark in AD pathology, the accumulation of neurofibrillary tau, more closely corresponds to the clinical expression of AD (Braak & Braak, 1997; Murray et al., 2015). Tau will be discussed in later sections of this chapter.

In addition to understanding the specific mechanisms of pathology, examining the cognitive phenotype associated with AD is useful for improving diagnosis and may help with the development of therapeutics. Episodic memory is one of the cognitive capacities most vulnerable in AD, accompanying extensive atrophy in medial temporal lobe structures. Because of this impairment in episodic memory and medial temporal lobe structures, some researchers have examined pattern separation in AD patients and

found evidence that a specific impairment in pattern separation underlies, at least in part, some of the amnestic symptoms exhibited by the patients.

6.1.1. Pattern separation and Alzheimer's disease

Evidence from functional Magnetic Resonance Imaging (fMRI) combined with cognitive tasks designed to evaluate pattern separation, suggests that humans exhibit age-related deficits of pattern separation that are more pronounced in patients diagnosed with Mild Cognitive Impairment (MCI) or AD (Yassa et al., 2010; Ally et al., 2013).

Kirwan and Stark (2007) developed a continuous recognition paradigm that presents subjects with a series of photographs, and asks participants to identify which pictures had been presented before, by differentiating them from other similar (i.e., lures) and dissimilar pictures. Lures were hypothesized to require an increased need for pattern separation due to overlapping object features that increase interference. Using this paradigm, it was shown that older adults are more likely to commit false positive errors and wrongly identify the lures as familiar (Toner et al., 2009; Yassa et al., 2011) and suggested an age-related deficit in pattern separation. This age-related impairment was replicated using a delay-match-to-sample task that varied the distance between dots presented on a screen (Holden et al., 2012) and an object-location task that varied the spatial displacements of images of everyday objects on the screen (Reagh et al., 2014). In all of these memory tasks, older subjects performed worse than younger subjects when similarity between the stimuli was high.

To evaluate whether this age-related impairment was more pronounced in elderly participants with early signs of cognitive impairment, Yassa and colleagues (2010) used a similar continuous pattern discrimination task to compare healthy older adults with patients diagnosed with amnestic MCI (aMCI). As before, the subjects were asked whether an image had been previously presented, and had to discriminate between photographs, perceptually similar lure items, and novel items. The aMCI patients were unable to effectively discriminate between repeated and lure items. These findings have been replicated (Stark et al., 2013) and extended to show that patients diagnosed with mild AD perform even worse than patients diagnosed with aMCI (Ally et al., 2013).

Furthermore, cerebrospinal fluid concentration of amyloid β42 is correlated with performance in this paradigm, and specifically with the ability to make difficult discriminations but not easier discriminations (Wesnes et al., 2014).

Performance on these memory tasks has been shown to be associated largely with hippocampal activity (Kirwan & Stark, 2007). In contrast, performance on another behavioural task developed by Barense and colleagues (2012a) to evaluate susceptibility to interference, has been shown to recruit activity in the perirhinal cortex but not the hippocampus. This may be partly because the task developed by Barense and colleagues is a visual discrimination task that does not explicitly involve memory. In their task, the stimuli used were abstract blob-like objects consisting of three distinct features: an inner shape, outer shape, and a fill pattern. On each trial, two objects were presented simultaneously but rotated to prevent a matching strategy, and the participants were asked if the objects were identical. The task had two conditions: (1) high interference, which contained consecutive trials of high ambiguity object discriminations, and (2) low interference, which contained mostly photographs of everyday objects that were easily discriminable and intermixed with high ambiguity blob-like images. This paradigm was inspired by the Representation-Hierarchical perspective, which was discussed in Chapter 1. Using this paradigm it was shown that patients at risk for MCI and patients diagnosed with aMCI were impaired on the high interference condition when compared to healthy older adults (Newsome et al., 2012). Notably, performance improved when the number of similar features viewed across trials was reduced to minimize perceptual interference.

Yeung and colleagues (2013) followed this up by designing another task to evaluate how this increased susceptibility to interference affected recognition memory in older adults at risk for MCI (Yeung et al., 2013). Their study used an eye-tracking-based methodology and presented subjects with photographs of everyday objects belonging to 12 semantic categories (e.g., coffee mugs, diamond rings, and socks). Participants were shown images from one semantic category during each testing block. Within each block, half of the images were shown during the study phase and then the other images were shown during the test phase. The images shown during the test phase were categorized as high interference foils if they were perceptually similar objects with high feature overlap and within the same semantic category, or low interference foils if they

were not perceptually similar but belonged to the same semantic category. Interestingly, because the images were presented in a continual stream, the participants were unaware when the sample phase ended and the choice phase began. Eye movements associated with novelty detection were used as an indirect measure of memory. The results revealed that patients at risk for MCI, falsely recognized the high interference novel objects as previously viewed, when compared to healthy older and young adults.

Although these studies conducted by Barense and colleagues (Barense et al., 2012; Newsome et al., 2012; Yeung et al., 2013) did not explicitly evaluate pattern separation processes, the increased susceptibility to interference may reflect an underlying deficit in pattern separation. As discussed in Chapter 1, pattern separation is a process that reduces interference among representations and is hypothesized to be necessary when having to disambiguate similar input.

To my knowledge pattern separation has not been directly tested in a mouse model of AD; however, there is some evidence from our lab that the tgCRND8 mouse model, which overexpresses Aβ, exhibits memory impairments resulting from enhanced encoding of interfering information and leads to false memories (Romberg et al., 2012). It was hypothesized that a selective deficit in pattern separation increased the mouse model's susceptibility to interference, which is similar to the false memories exhibited by aMCI and AD patients (Balota et al., 1999; Budson et al., 2001; 2006; Newsome et al., 2012; Dewar et al., 2012; Yeung et al., 2013). The memory paradigm used in the study by Romberg and colleagues (2012) to identify a false memory in the tgCRND8 mouse model, was used in the present experiment to evaluate whether the TgTau^{P301L} mouse model exhibited a similar susceptibility to interference. Furthermore, it was this paradigm that inspired the study by Yeung and colleagues (2013) in patients at risk for MCI, described above. This object recognition paradigm will be described in more detail in later sections.

6.1.2. TgTau(P301L) mouse model

AD and frontotemporal dementia (FTD) belong to a class of neurodegenerative disorders referred to as tauopathies. Tauopathies are histologically characterized by abnormal intracellular accumulation of hyperphosphorylated tau. Encoded by the

MAPT gene, tau is a microtubule-associated binding phosphoprotein involved in the assembly and stabilization of the cytoskeleton, which regulates neuronal processes and axonal transport. During pathogenesis, brain dysfunction and degeneration is linked to the progressive accumulation of hyperphosphorylated tau aggregates that form intracellular, filamentous inclusions, and neurofibrillary tangles (NFT; see Wang et al., 2013 for a review). Patients diagnosed with tauopathies often experience impairments in multiple mnemonic and non-mnemonic cognitive domains, such as attention and executive control.

In human patients, abnormal tau aggregates are observed in brain regions exhibiting neuronal loss, suggesting that dysregulation of tau may cause the neuronal cell death associated with the disease pathology (Gomez-Isla et al., 1997; Spires-Jones et al., 2009). The intracellular accumulation of tau aggregates also parallels memory disturbances and AD diagnosis criteria (Braak & Braak, 1995; Ohm et al., 1995). Because it may take up to 40 years from the first appearance of NFTs for a clinical diagnosis of AD, there is great interest in the role of tau in the earliest cellular changes that lead to functional deficits (Ohm et al., 1995).

Tau abnormalities alone are sufficient to cause neurodegenerative disease (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). Both NFTs and smaller tau oligomers are associated with neurotoxicity and cognitive deficits (see Ren & Sahara, 2013 for a review), and abnormal tau can contribute to neuronal dysfunction independently and prior to NFTs forming (Wittman et al., 2001; SantaCruz et al., 2005; Berger et al., 2007; Rocher et al., 2010). For example, a mouse model expressing a repressible mutant form of tau, showed improved memory and less neuronal cell loss when tau expression was suppressed, even though NFTs remained unaffected (SantaCruz et al., 2005).

Patients with tauopathies, such as AD and FTD, show central executive functioning impairment, demonstrating compromised performance on tasks assessing working memory, attention, and executive control (Nedjam et al., 2004; Stopford et al., 2012). Clinically, AD can be different than FTD. FTD patients often display more socially inappropriate behaviour and apathy during cognitive testing and FTD and AD patients can have qualitatively distinct cognitive performance profiles. For example, Stopford

and colleagues (2012) reported that FTD patients exhibited greater executive deficits in attention, set shifting, and response inhibition, while working memory was more prominently compromised in AD patients. Although these differences between FTD and AD patients have been independently reported (Thompson et al., 2005) they are not universally observed (e.g., Grossi et al., 2002; Nedjam et al., 2004).

The difference in symptoms detected between the various tauopathies is likely because the specific strains of misfolded tau species generated in each disorder selectively affect distinct brain regions, which are vulnerable to different forms of inclusions (Sanders et al., 2014; Clavaguera et al., 2013a,b). These differential effects also explain some of the variance in presentation within the same tauopathy.

For example, FTD patients with Parkinsonism linked to chromosome 17 (FTDP-17) show severe atrophy in the frontotemporal lobe, varying degrees of neurodegeneration in subcortical nuclei, and tau-positive pretangles, neurofibrillary tangles, and glial fibrillary tangles (Foster et al., 1997). However, the precise clinical and histological profile of FTDP-17 is dependent on the specific MAPT mutation expressed by an individual patient. A number of mutations in the MAPT gene have been associated with FTDP-17. Among these, the P301L mutation in MAPT exon 10 that results in a Pro→ Leu change at amino acid 301 (Bird et al., 1999; Nasreddine et al., 1999; Hutton et al., 1998; Dumanchin et al., 1998; Rizzu et al., 1999) is most frequently observed in FTDP-17 patients (Poorkaj et al., 2001) and is also a mutation associated with familial AD.

Developing transgenic animal models that recapitulate tau pathology is important for developing effective therapeutics. Some mouse models of FTDP-17 show hyperphosphorylation of tau, memory impairments, and increased mortality (Lewis et al., 2000; Terwel et al., 2005; Tatebayashi et al., 2002; Pennanen et al., 2006). For example, transgenic ($TgTau^{P301L}$) mice expressing the P301L mutation within the longest form of tau (2N, 4R) have previously been shown to exhibit tau pathology development in the hippocampus, amygdala, and cerebral cortex by 3 months of age, tau-positive pre-tangles by 10 months of age, and extensive NFTs throughout the frontotemporal cortex at 18 to 24 months of age (Murakami et al., 2006). This progressive neuronal impairment and accumulation of NFT, is associated with age-

related cognitive deficits, recapitulating the pathology seen in patients with FTD and AD (Murakami et al., 2006; Wakasaya et al., 2011).

Although TgTau^{P301L} is a model of FTD and not specifically AD, the mouse does exhibit similar histopathological features to AD patients (Sasaki et al., 2008; Wakasaya et al., 2011), which is not surprising given the overlap of pathological features between the taupathies and that the P301L mutation is associated with both FTD and AD. For example, the TgTau^{P301L} model develops tau tangles in many of the same brain regions as AD patients, such as the hippocampus, basal forebrain, and cerebral cortex (Murakami et al., 2006), and a comparative study using immunocytochemistry to analyze AD patients and the TgTau^{P301L} model revealed similar microglial activation in the grey matter associated with phosphorylated tau deposition (Sasaki et al., 2008). Furthermore, it is reasonable to assume that the cognitive effects of tau tangles in certain brain regions may generalize between the tauopathies and thus the TgTau^{P301L} mouse does represents a partial model of AD.

6.1.3. Overview

To further examine the effects of P301L mutant tau, the aim of the following experiment was to provide a longitudinal assessment of the TgTau^{P301L} mouse model across three cognitive domains.

Firstly, the TgTau^{P301L} model was evaluated using the 5-choice serial reaction time test (5-CSRTT) to assess executive function and attention. This was partly chosen because of the regional specificity of tau pathology in the TgTau^{P301L} model (i.e., frontotemporal lobe structures), and also because some evidence suggests that executive and attentional deficits may be the earliest cognitive symptoms of AD, occurring prior to deficits in spatial memory and language impairments (Lawrence & Sahakian, 1995; Perry et al., 2000; Collette et al., 1999; Baddeley et al., 2001). Impairments in executive and attentional processes may also be a predictive preclinical feature of AD (Albert et al., 2001). Because of this possible utility in early detection, 5-CSRTT was the first task tested in the longitudinal design.

Frontal cortex-dependent executive function and attention were examined at 4, 7, 12, and 16 months of age using a touchscreen version of the 5-CSRTT. 5-CSRTT is typically performed in rats (Muir et al., 1994; Mirza & Stolerman, 2000) and mice (Humby et al., 1999; de Bruin et al., 2006; Patel et al., 2006; Lambourne et al., 2007; Pattij et al., 2007) using behavioural chambers equipped with arrays of 5 or 9 nose poke apertures. However, the touchscreen version, which our laboratory has previously used successfully with the 3xTgAD and TgCRND8 models (Romberg et al., 2011; Romberg et al., 2013), is more similar to touchscreen-based tasks used to study this construct in patients (Sahakian et al., 1993ab), and thus assumed to be more translational than the nose poke aperture boxes.

Secondly, the TgTau^{P301L} model was evaluated using object recognition tasks. Recognition memory represents a fundamental ability to identify an object and judge whether it has been previously encountered. It is the memory required to detect a repeat occurrence of an object, which requires the capacity for both identification of an object and judgment of familiarity. Performance on visual recognition memory tasks is highly predictive of conversion to AD and impairments are considered by some to be an early cognitive biomarker of disease (Didic et al., 2013).

The impairments in recognition memory exhibited by AD patients, are consistent with the extensive atrophy in the perirhinal cortex associated with AD (Juottonen et al., 1998). Histopathological studies suggest that AD pathology in the perirhinal cortex may precede the pathology in the hippocampus (Braak & Braak, 1991) and that visual recognition memory impairments may be experienced prior to deficits in hippocampal-dependent processes. Because of this, perirhinal cortex-dependent tasks were prioritized over hippocampal-dependent tasks in the following experiments. Furthermore, although the earliest pathological markers in the TgTau^{P301L} model are not specified as to the region of the cerebral cortex, the entorhinal cortex, which neighbours the perirhinal cortex, and the temporal cortex, which is where the perirhinal cortex is located, are both identified as sites of extensive pathology in this model at later ages; thus, it seems reasonable to assume that the early markers in the cerebral cortex do occur in the perirhinal cortex.

Perirhinal cortex-dependent recognition memory was assessed in the same cohort of TgTau^{P301L} mice using the Forced-choice or Decoupled version of the object recognition (OR) task at 5, 8, 13, 17, 19 and 21 months of age. The Decoupled variant of OR used here was developed by our lab group and has been used successfully to identify memory impairment in the TgCRND8 mouse model of AD (Romberg et al., 2012). As mentioned previously, a benefit of the Decoupled version is that if a mouse exhibits an impairment in recognition memory, the task allows the experimenter to differentiate between forgetting and false memory. This task inspired the previously mentioned study by Yeung and colleagues (2013) that identified an increase of false memories in patients at risk for MCI. A false memory may reflect a higher susceptibility to interference due to a deficit in pattern separation. Patients diagnosed with AD do not necessarily have accelerated rates of forgetting, even though the patients exhibit profound memory deficits (Christensen et al., 1998; Money et al., 1992), which is why differentiating between errors resulting from forgetting and false memory is important.

Thirdly, the TgTau^{P301L} model was evaluated using hippocampus-dependent spatial memory tasks. The T-maze and Location Recognition (LR) memory tasks were conducted at 18 to 20 months of age. Previous studies had demonstrated impairments on the Morris water maze (MWM) and radial arm maze between 9 and 13 months of age in this model (Murakami et al., 2006). As discussed in Chapter 1, the hippocampus is critically important for episodic memory (Squire & Zola-Morgan, 1991), impairment of which is an early and prominent manifestation of AD. Hippocampal atrophy occurs at an estimated rate of 8% over the two years when symptoms first appear in patients and volume declines in parallel with verbal and visual memory (Fox et al., 1996). I prioritized tasks that evaluated attention and recognition memory at earlier ages, because of the evidence that those functions were disrupted prior to hippocampal memory in patients and the goal was to detect the earliest cognitive symptoms.

Although the Decoupled OR paradigm allowed susceptibility to interference and false memories to be studied - hypothesized to be caused by a failure in pattern separation-pattern separation mechanisms were not explicitly evaluated in this longitudinal study for a number of reasons. Firstly, I prioritized the 5-CSRTT to detect any early attentional deficits. Secondly, I was concerned that the touchscreen tasks developed to assess pattern separation, such as Location Discrimination (LD) and Trial-unique

Nonmatching-to-Location (TUNL; Oomen et al., 2013), would interfere with the 5-CSRTT because the stimuli are very similar. Thirdly, we have not yet developed a robust version of the Spontaneous Location Recognition (SLR) task for mice, which will be discussed in Chapter 7. Fourthly, once the memory impairment was observed (i.e., at 18 to 22 months of age), there was high attrition rate, low sample sizes (particularly in the Tg+ group), and high variability in performance, which together resulted in very low power to detect any differences between the groups. Because of this, when the mice were 22 months of age, I decided to prioritize the histological analysis over continuing behavioural testing, which could have determined whether the deficits observed reflected a specific impairment in pattern separation. However, as discussed, the Decoupled version of the novel OR paradigm allowed me to assess vulnerability to interference at 5, 8, 13, and 17 months of age, which was an indirect method to evaluate pattern separation in the object domain.

Following completion of the behavioural evaluation, a detailed histological analysis was performed by Professor Paul Fraser's lab to enable the anatomical basis of the identified behavioural effects to be characterized. The histological analysis is not yet complete and is not included in this chapter.

Taken together, this study represents the first comprehensive longitudinal analysis of cognition in the TgTau^{P301L} mouse model. No deficits in executive function or attention were detected; however, spatial and object recognition memory impairments were observed between 18 and 21 months of age.

6.2. Methods

6.2.1. Animals

Professor Paul Fraser's lab provided 26 male mice expressing a P301L mutant version of the longest form of human tau [denoted TgTau(P301L)23027, for brevity TgTau^{P301L}] on the 129SvEvxFVB/N genetic background and non-Tg littermates (Murakami et al., 2006). The mice were bred at the Tanz Centre for Research in Neurodegenerative Diseases, University of Toronto, Canada. Mice were acclimatized to

the Combined Animal Facility at the University of Cambridge for at least seven days following delivery.

At the start of behavioural testing, mice (12 Tg^+ and 14 Tg^-) were 8 to 10 weeks of age. 9 mice died prematurely during the study, with 17 mice (6 Tg^+ and 11 Tg^-) remaining at the final evaluation at 22 months of age. 1 Tg+ died for unknown reasons in the home cage, 1 non-Tg died from a home-cage accident, and the other 7 mice were developing what appeared to be hind limb paralysis and sacrificed for humane reasons at various points throughout the study. See Table 6.1 for sample sizes during each phase of testing.

Mice were housed in groups of 2 to 3 on a 12 h light cycle (lights on 19:00 - 07:00). All behavioural testing was performed during the dark phase of the cycle. Mice were provided with *ad libitum* access to water, but food was restricted prior to the start of behavioural testing to maintain body weight at 85 – 90 % of free-feeding weight (average 29 g). Restricted feeding was maintained throughout the entire longitudinal study but weights were allowed to increase with age (to an average 34 g). There were no differences between the average weights of the Tg^+ and Tg^- groups throughout the study (data not shown; $p > 0.05$).

All procedures were performed in strict compliance with the animal care guidelines of the University of Toronto and the United Kingdom Animals (Scientific Procedures) Act 1986 and the Amendment Regulations 2012.

Table 6.1. Timeline of testing and experimental design

Age (months)	Behavioural Task	Sample Size
0-2		
3-4	Pre-training	N = 26 (12 Tg^+ and 14 Tg^-)
5	5-CSRTT	N = 25 (12 Tg^+ and 13 Tg^-)
6	Decoupled SOR (1 h and 24 h delay)	N = 24 (11 Tg^+ and 13 Tg^-)
7	5-CSRTT	N = 24 (11 Tg^+ and 13 Tg^-)
8	Decoupled SOR (1 h and 24 h delay)	N = 23 (11 Tg^+ and 12 Tg^-)
12	5-CSRTT	N = 23 (11 Tg^+ and 12 Tg^-)
13	Decoupled SOR (1 h and 24 h delay)	N = 23 (11 Tg^+ and 12 Tg^-)
16	5-CSRTT	N = 22 (10 Tg^+ and 12 Tg^-)
17	Decoupled SOR (1 h and 24 h delay)	N = 22 (10 Tg^+ and 12 Tg^-)
18	SLR (1 h delay)	N = 21 (9 Tg^+ and 12 Tg^-)
19	Forced-choice OR (3 h and 8 h delay)	N = 19 (7 Tg^+ and 12 Tg^-)
20	T-maze	N = 18 (6 Tg^+ and 12 Tg^-)
21	Forced-choice OR (8 h delay)	N = 17 (6 Tg^+ and 11 Tg^-)
22	Testing complete. Histology performed.	N = 17 (6 Tg^+ and 11 Tg^-)

6.2.2. Design and timeline

To provide a longitudinal cognitive profile of the TgTau^{P301L} mouse model, several behavioural tasks were conducted from 2 to 22 months of age. Table 6.1 shows the details of the design and sample sizes included at each time point.

6.2.3. Touchscreen 5-choice serial reaction time test (5-CSRTT)

Touchscreen 5-CSRTT (Bartko et al., 2011; Romberg et al., 2011) was used to evaluate attention and executive function and was conducted as previously described (see Horner et al., 2013 and Mar et al., 2013 for detailed protocols of pre-training and 5-CSRTT testing). Briefly, mice were trained to respond to a white square stimulus on the screen using a 2 s stimulus duration for a maximum of 40 trials or 60 min as the baseline measure. Once acquired, the subjects were assessed using a series of probe tests, in which stimulus duration, delay, and trials per session were systematically adjusted.

5- CSRTT apparatus

Mice were tested in sound and light- attenuating boxes with a ventilation system, house light, tone generator, and infra-red light camera. The testing box enclosed a touchscreen operant chamber and reward delivery system (Campden Instruments Ltd., Loughborough, UK). Black plastic masks with five response windows were placed on the touchscreen to minimize unintended screen contact and to help focus attention. Figure 6.1 provides an illustration of the testing mask**.** The system was controlled by Whisker and ABETII software (Campden Instruments Ltd.). Each mouse was assigned to a particular chamber for the entire duration of the study.

Figure 6.1. Illustration of the 5-CSRTT mask (from Mar et al., 2013). During 5-CSRTT training and probe trials, one of the response windows would illuminate for a specified duration.

5- CSRTT habituation (Stage 1)

Mice were habituated to the food reward (Yazoo® strawberry milkshake, FrieslandCampina UK Ltd) for two daily sessions by placing a sample in a small dish inside the home cages. This was followed by two additional daily sessions of habituation to the chamber, which consisted of placing each subject in their assigned testing chamber for 20 min and providing 0.2 ml of the food reward in the magazine.

5- CSRTT pretraining (Stage 2 – 5)

To gradually shape screen-touching behaviour, mice went through pretraining (Stages 2 to 5) previously described in detail by Horner and colleagues (2013) and Mar and colleagues (2013). Each session consisted of up to 30 trials and was a maximum of 60 min duration, unless otherwise specified (e.g., the *Vigilance Probe*).

Stage 2: "Initial Touch" is a Pavlovian phase, where a reward is delivered in the magazine with a corresponding audible tone. The criterion used for Stages 2 to 4, was completing 30 trials within 60 min.

Stage 3: "Must Touch" required the mice to touch the white square stimulus, which was pseudorandomly presented in one of the five screen locations. Touches were rewarded.

Stage 4: “Must Initiate” required the mice to touch the stimulus, retrieve the reward, and then initiate the next trial after a 5 s inter-trial interval (ITI), by poking their nose back into the magazine. Initiation was indicated by a click (100 ms) and the extinction of the magazine light.

Stage 5: “Punish Incorrect” was the final step of pre-training. This was the first phase when the mice received a *time-out* if an incorrect location (i.e., response window with no stimulus) was touched. During a time-out the stimulus disappeared from the screen and the house light came on. The criterion for this phase was completing 30 trials within 30 min, with 80 % accuracy, for two consecutive days.

5- CSRTT training

Once each mouse completed pretraining, sessions were increased to 40 trials each, and stimulus duration was systematically reduced from 8 s, to 4 s, to 2 s. Figure 6.2 provides a flowchart of the stages involved in a single trial of the 5-CSRTT training. Stimulus presentation was followed by a 5 s *limited hold* period when responses were still counted. Responses during the stimulus presentation or the limited hold period were registered as *correct* if in the location of the stimulus or *incorrect* if in one of the other four locations. After a response, if there was still time remaining in its presentation, the stimulus was immediately removed from the screen. If no response was made, an *omission* was recorded and the mouse received a 5 s time-out. Once the reward was collected, and following the 5 s ITI, the next trial could be initiated. After initiation and a 5 s fixed delay period, the next trial started. If a response was made during the 5 s delay between initiation and stimulus onset, it was recorded as a *premature* response and the mouse received a 5 s time-out. Once stimulus duration was 2 s, and mice were performing at greater than 80 % accuracy and less than 20 % omissions, for 3 out of 4 consecutive sessions, they were moved onto 5-CSRTT probe testing.

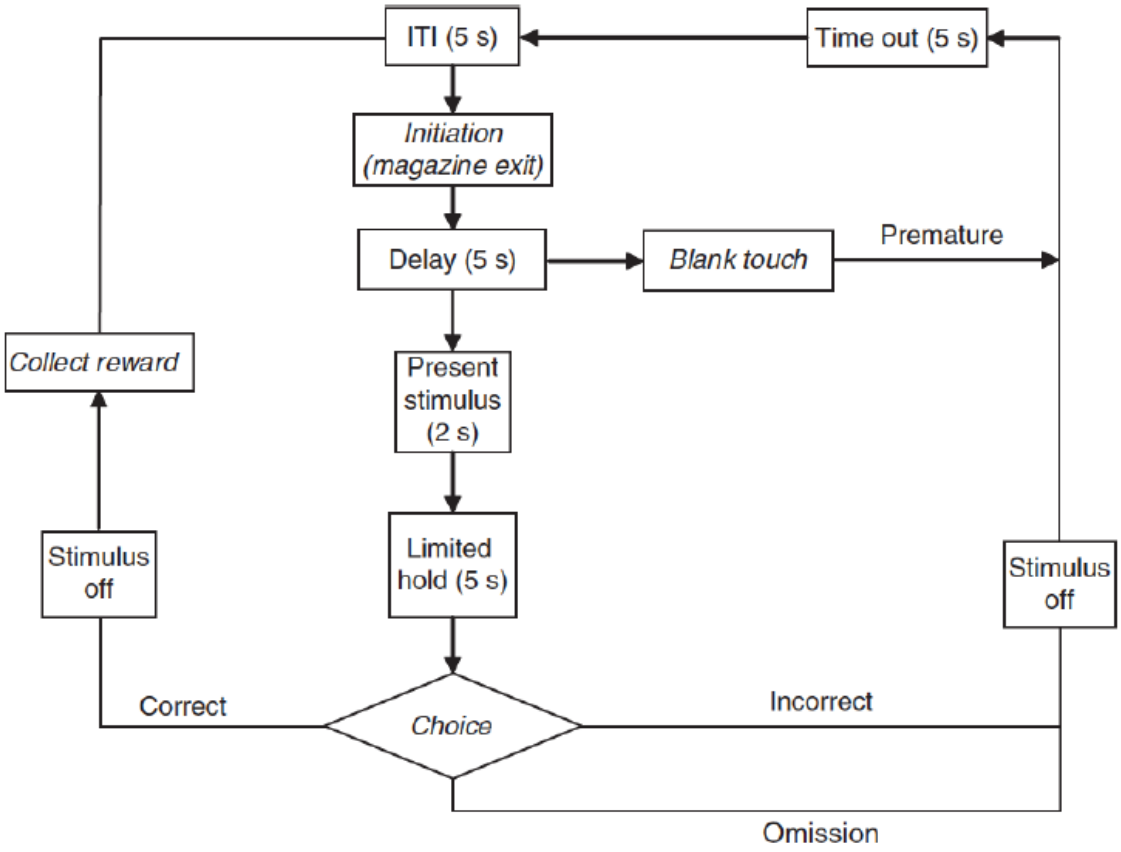

Figure 6.2. Flowchart of 5-CSRTT (from Mar et al., 2013). The flowchart was made by Dr. Chi Hun Kim and illustrates the stages that occur during one trial of 5-CSRTT.

5-CSRTT probe trials

Probe testing sessions were identical to the 5-CSRTT training sessions with the exception of stimulus duration, which was reduced from 2 s (baseline stimulus duration) to 1.6 s, 1.0 s, 0.8 s, and 0.6 s. Each stimulus duration was tested for two consecutive days, followed by 1 to 2 consecutive days of the 2 s baseline stimulus duration to ensure stable baseline performance.

At 7, 12, and 16-month time points, four additional probes were used. The stimulus duration was reduced to 0.4 s and 0.2 s and a *Vigilance Probe* and an *Impulsivity Probe* were included. The Vigilance Probe used a 2 s stimulus duration over 200 trials, for a maximum of 90 min. Because of the extended length of the session, the Vigilance Probe is a more sensitive measure for sustained attention. The Impulsivity Probe used a 2 s stimulus duration and 10 s delay, instead of the 5 s baseline delay. Because of the longer

delay, the Impulsivity Probe is a sensitive measure for assessing premature responding (Dalley et al., 2007).

5- CSRTT data analysis

The number of sessions to reach the criterion performance at each stage of pretraining and 5-CSRTT training was recorded. For the *5-CSRTT Probes* the following behavioural variables were evaluated: *accuracy, omissions, premature responding, perseverative responding, reward response latency, correct response latency, incorrect response latency, beam breaks front,* and *beam breaks back*.

Accuracy was defined as percentage correct, and was calculated as the number of trials on which a response was made to a correct location, divided by the total number of both correct and incorrect trials.

Omissions were defined as the percentage of all trials (i.e., correct + incorrect + omissions) on which the animal made no response.

Premature responses were defined as the number of touches made during the delay period prior to a stimulus appearing, and was used as a measure of impulsivity.

Perseverative responding was defined as the number of screen touches after a correct response, prior to collecting the reward, and was used as a measure of compulsivity.

Response latency was defined as the time between a stimuli appearing on the screen and the animal making a response.

Reward response latency was defined as the time taken to collect the reward after a correct response.

Beam breaks were defined as the number of times the mouse crossed the infrared beams near the screen (i.e., *beam breaks front*) or magazine (i.e., *beam breaks back*).

Data were analysed by converting trial data to group means on all of the performance measures described above, and analysed using repeated measures ANOVA, with a within-subject factor of stimulus duration and a between-subject factor of genotype. Each time point was analysed separately. Performance across ages was not compared or statistically analysed because of the variability in sample sizes and variation in the conditions used during testing.

All statistical analyses were conducted with SPSS version 22 and Microsoft Excel version 14.4.5. Statistical significance was set at $p < 0.05$, unless running a *post-hoc* comparison. To control for Familywise error, the level of statistical significance (i.e., α) for *post-hoc* comparisons was calculated using the Bonferonni correction, which was calculated as 0.05 divided by the number of statistical comparisons. All data are presented as mean ± standard error of the mean (SEM).

6.2.4. Object recognition (OR): Decoupled and Forced-choice

To evaluate perirhinal-dependent recognition memory, two versions of the OR paradigm were used during this study: *Decoupled OR* and *Forced-choice OR* (McTighe et al., 2010). These are both spontaneous tasks that do not require training. Much like the Spontaneous Location Recognition (SLR) task described in Chapters 2 to 5, OR takes advantage of a rodent's natural preference towards novelty. The spontaneous novel OR task was first developed by Ennaceur and Delacour (1988). Time spent exploring the novel and familiar objects is analysed and used to infer memory.

At least 30 min prior to testing, mice were brought into a holding room that was illuminated by a red light and adjacent to the testing room. All OR testing was carried out under dim white light conditions. Mice were individually transported in a cardboard carrying box between the holding room and the testing room.

OR apparatus

OR testing took place in a Y-maze (previously described in Romberg et al., 2012) made of homogenous opaque white Perspex. Walls were 30 cm high and each arm was 16 cm in length and 8 cm wide. A digital video camera was mounted above the Y-maze to

record all trials. One arm was used as the start arm, and the other two arms were used to present the testing stimuli, which were randomly shaped objects (dimensions approximately 10 cm x 4 cm x 4 cm) secured to the floor of the maze using Blu-tack ™. The maze and objects were wiped with a 50 % ethanol solution and dried between trials. The objects used and side of the maze in which the novel object was presented were counterbalanced.

OR habituation

Mice received two daily 5 min sessions of habituation to the empty maze prior to the first trial of OR. At later time points (i.e., 8, 13, 17, 19, and 21 months of age), only 1 day of habituation for 5 min was conducted prior to testing.

Decoupled OR

For Decoupled OR, testing was divided into two phases: *sample* phase and *test* phase. During the sample phase, the mouse was placed in the start arm of the Y-maze and allowed to explore two identical objects located at the ends of the other two arms for 5 min. The mouse was then removed from the maze and taken back to the holding room and placed in their home cage for either a 1 h or 24 h delay period. For the test phase, the mice were placed back into the same Y-maze apparatus and presented with one of two conditions for 5 min: *repeat* condition or *novel* condition. For the repeat condition, the same two identical objects (i.e., familiar) seen during the sample phase were presented. For the novel condition, two new (i.e., novel) identical objects were presented.

For each delay, mice were tested in both the repeat and novel conditions, using distinct object pairs, for a total of four trials at each time point. Trials were separated by at least 48 h to prevent interference and to prevent declining motivation. Objects were counterbalanced between mice to control for object bias.

Forced-choice OR

For Forced-choice OR, testing also consisted of two phases: *sample* phase and *test* phase. The 5 min sample phase was identical to Decoupled OR described above. However, during the test phase, mice were presented with one copy of the object used previously during the sample phase (i.e., *familiar* object) and a new object (i.e., *novel* object). Mice were allowed to explore the maze and objects for 5 min. Forced-choice OR used 3 h or 8 h delays between the sample and test phases, in which the mouse was returned to their home cage.

OR data analysis

Exploration was defined as a mouse directing its nose to an object at a distance of 2 cm or less. Climbing, sitting, or chewing on the object was not included as exploration. Exploration was scored blind to genotype and condition, using the same computer program, JWatcher_V1.0, written in Java[TM] (JWatcher, USA), described in Chapters 4 and 5. The program had two keys corresponding to the two objects. Exploration was recorded by pressing the appropriate keys at the onset and offset of a bout of exploration.

For Decoupled OR, discrimination ratios (D2) were calculated for both the *repeat* and *novel* conditions for each time delay, and calculated as follows:

$$D2 = \frac{Test\ Phase\ exploration(s)}{Sample\ Phase\ exploration(s)}$$

D2 scores < 1 on the *repeat condition* suggested that the mouse viewed the test objects as familiar, which is why they explored the objects less during the test phase than during the sample phase. This is interpreted as a subject remembering the sample objects. It was hypothesized that D2 scores would be ~1 for the *novel condition*. A D2 < 1 in the novel condition is interpreted as a false memory, such that the mouse saw the new object as familiar. This has been shown to be caused by interference, which may reflect impairment in pattern separation (McTighe et al., 2010).

For *Forced-choice OR,* preference for the novel object was calculated as:

$$D2 = \frac{novel\ object\ exploration - \ familiar\ object\ exploration(s)}{novel\ object\ exploration + \ familiar\ object\ exploration\ (s)}$$

D2 scores > 0 represent a novelty preference.

Sample data were compared using independent Student's *t*-tests, to ensure total exploration during the sample phase was equal between the genotypes for each condition. This was run as a control measure to assess motivation to explore. Choice data from Decoupled OR were analysed using a repeated measures ANOVA, with *post-hoc* Student's *t* contrasts. Choice data from Forced-choice OR were analysed using independent Student's *t*-tests comparing genotype in each condition. Each time point was analysed separately. Performance was not compared across ages because of the variable sample sizes and because the analysis was intended to match the 5-CSRTT analysis, which was not compared across ages. All data are presented as mean ± SEM.

All statistical analyses were conducted with SPSS version 22 and Microsoft Excel version 14.4.5. Statistical significance was set at $p < 0.05$, unless running a *post-hoc* comparison. To control for Familywise error, the level of statistical significance (i.e., α) for *post-hoc* comparisons was calculated using the Bonferonni correction, which was calculated as 0.05 divided by the number of statistical comparisons.

6.2.5. Location Recognition (LR)

To evaluate spatial memory we used the LR task (previously described by Warburton et al., 2000). Much like SLR described in previous chapters and OR described above, LR takes advantage of a rodent's innate preference towards novelty. Because of this, no training is required. The protocol was developed by Dr. Stephanie McTighe, who had previously been a graduate student in our lab.

At least 30 min prior to testing, mice were brought into a holding room, which was illuminated by a red light and adjacent to the testing room. The testing room was dimly lit with white light and had distinct distal and proximal spatial cues. Mice were

individually transported in a cardboard carrying box between the holding room and the testing room.

LR apparatus

LR testing took place in a black plastic circular arena (43 cm diameter, 17 cm tall walls) with 1 cm of bedding on the floor. A digital video camera was mounted above the maze to record all trials. The stimuli used were randomly shaped objects (dimensions approximately 10 cm x 4 cm x 4 cm). All objects presented were new and had not been seen previously. The objects were wiped with a 50 % ethanol solution and dried between trials and secured to the floor of the maze using Blu-tack ™.

LR habituation

Each mouse was given 4 consecutive daily sessions of habituation to the maze, where they were allowed to freely explore the empty maze for 5 min. This was intended to reduce anxiety and allow the mice to become familiar with the distal spatial cues in the room.

LR behavioural testing

Each trial consisted of two phases: *sample* phase and *test* phase. During the sample phase, each mouse was given 5 min to explore the arena and was shown two identical objects spaced 20 cm apart. After being placed back in their home cage for a 1 h delay, the mice were returned to the arena for the test phase and allowed to explore for 5 min. For the test phase mice were presented with the same two identical objects previously used during the sample phase: one in its previous (i.e., familiar) location and one in a new (i.e., novel) location. The novel location was always directly across from the object in the familiar location.

LR Data analysis

Exploration was defined as a mouse directing its nose to an object at a distance of 2 cm or less. Climbing, sitting, or chewing on the object was not included as exploration. The experimenter scored exploration using the same computer program used for OR analysis described above, JWatcher_V1.0, written in Java[TM] (JWatcher, USA). D2 scores were calculated as follows:

$$D2 = \frac{novel\ location\ exploration - \ familiar\ location\ exploration(s)}{novel\ location\ exploration + \ familiar\ location\ exploration\ (s)}$$

Data were analysed using independent Student's *t*-test. All data are presented as mean ± standard error of the mean. All statistical analyses were conducted with SPSS version 22 and Microsoft Excel version 14.4.5. Statistical significance was set at $p < 0.05$.

6.2.6. T-Maze

To evaluate spatial memory using an additional task, mice were tested using the hippocampal-dependent T-Maze. Details of the testing protocol have been previously published (Sigurdsson et al., 2010) and were provided by Dr. Simon Nilsson, a postdoctoral researcher in our lab.

T-Maze apparatus

Testing took place in a T- shaped three-arm maze made of Perspex. Each arm was 30 cm long, 10 cm wide, and 20 cm high. The maze had a white floor and black walls, and was placed on a table 43 cm above the floor in a room lit with white light and prominent distal visual cues. A digital video camera was mounted above the apparatus to record all trials. I sat behind the start arm throughout testing.

T-Maze habituation

Mice received two days of habituation to the maze. During habituation, all three arms

were baited with a single sucrose reward pellet (14 mg, Sandown Scientific, Middlesex, UK). The mouse was placed in the start arm with the door lowered until the pellet was consumed. The door was then removed and the animal was free to explore the maze. Once the animal had consumed the two remaining pellets, the start-arm was re-baited. When the animal had consumed the pellet in the start arm, the two choice arms were re-baited. This continued for 10 min.

T-Maze shaping

Mice received two days of shaping using one open choice arm while the second choice arm was blocked. The animal was placed in the baited start arm with the door lowered. Once the mouse had consumed the pellet, the start door was removed and the mouse was allowed to explore only one of the choice arms. Once the animal returned to the re-baited start arm, the door was lowered and the alternate choice arm was baited and opened for the next trial. Each session had 10 trials with the order of the open-baited arm presented in a pseudorandom order.

T-Maze acquisition

Acquisition training on delayed non-match to location began on the fourth day. Training consisted of a *sample* phase and a *choice* phase. For the sample phase, the mouse was placed in the baited start arm, with the door lowered. Once the pellet was consumed, the start arm was opened and the mouse was allowed to enter only one of the choice arms. Once the animal consumed the pellet in the choice arm, the start arm was re-baited. When the animal returned to the start arm, the start door was closed, and the maze was wiped with a 50 % ethanol solution and the other choice arm door was opened. After a 10 s delay, the choice phase began. The start arm door was opened and the mouse could choose to enter either the left or right choice arms. The *correct* choice was the arm not previously visited during the sample phase. Once the mouse entered a choice arm, the opposite arm was immediately closed. The start arm was re-baited and the next trial began once the mouse returned to the start arm. Each daily session had 10 trials. Testing was conducted for 24 consecutive days.

T-Maze data analysis

An accuracy score for each daily session was calculated as the number of correct trials out of 10. Data were analysed using a repeated measures ANOVA comparing genotype across trials. The number of trials to criterion was also calculated. Criterion was an accuracy score of 70 % for three consecutive days. An independent samples Student's *t*-test was used to analyze trials to criterion. All data are presented as mean ± standard error of the mean. All statistical analyses were conducted with SPSS version 22 and Microsoft Excel version 14.4.5. Statistical significance was set at $p < 0.05$.

6.2.7. Histology

At the end of the experiment, mice were anaesthetized by intraperitoneal (IP) injection of Dolethal (0.3 ml; Vetoquinol UK Ltd., Buckinghamshire, UK) and perfused transcardially with phosphate buffered saline (PBS) for 2 min, followed by 4% neutral buffered formalin (NBF) for 5 min. Brains were removed and post-fixed in NBF for at least 24 h at 4 °C, followed by immersion in 70 % ethanol at 4 °C and shipped from the University of Cambridge to Professor Paul Fraser at the Tanz Centre for Research in Neurodegenerative Diseases, University of Toronto, Canada. Histological analysis is not yet complete.

6.3. Results

6.3.1. No differences between Tg+ and Tg- on 5-CSRTT measures of attention and executive control at 5, 7, 12, and 16 months of age

As shown in Figure 6.3, during 5-CSRTT *pretraining* there were no differences between Tg+ and Tg- for sessions to criterion. There were also no statistical differences in baseline 5-CSRTT performance (2 s stimulus duration) prior to the start of probe testing, or throughout probe testing ($p > 0.05$, data not shown). Figure 6.4 shows baseline accuracy at the start of 5-CSRTT probe trials.

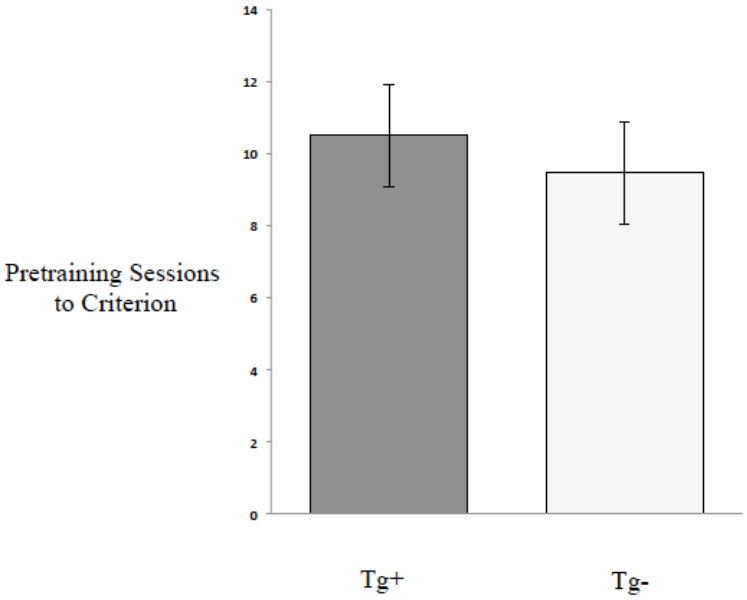

Figure 6.3. 5-CSRTT pretraining sessions to criterion. The y-axis is the mean number of sessions during pretraining that it took each group to reach criterion performance and move onto probe trials. There was no difference between Tg+ and Tg-. Data are expressed as the mean ± SEM.

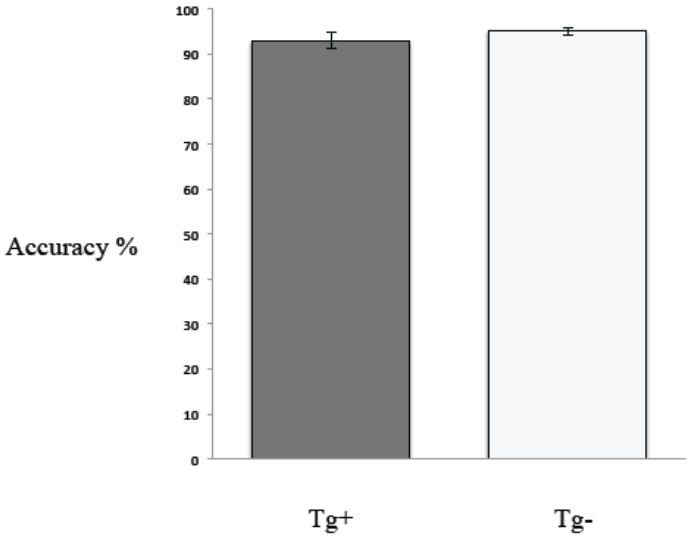

Figure 6.4. Baseline accuracy at the start of 5-CSRTT probe trials. The y-axis represents the mean accuracy (%) for the final two baseline sessions prior to the start of probes. There was no difference between Tg+ and Tg-. Data are expressed as the mean ± SEM.

Attention and executive control were evaluated using 5-CSRTT at 5, 7, 12, and 16 months of age. For probes of decreasing stimulus duration (i.e., 1.6 s, 1.0 s, 0.8 s, 0.6 s,

0.4 s, 0.2 s), measures of performance used to compare Tg+ and Tg- were: accuracy, omissions, premature responding, perseverative responding, reward response latency, correct response latency, incorrect response latency, beam breaks front, and beam breaks back. Repeated measures ANOVAs showed no statistically significant interactions between genotype and stimulus duration on any of the performance measures, at any of the time points (data are expressed as the mean ± SEM). Figure 6.5 provides line graphs illustrating *accuracy*, *omissions, premature responses*, and *perseverative responses* at 5, 7, 12, and 16 months of age. Tables 6.2 to 6.5 provide means (M) and SEM for reward response latency, correct response latency, incorrect response latency, beam breaks front, beam breaks back at 5, 7, 12, and 16 months of age. Each probe was run for two consecutive days, so each data point is an average of the two days.

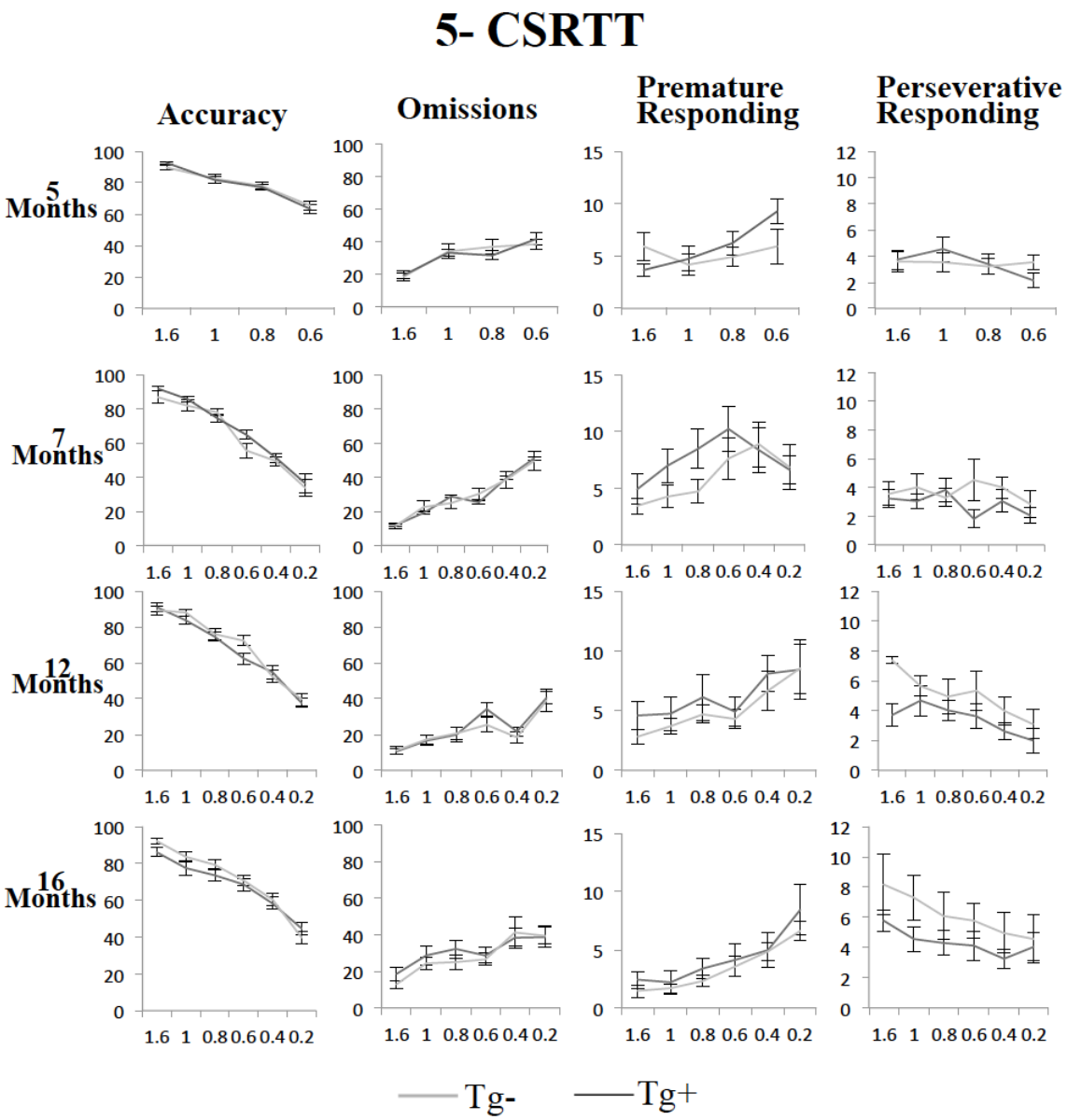

Figure 6.5. 5-CSRTT performance on probe trials using decreased stimulus duration. Line graphs showing *accuracy (%)*, *omissions (%),*number of *premature responses*, and number of *perseverative responses* for Tg+ and Tg- on 5-CSRTT at 5, 7, 12, and 16 months of age. The y-axis represents the mean performance value and the x-axis represents the stimulus duration for each probe. There were no statistically significant interactions between genotype and stimulus duration for any performance measure, at any time point ($p > 0.05$). Data are expressed as the mean ± SEM.

Table 6.2. Extra 5- CSRTT measures at 5 months of age

Probe	Reward Response Latency	Correct Response Latency	Incorrect Response Latency	Beam Breaks Front	Beam Breaks Back
1.6s	**Tg+** (M=1.23; SEM=0.039) **Tg-** (M=1.18; SEM=0.035)	**Tg+** (M=1.28; SEM=0.038) **Tg-** (M=1.24; SEM=0.041)	**Tg+** (M=2.36; SEM=0.32) **Tg-** (M=2.75; SEM=0.47)	**Tg+** (M=264.90; SEM=26.33) **Tg-** (M = 260.79; SEM=17.36)	**Tg+** (M=89.86; SEM=7.52) **Tg-** (M=97.45; SEM=10.12)
1.0s	**Tg+** (M=1.43; SEM = 0.28) **Tg-** (M=1.20; SEM= 0.04)	**Tg+** (M=1.13; SEM=0.05) **Tg-** (M=1.14; SEM=0.44)	**Tg+** (M=2.75; SEM=0.23) **Tg-** (M=2.50; SEM=0.35)	**Tg+** (M=259.36; SEM=25.95) **Tg-** (M=251.12; SEM=21.08)	**Tg+** (M=93.86; SEM=9.16) **Tg-** (M=96.58; SEM=10.65)
0.8s	**Tg+** (M=1.40; SEM=0.19) **Tg-** (M=1,23; SEM=0.04)	**Tg+** (M=1.18; SEM=0.070) **Tg-** (M=1.01; SEM=0.048)	**Tg+** (M=2.50; SEM=0.27) **Tg-** (M=2.44; SEM= 0.22)	**Tg+** (M=226.27; SEM=20.26) **Tg-** (M=283.54; SEM=27.11)	**Tg+** (M=90.27; SEM=12.19) **Tg-** (M=105.37; SEM=13.59)
0.6s	**Tg+** (M=1.31; SEM=0.067) **Tg-** (M =1.47; SEM= 0.24)	**Tg+** (M=1.15; SEM=0.093) **Tg-** (M=1.02; SEM=0.075)	**Tg+** (M=2.55; SEM= 0.20) **Tg-** (M=2.13; SEM= 0.20)	**Tg+** (M=281.40; SEM=53.42) **Tg-** (M=283.16; SEM=24.15)	**Tg+** (M =105.40; SEM=15.10) **Tg-** (M=118.16; SEM=11.0)

Table 6.3. Extra 5- CSRTT measures at 7 months of age

Probe	Reward Response Latency	Correct Response Latency	Incorrect Response Latency	Beam Breaks Front	Beam Breaks Back
1.6s	**Tg+** (M=1.27; SEM=0.05) **Tg-** (M=1.20; SEM=0.037)	**Tg+** (M=1.10; SEM=0.03) **Tg-** (M=1.15; SEM=0.046)	**Tg+** (M=2.00; SEM=0.36) **Tg-** (M=1.48; SEM=0.25)	**Tg+** (M=233.81; SEM=17.96) **Tg-** (M=249.92; SEM=19.63)	**Tg+** (M=84.95; SEM=13.32) **Tg-** (M=99.91; SEM=9.68)
1.0s	**Tg+** (M=1.22; SEM=0.042) **Tg-** (M=1.19; SEM=0.034)	**Tg+** (M=0.96; SEM=0.041) **Tg-** (M=0.99; SEM=0.026)	**Tg+** (M=1.83; SEM=0.29) **Tg-** (M=1.88; SEM=0.22)	**Tg+** (M=251.36; SEM=27.53) **Tg-** (M=254.69; SEM=16.78)	**Tg+** (M=99.59; SEM=9.52) **Tg-** (M=106.62; SEM=11.25)
0.8s	**Tg+** (M=1.20; SEM=0.048) **Tg-** (M=1.19; SEM=0.045)	**Tg+** (M=0.96; SEM=0.049) **Tg-** (M=0.95; SEM=0.034)	**Tg+** (M=1.90; SEM =2.15) **Tg-** (M=2.15; SEM=0.21)	**Tg+** (M=268.13; SEM=33.36) **Tg-** (M=281.26; SEM=29.24)	**Tg+** (M=110.86; SEM=14.61) **Tg-** (M=117.25; SEM=16.06)
0.6s	**Tg+** (M=1.22; SEM=0.044) **Tg-** (M=1.41; SEM=0.096)	**Tg+** (M=0.98; SEM =0.16) **Tg-** (M=1.16; SEM=0.14)	**Tg+** (M=1.81; SEM =0.16) **Tg-** (M=2.19; SEM=0.23)	**Tg+** (M=302.63; SEM=25.41) **Tg-** (M=278.07; SEM=29.5)	**Tg+** (M=123.54; SEM=15.03) **Tg-** (M=113.70; SEM=16.02)
0.4s	**Tg+** (M=1.43; SEM=.19) **Tg-** (M=1.38; SEM=0.071)	**Tg+** (M=1.02; SEM=0.089) **Tg-** (M=1.07; SEM=0.099)	**Tg+** (M=1.81; SEM =0.12) **Tg-** (M=1.90; SEM=0.12)	**Tg+** (M=300.77; SEM=36.76) **Tg-** (M=382.80; SEM=62.20)	**Tg+** (M=130.5; SEM=19.15) **Tg-** (M=123.5; SEM=17.28)
0.2s	**Tg+** (M=1.30; SEM=0.059) **Tg-** (M=1.81; SEM=.26)	**Tg+** (M=1.345; SEM =0.14) **Tg-** (M=1.42; SEM=0.16)	**Tg+** (M=1.88; SEM=0.17) **Tg-** (M=2.12; SEM=0.15)	**Tg+** (M=279.68; SEM=33.86) **Tg-** (M=328.42; SEM=48.01)	**Tg+** (M=143.0; SEM =19.2) **Tg-** (M=129.0; SEM=19.08)

Table 6.4. Extra 5- CSRTT measures at 12 months of age

Probe	Reward Response Latency	Correct Response Latency	Incorrect Response Latency	Beam Breaks Front	Beam Breaks Back
1.6s	**Tg+** (M=1.21; SEM=0.045) **Tg-** (M=1.53; SEM=0.94)	**Tg+** (M=1.08; SEM=0.023) **Tg-** (M=1.11; SEM=0.006)	**Tg+** (M=1.52; SEM=0.30) **Tg-** (M=1.72; SEM=0.23)	**Tg+** (M=302.77; S.E =25.05) **Tg-** (M=284.22; SEM=21.88)	**Tg+** (M=115.15; SEM=10.32) **Tg-** (M=123.12; SEM=13.45)
1.0s	**Tg+** (M=1.23; S.E =0.052) **Tg-** (M=1.29; SEM=0.036)	**Tg+** (M=0.933; SEM=0.029) **Tg-** (M=0.98; SEM=0.023)	**Tg+** (M=1.76; SEM=0.23) **Tg-** (M=1.55; SEM=0.21)	**Tg+** (M=380.0; SEM=91.33) **Tg-** (M=276.96; SEM=18.14)	**Tg+** (M=140.31; SEM=29.52) **Tg-** (M=123.64; SEM=12.49)
0.8s	**Tg+** (M=1.19; SEM=0.035) **Tg-** (M=1.32; SEM=0.053)	**Tg+** (M=0.89; SEM=0.033) **Tg-** (M=0.96; SEM=0.056)	**Tg+** (M=1.77; SEM=0.19) **Tg-** (M=1.81; SEM=0.13)	**Tg+** (M=346.31; S.E =64.25) **Tg-** (M=294.0; SEM=20.96)	**Tg+** (M=131.31; SEM=22.33) **Tg-** (M=125.91; SEM=14.30)
0.6s	**Tg+** (M=1.85; SEM=0.60) **Tg-** (M=1.57; SEM=0.27)	**Tg+** (M=0.89; SEM=0.05) **Tg-** (M=1.00; SEM=0.078)	**Tg+** (M=2.01; S.E =0.23) **Tg-** (M=1.80; SEM=0.15)	**Tg+** (M=309.86; S.E =45.15) **Tg-** (M=262.72; SEM=22.33)	**Tg+** (M=147.95; SEM=26.05) **Tg-** (M=117.40; SEM=14.61)
0.4s	**Tg+** (M=1.19; SEM=0.054) **Tg-** (M=1.41; SEM=0.067)	**Tg+** (M=0.95; SEM=0.075) **Tg-** (M=1.01; SEM=0.082)	**Tg+** (M=1.69; SEM=0.15) **Tg-** (M=1.72; SEM=0.13)	**Tg+** (M=340.0; SEM=53.78) **Tg-** (M=313.62; SEM=25.88)	**Tg+** (M=144.10; S.. =20.65) **Tg-** (M=135.75; SEM=17.93)
0.2s	**Tg+** (M=1.28; SEM=0.091) **Tg-** (M=1.50; SEM=0.10)	**Tg+** (M=1.09; SEM=0.13) **Tg-** (M=1.18; SEM=0.19)	**Tg+** (M=1.64; SEM=0.12) **Tg-** (M=1.71; SEM=0.10)	**Tg+** (M=289.90; SEM=56.12) **Tg-** (M=284.70; SEM=27.88)	**Tg+** (M=141.31; SEM=24.21) **Tg-** (M=126.36; SEM=15.48)

Table 6.5. Extra 5- CSRTT measures at 16 months of age

Probe	Reward Response Latency	Correct Response Latency	Incorrect Response Latency	Beam Breaks Front	Beam Breaks Back
1.6s	**Tg+** (M=1.36; SEM =0.095) **Tg-** (M=1.36; SEM=0.95)	**Tg+** (M=1.066; SEM =0.038) **Tg-** (M=1.11; SEM=0.042)	**Tg+** (M=1.56; SEM =0.27) **Tg-** (M=1.53; SEM=0.15)	**Tg+** (M=241.35; SEM =31.34) **Tg-** (M=232.66; SEM=17.21)	**Tg+** (M=107.55; SEM =13.94) **Tg-** (M=109.95; SEM=9.60)
1.0s	**Tg+** (M=1.36; SEM =0.087) **Tg-** (M=1.36; SEM=0.087)	**Tg+** (M=0.96; SEM =0.039) **Tg-** (M=1.055; SEM=0.036)	**Tg+** (M=2.06; SEM =0.30) **Tg-** (M=1.74; SEM=0.18)	**Tg+** (M=226.08; SEM =30.07) **Tg-** (M=225.04; SEM=21.90)	**Tg+** (M=121.34; SEM =17.29) **Tg-** (M=129.83; SEM=12.97)
0.8s	**Tg+** (M=1.28; SEM =0.070) **Tg-** (M=1.27; SEM=0.040)	**Tg+** (M=0.94; SEM =0.048) **Tg-** (M=0.98; SEM=0.037)	**Tg+** (M=1.78; SEM =0.15) **Tg-** (M=1.70; SEM=0.20)	**Tg+** (M=231.75; SEM =21.42) **Tg-** (M=219.0; SEM=12.54)	**Tg+** (M=136.80; SEM =15.79) **Tg-** (M=123.29; SEM=11.91)
0.6s	**Tg+** (M=1.34; SEM =0.096) **Tg-** (M=1.30; SEM=0.045)	**Tg+** (M=0.88; SEM =0.040) **Tg-** (M=0.94; SEM=0.045)	**Tg+** (M=1.67; SEM =0.19) **Tg-** (M=1.62; SEM=0.14)	**Tg+** (M=245.85; SEM =28.95) **Tg-** (M=251.79; SEM=21.52)	**Tg+** (M=144.45; SEM =21.47) **Tg-** (M=136.20; SEM=12.32)
0.4s	**Tg+** (M=1.33; SEM= 0.079) **Tg-** (M= 1.28; SEM=0.069)	**Tg+** (M=0.87; SEM =0.062) **Tg-** (M=0.91; SEM=0.050)	**Tg+** (M=1.74; SEM =0.12) **Tg-** (M=1.72; SEM=0.095)	**Tg+** (M=241.89; SEM =34.66) **Tg-** (M=277.50; SEM=23.67)	**Tg+** (M=132.39; SEM =17.68) **Tg-** (M=145.5; SEM=12.79)
0.2s	**Tg+** (M=1.35; SEM =0.075) **Tg-** (M=1.38; SEM=0.086)	**Tg+** (M=1.017; SEM =0.10) **Tg-** (M=1.089; SEM=0.086)	**Tg+** (M=1.82; SEM =0.19) **Tg-** (M=1.69; SEM=0.12)	**Tg+** (M=252.12; SEM =32.88) **Tg-** (M=297.54; SEM=28.55)	**Tg+** (M=146.35; SEM =17.97) **Tg-** (M=161.91; SEM=18.62)

Similarly, there were no statistically significant effects on any of these performance measures for the *Impulsivity Probe* (10 s delay) or *Vigilance Probe* (200 trials) tested at 7, 12, and 16 months of age ($p > 0.05$). For the *Impulsivity probe* that used a 10 s delay, means (M) and SEM are presented in Figure 6.6 and Table 6.6. At 12 months of age Tg- did make more perseverative responses than Tg+ but it is not statistically significant ($p = 0.09$). *Post-hoc* analysis of the premature responses revealed a decrease with age, which may be a result of learning (main effect of age, $F_{2,44} = 6.313$; $p = 0.04$). Omission rate at 16 months of age was higher for Tg+ than Tg- but not statistically different ($p = 0.052$). For the *Vigilance Probe* that used 200 trials per session, means (M) and SEM are presented in Figure 6.7 and Table 6.7. At 12 months of age, Tg+ show more premature responses than Tg- ($p = 0.09$) and Tg- show more perseverative responses ($p = 0.07$), but these are not statistically significant.

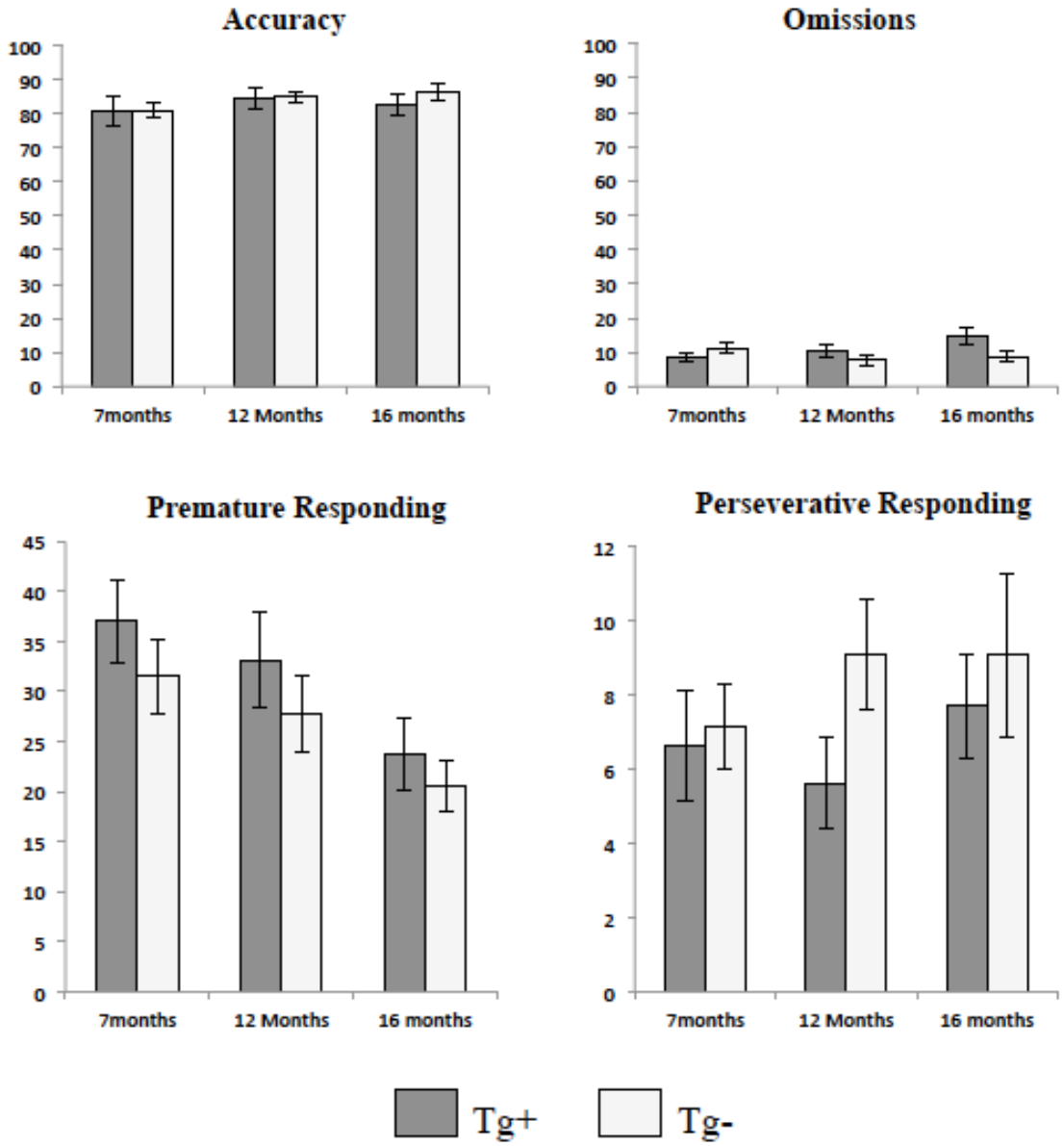

Figure 6.6. 5-CSRTT Impulsivity Probe. *Accuracy* (%), *omissions* (%), mean number of *premature responses,* and mean number of *perseverative responses* for Tg+ and Tg- during the 5-CSRTT Impulsivity Probe with a 10 s delay. Bar graphs show the performance measures at 3 time points: 7 months, 12 months, and 16 months of age. There were no statistically significant differences in accuracy between Tg+ and Tg- at any of the time points. Omission rate at 16 months of age is higher for Tg+ than Tg- but not statistically different (p = 0.052). Premature responses decrease with age, which may be a result of learning (main effect of age p = 0.04). No statistically significant differences in perseverative responding between Tg+ and Tg- were detected. At 12 months of age Tg- make more perseverative responses but it is not statistically significant (p = 0.09). Data are expressed as the mean ± SEM.

Table 6.6. Extra 5- CSRTT measures for the Impulsivity Probe (10 s delay)

Age (months)	Reward Response Latency	Correct Response Latency	Incorrect Response Latency	Beam Breaks Front	Beam Breaks Back
7	**Tg+** (M=1.38; SEM=0.076) **Tg-** (M=1.42; SEM=0.082)	**Tg+** (M=1.17; SEM=0.050) **Tg-** (M=1.26; SEM=0.053)	**Tg+** (M=1.21; SEM =0.12) **Tg-** (M=1.71; SEM=0.27)	**Tg+** (M=456.81; SEM=56.33) **Tg-** (M=599.61; SEM=66.25)	**Tg+** (M=179.00; SEM=19.10) **Tg-** (M=197.30; SEM=18.28)
12	**Tg+** (M=1.14; SEM =0.04) **Tg-** (M=1.30; SEM=0.07)	**Tg+** (M=1.24; SEM=0.042) **Tg-** (M=1.24; SEM=0.29)	**Tg+** (M=1.02; SEM =0.15) **Tg-** (M=1.54; SEM=0.19)	**Tg+** (M=520.72; SEM =57.7) **Tg-** (M=433.75; SEM= 53.5)	**Tg+** (M=205.72; SEM=20.15) **Tg-** (M=195.5; SEM=22.13)
16	**Tg+** (M=1.28; SEM=0.085) **Tg-** (M=1.24; SEM=0.057)	**Tg+** (M=1.25; SEM=0.055) **Tg-** (M=1.21; SEM=0.046)	**Tg+** (M=1.74; SEM =0.20) **Tg-** (M=1.11; SEM=0.20)	**Tg+** (M=339.90; SEM=32.02) **Tg-** (M=339.08; SEM=22.11)	**Tg+** (M=187.8; SEM=21.73) **Tg-** (M=179.91; SEM=22.71)

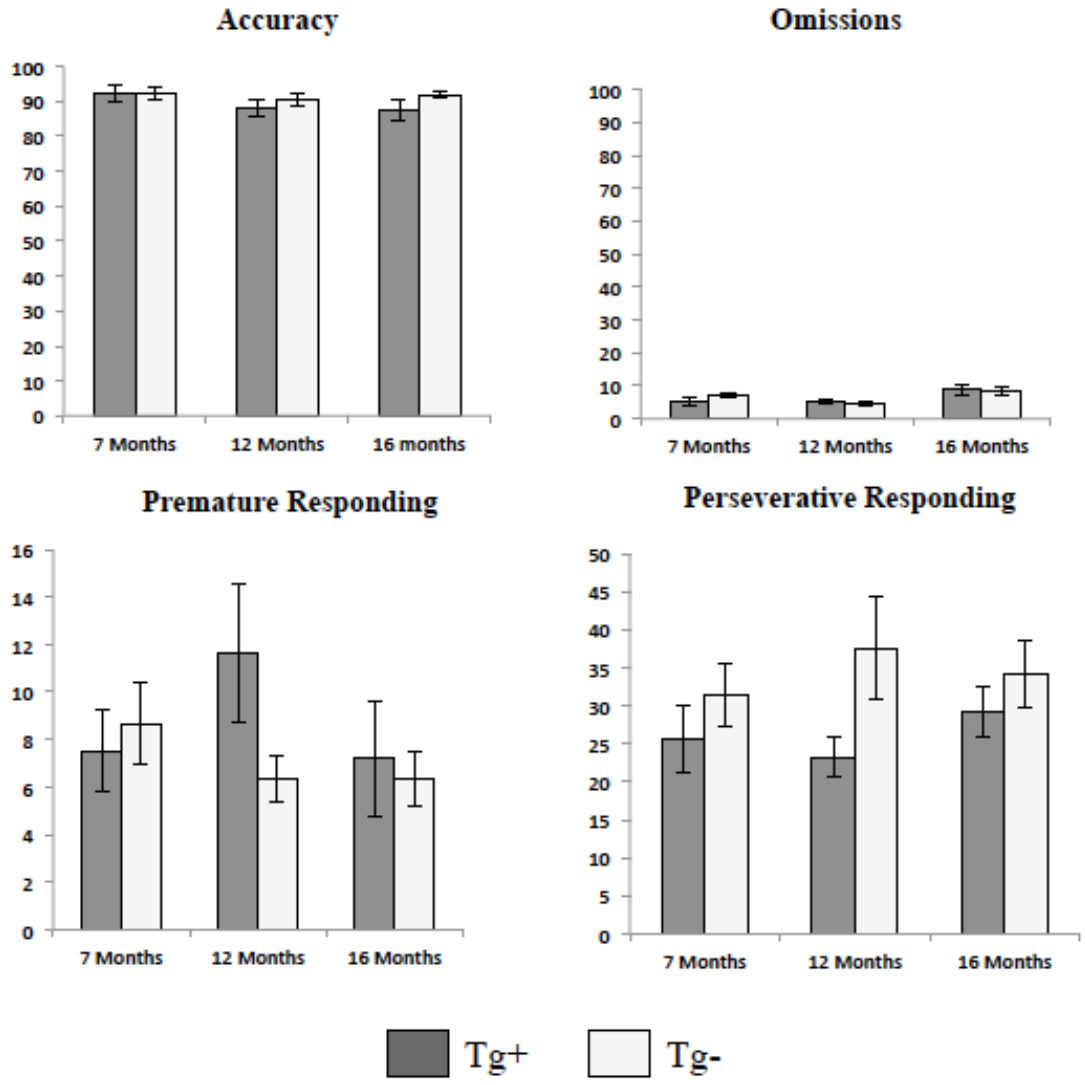

Figure 6.7. 5-CSRTT Vigilance probe. *Accuracy* (%), *omissions* (%), mean number of *premature responses,* and mean number of *perseverative responses* for Tg+ and Tg- during the 5-CSRTT *Vigilance probe* with 200 trials. There were no statistically significant differences on any of the measures, at any of the time points. At 12 months of age Tg+ show more premature responses than Tg- (p = 0.09) and Tg- show more perseverative responses than Tg+ (p = 0.07). Data are expressed as the mean ± SEM.

Table 6.7. Extra 5- CSRTT measures for the Vigilance Probe (200 Trial)

Age (months)	Reward Response Latency	Correct Response Latency	Incorrect Response Latency	Beam Breaks Front	Beam Breaks Back
7	**Tg+** (M=1.23; SEM =0.043) **Tg-** (M=1.28; SEM=0.050)	**Tg+** (M=1.09; SEM =.033) **Tg-** (M=1.16; SEM=.033)	**Tg+** (M=1.56; SEM =0.30) **Tg-** (M=1.85; SEM=0.22)	**Tg+** (M=1028.81; SEM =90.39) **Tg-** (M=1190.46; SEM=110.19)	**Tg+** (M=407.63; SEM =38.17) **Tg-** (M=514.0; SEM=42.38)
12	**Tg+** (M=1.26; SEM =0.050) **Tg-** (M=1.38; SEM=0.050)	**Tg+** (M=1.05; SEM =0.028) **Tg-** (M=1.10; SEM=0.035)	**Tg+** (M=1.28; SEM =0.087) **Tg-** (M=1.51; SEM=0.11)	**Tg+** (M=1203.73; SEM =102.37) **Tg-** (M=1134.5; SEM= 86.33)	**Tg+** (M=481.09; SEM =48.42) **Tg-** (M=535.5; SEM =48.04)
16	**Tg+** (M=1.43; SEM =0.11) **Tg-** (M=1.23; SEM=0.034)	**Tg+** (M=1.11; SEM =0.049) **Tg-** (M=1.15; SEM=0.038)	**Tg+** (M=1.46; SEM =0.15) **Tg-** (M=1.73; SEM=0.18)	**Tg+** (M=999.20; SEM =143.28) **Tg-** (M=1061.92;SEM=107.14)	**Tg+** (M=483.3; SEM =34.43) **Tg-** (M=536.58; SEM=61.03)

6.3.2. No differences between Tg+ and Tg- on Decoupled OR with 1 h or 24 h delays at 6, 8, 13, or 17 months of age

Mice were tested on Decoupled OR with a 1 h and 24 h delay at 6, 8, 13, and 17 months of age. There were no statistically significant differences in sample exploration between Tg+ and Tg- at any time point. Table 6.8 provides the means (M) and SEM for the sample data averaged across trials. Repeated measures ANOVAs revealed no statistically significant interactions ($p > 0.05$) between D2 scores of Tg+ and Tg- on *Repeat* or *Novel* conditions at 6, 8, 13, and 17 months of age. Figure 6.8 shows the D2 scores for each trial of Decoupled OR.

Table 6.8. Sample exploration for Decoupled Object Recognition

Age (Months)	1 h Delay (Trials Averaged)	24 h Delay (Trials Averaged)
6	**Tg+** (M=44.59; SEM=3.10**)** **Tg-** (M=44.31; SEM=2.04)	**Tg+** (M=41.90; SEM=2.54) **Tg-** (M=35.37; SEM=2.83)
8	**Tg+** (M=21.31; SEM=0.96) **Tg-** (M=21.94; SEM=1.52)	**Tg+** (M=19.18; SEM= 1.18) **Tg-** (M=17.97; SEM=1.33)
13	**Tg+** (M=19.23; SEM=0.77) **Tg-** (M=17.16; SEM=1.29)	**Tg+** (M=15.73; SEM= 1.12) **Tg-** (M=15.84; SEM=1.06**)**
17	**Tg+** (M=20.2; SEM=1.24) **Tg-** (M=18.5; SEM= 0.98)	**Tg+** (M=20.13; SEM=1.89) **Tg-** (M=19.93; SEM=1.27)

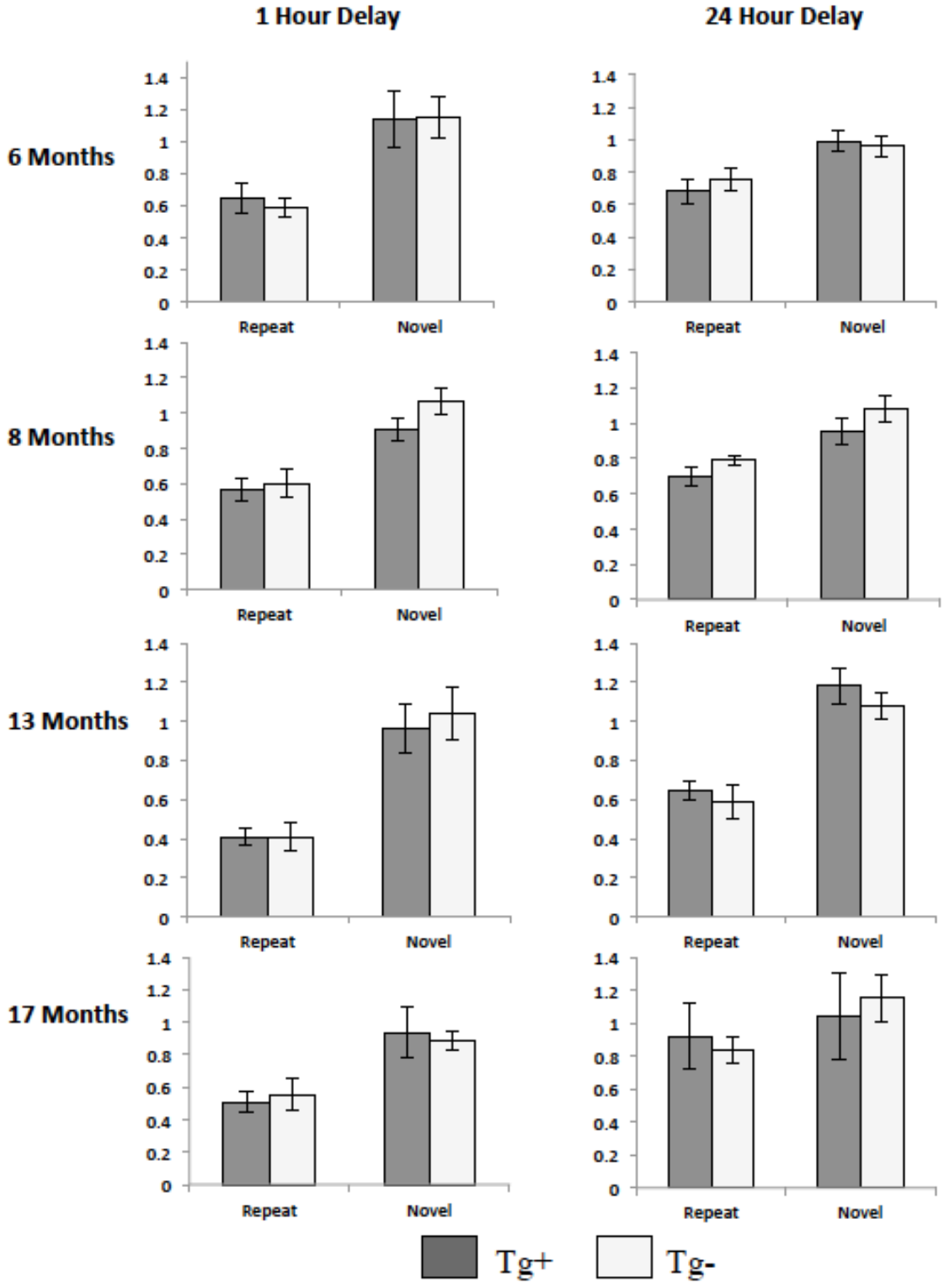

Figure 6.8. Decoupled OR at 6, 8, 13, and 17 months of age. Bar graphs showing D2 on the y-axis, comparing Tg+ and Tg- on *Repeat* and *Novel* conditions. There were no statistically significant differences between Tg+ and Tg- at any time point. Data are expressed as the mean ± SEM.

6.3.3. Tg+ show spatial memory impairment on LR at 18 months of age

At 18 months of age, mice were tested on the LR task using a 1 h delay. Due to highly variable performance, each mouse was tested on 4 trials. An independent Student's *t*-test comparing the average D2 scores across the four trials revealed that the Tg+ showed less of a preference for the novel location than the Tg- group (p = 0.018). Figure 6.9 shows combined data across the four trials. Analysis of the individual trials reveals the high variability in performance: Trial 1 (Tg+ M = 0.20, S.E = 0.045; Tg- M = 0.043, SEM= 0.074), Trial 2 (Tg+ M = -0.25, S.E = 0.086; Tg- M = 0.46, SEM = 0.058), Trial 3 (Tg+ M = -0.073, S.E = 0.059; Tg- M = 0.074, SEM = 0.069), and Trial 4 (Tg+ M = 0.022, S.E = 0.10; Tg- M = 0.26, SEM = 0.14). There were no differences in sample exploration ($p > 0.05$).

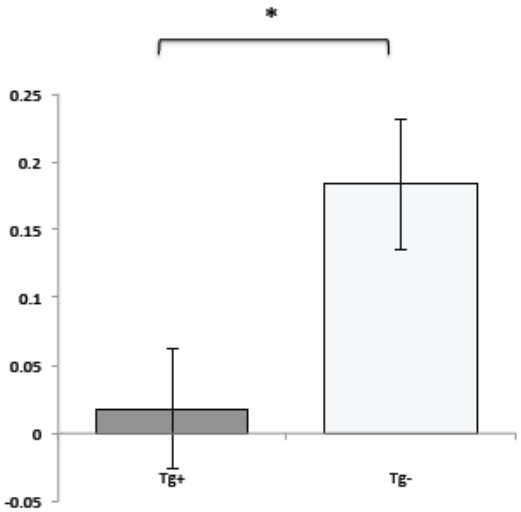

Figure 6.9. LR with 1 h delay at 18 months of age. Y-axis shows the average D2 scores for Tg+ (left) and Tg- (right) after a 1 h delay at 18 months of age. Tg+ showed less of a preference for the novel location (i.e., lower D2 score) than the Tg- group (p = 0.018). Means and ± SEM shown.

6.3.4. Tg+ show recognition memory impairment on Forced-choice OR with an 8 h delay that develops between 19 and 21 months of age

Mice were tested on Forced-choice OR at 19 months of age using a 3 h and 8 h delay. There was no statistically significant difference in D2 scores between Tg+ (M= 0.20; SEM= 0.063) and Tg- (M= 0.20; SEM= 0.086) after a 3 h delay. Three trials of the 8 h delay were run because of high variability; however, there was no statistically significant difference in D2 scores between Tg+ and Tg- after the 8 h delay at 19

months of age. At 21 months of age, mice were re-tested with two trials of Forced-choice OR with an 8 h delay. The Tg+ group showed no preference for the novel object, and significantly less preference for the novel object than the Tg- group (p = 0.022), suggesting that Tg+ had impaired recognition memory at 21 months of age. Figure 6.10 shows the D2 scores at 19 and 21 months of age for Forced-choice OR with an 8 h delay.

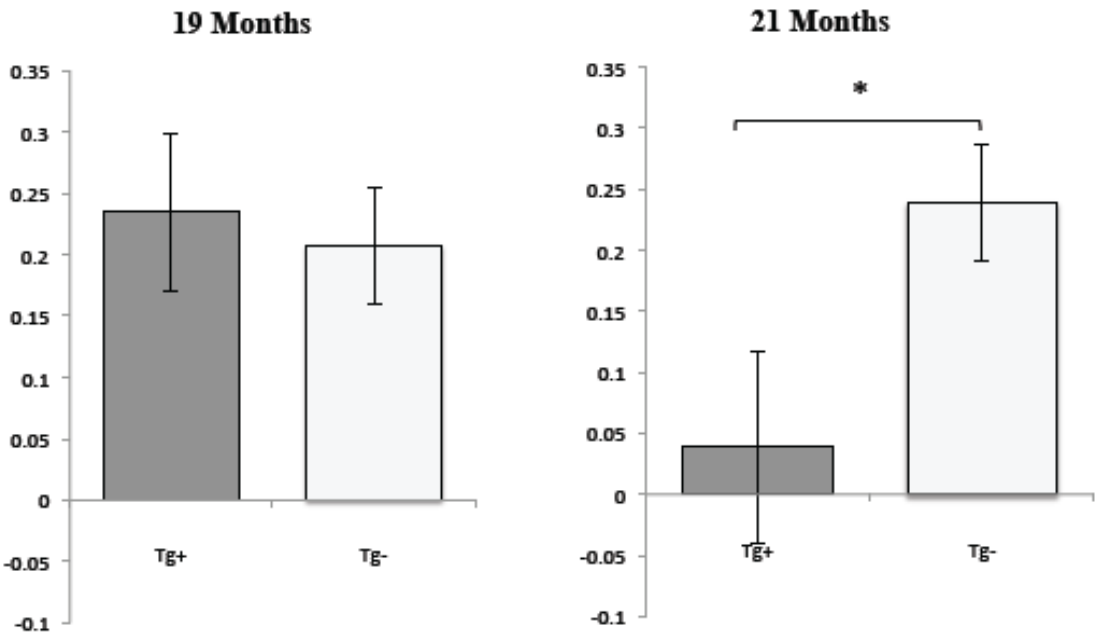

Figure 6.10. Forced-choice OR with 8 h delay. Bar graphs showing D2 scores on the y-axis for Tg+ and Tg- at 19 and 21 months of age. There was no difference between D2 scores at 19 months of age; however, at 21 months of age, the Tg+ group showed significantly less preference for the novel object than the Tg- group (p = 0.022). Data are expressed as the mean ± SEM and combined data from three trials at 19 months of age and two trials at 21 months of age.

6.3.5. Tg+ and Tg- show highly variable and inconclusive spatial memory performance on T-Maze at 20 months of age

Tg+ and Tg- were tested on the T-maze for 24 consecutive days. The mice showed highly variable and inconclusive performance. Criterion was an accuracy score of 70 % for three consecutive days, but only 4 Tg+ and 10 Tg- reached the performance criterion. Of these, there was no statistically significant difference between the number of trials Tg+ (M = 6.0; SEM = 1.47) and Tg- (M = 9.1; SEM = 1.30) took to reach criterion. Because of the low and highly variable performance, all mice were maintained

on the 10 s delay and never tested on probe trials with longer delays. On the baseline performance, there was no statistically significant interaction between genotype and block or main effect of genotype on performance accuracy. Figure 6.11 presents the performance data in blocks of three consecutive days.

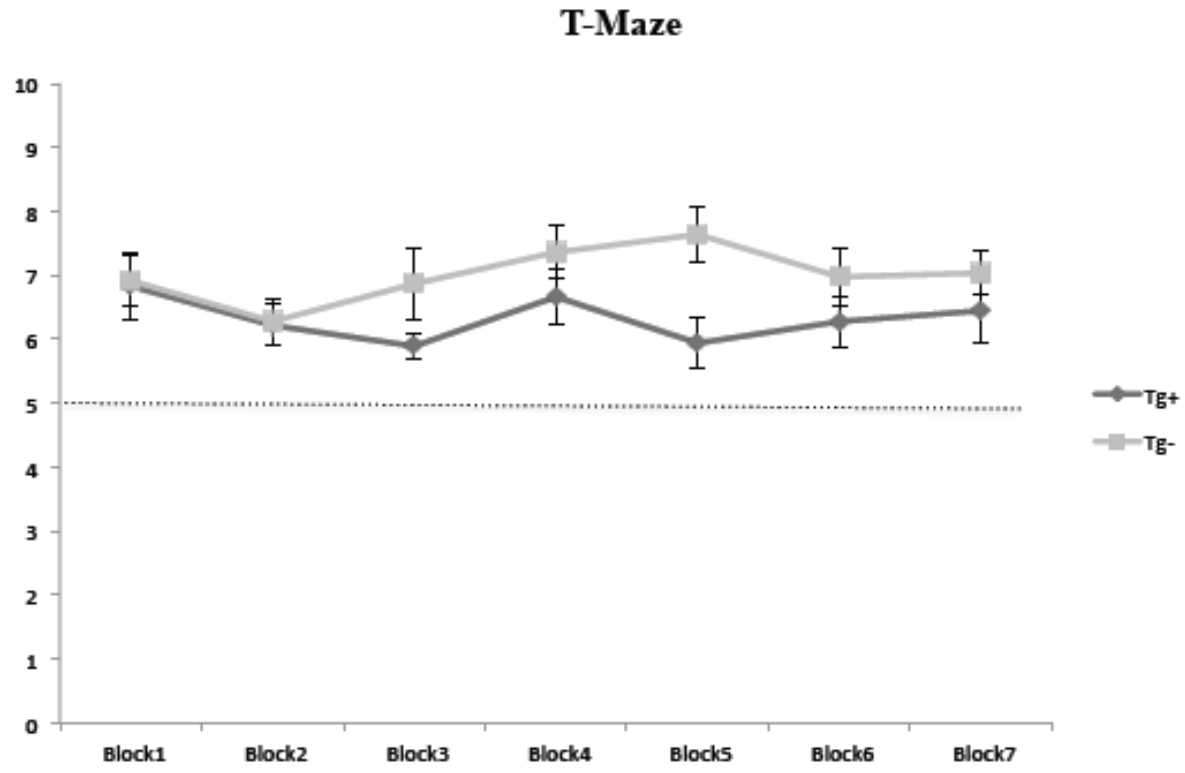

Figure 6.11. T-maze task at 20 months of age. No statistically significant effect of genotype on performance was detected. Each block consisted of three consecutive daily sessions (10 trials per session). The y-axis shows the mean number of correct trials (out of 10). Chance performance is a score of 5 and illustrated by the dotted line. Data are expressed as the mean ± SEM.

6.4. Discussion

The experiments described in this chapter provide the first longitudinal cognitive profile of the TgTau^{P301L} mouse from 5 to 21 months of age, using behavioural tasks to evaluate attention, executive functioning, object recognition memory, and spatial memory. Frontal cortex-dependent executive function and attention were evaluated using the touchscreen version of the 5-CSRTT at 4, 7, 12, and 16 months of age. Perirhinal cortex-dependent recognition memory was assessed using an OR task at 5, 8, 13, 17, 19, and 21 months of age. Hippocampus-dependent spatial memory was

evaluated using the T-maze and LR tasks between 18 and 20 months of age. The results were variable but suggest that the TgTau^{P301L} model develops a spatial memory and recognition memory impairment by 18 to 21 months of age and exhibits the relatively extended age of onset of behavioural symptoms often associated with neurodegenerative diseases such as FTD and AD.

P301L is the tau mutation most frequently observed in patients with FTDP-17 and one of the mutations associated with familial AD (Poorkaj et al., 2001). The TgTau^{P301L} transgenic mouse model has been previously shown to recapitulate the progressive development of glial fibrillary (GFT) and NFT, cerebral atrophy, and age-related cognitive impairments observed in patients (Sasaki et al., 2008; Murakami et al., 2006; Wakasaya et al., 2011). For example, Sasaki and colleagues (2008) compared immunocytochemical analyses of brains from six patients with tauopathies (including AD) and TgTau^{P301L} mice at 11 to 27 months of age. The TgTau^{P301L} mice showed microglial activation in grey matter associated with phosphorylated tau deposition, which was similar to samples from the human patients diagnosed with tauopathies. There are also many similar factors responsible for NFT formation and neuronal cell loss between the TgTau^{P301L} mice and both AD and FTD patients, as demonstrated by comparing oligonucleotide array expression (Wakasaya et al., 2011). These comparative studies validated the TgTau^{P301L} mice as a model of tauopathies, including both FTD and AD.

The TgTau^{P301L} model was first characterized by Murakami and colleagues (2006), who reported initial tau pathology development in the hippocampus, amygdala, and cerebral cortex at approximately 3 months of age and tau-positive pre-tangles at 10 months of age. Although not specified explicitly in the histological report for this cohort at 3 or 10 months of age, it was assumed that 'cerebral cortex' was referring to the frontotemporal cortex because that was the site of the most extensive pathological markers at later ages. For example, extensive NFTs were identified throughout the frontotemporal cortex at 18 to 24 months of age. Histological analysis of another cohort of TgTau^{P301L} at 13 months of age showed that 37 % had pretangles, 42 % had pretangles and GFTs, and 21 % had pretangles, GFTs and NFTs. NFTs were found in the cerebral cortex, hippocampus, amygdala, basal forebrain nucleus, locus ceruleus, and substantia nigra. Glial tau pathology developed independently and preceded neuronal cytopathology.

Mice showed brain atrophy by 18 months of age, in the temporal lobe and hippocampus. Tau-positive glial tangles were also observed in the spinal cord.

This summary illuminates the high within cohort variance in pathology. The authors suggest that this variance may be caused by genetic modifiers, environmental parameters, or stress (Murakami et al., 2006). The high variability in pathology may explain the variability in behaviour demonstrated by the cohort described in this chapter. Additionally, the presence of glial tangles in the spinal cord may explain the cases of hind limb paralysis, seen in our cohort. Histological analysis is not yet complete so the exact pathology is not yet known.

Because of the regional specificity of tau pathology in the TgTau^{P301L} model, the present study prioritized tasks dependent upon the frontotemporal lobe structures. The cohort was first tested on 5-CSRTT because evidence suggests that executive and attentional deficits may be the earliest cognitive deficits in AD, prior to deficits in spatial memory and language impairments (Lawrence & Sahakian, 1995; Perry et al., 2000; Collette et al., 1999; Baddeley et al., 2001), and may be a predictive preclinical feature of AD (Albert et al., 2001). Given the importance of early detection and the slow progression of pathology in this model, examining executive and attentional deficits was thought to provide the best chance at detecting the earliest cognitive changes. Although TgTau^{P301L} is a model of FTD-17 and not specifically modelling AD, as mentioned above the model has been shown to recapitulate the histopathological features of AD patients and the P301L mutation is associated with AD (Sasaki et al., 2008; Wakasaya et al., 2011). Furthermore, the cognitive effects of tau tangles may generalize between the tauopathies.

To my knowledge, only limited behavioural characterisation of these mice has been performed, which did not investigate the earliest cognitive changes. Murakami and colleagues (2006) evaluated the TgTau^{P301L} mouse model on the MWM at 9 and 12 months and the eight-arm radial maze at 9 and 13 months of age. Older cohorts were tested on the open-field test, MWM (reference memory and visible cued platform test), and conditioned taste aversion. The results showed impaired working memory at 12 and 13 months of age, and impaired conditioned taste aversion at 16 to 18 months of age.

Importantly, unlike the present study, these data were collected from a cross-sectional rather than longitudinal design.

The present longitudinal study evaluated three cognitive domains. Firstly, using a touchscreen version of the 5-CSRTT, I examined frontal cortex-dependent executive function and attention at 4, 7, 12, and 16 months of age in the TgTau^{P301L} mice. By comparing these data to the results of studies of other rodent models of dementia using the same testing method, an interesting profile of behavioural differences emerges, which may be related to the precise pathological insult experienced. Specifically, Romberg and colleagues (2011) tested attention and executive control in 3xTgAD mice, which express the APPswe, and PS1 M146V mutations in concert with the tau P301L mutation. Subsequently, the TgCRND8 mouse, a widely used model of Aβ pathology, expressing the Appswe/ind mutation was also evaluated in this paradigm (Romberg et al., 2013).

The 3xTgAD model was found to perform with less accuracy and make more perseverative responses, than the control mice at 9 months of age in this task. In contrast, 4 to 5 month old TgCRND8 mice exhibited lower accuracy, but no differences in other measures including perseverative responding. The results presented in this chapter now add to this profile, reporting that expression of the tau P301L mutation alone has no effect on touchscreen 5-CSRTT performance across a wide range of ages.

Taken together, these studies suggest that in rodent models of dementia, mutated amyloid may be required to disrupt touchscreen 5-CSRTT accuracy. In addition, as no effect on perseveration was detected in the TgTau^{P301L} animals here or in the TgCRND8 mice previously (Romberg et al., 2013), it is reasonable to speculate that PS1 mutations potentially lead to the increased perseverative responding in this task observed in the 3xTgAD model (Romberg et al., 2011). However, this hypothesis should be directly addressed by examination of PS1 mutant animals in the touchscreen 5-CSRTT. In addition it should also be noted that critical differences in background strain and the promoter used to express the various mutations could contribute to the phenotypes observed and will need to be systematically addressed for robust conclusions to be drawn. Furthermore, Seino and colleagues (2010) demonstrated that Aβ partially enhances tauopathy by crossing TgTau^{P301L} and Tg2576 mice, suggesting that it could

be the interaction between Aβ and P301L in the 3xTgAD model that resulted in attentional impairments.

Secondly, the longitudinal study presented in this chapter examined the TgTau^{P301L} mouse model using perirhinal cortex-dependent OR tasks, both the Decoupled and Forced-choice versions. Consistent with the progressive degenerative nature of this model, task performance was unaffected at 5, 8, 13, 17 and 19 months but became compromised at 21 months of age in the Forced-choice paradigm. This suggests a remarkable functional resilience of the perirhinal cortex, given the likely extensive nature of the pathological insult experienced at the 17 and 19 month time points.

As with the 5-CSRTT assessment, our laboratory has previously examined the performance of the TgCRND8 amyloid model in the Decoupled OR paradigm. Romberg and colleagues (2012) found that the Tg+ did not perform differently on the repeat and novel conditions in the Decoupled OR task, whereas the littermate controls showed higher D2 scores in the novel condition. This was interpreted as the Tg+ exhibiting a recognition memory impairment due to false recognition instead of forgetting. False recognition has been reported as a cause of memory impairments in AD patients and those with MCI (Hart et al., 1985; Budson et al., 2001; Gold et al., 2007; Hildebrandt et al., 2009; Plancher et al., 2009; Abe et al., 2011), and may represent a deficit in pattern separation. While no impairments were detected on performance of the Decoupled OR task here, the TgTau^{P301L} mice did exhibit a deficit in the Forced-choice OR paradigm when aged further (21 months), suggesting that the same perirhinal cortex-dependent process is compromised by either amyloid or tau accumulation. However, due to the limitations of the Forced-choice technique, it is not possible to determine if the deficit in the TgTau^{P301L} animals is due to false memory or forgetting.

The Forced-choice paradigm was chosen for later time points because I wanted to test different delays and the Forced-choice paradigm requires half as many trials as the Decoupled paradigm. At 17 months of age, performance at the 24 h delay on the repeat condition of the Decoupled version was approaching a D2 of 1, which suggested that both groups were having trouble remembering at such a long delay. This was one reason why I changed to the Forced-choice paradigm and tested the mice using a 3 h and 8 h

delay. As the mice were becoming aged, and the attrition rate was increasing, it was important to test different delays as quickly as possible. Additionally, the Forced-choice paradigm is more analogous than the Decoupled version to the LR spatial paradigm that was used around the same time. The similarity in design enabled direct comparisons.

Therefore, future studies should evaluate TgTau^{P301L} at 21 months of age on the Decoupled version of OR to confirm the nature of this deficit and enable comparison with the previous TgCRND8 study (Romberg et al., 2013). The decision to terminate the behavioural testing at 21 months of age was because of the uneven sample sizes, the increasing attrition rate of the TgTau^{P301L} sample, and the intention to conduct histological analysis. Future studies should use larger sample sizes to investigate the specific impairments at such late time points.

Interestingly, Boekhoorn and colleagues (2006) characterized another tau-P301L mouse model and found improved recognition memory at a young age, using a 3.5 hour delay. It is unclear why the control mice were unable to perform OR after only a 3.5 hour delay, particularly because the sample phase had a 10 min duration which presumably would increase the ability of mice to remember the objects. Because the study did not use littermate controls it is difficult to draw conclusions about the role of the transgene.

Thirdly, the longitudinal study presented in this chapter assessed spatial memory using the hippocampus-dependent LR and T-maze tasks. The results were variable but suggestive of mild spatial memory impairment in the TgTau^{P301L} mice at 18 months of age. This is consistent with the temporal lobe and hippocampus focused atrophy observed in mice of the same age. Due to high levels of sub-criterion performance, it was not possible to draw conclusions from the data collected using the T-maze task. Unlike the other tasks used in this study, the animals were first exposed to these tasks at a relatively extended age. It is therefore likely that the advanced age contributed to the low performance levels observed in the T-maze.

Considering the LR deficit observed here and the fact that spatial deficits have been reported in these mice at younger ages (Murakami et al., 2006), future studies of this model should prioritize early detection of hippocampus-dependent deficits. For

example, there is some evidence from other mouse models expressing the P301L transgene that trace fear conditioning is particularly sensitive to the hippocampal dysfunction, more so than other hippocampal-dependent tasks such as the MWM (Hunsberger et al., 2014), thus future studies characterizing TgTau^{P301} should consider employing trace fear conditioning tasks in order to detect the earliest memory deficits.

Furthermore, it is possible that specifically examining pattern separation by manipulating the similarity between the spatial locations, analogous to the SLR task described in Chapters 2 to 5, may identify a subtle spatial memory impairment. By moving the objects closer together, and making the task more challenging, the Tg+ and Tg- may exhibit differential performance at an earlier age. Because the 5-CSRTT was unsuccessful at detecting early attentional impairments, future studies could use the LD and TUNL touchscreen tasks to evaluate pattern separation mechanisms at early ages (Oomen et al., 2013). LD and TUNL were not used in the present study because the setups are similar to 5-CSRTT, and interference was a concern.

In summary, this study represents the first longitudinal behavioural evaluation of the TgTau^{P301L} mouse model of tauopathy. This model recapitulates the relatively extended age of onset of behavioural symptoms often associated with neurodegenerative diseases such as FTD and AD. There were no apparent changes in executive function or attention in these animals as measured in the touchscreen 5-CSRTT. However, spatial and object recognition memory impairments were observed in the OR and LR tasks, consistent with a dementia-like phenotype in these mice when aged.

Pattern separation was not directly evaluated in this model, although it is possible that a specific pattern separation impairment underlies the spatial and object recognition memory deficits. Employing tasks that are more sensitive to earlier cognitive changes is necessary to examine these specific impairments. The overarching goal of this study was to evaluate the TgTau^{P301L} as a model for tauopathy, such as AD, which are characterized by impaired episodic memory, possibly indicative of pattern separation deficits. Currently, no mouse model of AD is adequate at reproducing the full spectrum of AD phenotypes (Laurijssens, Aujard, & Rahman, 2013; Selkoe, 2011). Some current models have face validity, where the animals share phenomenological similarities with

AD; however, researchers have yet to find a model that provides good predictive validity.

Chapter 7: General Discussion

The goal of this dissertation is to elucidate the mechanisms underlying pattern separation in medial temporal lobe structures. Pattern separation was first described as a specific computational mechanism that transforms similar input patterns into more orthogonal representations in neural networks. Over the past 40 years this concept has been studied using computational modelling, electrophysiology, neuroimaging, and behaviour. The work described in this dissertation rests on the assumption that behavioural tasks can be designed in such a way as to evaluate the effectiveness of pattern separation; however, this assumption has been criticized and challenged by others in the field. Some argue that behavioural approaches are only able to study a process of discrimination and it is inappropriate to refer to any behavioural approach as studying pattern separation. This final chapter will (1) summarize the full body of work presented in this dissertation, (2) discuss the debate occurring within the 'pattern separation field' and argue that we can study pattern separation behaviourally, and (3) briefly mention some general future research directions.

7.1. Summary

Keeping memories distinct and separated is essential for successful memory. Pattern separation is proposed as a computational mechanism that enables events to be distinguished in memory, by transforming similar inputs into discrete non-overlapping representations. This process increases the likelihood of accurate encoding and subsequent retrieval by reducing interference among stored memories. Because forming distinct memories and being able to differentiate between similar events is essential for episodic memory, identifying the mechanisms underlying pattern separation is important for both our basic understanding of the fundamental processes underlying memory formation as well as having potential clinical relevance to amnestic disorders that specifically disrupt episodic memory, such as Alzheimer's disease (AD).

As discussed in Chapter 1, following the pioneering work of Marr (1971), O'Reilly and McClelland (1994) and others provided models of the hippocampal memory system in

which pattern separation was identified as a process that amplifies discrepancies between similar input patterns. Experimental evidence from electrophysiology (Leutgeb et al., 2007; Neunuebel & Knierim, 2014), immediate early-gene (Vazdarjanova & Guzowsi, 2004; Kubik et al., 2007), and behavioural studies (Gilbert, Kesner, & DeCoteau, 1998; Gilbert, Kesner, & Lee, 2001; McHugh et al., 2007; Hunsaker, Rosenberg, & Kesner, 2008; Clelland et al., 2009; McTighe et al., 2009; Creer et al., 2010; Lee & Sollivan, 2010; Sahay et al., 2011a; Nakashiba et al., 2012; Tronel et al., 2012; Bekinschtein et al., 2013; 2014; Kent et al., 2015) have accumulated in support of a pattern separation process occurring in the hippocampus. The process that enables spatially proximate stimuli to be encoded and recalled as distinct appears to be particularly dependent upon plasticity-related mechanisms in the dentate gyrus (DG) (McHugh et al., 2007; Bekinschtein et al., 2013) and adult-born hippocampal neurons (Clelland et al., 2009; Creer et al., 2010; Sahay et al., 2011; Nakashiba et al., 2012; Tronel et al., 2012; Kheirbek et al., 2012; Bekinschtein et al., 2014; Kent et al., 2015), when forming spatial or contextual representations.

Although much of the literature, and the experiments described in this dissertation, focus on pattern separation in the DG, there is some evidence of pattern separation occurring in the perirhinal cortex and throughout the ventral visual stream (Bussey, Saksida, & Murray, 2002; 2003; Eacott, Machin, & Gaffan, 2001; Bartko et al., 2007a,b), the dorsal visual stream (Goodale, 2011), cortical auditory processing stream (Rauscheker & Scott, 2009), CA1 region of the hippocampus (Gilbert et al., 2001), amygdala (Gilbert & Kesner, 2002), and olfactory bulbs (Mandairon et al., 2006; Moreno et al., 2009; Valley et al., 2009; Breton-Provencher et al., 2009). The Representational-Hierarchical perspective (Bussey & Saksida, 2002; 2005; 2007) provides a theoretical framework for understanding how different regions perform pattern separation at the level of stimulus complexity that is represented in that specific region. From this perspective, pattern separation can be thought of as a process for discriminating between ambiguous stimuli by reducing overlap in representations; by reducing similarity, pattern separation reduces interference.

7.1.1. Summary of the SLR task

Chapters 2 to 5 describe experiments that employed the Spontaneous Location Recognition (SLR) task. The SLR task was designed to enable the parametric manipulation of similarity between spatial landmarks. The task rests on the assumption that it is more challenging to form representations that are distinct and resistant to confusion when objects to be encoded are closer together in the arena. This is supported by the observation that the extra small separation condition (objects separated by a 40° angle) is the most difficult condition for rats (Bekinschtein et al., 2013; 2014; Kent et al., 2015). Specifically, in the extra small separation condition, naïve wildtype rats do not show a preference for an object in a novel location, suggesting that they do not recognize the location as different from the object's positioning during the sample phase. Furthermore, because DG manipulations impair performance only in the small (objects separated by a 50° angle) and not the large separation condition (objects separated by a 120° angle), it suggests that when the load on pattern separation varies between conditions, different mechanisms are engaged when encoding spatial representations that need to be separated. The work described in this dissertation was designed to further elucidate the mechanisms involved in DG-dependent spatial pattern separation.

SLR has been previously validated to study pattern separation and has several inherent benefits compared to other behavioural methods (Bekinschtein et al., 2013). Firstly, because it is a spontaneous task and takes advantage of a rodent's innate preference for novelty, there is no training required. In contrast, tasks such as the radial arm maze and the automated touchscreen tasks- Location Discrimination (LD) and Trial-unique Nonmatching-to-Location (TUNL) - require several days of training before being able to assess pattern separation (Clelland et al., 2009; Oomen et al., 2013). Secondly, the design of SLR allows experimenters to discriminate between the stages of memory (i.e., encoding, consolidation, or retrieval). This is possible because of its single trial nature and because there is a clear distinction between the sample phase and the test phase. The distinct phases enable a comparison between the effects of manipulations occurring during different stages of memory processing. Other tasks that run several trials within one session (e.g., radial arm maze and the touchscreen LD and TUNL tasks) make it difficult to disrupt specific stages of memory; however, with the relatively recent

development of optogenetics (Deisseroth, 2011), experimenters may now have a method that is time-sensitive enough to selectively disrupt encoding and retrieval on other tasks. The third advantage of SLR, compared to other tasks, is that SLR uses identical choice phases in every condition and manipulates similarity during sample. This is important because pattern separation is hypothesized to occur during encoding. Other tasks, such as those that use a contextual fear-conditioning paradigm (e.g., McHugh et al., 2007), manipulate the difference in stimuli during retrieval. A final benefit of SLR is that no appetitive rewards or aversive stimuli are used. This means that the rodents do not need to be under strict food–restriction or experience unpleasant shocks.

Although SLR has clear advantages over other tasks and has allowed us to uncover some of the mechanisms underlying pattern separation, it also has some disadvantages. Firstly, the spontaneous exploratory behaviour is highly sensitive to environmental cues. Unusual noises in the testing environment can distract the subject. These distractions are particular disruptive because of the single trial nature of the task. Secondly, the affective state of the subject can affect willingness to explore and the preference for novelty. When running SLR it is critical that the subject be fully habituated to the experimenter and the experimental room. Thirdly, as with all hand-testing tasks, SLR requires a lot of experimenter involvement, and increases the possibility of experimenter-effects. Finally, because the exploratory behaviour is scored by hand, inter-rater variability can be a problem. Thus, although SLR does not require time-consuming training for the subject, it does require that the experimenters undergo a lot of training to achieve consistent and reliable scoring criteria. However, despite these disadvantages, SLR has proven to be a useful, replicable, and valid task for evaluating pattern separation.

7.1.2. Summary of experiments

The SLR task was used in the experiments discussed in Chapters 2 to 5 to elucidate the mechanisms important for pattern separation. The first experiment provided further support for the role of plasticity-related mechanisms for pattern separation by demonstrating that performance on SLR was influenced by BDNF acting on NMDA receptors in the DG (Bekinschtein et al., 2013). I showed that pre-sample infusions of

an NMDA antagonist blocked the beneficial effect of a subsequent post-sample injection of recombinant BDNF. This suggested that BDNF enhances pattern separation in an NMDA-dependent manner, such that BDNF in conjunction with NMDA receptors promote plasticity in the activated neural networks to encode unique representations of similar spatial locations.

After identifying the importance of plasticity-related mechanisms in the DG for performance on SLR, I sought to explore the possible DG cell types involved in pattern separation. Previous work elucidated an important role for adult-born neurons for pattern separation on a different behavioural task (Clelland et al., 2009), but the effects of manipulating neurogenesis on SLR performance had not yet been examined. The experiments described in Chapter 3 demonstrated that reducing DG neurogenesis using a lentiviral approach impaired performance on SLR and blocked the beneficial effect of BDNF infusions. This suggests that BDNF acts on adult-born neurons during pattern separation.

How NMDA receptors are involved in this effect is unclear. It is possible that BDNF acts on NMDA receptors located on young DG neurons. BDNF-induced plasticity and memory have been linked to NMDA activation in the hippocampus (Mizuno et al., 2003; Suen et al., 1997) and BDNF and its receptor TrkB are widely expressed in association with glutamatergic synapses (Bramham & Messaoudi, 2005). NMDA receptors develop within the first 14 days after neuronal birth (Nacher & McEwen, 2006; Tashiro et al., 2006), so it is possible that BDNF is acting on NMDA receptors located on adult-born cells. It would be interesting to examine the specific pathway of action using a rat with NMDA receptors blocked selectively on newborn granule cells to determine whether BDNF is acting on NMDA receptors located on the immature neurons.

To further examine the role of hippocampal neurogenesis in pattern separation, the next experiment described in this dissertation was designed to evaluate the effect of increasing neurogenesis on SLR performance. Rats were administered a sub-chronic ghrelin treatment for two weeks, which resulted in an increase in the number of new neurons in the DG and improved performance on SLR. The dose chosen was equivalent to the level of ghrelin in circulation after a 24 h fast in rats (Wren et al., 2001). It is

impossible to know from this study, how the ghrelin treatment exerted its effect on neurogenesis and pattern separation; however, previous research has shown that upregulating neurogenesis is sufficient to improve pattern separation (Clelland et al., 2009), thus the increase in neurogenesis may be the mediating factor. Although ghrelin may be acting directly in the DG to increase neurogenesis (Diano et al., 2006; Li et al., 2013), there are also several indirect pathways by which ghrelin can increase neurogenesis, such as stimulating the growth hormone-IGF-1 axis (Aberg et al., 2000). Future research should investigate the specific mechanism by which ghrelin affects neurogenesis and pattern separation, to determine whether ghrelin is acting directly in the DG to stimulate neurogenesis and whether the upregulation of neurogenesis is necessary for the improvements in pattern separation. Together with the broader literature, the experiments from Chapters 3 and 4 reveal that the level of hippocampal neurogenesis is critical for encoding similar spatial inputs as distinct representations that are necessary for accurate retrieval.

The final set of experiments using SLR was designed to investigate whether the process of pattern separation has reciprocal effects on hippocampal neurogenesis and subsequent SLR performance. The hypothesis was founded in the confusing literature surrounding the effects learning and environmental enrichment have on neurogenesis and performance on subsequent cognitive tasks. Chapter 5 described a series of experiments demonstrating that repeatedly exposing rats to spatial landmarks placed close enough together to engage a pattern separation-like process, results in an increase in adult-born neurons in the DG and improved performance on SLR. Furthermore, rats that had reduced neurogenesis after being treated with one of two methods that inhibited cell division in the DG, did not show improvements on SLR after the exposure treatment, suggesting that the upregulation of neurogenesis resulting from the exposure treatment is necessary for improvements in pattern separation.

The results of these experiments described in Chapters 3 to 5 suggest that a key function of adult hippocampal neurogenesis is pattern separation of spatially proximate stimuli and that some of the contradictory findings in the literature may be explained by examining the load on pattern separation as the important parameter (Bekinschtein et al., 2011).

Moving away from the SLR task, Chapter 6 described a 22-month longitudinal study that aimed to provide a cognitive profile of a mouse model of dementia. Specific deficits in pattern separation are thought to underlie some of the memory impairments experienced by AD patients, so establishing valid mouse models of disease will help researchers identify the mechanisms underlying disease progression and aid in the development of effective therapeutics.

The aim of the study was to evaluate whether the TgTau^{P301L} mouse was a suitable model of tauopathies, such as FTD and AD, with particular emphasis on early detection. The TgTau^{P301L} mouse model was evaluated on a battery of cognitive tasks. Frontal cortex-dependent executive function and attention were assessed using the touchscreen 5-choice serial reaction time test (5-CSRTT) at 4, 7, 12, and 16 months of age, but no effect of the transgene was identified. Similarly, no differences were detected on perirhinal cortex-dependent recognition memory, which was assessed using object recognition (OR) tasks at 5, 8, 13, 17 and 19 months of age. However, consistent with the progressive degenerative phenotype of this mouse, a robust deficit was observed in OR at 21 months of age. I also examined hippocampus-dependent memory using the T-maze and Location Recognition memory tasks at 18 to 20 months of age, which revealed spatial memory impairment.

This model shows high levels of heterogeneity in pathology and performance, and late onset of cognitive impairment. Future studies will require larger sample sizes and should focus on hippocampal-dependent tasks at earlier ages. It is possible that the load on pattern separation is a key parameter in the cognitive effects of pathological tau in the hippocampus; although, pattern separation was not explicitly evaluated in this study for a variety of reasons. Firstly, the 5-CSRTT was prioritized because attentional deficits are thought to occur prior to hippocampal-dependent memory impairments in patients (Lawrence & Sahakian, 1995; Perry et al., 2000; Collette et al., 1999; Baddeley et al., 2001). Secondly, other touchscreen tasks developed to assess pattern separation, such and LD and TUNL (Oomen et al., 2013), are very similar to the 5-CSRTT setup, and may interfere with task performance. Thirdly, the SLR task has not yet been verified for studying pattern separation in mice, and will be discussed in a later section of this chapter. Fourthly, once memory impairment was observed, there was a high attrition rate, low sample size, and high variability in performance. Because of this, I

decided to prioritize the histological analysis over continuing behavioural testing. However, the Decoupled version of the OR paradigm allowed me to assess vulnerability to interference, which is an indirect method to evaluate pattern separation in the object domain.

7.2. Critiques of pattern separation research

The work described in this dissertation rests on a fundamental tenet of behavioural neuroscience: behavioural analysis can be used to evaluate biological processes underlying specific psychological constructs. In other words, behavioural neuroscientists use tasks as assays of a postulated putative process. The main focus here has been to use the SLR task to evaluate the postulated process, pattern separation. Although several independent research groups have used behavioural tasks to evaluate pattern separation, there is some debate about whether these tasks can or should be used to study pattern separation. In particular, there is some disagreement over the operational definition of pattern separation and how it is best studied experimentally.

For example, Santoro (2013) argues that the term pattern separation should not be used interchangeably to describe computational processes, changes in cell ensemble activity, and behaviour. Santoro goes as far as to say that the term pattern separation should never be used when describing behaviour, and instead that we should refer to the process as discrimination. According to Santoro, discrimination describes behaviour, whereas pattern separation describes a neurocomputational process. He argues that the term pattern separation should be reserved for computational models or when directly measuring the inputs to a brain region and its outputs.

Although I agree that behavioural tasks, such as SLR, do evaluate the subject's ability to discriminate between similar stimuli, I disagree that discrimination is as specific as we can get to describe the observed phenomena. I think we can move beyond the more general concept of discrimination and use behavioural paradigms to study the potential functional output of the underlying process of pattern separation.

As the data presented in Chapters 2 and 3 suggest, discriminating between highly similar spatial locations requires the engagement of qualitatively different processes

than discriminating between dissimilar spatial locations. Because the mechanisms required vary depending on the similarity of stimuli, it suggests that a specific type of discriminatory process is being engaged under certain conditions. Thus, this is not simply evaluating discrimination, it is evaluating the mechanisms required to discriminate under conditions of high interference.

These behavioural findings are in keeping with the type of pattern separation processes postulated by the computational models. As described in Chapter 1, pattern separation is proposed as a process that transforms overlapping input into distinct representations, which reduces interference and enables the representations to be retrieved as unique events in memory. I cannot be sure that pattern separation is the putative mechanism underlying the differential performance on the SLR conditions, but by increasing the similarity of stimuli, it is reasoned that this also increases the similarity and overlap of neural inputs, and thus interference. Although pattern separation - a transformation of input representations to output representations that are less correlated- has not been directly observed, it seems reasonable to claim that the results of these experiments are consistent with a pattern separation-like process. The behavioural tasks do not measure pattern separation, but they measure behaviours that are consistent with the postulated mechanism and provide experimental evidence for its existence.

It is my opinion that even though we are not measuring pattern separation directly and cannot be certain that the behavioural effects are a result of pattern separation, using this terminology is not only appropriate but also beneficial. By using the same terminology across levels of analysis, it allows for interdisciplinary investigation into the mechanisms underlying hippocampal encoding of similar input patterns that would engage overlapping representations, if not for a process to reduce similarity during encoding. Sharing terminology is especially important because until recently, we only had indirect evidence of pattern separation.

The strongest evidence of pattern separation at the neuronal level comes from a recent study by Neunuebel and Knierim (2014). In their study the experimenters simultaneously recorded single-unit activity from DG and CA3 and demonstrated that DG outputs are less correlated than the inputs. By measuring both input and output representations they were able to for the first time explicitly test whether outputs were

less similar than inputs. This is the most direct evidence of pattern separation in the DG and provides further evidence that the behavioural outcomes seen through tasks designed to manipulate pattern separation may in fact be doing just that.

Because of the debate surrounding the operational definition of pattern separation, Dr. Michael Yassa created a website to be used as a platform for discussion, with a mission to develop an appropriate operational definition (see www.patternseparation.com). The initial proposal for the operational definition was:

"the process of reducing interference among similar inputs by using non-overlapping representations. In the brain, this is represented by using distinct neural codes."

There was general agreement that studies using behavioural paradigms to study pattern separation should include an explicit caveat proposed by Professor Timothy Bussey:

"We are aware the term 'pattern separation' refers, in the original computational literature, to a specific proposed mechanism involving the transformation of an input representation to an output representation, in which the output is less correlated than the input, resulting in non-overlapping stimulus representations. Our behavioural tests assess the use of such representations. However it should be emphasised that our tests do not assess the mechanism of pattern separation, as defined by the computational modellers, directly."

Evidence from computational models, electrophysiology, neuroimaging, and behaviour are consistent with a process of pattern separation occurring in the DG. It is useful to use this specific terminology in order to connect these different levels of analysis, particularly because one of the ultimate goals of computational modelling and theory is for the resulting hypotheses to be tested at the whole animal level, which requires an ability to translate across scales.

Other critiques about the pattern separation literature have come from Aimone, Deng, and Gage (2011) who have questioned the interpretation of some of the behavioural studies. They suggest that some 'pattern separation impairments' could result from deficits in inhibitory learning (e.g., McHugh et al., 2007; Sahay et al., 2011), and that

tasks which require working memory could show variations in performance because the animal solves the task using different neural pathways according to the degree of dissimilarity between input patterns (e.g., Clelland et al., 2009; Creer et al., 2010; Gilbert et al., 2001). Their main argument is that with many of these tasks, deficits in other processes that are not pattern separation, may explain the pattern of results, such as impaired inhibitory learning or working memory. Although this is a possible explanation for some of the behavioural evidence, these critiques do not apply to impairments demonstrated using SLR, which is not a working memory task, and do not explain separation-dependent impairments on working memory tasks, such as the radial arm maze.

Similarly, the authors point out the potential for circularity of interpretation, such that we only found evidence for pattern separation because it was proposed in the computational models (Aimone, Deng, & Gage, 2011). They argue that if we were presented with the full body of evidence, without a priori assumptions, then we may propose another explanation for the data that does not involve a pattern separation process. This is a valid concern. As stated above, we are not observing pattern separation directly but rather inferring that a pattern separation process underlies the various behaviours that we observe. It is possible that the pattern of behaviour demonstrated using the SLR task and other tasks result from a process unrelated to pattern separation as proposed by computational models. The modellers and theorists have provided us with a postulated mechanism that fits with our behavioural observations. As research continues to identify the specific mechanisms underlying pattern separation, the validity of these critiques will be determined.

7.3. Future directions

Moving forward, there are still a lot of questions remaining. Many of these were mentioned in the discussion sections of each chapter, such as ‘Does BDNF act on NMDA receptors that are located on newborn neurons?” and “How does the systemic treatment of acyl-ghrelin increase neurogenesis?” The following section will discuss at a more general level, why future work should focus on developing an analogous SLR task for mice.

Developing a version of the SLR task for mice would provide a novel task for studying pattern separation in transgenic mouse models of disease. The spontaneous nature of SLR would complement other tasks often used in cognitive batteries to phenotype transgenic models. For example, in Chapter 6 I described a longitudinal study that had the aim to provide a cognitive profile of the TgTau^{P301L} mouse. If SLR had been validated, it could have easily been incorporated into the battery without worry of interfering with other tasks, such as the touchscreen version of 5-CSRTT.

Our lab has worked on developing a mouse version of SLR with mixed success. The task used a very similar protocol to the one that had been developed for rats, but using a smaller arena and using smaller objects, and was tested with several strains of mice. Unfortunately, performance of the mice on SLR was highly variable and exploration levels were low. Figures 7.1 and 7.2 show example exploration times during the choice phase of SLR for two groups of mice: the C57BL/6 and TgCRND8 mouse models. For mice, the average total exploration ranges between approximately 7 to 15 s, which is a third to a quarter of the average exploration of rats. Figure 7.3 provides some example exploration times from rats during the choice phase of SLR from experiments described in previous chapters. For rats, the average total exploration ranges from approximately 40 to 55 s.

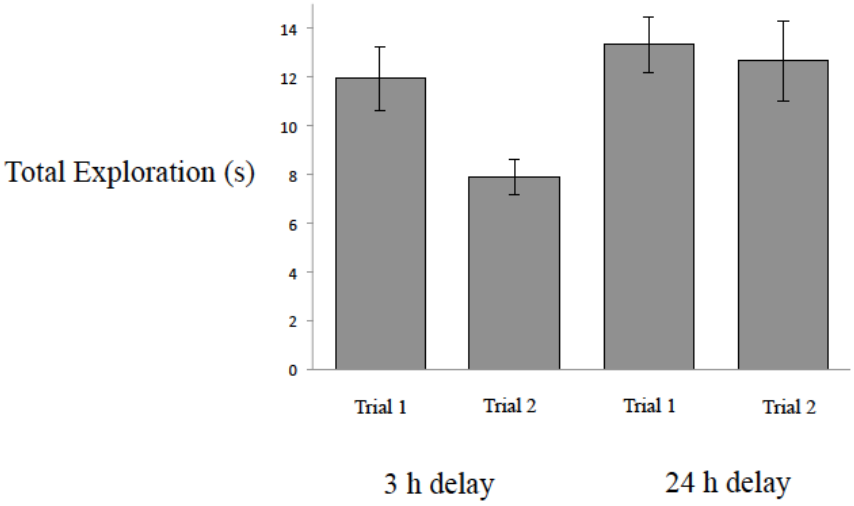

Figure 7.1. Exploration of C57BL/6 on mouse SLR. The y-axis presents the mean total exploration times for C57BL/6 mice (n = 10) during the choice phase of SLR. Mice were tested on SLR with a 3 h and 24 h delay. Two trials of each delay were tested. Data presented as means and ± standard error of the mean (SEM).

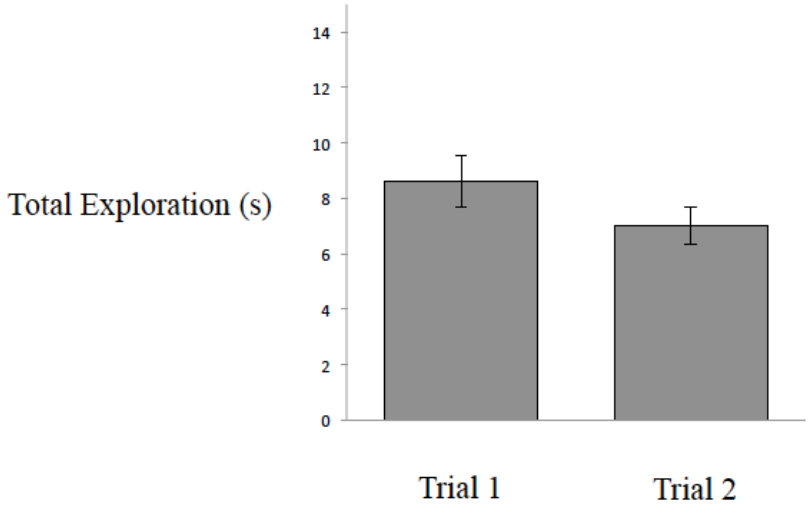

Figure 7.2. Exploration of TgCRND8 on mouse SLR. The y-axis represents the total exploration time for TgCRND8 (n = 24) mice during choice phase of SLR. Mice were tested on two trials of SLR using a 3 h delay. Data shown combined the littermate controls and Tg+. Data presented as means and ± SEM.

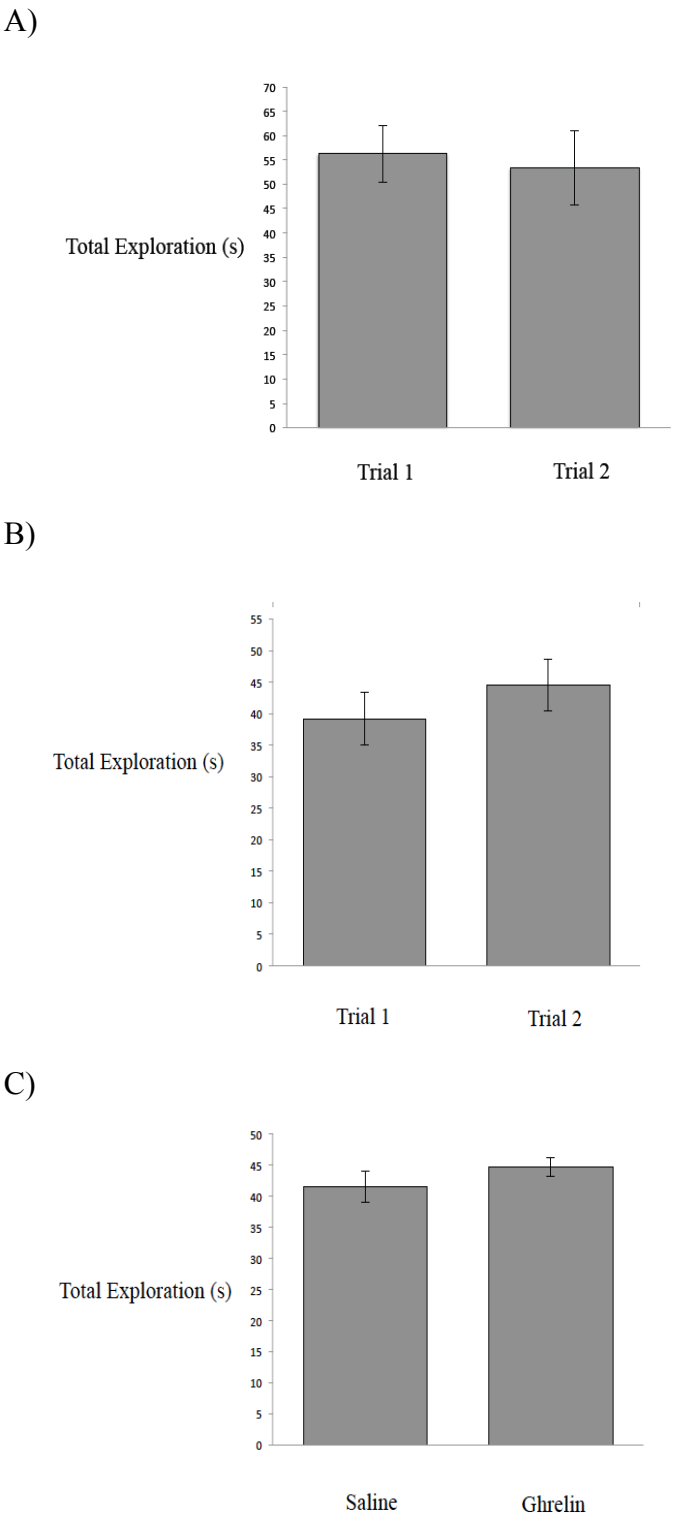

Figure 7.3. Total exploration during the choice phase of SLR from experiments using rats. The y-axis represents total exploration time during the choice phase of rat SLR. (A) Exploration data from an experiment described in Chapter 2. Rats were treated with AP5 and/or BDNF infusions (B) Exploration data from an experiment described in Chapter 3. Rats were treated with a lentivirus. (C) Exploration data from an experiment described in Chapter 4. Rats were treated with acyl-ghrelin or saline. Data presented as means and ± SEM

These figures are provided to help illustrate the large difference in exploration between mice and rats on the SLR task, and are not an exhaustive review of the data. Variations in the size of the objects and the time delays have been used to encourage exploration in mice, but with limited success.

Another challenge was preventing mice from jumping out of the arena. As object size increased, it also increased the ease at which mice were able to escape the testing arena. It was difficult to keep the walls of the arena low enough so that the mice could see spatial cues around the testing room, but to keep the walls high enough to prevent mice from escaping.

Future work should continue modifying the parameters of SLR to use with mice. As discussed in Chapter 6, there is some evidence that AD patients have impaired pattern separation, which may underlie some of their memory impairment. A major challenge in developing effective therapies for AD is validating an appropriate model. By developing SLR for mice, we would have a relatively fast method for evaluating pattern separation in transgenic mouse models of disease.

7.4. Conclusions

Studying pattern separation emphasizes the important but often overlooked fact that successful memory involves more than just remembering events over a period of time. Successful memory also involves differentiating between similar memories. The work described in this dissertation adds support to the literature that the DG region of the hippocampus is important for pattern separation when encoding spatial and contextual inputs. Using the SLR task it was shown the BDNF can improve performance by acting on NMDA receptors in the DG and adult-born neurons. Manipulating the level of hippocampal neurogenesis by inhibiting Wnt signalling or by administering acyl-ghrelin systemically was shown to impair and enhance performance on SLR, respectively. Using a novel exposure paradigm in combination with SLR, it was demonstrated for the first time that the relationship between pattern separation and neurogenesis may be reciprocal, such that inhibiting neurogenesis impairs pattern separation, enhancing neurogenesis improves pattern separation, and performing pattern separation enhances the production or survival of adult-born hippocampal neurons. Finally, it was shown

that a mouse model of dementia ($TgTau^{P301L}$) exhibited spatial and object recognition memory impairments at a late age. Understanding the mechanisms that contribute to effective pattern separation may help elucidate the processes underlying some of the memory impairment experienced by AD patients.

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