

*EXAMINING THE CONTRIBUTION OF  
DENTATE GYRUS GRANULE CELLS AND  
AMBIGUITY TOWARD THE STRESS  
RESPONSE AND BEHAVIOUR OF THE  
RODENT*

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## DECLARATION

This thesis is the result of my own work and collaborative efforts are mentioned here. It has not been previously submitted to any university or institution for any degree, diploma, or other qualification.

Collaborations on aspects of this thesis include: Dr. Stephen B. McHugh (Oxford), Dr. Chris Barkus (Oxford), Dr. Rob Deacon (Oxford), and myself performed hippocampal and sham lesions and behaviour; Greg Daubney (Oxford) assisted with hippocampal lesion histology; Dr. Rose-Marie Karlsson (NIH) assisted with the adrenalectomy surgeries and other endocrine measures along with Dr. Heather Cameron (NIH); Dr. Timothy J. Schoenfeld (NIH) assisted with the immediate-early gene analysis; Michelle Brewer (NIH) assisted with genotyping.

Signed:



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SHORT ABSTRACT

The hippocampus and adult-born neurons have been implicated in regulating the stress response and disambiguating conflicting goal-related information. How these functions interact to promote adaptive behaviour has not been investigated. In this thesis, hippocampal lesions or pharmaco-genetic ablation of adult neurogenesis in mice ('TK mice') was used to dissect this relationship. Hippocampal lesions or selectively ablating adult neurogenesis decreased defensive behaviours toward a partially reinforced fear conditioned cue ('ambiguous fear conditioning'). Conversely, these animals did not show these impairments when a cue reliably predicted a footshock ('reliable fear conditioning'). In TK mice, this same pattern of results was also seen in Fos expression levels in the dentate gyrus and CA3. In additional experiments, baseline novelty-suppressed feeding behaviour (latency to eat a familiar food) did not differ between genotypes. After reliable fear conditioning, TK mice took longer to eat the food, suggesting increased anxiodepressive-like behaviour. After ambiguous fear conditioning, however, TK showed quicker latencies to eat, suggesting decreased anxiodepressive-like behaviour, as compared to wildtype mice. Clamping corticosterone levels was used to determine its role in adaptive responding to prior stress. Preventing experience-induced increases in corticosterone did decrease the latency for wildtype mice to eat after ambiguous fear conditioning, whereas TK mice were unaffected. Finally, another set of experiments were designed to see if ambiguity would affect appetitive behaviour in a non-spatial T-maze task with conflicting goal responses. It was found that mice with hippocampal lesions, but not TK mice, tended to choose an arm associated with a large reward less often if the other arm contained a small reward, as compared to their respective control groups. Similarly, hippocampal-lesioned mice, but not TK mice, chose an arm that was always associated with the presence of a reward less often if the other arm ambiguously predicted a reward. All of the above results suggest that the hippocampus, especially adult-born neurons, is necessary to disambiguate salient conflicting memories that underlie goal responses and that this disambiguation biases goal-related behaviour in an adaptive manner.

## LONG ABSTRACT

In this thesis, the function of the hippocampus and adult neurogenesis are investigated, utilising a variety of measures to investigate the effects of ambiguity/uncertainty of aversive and appetitive experiences on the behaviour of the animal. Extensive behavioural tasks are used to probe these questions, along with neuroendocrine and neural activity using immediate-early gene activity levels. It is shown that there is a complex interaction between the hippocampus and adult-born neurons and the type of uncertainty experienced previously by the animal. In particular, the ambiguity about whether a stimulus predicts an aversive outcome has effects on future adaptive responding, and this successful adaptive behaviour is dependent on adult neurogenesis and its interaction with stress hormones. Furthermore, the hippocampus as a whole, and not adult-born neurons selectively, is necessary to successfully perform a decision-making task involving uncertainty of reward outcome or the magnitude of reward. These results suggest that the hippocampus and adult-born neurons are necessary to disambiguate overlapping goals, or memories that support these goals, that aid in adaptive responding to future aversive and appetitive decisions.

In Chapter 1, the basic anatomy and functions of the hippocampus and adult neurogenesis are laid out throughout this chapter. First, the anatomical connectivity of the hippocampus, with specific references to the new neurons' connectivity, is discussed. Second,

memory and emotion are discussed in regard to hippocampal functionality as a whole. These topics range from more cognitive functions, referencing prominent theories regarding declarative memory, associative memory, and spatial processing to more emotive functions regarding endocrine responses to stress, J A Gray's theory on the behavioural inhibition system, and anxiety and depression as the phenomenological and behavioural emergences. The third portion of this chapter deals with specific functions of adult neurogenesis that have been discovered over the past few decades and how they relate to the broader functions of the hippocampus.

Chapter 2 begins the experimental portion of this thesis. Here, complete, excitotoxic hippocampal or sham lesions were made to mice and mice were subsequently tested in a fear-conditioning paradigm. The aim of this chapter, then, was to examine how hippocampal-lesioned mice interpret and respond to distinct predictor cues about an aversive event. In addition, context-conditioning effects are investigated, because many studies have shown context effects with these animals. In the within-subject design, distinct tones were used as the conditioned stimuli to represent three different cue associative, predictive outcomes of an aversive event that were all intermixed with one another. The first type of tone fully predicted a footshock; a second tone that was never associated with a footshock; and a third that partially predicted footshock with a contingency of 20%. This last condition,

because it was only partially reinforced, created ambiguity with regard to the tone's outcome and associative structure. Briefly, mice with hippocampal lesions showed a selective deficit in processing, as measured by freezing levels, the ambiguous tone but did not show a deficit, compared to sham-lesioned mice, when the different tones were either reliably/fully predictive of the presence or absence of footshock. These mice, using the lead-in period to each conditioning and testing session, show normal context conditioning effects but did not differ between groups.

In Chapter 3, a set of experiments was performed to investigate the effects of ablating adult neurogenesis on the processing and responding to cues that were full or partial (reliable or ambiguous, respectively) predictors of the aversive footshock. In this, a between-subjects design was used to assess the cue predictability while minimizing interference effects caused by competing cues, as was done in the previous chapter. Transgenic GFAP-TK, who lack adult neurogenesis under antiviral treatment, and wildtype mice were tested in this fear conditioning paradigm using a tone and a light as conditioned stimulus cues in two different experiments and using difference performance measures, namely freezing and startle responding using all separate cohorts of littermates. TK mice showed a selective freezing and startle impairment to the ambiguous, but not reliable, predictor cue. It was confirmed that these TK mice have normal unconditioned sensorimotor responding to the first presentation of all the cues

and footshock, ruling out a sensory/motor deficit. In further studies, it was confirmed that these mice also showed diminished Fos reactivity after experiencing ambiguous, but not reliable, cue training as compared to their wildtype counterparts, that was not reflective of overall activity levels.

Chapter 4 describes a set of experiments that first demonstrates that unconditioned, innate anxiety-like behaviours are not affected in mice with ablated adult neurogenesis using the elevated-plus maze or the light/dark exploration box, and anxiodepressive-like behaviours are unaltered in the novelty-suppressed feeding task. In the elevated-plus maze and the light/dark exploration box, there was no observable phenotypic difference between mice with and without adult neurogenesis. Specifically, both groups showed similar percentages of time spent in the anxiogenic regions of both tasks and faecal boli throughout. Additionally, both groups showed similar locomotor behaviours, as indicated by indistinguishable distances travelled and velocities on both tasks. In the novelty-suppressed feeding, under unconditioned circumstances, all mice displayed a similar latency level to start eating a familiar food in a novel environment. In addition, after fear conditioning, proper adaptation to a novel, potentially dangerous, situation is dependent on the interaction between new neurons and normal glucocorticoid signalling, especially when followed by prior fear conditioning using an ambiguous predictor cue about footshock. Specifically, all mice were first fear conditioned using a reliable or

ambiguous predictor cue. Then, days later, these mice, and their nonshocked control counterparts, underwent novelty-suppressed feeding and latency to eat a food pellet is measured. Under control conditions where no prior fear conditioning occurred, mice with and without neurogenesis showed similar latencies to eat food, indicating normal innate anxio-depressive and motivational behaviours in this task. In reliable or ambiguous fear conditioned mice, however, mice with intact adult neurogenesis showed robust, adaptive defensive behaviour in the novelty-suppressed feeding task if they previously received fear conditioning to an ambiguous predictor cue but not if their prior experience was with a reliable predictor cue. Mice without adult neurogenesis, however, showed uniform, intermediate increases in latency, irrespective of prior fear conditioning type received, showing increased anxio-depressive and decreased anxio-depressive behaviour after reliable and ambiguous fear conditioning compared to the wildtype littermates, respectively. When a separate cohort of mice underwent adrenalectomy and corticosterone levels were clamped at baseline levels, thereby preventing experience-induced surges in these levels, and then fear conditioned using the same predictor cues, a slightly different set of novelty-suppressed feeding latencies emerged. In particular, adrenalectomized-wildtype mice no longer showed this adaptive responding and were similar in latency to those that experienced a reliable predictor cue. Adrenalectomized mice

without neurogenesis, however, were largely unaffected, showing a similar pattern of results as their adrenal-intact counterparts.

Chapter 5 describes experiments examining the contribution of hippocampus as a whole and adult neurogenesis toward reward-based decision-making processes under conditions of ambiguity or uncertainty in a non-spatial T-maze task. In the first set of experiments, ambiguity of reward delivery was investigated. Specifically, mice with complete lesions of the hippocampus or sham controls were given 8 forced trials to (4 trials to each arm) to control for exposure effects of each arm and reward presence. After these trials, 4 free-choice trials were given, in which both arms were open, to choose from, to look at effects of competing reward certainties (50% vs. 100%) on choice behaviour. Mice with hippocampal lesions chose an arm that was ambiguously predictive of reward longer as the test progressed. In a control visual discrimination experiment, using the same trial design, sham mice performed well above chance performance on free-choice trials and hippocampal-lesioned mice quickly learnt this rule and were nearly indistinguishable from their sham counterparts. Again, using the same design, mice with ablated adult neurogenesis were indistinguishable from wildtype mice, with both groups rapidly increasing their choice performance to pick the certain reward over the arm ambiguous predictive arm. In a second set of experiments, the effects of different reward size (magnitude) were performed. One arm was associated with a low

amount of reward while a higher amount (5x) of reward was given in the competing arm. Using the same non-spatial T-maze task but given only 2 forced-choice trials and 5 free-choice trials, hippocampal-lesioned mice showed a selective deficit in terms of their percentage correct over blocks, meaning they persisted in choosing the small-reward arm, whereas shams rapidly climbed to preferring the high-reward arm. Mice with ablated adult neurogenesis, however, were relatively indistinguishable from wildtype mice. However, performance of the sham vs. wildtype mice showed a relative difference in percent accuracy achieved in the same amount of time.

Finally, in Chapter 6, the results are summarised and a broader interpretation is presented. The final section discusses how these results relate to aspects of how goal conflict aids in predictions and accurate memories.

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## List of Publications

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## LIST OF ABBREVIATIONS

ACTH = adreno-corticotropic hormone

ADX = adrenalectomy

A/P = anterior/posterior

CA1 = Cornu Ammonis region 1

CA3 = Cornu Ammonis region 3

CRF = corticotrophin releasing factor

CS - = conditioned stimulus not paired with shock

CS = conditioned stimulus

CS+ = conditioned stimulus co-terminating in shock

CSa = ambiguously conditioned stimulus

D/V = dorsal/ventral

DNA = deoxyribonucleic acid

EPM = elevated-plus maze

GABA =  $\gamma$ -aminobutyric acid

HP arm = high-probability arm

HPA axis = hypothalamic-pituitary-adrenal axis

HPCx = hippocampal lesion

HR arm = high-reward arm

ISI = inter-stimulus interval

ITI = inter-trial interval

LDB = light/dark exploration box

M = mean

M/L = medial/lateral

n = number of subjects per group

NMDA = *N*-methyl-D-aspartate

NSF = novelty-suppressed feed task

SEM = standard error of the mean

SSRIs = selective-serotonin reuptake inhibitors

thymidine-<sup>3</sup>H = tritiated (radioactive) version of thymidine

TK = valganciclovir-treated GFAP-thymidine kinase (GFAP-TK)  
transgenic mice

UCS = unconditioned stimulus

µm = micrometre

vGCV = valgancilovir

VTE = vicarious trial-and-error

WT = valganciclovir-treated GFAP-TK wildtype mice

This thesis is dedicated to C. Ray Church, Jr. (1921 - 2010)

# 1 INTRODUCTION

The hippocampus is one of the most widely studied brain structures in terms of its anatomy and functionality, and has proved itself far more complex than thought in both of these realms. The hippocampus, and indeed the entire brain, is not an isolated, static machine; it is a dynamic piece of tissue that continuously communicates with itself and other areas via local, regional, and functional connectivity. Contributing to this dynamic nature, the hippocampus has an extraordinary amount of plasticity within itself. One of the most intriguing changes that occur within this structure is the process of adult neurogenesis, or the birth of new neurons in adulthood. The degree of plasticity is typified by a dual interaction: new neurons can modulate behaviour, and experiences can modulate new neurons. For example, the environment in which the organism lives influences dendritic branching and the birth and death of cells, and this in turn influences cognition and emotionality, which feeds back into this cycle of plasticity. Both these regulatory avenues dictate the (future) behavioural output of the animal.

One of the most widely studied interactions with the environment that influences the brain is with stress. Stress has profound effects on the developing and adult animal with distinct, yet often paradoxical effects. During perinatal development, the brain is producing an overabundance of new cells, solidifying connectivity with other brain regions, and hormone shifts are programming responses that will serve the animal for later in life.

Thus, this is one of the most plastic times for brain development. This early plasticity within the animal's life allows for adaptive changes in brain and behaviour. An animal's stress response is continually being modified by both bottom-up and top-down mechanisms, and new experiences influence how our brain processes information related to stress and stress-related situations. What in the brain allows for these degrees of freedom in responding to particular stressors? How does the brain handle both aversive and appetitive experiences that have ambiguity as an inherent feature in their ontogeny? Likewise, does this ambiguity, particularly for aversive experiences, promote adaptive responding in future uncertain situations?

The aim of this introduction is to provide a brief history of the hippocampus and adult neurogenesis, and how both contribute to cognition and emotion in the rodent. For a more complete history and description of hippocampal anatomy and function, see 'The Hippocampus Book' (Andersen et al. 2006). In the first section of this introduction, the local, regional, and functional connectivity of the hippocampus is reviewed. The role of the hippocampus in memory is discussed in the second section, from complex episodic and spatial memories to configural and relational processing theories that may underlie these types of memories. Along with memory, however, the role of the hippocampus in emotion is reviewed in the third section, particularly how it relates to the neuroendocrine stress response

and how this translates into the behaviour of the animal. In the fourth section, a brief history of the field of adult neurogenesis is presented along with techniques used to ablate adult neurogenesis experimentally. Next, the functional role(s) that these new, adult-born neurons may contribute to cognition and emotion is explored in the fifth section. Finally, in the sixth section, the experimental aims of this thesis are described. Finally, how these specific experiments provide insights into how the hippocampus, and particularly the dentate gyrus, disambiguates conflicting goals or memories is discussed, along with how this then translates into the behaviour of the rodent.

## 1.2 Anatomy of the Hippocampus

In order to understand the functionality of the hippocampus, one must understand and appreciate the intricate connectivity of this structure. This section will give a brief review of our current understanding of the local architecture and connectivity within the hippocampus.

### **Local Connectivity**

The hippocampus is a structure within the medial temporal lobe of both hemispheres. This structure comprises the dentate gyrus (dentate) and the cornu ammonis (CA) subfields (CA1-3) (Figure 1.1). It is classically described as a tri-synaptic pathway.

Information flow first comes into the dentate from the adjacent entorhinal cortex via the perforant pathway. The dentate sends mossy-fibre projections to area CA3, which sends Schaffer collaterals to CA1. This connectivity, however, is simplistic and does not take into account many features. For example, the CA3 alone contains a large number of recurrent collaterals, back projections to the dentate, and additional connectivity to the fimbria-fornix. Likewise, CA1 receives direct connections from the dentate and from the entorhinal cortex, known as the temporoammonic pathway (Andersen et al. 2006). In addition, new neurons born within the dentate gyrus receive local, monosynaptic connections from a variety of local regions, including interneurons and excitatory neurons within the dentate and back-projections from CA3 (Deshpande et al. 2013, Vivar et al. 2012).

Within the CA subfields of the hippocampus, a laminar organisation is present and it is cytoarchitectonically defined into different strata. The major excitatory neurons within the CA subfields are the pyramidal neurons, so named because of their pyramid-shaped soma that measure approximately 15-20  $\mu\text{m}$ . These neurons are located in the stratum pyramidale, or simply the pyramidal layer. Located above this layer are the stratum oriens and the alveus. The stratum oriens is a superficial layer and houses the basal dendrites of the pyramidal neurons, named because of their branching from the basal end of these neurons,

and a substantial population of interneurons, largely basket cells, that target the dendritic area close to the pyramidal cell body. This stratum's connectivity includes fibres from the septum and commissural fibres. In addition, the alveus contains commissural fibres. Below the pyramidal layer is the stratum lucidum, which is the thinnest layer, in general, and, in the CA3, receives input projections from the dentate gyrus. Below the stratum lucidum is the stratum lacunosum. In CA1, this stratum contains synaptic connections from the Schaffer collaterals and the entorhinal cortex. The stratum radiatum contains apical dendrites and forms synaptic connections with the Schaffer collaterals. The stratum lacunosum-moleculare is divided into two sub-strata. The stratum moleculare also houses apical dendrites that extend from the pyramidal layer and, in CA3, form synaptic connections with the entorhinal perforant pathway.

The dentate gyrus is made of two major 'blades' that form its characteristic 'V' shape: the supra- and infra-pyramidal blades. The neurons' dendritic trees in the supra-pyramidal blade generally have a greater length, greater transverse spread, and a higher count of dendritic segments as compared to the infra-pyramidal blade counterparts (Claiborne et al. 1990). Within each blade, a different set of strata can be identified. The main excitatory neurons in the dentate are the granule cells and measure approximately 10- to 18- $\mu\text{m}$  in diameter and innervate the CA3 subfield among the cells in the hilus (discussed below).

The stratum granulosum, or the granule cell layer, is the layer where these cells reside and it contains roughly  $0.89 \times 10^6$  granule cells in the mature rat (Bayer 1982).

New granule cells are continuously added to the subgranular zone throughout life and these new neurons are synaptically and functionally integrated into networks over several weeks (Deshpande et al., 2013; Snyder, Glover, Sanzone, Kamhi, & Cameron, 2009; Toni et al., 2008; Vivar et al., 2012). The dendrites of mature granule cells create a mosaic of first-, second-, and third-order branching that extends superficially. Here, the stratum moleculare receives these dendrites and is divided into an inner and outer portion. When newborn neurons start to project dendrites into this area, interneurons that release  $\gamma$ -amino butyric acid (GABA; GABAergic interneurons) form synaptic connections early after the neurons' proliferation (within two weeks) serving as the new neurons' first neurotransmitter experience, which is excitatory and recapitulates embryonic-derived granule cell maturation (Deshpande et al. 2013, Espósito et al. 2005). The outer stratum moleculare houses the distal ends of the dendrites and entorhinal axonal connections, near the hippocampal fissure, whereas the inner section primarily receives commissural afferents. Inferior to the stratum granulosum is the stratum multiforme, usually referred to as the polymorphic cell layer, or simply the hilus. This region is where the unmyelinated mossy fibre projections pass through *en route* to CA3. Each granule

mossy fibre communicates with up to 15 CA3 pyramidal neurons and approximately 12 hilar cells (Acsády et al. 1998).

The hilus also contains a number of interneurons and hilar cells. Compared to roughly 0.5 to 1.0 x 10<sup>6</sup> granule cells, there is only a small fraction of hilar cells, approximately 30,000 in total. These cells likely have an important function in modulating mature and young granule cell activity in addition to CA3 pyramidal neurons, because they receive a large amount of mossy fibre terminals from the granule cells and communicate extensively with this region's interneuron population (Acsády et al. 1998, Jinde et al. 2013). In addition, the first connections that newborn neurons receive are from this layer and are GABAergic interneurons, at approximately 10 days post-proliferation (Deshpande et al. 2013, Jinde et al. 2013, Vivar et al. 2012)

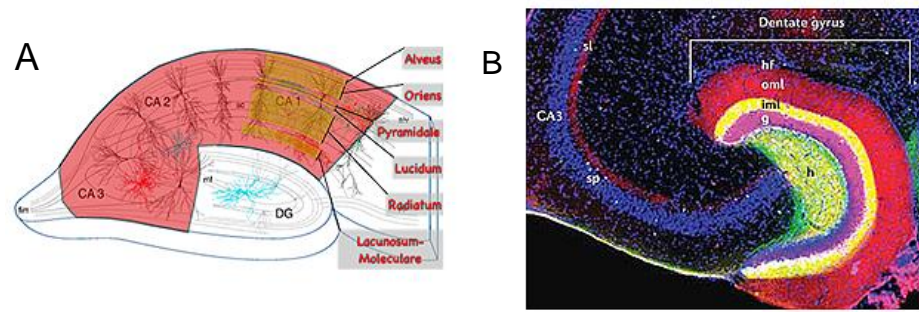


Figure 1.1 Sections through the hippocampus showing the laminar organisation within the CA subfields shaded in red (A) and the dentate gyrus (B); oml (outer molecular layer); h (hilus); iml (inner molecular layer); sp (pyramidal layer); g (granule cell layer); hf (hippocampal fissure); sl (stratum lucidum). Left: originally from <http://neuralnetoff.umn.edu>. Right: adapted from Förster, Zhao, & Frotscher (2006) with permission.

## Regional Connectivity

In terms of extrahippocampal connectivity, the major excitatory input to the dentate comes from the adjacent entorhinal cortex (Figure 1.2), which receives highly processed sensory data from other cortical areas. There appears to be a strict topology of the entorhinal-hippocampal connectivity in the longitudinal and transverse planes. By using different retrograde tracers injected in to varying levels of the dentate along the septo-temporal axis, it is possible to visualise the origins of these projections. Injections into the septal dentate produced labelling in the lateral and caudo-medial portions of the entorhinal. Injections into the mid-septotemporal level down to the most temporal aspect of the

dentate labelled cells in the medial portion of the lateral entorhinal cortex, along with rostral sections of the medial entorhinal cortex (Dolorfo & Amaral 1998). Transversely, those fibres projecting to the dentate and CA3 originate in entorhinal layer II, whereas those projecting to the CA1 and subiculum originate in layer III (Lavenex & Amaral 2000). By using a retrograde tracer-mediated rabies virus, direct connections to newborn neurons within the dentate have been found to come from the perirhinal/lateral entorhinal cortical layers II/III (Vivar et al. 2012). Those fibres projecting to the CA1 and subiculum can originate from the perirhinal and postrhinal cortices, respectively. In addition, with respect to transverse topology, fibres from layer III of the medial entorhinal connect to the portion of the CA1 closest to CA3, and those from the layer III of the lateral section project to CA1 regions closer to the subiculum, thereby connecting each region with its closest neuroanatomical neighbour (Witter et al. 2000).

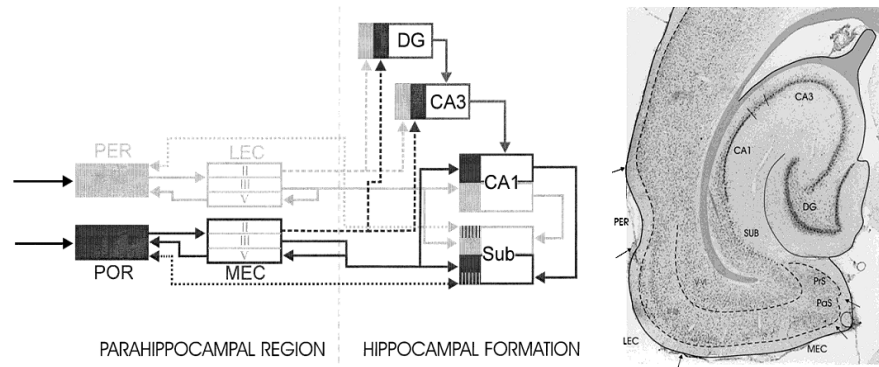


Figure 1.2 Schematic of adjacent cortical connectivity into the hippocampal formation. Left: Both MEC and LEC layer II largely project to the dentate gyrus and CA3 region, while layer III connects with the CA1 and subiculum, both in a topographic manner (see text for details). Grey/black distinctions also represent a level of topographic organisation. Right: Horizontal section through the rat brain, which is used to illustrate the proximity of neuroanatomical locations and their topography pattern of connectivity with those neighbouring regions. CA1-3 (fields of Ammon's horn); DG (dentate gyrus); LEC (lateral entorhinal cortex); MEC (medial entorhinal cortex); PaS (parasubiculum); PER (perirhinal cortex); PrS (presubiculum); Sub (subiculum). Adapted with permission from Witter et al. (2000).

Subcortical projections into the hippocampus are numerous. For example, the medial septal nucleus and diagonal band of Broca provide cholinergic, GABAergic, and glutamatergic modulation into the hippocampus. Adult newborn neurons within

the dentate also receive direct cholinergic projections from the medial septum (Deshpande et al. 2013, Vivar et al. 2012). Inputs into the hippocampus affect granule cells, pyramidal cells, and interneurons, and thus influence local rhythmicity, via projections from the fimbria-fornix, amygdaloid complex, and supracallosal stria (Amaral & Kurz 1985, Freund 1989, Huh et al. 2010). In addition, connectivity between the paraventricular nucleus and the ventral CA1/subiculum, via glutamatergic and GABAergic connectivity, regulates neuro-endocrine hormonal production and information into the dentate serves as part of the feedback mechanism in this stress-regulation process (Canteras & Swanson, 1992; Cullinan, Herman, & Watson, 1993; see for review Jankord & Herman, 2008). Similarly, one study has found that newborn neurons are also monosynaptically innervated by the subiculum (Deshpande et al. 2013), and this connection may be important in this neuro-endocrine feedback. Serotonergic projections from the median raphe nucleus project to the hippocampus, particularly the hilus of the dentate and stratum moleculare-lacunosum and radiatum of CA subfields largely via the fimbria-fornix pathway (Azmitia & Segal 1978, Vertes et al. 1999). Further subcortical modulation comes from the locus coeruleus and subcoeruleus, a pontine nucleus, and the ventral tegmental area, which provides noradrenergic and dopaminergic inputs, respectively, to the dentate. It is likely that all these signals influence newborn neurons but it is not clear how specific these

inputs are in relation to direct or indirect connectivity, and the specific functional consequences they have on these new neurons.

## 1.3 Memory and the Hippocampus

The hippocampus is most widely known for the role it plays in memory; however, there is debate on what type of memory. Two large bodies of literature dominate this field and the two are somewhat separate. One view is that the hippocampus deals with the mapping of space, or spatial memory. The second view is that the hippocampus is critically involved in episodic memories and the relational interaction between memories, such as experiences that happened in one's life. Here, these two lines of literature will be briefly reviewed.

### **Spatial Memory**

In the spatial memory, or the cognitive mapping, theory, the hippocampus is primarily viewed as mapping out the animal's space and processing spatial information that aids in navigation throughout that space. Tolman described rats as having a cognitive map in 1948. He, as a self-professed 'field theorist', believed that a mental representation of the environment is encoded in the rodent's brain while it is passively traversing a maze, rather than relying on simple stimulus-response

associations. This was largely established by the use of rewarded and non-rewarded trials to serve as motivators. In Tolman's experiments, rats were allowed to explore passively a complex maze. Some rats received a food reward at the end of the maze starting on the first trial and these rats showed quick learning rates. Other rats received food in their homecage for the first two days and explored the maze during those two days; they later received rewards at the end of the maze on the third and subsequent days. These rats, too, showed quick learning when the food reward was introduced at the end of the maze. The critical group of rats received food in their homecage for six days and passively explored the maze for those six days and showed little learning of the maze; on the seventh day, food was introduced at the end of the maze and these rats showed a very steep learning curve to complete the maze. Because of this steep learning curve, it was suggested that the rats were incidentally encoding the maze routes during the initial six passive exploration days but had no motivation to run it very fast and complete the maze. Upon the introduction of food to motivate the rats to run the maze, these rats displayed a remarkable amount of incidental learning (Tolman 1948). This suggested to Tolman that a mental representation of the maze was being constructed somewhere in the brain and that motivators were important to 'draw out' the behaviour that reflected learning about the environment.

Following from this, O'Keefe & Dostrovsky (1971) successfully recorded cells in CA1 of the hippocampus, called place cells, in the rodent brain that responded selectively to aspects of the environment (specific 'place fields') and slowed down their firing in other parts of the environment. These cells seemed to be the neural correlates of Tolman's cognitive map theory and could provide or underlie the mapping of the environment. The theory of spatial memory is based on locale, or allocentric, information that is used to guide behaviour throughout the environment and taxon, or egocentric (animal-centred), information that can also be used to guide behaviour throughout the environment. The discovery of these place cells has spawned many studies that have probed their workings and the underlying working of other sets of 'spatial cells', e.g. head-direction cells; boundary cells (see Moser et al. 2008). Of importance, many years later, the discovery of grid cells in the entorhinal cortex (Hafting et al. 2005) helped solidify the foundation onto which the mental representations of space might be formed and laid down.

The observation of place cells in the hippocampus is consistent with the idea that the hippocampus represents space. The functional importance of the hippocampus for performance in spatial memory tasks was then demonstrated using lesioning techniques in combination with clever maze designs that probed this mnemonic process. Morris and colleagues devised a watermaze task where the rodent must use the environmental (i.e.

allocentric) cues to navigate to a hidden platform to escape the water. Using this task, it has been shown that hippocampal lesions impair this type of spatial memory (Morris 1984). In spatial reference memory, the platform is in a consistent location in the maze on every trial and the rodent starts at various starting points to navigate to the platform. Animals are trained across several days, and they gradually learn the location of the platform. Hippocampal lesions using aspiration (Morris et al. 1982) and later a fibre-sparing neurotoxin (ibotenic acid) (Morris et al. 1990) impaired this spatial memory task.

Another similar task that is reported to test the same memory is the radial arm maze. In the radial arm maze, separate arms radiate outward from a central zone. In this task, the animal must learn, based on allocentric cues, which arms are rewarded and which are not (in one version of this maze). Errors into never-rewarded arms are considered reference memory errors. Revisiting of the arms were considered working memory errors, because the animal needs to keep in mind what arms it already visited in order to efficiently receive all the rewards (Olton et al. 1977). Olton and colleagues further found that hippocampal lesions did not impair the reference memory portion of the task but only the working memory (or the revisiting of arms) aspects of the task (Olton et al. 1979); however, explicit hippocampal lesions were not tested, only the extrinsic connectivity with this region.

Furthermore, this task can be used to delineate place vs. cue memory by using inserts to serve as intramaze cues. Rats with hippocampal lesions appear to be impaired at the learning of this complex radial maze task. Lesions made after learning, resulting in studying the retention of the task, resulted in hippocampal lesions producing making both reference and working memory errors in the place version of the task but only temporarily but, importantly not on the arms where intramaze cues were used (Jarrard 1983, 1993), suggesting the hippocampus is not needed for cue-response strategy. However, rats can adopt varying strategies to solve these tasks that can compensate for lost hippocampal tissue (see Hodges 1996 for comparison of mazes).

### **Episodic Memory**

The hippocampus also has a long history of being involved more broadly with episodic memories. Episodic memories are classified under declarative memories, and these memories often are self-referential and experience based (e.g. 'I remember when...') that are generally more vivid than semantic memories (i.e. fact-based memory without contextual information; e.g. 'The capital of England is London'). Because episodic memories can be vivid in detail, they can often elicit emotional reactions.

In one of the most famous cases to shed light onto the neuroanatomical basis of episodic memory, Scoville (1954), as

cited by Scoville and Milner (1957), performed a “radical” and “experimental” surgery to try and ameliorate psychotic symptoms and extreme seizures in two separate individuals. Bilateral resections of the medial temporal lobe were performed on both patients, and some of resected tissue included the hippocampus. Scoville, almost immediately, reported a profound impairment in memory abilities in the patients. A similar surgery was performed on patient HM in an attempt to decrease major, intractable seizures for which a central focus could not be found (Scoville & Milner 1957). What made this case famous was the extensive neuropsychological assessments performed after the surgery for the next 50 years, and this has allowed us to peer inside the working brain at how memory operates and what happens when it goes wrong. The memory effects, as in the previous patients, were apparent directly after the surgery and, as described in the paper, HM had no recollection of the specific episode of talking to Dr. Karl Pribram and denied doing such a thing. Further testing revealed nearly complete anterograde and at least partial retrograde amnesia (Corkin 2002, Scoville & Milner 1957, Steinworth et al. 2005). Despite memory impairments, HM exhibited quite normal intelligence, intact language abilities, and could recall immediate memories (Corkin 2002).

Upon HM’s death, extensive post-mortem histological examinations and 3D reconstructions confirmed that significant portions of the parahippocampal gyrus, including nearly all the

entorhinal cortex and the amygdala, were removed along with damage that included the hippocampus, albeit to a lesser extent than originally thought (Annese et al. 2014). HM taught us that there are multiple memory systems and memory is not a monolithic psychological process (Corkin 2002). For example, HM had severe memory impairments about experiences in his life but not about facts that were learnt (i.e. episodic but not semantic memory, respectively). HM could learn to perform difficult tasks (e.g. mirror-drawing) and obtain high proficiency in these tasks but with no recollection of learning the task/skill itself. The ability to learn the skill required for the task is a form of memory that is called procedural or habit memory, which is independent of the hippocampus.

The extensive neuropsychological testing performed on patient HM led investigators to further explore the underlying neural mechanisms and regions involved in, for example, the recognition memory deficit that HM displayed. They used animal models to do this, because animals are more amenable to invasive experimentation. Researchers working with non-human primates and rodents were eventually successful in mirroring a visual object recognition deficit, similar to HM's, but the path to success involved multiple experiments.

Early studies produced conflicting results with no immediate answer present, suggesting that resection of the hippocampus alone was sufficient to induce an amnesia-like

syndrome in monkeys. Further studies that used fibre-sparing injections of excitotoxins into the hippocampus failed to find this result and selective lesioning of other medial temporal lobe structures around the hippocampus, such as the amygdala, did not readily recreate this amnesia-like syndrome (Murray & Mishkin 1998, Zola-Morgan et al. 1989a). However, lesions to the adjacent rhinal cortices, tissue that was resected when gaining access to the hippocampus, mirrored this amnesia syndrome (Meunier et al. 1993, Zola-Morgan et al. 1989b, 1993). Largely, the results of recognition memory studies, like HM remembering a visit with Dr. Pribram, in non-human primates found that a delayed non-match to sample task could be achieved in the absence of the hippocampus. However, the extent of hippocampal contribution is debated (Meunier et al. 1993, Murray & Mishkin 1998, Squire et al. 2004, Zola-Morgan et al. 1994) and things such as temporal delays and the depth (familiarity vs. recollection) of the original encoding likely dictate the hippocampus's involvement (Squire et al. 2007).

Nevertheless, animal models have allowed us to probe further into the underlying neural correlates of memory than we could ever do in humans. The hippocampus does appear to be necessary in other memory tasks when configural processing (i.e. the combining of the joint occurrence of separate elements) is needed and when relations among associations form an episodic memory.

Much of the impetus supporting a spatial account of the hippocampus has come from the place cells (described in the section above). The firing from these cells, however, also seems to support an episodic-like account of the hippocampus. In one study, rats were trained to dig for rewards based on different odours and if they matched or did not match a sample odour. Recording from hippocampal cells was performed throughout the task in dorsal CA1 and CA3. The task was designed in such a way as to control for spatial arrangement, contingencies of the odours, and several other factors were counterbalanced. It was found that roughly 40% of cells fired in response to non-spatial parameters and instead to the odours and trial types but not to the specific location of the odour. However, 31% did display firing to spatial parameters in addition to other non-spatial firing correlates (Wood et al. 1999) and similarly to left- or right-hand turns without any clear spatial component (Wood et al. 2000).

In addition to non-spatial firing of these 'place cells', they also have a role in 'preplay' of firing sequences to novel environments, along with showing 'replay' of sequences during rest periods along with modulating of these sequences based on starting points relative to goals (Dragoi & Tonegawa 2011, Foster & Wilson 2006). Also, dopaminergic neurons within the ventral tegmental area fire in response to novel environments, and this dopaminergic activity 'tagged' memories for subsequent 'replay'

and stabilisation of the CA1 memory traces (McNamara et al. 2014).

In sum, the hippocampus receives highly processed sensory information from the surrounding area (from entorhinal and perirhinal cortices) and encodes particular features about the surrounding area. These features include locations in space, the proposed trajectory of the animal based on goal-related information, and specific sequences of learnt associations. However, instead of encoding specific places within the environment like on a map, the hippocampus may be providing a mechanism that links together all of these elements to form a true *cognitive* map that is not only spatial in origin (Dudchenko & Wood 2015, Eichenbaum et al. 1999, Wood et al. 2000). In addition, these results further suggest a possible role for the hippocampus in predicting goal-related aspects, such as paths to a reward and the recombination of episodic memories in a flexible way (see Buckner 2010, Mullally & Maguire 2013)

## **Relation and Configural Processing**

The development of episodic memories relies on the linking of memories based on some common property between the memories. Eichenbaum and colleagues (1999) proposed that the hippocampus is necessary to link together sequences of places and events through their shared elements. This then gives rise to

episodic-like memories that can be used to infer outcomes, and termed this *relational processing theory* (see Eichenbaum et al. 1999). For example, rodents can infer relationships between overlapping odour associations that have previously been learnt (e.g. A-B and B-C). On a subsequent probe test, the rodents are given one sample and 'asked' to infer to the other relationship (e.g. A-? and given choices between C and Z, with the correct answer being C because A-B-C). Hippocampal-lesioned rats could learn the task but were impaired at inferring associations in both forward and reverse order, whereas sham-lesioned rats did not show any problems (Bunsey & Eichenbaum 1996). In a similar associative paradigm using houses and faces instead of odours, Preston and colleagues (2004) showed selective hippocampal activation upon asking participants to infer associations as was done in the previous rat study. These results point to an important role for the hippocampus in predicting, or inferring, relationships between sequential experiences or episodes.

Additional evidence for the hippocampus's role in memory comes from studies examining configural associative processing. Numerous behavioural studies using rodents have revealed a particularly important place for the hippocampus in the retrieval of types of associative memories. In the *configural association theory* (Rudy & Sutherland 1995, Sutherland & Rudy 1989) that was based on rodent studies, associations between two or more stimuli, via conjunctive encoding based on the co-occurrence of

the stimuli, are learnt and comprises of two different mechanisms. The first is a hippocampal-dependent form that deals with configural stimuli and the rapid formation of these conjunctive representations. The second is independent of the hippocampus and deals with associations between 'elemental' stimuli.

The configural association theory was first demonstrated using a negative patterning task. Negative patterning is where presentations of two different cues separately or individually yield reinforcement but not when the two cue are presented simultaneously (i.e. A+, B+ but AB-). Using this paradigm, animals cannot use individual associative strengths to solve the compound (configural) stimulus situations, creating a non-linear discrimination. Lesions to the hippocampus first appeared to disrupt this type of encoding but other experiments using non-linear discriminations (e.g. Davidson et al. 1993, Honey et al. 1998) did not find this effect. The theory was then reformulated to take into account these negative results. The new theory suggested that configural representations can be achieved by other (e.g. in the neocortex) but require much more time and many more trials. The hippocampus, on the other hand, allows for the rapid formation of these configural representations (see O'Reilly & Rudy 2001).

Other indirect evidence that may be interpreted to support the configural theory (but could also be interpreted as supporting the relational theory as well) has come from contextual fear

conditioning in which the configural encoding of the environment must occur to form a coherent representation of the 'context' itself. In this task, an animal is placed in an environment where they are given an aversive experience (e.g. random delivery of footshocks). Later, they are placed back into the fear-conditioned environment or in the novel one and the difference in freezing levels are compared. Rodents in the fear-conditioned environment should freeze significantly more than in the novel environment. The configural theory suggests that animals form a 'context' representation of the environment based on conjunctive encoding, or co-occurrence of polymodal associations. Using this method, it has been shown that the hippocampus is needed to form a contextual representation of the environment through learnt associations (discussed in more detail below, c.f. Gewirtz, McNish, & Davis, 2000; Maren, Anagnostaras, & Fanselow, 1998; McNish, Gewirtz, & Davis, 1997). Although other explanations based on spatial and contextual labelling processing have been proposed (Hirsh 1974, O'Keefe & Nadel 1978).

Hirsh (1974) suggested that performance was dictated by the past and current reinforcement history (including motivational states, e.g. hunger) and may not reflect direct context encoding of some representation or map, *per se*, but rather the reinforcement history experienced within that particular environment. Rodents with hippocampal lesions, according to Hirsch, are unable to form context labels to varying reinforcer contingencies (e.g. shocks in

fear conditioning, or internal states of the animal in other discriminative tasks) and thus fail at discrimination tasks. However, this does not seem to be true in all discriminative learning tasks. For example, hippocampal lesioned rats did not differ from control rats in their ability to select a correct object based on hunger, thirst, or visuo-spatial cues in an object discrimination task (Deacon et al. 2001) like Hirsch would predict.

## 1.4 Emotion and the Hippocampus

It is largely agreed that the hippocampus subserves aspects of memory. There are, of course, discussions on whether this is primarily spatial or episodic at its core; its role in processing, storage, (re)consolidation and/or retrieval of memory; and its role in long-term, short-term, and/or working memory. Earlier thinking about the role of the hippocampus, however, did not always focus on memory as its core component, but rather its role in aspects of emotion, motivation, and exploration (Altman et al. 1973, Amsel 1993, Douglas 1967, Gray & McNaughton 2000, Woodruff et al. 1975).

One of the oldest systematic theories about the hippocampus came from the pioneering works of Papez in 1937. Papez thought that the hippocampus itself was responsible for the control and expression of emotions. He argued that, based on patients' behavioural and neuropathological observations from

being inflicted with the rabies virus, that emotions were generated in the hippocampus and then relayed throughout a circuit containing the mammillary bodies, anterior thalamic nuclei, anterior cingulate gyrus, parahippocampal gyrus, and back to the hippocampus (Papez 1937). Despite the anatomical connectivity not being in dispute, the amygdala, instead of the hippocampus, is now thought to be the central hub for emotions (Dalglish 2004). Nevertheless, the hippocampus continues to play a prominent role in emotion and its regulation (Bannerman et al. 2014, Barkus et al. 2010).

## **Endocrine Responses**

The hippocampus has strong interplay with the stress response system in general and the hypothalamic paraventricular nucleus in particular, which all regulate the future production of stress hormones. The dysregulation of the stress response and glucocorticoid production can have significant impacts of mental health. Broadly, the initiation of the stress response occurs in the paraventricular nucleus of the hypothalamus by the biosynthesis of corticotrophin releasing factor (CRF). Travelling through the median eminence, CRF is released from nerve terminals in the anterior pituitary gland. Here, CRF stimulates the production and secretion of adrenocorticotropin-releasing hormone (ACTH). ACTH is then transported in the bloodstream and stimulates

corticosterone production (in rodents) in the adrenal glands -- specifically the cortex region of these glands. Corticosterone, among others released, has widespread effects all throughout the body (McEwen 1998). One target of corticosterone in the brain is the hippocampus, which contains a large density of glucocorticoid receptors (McEwen et al. 1968). Binding to these receptors provides a negative feedback loop which dampens hypothalamic-pituitary-adrenal (HPA) axis activity (Sapolsky et al. 1984), further evidenced by lesioning and stimulation studies. Lesioning the hippocampus in rodents produces an increase in circulating glucocorticoid hormone levels, whereas stimulating this region produces a decrease (Knigge 1961, Knigge & Hays 1963, Slusher & Hyde 1961). Similar effects have been found in humans through stimulation of the hippocampus (Mandell et al. 1963, Rubin et al. 1966). The connectivity has only recently been expounded.

The ventral CA1/subiculum is disynaptically connected to the paraventricular nucleus. These connections are mainly glutamatergic (excitatory) and synapse onto GABAergic (inhibitory) interneurons in the bed nucleus of the stria terminalis, preoptic area, and the basolateral hypothalamus, which then inhibit the paraventricular nucleus. The efferents of the CA1/subiculum can travel through two different pathways. The first is through the fimbria-fornix that then projects to the hypothalamic areas and basal forebrain regions. The second

travels through the amygdala and connects to the bed nucleus of the stria terminalis (Herman et al. 1995, 2005).

It appears that the hippocampus is more sensitive to psychogenic and anticipatory stressors (those in which there is a perceived chance of danger, e.g. anticipatory footshock, predator odour exposure). This is opposed to threats that are immediately apparent or systemic stressors (e.g. ether inhalation, hypoxia), and psychogenic stressors have significant effects on glucocorticoid recovery rates (Jankord & Herman 2008, Koolhaas et al. 2011). This suggests that the hippocampus may be important in assessing or evaluating threatening situations rather than responding directly to the threat itself.

Several studies have looked for parallels between glucocorticoid levels, anxiety states and the hippocampus. For example, rats that are genetically bred for high-anxiety-like behaviours show a decrease in exploration of open arms on the elevated-plus maze (EPM) but show similar glucocorticoid levels as compared to low-anxiety rats. However, when these highly anxious rats are forced into an open arm and sit there, their glucocorticoid levels suddenly rise to higher levels as compared to their low-anxiety counterparts (Landgraf et al. 1999). All together, these results suggest that voluntary behaviour, such as exploration, may be important in modulating or maintaining glucocorticoid levels, which normally do not differ between these two groups of rats. Likewise, ventral CA1/subicular lesions

increase the amount of time spent in open arms on the EPM and also raise glucocorticoid responses (Mueller et al. 2004), again suggesting that the hippocampus is important in setting the tolerance/threshold levels of stress, and this affects willingness to engage in anxiogenic activities or go into these types of situations.

In rodents, chronic glucocorticoid production leads to remodelling of CA3 dendrites, inhibition of adult neurogenesis, and an overall reduction in hippocampal volume (McEwen & Magarinos 2001). The link between behaviour and stress hormone levels is not fully understood. Stress-induced changes in spatial memory and hippocampal-structure do not always vary together. Inhibiting stress-induced CA3 dendritic shrinkage (with tianeptine) also impairs spatial memory in the watermaze but does not inhibit the facilitation of contextual fear conditioning. Additionally, chronic injections of corticosterone, thereby mimicking chronic stress, affect CA3 dendrites but do not consistently impair spatial memory (Conrad 2006).

The above results suggest that the stress response and their stress-related behaviours are not secondary to learning or memory effects because the stress effects can be present on the first exposure to the experience.

## **Behavioural Inhibition**

Rats display a flexible array of defensive behaviours that are used to collect information, hide from danger, or escape from potential threatening situations. The range of behaviours is dependent on the magnitude of the danger (i.e. the nature of the threat or danger and also whether escape is possible) (Blanchard et al. 2001b, Gray & McNaughton 2000, McNaughton & Corr 2004). The initial response when a (potential) threat is encountered is to inhibit ongoing behaviours, the process of behavioural inhibition. The lack of activity that signifies risk-assessment behaviour, such as only passively scanning the environment with the head, can be part of this behavioural inhibition. This can be used to collect more information about potential threat situations, but other risk-assessment behaviours, such as rearing on the hind limbs, also play an important role in threat detection and assessment (Blanchard & Blanchard 1989, Gray & McNaughton 2000).

After information has been gathered, these risk assessment behaviours are modified to reflect the specific situation. In a closed space where no escape is possible, adult rats will show complete immobility (freezing) when confronted by a cat; however, hippocampal-lesioned rats display less immobility. When an escape is possible, these hippocampal-lesioned rats show enhanced avoidance behaviours, as compared to controls, and when these hippocampal-lesioned rats are exposed to cat

odour, they show less freezing and risk assessment behaviours (Blanchard & Blanchard 1972, Pentkowski et al. 2006). These observations are consistent with a role of the hippocampus in mediating situation-specific responses to potentially threatening situations unless there is clear escape possible, in which case other behaviours along the defence hierarchy take control (Gray & McNaughton 2000).

Similar behavioural inhibition can be seen in preweanling rat pups. When pups are exposed to an unfamiliar adult male rat, their natural behaviour is to freeze (Takahashi, 1992). Injections of glucocorticoids into the dorsal dentate facilitates this behavioural inhibition response and, interestingly, also the maturation of the granule cells (Gould, Woolley, & McEwen, 1991; Takahashi, 1995). On the other hand, lesioning the dorsal dentate gyrus or giving drugs that disrupt granule cell maturation inhibit this behavioural inhibition (Gould & Cameron, 1997; Takahashi, 1992). This is consistent with (i) a hippocampal role in mediating behavioural inhibition in response to a threat (e.g. the adult male rat) in both young and older rats; (ii) demonstrates an effect of stress hormones on the development of this behaviour; and (iii) shows a role for the maturation of the developing dorsal dentate gyrus in this behaviour.

Behavioural inhibition is also observed in learning situations, in which incorrect responding must be suppressed (Bannerman et al. 2012). Lesions of the ventral hippocampus, or

disconnection from the frontal cortex, produces impulsive responding, such that rats press a touchscreen prematurely, as well as perseverate on this response by repeatedly touching the screen even though only the first response is rewarded (Abela & Chudasama 2013, Chudasama et al. 2012). The initial correct response demonstrates that the animal has been able to form an association but appears less able to withhold responses at times when responding is not in their best interest. This suggests that the hippocampus has a role in behavioural inhibition of reward-motivated tasks as well as aversive tasks that may be separate from its role in associative learning and memory.

In sum, the hippocampus increases inhibition of prepotent but inappropriate behaviours in both appetitive and aversive situations. It is important to note that inhibition may result in the reduction of activity levels in certain situations but also may inhibit specific behaviours to allow alternative and potentially more adaptive behaviours to emerge in other situations (e.g. perseverative behaviour at the expense of exploration).

## **Anxiety and Fear**

The topics in the previous sections play an integral part in the assessment of danger, all of which feed into stress-related conditions, particularly clinical or pathological anxiety and depression. One role that anxiety or fear serve is to allow animals

to learn from the experience and adapt future behaviour in similar situations. Clinical anxiety or fear, however, is not adaptive and is a hindrance to normal day-to-day functioning.

In anxiety, attentional shifting after behavioural inhibition and the neuroendocrine stress response are all involved in the appraisal and reactivity toward a potentially dangerous situation. These behaviours aid in the animal's ability to deploy appropriate defensive behaviours in order to adapt to the situation. Early studies using activity levels as a proxy for anxiety levels found that rodents with hippocampal lesions showed hyperlocomotion in novel environments and increased eating if food deprived (Jarrard 1968), consistent with decreased anxiety levels, although one cannot rule out an effect of decreased habituation. Selective lesions to the ventral hippocampus also produced behaviours consistent with decreased anxiety, such as increased exploration of novel environments and faster eating times in a neophagia task (Bannerman et al. 2002, 2003; Kjelstrup et al. 2002, McHugh et al. 2004). The decreased anxiety-like behaviours displayed by these lesioned animals may be explained by a shift in the approach-avoidance (danger-safety) conflict, where approach behaviours (explore, eat novel food) are favoured over avoidance ones (stay in corner). In other words, there is a failure to inhibit these approach behaviours.

Similar effects have been seen in mice lacking functional *N*-methyl-D-aspartate (NMDA) receptors in the dentate gyrus and

in rats with ventral dentate gyrus lesions (Barkus et al. 2010, Niewoehner et al. 2007, Weeden et al. 2014). Additionally, rats with ventral hippocampal lesions show decreased immobility in response to cat odour, an innate anxiety-provoking stimulus, and in both delayed cue or contextual fear conditioning (Pentkowski et al. 2006, Richmond et al. 1999). Importantly, these results do not reflect a deficit in freezing, *per se*, because they can freeze normally to the presence of an actual cat. Additionally, they show other changes in behaviour -- such as increase sniffing, increased rearing, decreased crouching -- in response to cat odour that are indicative of increased anxiety (Pentkowski et al. 2006). These changes in innate, unconditioned anxiety-like behaviours, like those to cat odour, are unlikely to reflect a deficit in any specific memory for a particular past experience and underscores a role for the hippocampus in emotionality.

Recently, targeted inactivation or activation of particular cluster(s) of neurons or ventral hippocampal inputs through optogenetic technology also points to a role for the hippocampus in anxiety. Inhibition of basolateral amygdalar inputs to the ventral hippocampus increased open-arm and centre exploration on the EPM and OF, respectively (Felix-Ortiz et al. 2013). In the same study, activation of these inputs has the opposite effect: activation decreased open arm and centre exploration in the EPM and OF, respectively, and increased the latency to eat a food reward in novelty-suppressed feeding task. Additionally, stimulation of the

ventral dentate gyrus produced more open field and elevated-plus maze exploration, mirroring lesion studies (Kheirbek et al. 2013).

This effect may be because massive granule cell stimulation innervates more inhibitory interneurons (almost 10x as many) than the principal neurons and thus can suppress overall CA3 collateral discharge to CA1 and back-projection to the dentate (Acsády et al. 1998).

Both fear and anxiety can elicit similar emotional responses. There is, however, a conceptual and neural distinction between the two states (McHugh et al. 2004). Fear is typically generated when an adverse event is imminent, whereas anxiety is generated when the potential for threat is present (Bannerman et al. 2004, McNaughton & Corr 2004). According to this definition, through studies using lesioning or inactivation of these different brain regions, the amygdala processes fear while the ventral hippocampus processes anxiety (Bannerman et al. 2004, McHugh et al. 2004). These two states can elicit similar behaviours but can often be distinct from one another. In the case of fear, animals will almost always try to rapidly escape the threatening situation; if escape is not possible, they will attempt to protect themselves with other behaviours (e.g. attacking or freezing). In the case of anxiety, animals will display risk-assessment behaviours that are often characterised as being anxiety-like, in an attempt to gather more information regarding the potential threat stimulus or

situation (Blanchard et al. 2011, Lever et al. 2006, McHugh et al. 2004).

In rodents without adult neurogenesis, unlike those with large hippocampal lesions, there is typically no alteration in baseline anxiety-like behaviours. Classic tests of anxiety, such as the open field and the elevated-plus maze, have been used to study anxiety-like behaviour under baseline or innate conditions and have generally found no effect (see Groves et al. 2013, c.f. Revest et al. 2009). Similarly, restraint-induced stress did not elicit genotypic changes between those with and without adult neurogenesis on the elevated-plus maze despite the stress affecting other unconditioned novelty-suppressed feeding behaviour (Snyder et al. 2011). One recent study, however, has shown that prior history of fear conditioning can affect anxiety-like behaviour on the open field task but this effect is not present without prior fear conditioning (Seo et al. 2015). This issue is further discussed in more depth below (Section 1.5, Adult Neurogenesis)

## 1.5 Adult Neurogenesis

The fact that new neurons continue to be born in adulthood is now well established; however, the early research showing adult-born neurons was met with scepticism (see Altman 2011 for a thorough historical view). The first evidence of newly dividing

cells in young-adult rats and cats came from injections of a radioactive nucleoside (tritiated thymidine; thymidine-<sup>3</sup>H) (Altman 1963), who reported newly divided glia and neurons within the neocortex and granule layer of the dentate gyrus based on morphological investigation. Tritiated thymidine incorporates into new DNA synthesis during the S-phase of mitotic cell division into the daughter cells. Using autoradiography to detect these radioactive particles, it is possible to visualise where these particles are located within the brain. This was then followed up with more extensive studies using larger sample sizes and also used a longitudinal approach to examine how long this postnatal neurogenesis continued (Altman & Das 1965, 1966). These initial studies were very controversial, mainly because the central dogma at the time was that no new neurons were born soon after birth. A few years later, using tritiated thymidine, it was confirmed again, that in 3-month old rats, adult neurogenesis persisted. Using electron scanning microscopy that allows for detailed morphological investigation, it was found that new neurons were born in the granule cell layer and the olfactory bulb (Kaplan & Hinds 1977). Shortly thereafter, it was found that adult female canaries also produced new neurons in the ventricular zone overlying the hyperstriatum ventrale which correlated when they learnt new songs (Goldman & Nottebohm 1983). The use of tritiated thymidine, however, was found to be problematic because it could only be visualised from above, leaving open the possibility

that the cells labelled were 'satellite' glial cells laying over already existing neurons.

Several years later, after advances in immunohistochemistry and confocal microscopy, this basic principle of using thymidine to detect new cell division in the adult brain still served this purpose; however, Bromodeoxyuridine (BrdU) is used in place of tritiated thymidine to detect new cell division. BrdU is a thymidine analogue that also incorporates into the S-phase of new DNA synthesis. During the synthesis phase, a bromine atom is added to thymidine's methyl group. Using immunohistochemistry instead of autoradiography, antibodies are used to detect the presence of BrdU. The use of confocal microscopy has added significant advantages in characterising the precise identity of cells and allowing for 3D confirmation that the BrdU is in the cell nuclei. Specifically, when used in conjunction with neuronal markers like NeuN or doublecortin, confocal microscopy allows for co-labelling to be visualised. This means that, one can visualise cells that express BrdU and see if they are also positive for specific neuronal markers (e.g. NeuN, doublecortin), allowing one to confirm their identity.

The first identification of new neurons within higher-order mammals came in the late 1990s. It was found that monkeys produced new neurons in the granule cell layer of the dentate gyrus and the subventricular zone around the lateral ventricle which migrated to the olfactory bulb via the rostral migratory

stream (Gould et al. 1999a, Kornack & Rakic 1999). Soon after, it was reported that new neurons also continued to be generated in the neocortex (prefrontal cortex, inferior temporal cortex, and posterior parietal cortex) whose origin also appeared to be from the subventricular zone (Gould et al. 1999b). This adult neocortical neurogenesis in the primate was met with great scepticism (Rakic 2002), largely focusing around methodological issues in what exactly BrdU is labelling, as it can label damaged and/or repairing DNA as well.

Around the same time as reports about primate adult neurogenesis were first circulated at meetings, another seminal report came out showing adult neurogenesis in the human (Eriksson et al. 1998). In this unique study, cancer patients were given BrdU to label and track proliferating tumour cells. Using additional neuron-specific immunohistochemical markers on the post-mortem brain tissue, it was found that these patients had new neurons being born throughout adulthood in the granule cell layer of the dentate gyrus and subventricular zone.

From here, much research has gone into what exactly these adult-born neurons functionally do, how they contribute to behaviour of the animal, and what regulates their proliferation, survival, maturation, and death (see Gage et al. 2008). In one study that quantified the amount of adult neurogenesis, it was estimated that approximately 9000 newly divided cells are born each day (Cameron & McKay 2001), suggesting that this

continual proliferation is likely an important feature of the dentate gyrus and not a perinatal 'left-over' of cell proliferation. Many physiological and environmental conditions appear to regulate these new neurons. These conditions range from stress and adrenal hormones (Cameron & Gould 1994, Cameron & McKay 1999, Cameron et al. 1993, 1998; Gould et al. 1991a, 1992; Snyder et al. 2009b), environmental enrichment (Kempermann et al. 1997, 2002) and running (Snyder et al. 2009b, van Praag et al. 1999b, 2005). Following on from this, it was later found that selective-serotonin reuptake inhibitors (SSRIs) could also stimulate adult neurogenesis (Santarelli et al. 2003).

The above observations, however, are correlation studies and lack a more causal explanation. Fortunately, with advances in genetics, it is now possible to selectively target and manipulate (i.e. inhibit or promote) these adult-born cells using specific loss-of-function or gain-of-function experimental designs. The following section will describe different methods for ablating adult neurogenesis and the later sections (about the function of these new neurons) will largely include findings that used these methods.

### **Methods for Ablating Adult Neurogenesis**

The functional role(s) of adult-born neurons within the dentate gyrus of the hippocampus remains unclear. Behavioural tasks that increase the survival and maturation of new neurons,

e.g. by exercise (Snyder et al. 2009b, van Praag et al. 1999a), likely reflect their importance in functioning within the dentate. Additionally, experimental manipulations of experiences that affect adult neurogenesis may provide hints at a causal role but parallels in behavioural performance and levels of adult neurogenesis can be spurious (Lazic 2012). In this section, methods for ablating adult neurogenesis are mainly discussed, but methods for enhancing adult neurogenesis are mentioned as well. Later, their functional implications of adult neurogenesis (using these methods) are further discussed.

Today, direct manipulation of adult neurogenesis levels, either by increasing or decreasing the levels in a controlled manner, occurs without the need for experiential modulation (e.g. running, stress) to be used. The first method to directly inhibit the proliferation of adult-born neurons used the chemotherapeutic drug, methylazoxymethanol acetate (MAM) (Shors et al. 2001). The advantage to the use of these pharmacological agents, like MAM, are that these can be used with any strain of rodent and can be administered by systemic injection. These drugs, however, have side-effects that may impact adult neurogenesis, such as immunological or gastrointestinal effects that may also impact state-dependent learning.

The most common, at least in the early years of this field, used ionizing x-irradiation to ablate adult-born neurons by damaging DNA and affecting mitotic cell division, with high doses

preventing almost any recovery of further mitotic division (Bayer & Altman 1975, Gazzara & Altman 1981, Snyder et al. 2005, Winocur et al. 2006). However, whole-body x-irradiation affects many cells all throughout the body (e.g. gastrointestinal cell division) that can have major effects on the health of the animal, and thus can alter behaviour in ways that are independent of the prevention of adult neurogenesis. Specific cranial x-irradiation can be used to limit exposure to the brain, and now even more selectivity to the hippocampal region using cranial shielding techniques can be used and thus sparing subventricular zone/olfactory bulb adult neurogenesis (Santarelli et al. 2003, Tan et al. 2011, Wu & Hen 2014). Several disadvantages with this technique, however, exist. It is very expensive and requires special knowledge and training, lacks fine-scale temporal specificity and (to an extent) spatial specificity, and can have side-effects that are not necessarily related to adult neurogenesis. With all of these techniques, though, each has its advantages and disadvantages, especially potentially affecting neurogenesis outside the two conventional neurogenic regions, including hypothalamic, striatal, or neocortical adult neurogenesis (Dayer et al. 2005, Ernst et al. 2014, Robins et al. 2013).

There are several techniques that take advantage of pharmaco-genetic strategies in order to target progenitor cells (those that give rise to new neurons in the adult brain). The advantage to these models is that they are less susceptible to off-

target effects on the body (although not always) and can be used to target these cells at specific timepoints. One model is the herpes simplex virus thymidine kinase (HSV-TK) transgenic rodent model that targets the cells that express the glial fibrillary acidic protein (GFAP) or nestin expression. This causes the expressing cells to produce this herpes virus and, when the antiviral drug valganciclovir is administered, DNA damage occurs and cell death ensues. Using this method, an approximate knockdown of ~99% occurs and the number of astrocytes in the granule cell layer and subventricular zone are not altered (Garcia et al. 2004, Groves et al. 2013, Saxe et al. 2006, Snyder et al. 2011).

Other models take advantage of Cre recombinase to manipulate gene expression, specifically targeting these progenitor cells via the nestin promoter (Imayoshi et al. 2008). First, crossing transgenic mice in which a tamoxifen-inducible Cre recombinase is expressed under a nestin promoter with mice that express an inducible version of the diphtheria toxin receptor in a Cre-recombinase manner occurs. Then, when these progeny mice grow up into adults, an injection of tamoxifen is given which, in turn, induces diphtheria toxin receptor expression (via Cre recombinase) in the nestin-specific progenitor cells. Subsequent injection of the diphtheria toxin, then, binds to these receptors that are only present on newly divided neurons, or the "tagged" population. This allows for normal maturation of adult-born

neurons into established circuitry during behavioural training. Later, the toxin is injected and these adult-born neurons are killed, allowing one to examine the particular function of these cells toward behaviour (Arruda-Carvalho et al. 2011) and those cells that were possibly involved during initial learning of a task. Another model using Cre technology targets and eliminates the NR2B-containing NMDA receptors of young neurons and disrupts plasticity (long-term potentiation and dendritic branching complexity) (Kheirbek et al. 2012).

Another Cre model targets the elimination of the proapoptotic gene, *Bax*, and therefore prevents cell death. When new neurons are targeted via promoters, and apoptosis of newly generated neurons is prevented, this enhances specifically new, adult-born neuronal proliferation (Sahay et al. 2011). Using Cre models allows for great flexibility and temporal specificity but can have effects independent of mitosis, and the expression of Cre occurs in other brain regions (Sun et al. 2014).

## 1.6 Adult Neurogenesis and Behaviour

During development, most neurons within the brain are added during very specific time-windows and cessation of new neuronal birth occurs during pre- and peri-natal development. However, continual adult neurogenesis seems to be functionally important and relevant to the animal's behaviour. These adult-

born neurons become electrophysiologically active, integrate into existing dentate gyrus circuitry, and contribute to behaviour (see Zhao et al. 2008). The rate at which these new neurons are born is regulated by environmental factors and the experiences of the animal (as discussed in Section 1.5, "Adult Neurogenesis"), providing indirect evidence that these neurons are situated to act as an interface between brain and environment. The specific role that these neurons play in cognition and emotion, however, remains unclear. Elucidating the role that new granule neurons play in behaviour, and indeed the specific neuronal function(s) that contribute to these behaviours, is of fundamental importance to understanding the brain-behaviour-environment interaction and for a complete understanding of hippocampal function. In this section, particular behavioural functions affected by the ablation or enhancement of adult neurogenesis, using the techniques mentioned in the previous section, are reviewed.

### **Trace Fear Conditioning**

The first study to show a behavioural deficit in mice lacking adult neurogenesis used eyeblink conditioning in rats (Shors et al. 2001). They found that MAM-treated rats lacking adult neurogenesis showed a decrease in cue responding in a trace cue condition when a 500-ms trace interval separated the cue offset and the onset of the unconditioned stimulus (air-puff to the

eye) as compared to non-treated control rats. These MAM-treated rats, however, were not impaired in delay eyeblink conditioning when the air puff occurred with the offset of the cue. Similarly, inhibiting adult neurogenesis by MAM produced a deficit in freezing behaviour in a cued fear-conditioning task with a trace interval, as compared to control rats (Shors et al. 2002).

### **Contextual and Spatial Memory**

It is not surprising that the best-known function (i.e. spatial memory) of the hippocampus was also one of the first tested in rodents lacking adult neurogenesis. Some studies have shown a deficit in spatial memory in different spatial paradigms but not always (see Groves et al. 2013). In a meta-analysis, Groves and colleagues found a minimal (uncorrected for multiple comparisons) effect on contextual fear conditioning in studies using animals with ablated adult neurogenesis. In the same analysis, there was no overall evidence for an effect on cued fear conditioning, Morris watermaze memory, or anxiety-like behaviour on the elevated-plus maze or in the open field (Groves et al. 2013). Nevertheless, individual studies do report differences using different techniques to eliminate new neurons.

In contextual fear conditioning using irradiated rats, decreased freezing in contextual fear conditioning (i.e. fearing a context that had previously been associated with shocks) has

been found (Snyder et al. 2009a, Winocur et al. 2006, Wojtowicz et al. 2008). However, two other studies did not find this effect using different ablation techniques (Groves et al. 2013, Shors et al. 2002). This questions the external validity of these tasks across methods, assuming a successful or selective elimination of adult-born neurons. In rats, it was found that a deficit in contextual fear conditioning developed between three and four weeks after inhibition of neurogenesis but no effect on freezing levels was found even after eight weeks in mice (Snyder, Choe, et al., 2009). Interestingly, using mice, a general deficit in contextual fear conditioning only manifested itself after a single shock but not multiple shocks (Drew et al. 2010), suggesting that it may be the weak context-shock association that is responsible, or possibly the task difficulty (Beylin et al. 2001), that determines when new neurons in the hippocampus become engaged. Nevertheless, it does appear that new neurons are required in aspects of successful contextual fear conditioning in certain situations, although the precise experimental conditions needed in order to see an effect remain unclear.

Spatial deficits in the Morris watermaze have been equally equivocal as those in contextual fear conditioning. Although, it has been found that in watermaze protocols, spatial learning and testing during the probe trial have been spared (Arruda-Carvalho, Sakaguchi, Akers, Josselyn, & Frankland, 2011; Ben Abdallah et al., 2013; Snyder, Hong, McDonald, & Wojtowicz, 2005;

Wojtowicz et al., 2008), other more difficult versions of this task have shown more subtle impairments and a complex interplay between the cells' birth and death. For example, using longer delays between sessions taxes memory more and impairments have been found using this methodology. In addition, more careful analysis of behavioural strategies (such as swim-path strategies) used by these mice also show an impairment relative to control animals. For example, mice without adult neurogenesis tend to use "chaining" behaviour to locate the platform during watermaze testing rather than more spatially precise methods, but, again, this is not always consistent across studies (Ben Abdallah et al. 2013, Garthe & Kempermann 2013, Snyder et al. 2005). Along these lines, in the Barnes maze, mice lacking adult neurogenesis are slower, as compared to control mice, to use a spatial memory strategy but instead use a random search strategy to find the hidden tunnel (Raber et al. 2004). This is consistent with other altered search strategies used to solve the watermaze, e.g. thigmotaxis and chaining, to eventually find the platform on reversal trials (Garthe & Kempermann 2013). In addition, Dupret et al. (2007) found that both the addition and removal of new neurons was required for precise spatial learning. Specifically, successful learning of the watermaze depended on the death of cells at seven and nine days post-proliferation but not of cells younger than five days or older than 13 days post-proliferation. Indeed, blockade of this learning-induced cell death actually

impaired spatial learning (Dupret et al. 2007). All the above results demonstrate that, while the elimination of new neurons appears to hinder spatial learning, memory, and strategies, it is equally important that the addition of these cells occurs to promote flexibility for new learning and stabilization of memories to occur (Dupret et al. 2008)

### **Pattern Separation and Interference**

Another function ascribed to the dentate gyrus, based mainly on neuro-computational models (Aimone, Wiles, & Gage, 2009; Becker, Macqueen, & Wojtowicz, 2009), is pattern separation (Aimone & Gage, 2011; Groves et al., 2013; Sahay et al., 2011). Pattern separation, in broad terms, is the disambiguation or separation of two or more overlapping inputs into more orthogonalised outputs. Although this function was originally conceptualised as a neuronal network process, it is now applied to situations involving animal behaviour. Impairments in behavioural pattern separation are caused by seemingly overlapping sensory and/or memory inputs, such as similar contexts or objects, in which behavioural discriminations need to be made. Adult-born granule cells instead of, or in addition to, the entire granule cell population may play an important role in pattern separation.

One task used to assess behavioural pattern separation is contextual fear conditioning. In these studies, mice or rats learn to associate a certain context or environment with an aversive event, e.g. shock. The rodent is then placed in a safe, non-shocked context and needs to discriminate this environment from the other shock environment, as measured by differential freezing levels. Mice without adult neurogenesis are slower to show differential freezing levels between two highly similar contexts as opposed to two drastically different contexts (Kheirbek et al. 2012, Nakashiba et al. 2012). Mice with increased levels of adult neurogenesis, through the elimination of the *Bax* pro-apoptotic gene, are quicker to show differential freezing levels between these similar contexts (Sahay et al. 2011).

Behavioural pattern separation has also been extended to the spatial realm. Specifically, spatial locations with a high degree of separation (i.e. places that are far apart) are more likely to be discriminated than those with a small degree of spatial separation (i.e. places that are close together). For example, in the radial arm maze where 8 arms radiate from a central platform, mice lacking adult neurogenesis made significantly more errors when two of the arms had a low degree of separation as compared to arms with a high degree of separation (Clelland et al. 2009). However, in studies using rats or mice that lack adult neurogenesis, using a similar radial maze protocol, others were unable to find this impairment (Groves et al. 2013, Piatti et al. 2014).

The role of the hippocampus, and in particular the dentate gyrus, in pattern separation may occur when two competing or overlapping memories need to be separated as opposed to overlapping sensory or spatial inputs, as has been suggested above. Specifically, instead of disambiguating similarities based on sensory inputs (e.g. similar environments), it may be that the animal has overlapping memories upon, for example, retrieval of these environments. Some studies in rodents lacking adult neurogenesis find that these animals are able to learn the initial platform location in the water maze (Groves et al. 2013, Saxe et al. 2006). However, these mice are sometimes impaired on reversal stage of the watermaze task, such that these mice show less precise spatial strategies to locate a second hidden platform, but not initial learnt platform location, whereby they tend to show more thigmotaxic and chaining behaviours (Garthe et al. 2009). Similarly, during the probe trial after a second acquisition, mice lacking adult neurogenesis searched in both the new and original platform location, whereas control mice focused more on the newly learnt spatial location (Arruda-Carvalho et al. 2011). Similarly, in an active place-avoidance spatial reversal task, mice lacking adult neurogenesis show impairments when a shock zone is switched to a new location (Burghardt et al. 2012). In these studies, the previously learnt spatial location interferes with the new memory, similar to studies on proactive interference. This could be taken to reflect a failure in pattern separation during the

memory retrieval of two similar memories. This failure to disambiguate these competing memories, then, may cause less precise spatial strategies based on memory interference. Taken together, these studies indicate that adult-born neurons are involved in behavioural tasks involving high amounts of interference.

### **Non-mnemonic Behaviours**

Exploration is a typical proxy of anxiety-like behaviours in, for example, the open-field test and the EPM, and rodents with adult neurogenesis inhibited normally do not show altered exploration on these tasks (see Groves et al., 2013 for a meta-analysis), at least under some conditions. The exception to this, at least to some degree, is that mice lacking adult neurogenesis are impaired at the novelty-suppressed feeding (NSF) task after restraint stress or chronic unpredictable stress (Santarelli et al., 2003; Snyder, Soumier, Brewer, Pickel, & Cameron, 2011; Surget et al., 2008), though many factors contribute to this behaviour that may not necessarily be exploration-related. This NSF task measures the latency to eat a familiar food in a novel environment and is influenced by internal conflicting hunger, safety, and exploratory signals (Britton & Britton 1981). Mice with inhibited adult neurogenesis show normal latencies, compared to wild-type mice, under baseline conditions, but after acute restraint stress

the adult neurogenesis-deficient mice increase their latency relative to wild-type mice (Snyder et al. 2011). Thus, the mice lacking adult-born neurons look more anxious and/or depressed ("anxio-depressive") than their wildtype control littermates.

The recent advances in the field of adult neurogenesis suggest that new neurons have a particular role in the stress response and interact closely with HPA axis release of glucocorticoids (Lehmann et al. 2013, Schloesser et al. 2009, Snyder et al. 2011, Surget et al. 2008). The direct mechanism that mediates this negative feedback provided by adult-born neurons to the HPA axis is not clear; however, it seems plausible that these new neurons act indirectly through the ventral subiculum which acts by attenuating HPA axis response (Mueller et al. 2004; see above under 'Endocrine Response' for general hippocampal involvement).

Another recent idea related to the stress response and the hippocampus's role in HPA axis control suggests that adult neurogenesis may be involved in anxiety and/or depression. The adult neurogenic theory and its potential pathophysiological relation to depression has been proposed along with theories relating to the generalisation of anxiety (Besnard & Sahay 2015, Jacobs et al. 2000, Miller & Hen 2014). These specific correlations that prompted the adult neurogenic theory are that elevated glucocorticoid levels inhibit adult neurogenesis and can exacerbate anxiety and depressive symptoms; people with these

disorders display smaller hippocampal volume; and antidepressants that treat anxiety and depression can enhance adult neurogenesis under specific conditions. (Jacobs et al. 2000, Miller & Hen 2014). A more direct link comes from studies that selectively inhibit adult neurogenesis and test the animals' stress response in anxiodepressive-like tasks. Acute restraint stress, for example, produces no altered novelty-suppressed feeding behaviour in normal mice; however, when mice lacking adult neurogenesis are restrained just before the novelty-suppressed feeding testing, they show increased anxiodepressive-like behaviour and heightened glucocorticoid response (Snyder et al. 2011).

## 1.7 Aim of Thesis

It is clear that ablating adult-born neurons produces some similar deficits as hippocampal lesions but, not surprisingly, the effects appear to be more specific and are not as broad of an array as those caused by large lesions. The specificity of the tasks that tax adult-born neurons and the underlying commonalities between the tasks is unclear. Many theories seem to converge on emotionality and the stress response as a key role for these adult-born dentate gyrus neurons and the hippocampus in general. In addition, the hippocampal formation has a long history of detecting and resolving conflicting goal responses or the

memories that underlie the goals (Gray & McNaughton 2000).

Evidence points to the role of the dentate gyrus in separating out, or disambiguating, overlapping/conflicting inputs (e.g. memories).

These two functions in combination suggest a role for the hippocampus, and possibly adult-born neurons, in the resolution of conflicting memories to help promote behavioural adaptation in stressful situations.

Specifically, the experiments in this thesis aimed to provide a clearer understanding about the role that the hippocampus and adult-born neurons play in the behavioural response toward ambiguous (or conflicting) memories that may underlie the animals' goals and their behavioural responses. Fear conditioning experiments were designed to introduce a cue that always predicted an aversive outcome (i.e. a reliable predictor about a shock outcome) and other cues in which the outcome was unable to be predicted due to partial reinforcement (i.e. an ambiguous predictor about a shock outcome).

Further experiments built on this theme and investigated how experiencing this ambiguity of threat outcome affected future behaviour. Specifically, it further clarified the importance of being able to disambiguate conflicting or overlapping memories in order to promote adaptive behaviour in future novel, potentially dangerous, situations. In addition, because of the stressful nature involved in the prior stress history of the animal, the role that

glucocorticoids play in adaptive behaviour, in conjunction with the presence or absence of adult-born neurons, was assessed.

The last set of experiments were designed to see if this ambiguity effect (i.e. overlapping or conflicting memories) about goal responses would also apply to the appetitive domain. In these experiments, a non-spatial version of the T-maze was used to produce conflict in goal responses and hippocampal-lesioned mice and mice lacking adult neurogenesis (and their respective control animals) were used. Specifically, the two experiments were designed so that mice had to discriminate between two rewarded arms: (i) one of the goal arms was only partially reinforced with a reward whereas the competing arm was always reinforced with a reward and (ii) having two conflicting reward magnitude differences in the competing arms and mice had to discriminate between the reward sizes.

All together, these sets of experiments utilising conflicting or ambiguous threat or reward-related outcomes provide important insights into how the hippocampus, and particularly the dentate gyrus and adult neurogenesis, disambiguates overlapping goal or memory inputs into the hippocampus.

# 2 THE HIPPOCAMPUS IS NECESSARY FOR APPROPRIATE BEHAVIOURAL RESPONSES TOWARD AMBIGUOUS THREAT CUES

## 2.2 Abstract

Learning about threats within the world often requires making decisions about ambiguous situations that may signal danger. In this study, it was found that complete excitotoxic hippocampal lesions produced deficits in fear conditioning to an ambiguous, but not a reliable, cue of threat. Specifically, mice with hippocampal lesions showed low cue-induced freezing when the tone was partially reinforced with shock, whereas sham-lesioned mice showed high cue-induced freezing levels. When a distinct tone was fully predictive (i.e. a reliable cue) of shock, hippocampal-lesioned mice were indistinguishable from sham-lesioned mice. Both groups also showed minimal, and equivalent, freezing in the presence of a third cue that was never reinforced. In addition, during extinction of the tone-shock associations, shams displayed higher freezing levels than hippocampal lesioned mice overall. To confirm lesion efficacy, mice with hippocampal lesions displayed reduced latency to eat food in a novel environment in the novelty-suppressed feeding task, as compared to sham-lesioned mice, demonstrating a decreased anxiety- and/or depressive-like response. Histological analysis confirmed near-complete destruction of the hippocampus. This study demonstrates the importance of the hippocampus in mediating appropriate defensive behaviours when a cue about threat cannot be

predicted (i.e. ambiguous) and how defensive responding is altered when the threat is made ambiguous.

## 2.3 Introduction

Learning about threatening cues in our environment is important to help us avoid dangerous situations in the future. If a cue is a reliable predictor of threat, fear learning occurs rapidly and stress levels are only slightly elevated. Oftentimes, however, the animal receives conflicting information about a potential threat, such as whether it is truly a threat to the animal or if it's a false alarm. This conflicting information hinders the ability to predict the nature and outcome of the potential threat. These ambiguous predictors about threat raise stress levels and impair decision making (Koolhaas et al. 2011).

During contextual fear conditioning, animals are placed into an environment and they receive electric footshocks. If these shocks are not signalled by a discrete stimulus, the animal has to rely on whatever information is available within its environment to establish predictors of the shock (e.g. the diffuse spatial cues in the chamber, often referred to as the context). The hippocampus is thought to be necessary for this type of contextual fear conditioning, because hippocampal lesions produce deficits in freezing behaviour in the training context but not in a neutral context. However, hippocampal lesions typically do not produce freezing deficits during standard 'delayed fear conditioning'

paradigms in which discrete, unimodal cues signal shock 100% of the time and CS offset and US onset are contiguous or co-terminus. The reason why the hippocampus appears to mediate contextual but not delayed fear conditioning remains contested (Maren et al. 1998, 2013; McNish et al. 1997, Zelikowsky et al. 2014).

One idea, the configural association theory, is that the hippocampus needs to form a representational 'whole', or a multimodal representation of environmental cues (O'Reilly & Rudy 2001). The co-occurrence of the elemental cues produces a unification into a single or configural representation and this is the 'context'. Fanselow argued that forming this configural representation requires sufficient time to encode and synthesize the individual elements into this new configuration. For example, to form a configural representation, rats are thought to require approximately two minutes to explore the fear conditioning chamber; shorter exploration times are likely insufficient to form this new representation (Fanselow 1986). By this theory, in the case of delayed fear conditioning, in which a discrete elemental cue (e.g. an auditory tone) is always paired with shock (and 100% predictive of shock), no configural representation is required. Hence, the hippocampus is not necessary for delayed fear conditioning (O'Reilly & Rudy 2001, Phillips & LeDoux 1992).

There is, however, another account for why the hippocampus may be needed for context but not delayed fear

conditioning. The context may represent a conditioning stimulus itself and it may not be that the hippocampus needs to form a multimodal representation. The conditioning context is present when the animal receives shock (and therefore is predictive of shock) but it is also present when the animal is not receiving shock (and therefore predicts the absence of shock, i.e. safety). This situation creates an inherent conflict (i.e. ambiguity) about what the context means or predicts. The hippocampus, and in particular the dentate gyrus, may be needed to detect this conflict and to mediate the appropriate response to this conflict. This idea predicts that the hippocampus will be necessary for encoding or retrieving ambiguous elemental cues (such as discrete auditory tones) and not just contexts. Conversely, the hippocampus should not be required for encoding or retrieving unambiguous elemental cues.

To test this hypothesis, a task in which mice learnt to discriminate between three discrete auditory cues (continuous tone, intermittent tone, white noise), each with a different predictive relationship with an aversive outcome (footshock), was used. In this study, one cue was paired with shock on 0% of trials (CS-), a second cue on 100% of trials (CS+), and a third cue on 20% trials (CSambiguous, CSa). Mice received excitotoxic lesions of the entire hippocampus or sham surgery before training began. After fear conditioning to the different cues, mice underwent extensive extinction to investigate whether a partial predictive or

reinforced cue (CSa) would produce faster or slower extinction as compared to the fully predictive or reinforced (CS+ and CS-) cues. To confirm lesion efficacy behaviourally, the well-validated hippocampal-dependent novelty-suppressed feeding test was given to examine anxiety- and/or depressive-like responses. Finally, histological examinations were carried out to determine if any extra-hippocampal damage occurred.

## 2.4 Methods

All procedures performed at University of Oxford adhered to the United Kingdom Animals Scientific Procedures Act (1986) under project license 30/3068 and were approved by local ethical review for the University of Oxford.

### **Animals**

All mice were female C57/Bl6 (Harlan, UK), group housed (6/cage), and the animal colony was on a 12 hr light/dark cycle (lights on at 7:00 AM). These animals had access to food and water ad lib.

## Surgery

Mice were anaesthetised with isoflurane and administered intra-peritoneal (i.p.) injections of meloxicam (1 mg/kg; a non-steroidal anti-inflammatory), chlordiazepoxide (10 mg/kg; a benzodiazepine) and atropine (10 mg/kg; anti-cholinergic to steady heart rate) for the first cohort of mice. For the second cohort of mice, i.p. injections of metacam (5 mg/kg; non-steroidal anti-inflammatory) and vetergesic (0.08 mg/kg; buprenorphine analgesic) were given, and marcaine (2 mg/kg; local anaesthetic) was given subcutaneously into the scalp. These injections were given prior to stereotaxic mounting (Kopf Instruments, CA; Clark Electromedical, Reading, UK).

A midline incision was then made down the scalp and four burr holes were drilled per hemisphere. The excitotoxin *N*-methyl-D-aspartic acid (NMDA, 10mg/ml) in phosphate-buffered saline (pH 7.4) was injected with a 5- $\mu$ l Hamilton syringe using a 34-gauge needle (see Table 2.1 for coordinates for both experiments). For every 0.1  $\mu$ l of toxin injected, the needle sat in place for 60 seconds to allow for diffusion away from the site and then was raised in a step-wise manner out of the tissue. After suturing, mice were given chlordiazepoxide (first cohort of mice; 10mg/kg i.p.; a benzodiazepine) or midazolam (second cohort of animals; 5 mg/kg; a benzodiazepine) and 1 ml saline subcutaneously (both cohorts). Sham-operated animals received

all drugs and anaesthesia, were placed in the stereotaxic frame, received midline incision, and then sutured.

After surgery, mice were placed into individual incubating cages (30°C) and given access to water and a mashed food mixture (regular food pellet mixed with water). These mice were monitored until they were freely moving around the cage. Animals were given at least one week to recover before behavioural testing.

Table 2.1 Stereotaxic coordinates and volume of NMDA excitotoxin at each site: A/P and M/L coordinates in the first cohort were made from the interaural line (indicated by green arrowheads) and from bregma for D/V coordinates. All coordinates from second cohort were made from bregma

Site	First Cohort				Second Cohort			
	1	2	3	4	1	2	3	4
Volume (µl)	0.2	0.1	0.15	0.1	0.1	0.15	0.1	0.2
A/P	+0.7	+1	+1.5	+2.1	-1.7	-2.3	-2.8	-3.1
M/L (±)	3	2.2	1.7	1.2	1.2	1.7	2.2	2.8
D/V (-)	4	2	1.9	1.9	1.9	1.9	2	4

## Handling and General Procedure

Mice were handled 3-5 min/day for at least 2 days prior to behavioural testing and allowed to rest for at least 20 minutes before testing. After fear conditioning, tested mice were placed directly back into their homecage.

## **Fear Conditioning and Extinction**

Mice were fear conditioned in one of two operant chambers (ENV-307A, Med Associates). Each chamber contained its own set of visual and olfactory cues. The timeline of the experiment consisted of: a pre-exposure day, three conditioning days, a test day (fear memory recall day), five extinction days, and one renewal day. Shocks were only given during fear conditioning sessions. For fear conditioning, three distinct tone types (white noise, a pure tone, and an intermittent sounding tone; all 72 dB, 15 s duration) served as one the conditioning stimuli (CS) and some tones co-terminated in a footshock (0.3 mA, 0.5 s duration). The CS- never co-terminated in footshock, while the CS+ co-terminated 100% of the time with footshock in the training sessions. A partially conditioned stimulus (CSa; CSambiguous) co-terminated in footshock 20% of the time, or 1 in 5 trials per day. The fully conditioned stimulus (CS+) was paired with footshock in all 5 trials per day. The CS-, however, was never paired with footshock during the 5 trials per day. All tones and presentations were counterbalanced across trial orders, tone allocation, and mice. For extinction, all tones were played in a novel, and therefore neutral, context without co-terminating in shock. For fear renewal, the mice were placed back into the original conditioning context and the tones were played without any co-terminating in shock.

At the beginning of each conditioning session, two mice were placed in their respective chamber (one lesion and one sham). Each session started with a 300-s lead-in period that served as a measure of context freezing. After this, 15 auditory cues (5 presentations of each type) were played (mean ITI was 80 s, standard deviation of 13.4 s) in a pseudorandom order. The same tone type never occurred on more than two consecutive trials. On fear conditioning days, footshocks were applied where assigned. At the end of each session, chambers were cleaned with 10% ethanol mixed with distinct odour (lavender or vanilla).

Videos were recorded and analysed by a script running in NIH Image that compares pixel changes from one frame to another at one second intervals (Schneider et al. 2012). To analyse %freezing difference scores, the amount of %freezing in the 15 s immediately preceding the CS was subtracted from the %freezing during the CS (i.e. CS - preCS). Positive difference scores meant that the mouse froze more during the CS than the preCS period. Negative scores meant the opposite. To examine contextual conditioning, the 300 s lead-in period before any CS presentation was examined for each conditioning day.

### **Novelty-Suppressed Feeding**

Mice underwent novelty-suppressed feeding to investigate the functional efficacy of the hippocampal lesions. Before the task,

all mice went through fear conditioning and several rounds of extinction sessions, along with a reactivation phase prior to the task. Eighteen hours before this task, all food was taken away from the mice with unlimited access to water. The novelty-suppressed feeding task is thought to test anxiety- and/or depressive-like behaviours (collectively referred to as anxiodepressive-like behaviours in this thesis), because it contains elements of anxiety (e.g. approach-avoidance conflict) and depression (e.g. motivation and can assess anhedonia-like behaviour). In addition, it is sensitive to (ventral) hippocampal lesions, anxiolytics, and SSRIs, all of which act to reduce anxiodepressive-like behaviours that rodents display on this and similar hyponeophagia tasks (Bannerman et al. 2002, Bodnoff et al. 1988, Santarelli et al. 2003, Surget et al. 2008). The novelty-suppressed feeding task includes an approach-avoidance conflict, such that the animal wants to explore the open arena and possibly find other rewarding opportunities (e.g. food or a mate) but generally wants to avoid open areas and bright lighting (avoid predation). The novelty-suppressed feeding task involved mice being tested in a white arena (50 x 50 x 40 cm) (*l x w x h*) that was made out of plastic with a cardboard-shape "+" separator used to create 4 smaller arenas. Novel bedding covered the floor. One pellet of their regular home cage food was placed on top of a 1-cm-high white weighing dish in the centre of each individual

arena. Latency to begin eating the familiar food was recorded (maximum trial time of 10min).

## **Histology**

At the end of behavioural testing, mice were overdosed with 200 mg/ml of sodium pentobarbital (Euthatal; 200 mg/kg i.p.) and perfused transcardially with physiological saline (0.9% NaCl) followed by 10% formol saline (10% formalin in physiological saline). The brains were removed and stored in the 10% formol saline. They were then transferred to a 30% sucrose-formolin solution for 24 hr. Brains were then frozen and coronally sectioned (50  $\mu$ m) on a freezing microtome. Sections were then stained with 0.5% Cresyl violet (diluted in deionised water) by initially washing the slides in histoclear before being dehydrated in descending concentrations of alcohol (100%, 96%, 80% and 70%). Slides were then washed in deionised water before being left in the stain for 6 min, washed again in water, then taken through ascending concentrations of alcohol (70%, 80%, 96% and 100%) to wash and set the stain. Cresyl violet selectively stains Nissl substance, the basophilic rough endoplasmic reticulum present in cytoplasm. Slides were scanned into a computer for examination using a Polaroid SprintScan 35Plus digital imager. Quantification of the spared cellular lamination of hippocampal tissue was performed by drawing a circle over the spared cellular

lamination of both shams and lesioned animals. The percentage of spared tissue was compared to shams.

## Statistics

The percentage of freezing data, after it was obtained from NIH Image (see directly above for more information), was analysed with GraphPad Prism and SPSS software. ANOVAs performed were two-way mixed ANOVAs (within-subject factors of days<sub>T1-3 & TEST</sub>, period<sub>preCS, CS</sub>, and cue type<sub>CS-, CS+, CSa</sub>; between-subject factor of lesion group<sub>HIPP LESION, SHAM</sub>). A three-way mixed ANOVA was performed on raw %freezing scores from the test day (i.e., fear memory recall) with a between-subject factor of lesion group<sub>HIPP LESION, SHAM</sub>, and within-subject factors of Cue type<sub>CS+, CSa</sub> and trials<sub>1-5</sub> of the test day. Another three-way mixed ANOVA was also performed on the extinction+renewal sessions with a between-subject factor of lesion group<sub>HIPP LESION, SHAM</sub> and within-subject factor of Cue type<sub>CS-, CS+, CSa</sub> and session<sub>E1-5 & Renewal</sub>. If the within-subjects factor did not meet Sphericity, the Greenhouse-Geisser corrected degrees of freedom, and its associated  $F$  and  $p$  value, was used. If homogeneity of variance (by Levene's test) was violated with fewer than 10 subjects per group, a modified  $p$  criterion was used ( $< 0.03$ ). All post-hoc testing used Bonferroni correction. All  $t$ -tests performed were independent-sample and were two-tailed. Data points in graphs represent means and error bars represent standard error of the mean.

## 2.5 Results

### Fear Conditioning

After recovery from surgery, mice underwent fear conditioning. During the pre-exposure (PE) session, onset of cue did not evoke a freezing response by either sham or hippocampal-lesioned mice. Analysis of these PE freezing responses revealed no main effect of lesion, cue type (CS-, CS+, or CSa), or an interaction between lesion and cue type (all  $F < 2.3$ ,  $p > 0.1$ ). On the first training session (i.e. day) (T1), freezing responses were higher in both the shams and hippocampal-lesioned mice compared to PE, but there was no effect of lesion group, cue type, or interaction (all  $F < 1.2$ ,  $p > 0.3$ ; Figure 2.2 A). A three-way mixed ANOVA on the training difference scores (factors: cue type, training day, and lesion type) revealed that there was a main effect of lesion ( $F_{1, 46} = 19.1$ ,  $p < 0.001$ ), such that the hippocampal-lesioned mice showed lower difference scores overall as compared to sham mice. There were no other significant main effects or interactions (all  $F < 1.63$ , all  $p$ 's  $> 0.05$ ). Two-way mixed ANOVAs for each training (conditioning) day's freezing levels toward each individual cue type revealed that there was an effect of lesion ( $F_{1, 46} > 6.53$ , all  $p < 0.05$ ), such that the

hippocampal-lesioned mice tended to freeze less overall as compared to shams, but no effect of each CS type ( $F_{2, 92} > 1.02$ , all  $p$ 's  $> 0.05$ ), and no interaction between the variables (all  $F < 1$ ) (Figure 2.1 A).

To investigate freezing responses during the test ("TEST") session alone, a two-way ANOVA was performed on the average freezing response across all trials on the test session. The focus of the analysis was on the possible differences between the CS+ and CSa. Analysis on these two cue types by lesion group revealed that there was a significant effect of cue type ( $F_{1, 46} = 14.2$ ,  $p < 0.0001$ ), lesion group ( $F_{1, 46} = 5.3$ ,  $p = 0.025$ ), and importantly a cue type x lesion group interaction ( $F_{1, 46} = 5.7$ ,  $p = 0.02$ ). Bonferroni post-hoc testing of the interaction revealed that only during the CSa did shams have significantly higher freezing score compared to hippocampal-lesioned mice ( $p = 0.005$ ); their freezing levels did not differ during the CS+ presentations. Similarly, the hippocampal-lesioned mice, and not the shams, froze more during the CS+ presentation as compared to their freezing during CSa presentations ( $p < 0.0001$ ), while shams did not differ across cue type ( $p < 0.05$ ) (Figure 2.1 A, right).

Differences between sham and hippocampal-lesioned mice were most apparent on the test day when no shocks were given. Figure 2.1 (B, C) show pre-CS and during-CS freezing levels in shams and hippocampal-lesioned mice, respectively. In the shams, both CS+ and CSa onset produced a significant increase

in freezing (both  $p < 0.001$  for pre-CS vs. CS freezing (Figure 2.1 B). In hippocampal-lesioned mice, CS+ onset produced a significant increase in freezing ( $p < 0.001$ ) but CSa onset did not ( $p = 0.8$ ; Figure 2.1 C). When the pre-CS and CS-induced freezing scores were subtracted (resulting in difference scores) and plotted (Figure 2.1 D), there were no differences between sham and hippocampal-lesioned mice in freezing to the CS- ( $p = 0.2$ ) or the CS+ ( $p = 0.6$ ) but a significant difference in freezing to the CSa cue ( $p = 0.008$ ). Thus, hippocampal lesions selectively reduced freezing to the ambiguous CSa cue.

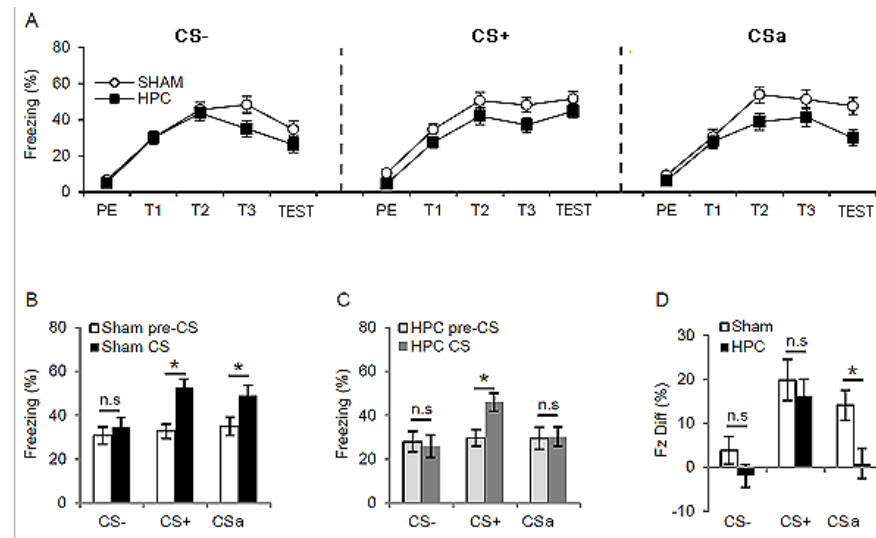


Figure 2.1 Freezing percentages across cue types and by group.

(A) The raw percent freezing across the pre-exposure day (PE), all training days (T1-T3), and the Test day is given by CS type. Both sham and hippocampal-lesioned mice showed normal cue-induced freezing across time but only during the CSa do the hippocampal-lesioned mice show reduced freezing toward the CSa starting on T2 - TEST.

(B) During the test day when examining the sham mice's freezing scores, freezing was lower before the CS+ and CSa cues' onset but not for the CS-. (C) During the test day when examining the hippocampal mice's freezing, freezing was lower before the CS+ cues' onset only but not for the CS- or CSa.

(D) The differential freezing scores ('Fz Diff (%)') between CS types and pre-CS periods showed that hippocampal and sham mice did not differ in their response to the CS- or CS+, and only hippocampals did not show a difference between the pre-CS and CSa-induced freezing. \*  $p > 0.05$ ; n.s. = not significant.

## Context Conditioning Test

Animals with hippocampal lesions have been known to show a selective deficit toward conditioning to the context (Maren & Holt, 2000). Therefore, to examine any effects on contextual freezing, each conditioning session's 300-s lead-in time to the first CS presentation was examined (Figure 2.2). There was no effect of hippocampal lesion on context conditioning in any of the conditioning sessions (lesion effect:  $F_{1, 46} < 1$ ). Both sham and hippocampal mice exhibited higher levels of context-related freezing during T2 and T3 compared to PE, T1 or Test session, suggesting that both exhibited fear to the training context but minimal context conditioning to the novel fear recall (Test) context (session effect:  $F_{4, 184} = 61.6, p < 0.0001$ ). Importantly, both sham and hippocampal-lesioned mice froze significantly less during Test than T2 or T3 (all  $p < 0.002$ ), suggesting that both groups were aware of the change in context. Hippocampal-lesioned mice did freeze less than shams during T1 ( $p = 0.005$ ), but this was before the mice had received any footshocks and thus is unlikely to reflect any contextual conditioning. It is likely that the reduced levels of freezing in hippocampal-lesioned mice during T1 reflect hyperactivity (and/or reduced habituation to the conditioning chamber), which is a common consequence of hippocampal lesions (Good & Honey 1997).

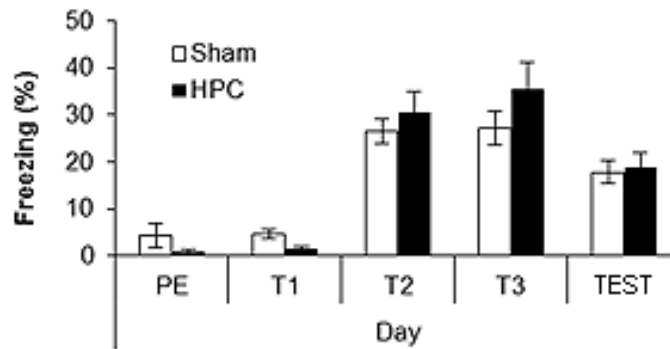


Figure 2.2 Sham and hippocampal-lesioned mice show normal conditioned freezing to the context during the 300 s lead-in period to the first cue presentation on each session. PE=pre-exposure; T1-3 = fear conditioning training 1-3; TEST = fear memory recall test to cues in a novel context.

### **Extinction & Renewal of Conditioned Responding**

When an animal is repeatedly exposed to the conditioned cue only (without the co-termination in shock), they begin by showing a natural conditioned response (i.e., freezing in this experiment). Subsequent presentations of this kind over many trials, however, creates a new type of learning that may inhibit the conditioned stimulus-response association and promotes a decrement in generalisation in that new context (Bouton & Bolles 1979, but see Bouton 2004). To examine extinction of the freezing response through difference scores (CS - preCS) toward each

cue type, multiple extinction session were given in a second context (same context as tested during the memory recall test session) and then returned to the original fear conditioned context for a fear renewal session. Only half of the original experimental mice went through this paradigm (hippocampal mice  $n = 12$ ; shams  $n = 12$ ).

First, a three-way ANOVA was performed (as described in Statistics section) with lesion group, session, and cue type as factors on the data presented in Figure 2.3. Overall, there was main effects of session ( $F_{5, 330} = 2.3, p = 0.04$ ), lesion group ( $F_{1, 66} = 4.3, p = 0.04$ ), and cue type ( $F_{2, 66} = 32.7, p < 0.0001$ ). There were, however, no interactions between any of these variables (all interactions  $F < 1.59, p > 0.05$ ). Bonferroni post-hoc analyses revealed no statistical differences overall in the sessions (all  $p > 0.05$ ). However, they did reveal that hippocampal-lesioned mice tended to show a lower difference scores (i.e., lower freezing toward the CS compared to preCS levels) than the sham mice ( $p = 0.04$ ). When examining the cue type for post-hoc differences overall, the CS- elicited a lower difference scores compared to the CS+ ( $p < 0.0001$ ) and the CSa ( $p = 0.003$ ). Additionally the CS+ tended to have a higher freezing difference score as compared to the CSa ( $p < 0.0001$ ).

Because we obtained main effects in the three-way ANOVA, individual two-way mixed ANOVAs were performed on each cue type separately. For the freezing responses toward the

CS- during extinction and renewal, no differences were found between lesion groups ( $F_{1, 22} = 1.8, p = 0.2$ ) or across sessions ( $F_{5, 110} < 1$ ), and there was no interaction between these two variables ( $F_{5, 110} < 1$ ) (Figure 2.3, A). When exposed to the CS+, no statistical differences were found between each lesion group ( $F_{1, 22} = 2.9, p = 0.1$ ) or across sessions ( $F_{5, 110} < 1$ ), and no interaction ( $F_{5, 110} = 1.7, p = 0.1$ ) (Figure 2.3, B). Bonferroni post-hoc analyses revealed that there were no differences between lesion groups across any of the sessions (all  $p > 0.05$ ). For the extinctions and renewal session of the CSa, there was a clear effect of session ( $F_{5, 110} = 3.8, p = 0.003$ ) but no difference between lesion groups ( $F_{1, 22} < 1$ ). There was, however, a trending interaction between group and sessions ( $F_{5, 110} = 2.1, p = 0.066$ ) (Figure 2.3, C). This trend was likely due to the fact that there was significant differences in sham lesioned mice between E1 and E3 and E5 (not E4; (extinction days) (all  $p < 0.05$ ) but no significant session differences for hippocampal-lesioned mice.

To demonstrate any renewal effects as compared to the last session of extinction, separate two-way mixed ANOVAs were performed on the last extinction session (E5) and the renewal session separately for each cue type. For the CS-, there was no main effect of session or lesion group, or interaction between these variables (all  $F_{1, 22} < 1, p > 0.05$ ) signifying no renewal of the CS- response. When examining the CS+, surprisingly, there were trends but no significance. There was no main effect of lesion

group ( $F_{1, 22} = 2.7, p = 0.1$ ) or of session ( $F_{1, 22} = 3.1, p = 0.09$ ).

Additionally, there was no lesion group x session interaction ( $F_{1, 22} < 1, p > 0.05$ ). When examining the CSa response, there was also no main effect of lesion ( $F_{1, 22} < 1, p = 0.13$ ) but a trend for session ( $F_{1, 22} = 3.8, p = 0.06$ ). There was, however, an interaction between lesion group and session ( $F_{1, 22} = 5.4, p = 0.03$ ), such that sham mice did renew their fear upon hearing the CSa 'ambiguous' tone as compared to their last extinction session ( $p = 0.01$ ) while the hippocampal-lesioned mice did not ( $p > 0.05$ ). No post-hoc differences existed between shams and hippocampal-lesioned mice during each session ( $p > 0.05$ ).

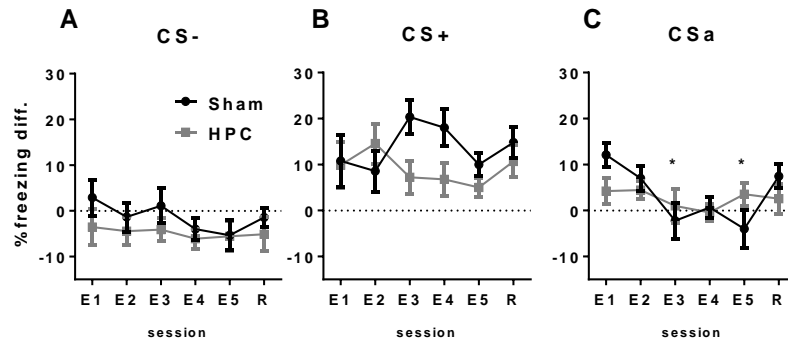


Figure 2.3 Extinction and renewal of fear conditioned responding toward the CS-, CS+, and CSa. A: all mice showed minimal freezing and renewal to the CS-, which never co-terminated with a shock during fear conditioning. Additionally, no evidence of fear renewal was shown by either group of mice. B: both groups showed no differences in extinguishing their freezing response during extinction or fear renewal, although sham mice did appear (numerically) to show an increase in freezing during E3-4, which returned to normal by E5 and a slight increase during the renewal session. C: sham mice displayed some extinction on sessions on E3 and E5 as compared to E1, while hippocampal-lesioned mice did not readily show a difference in freezing scores. However, sham mice did renew their fear memory upon hearing the ambiguous tone, as compared to their last extinction session, whereas hippocampal-lesioned mice did not. Note: E1-5 = extinction sessions; R = renewal session; \*  $p < 0.05$  compared to E1 for sham mice.

## **Novelty-Suppressed Feeding**

To confirm lesion efficacy (and particularly ventral hippocampal dysfunction) using a behavioural task in mice from the second cohort that went through extinction and renewal, a novelty-suppressed feeding task was conducted. This was done after additional extinction and renewal with only a single cue (either a CSa or a CS+ without shock) 30 minutes prior to this testing (no effects emerged based on the type of previous 'renewal' cue experienced). This task, like similar hyponeophagia tasks, is sensitive to (ventral) hippocampal lesions with these mice showing decreased latency to start eating food in a novel environment (Bannerman et al. 2002, McHugh et al. 2004). Mice were placed in a large open field and allowed to explore the arena for 10 min with a familiar food pellet in the middle of the arena. Hippocampal-lesioned mice showed a decreased latency to start eating compared to sham animals (Figure 2.4), confirmed by *t*-test ( $t_{22} = 3.2, p = 0.003$ ). This suggested successful lesioning of the hippocampus, as later indicated with histological analysis (see Figure 2.5).

#### Novelty-Suppressed Feeding

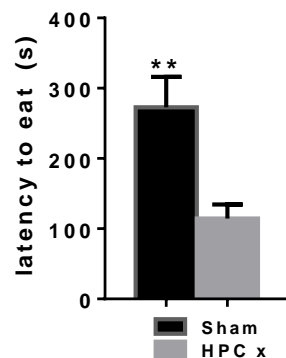


Figure 2.4 Novelty-suppressed feeding was induced in sham-lesioned animals more readily than hippocampal-lesioned mice. Hippocampal-lesioned mice showed a lower mean latency to begin eating a familiar food in a novel environment. \*\*  $p = 0.003$ .

### Histology

Lesions throughout the hippocampus were made with *N*-methyl-d-aspartic acid (NMDA) excitotoxin using the coordinates taken from the interaural line or from bregma in two different cohorts of mice (volumes of NMDA injected and stereotaxic coordinates are listed in Table 2.1).

After post-fixing and staining of the brains, the hippocampus and areas surrounding it were analysed. Figure 2.5 shows reconstructions (left) of the hippocampus and shows the largest (orange) and smallest (blue) lesion size. In most mice, there was little sparing of cell bodies in hippocampal-lesioned

animals and complete sparing in sham animals. Most sparing of cell body lamination from the hippocampal lesions occurred in the very ventral-most portion and spared portions of CA1/subiculum border. The smallest lesions sometimes included tissue sparing but little, if any, discernible cellular lamination could be found. Quantification of each lesion revealed that hippocampal-lesioned mice (second cohort) had near-complete destruction of the hippocampus ( $M = 6.2\% \pm SEM = 1.2\%$  sparing) compared to sham's hippocampus measurements.

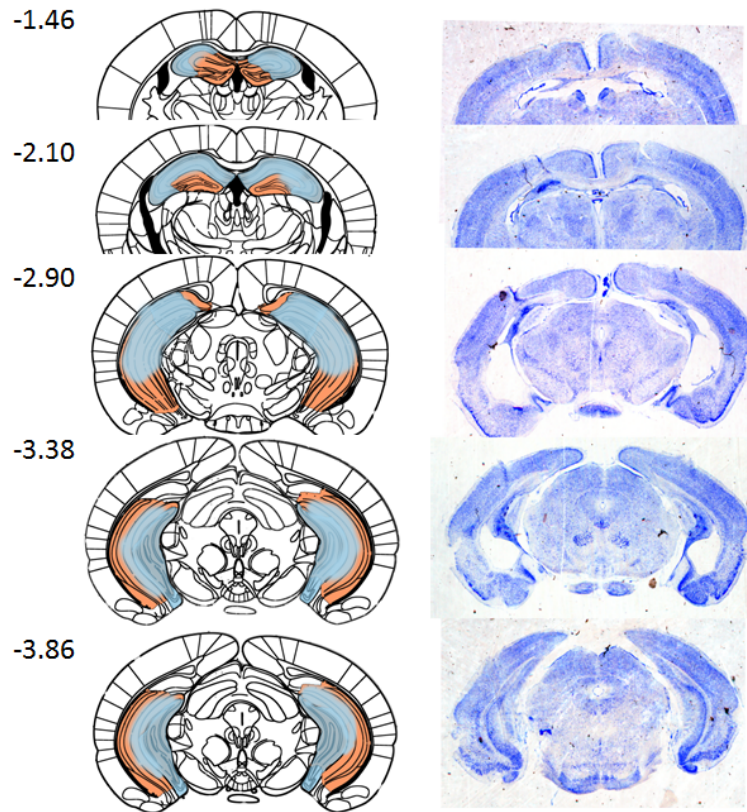


Figure 2.5 Reconstructions and photomicrographs throughout the hippocampus in a typical lesioned animal from the second cohort of mice. On the reconstructions, the area shaded orange (underlying the blue) represents the largest lesion and blue (overlying the orange) represent the smallest lesion.

## 2.6 Discussion

### Summary of Results

In this study, hippocampal-lesioned mice showed lower freezing levels, as compared to shams, toward an ambiguous cue (CSa) that was 20% predictive of shock during initial fear conditioning and during the critical test (i.e. fear memory recall) session in a new context. These mice, however, did not differ from sham controls in their freezing responses toward a cue that was either perfectly predictive of shock (CS+) or non-shock (CS-). The hippocampal-lesioned mice treated the CSa more like the CS- (see Figure 2.1 A). Shams exhibit similar freezing levels toward the CSa as they did to the CS+ (see Figure 2.1 A). Similar freezing responses between sham and hippocampal-lesioned mice were seen during training sessions (T1-T3) when conditioned to a CS- and the CS+ (see Figure 2.1, left and middle). The hippocampal-lesioned mice showed lower freezing from training sessions 2, 3 (T2, T3), and the test session (see Figure 2.1 A, right) during training with an ambiguous cue (the CSa). During all training sessions, the 300 s lead-in period before any cue was played was evidence for any contextual conditioning that may have occurred to the conditioning context. Hippocampal-lesioned mice showed no evidence of this in any of the training sessions or on the critical test session, as compared to shams.

When extinguishing their conditioned responses toward the tones, these two groups did display slightly different behavioural profiles. There were no differences between sham and hippocampal-lesioned mice in their response to extinction sessions of the CS-, which was expected, or renewal of any fear memory (Figure 2.3, A). In addition, we found no statistical difference between sham and hippocampal-lesioned mice during the CS+ extinction or renewal session (Figure 2.3, B). During extinction to the CSa, however, sham mice did exhibit extinction compared to their first extinction session (on E3 and E5) but hippocampal-lesioned mice did not show this extinction across sessions; however, there was an interaction between the groups as sessions progressed, likely reflecting the differences in extinctions across sessions for shams but not lesioned mice (Figure 2.3, C). Individual analysis of freezing behaviour in the original conditioning context, the so-called 'renewal session', as compared to the last extinction day (E5) showed that sham mice did renew their fear, whereas hippocampal-lesioned mice did not display this fear renewal.

When behaviourally confirming lesion effectiveness, it was found that hippocampal-lesioned animals showed a hyponeophagic response in the novelty-suppressed feeding task which is sensitive to (ventral) hippocampal lesions, consistent with other studies showing that these lesions affect hyponeophagic responding (Bannerman et al. 2002, 2003; McHugh et al. 2004).

## **Hippocampus and Ambiguity**

The hippocampus is a structure important in memory and emotion (Barkus et al. 2010) but the relationship between these two functions is not clear. It is clear, however, that a complete understanding of the hippocampus must take into account these two roles. In studies of fear conditioning, an animal must learn that a specific cue predicts an aversive event. There are, however, variations of this task that more or less engage the hippocampus (Anagnostaras et al. 2001, Beylin et al. 2001, Phillips & LeDoux 1992, Quinn et al. 2008).

In this fear conditioning paradigm, the utilisation of an ambiguous conditioned stimulus, the CSa, introduces specific conflict into this task, which may not be present in classic fear conditioning studies that use only a cue that always or never signals the threat (shock). Similar tasks have used ambiguous cues in fear conditioning and the interpretation about how animals respond to these cues has been more from an emotion perspective as it pertains to the negative cognitive biasing of information, or the tendency to interpret ambiguous cues as threatening if the outcomes are uncertain (McHugh et al. 2015, Tsetsenis et al. 2007).

Indeed, the results gathered from this experiment, namely that hippocampal-lesioned animals show weaker freezing responses toward the ambiguous predictor cue of threat

compared to shams, are consistent with a previous study also looking at ambiguity and hippocampal dysfunction. Mice with silenced dentate gyrus granule cells in serotonin receptor 1A knockout mice show selective diminished freezing toward an ambiguous cue predictor but show normal freezing behaviour toward a fully predictive cue (Tsetsenis et al. 2007). Additionally, using a similar paradigm in mice over-expressing the serotonin transporter (and which therefore have reduced levels of synaptic serotonin), it was found that these mice similarly display this freezing deficit toward an ambiguous cue but not toward a cue that was perfectly predictive of shock (McHugh et al., 2015). These studies, in conjunction with the results presented here, suggest that unaltered serotonergic transmission to the hippocampus could possibly be required for proper defensive responding toward this type of ambiguous threat cue, while behaviour toward a perfect predictor cue remains unaltered by these manipulations.

### **Context as an Ambiguous Predictor of Threat**

The hippocampus is necessary for context fear conditioning, whereby lesions of this area decrease freezing behaviour when animals are re-exposed to the context that has been previously associated with shock (Anagnostaras et al. 2001, McNish et al. 1997, Zelikowsky et al. 2014). In these studies, an

animal is usually placed into a conditioning chamber and delivered unsignalled shocks. Later, when they are tested in this original conditioning environment as compared to a novel one, they show heightened freezing behaviour. It has been suggested that configural learning about the context, such that it becomes a multimodal cue, i.e. "context", which can then be associated with the shock, underlies the hippocampus's involvement.

Alternatively, it could be that the ambiguity about what the context engages the hippocampus. Specifically, what is common to this context conditioning and the ambiguous cue used in this study is that they both sometimes signal shock while other times signalling the absence of shock, or safety. This makes both the context in contextual fear conditioning and the discrete cues partially reinforced by shock in the present study's ambiguous predictors about an aversive event. Also in the current study, there was no evidence of contextual conditioning when examining the lead-in periods during the training, possibly owing to the fact that there were three distinct tones that all represented different outcomes and were possibly better predictors of shock or non-shock than the context itself.

### **Fear or Anxiety?**

When studying fear conditioning, especially using an ambiguous cue like that used here, studies should take into

account the potential theoretical differences that an ambiguous predictor about shock (and by extension the context) represents as compared to a cue that is un-ambiguous, or always associated with a shock outcome. For example, the ambiguous cue may generate anxiety; by contrast, a fully predictive cue of shock may generate fear. These two different states may, and likely do, elicit different defensive behaviours and brain areas processing these different cues. Indeed, there appears to be dissociation in structures relating to fear and anxiety (McHugh et al., 2004; McNaughton & Corr, 2004). Fear is mainly controlled by the amygdala and is associated with a current or immediate threat. Anxiety, however, is related to the anticipatory angst or the potential presence of threat and has been ascribed to the hippocampus, particularly the ventral portion (Bannerman et al. 1999, Blanchard & Blanchard 1988, Gray & McNaughton 2000, McHugh et al. 2004). The CS+ represents a perfect predictor of an aversive outcome and may represent an immediate or imminent threat, generating freezing behaviour and that is processed in extrahippocampal structures, such as the amygdala. Conversely, the ambiguous CSa cue may be associated with potential threat, mainly because the animal is unsure of its exact relation to threat, and therefore mediated by the ventral hippocampus (and extrahippocampal areas). Other threat-related behaviours, such as risk assessment and behavioural inhibition, are exhibited instead (Blanchard et al. 2001a).

## **Altered Learning?**

Traditionally, it has generally been found the hippocampus is not needed for fear conditioning where a cue is 100% predictive of shock, as is the case in delayed fear conditioning (Phillips & LeDoux 1992 but see Quinn et al. 2008, 2009; Richmond et al. 1999) . The training data in this study does suggest that the hippocampal-lesioned mice generally do show lower freezing responses to the ambiguous (CSa) cue (Figure 2.1 A). During the testing session (i.e. fear memory recall), which freezing was assessed toward each type of cue without shock, however, particularly the CS+ tone, these mice do show a robust increase in freezing response. It is possible that these lesioned animals exhibit slightly different defensive behavioural responses on days when shock is present (training sessions) compared to days when the shock is not present (fear memory recall testing day).

One group that looked at contextual fear conditioning (with unsignalled shocks) used both behavioural freezing and startle responses to measure performance. In one of their studies (McNish et al. 1997), rats that received dorsal hippocampal lesions retained their ability to show fear-potentiated startle but did show deficits in freezing toward the conditioned context. In a follow-up study (McNish et al. 2000), these authors used contextual blocking to indirectly assess the involvement of the dorsal hippocampus in contextual fear-potentiated startle. Using a

modification of Kamin's blocking effect in which conditioning to one stimulus is impaired if a prior conditioned stimulus is present at the same time, they showed that pre-exposure to the context resulted in blocking of conditioning to a light presented in that same context as measured by fear-potentiated startle (i.e. the prior exposure to the context blocked the conditioning to the light) (McNish et al. 2000). These studies provided evidence that the hippocampal-lesioned rats possibly still retained a contextual representation but had different defensive behavioural (performance) responses (Gewirtz et al. 2000, McNish et al. 1997, 2000).

While it is possible that these mice in this chapter may have learnt the CSa slower in general, there is precedence, albeit indirectly, that suggests that lesioning of the hippocampus can produce or modify defensive behavioural responses. It may be necessary to measure fear-potentiated startle instead of a freezing response toward the cues, which may provide a different set of results.

## **Conclusion**

Together, these results raise interesting questions relating to the hippocampus's specific involvement in this type of fear conditioning. The results presented here could be taken to mean that the hippocampus is simply playing a subordinate role in delayed fear conditioning with an elemental cue (i.e. tone) and

that the fully or partially predictive cue is relying more heavily on other structures, such as the amygdala. Similarly, as it pertains to the CS+, a ceiling effect may be masking any freezing deficit. These possibilities cannot be ruled out in this study. Moreover, in line with the current interpretation, is that the hippocampus is needed to separate out or resolve conflicting goals, or the associative structures of the memories that underlie them. The ambiguous cue forms associations in parallel (i.e. a CSa-shock and a CSa-non-shock association), and in normal animals the hippocampus may bias further processing toward treating it as a pure CS-shock, as is the case in the sham-lesioned mice. This biasing of processing, then, plays out behaviourally as defensive behaviour (freezing).

Moreover, because the ambiguous cue represents a sort of uncertainty with regard to its outcome, the ambiguous cue may produce a state of anxiety that is, at least in part, mediated by the ventral hippocampus. A fully predictive cue, however, is treated like a fear-inducing stimulus and the amygdala may primarily dictate the behavioural output. Although, it is likely that the hippocampus is constantly monitoring for conflicting information and is therefore always involved in these types fear conditioning trials with a fully predictive cue (Gray & McNaughton 2000).

# 3 ADULT NEUROGENESIS MEDIATES APPROPRIATE BEHAVIOURAL RESPONSES TOWARD AMBIGUOUS THREAT CUES

### 3.2 Abstract

Fear learning is critical to the survival of the organism. The ability to predict whether a threat is going to occur or not is necessary for continued well-being. In this chapter, mice with ablated adult neurogenesis and their wildtype littermates were tested on ambiguous and reliable fear conditioning (see Chapter 2), in which the cue signalled footshock 50% or 100% of the time, respectively. Mice with ablated adult neurogenesis showed selective impairments in defensive responding toward an ambiguous, but not toward a reliable, predictor cue of footshock. Specifically, these ablated mice showed diminished freezing and startle responding toward cues (lights and tones) that were partially reinforced with footshock. These mice, however, were indistinguishable from the wildtype mice on these performance measures when the cues were fully reinforced with footshock. In addition, these mice showed a decrease in hippocampal (dentate gyrus and CA3) Fos expression levels that mirrored the results seen from the behavioural measures. Finally, both mice with and without adult neurogenesis showed very little freezing to the conditioning context itself. Therefore, deficits in context conditioning are unlikely to underlie fear conditioning to the explicit cues.

### 3.3 Introduction

In the previous chapter, hippocampal lesions resulted in reduced defensive behavioural responding (freezing) when a discrete cue was made to be an ambiguous predictor about footshock. In that chapter, a within-subject design was used, whereby each mouse was exposed to all different cue conditions that were intermixed within each session. This design may promote cue generalisation and have more ecological validity at the expense of cue-specific effects. This generalisation may reflect a "spread of emotionality" after footshock and/or reactivity effects to other cues or future situations (Seo et al. 2015), and is of interest in many stress-related disorders, such as post-traumatic stress and panic disorders (Lissek et al. 2010).

More specific insults to the hippocampus have been shown to have similar effects on freezing toward an ambiguous-predictor cue when granule cells of the dentate gyrus were selectively silenced (Tsetsenis et al. 2007). This suggests that the granule cells in this region may be responsible for this processing of the ambiguous cue properties or that, because the dentate gyrus is a gate into the hippocampus proper, granule cell interference may impede the spread of the signal throughout the hippocampal network.

The lack of predictability of an aversive situation is a hallmark feature of what can constitute a stressor and has

psychological and physiological effects. Defining a stressor solely based on its influence on stress hormone levels is not sufficient and defining a stressor must encapsulate psychological states that co-occur with stress hormone elevation (Bonilla-Jaime et al. 2006, Bronson & Desjardins 1982, Koolhaas et al. 2011, Weiss 1970). Psychological factors, such as predictability (or lack thereof), play a role in how an animal perceives and categorizes a stimulus as a stressor or threat.

Predictability (or lack thereof) is, in some cases, more important than the objective intensity of the threat. In an elegant set of experiments, Weiss has shown that when rats are subjected to unpredictable shocks, as opposed to those where a signal warned of impending shock, they developed ulceration of the stomach, displayed higher body temperatures, and showed elevated corticosterone responses for a longer period of time (Weiss 1970, 1971). The unpredictability about a threat often can make animals become 'helpless', as Weiss also found, and can affect their appraisal process of stressors.

In the brain, not much is known about what regions are responsible for processing these unpredictable threats. Some clues, however, do exist. The hippocampus is a key neuroanatomical region that contributes to the physiological and behavioural stress response (McEwen et al. 2015). In addition, the hippocampus detects conflicting memories and plays a role in prediction about goal outcomes (Bannerman et al. 2014, Buckner

2010, Chudasama et al. 2009, Gray & McNaughton 2000, Tsetsenis et al. 2007). The unpredictability of shocks, for example, may therefore make the context (i.e. the immediate surroundings) in which the shocks occurred an ambiguous predictor about when shock occurs. The conflict generated by the context, such that it predicts both the presence and absence of shock, then, may hinder further predictions about when the shock occurs. Therefore, it seems at least reasonable that the hippocampus may be engaged in these types of situations where predicted outcomes cannot be generated due to conflicting, or overlapping, information.

In particular, recent work suggests that it is more specifically the dentate gyrus granule cells that mediate the behaviours toward ambiguous cues related to threat (Tsetsenis et al. 2007). Furthermore, adult-born granule cells seem to be particularly sensitive to stress effects and some animals with ablated adult-born neurons show anxiodepressive-like or defensive behaviours (David et al. 2009, Santarelli et al. 2003, Snyder et al. 2011). In line with this, it is plausible, then, that these adult-born neurons may mediate the current defensive behaviours when a cue is made to be an ambiguous predictor of threat.

Using transgenic mouse models to ablate adult neurogenesis (described more fully in the Introduction of Chapter 1), anxiodepressive-like behaviours have been observed.

Furthermore, the study of stress effects in the absence of new

neurons can shed new light on the regulation of stress and the pathophysiology of stress-related conditions, such as anxiety or depression (Miller & Hen 2014). Many of the animal models used to eliminate adult-born neurons are based on principles that were originally used in the detection of adult neurogenesis in normal rodents. The method of using a thymidine analogue in the labelling of cell division came from pioneering studies by Altman and colleagues, who injected a radioactive version (tritiated thymidine; thymidine-<sup>3</sup>H) into the animal. This tritiated thymidine is incorporated into the new DNA synthesis during the S-phase of cell division and labels the cell nuclei of newly divided cells. Using autoradiography, the radioactivity can be visualised as little grains located within cell nuclei (Altman & Das 1965, 1966).

Several years later, after advances in immunohistochemistry and confocal microscopy, this basic principle of using thymidine to detect new cell division is still in use today. Bromodeoxyuridine (BrdU) is used in place of tritiated thymidine to detect new cell division. BrdU is a thymidine analogue that also incorporates into the S-phase of new DNA synthesis. During the synthesis phase, a bromine atom is added to thymidine's methyl group. Using immunohistochemistry instead of autoradiography, antibodies are used to detect the presence of BrdU. The use of confocal microscopy has added significant advantages in characterising the precise identity of cells and allowing for confirmation that the BrdU is in the cell nuclei.

Specifically, when used in conjunction with neuronal markers like NeuN or doublecortin, confocal microscopy allows for co-labelling to be visualised. Meaning that, one can visualise cells that express BrdU and see if they are also positive for specific neuronal markers, allowing one to confirm its identity (neurons or glia).

Similarly, this basic principle is used in animal models that ablate adult neurogenesis using pharmaco-genetic manipulations. In this study, the GFAP-TK model (e.g. Delaney et al. 1996, Denny et al. 2012, Garcia et al. 2004, Groves et al. 2013, Imura et al. 2003, Lehmann et al. 2013, Saxe et al. 2006, Schloesser et al. 2009, Seo et al. 2015, Snyder et al. 2011), described in the Introduction chapter, is used to target adult-born neurons by the use of a transgene. Briefly, the herpes simplex virus thymidine kinase (HSV-TK) transgene targets the cells that express the glial fibrillary acidic protein (GFAP), causing these specific cells to express the herpes virus. Then, when the antiviral drug valganciclovir is administered, DNA damage occurs and cell death occurs in dividing cells (see Garcia et al. 2004).

In this study, using the GFAP-TK mouse model, we examined whether adult-born neurons play a necessary role in the processing of ambiguous cues of threat that mediate appropriate defensive behaviours. Moreover, it is hypothesized that, given that prior unpredictable shock can elicit long-lasting behavioural/adaptive effects and rodents prefer predictable

stressors (Parihar et al. 2011, Seo et al. 2015, van Dijken et al. 1993, Weiss 1970), an impairment in the processing (or disambiguation) of these ambiguous cues may also present lasting behavioural effects that aid in adaptation. This effect, like that seen in development, may rely on normal glucocorticoid signalling to help orchestrate appropriate behaviour and protect against anxiodepressive-like states (Champagne et al. 2009, Lehmann et al. 2013, Snyder et al. 2011, Takahashi 1996).

### 3.4 Methods

All procedures performed at the National Institutes of Health followed the Institute of Laboratory Animal Research guidelines and were approved by the Animal Care and Use Committee of the National Institute of Mental Health.

#### **Animals**

Transgenic male mice (TK mice) expressing herpes virus thymidine kinase under the human glial fibrillary acidic protein promoter and maintained on a CD-1 background (Snyder et al., 2011) were generated from heterozygous x wild type mating. They were weaned at 3 weeks of age, genotyped via PCR, and housed 3 to 4 per cage with mixed genotype siblings. Beginning at 8 weeks of age, mice were treated with valganciclovir in regular mouse diet (0.3%, 35 mg/kg per day), 4 days/week, for 8-9 weeks

before behavioural testing. Mice were housed under a 12 hour light:dark cycle (lights on at 7:00 AM for a minority of experiments; reverse lighting then occurred so the lights were on at 7:00 PM); all testing took place during their dark phase.

### **Handling and General Procedure**

Mice were handled for 3-5 min/day for at least 2 days prior to behavioural testing. On each testing day, mice were brought to a dark holding area and remained in their home cages for approximately 30 minutes prior to the start of the first session. After each mouse completed their session, it was placed in a holding bin (in a separate room) and then returned to the homecage once all mice in the cage were finished. Completed cages were in a separate room from those waiting to be tested.

### **Fear Conditioning and Extinction**

Fear conditioning was used in order to examine freezing toward cues that either reliably or ambiguously predicted a shock outcome 100% or 50% of the time, respectively. In traditional cued (delayed) fear conditioning that is fully reinforced, a conditioned stimulus (e.g. a tone) is paired with an unconditioned stimulus that is, in this case, inherently aversive (e.g. footshock). After sufficient pairings that produce an unconditioned response (e.g. bursting activity when the shock comes on), the animals

learn to predict that once it hears a tone, a shock will follow, producing a conditioned response (e.g. freezing) during the presentation of subsequent tones. In the case of partially reinforced or ambiguous fear conditioning, the conditioned stimulus is paired with the unconditioned stimulus partially (50% in this case).

### **Fear Conditioning Parameters**

Fear conditioning was conducted in a clear-walled chamber with internal dimensions of 30 x 30 x 24 cm (Coulbourn Instruments). A 0.5 mA, 1 second scrambled shock delivered through a grid floor was used as the unconditioned stimulus (US). A second, separate experiment using a weaker shock (0.3 mA) was also performed on a separate cohort of experimentally naive mice that experienced reliable fear conditioning. With the exception of the change to the shock intensity, the protocol was identical to that used for the reliable cue fear conditioning group. Each cue (Coulbourn bright cue light or 2 kHz, 85 dB(A) tone) lasted 20 s and served as the conditioned stimulus (CS). For half of the mice in each experiment, the cue always co-terminated with a shock ('reliable group'), resulting in 3 cue-shock pairings per session. The other half of the mice received the same 3 cue-shock pairings but also 3 additional cue-only trials in each

session, such that the cue only co-terminated with a shock 50% of the time ('ambiguous' group).

### **Fear Conditioning Procedure**

Two separate fear conditioning experiments were conducted using either a tone or a light as the conditioned stimulus. These experiments used separate cohorts of mice. In the tone fear conditioning experiment, mice were given 120 seconds to explore the chamber before any tone stimulus was presented. The conditioning session lasted 600 seconds. In the light fear conditioning experiment, mice were given 180 seconds to explore the chamber before any light stimulus was presented. The conditioning session lasted 660 seconds.

For both tone and light fear conditioning experiments, the inter-stimulus interval for the 'reliable conditioning' group on training days was variable ( $M = 170 \pm SD = 41$  s); and it was also variable for the 'ambiguous conditioning' group ( $M = 77 \pm SD = 18$  s).

### **Fear Memory Recall Test Procedure**

Twenty-four hours after the last session of fear conditioning, all mice were given a test day in a new context. On this test day, all mice experienced the same testing protocol. Six 20-second cues were played without co-terminating in a shock. All

groups had the same inter-stimulus interval parameters on the test day, with  $M = 80 \pm SD = 14$  s. The total percent of time spent freezing during the 6 cues was then compared to a baseline (the first 100 second lead-in period). Additionally, pre-CS freezing levels (in the tone fear conditioning experiment) were examined. These pre-CS levels were the immediate 20 seconds preceding each CS's onset.

Conditioning and testing contexts differed by the number of walls (4 vs. 5), distinct visual cues presented in and around the chambers, odour of the cleaning solutions (vanilla mixed with 70% EtOH or SimpleGreen cleaning solution (Sunshine Makers, Inc)), and textured floor (grid floor or smooth black panelling as the floor).

### **Fear Extinction Procedure**

Fear extinction was given to those mice in the reliable and ambiguous tone fear conditioning experiment. In the explicit extinction sessions in which they were presented with the six 20-second tones separated by an inter-stimulus interval of 40 seconds. They were considered extinguished when they displayed approximately 10% freezing toward the cue and no detectable differential freezing between the inter-trial interval and tone period. All testing sessions were separated by 24 hours.

## **Analysis of Freezing Behaviour**

Freezing was analyzed using FreezeView software (Coulbourn Instruments). Briefly, this software compared pixels from frame to frame and judged freezing as the absence of movement save that movement necessary for breathing. Baseline freezing was measured during the first 100 s of each trial and compared with freezing during the six 20-s presentations of the CS. Additionally, pre-CS freezing was assessed (20 s immediately preceding each CS).

## **Fear-Potentiated Startle**

Fear-potentiated startle has been a successful tool and is an alternative, objective measure of fear rather than freezing behaviour. In fear-potentiated startle, typically a pre-conditioning test is given to assess the startle reflex of the animal. A loud burst of white-noise is used to provoke this startle reflex behaviour from the animal. Trials where the white-noise burst is presented in isolation or an unconditioned tone immediately precedes the white-noise burst are intermixed with one another. The differences in startle behaviour toward these different trial types is measured. Next, the animal is fear conditioned to the tone (see the section above for a description). Then, a post-conditioning test is given that mirrors the test given during the pre-conditioning phase. Comparing startle behaviour pre- and post-conditioning describes

how the conditioned tone influenced the startle behaviour/startle reflex magnitude when the white-noise burst is played. Performing this experiment was very important, because it provides a separate performance measure of "fear". This independent measure of fear conditioning, as compared to freezing, has been used to dissociate contextual fear conditioning "deficits" in dorsal hippocampus-lesioned animals (McNish et al. 1997, 2000), suggesting a possible hierarchy of defensive behavioural responding.

### **Fear-Potentiated Startle Procedure**

Both fear conditioning and startle reflex testing were conducted using startle testing apparatus consisting of an acrylic tube attached to a load cell and amplifier (Med Associates Inc). The experiment was modelled after Falls (2002) and consisted of an explicit habituation day, two noise burst intensity (NBI) test days, a pre-conditioning test day, 3 days of "reliable" or "ambiguous" fear conditioning, and a post-conditioning test (8 total days), each separated by 24 hours except the second NBI test and pre-conditioning days, which were separated by 48 hours. Separate reliable and ambiguous fear conditioning groups were run on consecutive weeks.

## **Habituation**

During the explicit habituation day, mice were placed in the tube and chamber for 50 minutes with no stimuli except a background fan. Two days of NBI tests were carried out to identify the best intensities to serve as the startle noise (white noise) bursts. Various noise bursts ranging from 70 - 110 dB were given in a pseudo-random order. Startle stimuli based on these results were selected to serve as the white-noise bursts used during the actual experiment. In the experiment 75, 80, and 85 dB were selected and startle responses to 75 and 80 dB were averaged together. Behaviour toward the 85 dB produced a ceiling effect and was possibly a fearful stimulus itself without prior conditioning.

## **Pre-Fear Potentiated Startle Behaviour**

During pre-conditioning, mice were placed in the startle boxes for two minutes before any stimuli were presented. A “leader sequence” was recorded in which different intensities of the noise burst were played to assess startle responses in the absence of hearing any tones beforehand. This was used as an acclimation period to the noise bursts and to select appropriate noise-burst intensities. In the “follower sequence”, startle responses to noise-burst alone (NBA) and startle responses to noise burst preceded by a tone (TNB) trials were measured to

serve as a baseline for comparison with post-conditioning responses. Tones were 20 seconds in duration, 2 kHz, and 70 dB(A), with no rise/fall time. In the leader sequence, each noise burst intensity (75, 80, 85 dB) was presented three times alone (total of 9 trials) in a pseudo-random order. Immediately following this, a “follower sequence” was given consisting of NBA trials at the three intensities which were inter-mixed with TNB trials for each intensity (three per intensity). All ISIs were 30 seconds.

### **Fear Conditioning**

Conditioning followed a protocol similar to that used in the cued fear conditioning experiments with freezing as the dependent measure. Fear conditioning took place in the same startle apparatus and mice were confined in the acrylic tubes with a shock floor inserted into the tube. Mice were fear conditioned with 3 tone-shock pairings per session for the reliable group and 3 tone-shock pairings and an additional 3 tone-alone trials for the ambiguous cue group in pseudo-random order. However, the shock used as the unconditioned stimulus was 250 milliseconds in duration at 0.5 mA. The inter-trial interval was variable between 80-120 seconds for the reliable group and between 50-70 seconds for the ambiguous group to keep total time in the startle apparatus approximately equal.

### **Post-Fear Potentiated Startle Behaviour Test**

To test startle to the conditioned tone in the absence of a shock, the same protocol as in pre-conditioning was used. The absolute values of the maximum and minimum amplitudes were used to give a total peak-to-peak startle value. For all data, peak-to-peak values were collected 600 milliseconds following the startle stimulus, and the average of the difference scores from the 75 and 80 dB noise bursts were used. Fear-potentiated startle was defined as an increase from pre- to post-conditioning in startle difference. The startle difference was the difference between startle responses on trials with the tone presentation prior to the noise burst (TNB) and those when the noise burst occurred alone (NBA).

### **Corticosterone Measurements**

In order to validate that (i) fear conditioning is in fact stressful to the animal and (ii) to investigate if ambiguous fear conditioning is more 'stressful', we measured corticosterone levels. In stressful situations, corticosterone is released from the adrenal glands in an experience-dependent manner. The hippocampus and adult-born neurons play a major role in detecting and providing negative feedback to shut off further corticosterone production (McEwen et al. 1968, Sapolsky et al. 1984, Snyder et al. 2011). To examine these experience-

dependent effects of prior predictability of fear conditioning, a separate cohort of animals received ambiguous or reliable fear conditioning and submandibular blood samples were taken from the live mice using a 3-mm Goldenrod animal lancet (MEDpoint, Inc). Blood was collected in a non-heparinised 1.5 ml Eppendorf tube and allowed to clot. One to two drops of blood were collected 30 minutes after they completed their third training session of reliable or ambiguous fear conditioning. Once samples began clotting, they were spun in a centrifuge and 3-10  $\mu$ l of serum was pipetted from the tube and samples were stored in -80 °C freezer before measurement. Corticosterone was measured via double antibody corticosterone radioimmunoassay (MP Biomedicals). This assay is based on the competition for the limited binding sites of specific antibodies of corticosterone in the blood's sample and the radioactively labelled antibody (via attachment to iodine-125). The radioactively labelled corticosterone and rabbit anti-corticosterone were incubated with the samples (diluted with a 1:50 steroid diluent) for approximately two hours to form an antibody-antigen binding complex caused by a polyethylene glycol and goat anti-rabbit antibody reaction. This was then centrifuged to form a precipitant containing the excess or unbound antigen. This was then run through a  $\gamma$ -counter. The amount of radioactively labelled corticosterone were inversely related to the sample's corticosterone. Control measurements of corticosterone were provided by the manufacturer for comparison.

## Groups

To serve as a baseline control condition, one group of WT and TK mice was trained for the first two days on reliable fear conditioning, but on the third day, they were taken directly from their homecage to take blood samples. The other two groups experienced either reliable or ambiguous fear conditioning for the entire three days prior to blood sampling thirty minutes after completion of fear conditioning.

## Histology

### **Bromodeoxyuridine and Fos Expression**

In order to visualise activity within the hippocampus and see if this activity was related to the adult-born neurons after reliable or ambiguous fear conditioning, we used the immediate-early gene, *c-fos* (and stained for the protein it encodes, Fos) as a proxy of neuronal activity (see Guzowski et al. 2001). In order to examine whether adult-born neurons in the dentate gyrus were activated by fear conditioning, we first had to tag newly dividing cells by giving these experimentally naive mice BrdU (1 mg/ml; Roche, in 0.9% saline with 1% sucrose) in drinking water for 6 days. The mice then underwent tone-cued fear conditioning 4 weeks after the BrdU treatment began. Two hours following the start of the 3<sup>rd</sup> training session, mice were transcardially perfused

with 4% paraformaldehyde. Brains were post-fixed for 24 hours and cryoprotected in glycerol. Brains were then cut coronally (40  $\mu\text{m}$ ) on a sliding microtome in 1:8 sections throughout the entire rostral-caudal extent of the hippocampus. The tissue was then stained for the presence of BrdU, Fos expression, and the mature neuronal marker NeuN.

For BrdU-Fos-NeuN triple staining, free-floating sections were washed in 0.1M PBS, denatured in 2N HCl:PBS for 2 hours, washed in 0.1M PBS, immersed in blocking solution for 20 minutes, and directly incubated in primary solution for 3 nights using the following primary antibodies: rat anti-BrdU (1:200; Accurate Chemical), goat anti-Fos (1:250; Santa Cruz Biotechnology), and mouse anti-NeuN (1:250; Chemicon). After primary incubation, sections were washed and incubated in secondary solution (donkey anti-rat Alexa 488 to visualize BrdU, donkey anti-rabbit Alexa 555 or donkey anti-goat Alexa 555 to visualize Fos expression, and donkey anti-mouse Alexa 633 to visualize NeuN; all 1:200, Life Technologies) for 1 hr before being washed and mounted on slides. Slides were cover-slipped with PermaFluor (Thermo Scientific) and coded prior to data analysis.

For all estimates of immediate-early gene expression, all Fos+ cells on every eighth section in one hemisphere through the entire dentate gyrus and CA3 were counted. Counts were multiplied by 16 to estimate the cell number throughout the entire hippocampus. In addition, all Fos+ cells in every eighth section

through the entire dentate gyrus were counted and examined for co-labelling with BrdU. Each potential double-labelled cell was scanned by obtaining optical stacks of 1  $\mu\text{m}$  thick sections and analyzed using an Olympus FV300 confocal microscope and a 60X oil-immersion lens. To verify double-labelling, cells were examined in orthogonal planes.

### **Doublecortin**

To confirm genotypes obtained via PCR (see above under 'Animals') and the valganciclovir effects on neurogenesis, all WT and TK mice from all experiments were sacrificed and brains postfixed in 4% paraformaldehyde in phosphate-buffered saline (pH = 7.4) overnight. Brains were then transferred to 10% glycerol solution for 24 hours and then transferred to 20% glycerol solution for 48 hours. After cryoprotection, brains were sectioned coronally at 40  $\mu\text{m}$  on a freezing microtome. Free-floating sections were used. These sections were washed 3 times for 5 minutes each in PBS and placed in 0.6%  $\text{H}_2\text{O}_2$  in PBS and incubated for 30 minutes at room temperature. Sections were again washed with PBS. Next, sections were placed in blocking solution (0.5% tween-20, 3% normal donkey serum) in PBS, and incubated for at least 30 minutes. Doublecortin (primary antibody goat anti-doublecortin; Santa Cruz; 1:200) was added in to the blocking solution and left overnight. Sections the next day were washed in

PBS and secondary antibody (donkey anti-goat (Alexa fluorescent family)) in 1:250 PBS and incubated for 90 minutes. Sections were again washed and stored in PBS. The next day, a Hoechst (blue fluorescent family) counterstain (of deoxyribonucleic acid) in 1:1000 PBS solution was used and incubated for 10 minutes. Sections were then mounted onto ColorFrost or SuperFrost slides and cover-slipped with Paramount medium. Slides were examined using an Olympus FV300 confocal microscope. The lack of doublecortin-positive neurons demonstrated the effectiveness of the valganciclovir.

## Statistics

Data were analysed with SPSS and GraphPad Prism. All ANOVAs performed were two-way mixed ANOVAs, unless otherwise specified, and Bonferroni correction was applied for post-hoc analyses. The between-subject factor was genotype<sub>WT, TK</sub> and within-subject factor of period<sub>pre-CS or baseline, CS</sub> (for the fear conditioning data) or time<sub>seconds or days</sub> (for the bursting activity and extinction data, respectively). If the within-subjects factor did not meet Sphericity, the Greenhouse-Geisser corrected degrees of freedom, and its associated  $F$  and  $p$  value, was used. If homogeneity of variance (by Levene's test) was violated with fewer than 10 subjects per group, a modified  $p$  criterion was used ( $< 0.03$ ). All  $t$ -tests were unpaired (two-tailed). All bars in the

figures represent the mean and error bars represent standard error of the mean.

## 3.5 Results

### Doublecortin Cell Counts

Throughout the hippocampus, virtually all of the adult-born cells were eliminated (see Snyder et al. 2011 for other stereological counts, e.g. BrdU counts), evidenced by the absence of doublecortin-positive cells in treated TK mice in Chapter 5's experimental animals (see Figure 5.2, A) (one-tailed Man-Whitney nonparametric test,  $U < 1$ ,  $p = 0.0048$ , *a priori* assumption that TK mice would show very few young neurons and equal variance and distribution were not assumed). WT mice treated with valganciclovir showed high levels of doublecortin-positive cells ( $n = 4$  brains;  $M = 4282 \pm 441.2$  cells) compared to treated TK mice ( $n = 6$  brains;  $M = 9.33 \pm 4.34$  cells).

Representative images from the dorsal dentate gyrus are shown in Figure 5.2 (B) and doublecortin-positive cells in WT mice that were treated are easily visible (brown dots) in the subgranular zone, while none are visible in TK mice that were treated with valganciclovir (see Figure 5.2, B).

## **Tone Fear Conditioning**

To model ambiguity of a single stressor within the lab, fear conditioning was performed over four days, with the first three being training days with tone-shock pairings and the fourth serving as a tone test (fear memory recall) day in a novel context (Figure 3.1).

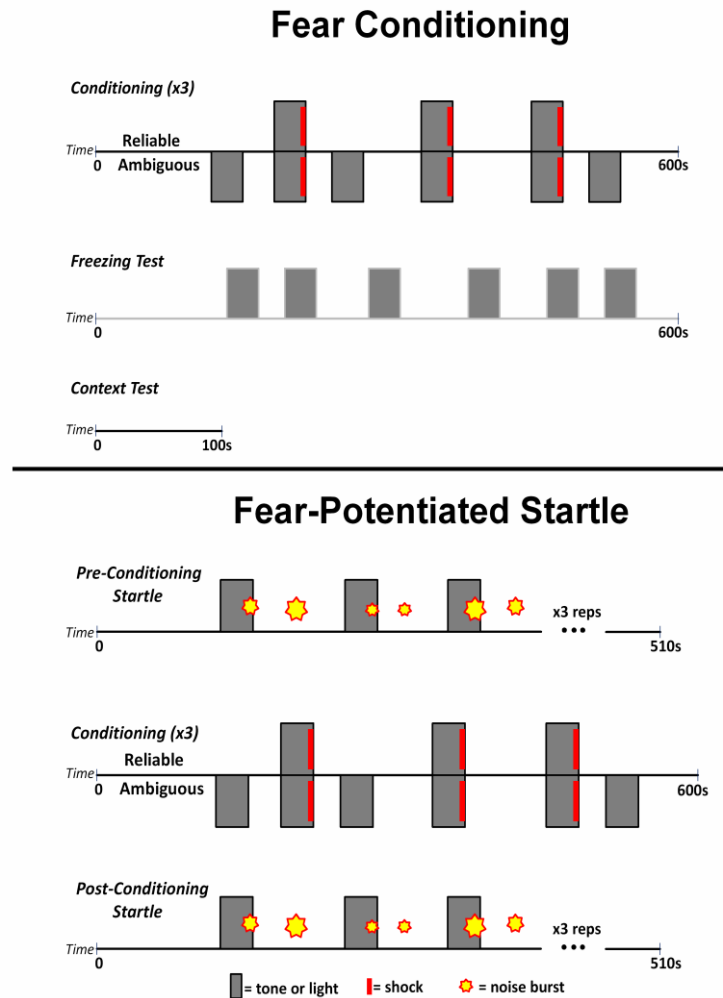


Figure 3.1 An example experimental timeline of fear conditioning and fear-potentiated startle experiments. Top: For Fear Conditioning, WT and TK mice were split into either a group that received reliable- or ambiguous-cue predictability about shock outcome. Bottom: For Fear-Potentiated Startle (bottom), mice received reliable or ambiguous fear conditioning and their post-conditioning startle responses were compared to their pre-conditioning startle responses. Different sizes of the star (noise burst) represent different decibel levels.

In fear conditioning, the percentage of time freezing during the conditioned stimulus is measured and compared to the baseline freezing levels. In one group of mice, both wildtype (WT;  $n = 7$ ) and transgenic mice lacking adult neurogenesis (TK;  $n = 8$ ) experienced three 20-s tones that always predicted an upcoming shock ('reliable' group). In littermates, WT ( $n = 7$ ) and TK mice ( $n = 9$ ) experienced six 20-s tones, where half co-terminated with a shock and the other half did not lead to a shock ('ambiguous' group) (Figure 3.1). During the first conditioning day, reactivity (unconditioned responses) to the first tone presentation (first 3 seconds) and the first shock in 3-second timebins were examined to see if sensorimotor abilities were similar between WT and TK mice. No differences were found between the genotypes in terms of freezing but freezing did increase significantly upon the first tone's onset (genotype effect:  $F_{1, 27} < 1$ ; period effect (baseline vs. tone):  $F_{1, 27} = 4.86$ ,  $p = 0.036$ ; interaction effect:  $F_{1, 27} = 2.87$ ,  $p = 0.10$ ) (Figure 3.2 A) (two WT mouse's videos did not get captured during this day but hardware for fear conditioning was working). Similarly, no differences were detected in terms of the unconditioned responses to the first shock between the genotypes but bursting activity in the 3 seconds after the shock's onset did steadily increase, with no interaction between the variables (genotype effect:  $F_{1, 27} < 1$ ; time effect:  $F_{2, 54} = 83.93$ ,  $p < 0.0001$ ; interaction effect:  $F_{2, 54} = 1.63$ ,  $p = 0.21$ ) (Figure 3.2 B) (one WT

and one TK mouse could not accurately be traced with the TopScan software due to shadows upon video capture).

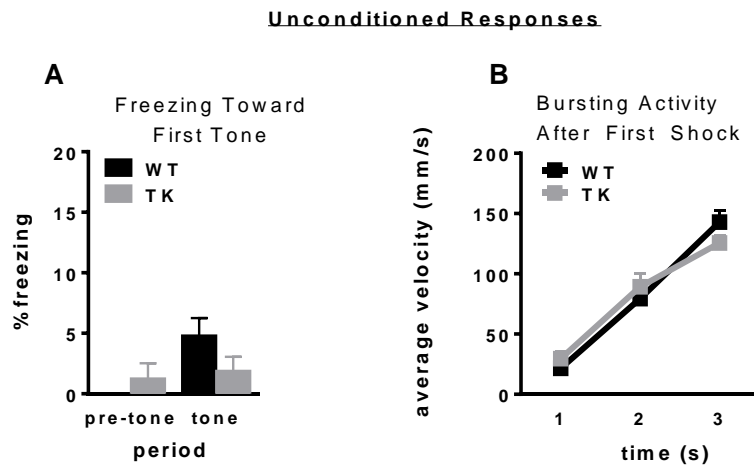


Figure 3.2 A: The freezing reaction to first tone did increase percent freezing as compared to the pre-tone period but these levels did not differ between WT and TK mice. B: The average velocity of bursting activity did increase across the total 1-s timebins after the first shock but did not differ between WT and TK mice (right).

After conditioning to the tone stimulus was complete, all mice were given a tone test (fear memory recall) day in a novel context, during which six tones were presented with no co-termination with shock. Freezing toward the tone period was compared to the baseline period leading up to the first tone's onset. In the reliable group (Figure 3.3, A), there was no difference between WT and TK mice but both groups did show increased freezing levels when the tones came on relative to

baseline (period effect (baseline vs. tone):  $F_{1, 12} = 48.56$ ,  $p < 0.0001$ ; genotype effect:  $F_{1, 12} < 1$ ; interaction effect:  $F_{1, 12} < 1$ ).

In the ambiguous condition, both groups showed an increase in freezing as compared to baseline but there was a trending overall effect of genotype, and, importantly, a significant interaction between these variables (period effect (baseline vs. tone):  $F_{1, 14} = 55.93$ ,  $p < 0.0001$ ; genotype effect:  $F_{1, 14} = 3.65$ ,  $p = 0.08$ ; interaction effect:  $F_{1, 14} = 5.0$ ,  $p = 0.04$ ). During the 'tone' period, TK mice showed a decrease in freezing as compared to their WT counterparts (Bonferroni post-hoc:  $p = 0.013$ ) (Figure 3.3, B).

The next day, mice were placed back into the original conditioning context and experienced the same protocol as the day before (i.e. tone presentations without shocks). The first 100 seconds served as a test for contextual conditioning. To this end, there was no genotype effect but a trend for the cue/predictor (reliable vs. ambiguous fear conditioning) effect, and no interaction between the two variables (genotype effect:  $F_{1, 27} < 1$ ; predictor effect:  $F_{1, 27} = 3.7$ ,  $p = 0.07$ ; interaction effect:  $F_{1, 27} < 1$ ). Freezing levels were very low (means  $< 25\%$ ), suggesting very little conditioning to the context occurred (not graphed). This low level likely reflected general activity levels of the mice and not fear-induced freezing to the conditioning context.

Mice were next given explicit extinction sessions in the original conditioning context the following days. Only data from

the reliable conditioning group could be reasonably interpreted, because both genotypes started their extinction trials with the same freezing level to the tones (Figure 3.3, C). The data show that both genotypes extinguish freezing behaviour at a similar rate (extinction day effect:  $F_{5, 65} = 21.86$ ,  $p < 0.0001$ ; genotype effect:  $F_{1, 13} < 1$ ; interaction effect:  $F_{5, 65} < 1$ ).

It is possible that the effect of ablating adult neurogenesis on ambiguous cue fear conditioning but the lack of a genotype difference on the reliable cue task reflects the weaker/slower learning in the former training condition. Furthermore, it is possible that in the reliable cue condition there is a ceiling effect that may be obscuring a genotypic difference in freezing levels. A follow-up experiment using a weaker shock intensity (0.3 mA instead of the normal 0.5 mA) was therefore performed in WT ( $n = 12$ ) and TK mice ( $n = 15$ ) mice (Figure 3.3, D). The weak shock experiment under reliable fear conditioning resulted in no differences between genotypes but both genotypes did show an increase in freezing relative to baseline (genotype effect:  $F_{1, 25} = 2.61$ ,  $p = 0.12$ ; period effect (baseline vs. tone):  $F_{1, 25} = 48.37$ ,  $p < 0.0001$ ; interaction effect:  $F_{1, 25} < 1$ ). These results suggest that, like that with a stronger shock intensity, no genotype differences exist on the reliable fear condition task with a weak shock intensity.

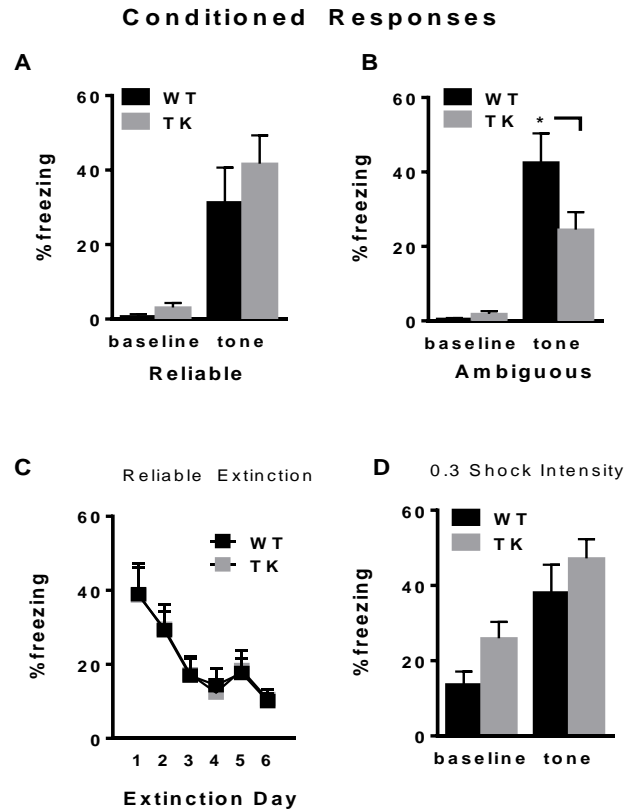


Figure 3.3 TK mice exhibited decreased freezing toward an ambiguous, but not a reliable, predictor of shock after fear conditioning. A: WT and TK mice similarly increased their percent freezing during the tone as compared to the baseline period on the fear memory recall test for the reliable tone. B: However, TK mice showed lower freezing during the tone period as compared to WT mice on the fear memory recall test for the ambiguous tone. C: The percent freezing during reliable cue extinction across six days was similar for both genotypes. D: A weaker shock intensity (0.3 mA) for reliable fear conditioning also revealed no genotypic difference. Bonferroni post-hoc: \*  $p < 0.05$  relative to wild-type in tone condition.

Some studies compare the level of freezing to the conditioned stimulus against a baseline (e.g. 'global baseline'; the first 100 seconds of the session) (see Drew et al. 2010, Tsetsenis et al. 2007). An alternative approach adopted in other studies (see Esclassan et al. 2009) is to take a "local" baseline for each CS cue (i.e. take the 20 seconds immediately before each tone CS onset (the pre-CS period) and the 20 second during CS presentation). Examination of the data using each individual period of pre-tone freezing levels as a 'local baseline' revealed a similar pattern of results as obtained using the 'global baseline' freezing. In the reliable condition (Figure 3.4, A), WT and TK mice showed markedly higher freezing to the tone compared to the local pre-tone period (period effect:  $F_{1, 12} = 56.06$ ,  $p < 0.0001$ ; genotype effect:  $F_{1, 12} < 1$ ; interaction effect:  $F_{1, 12} < 1$ ). Likewise, pre-tone freezing was markedly lower for the mice that received conditioning to an ambiguous predictor tone (Figure 3.4, B) and showed Bonferroni post-hoc differences during the tone period as compared to when the tone was presented (post-hoc  $p < 0.05$ ) (period effect:  $F_{1, 14} = 41.43$ ,  $p < 0.0001$ ; genotype effect:  $F_{1, 14} = 4.49$ ,  $p = 0.053$ ; interaction effect:  $F_{1, 14} = 2.68$ ,  $p = 0.12$ ). Although there was no significant interaction between genotype and period for the ambiguous cue condition (pre-tone vs. tone), an *a priori* hypothesis was that TK mice would freeze less to ambiguous cues based on the previous findings (e.g. see Chapter 2; Tsetsenis et al. 2007), which provided a basis for selected post-

hoc comparisons. Bonferroni post-hoc testing revealed that, indeed, there was a difference during the tone period between WT and TK mice ( $p < 0.05$ ) (Figure 3.4, B).

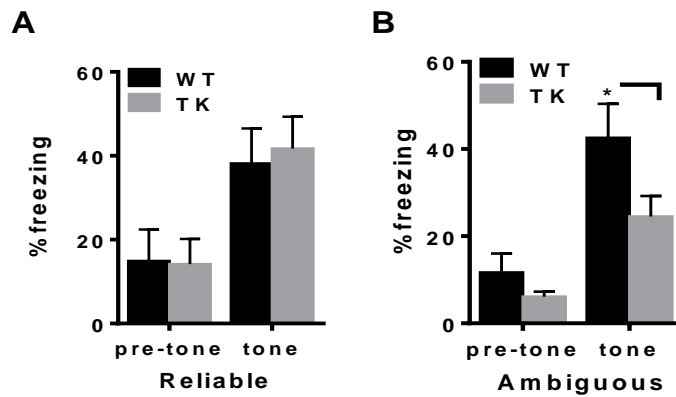


Figure 3.4 A: Both genotypes did not differ in their percent freezing during either period (pre-tone vs. tone) after reliable fear conditioning. B: The percent freezing did not differ between genotypes during the pre-tone period but did during the tone period for the ambiguous condition; TK mice showed diminished freezing when conditioned to an ambiguous predictor of shock. Bonferroni post-hoc: \*  $p < 0.05$ .

To understand how freezing developed over the course on the fear memory recall test, and how individual tones impacted behaviour, 20-second timebins throughout the test were analysed. In mice that received reliable conditioning (Figure 3.5, A), who showed similar freezing levels overall to all six tone presentations, there was no effect of genotype but an increase in freezing across

timebins, with no interaction between those variables (time effect:  $F_{32, 384} = 7.3, p < 0.0001$ ; genotype effect:  $F_{1, 12} < 1$ ; interaction effect:  $F_{32, 384} < 1$ ). In mice that received ambiguous conditioning (Figure 3.5, B), however, there was a general main effect of timebin but no overall difference between the genotypes (no main effect of genotype). However, there was a significant genotype x timebin interaction (genotype effect:  $F_{1, 14} = 3.2, p = 0.09$ ; timebin effect:  $F_{32, 448} = 9.4, p < 0.0001$ ; interaction effect:  $F_{32, 448} = 1.8, p = 0.005$ ). Bonferroni post-hoc analyses revealed that, at 440 seconds (i.e. during the fifth tone presentation of the session), WT and TK did differ significantly ( $p = 0.007$ ) (Figure 3.5, B).

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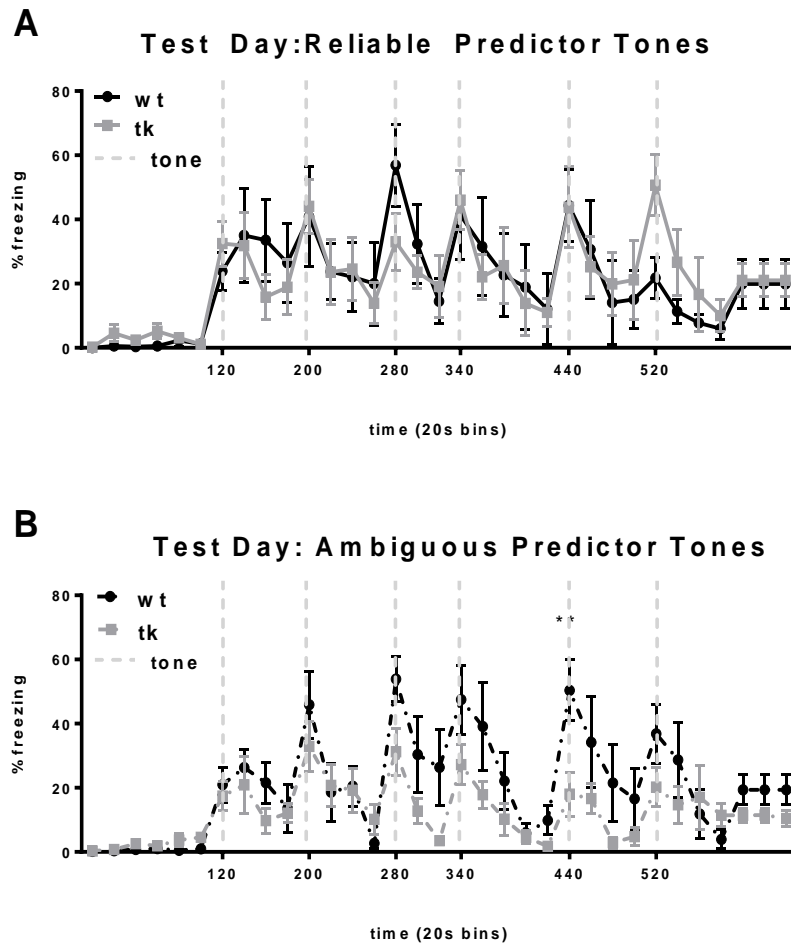


Figure 3.5 A: The percentage of freezing across 20-second timebins throughout the reliable fear memory recall test showed the WT and TK mice were indistinguishable across these timebins. B: TK mice, however, reduced their freezing during ambiguous fear memory recall test across timebins relative to WT mice. Vertical dotted grey lines represent the timebin when the tone was presented. Bonferroni post-hoc: \*\*  $p = 0.007$  between WT and TK mice at time 440 seconds.

In a separate cohort of animals, corticosterone levels were taken after they experienced this tone fear conditioning to examine their serum corticosterone levels (Figure 3.6). Both groups of mice that went through the entire ambiguous (*n*'s WT = 9; TK = 9) or reliable (*n*'s WT = 8; TK = 8) fear conditioning experienced elevated corticosterone levels relative to their experimental controls ('baseline'), who only went through the first two days of reliable fear conditioning (*n*'s WT = 7; TK = 7). There was no main effect of genotype but an overall effect of conditioning type received, with no interaction between the two (conditioning type<sub>baseline, reliable, ambiguous</sub> effect:  $F_{2, 42} = 16.7$ ,  $p < 0.0001$ ; genotype effect:  $F_{1, 42} < 1$ ; interaction effect:  $F_{2, 42} < 1$ ). Posthocs on conditioning type revealed significant differences between the baseline vs. reliable condition ( $p < 0.0001$ ) and baseline vs. ambiguous conditioning ( $p = 0.0006$ ). There was no difference between the reliable vs. ambiguous condition.

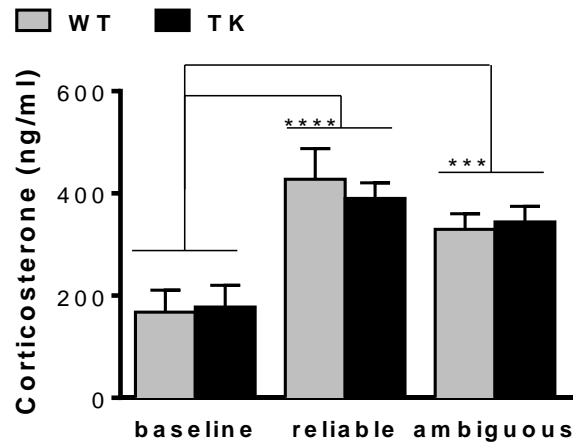


Figure 3.6 Fear conditioning using a 0.5 mA shock intensity increased serum corticosterone level measurements after the third fear conditioning day in the different conditions. Both reliable and ambiguous fear conditioning elevated these levels compared to homecage controls on the third conditioning day. \*\*\*\*  $p < 0.0001$ ; \*\*\*  $p = 0.0006$ .

### Light Fear Conditioning

Tone and light stimuli effects could potentially produce different behavioural reactions, especially if any modality-specific differences in the strain of mouse (e.g. age-related hearing loss) exist. To replicate our findings and to assess their generality to stimuli from a different sensory modality (i.e. visual stimuli), another experiment was conducted using light cues as the conditioned stimuli.

A separate cohort of mice was conditioned using the same basic design as used previously with acoustic tones but now with a different sensory modality for the conditioned stimulus. Similar to the previous results for tone fear conditioning, half of the WT ( $n = 9$ ) and TK mice ( $n = 8$ ) underwent reliable conditioning, whereas the other WT ( $n = 10$ ) and TK mice ( $n = 8$ ) were assigned to the ambiguous condition. On the first training day and during their first experience of seeing the light cue (first 3 seconds), there was a small increase in freezing behaviour relative to pre-light period for all mice (one TK mouse excluded because video was not captured correctly) but no genotype effect, and no interaction between the variables (effect of period (pre-light vs. light):  $F_{1, 33} = 249.40$ ,  $p < 0.0001$ ; genotype effect:  $F_{1, 33} < 1$ ; interaction effect:  $F_{1, 33} < 1$ ) (Figure 3.7, A). Both genotypes, again, were indistinguishable when it came to bursting activity to the shock's onset (activity measured for 3 seconds), showing only an overall effect of time (time effect:  $F_{2, 66} = 198.6$ ,  $p < 0.0001$ ; genotype effect:  $F_{1, 33} < 1$ ; interaction effect:  $F_{2, 66} = 1.87$ ,  $p = 0.16$ ) (Figure 3.7, B).

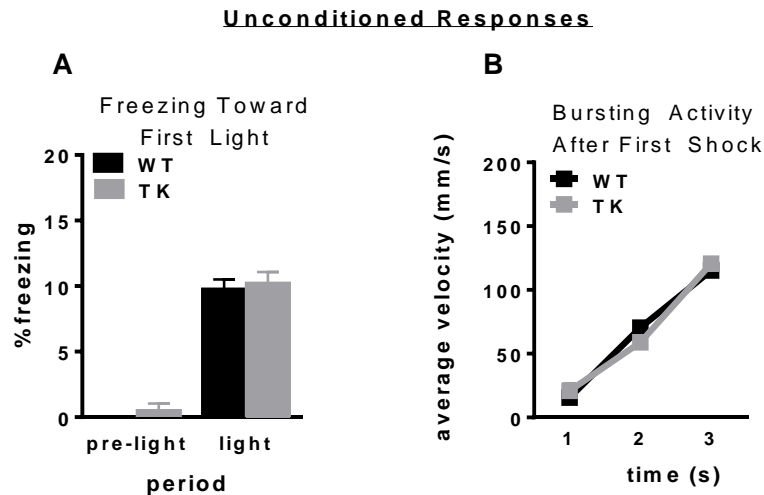


Figure 3.7 A: WT and TK mice showed similar increases in percent freezing upon exposure to the light compared to the pre-light period. B: Both WT and TK both showed similar increases in average velocity (bursting activity) when the first shock occurred.

After their reliable or ambiguous conditioning was complete, mice were tested on their freezing toward the light cue in a novel context (fear memory recall test). Consistent with the tone fear conditioning results above, mice that underwent reliable light conditioning showed no genotype difference in their freezing response but overall showed an increasing in freezing relative to baseline period, with no interaction between the variables (period effect (baseline vs. light):  $F_{1, 15} = 18.37$ ,  $p = 0.0007$ ; genotype effect:  $F_{1, 15} < 1$ ; interaction effect:  $F_{1, 15} < 1$ ) (Figure 3.8, A). Mice

that underwent ambiguous conditioning, however, showed a significant main effect of genotype, reflecting lower freezing levels overall in the TK mice, and mice exhibited a significant increase in freezing toward the light compared to the baseline period; there was no interaction between the two variables (genotype effect:  $F_{1, 15} = 9.13$ ,  $p = 0.0086$ ; period effect (baseline vs. light):  $F_{1, 15} = 10.40$ ,  $p = 0.0057$ ; interaction effect:  $F_{1, 15} = 1.44$ ,  $p = 0.25$ ). Overall, this suggests that TK mice froze less than WT mice in the ambiguous fear memory recall test, regardless of the presence/absence of the light. Nevertheless, based on our data from the prior fear conditioning study with the ambiguous tone cue, the *a priori* hypothesis was that TK mice would show diminished freezing during the ambiguous light cue. Bonferroni post-hoc testing revealed that, during the ambiguous light presentation, WTs froze more than TKs ( $p = 0.0075$ ), but not during the pre-light baseline period ( $p > 0.05$ ) (Figure 3.8, B).

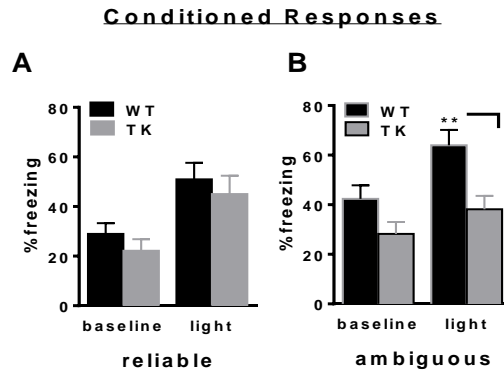


Figure 3.8 A: Both WT and TK mice increased their freezing similarly during reliable fear conditioning (fear memory recall test). B: TK mice overall froze less during ambiguous conditioning, an effect not seen after reliable conditioning but importantly showed a decreased freezing during the light period. Bonferroni post-hoc: \*\*  $p < 0.01$ .

To further examine if freezing toward the ambiguous cue was specific in any way to the stimulus type (light or tone), the percentage freezing data from both experiments (during the 'tone' or 'light' period only) were placed into a three-way, between-subjects ANOVA (genotype<sub>WT, TK</sub> x stimulus type<sub>tone, light</sub> x predictor cue<sub>reliable, ambiguous</sub>). The freezing to the cue period were only used because the baseline levels differed between stimuli substantially. ANOVA revealed a significant effect of genotype<sub>WT, TK</sub> ( $F_{1, 56} = 5.72, p = 0.02$ ), which reflected lower freezing levels in TK mice overall, and a significant main effect of stimulus type<sub>tone, light</sub> ( $F_{1, 56}$

= 6.99,  $p = 0.011$ ), which reflected that the light condition elicited higher freezing overall. There was, however, no main effect of predictor<sub>reliable, ambiguous</sub> ( $F_{1, 56} < 1$ ) and no predictor<sub>reliable, ambiguous</sub> X stimulus type<sub>light, tone</sub> interaction ( $F_{1, 56} < 1$ ). In addition, there was no interaction between genotype<sub>(WT, TK)</sub> X predictor<sub>(reliable, ambiguous)</sub> X stimulus type<sub>light, tone</sub> interaction ( $F_{1, 56} < 1$ ). Importantly, there was an interaction between genotype<sub>WT, TK</sub> X predictor cue<sub>reliable, ambiguous</sub> ( $F_{1, 56} = 4.61, p = 0.036$ ). This interaction is evidence that TKs in the ambiguous, but not reliable, condition selectively showed diminished freezing compared to WTs (Bonferroni post-hoc  $p = 0.002$ ), and that this effect was not dependent upon the conditioning stimulus type (light or tone; lack of a significant three-way interaction), but rather only the predictability of the cue.

## Fear-Potentiated Startle

Freezing is a situation-specific response and is only one possible performance measure of fear-related behaviour. To assess the generality of our findings from freezing assays but using a different performance measure, we used the startle response toward a noise-burst that was either preceded by a conditioned fear stimulus (i.e. tone) or not. We repeated the ambiguous and reliable cue fear conditioning experiments to see whether the same results would be obtained with the startle

measure. Alternatively, a possible dissociation in defensive behaviours (freezing vs. startle responding) may emerge.

In those WT ( $n = 11$ ) and TK mice ( $n = 11$ ) mice that received reliable fear conditioning, there was a main effect of period (pre- to post-conditioning sessions) but not genotype, with no interaction between the two variables (period effect:  $F_{1, 20} = 4.71$ ,  $p = 0.042$ ; genotype effect:  $F_{1, 20} < 1$ ; interaction effect:  $F_{1, 20} < 1$ ) (Figure 3.9, A). After WT ( $n = 11$ ) and TK ( $n = 10$ ) mice received ambiguous fear conditioning, however, there was an overall effect of genotype and no effect of period (pre- to post-conditioning sessions), but a significant interaction between these variables (genotype effect:  $F_{1, 19} = 4.65$ ,  $p = 0.044$ ; period effect:  $F_{1, 19} = 1.16$ ,  $p = 0.29$ ; interaction effect:  $F_{1, 19} = 4.52$ ,  $p = 0.047$ ). Bonferroni post-hoc tests revealed that TK mice showed a lower startle difference score (startle response to those trials where a tone preceded the noise burst minus those trials where a noise burst was presented alone) after ambiguous cue fear conditioning only, as compared to their WT counterparts ( $p = 0.0088$ ) (Figure 3.9, B). These results from a second performance measure (startle) suggest that, like freezing, startle responding also was diminished toward an ambiguous conditioned tone but was intact toward a reliable conditioned tone.

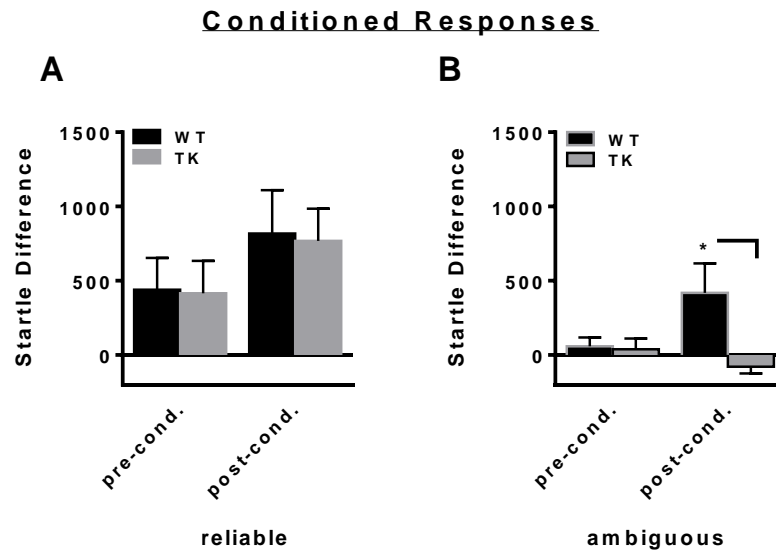


Figure 3.9 A: Both wildtype and TK mice showed similar increases in startle responding (difference scores) when conditioned with a reliable tone (pre- vs. post-conditioning) during fear conditioning. B: Mice without adult-born neurons, however, showed a diminished startle response (difference score) after being fear conditioned with an ambiguous tone (pre- vs. post-conditioning). Bonferroni post-hoc: \*  $p < 0.05$ .

### Dentate and CA3 Activity: Fos Expression

These results, thus far, have provided evidence that ablating a small population of adult-born neurons in the dentate gyrus can have strong effects on defensive behaviours, specifically to ambiguous cues of threat. What, though, are the wider downstream network effects of ablation of this population of

cells? To examine this, the neuronal activity-dependent immediate-early gene (IEG), *c-fos*, and its subsequent protein Fos, was used to tag activated cells (see Kubik, Miyashita, & Guzowski, 2007) after exposure to reliable or ambiguous fear conditioning. Separate cohorts of mice were used in this experiment.

On the last conditioning session, total freezing was not different between genotypes or predictors (reliable versus ambiguous), and there was no interaction between these two variables (genotype effect:  $F_{1, 25} < 1$ ; predictor effect:  $F_{1, 25} < 1$ ; interaction effect:  $F_{1, 25} < 1$ ; Figure 3.10, A). Thus, there was no difference in behaviour (freezing) between the groups of mice during the training session immediately prior to sacrifice, and thus any differences in Fos expression were not being driven by the differences in preceding behaviour. Fos expression levels, however, were not similar across reliable or ambiguous fear conditioning groups in mice where staining for Fos was readily visible. In the dentate gyrus, a main effect of predictor (reliable vs. ambiguous fear conditioning) was found but not an overall effect of genotype. Importantly, there was a significant genotype x predictor type interaction (predictor effect:  $F_{1, 22} = 4.76$ ,  $p = 0.04$ ; genotype effect:  $F_{1, 22} < 1$ ; interaction effect:  $F_{1, 22} = 6.82$ ,  $p = 0.016$ ). Bonferroni post-hoc analysis revealed that TK mice showed lower Fos expression, as compared to their WT counterparts, during the ambiguous condition only ( $p = 0.043$ )

(Figure 3.10, C). These results suggest that lower network activity occurred throughout the whole dentate gyrus (i.e. in the mature granule cells) after ambiguous, but not reliable, fear conditioning in the TK but not WT mice.

In the CA3 region, there was a similar pattern of Fos expression (Figure 3.10, D). There was both an effect of genotype and predictor type (reliable vs. ambiguous fear conditioning), with an interaction between the two variables (genotype effect:  $F_{1, 22} = 5.87$ ,  $p = 0.024$ ; predictor effect:  $F_{1, 22} = 4.23$ ,  $p = 0.052$ ; interaction effect:  $F_{1, 22} = 5.7$ ,  $p = 0.026$ ). (Figure 3.10, D).

Bonferroni post-hoc analyses revealed that there were differences between WT and TK mice after ambiguous, but not reliable, fear conditioning ( $p = 0.0025$ ). As suggested by the Fos expression in the dentate, this data set suggests that there is lower network activity occurring throughout the CA3 pyramidal cells after ambiguous, but not reliable, fear conditioning in the TK mice but not in WTs.

In addition, we gave wild-type mice BrdU in their drinking water 4 weeks prior to fear conditioning in order to examine if newly divided neurons showed a different Fos expression pattern depending on the type of fear conditioning. It was found that there was no effect of predictor conditioning type ( $t_{10} = 1.06$ ,  $p = 0.32$ ) (Figure 3.10, B) on WT BrdU+ young neurons that were also co-labelled with Fos expression. Confocal images show

representative neurons in the dentate gyrus, located near the hilus, and in the pyramidal cell layer of CA3 (Figure 3.11).

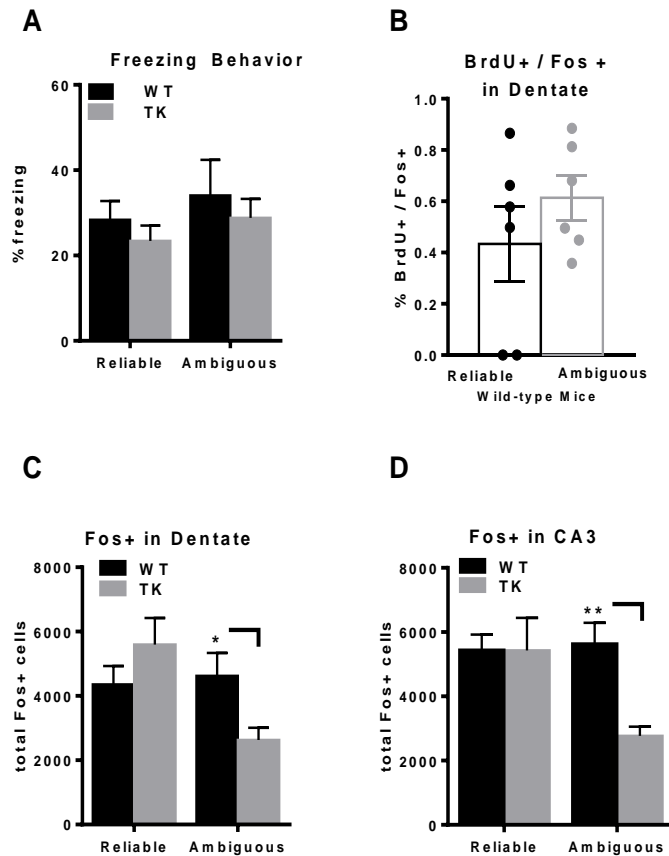


Figure 3.10 Freezing behaviour of mice and Fos expression analysis in the dentate gyrus and CA3. A: TK mice showed normal percent freezing on the last training day during reliable and ambiguous fear conditioning. B: There was no difference in the percentage of BrdU+ cells that also expressed Fos+ between reliable and ambiguous fear conditioning in WT mice. C: In the dentate gyrus, TK mice showed fewer Fos+ cells than WT mice after ambiguous, but not reliable, fear conditioning. D: Similarly, in CA3, TK mice showed fewer Fos+ cells than WT mice after ambiguous, but not reliable, fear conditioning. Bonferroni post-hoc: \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

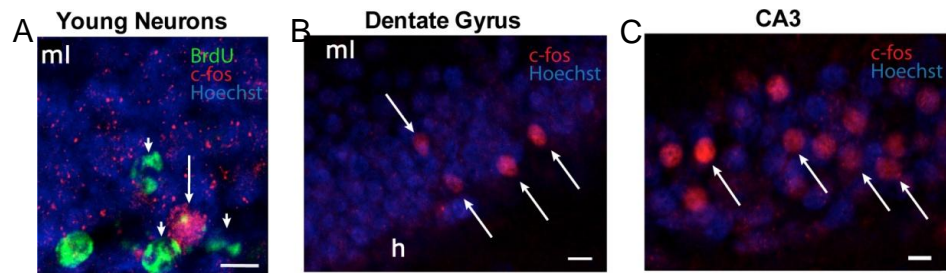


Figure 3.11 Example confocal images of BrdU+ and Fos+ cells located within the dentate gyrus and CA3. A: A young granule (arrow) cell triple-labelled for Hoechst, BrdU, and Fos expression located in the subgranular zone of the dentate gyrus with BrdU+ cells labelled alone (arrow heads). B: Example of three (Fos+) activated neurons located in the subgranular zone and one likely more mature cell that migrated toward the molecular layer of the dentate. C: Finally, pyramidal neurons in CA3 are also activated (Fos+ expression) in the pyramidal cell layer. Hilus (h) and molecular layer (ml) of dentate gyrus are labelled. Measuring bar represent 10  $\mu$ m.

## 3.6 Discussion

### Summary of Results

TK mice (TKs) showed decreased fear-related defensive behaviours toward a cue that was only 50%, and therefore ambiguously, predictive of a shock outcome, compared to wildtype (WT) mice. TK mice, on the other hand, did not show differences in defensive behaviours when a separate cue was

100%, or reliably, predictive of a shock. These results were consistent when using two different sensory cues (tone and light) and across different performance measures (freezing and startle reflex). In contrast to cue conditioning, there were very low levels of freezing to the training context and this did not differ between genotypes or predictor type, suggesting that it provided low competition for conditioning strength with the punctate cue. TK and WT mice also did not show any differences in unconditioned responding to “would-be” conditioned (light or tone) or unconditioned (shock) stimuli before any fear conditioning occurred. These results suggest that there are normal sensorimotor abilities in TK mice and they likely do not explain diminished defensive behavioural responses observed after conditioning to ambiguous cues.

Additionally, the stress hormone corticosterone was elevated in WT and TK mice equally after reliable or ambiguous fear conditioning, relative to a baseline control condition. This result reflects that, at least 30 minutes after ending their respective fear conditioning sessions, there are no significant differences in corticosterone recovery levels that vary between genotypes (WT vs. TK) or conditioning type (reliable vs. ambiguous).

It was also observed that when mice's freezing behaviour was extinguished after receiving reliable fear conditioning, there were no differences between mice with and without adult

neurogenesis. This suggests that a ceiling effect did not obscure any freezing deficits between the genotypes during the fear memory recall test day in the reliable condition. In addition, this result also suggests that the underlying associative structures and precisely how the initial association(s) are formed may be important in determining when new neurons in the hippocampus becomes engaged in situations of uncertainty in these different fear conditioning experiments (e.g. acquisition of fear conditioning with an ambiguous cue vs. extinction of a previously reliable predictor of shock; more detail given below).

When examining the activity-dependent neural responses in the dentate gyrus and CA3 subfields, TK mice showed decreased Fos expression in both mature granule cells and CA3 pyramidal cells compared to the WT mice specifically toward the ambiguous cue predictor. Conversely, TK mice did not show differences in Fos expression when a cue reliably predicted the shock. Collectively, these results suggest that TK mice are impaired at the processing of cues that are ambiguously predictive of shock outcome but show normal defensive responding to cues that reliably predict a shock, and this pattern of behavioural results is reflected in the activity of the dentate gyrus as a whole and in the neuronal activity of the downstream target area CA3.

## **Emotionality and Ambiguous Cues**

Humans that have altered emotional or affective responses often show differences in information processing. Persons with stress-related conditions, such as anxiety and depression, often show a negative cognitive bias in the way they interpret stimuli, especially those related to perceived threats, and they show enhanced attention to threat stimuli (Beck & Clark 1988, Clark & Beck 2010). This negative cognitive bias is the tendency to interpret ambiguous cues in a pessimistic way. Likewise, persons that appear to be less susceptible to anxio-depressive conditions (anxiety and/or depression) are more likely to attend and process positive stimuli, as opposed to negative/threat stimuli (Fox et al. 2009).

Generally, with regard to ambiguity and the specific qualitative characteristics of a cue (i.e. presenting an intermediate frequency that is half-way between distinct conditioned frequencies that represent positive and negative cue), rats showed a particular altered emotional bias toward ambiguous cues that signalled different outcomes in a go/no-go task after they experience unpredictable housing conditions (Harding et al. 2004). In addition, using congenitally helpless (vs. non-helpless) rats, representing a stress-like phenotype, and rats given corticosterone and noradrenalin reuptake inhibitor, these animals showed increased negative responding to an ambiguous cue

(Enkel et al. 2010). These results suggest that, in rodents that are susceptible, or have altered emotional responses, to stress effects, an interaction between serotonin, corticosterone, and granule cells within the dentate gyrus may play a particular role in the processing and behavioural response to ambiguous cues.

Similar to the design used here, other studies have used fear conditioning to a particular cue that has a certain probability of resulting in a shock or resulting in no shock. With respect to the cue's probability about predicting an outcome, in one study it was shown that when the dentate gyrus granule cells are silenced in serotonin receptor 1A knockout mice, who show enhanced anxiety-like behaviours, selective freezing deficits are observed in an ambiguous, but not reliable, predictor cues of shock (Klemenhausen et al. 2006, Tsetsenis et al. 2007). In addition, genetically modified mice over-expressing the serotonin transporter, and thus displaying decreased anxiety-like behaviour, showed decreased freezing behaviour toward an ambiguous, but not reliable, predictor cue about a shock outcome (McHugh et al. 2015).

Here, the results from using a tone or light as the conditioned stimulus in fear conditioning resulted in lower levels of defensive behaviour responding toward an ambiguously predictive, but not a reliable predictor, of threat in TK mice. So how could negative bias fit in with these results? If one compares how mice respond to the ambiguous cue, which signals shock

50% of the time and safety the other 50% of the time, against how their counterparts respond to the reliable cue, we can gain a sense of how the animal may be interpreting the cue. Specifically, WT mice respond similarly to both ambiguous and reliable cues, which may reflect a negative bias interpretation of the ambiguous cue. TK mice, however, show decreased responding to the ambiguous cue relative to the reliable cue. This, then, may represent decreased negative bias in TK mice in aversive situations, an unexpected finding of this study. This interesting topic is followed-up in the next chapter and later in Chapter 6 (General Discussion).

The behavioural results are also reflected in the Fos expression data. The TK mice showed lower expression levels in mature granule and pyramidal cells after experiencing ambiguous cues but showed normal levels after conditioning to a reliable cue. WT mice, on the other hand, showed no expression difference in the ambiguous vs. reliable cue, with equally high expression levels. It is not precisely clear how this relates to any sort of negative (cognitive) bias but, if one were to make an inference between levels of analysis, increased Fos expression may indicate that the role of the dentate is to preferentially bias information that is relevant to survival and therefore is passed on to other hippocampal regions for further processing. This bias in what information receives further processing, then, may be reflected in behavioural output. However, behaviour is usually not

linearly related to degree of fear and/or anxiety. It may be that TK mice have a different behavioural responses or strategy to cope with threat to their well-being.

The results from fear-potentiated startle were consistent with an interpretation of impaired ability to process cues that are ambiguous, but not reliable, predictors of threat. Mice without adult neurogenesis startled less in response to a tone that was an ambiguous predictor of threat but not toward a highly predictive one, as compared to their wildtype counterparts. Because we have shown similar results in freezing and startle behaviour, two independent measures of performance (Maren et al., 1998; McNish et al., 1997), this attenuates the possibility of a dissociation between freezing and startle responding in this situation.

Though not strictly explored here, there is a difference in what the term "ambiguous" means, with respect to cue quality and threat outcome. In ambiguous cue quality experiments, two distinct tones (high and low frequency tones) are used during initial conditioning that have two distinct behavioural responses. On the critical test day, an intermediate tone (i.e. a tone frequency between the original conditioning tones) is played and the animals response is examined to see if it interprets that ambiguous cue as relating more to the high or low frequency tone. The other form of ambiguity or uncertainty, that of which is ambiguous about a threat's outcome (i.e. ambiguous predictor cues about the

occurrence of a shock) also exists and is what is used in this thesis. These two forms may relate to the underlying development of associative structures and the specific construction of these associations may be the determining factor that engages the hippocampus.

The extinction of reliable cue conditioning used in this chapter could represent another form of ambiguity. It is interesting to wonder whether these characteristics are dissociable or have a common underpinning. The data presented here possibly suggest that, at least with regard to the testing vs. extinction data, these are at least separable. In the case where there is the simultaneous development of conflicting associative structures, such that a tone sometimes signals shock and other times non-shock, the hippocampus appears to be engaged. In the case of extinction, where the reliable tone now comes to be paired with non-shock, it appears that the hippocampus may not be involved (or may act in a subordinate role) in this type of ambiguity. The reason may be due to the fact that there are no conflicting cues during the initial formation of the association that engage the hippocampus. For example, during formation of the reliable conditioning (tone-shock) and subsequently the development of the extinction (tone-no shock) association are two distinct associations that form at separate times (however, see Bouton 2004). This issue is further discussed in the General Discussion at

the end of this thesis. More studies are needed to flesh out the precise similarities and differences.

### **Context, Ambiguity, and Adult Neurogenesis**

Several studies investigating the function of adult-born neurons relate to contextual fear conditioning. Studies have shown that rodents lacking adult neurogenesis show impaired learning abilities, especially in contextual fear conditioning where a context signals a shock, but equally show spared learning in several other tasks (e.g. Drew, Denny, & Hen, 2010; Saxe et al., 2006; Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002; Snyder et al., 2009; Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006 but see Groves et al., 2013 for meta-analysis).

In this chapter, after testing to the conditioned stimulus in a novel context, mice were put back into the conditioning context and freezing was assessed in the absence of the cue. Importantly, there were no difference found between genotypes; TK mice froze just as little to the context as the WT mice. This lack of an effect, and the overall low freezing levels, represents that the conditioning context possibly posed little competition for conditioning strength that may be the cause of the decreased freezing during the test day to the ambiguous tone. The low freezing to the context likely reflected a general inactivity rather than fear-induced freezing.

One important study showed that, as it related to contextual conditioning and discrete cues were not involved, a weak shock was sufficient to produce impaired freezing but not if the mice lacking adult neurogenesis were conditioned with a strong shock or had more extensive training (Drew et al. 2010). This study possibly converges on an idea that the hippocampus is necessary when conditioning produces a weak association and proves to be more difficult (Beylin et al. 2001, Curlik et al. 2011, Quinn et al. 2008). However, these studies of weak conditioning may reflect the ambiguity in what precisely signals a shock.

In studies of context conditioning in rodents with ablated adult neurogenesis, there is a slight effect that comes out in a meta-analysis (see Groves et al. 2013). Most studies looking at context conditioning effects and hippocampal involvement have attributed it to conjunctive coding such that elemental cues in the environment form a multimodal representation, and this is called 'context'. However, also possible, is that the conditioning context may serve as an ambiguous predictor about shock in and of itself. Specifically, the context sometimes signals shock and other times not. This inherent partial reinforcement provided by the context may be similar to what is happening with the mice in this chapter who show a decreased freezing to ambiguous fear conditioned cues. This context, then, forms a context-shock and context-no shock associative memory. The *simultaneous formation* (Figure 3.12, A) of this associative structure produces conflicting

information and therefore the subsequent retrieval of this associative memory generates conflict in its meaning. This may be when the hippocampus is needed to parse apart competing associative memories that were formed at the same time. The reliable cue, on the other hand, contains no such conflicting information during the formation of that associative structure. Therefore, there is no conflict generated upon its retrieval (Figure 3.12, B).

During the extinction of the reliable cue, a new association is being formed that, at face value, possibly makes the reliable tone ambiguous and generates conflict. However, an independent association is being formed during extinction, namely that a tone now does not signal shock. As extinction progresses, this new association strengthens and may inhibit the original association (Figure 3.12, C), and the animal may indeed give this 'new' tone a different *meaning* and thus represents something different to the animal. Since these are two independent associative memories, they are retrieved independently of one another, potentially processed sequentially during the early phases of extinction, and thus no conflict arises. Perhaps, in this instance, the hippocampus is not necessary to parse apart anything. Other brain structures, potentially the amygdala or prefrontal regions, may be capable of processing this information.

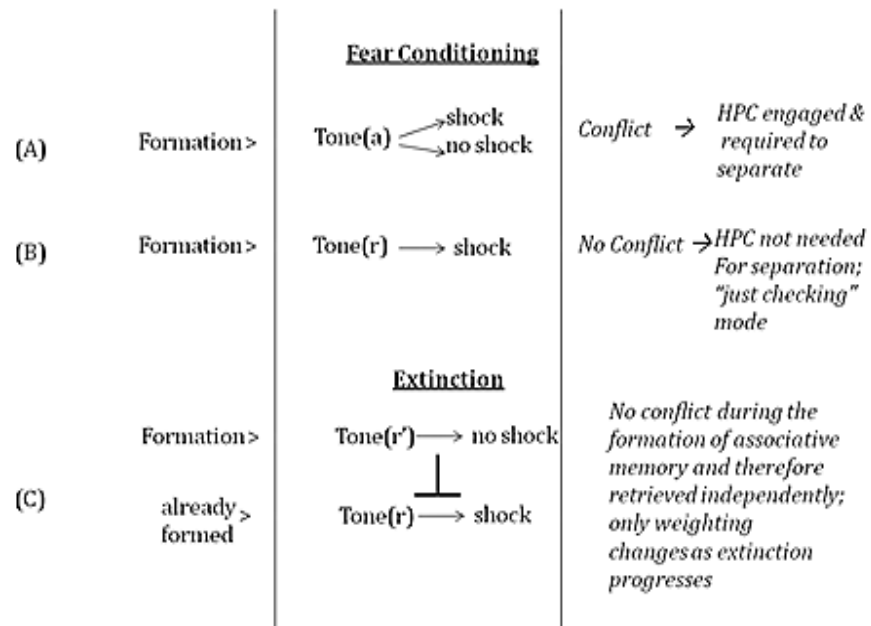


Figure 3.12 The structural formation of associative memories at end of fear conditioning and extinction and involvement of the hippocampus. (A) During the formation of the ambiguous cue (e.g. tone; Tone(a)) associative memory, the tone signals both shock and no shock. When this associative structure is subsequently retrieved, the hippocampus detects conflict and is needed to separate out these signals and preferentially allows information related to threat to continue. (B) During the formation of the reliable cue (e.g. tone; Tone(r)) associative memory, the tone signals shock and presents no conflict in its initial formation. When this associative structure is retrieved, no conflict is detected and the hippocampus is in 'just checking' mode. (C) During extinction, a new reliable cue (e.g. tone; Tone(r')) associative memory is formed that, as extinction progresses, gains conditioning weight and inhibits the already-formed associative

Tone(r) memory. Because these were formed independently, they are recalled independently and no conflict arises.

In addition, the extinction data also potentially helps to rule out the possibility of a ceiling effect on the fear conditioning test day (fear memory recall day; Figure 3.3, A) which could potentially explain the differential effects in the TK mice with reliable and ambiguous cue conditioning. The extinction data (Figure 3.3, B) in this chapter showed similar levels of freezing for the two genotypes as extinction progressed over 6 days. At no point during the extinction process, across various different levels of freezing behaviour, did the two genotypes differ. Therefore it seems unlikely that a ceiling effect can explain the lack of a deficit in the TK mice in the reliable condition. An additional reliable cue experiment using a weaker shock (0.3 mA) was also performed. There was no effect of genotype in this experiment. Both of these results strongly suggest that a ceiling effect is not obscuring genotypic freezing difference to the reliable cue, and thus a ceiling effect cannot explain the dissociation between reliable and ambiguous cues.

## **Hippocampus-Amygdala Interaction?**

It was found that, in both the dentate gyrus and CA3, Fos expression patterns were lower in mice without new neurons but only during the ambiguous conditioning paradigm but not when the cue was a perfect (reliable) predictor of threat. Importantly, freezing levels taken the same day in these mice revealed that this Fos expression was not related to freezing/activity levels in these mice in the immediately preceding behavioural session, signifying that freezing/activity differences did not solely drive Fos-measured activation. Exactly what this Fos signal represents is not clear, as immediate-early genes are activated during both hippocampal-dependent and -independent tasks, as defined by hippocampal lesions, (Guzowski et al. 2001), and reliable cue fear conditioning can be successfully performed in hippocampal-lesioned animals (see Chapter 2). The equal elevation in Fos expression in WT mice during reliable and ambiguous fear conditioning suggests that the hippocampus is similarly engaged during reliable fear conditioning but the functional role that this signal plays is not completely known, and the lesion data would suggest that this hippocampal signal is not essential for reliable cue fear conditioning. One possibility is that the Fos signal in the reliable cue condition reflects the hippocampus in a sort of “just-checking” mode during which on-going inputs are monitored (see Gray 1982; Gray & McNaughton, 2000). It is clear, though, that during ambiguous cue fear conditioning the signal is necessary

and is transmitted from the dentate to CA3, which is affected in TKs, because both the dentate and CA3 showed decreased Fos expression.

It may be that the amygdala and hippocampus serve similar threat-related roles by monitoring information relevant to the animal's survival and the two structures have a balance between them regarding which area primarily processes these cues about a threat. When a reliable cue-shock association is present, the amygdala may take control and dictate defensive behaviours deployed (Phillips & LeDoux 1992) and the dentate is acting more in a supervisory role. Once a cue about a threat is made ambiguous, or conflicting information is involved, the hippocampus may handle this ambiguity of threat and new neurons are necessary to initiate defensive behaviours. This may be due to the ambiguous cue retrieving two distinct and conflicting memories and the need to resolve this cue-shock /cue -non-shock conflict, in order to evoke some defensive responses.

Similarly, the differential engagement of the amygdala or hippocampus during reliable and ambiguous fear conditioning may relate to the defensive distance or defensive direction of the threat (i.e. an internal cognitive construct related to the intensity and degree of threat) (see McNaughton & Corr 2004). The different degrees of defensive distances that each cue represents may map onto different psychological processes that engage different levels of a defensive hierarchy and likely have differing

underlying neural structures (McHugh et al. 2004, 2013). For example, the reliable cue is a certain threat that poses immediate danger, while the ambiguous cue signals potential danger.

The reliable cue, based on its immediacy of danger, may be processed predominantly in the amygdala, and this may be sufficient to engage defensive behaviours, classically known to be fear-like behaviours (Gray & McNaughton 2000). The hippocampus's role during reliable fear conditioning, then, may be supervisory in the form of constant monitoring for conflicting threat-related information; however, no threat-related conflict exists in the reliable cue's associative structure (as discussed above) and therefore the hippocampus does not usurp control from the other brain areas. This would account for why hippocampal lesions sometimes do not affect behaviour in this type of fear conditioning (Phillips & LeDoux 1992). Conversely, because of the ambiguous cue's signalling of potential danger, the hippocampus along with possibly the prefrontal cortex, may be necessary to engage and mediate these alternative defensive behaviours, classically known to be anxiety-like behaviours (e.g. behavioural inhibition) which are affected by ventral hippocampal lesions (Deacon et al. 2002a, Gray & McNaughton 2000).

The decreased level of Fos expression in TK mice during ambiguous fear conditioning may give insights into how this decreased activity is sustained within the hippocampal network. This may reflect that the hippocampus is receiving less valence-

like information from the amygdala (Felix-Ortiz et al. 2013, Tye et al. 2011) and/or that the recurrent collaterals within the CA3 subfield and the back-projections to the dentate are not being engaged as strongly. Subsequently, then, less information is being sent out of the hippocampus to the amygdala and other extra-hippocampal structures that again feedback into the hippocampus, thereby sustaining decreased activity levels within the amygdalo-hippocampal and/or intra-hippocampal recursive loops.

Pattern separation mechanisms may be needed to parse apart these conflicting associative structures (see next section), as mentioned above, but this is not clear and it is not clear as how this sparse coding relates to behavioural output (Aimone, Deng, & Gage, 2011; Clelland et al., 2009; Groves et al., 2013; Piatti et al., 2014). The dentate gyrus, via pattern separation at a computational level, may play a role in separating conflicting goal information and the dentate itself may add more weighting to negative valence information that is then passed on to the other hippocampal subfields. This initial biasing of the information is then reflected in dentate and CA3 activity levels and finally in defensive behaviours that the animal exhibits.

## **Pattern Separation and Associative Memories**

An idea that has gained prominence, first relating to the dentate gyrus as a whole and then extending to adult-born dentate granule cells, is that of pattern separation. Pattern separation, first conceptualised as a computational process, is defined as the orthogonalisation of redundant signals that are coming into a structure. The dentate gyrus has been suggested to play an important role in pattern separation based on the high amount of information coming into the structure via the entorhinal cortex and its relatively sparse output to the CA3 subfield due in part to the inhibitory feedforward and feedback information to the dentate. This computational process has been extended to the behavioural realm where discrimination needs to occur between similar places or events in order to orthogonalise outputs that translate into behaviour (Clelland et al. 2009, Creer et al. 2010, Groves et al. 2013, Kesner 2007, Leutgeb et al. 2007, Nakashiba et al. 2012, Sahay et al. 2011). As an example, contextual fear conditioning tasks have frequently been used to show impairments in discriminating between a previously shocked environment and a neutral environment.

The closer these environments are in their appearance, rodents with impaired dentate gyrus function, and specifically adult neurogenesis, show lower freezing behaviour (Leutgeb et al. 2007, Nakashiba et al. 2012). These lower freezing behaviours

supposedly reflect a decreased ability in discriminating these two contexts (Kheirbek et al. 2012, Leutgeb et al. 2007, Nakashiba et al. 2012). This, however, may reflect that the context is an ambiguous predictor about threat, as discussed earlier. The associative retrieval of what the context means primes a shock and a non-shock outcome/relationship. Instead of the sensory qualities about the environment that need to be discriminated, it may be that the continual overlap about associations may be what is engage the hippocampus and why new neurons are needed. Similarly, in the experiments presented in this chapter, the ambiguous tone represents conflicting associative information. Upon hearing this tone and retrieving the memories, the animal may need to bias, or pattern separate, the memories that are most relevant to the survival of the organism. If the cue is paired with an aversive situation, the animal may be more likely to associate it with a negative outcome and treat it like a reliable cue, and give less weighting to its positive association (signalling non-shock), like the wildtype animals appears to do in this chapter.

This suggests that it may be something about the initial formation of conflicting associative structures when formed simultaneously that produces the type of conflict that engages the granule cells and pattern separation mechanisms, and not when associations are formed separately. When a non-conflicting cue-shock relationship is first encoded, as is the case with the reliable

cue during fear conditioning, further extinction represents new encoding with its own associative structure.

### **Pattern Separation and Fos Expression**

The decreased level of Fos expression in TK mice during ambiguous fear conditioning may give insights into how this decreased activity is sustained within the hippocampal network. Additionally, the decreased Fos expression may relate to the decreased pattern separation used when retrieval of conflicting associative memories occurs; however, it is difficult to know what direction this correlational data represents. Is it the decreased Fos expression that is driving a failure in pattern separation, or is it a failure in pattern separating that is driving decreased Fos expression? A third variable, too, could be producing this decreased Fos expression and/or pattern separation failure. This decreased expression pattern in the dentate and the CA3 may reflect that the hippocampus is initially receiving less valence-like information from the amygdala (Felix-Ortiz et al. 2013, Tye et al. 2011) and/or that the recurrent collaterals within the CA3 and the back-projections to the dentate are not being engaged as strongly, as discussed above.

## **Conclusion**

In this chapter, it is shown that TK mice show diminished behavioural responses and hippocampal network activity after fear conditioning with cues that ambiguously predict an aversive event but not if the event is fully predictable. This suggests that adult-born neurons are necessary for appropriate defensive responding to ambiguous cues. It may be, however, that TK mice show a difference in the way they respond to the aversive environment that suits their genetic and/or psychological predispositions. In some instances, the way to assess with this altered defensive behavioural responding is beneficial to well-being is to examine whether this type of fear learning leads to adaptive changes in behaviour to future threatening situations. In other words, does prior fear learning, whether it be about predictable or unpredictable threats, bias the perception of future threats and modify behaviour to adapt optimally to adverse environments? If it does, is this reliant on stress hormones that may have been elevated during the initial experience and does it rely on new neurons?

# 4 ADULT NEUROGENESIS AND GLUCOCORTICIDS MEDIATE FUTURE BEHAVIOURAL STRESS RESPONDING

## 4.2 Abstract

In this chapter, it is shown that a prior stress history has a large impact on how the animal behaves in future uncertain situations. It is additionally shown that removal of circulating glucocorticoids affects this future responding. In non-fear conditioned mice, both mice with and without adult-born neurons show similar innate anxiety-like behaviours in the elevated-plus maze and light/dark exploration box; mice with and without adult neurogenesis showed similar percentages of time spent in anxiogenic regions and similar locomotor behaviours in both of these tasks. A new cohort of animals was also tested in an alternative test of anxiodepressive-like behaviour, the novelty-suppressed feeding task. In the novelty-suppressed feeding task, both non-fear conditioned genotypes again showed similar latencies to start eating a familiar food (neophagia). In separate mice, prior fear conditioning with reliable or ambiguous predictor cues of the shock resulted in increased and decreased anxiodepressive-like behaviour, respectively, in TK mice as compared to their wildtype counterparts. Specifically, TK mice showed increased latencies to feed relative to their wildtype counterparts if both groups experienced reliable fear conditioning. This pattern of results, however, was reversed if they experienced ambiguous fear conditioning. Wildtype mice now showed an increase in their latencies to eat compared to those mice without adult

neurogenesis. These results suggest that ambiguous fear conditioning promotes adaptive behaviour in wildtype mice but does not produce this same behaviour in those TK mice. To investigate the role that glucocorticoids play in this adaptive behaviour, a new cohort of mice underwent adrenalectomy and corticosterone levels were clamped to prevent experience-induced increases in their levels. The main difference in this set of results is that wildtype mice that experienced ambiguous fear conditioning no longer showed this adaptive behaviour and now showed similar latencies as those mice that experienced reliable fear conditioning. Mice without adult neurogenesis remained unaffected, showing similar latencies irrespective of the prior type of fear conditioning received. These results suggest that adult neurogenesis, in conjunction with glucocorticoids, bias future behaviour depending on the prior predictability of fear conditioned cues (i.e. to ambiguous predictors of threat).

### 4.3 Introduction

In many stress-related conditions, exposure to a brief stressor can enhance future stress responding, such as that seen in post-traumatic stress disorder. What neuroendocrine mechanisms and neuroanatomical regions mediate this adaptive, enhanced reactivity is not clear. Previous studies have shown that short-term exposure to stressors, like social-defeat stress,

predator odours, or unsignalled footshocks, can have short- and long-lasting consequences on anxio-depressive behaviours and hippocampal stress hormone receptor binding (Daviu et al. 2010, Koolhaas et al. 1990, van Dijken et al. 1992a,b, 1993).

The hippocampus plays an important role in the feed-back regulatory response that terminates further glucocorticoid production from the adrenal glands (Sapolsky et al. 1984) and also mediates contextual fear conditioning (Anagnostaras et al. 2001). It has been suggested that successful contextual fear conditioning results in long-lasting changes in behaviour, such that the rodent is more cautious in future environments (Daviu et al. 2010). This change may be necessary to avoid future uncertain contexts that may pose a similar risk.

In addition, glucocorticoids, a class of adrenal steroid hormones, play a fundamental role in the physiological and behavioural stress response. Their receptors densely populate the hippocampus and, in particular, the dentate gyrus (McEwen et al. 1968). Aversive experiences (e.g. shocks) that elevate glucocorticoid levels produce long-lasting behavioural changes and increased numbers of glucocorticoid receptors as measured by binding capacity levels within the hippocampus (van Dijken et al. 1993). During perinatal development, these stress hormones also play a fundamental role in normal neuroanatomical development and the emergence of defensive behaviours.

Circulating levels and the binding of these hormones is essential

in the perinatal development of the dentate gyrus and contribute to emergence of behavioural inhibition (Cameron & Gould 1994, Gould et al. 1991b, 1992; Sapolsky et al. 1984, Sloviter et al. 1989, Spanswick et al. 2011, Takahashi 1995, Takahashi & Kim 1995).

Mice lacking adrenal glands (which prevent experience-induced increases in circulating glucocorticoid levels) and adult neurogenesis do not regulate glucocorticoid levels properly and also do not benefit from their protective effects when stress occurs (Lehmann et al. 2013, Snyder et al. 2011). It is not yet known how these altered responses are mediated and how prior experiences of stress affect future stress responding in mice that lack adult neurogenesis and adrenal glands. New neurons may be necessary for the adaptive, future stress response in subsequent unconditioned, ethological tests of anxiety (e.g. the elevated-plus maze and novelty-suppressed feeding test) after experiencing a history of stress (shock) that was either ambiguously or reliably predicted via partial or full reinforcement of a tone, respectively. Classical studies of anxiety have used the elevated-plus maze to look at the efficacy of antidepressants and classic anxiolytics (Lister 1987). Furthermore, many studies of altered dentate gyrus function, including ablating adult neurogenesis, have successfully employed the novelty-suppressed feeding task (NSF) to show an anxiodepressive-like phenotype after stress or treatment with some antidepressants (Miyamoto, Iijima, Yamamoto, Nomura, &

Matsuki, 2011; Santarelli et al., 2003; Snyder et al., 2011; Surget et al., 2008; Wang, David, Monckton, Battaglia, & Hen, 2008).

In this study, we used the EPM and NSF tasks to investigate if a prior stress history after ambiguous or reliable fear conditioning has lasting, adaptive behavioural changes on anxiodepressive-like behaviour. We further investigated the role that glucocorticoids may play in any lasting adaptive behavioural changes in the NSF task by preventing experience-induced surges in corticosterone levels in WT and TK mice.

## 4.4 Methods

All procedures performed at the National Institute of Mental Health followed the Institute of Laboratory Animal Research guidelines and were approved by the Animal Care and Use Committee of the National Institute of Mental Health.

### **Animals**

Mice used were of the same GFAP-TK model described in the previous chapter. Transgenic male mice (TK mice) expressing the herpes virus thymidine kinase under the human glial fibrillary acidic protein promoter were maintained on a CD-1 background (Snyder et al., 2011) and were generated from heterozygous x

wildtype mating. Pups were then weaned at 3 weeks of age, genotyped via PCR, and housed 3 to 4 per cage with mixed genotype siblings. Beginning at 8 weeks of age, mice were treated with valganciclovir in their normal (crushed) food pellets (0.3%, 35 mg/kg per day), 4 days/week, for 8-9 weeks before behaviour testing. Mice were housed under a 12 hour reversed light:dark cycle (lights on at 7:00 pm), and all testing took place during the dark phase.

### **Handling**

Mice were handled for 3-5 min/day for at least 2 days prior to behavioural testing. On each testing day, mice were brought to a dark holding area and remained in their homecages for approximately 30 minutes prior to the first trial.

### **Elevated-Plus Maze**

The elevated-plus maze (EPM) was assessed twice (different experiments). The first EPM testing occurred in experimentally naive mice. In a new cohort of mice, the second EPM testing took place twenty-four hours after completing fear conditioning (see Chapter 3, Methods, Fear Conditioning; or below for brief details), mice were tested on the elevated-plus maze. The maze had four arms, two high-sided 'closed' arms measuring 40 x 10 x 50 x 10 cm (*l x w x h*) and two 'open' arms

without the high-sided walls and a clear drop-off edge. The centre zone measured 10 cm<sup>2</sup>. The maze was elevated 50 cm above the ground, surrounded by black curtains, illuminated to ~700 lux, and cleaned with 70% EtOH between trials. The maze was made of metal and painted dark grey. Mice were placed near the end of a closed arm and were allowed to explore freely for 5 minutes. Percent time spent in the open arms, total distance (mm) travelled on the maze, and average velocity (mm/s) were determined using TopScan (CleverSys, Inc).

### **Light/Dark Exploration Box**

The Light/Dark Exploration Box (LDB) was conducted on experimentally naive mice. The LDB was conducted in a white plastic open field box measuring 50 x 50 x 40 cm (*l x w x h*), which was surrounded by black curtains, and the room was dark (except for the light in the light zone of the light/dark box). A black wall separated the lit from the dark zone and a passageway to the other zone was located at one end of the separator. The dark zone measured 20 x 50 x 40 cm (*l x w x h*) and was illuminated to ~25 lux, while the lit zone measured 30 x 50 x 40 cm (*l x w x h*) and was illuminated to ~450 lux by an overhead spot light directly above the lit zone. The mouse was placed in the dark zone at the beginning of the trial and access to the lit area was blocked for five seconds, after which the mouse was free to explore for five

minutes. The box was cleaned with 70% EtOH after each trial. Percent time spent in the lit zone, total cross-overs between compartments, and latency to first cross-over into the lit zone were recorded by hand. Cross-overs were only considered when all four paws crossed into the new zone.

### **Fear Conditioning**

After habituation to handling, mice underwent fear conditioning according to their respective groups (ambiguous or reliable fear conditioning). There were additional non-shocked control groups (WT and TK mice). The same fear conditioning procedure and apparatus was used as described in Chapter 3. Briefly, a 0.5 mA, 1 second scrambled shock was used as the unconditioned stimulus (US). Each tone (2 kHz, 85 dB(A) tone) lasted 20 s and served as the conditioned stimulus (CS).

For half of the mice in each experiment, the tone always co-terminated with a shock ('reliable group'), resulting in 3 tone-shock pairings per session. The second group of the mice received the same 3 cue-shock pairings and 3 additional tone-only presentations in each session, such that the cue only co-terminated with a shock 50% of the time ('ambiguous' group). The third group served as a non-shock control groups that received the same protocol as the reliable group but none of the tones ended in shock. Total session time lasted 600 seconds. Each

mouse was trained in 3 separate sessions, each separated by 24 hrs; the timing of cues and order of CS-US and CS only trials (for the ambiguous group) differed from the previous day's protocol and were pseudo-randomly delivered in order (see Chapter 3 for timing details). Freezing behaviour was analyzed using FreezeView software (Coulbourn Instruments).

After each mouse completed their session, it was placed in a large tinted holding bin in a separate room (with other already-completed cagemates) and then returned to the homepage once all mice in the cage were finished. Completed mice/cages were kept in a separate room from those waiting to be tested. In sum, all 'stressed' mice that experienced fear conditioning were separate (in different rooms) from the 'non-stressed' mice waiting to go through fear conditioning. This limited the exposure they had to stress-related odours or vocalizations.

### **Novelty-Suppressed Feeding**

The novelty-suppressed feeding task (NSF) is thought to test anxiety- and/or depressive-like behaviours (collectively referred to as anxiodepressive-like behaviours in this thesis). It is sensitive to hippocampal lesions, anxiolytics, and SSRIs all of which reduce anxiodepressive-like behaviours that rodents display on this task. Like the elevated-plus maze, this task includes an approach-avoidance conflict but adds other

psychological factors, such as hunger/motivation (and thus can model aspects of anhedonia) and contains no hiding locations for the mouse to hide from danger (see Discussion for more detail).

The novelty-suppressed feeding task consisted of the mice being tested in a white arena (50 x 50 x 40 cm) (*l x w x h*) that was made out of plastic. Novel bedding covered the floor. One pellet of their regular homecage food was placed on top of a 1-cm-high white weighing dish in the centre of the arena. Latency to begin eating the familiar food was recorded (maximum trial time of 10min). In one cohort, right after the completion of the elevated-plus maze, all food was taken away from the mice for 20 hours before NSF testing and they were only allowed access to water. A second cohort of mice did not go through the EPM testing and instead received an extra day off (but also had food taken away 20 hours before NSF testing). Thus, the NSF test was again performed 48 hr after the prior fear conditioning, as with the first cohort.

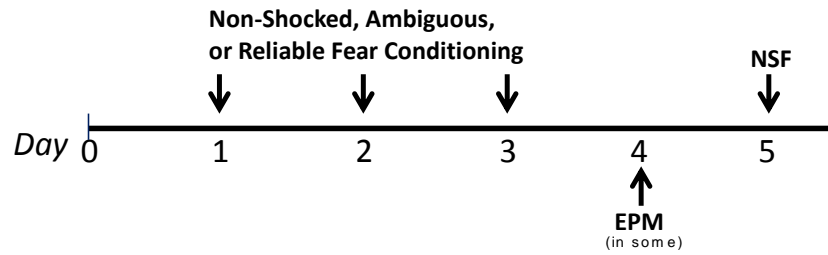


Figure 4.1 Example timeline of the study examining the effects of prior fear conditioning on subsequent unconditioned anxiety- and/or depressive-like responses. The different fear conditioning groups were a non-shocked control group, ambiguous fear conditioning group, and reliable fear conditioning group. Day 0 represents the day before fear conditioning started.

### **Corticosterone Manipulation**

To clamp corticosterone levels at a fixed basal level and prevent stress-evoked elevations in corticosterone levels, adrenal glands were removed from mice under isoflurane anaesthesia 5 days prior to behavioural testing. The two sides of the mouse were shaved (approximately near the end of the rib cage and verified by palpating the kidneys) and small bilateral incisions were made through the tissue and muscle. Adrenal glands were clamped with ringed-forceps and pulled gently away from the kidneys. After removal, incisions were stapled and the mice were placed in a heated incubator. Adrenalectomized mice were given saline (0.9% NaCl) with low-dose corticosterone replacement (25

µg/ml with 0.15% v/v ethanol) in drinking water to maintain a low-dose baseline level of corticosterone (Gould et al. 1991b).

### **Corticosterone Measurement**

After behavioural testing, corticosterone replacement in the drinking water was discontinued for 4 days, blood was then sampled under isoflurane anaesthesia, and serum corticosterone measured via radioimmunoassay (MP Biomedicals; see Chapter 3, Methods, Corticosterone Measurement for more detail). Blood was collected in a non-heparinised 1.5 ml Eppendorf tube and allowed to clot. Once samples began clotting, they were spun in a centrifuge and 3-10 µl of serum was pipetted from the tube and the sample stored in a -80 °C freezer before measurement by  $\gamma$ -counter (see Chapter 3, Methods, Corticosterone Measurement for details). Two mice were excluded (from all endocrine and behavioural results) due to incomplete adrenalectomy (levels >40 ng/ml).

### **Histology**

As in the previous chapter, 3-6 sections were taken from the hippocampus of each mouse. Doublecortin staining for young neurons was performed to confirm that valganciclovir treatment

ablated new neurons in the GFAP-TK+ mice (see Chapter 3, Methods, Doublecortin for more details).

## Statistics

Data were analysed with SPSS and GraphPad Prism. All ANOVAs performed were two-way ANOVAs with Bonferroni correction for post-hoc analyses. The between-subject factors were predictor type<sub>reliable, ambiguous</sub> and genotype<sub>WT, TK</sub>. If the within-subjects factor did not meet Sphericity, the Greenhouse-Geisser corrected degrees of freedom, and its associated  $F$  and  $p$  value, was used. If homogeneity of variance (by Levene's test) was violated with fewer than 10 subjects per group, a modified  $p$  criterion was used ( $< 0.03$ ). All  $t$ -tests were for independent-samples and two-tailed. All bars in the figures represent the mean and error bars represent standard error of the mean.

## 4.5 Results

### **Innate Anxiety-like Responses on the Elevated-Plus Maze and Light/Dark Exploration Box**

Unconditioned, ethological anxiety-like behaviours were first tested on the elevated-plus maze, a test that is sensitive to many anxiolytics and highly anxious mouse strains (Deacon et al. 2002b, Ramos et al. 1997). Here, TK mice did not show altered innate anxiety levels or exploratory behaviours, as compared to their wildtype littermates. On the EPM, TK mice ( $n = 16$ ) spent roughly an equivalent percentage of time in the open arms as compared to WT. Statistically, there was no difference in percent time in the open arm (Figure 4.2a;  $t_{31} = 0.50$ ,  $p > 0.05$ ). Likewise, both genotypes were indistinguishable from one another on the total distance traversed ( $t_{31} = 1.89$ ,  $p > 0.05$ ) and average velocity ( $t_{31} = 1.23$ ,  $p > 0.05$ ) on the EPM (Figure 4.2b, c), both indicating that TK mice do not show altered exploratory/locomotor behaviour under normal, basal conditions.

In a separate cohort of mice, TK ( $n = 15$ ) and WT ( $n = 20$ ) mice were tested in a different anxiety-provoking test, the Light/Dark Exploration Box. TK mice did not differ in their anxiety-like or locomotor/exploratory behaviours, as compared to their WT littermates. TK mice did not differ in their time spent in the lit zone ( $t_{33} = 0.44$ ,  $p > 0.05$ ) compared to WT mice (Figure 4.2d).

Similarly, their exploratory drive to enter the lit zone from the dark zone, as reflected in the latency to cross into the lit zone, did not differ ( $t_{33} = 1.36, p > 0.05$ ) (Figure 4.2e). Furthermore, the total number of crossovers between the zones also did not differ between genotypes ( $t_{33} = 1.02, p > 0.05$ ) (Figure 4.2f), indicating similar locomotor activity levels as compared to WT mice.

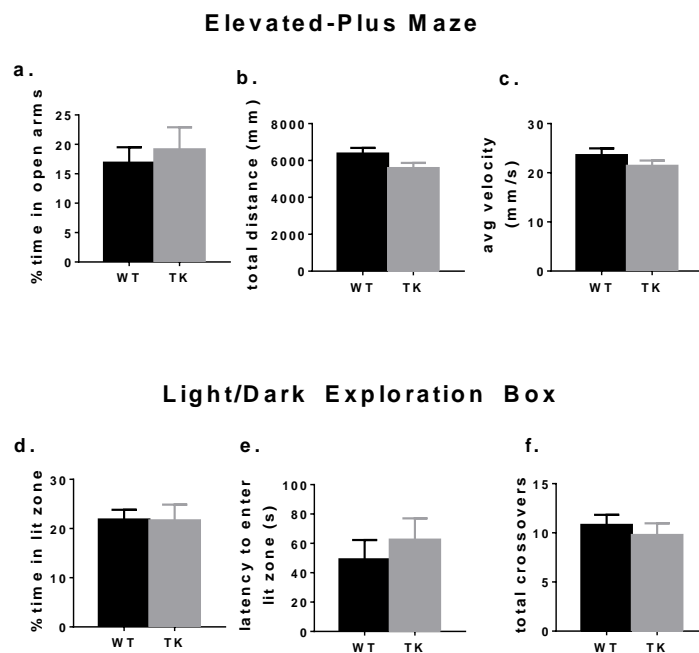


Figure 4.2 TK mice do not show altered unconditioned, innate anxiety in the elevated-plus maze or the light/dark exploration box. On the EPM (a-c), there was no difference between genotypes in the percentage of time in the open arms, total distance travelled, or average velocity. On the LDB (d-f), there was no difference between genotypes in the percentage of time spent in the lit zone, first latency to cross-over to the lit zone, or total number of cross-overs between zones.

## **Prior Fear Conditioning Effects on Elevated-Plus**

### **Maze Behaviour**

The elevated-plus maze and novelty-suppressed feeding tasks were used to examine how prior fear conditioning (non-shocked, ambiguous, or reliable cue conditions) affects subsequent unconditioned behaviours (Methods, Figure 4.1).

In a first experiment, WT and TK mice that went through prior fear conditioning with a reliable cue were compared to groups of mice that received ambiguous cue fear conditioning (as in Chapter 3). In mice that went through reliable (WT, TK  $n$ 's = 20, 20) or ambiguous (WT, TK  $n$ 's = 21, 20) fear conditioning, there were no differences detected on the EPM (Figure 4.3). All groups showed a similar percentage of time spent in the open arms (predictor effect:  $F_{1, 77} < 1$ ; genotype effect:  $F_{1, 77} < 1$ ; interaction effect:  $F_{1, 77} = 1.98$ ,  $p = 0.16$ ). In a subset of these mice that were tested on the EPM after reliable fear conditioning (WT, TK  $n$ 's = 9, 9) or ambiguous fear conditioning (WT, TK  $n$ 's = 10, 9), the EPM data were examined with a separate computer programme which allowed average velocity to be determined. The average velocity traversing the maze also did not differ between the groups (predictor effect:  $F_{1, 33} < 1$ ; genotype effect:  $F_{1, 33} < 1$ ; interaction effect:  $F_{1, 33} = 1.4$ ,  $p = 0.24$ ).

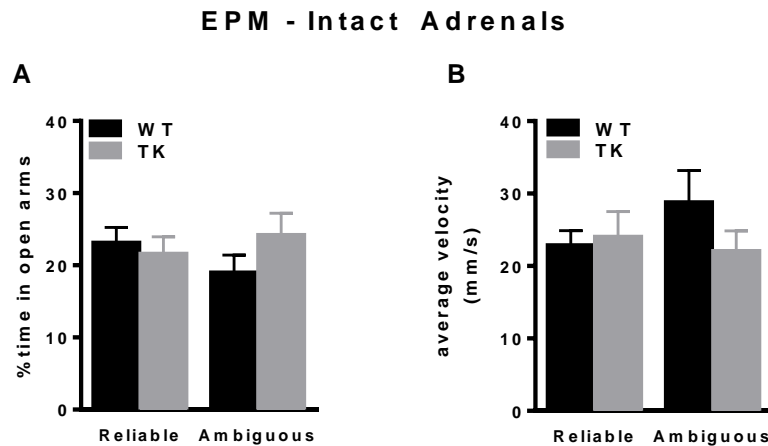


Figure 4.3 A: The percentage of time spent in the open arms of the elevated-plus maze did not differ between genotypes or by prior type of fear conditioning. B: Similarly, average velocity did not differ between genotypes or by prior type of fear conditioning.

### **Prior Fear Conditioning Effects on Novelty-Suppressed Feeding Behaviour**

Twenty-four hours later anxiodepressive-like behaviours were also tested in the NSF task. In the NSF test, the latency to start eating a familiar food in a novel environment was measured. The first experiment used the same group of mice as previously tested on the elevated-plus maze 24 hr earlier (i.e. 48 hr after the last day of fear conditioning training) but was combined with another cohort that did not experience the elevated-plus maze (instead received a day off). WT mice that received ambiguous

cue fear conditioning showed much longer latencies compared to WT mice that received reliable cue fear conditioning. TK mice in both the reliable and ambiguous cue fear conditioning groups showed similar latencies irrespective of the cue predictor type (Figure 4.4, A), resulting in a significant genotype x predictor interaction (genotype effect:  $F_{1,77} < 1$ ; predictor effect:  $F_{1,77} = 12.90$ ,  $p = 0.0006$ ; interaction effect:  $F_{1,77} = 12.32$ ,  $p = 0.0008$ ). Specifically, TK mice either showed increased or decreased latencies to begin eating compared to their WT mice after reliable or ambiguous cue fear conditioning respectively (Bonferroni post-hoc:  $p = 0.019$  and  $0.048$ , respectively).

A second experiment was performed to investigate baseline NSF behaviour in the absence of any prior fear conditioning, as compared to their reliable cue fear conditioning/shocked counterparts. In non-shocked TK ( $n = 19$ ) and WT ( $n = 19$ ) mice, there were no differences in their baseline latencies to start eating during the NSF test. After reliable fear conditioning, it is notable that TK ( $n = 20$ ) mice again showed longer latencies to begin feeding compared to WT controls ( $n = 19$ ), showing parallel results with the previous reliable fear conditioning experiment (predictor effect:  $F_{1,73} = 4.06$ ,  $p = 0.048$ ; genotype effect:  $F_{1,73} = 3.02$ ,  $p = 0.086$ ; interaction effect:  $F_{1,73} = 1.97$ ,  $p = 0.17$ ). Although the genotype by conditions failed to reach significance, based on an *a priori* prediction from the previous NSF experiment's results (reliable cue vs. ambiguous

cue), Bonferroni post-hoc analysis was performed to compare the TK and WT mice that received reliable fear conditioning. This showed that TK mice did again in fact show significantly longer latencies than WT mice (Bonferroni post-hoc:  $p = 0.05$ ) (Figure 4.4, B).

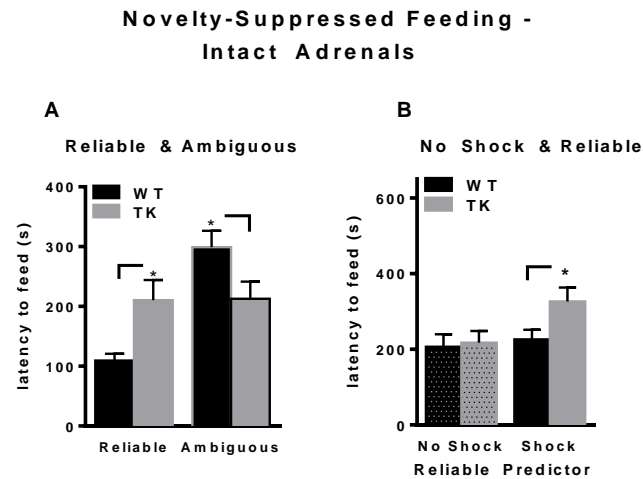


Figure 4.4 TK mice show both increased and decreased anxiodepressive-like behaviour. A: TK mice do show similar latencies to eat after reliable or ambiguous fear conditioning, whereas wildtype mice modulate their anxiodepressive-like behaviours in an adaptive manner in accordance with prior shock predictability. B: Latencies to begin eating in the novelty-suppressed feeding task after non-shocked or reliable fear conditioning; TK mice only show increased anxiodepressive-like behaviour (latency to eat) relative to their non-shocked control condition. Bonferroni post-hoc: \*  $p \leq 0.05$ .

When combining the reliable "Shock" data from the "No Shock & Reliable" condition panel (Figure 4.4 B) and the "Reliable" group's data from the "Reliable & Ambiguous" condition panel (Figure 4.4 A), a separate two-way ANOVA revealed a genotype effect ( $F_{1, 75} = 12.4, p = 0.0007$ ), meaning that TK mice showed increased latencies overall compared to WT (Bonferroni post-hoc tests for both conditions: both  $p < 0.05$ ). A main effect of experiment emerged ( $F_{1, 75} = 16.6, p = 0.0001$ ), meaning that mice in the control NSF experiment that received reliable shocks had longer latencies overall. There was no interaction between the two variables. These results confirm that, indeed, in both instances, there was a robust genotypic effect such that TKs always had longer latencies (anxiodepressive-like behaviour) to begin feeding in the NSF task after reliable fear conditioning.

### **Novelty-Suppressed Feeding Behaviour in Adrenalectomized Mice**

In the previous set of experiments, fear conditioning was found to have very different effects on future behaviour based on the predictability of the shock. If wildtype mice experienced ambiguous fear conditioning, they displayed adaptive behavioural responding in terms of increased anxiety in the novelty-suppressed feeding task. Glucocorticoids mediate, at least in part, the ontogeny of defensive behavioural inhibition and adaptive

effects of stress during perinatal development (Champagne et al., 2008; Gluckman et al., 2005; Takahashi, 1995). Glucocorticoids may also mediate this adulthood adaptive responding.

To test this possibility, mice were given adrenalectomy (ADX) and corticosterone was clamped at low levels. After recovery from surgery, mice were given fear conditioning. Forty-eight hours after mice received reliable ( $n$ 's: ADX-WT = 7, -TK = 9) or ambiguous ( $n$ 's: ADX-WT = 6, -TK = 7) cue fear conditioning, they were tested in the novelty-suppressed feeding task (thus paralleling the protocol used in the previous experiment in this chapter with the exception that EPM was not included). Overall ADX-TK mice showed longer latencies to begin feeding as compared to their ADX-WT counterparts. ADX-TK mice showed similarly increased latencies irrespective of prior type of fear conditioning (reliable vs. ambiguous) (genotype effect:  $F_{1,25} = 11.54$ ,  $p = 0.0023$ ; predictor effect:  $F_{1,25} < 1$ ; interaction effect:  $F_{1,25} < 1$ ) (Figure 4.5, A). Notably, ADX-TK mice showed a longer latency to begin feeding than ADX-WT mice after reliable fear conditioning (Bonferroni post-hoc:  $p = 0.01$ ), resembling the previous experiment in adrenal intact animals. Interestingly, however, ADX-TK mice now also exhibited longer latencies than WT mice on the NSF task after ambiguous cue fear conditioning, in contrast to the previous experiment in adrenal intact animals (see Figure 4.4). Confirming the success of the adrenalectomies,

all mice had low levels of corticosterone ( $M = 18.08$ ,  $SEM = 1.53$  ng/ml), indicating successful adrenalectomy (Figure 4.5, B).

The general pattern after reliable fear conditioning in ADX mice mirrored the pattern after reliable fear conditioning in the previous experiment with adrenal-intact mice (Figure 4.4, B, reliable condition). In contrast, as compared to the previous experiment, ADX-WT mice did not show adaptive increases in anxiodepressive-like behaviour after ambiguous conditioning like they did in the adrenal-intact NSF result (Figure 4.4, B).

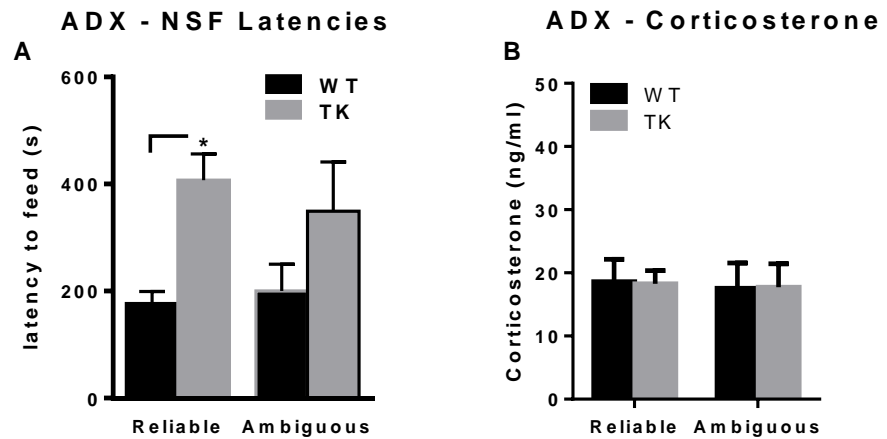


Figure 4.5 Modulation of adaptive behaviour is dependent on intact adrenal glands and adult neurogenesis. A: After reliable or ambiguous fear conditioning, ADX-TK mice showed increased anxiodepressive-like behaviours in the NSF task compared to their ADX-WT counterparts. B: Corticosterone measurements show similar low levels between WT and TK mice irrespective of prior reliable or ambiguous fear conditioning. Bonferroni post-hoc: \*  $p < 0.05$ .

## Histology

All mice were sacrificed at the end of their experiment and brains were excised. Doublecortin staining was performed to confirm the genotypes previously obtained via PCR tailsnips. Mice with few or no doublecortin+ cells were presumed TK mice, while those with many doublecortin+ young neurons were presumed WT mice. The information gathered from histological analysis was cross-checked and agreed with the PCR genotyping results (see Chapter 3, Methods, Histology, Doublecortin for more details).

Throughout the hippocampus, virtually all of the adult-born cells were eliminated (see Snyder et al. 2011 for other stereological counts, e.g. BrdU counts), evidenced by the absence of doublecortin-positive cells in treated TK mice in Chapter 5's experimental animals (see Figure 5.2, A) (one-tailed Man-Whitney nonparametric test,  $U < 1$ ,  $p = 0.0048$ , *a priori* assumption that TK mice would show very few young neurons and equal variance and distribution were not assumed). WT mice treated with valganciclovir showed high levels of doublecortin-positive cells ( $n = 4$  brains;  $M = 4282 \pm 441.2$  cells) compared to treated TK mice ( $n = 6$  brains;  $M = 9.33 \pm 4.34$  cells).

Representative images from the dorsal dentate gyrus are shown in Figure 5.2 (B) and doublecortin-positive cells in WT mice that were treated are easily visible (brown dots) in the subgranular

zone, while none are visible in TK mice that were treated with valganciclovir (see Figure 5.2, B).

## 4.6 Discussion

### **Summary of Results**

Experiments in this chapter produced four main results. First, wildtype mice and those with ablated adult neurogenesis did not show differences in unconditioned, innate anxiety-like behaviours on the elevated-plus maze, the light/dark exploration box, or the novelty-suppressed feeding task, in agreement with previous studies using these mice (Snyder et al., 2011). Second, wildtype mice increase their anxiodepressive-like behaviour in the novelty-suppressed feeding task (but not the EPM) after experiencing ambiguous, but not reliable, predictors about shock in fear conditioning. After ambiguous cues of shock, wildtype mice increased (adaptive) anxiodepressive-like behaviour relative to their wildtype counterparts that had previously received reliable predictors of shock. Third, adult neurogenesis was needed in order to modulate these behaviours to future, novel and potentially dangerous situations, and TK mice showed increased and decreased anxiodepressive-like behaviours, depending on the nature of the prior fear conditioning (reliable vs. ambiguous).

After reliable fear conditioning, TK mice show higher latencies (i.e. more anxiodepressive-like behaviour) on the novelty-suppressed feeding task compared to non-shocked TK mice, and to wildtype mice that also experienced reliable fear conditioning. Conversely, TK mice that received ambiguous cue fear conditioning now showed lower latencies (i.e. less anxiodepressive-like behaviour) on this NSF task as compared to wildtype mice who also received ambiguous fear conditioning. There was no difference in the latency to eat for TK mice that received reliable or ambiguous cue fear conditioning training prior to NSF testing. Fourth, clamping corticosterone levels, in order to eliminate any experience-induced surge in corticosterone levels during fear conditioning, abolished this adaptive anxiodepressive-like responding in wildtype mice on the novelty-suppressed feeding task. After adrenalectomy, wildtype mice that received ambiguous predictors of shock no longer showed increased latencies in this task, showing similar latencies as their WT counterparts that had received reliable conditioning, thus eliminating this experience-induced behavioural adaptation. In TK mice, however, there were still similar latencies on this task, irrespective of the prior conditioning type, and these animals were not affected by adrenalectomy (i.e. TK mice do not show corticosterone-dependent changes in their anxiodepressive-like responding).

## **Innate Anxiety-like Responses Before Fear**

### **Conditioning**

In this study, TK mice do not show altered behavioural responses on the elevated-plus maze, light/dark exploration box, or the novelty-suppressed feeding task (discussed later in the discussion about future behaviours below). These animals spend the same amount of time in the open arms of the elevated-plus maze and the lit zone of the light/dark exploration box, reflecting similar levels of anxiety as their wild-type littermates. In addition, mice without new neurons travelled the same distance and explored at a similar velocity as wild-types on the EPM and showed normal latencies to leave the dark area of the LDB in addition to showing no hyperlocomotion, all indicative of normal exploratory tendencies.

Some previous studies have shown altered innate, unconditioned anxiety levels in mice with reduced adult neurogenesis (Bergami et al., 2008; Revest et al., 2009). Revest and colleagues (2009) found that mice with decreased hippocampal neurogenesis showed increased anxiety-like behaviour in these two tasks used here. While similar, these tasks did differ in their dimensions, luminosity of each apparatus, and the housing conditions of the mice (singly housed vs. group housed), all of which may affect these behaviours. The bigenic mice used in that study may also just be more susceptible to

unspecified, natural prior stressors (e.g. cage changes, noise) and the added stress of these particular paradigms could have resulted in altered anxiety-like behaviours in these animals. This is plausible given that we know the importance of new neurons in the regulation of stress levels and stress behaviours (Lehmann et al., 2013; Schloesser, Manji, & Martinowich, 2009; Shors et al., 2002; Snyder et al., 2011), and that previous stress can affect future behaviours (Snyder et al., 2011; this chapter).

In another study, stimulating adult-born neurons (via optogenetic strategies) in the ventral dentate gyrus decreased innate anxiety levels (Kheirbek et al. 2013). This general pattern of results does not necessarily conflict with the current data because we did not activate these neurons in the current study, which may have strong inhibitory effects on the dentate as a whole (Acsády et al. 1998), which then may translate to decreased anxiety-like behaviours. In addition, these decreased anxio-depressive behaviours may be due to hippocampal output targets (e.g. amygdala and prefrontal cortex), modulating the incoming information based on the processing derived from hippocampal output (Adhikari et al. 2011, Felix-Ortiz et al. 2013, Tye et al. 2011).

Of course, the results here are contrary to studies with much larger hippocampal lesions. Frequently, animals with these lesions will show increased locomotor and/or exploratory behaviour in several tasks that test anxiety-like behaviours

(Bannerman et al. 1999, Good & Honey 1997, Jarrard 1968).

Although ablating adult neurogenesis in the dentate gyrus is a much smaller disruption to hippocampal functioning, it is still possible that such a slight manipulation could have large effects. Nevertheless, it is not obvious how meaningful it is to compare the TK mice who have a much smaller ablated cell population compared to animals with large hippocampal lesioned mice.

That said, the dentate gyrus is a major conduit to the rest of the hippocampus proper and irregular processing at this stage could have effects downstream in the hippocampal circuit (see Chapter 3). Thus, it may be sufficient to disrupt this region and effectively cause altered anxiety levels, locomotor activity, and/or exploratory behaviours. In this study, however, we did not observe such effects when ablating new neurons. It may be that, in this GFAP-TK model, new neurons are not necessary for baseline anxiety responding and that mature granule cells are quite capable of modulating these behaviours. In addition, it may be that through connectivity with other anxiety-related areas, such as the prefrontal cortex and amygdala (Adhikari et al. 2011, Felix-Ortiz et al. 2013), other structures may adjust their processing to cope with the altered processing/output of the hippocampus. Alternatively, adult-born neurons may have a secondary role, such as separating competing goal signals or memories which then help determine anxiety-like behaviours in downstream

hippocampal subfields and in conjunction with the other brain regions (see Besnard & Sahay 2015).

## **Effects of Prior Fear Conditioning on Subsequent Unconditioned Tasks of Emotionality**

Given the results seen here, what is the role for adult neurogenesis in the stress response? In a previous study, it was shown that TK mice showed prolonged glucocorticoid levels after experiencing a stressor (restraint stress) and also showed different sets of anxiodepressive-like behaviours (Snyder et al. 2011). In addition, this chapter adds evidence that not only does adult neurogenesis 'buffer' stress and defensive behaviours in the immediate future but it also contributes to longer-term changes in these behaviours. Days after experiencing fear conditioning, mice with normal adult neurogenesis showed adaptive anxiodepressive-like behaviours in the novelty-suppressed feeding task, but only if prior conditioning used ambiguous predictor cues. This adaptive responding was not, however, present when tested on the elevated-plus maze.

The elevated-plus maze tests anxiety-like behaviours using different dependent measures from those used during the NSF test, including travelling distance, speed, latency to explore the open arms, time spent in the open arms, etc. (Kjelstrup et al. 2002, Walf & Frye 2007). This test is considered to tap into

anxiety for two reasons: mice who receive anxiolytic drugs explore the open arms more (the 'anxiogenic regions') and the task has a clear approach-avoidance conflict point. This approach-avoidance conflict, especially at the centre zone, or the decision point, is thought to underlie some aspects of anxiety. Mice generally want to explore the maze and potentially find rewards but they also want to stay safe by avoiding the open areas where potential predators might be present. It may be that this EPM is just not sensitive enough to pick up experience-dependent changes in anxiety-like behaviours in these TK mice with this ablated cell population (via inhibition of adult neurogenesis). Furthermore, the NSF test may simply be more sensitive than the EPM. A common example in humans may be that we are in fact anxious but are still able to walk out the door and give a presentation in front of colleagues; however, we couldn't eat breakfast that morning because of the anxiety. The EPM does, however, detect such decreases in anxiety-like behaviours with much larger insults to the hippocampus, such as through complete or ventral hippocampal lesions (see Barkus et al. 2010). Alternatively, the EPM and NSF tests may, at least in part, tap into qualitatively different psychological processes. For example, the mouse is unable to hide from potential danger in the NSF test (no clear safe areas like the 'closed arms' of the EPM). Additionally, and may be more important, the NSF may be more sensitive to measuring underlying motivation, because animals are typically food

deprived for hours beforehand and food is used as an explicit motivator to venture out into the centre of the field (but see next section for details). The EPM, on the other hand, lacks these features.

In other studies examining dentate gyrus manipulations, Snyder and colleagues found that mice with ablated adult neurogenesis did not show an innate impairment in elevated-plus maze exploration as compared to wildtypes. These mice, even after experiencing restraint stress, were still similar to wildtype mice who also experienced restraint stress prior to the EPM task (Snyder et al. 2011). Normal mice that were previously exposed to shocks also did not show an experience-dependent change in this task compared to non-shocked mice (Daviu et al. 2010). Furthermore, in male mice after a rewarding experience, reactivating dentate gyrus granule cells that were activated during exposure to a female mouse also did not alter elevated-plus maze behaviour (Ramirez et al. 2015).

The novelty-suppressed feeding task is also considered an anxiety-like test which could be considered in terms of an approach-avoidance conflict, and is thus similar to the elevated-plus maze. Rodents with hippocampal lesions, and specifically ventral hippocampal lesions show less anxiety-like behaviours in this task or on related neophagia tasks (Bannerman et al. 2002, 2003; McHugh et al. 2004; chapter 2). Novelty-suppressed feeding has been used successfully to show differences in

behaviour in mice lacking adult neurogenesis in a number of studies (e.g. Santarelli et al. 2003, Snyder et al. 2011, Surget et al. 2008).

What are the main differences between the two tasks? In the version of the novelty-suppressed feeding task used in this thesis, a familiar food was placed in the middle of an open arena that was in a novel room. Lights illuminated the maze and the mouse had nowhere to go that allowed for avoidance of the 'anxiogenic' (potentially dangerous) region, although it may be the centre zone where an approach-avoid conflict exists. In the elevated-plus maze, the mouse can choose to stay in the closed arm and avoid the potentially dangerous open arms or centre zone if it so chooses. In the novelty-suppressed feeding task, however, the only less anxiogenic areas, arguably, are the sides of the open arena, yet the animal is still exposed to bright lights and open spaces.

Another feature that differs in this novelty-suppressed feeding task is that food is taken away from the animal overnight and so the animal is in a state of hunger when it is first exposed to this task. This not only may change the dynamics between the approach-avoidance priorities, but also may tap into other emotional processes. For example, animals are food restricted before performing this task in order to measure latency to eat a food reward in the middle of the arena. This food restriction modifies the approach-avoidance balance of the animal. The

novelty-suppressed feeding task is arguably more biased by motivational approach factors rather than factors related to avoiding danger, whereas the elevated-plus maze may be the opposite in terms of an approach-avoidance balance framework.

Additionally, a decrease in motivation to eat rewarding food is a hallmark feature of depression (anhedonia). Indeed, in mice lacking adult neurogenesis, Snyder et al. (2011) found that mice display some features related to a depressive-like phenotype, including anhedonia in certain situations. However, one argument against an anhedonic-like phenotype in this novelty-suppressed feeding task is that there is no genotypic difference under baseline conditions on this task and these mice display normal latencies to eat food in their homecage after the task (Snyder et al. 2011).

It is clear that there are fundamental differences in the EPM and NSF behavioural tasks that may recruit different neural and psychological processes that engage the hippocampus to different degrees. This may depend on the size of the hippocampal insult, the task parameters that may affect the approach-avoidance conflict, and the genetic and phenotypic background of the animals (e.g. a strain more susceptible to stress) that may affect the emotional responding of the animal.

## **Adult Neurogenesis and Interference**

In this study, TK mice showed similar novelty-suppressed feeding behaviours irrespective of the prior fear conditioning received. Wildtype mice, however, showed adaptive behaviours that were dependent on the nature of the prior type of fear conditioning received. The difference between WT and TK mice may be due to a lack of sufficient learning during the initial fear conditioning in the TK mice. Two points suggest that a general deficit in learning is unlikely to be the explanation. Mice with adult neurogenesis extinguish at the same rate as mice without adult neurogenesis and also show normal associative learning to the reliable predictor, including in a version of the task using a weaker shock (see previous Chapter 3 and see Chapter 3's discussion for more detail).

Related to a learning interpretation, this set of results may reflect a general deficit of TK mice to form episodic-like representations based on prior fear conditioning's cue predictability. The inability to form or retrieve clear episodic-like representations may be a failure to separate out incoming signals (e.g. memories). This inability to separate out, for example, memories may create overlap amongst memories. A 'fear memory' that is unable to be appropriately assigned to a specific context, or overlaps with aspects of a 'healthy memory' of a different context may promote fear generalisation to inappropriate

situations, such as that seen in post-traumatic stress disorder (Besnard & Sahay 2015, Sahay et al. 2011). More specific to this thesis, during the initial fear conditioning, the ambiguous predictor cue may generate two overlapping associative excitatory 'links', one a cue-shock and the other a cue--no shock relationship. The ability to separate these events, then, may let the animal stay on the side of caution and freeze upon hearing the ambiguous tone. Additionally, it may be that the interference caused by the overlap of these two associative 'links' may hinder the accurate retrieval of an episodic-like memory. If the mice are unable to reduce interference through pattern separation mechanisms during conditioning, future behaviour may adopt a more general defensive or cautious strategy rather than situation-specific strategies that relate to different experiences (i.e. the animals will be more inclined to generalise). This may account for the uniform latencies in novelty-suppressed feeding behaviour (i.e. a general cautious strategy) irrespective of prior fear conditioning type in mice that lack adult neurogenesis. Wildtype mice show clear adaptive behaviour (i.e. a situation-specific strategy) that is based on the nature of the prior fear conditioning type. Put simply, they experienced uncertainty related to threat (ambiguous fear conditioning) and generalised this to future experiences that contain elements of uncertainty related to threat, and the exact behavioural response is related to an increase in anxiodepressive-like behaviour.

## **Adult Neurogenesis and Glucocorticoids**

Related to these points, glucocorticoids play a specific role in stress response and adaptation, and also influence behavioural strategies in order to cope with stressors. New and mature granule cells are particularly sensitive to the effects of circulating glucocorticoid levels (Cameron & Gould 1994, Gould et al. 1991a, Sloviter et al. 1989). During the perinatal stages of life, glucocorticoids are necessary for normal dentate gyrus development and normal development of defensive behaviours that rely on the structural integrity of the dentate gyrus (Gould & Cameron 1997, Takahashi 1992, 1995, 1998; Takahashi & Rubin 1993).

Additionally, early-life exposure to glucocorticoids programs a general stress (coping) response that is used throughout life in order to allow one to adapt to (predicted) future environmental conditions. The 'match/mismatch hypothesis' is based on predictive adaptive responding and suggests that the environment in which the organism is born programs a general stress response that serves them throughout life. This response is beneficial if the future environment matches the perinatal one but not if there is a 'mismatch' in adversity conditions. For example, low maternal care (increasing perinatal stress levels) during development results in decreased glucocorticoid receptor

expression and shorter dendritic spines, affecting CA1 long-term potentiation levels in adulthood. These effects on plasticity are reversed upon exogenous corticosterone treatment, thereby mimicking the original stress environment experienced during development. In addition, mice reared in low-maternal care environments had enhanced object recognition abilities after experiencing footshock stress, whereas those in high-maternal care environments showed this effect only under non-stressful conditions. Similarly, rats reared in a low-maternal care environment displayed enhanced contextual fear conditioning, a task which is highly stressful due to unpredictable shocks. Thus, animals born into a high stress environment perform better in high stress environments in adulthood compared to those born into a low stress environment (see for review Champagne et al. 2008, Oitzl et al. 2010). These studies suggest that, like during perinatal development when new neurons are at the peak of proliferation and endow high amounts of plasticity within the brain, those new neurons born in adulthood may continue to influence how the animal responds to its stressful environment and promote adaptive changes in behaviour.

The results in this chapter are potentially consistent with glucocorticoids' protective role in promoting adaptive behaviour in the face of stressful experiences. Specifically, in mice with new neurons, adrenalectomy and the clamping of corticosterone shortened latencies in the novelty-suppressed feeding task after

ambiguous fear conditioning. Compared to the adrenal-intact mice with continual adult neurogenesis, these mice failed to show adaptive responding. Mice without new neurons, by contrast, did not show this adaptive responding after ambiguous fear conditioning with or without adrenalectomy. The set of results points to glucocorticoids pivotal role but adds that a continual supply of new neurons must be added in order to reap this protective role from the experience-induced glucocorticoids.

## **Conclusion**

The results in this chapter suggest that new neurons, in conjunction with glucocorticoids produced during fear conditioning, may be necessary for appropriate adaptive defensive behaviours that are dependent upon by the predictability of a previous threat. Furthermore, rates of adult neurogenesis do not correlate directly with levels of anxiodepressive-like behaviour but instead with the ability to adapt appropriately the levels of these behaviours to new, potentially dangerous, situations.

# 5 HIPPOCAMPAL LESIONS AND ABLATING ADULT NEUROGENESIS DIFFERENTIALLY AFFECT BEHAVIOUR TOWARD AMBIGUOUS CUES OF REWARD

## 5.2 Abstract

In this set of experiments, the contribution of (i) the hippocampus and (ii) adult-born neurons are assessed in reward-based decision-making tasks in which conflict arises between opposing choices. The first set of experiments examined how reward uncertainty (varying the probability of reward) influenced choice preference behaviour while the second set of experiments examined how differing reward sizes influenced this choice behaviour. In the first set of experiments, a non-spatial version of a T-maze task was used to vary the certainty/uncertainty of reward delivery. One arm was partially reinforced with a reward (50% of the time; and therefore ambiguous) while the opposite arm was continuously reinforced with a reward. Sham animals showed a clear preference for the certain (continually reinforced) arm. Hippocampal-lesioned mice tended to show a decrease in choice preference behaviour for the continuously reinforced arm and continued to make incorrect entries into the partially reinforced arm. Mice without new neurons were indistinguishable from wildtype mice on this task, both showing a preference for the certain arm. In the second set of experiments using a different cohort of mice, the same non-spatial T-maze arms were now manipulated such that one arm was associated with a low magnitude of reward and the other associated with a high magnitude of reward (5x more milk reward). In a previous study, sham animals preferred the high-magnitude reward compared to the low-magnitude reward. Hippocampal-lesioned mice, however, did not show

such a clear preference, and instead continued to enter into the arm containing the low-magnitude reward to a significant degree. Mice with ablated adult neurogenesis did not differ from wildtype mice with respect to preferring the high-magnitude rewarded arm. These results suggest that the hippocampus, but not adult born dentate gyrus granule cells, may be involved in non-spatial decision-making tasks, particularly when there is a conflict of goal responses, and when one conditioned prepotent response needs to be inhibited. As a control task for these different decision-making tasks, separate groups of mice were trained on versions of a simple visual discrimination on the non-spatial T-maze task, during which one coloured goal arm was always associated with reward, and the other was never associated with reward. In two different versions of visual discrimination control task that varied with regard to the number of forced-trials given before free-choice decision-making, it was found that, in shams, the number of forced-choice trials given before the choice trials influenced how well they performed on subsequent free-choice trials. Sham mice displayed a clear preference by the end of the first block if trained initially with high numbers of forced-choice trials; hippocampal-lesioned mice did not show this clear preference until later in training. These results may reflect differences in then attention paid to stimuli in the environment in sham and hippocampal lesioned animals during the forced trials.

## 5.3 Introduction

In the previous chapters, it was shown the hippocampus and new neurons in the dentate gyrus are necessary for normal defensive behavioural responses and immediate-early gene expression levels, as compared to control mice, towards ambiguous predictor cues in fear conditioning. These defensive behavioural responses, however, are only one aspect of experiencing ambiguity or uncertainty within the world. Predicting outcomes of reward from ambiguous cues is also necessary to optimize well-being.

Given the hippocampus's well-known role in memory, it also appears to play an integral role in executive processes, like decision-making. Hippocampal-lesioned animals tend to show intolerance in waiting to receive a reward (with temporal delays) and when reward is uncertain to occur. In one study, Rawlins and colleagues (1985) tested rats with aspiration lesions of the hippocampus on a Y-maze decision-making task. They found that these animals tended to prefer choosing an arm of the Y-maze that produced immediate access to a reward that was only present 25% of the time over the other arm that provided reward but which was delayed, although the reward was always guaranteed to be present. Sham-lesioned animals, however, continuously showed a clear preference for choosing the arm with delayed access to the guaranteed reward (Rawlins et al. 1985). These results were consistent with a previous study in which septal lesions were made to rats, using an identical task

(Newman et al. 1983), suggesting that a septo-hippocampal system plays an important role in inhibitory control of decision-making behaviour.

In a similar study, rats with cytotoxic lesions to the hippocampus tended to prefer an immediate small reward over a delayed large reward, whereas sham animals preferred the delayed large reward in a non-spatial T-maze decision-making task (Mariano et al. 2009). What made the task non-spatial was that one arm was black/white striped and the other arm was uniform grey and these arms were counterbalanced across lesion and sham groups and within each group (Mariano et al. 2009). Similarly, hippocampal-lesioned rats show this same effect (intolerance to delay) in an operant box where instrumental responding must occur for a reward (Cheung & Cardinal 2005). Further studies have suggested that both the dorsal and ventral hippocampus (McHugh et al. 2008) are critically important in a spatial version of the delay discounting T-maze task. It has been suggested that the ventral hippocampus's interaction with regions of the ventral prefrontal cortex, like the orbitofrontal cortex, and the nucleus accumbens is necessary to regulate impulsive choice behaviour and also the tolerance of uncertainty in touchscreen tasks (Abela & Chudasama 2013, Abela et al. 2013, 2015).

Using a variety of spatial and non-spatial tasks clearly demonstrates that the hippocampus is important in decision-making tasks. In addition to temporal delays, the detection of reward size magnitudes may also be impaired in these rats. Bett and colleagues (2014) have

shown that rats with hippocampal lesions spent more time investigating the reward size differences during the investigative phase of an adjustable delay-discounting task, displayed less sensitivity toward reward magnitude shifts during this task, and showed more variability in their choice performance on the non-adjustable delay-discounting task (Bett et al. 2014). Similarly, underlying vicarious trial-and-error sampling appears to be impaired in these animals. Rats with hippocampal lesions display less vicarious trial-and-error behaviour in a modified Y-maze task when reward location must be determined (Bett et al. 2012).

The above results suggest that the hippocampus is intricately involved in decision-making tasks that involve elements of conflict, whether they are between choices related to reward sizes or related to temporal cost-benefit trade-offs (delay-discounting). In this set of experiments, mice with hippocampal lesions and mice with a selective ablation of adult neurogenesis were tested in two non-spatial, reward-based decision-making non-spatial T-maze tasks. These tasks were devised in such a way that visually distinct, non-spatial arm choices were either associated with (i) the uncertainty or certainty of obtaining a reward, meaning that one arm was an ambiguous predictor of reward, or (ii) a variation in reward magnitude (large vs. small reward). Both of these situations create conflict between the possible behavioural responses available and disambiguation of these goal-related conflicts must be resolved in order to perform maximally in these tasks.

## 5.4 Methods

All procedures were performed at the University of Oxford and adhered to the United Kingdom Animals Scientific Procedures Act (1986) under project license 30/3068, and they were approved by local ethical review for the University of Oxford.

### **Animals**

For hippocampal lesion experiments, all mice (aged 4-8 months) were female C57/Bl6 mice (Harlan, UK), group housed (4-8/cage) and the animal colony was on a 12 hr light/dark cycle (lights on at 7:00 AM). Hippocampal- and sham-lesioned mice were food restricted to approximately 85% of their free-feeding weight. During the food deprivation schedule, crushed food was sprinkled around the cage in order to ensure equal food distribution among the mice when administering the allotted food ration. The sham and hippocampal-lesioned mice used in Expt. 1a and 1b had a history of fear conditioning (see Chapter 2) but were tested here approximately two months later. The sham and hippocampal lesioned mice used in the simple visual discrimination experiment (Expt. 2a) had prior experience of behavioural tests, such as the elevated-plus maze, neophagia task, burrowing task,

nesting task, and the spontaneous alternation task. Mice in the reward discrimination experiment (Expt. 2b) had previously performed a water Y-maze reference memory task.

For adult neurogenesis experiments, all mice were experimentally naïve and were re-derived here in Oxford from the mice generated at NIH that carried the herpes simplex thymidine kinase transgene (TK mice). The re-derived mice were therefore of the same strain and genetic background as those described in Chapters 3 and 4 methods under "Animals", and all mice tested were males in order to provide consistency with the animals used in the experiments described in Chapters 3 and 4. They were also maintained on the same feeding schedule with the drug valganciclovir in the same concentration of drug-to-food ratio. All environmental and caging conditions were similar as that described above for the hippocampus experiments. These mice were food deprived in the same manner as the above sham and hippocampal-lesioned mice to approximately 85% of their free-feeding weight. Because mice received valganciclovir mixed with powdered food, mice were given their own independent food pots in each corner of the cage in order to help and ensure equal food distribution and hence valganciclovir intake among the mice.

## Hippocampal Lesions

Mice were anaesthetized with isoflurane and administered intraperitoneal (i.p.) injections of metacam (5 mg/kg), vetergesic (0.08 mg/kg), and marcaine (2 mg/kg). These injections were given prior to stereotaxic mounting (Kopf Instruments, CA; Clark Electromedical, Reading, UK). A midline incision was made down the scalp and four burr holes were drilled per hemisphere. The excitotoxin *N*-methyl-d-aspartic acid (NMDA, 10mg/ml) in phosphate-buffered saline (pH 7.4) was injected with a 5- $\mu$ l Hamilton syringe using a 34-gauge needle (see Table 2.1 for coordinates; second cohort). For every 0.1  $\mu$ l of toxin injected, the needle sat in place for 60 seconds to allow for diffusion away from the site and then was raised in a step-wise manner out of the tissue. Sham-operated animals received all drugs and anaesthesia, were placed in the stereotaxic frame, received midline incision, and then the incisions were sutured. There was no penetrating the needle into brain tissue. After suturing, mice were given midazolam (5 mg/kg) and 1 ml saline subcutaneously. After surgery, mice were placed into individual heated cages and given access to water and a mash mixture (regular food pellets mixed with water). These mice were monitored for at least 30 minutes before being returned to their homecages.

## **Histology**

The same procedures were used as in Chapter 2 for hippocampal Cresyl violet staining (a subset of the animals here were the same mice as described in Chapter 2; second cohort). This histology from Expt. 2a (Simple Visual Discrimination) was used in C. Barkus's DPhil thesis (Barkus 2010). The histology from Expt 2b/ (Reward Magnitude Discrimination) was published (Sanderson et al. 2012). To confirm that the TK mice were receiving sufficient valganciclovir in order to ablate the new neurons under the food restriction protocol, 50 micron sections were taken using a sliding microtome, and were stained for doublecortin (Santa Cruz Biotechnology) using a 1:2000 blocking solution. The protocol and procedures used for this staining were similar to those used in Chapter 4 methods under "Histology". A 1:8 series throughout the hippocampus was taken and stereological counts were taken at 10x and 40x magnification using a bright-field (light) Nikon microscope. Cell counts were multiplied by 8 to achieve estimates throughout the entire dentate gyrus. Representative photos were imaged at 10x magnification and 40x magnification was used to examine cells.

## **Handling and General Procedure**

Mice were handled for 3-5 min/day for at least 2 days prior to behavioural testing and allowed to acclimate to the mouse holding are for at least 20 minutes prior to any behavioural testing.

## **Experiment I: Ambiguous Predictor of Reward**

### **Apparatus**

A wooden T-maze was made of two goal arms, with one painted uniform grey and the other black/white striped, and a plain wooden-coloured (light brown) start arm. During habituation, the two goal arms were replaced by two plain wooden arms. Each arm measured 10 x 30 x 30 cm with a stainless steel reward well (1 cm diameter) located 2 cm from the distal ends of the goal arms.

### **Habituation**

All mice (both Simple Visual Discrimination and Ambiguous Reward groups, see below) in this experiment were habituated to the reward (50% sweetened-condensed milk:water) and to the T-maze for 3 days. All habituation days consisted of multiple sessions and mice were allowed to

explore the maze freely with multiple rewards (refills of diluted sweetened-condensed milk) given in the wells for approximately 5 minutes each session. On the first day, mice were placed on the maze with their cagemates. On subsequent days, if still anxious, they were re-placed on the maze with cagemates. They were then placed in the maze singly. All mice were habituated to a degree such that they were readily running down the arms and drinking the reward with little or no hesitation. After habituation, mice were either allocated to the visual discrimination or ambiguous reward groups listed below.

### **Exp. 1a: Simple Visual Discrimination Group**

#### ***Procedure***

After habituation, half of the hippocampal- and sham-lesioned mice were trained on the T-maze with the removable goal arms, which were either grey or black/white striped. One arm signalled 100% chance of reward, while the other was never rewarded (0% chance of reward). The reward was diluted 50/50% sweetened-condensed milk/water mix (0.1 ml per reward). Each mouse was allocated a particular goal arm (grey or black/white striped) in which they always received reward. They were never rewarded in the other arm. Allocation of mice to particular coloured goal arms for reward was fully counterbalanced across groups. Mice were placed on the maze at the beginning of the start arm facing the experimenter and allowed to run and collect a reward. On correct choices,

mice were allowed to drink all the milk before being removed from the arm. The next mouse was then run on their respective trial. The inter-trial interval was variable, because it depended on how fast the other mice took to eat; however, no mouse took longer than five minutes to consume the reward. For incorrect choices to the never-rewarded arm, mice were removed from the maze when they reached the empty reward-well. The position of the rewarded arm (left or right) was pseudo-randomly generated, such that no more than two consecutive arm positions/rewards were in the same spatial location, and each position was rewarded equally within each session, in order to remove any spatial component to the task.

### ***Design***

Mice experienced eight forced-choice trials (by blocking access to one of the goal arms; they received a total of four forced trials to the black/white striped arm and four to the grey arm), and then four free-choice trials (with both goal arms open) on each day of testing. Only data from the four free-choice trials per session were graphed and analysed. The same reward/non-reward contingencies applied on both the forced and choice trial (i.e. animals received reward in the same goal arm on forced trials as they did on the choice trials). The eight forced-choice trials were used as training trials and were introduced to ensure exposure to both goal arms and their contingencies during training, and to provide continuity with the experimental paradigm below (Ambiguous Reward Group). On the last day of behavioural testing, mice received eight forced-

choice trials and then eight free-choice trials in which the rewarded goal arm was baited but only after the choice had been made (post-choice baiting) in order to investigate whether odour cues from the reward biased choice behaviour. Allocation to a particular goal pattern (black/white striped or uniform grey), the trial order, and arm positions were pseudo-randomly assigned across sham- and hippocampal-lesioned mice, and for mice with (WT) and without (TK) adult neurogenesis. Mice were run on this task for 14 days. Data were blocked into two days/block.

### **Expt. 1b: Ambiguous Reward Discrimination Group**

#### ***Procedure***

To examine the ability of mice (lesioned and those lacking adult neurogenesis) to discriminate between one arm that reliably predicts (100% of the time) a reward and one that ambiguously predicts reward presence (50% of the time), a modified version of the above Simple Visual Discrimination T-maze task was performed. Again, the reward was diluted 50/50% sweetened-condensed milk/water mix (0.1 ml per reward). After habituation, the other half of the mice began T-maze testing with a similar procedure as above; however, the reward probability differed. One goal arm was rewarded 100% of the time (high-probability arm, 'HP arm') and the other was rewarded in half of the trials (50%) only. On correct choices to the HP arm, mice were allowed to drink all the milk before being

removed from the arm. For choices into the low-probability arm, mice were allowed to drink the milk on rewarded trials or were removed from the maze when they reached the empty reward-well on non-rewarded runs. The next mouse was then run on their respective trial. The inter-trial interval was variable, because it depended on how fast the other mice to eat; however, no mouse took longer than five minutes to eat.

### ***Design***

Similar to the Simple Visual Discrimination design above, the mice were given eight forced-choice trials (by blocking access to the other goal arm; they received a total of four forced trials to the black/white striped arm and four to the grey arm) and then four free-choice trials (with both goal arms open). The forced-choice trials were necessary to ensure exposure to each arm-reward association. On the last day of behavioural testing, mice received eight forced-choice trials and then eight free-choice trials (as above) during which wells were baited only after the choice had been made in order to investigate whether odour cues from the reward biased choice behaviour (post-choice baiting). Allocation to a particular goal pattern (black/white striped or uniform grey), the trial order, and arm positions were pseudo-randomly assigned across sham- and hippocampal-lesioned mice. Mice were run on this task for 14 days. Data were blocked into two days/block.

## **Experiment II: Reward Magnitude Discrimination**

### **Apparatus**

The same wooden T-maze with the same goal arms (black/white striped and uniform grey) and reward type (diluted sweetened-condensed milk) were used in this experiment as in Experiment I.

### **Habituation**

All mice (both Simple Visual Discrimination and Reward Magnitude Discrimination groups, see below) in this experiment were habituated to the maze and reward in a similar way as described in Experiment I.

### **Expt. 2a: Simple Visual Discrimination Group**

#### ***Procedure***

After habituation, half of the hippocampal- and sham-lesioned mice were trained on a simple visual discrimination task with the same general procedure as used in the above Simple Visual Discrimination portion of Experiment I. Briefly, on correct choices to the always-rewarded arm, mice were allowed to drink all the milk before being removed from the arm. The reward was diluted 50/50% sweetened-condensed milk/water mix (0.1 ml per reward). For incorrect choices into the never-rewarded arm, mice were

removed from the maze when they reached the empty reward-well. The next mouse was then run on their respective trial.

### ***Design***

Mice experienced 10 free-choice trials each day. Allocation to a particular goal arm pattern (black/white striped or uniform grey) and arm positions (i.e. right/left allocation of the rewarded goal arm) were pseudo-randomly assigned across sham- and hippocampal-lesioned mice. Mice were run on this task for eight days.

### **Expt. 2b: Reward Magnitude Discrimination Group**

#### ***Procedure***

To examine the ability of mice (lesioned mice and those lacking adult neurogenesis) to discriminate between two goal arms that both contain a reward but of different magnitudes, a modified version of the Visual Discrimination, non-spatial T-maze task was performed. The remaining half of the mice were trained on the T-maze task with different reward magnitudes associated with each goal arm. Specifically, one goal arm was allocated a reward size of 0.25 ml (high-reward arm, or 'HR arm') of the 50/50% diluted sweetened-condensed milk/water mix, while the other contained 0.05 ml (low-reward arm). On correct choices to the HR

arm, mice were allowed to drink all the milk before being removed from the arm. For choices into the low-reward arm, mice were also allowed to drink the reward and were then removed from the maze. The next mouse was then run on their respective trial.

### ***Design***

To ensure exposure to both reward magnitudes in their respective goal arms, mice began each session with two forced-trials (one to each reward arm). The forced-trials were conducted in such a way that the mouse had to run into the one arm while the other arm was blocked, and then on its subsequent trial run into the other arm while the other was blocked; this was counterbalanced across two days. Then, they were run on five free-choice trials per day (session). Data were blocked into 10 trials for analysis (i.e. two consecutive days (sessions) were combined). Mice were run on a total of six blocks. Only the free-choice trials were analysed.

### **Statistics**

Data were analyzed in SPSS and GraphPad Prism. The two-way ANOVAs were mixed model with block as the within-subjects factor and genotype or lesion as the between-subjects factor. The three-way

ANOVAs were also mixed models with session or block, as the within-subjects factor. Lesion/genotype and/or high-reward arm (i.e. whether the high reward was assigned to the grey arm or whether it was assigned to the black/white arm) were the between-subject factors. The four-way ANOVA was mixed model with block as the within-subject factor and lesion, group (visual discrimination or ambiguous reward), and high-reward arm pattern (i.e. whether the high reward was assigned to the grey arm or whether it was assigned to the black/white arm) as the between-subjects factors. Greenhouse-Geisser correction was applied when homogeneity of variance could not be assumed for the within-subjects factors or a stricter  $p$ -value introduced ( $p < 0.03$ ) when homogeneity of variance assessed by Levene's test could not be assumed. All  $t$ -tests are independent sample two-tailed tests except for the stereological cell counts, which were one-tailed, non-parametric Man-Whitney  $U$  tests where equal variance was not assumed. All data are expressed as the mean  $\pm$  standard error of the mean. All post-hoc analyses used Bonferroni correction.

## 5.5 Results

### **Experiment I: Ambiguous Predictor of Reward**

#### **Histology**

For lesions throughout the hippocampus, the excitotoxin *N*-methyl-d-aspartic acid (NMDA) was injected using coordinates either from the interaural line or from bregma in two different cohorts of mice (volumes and coordinates are listed in Table 2.1 but are also given in the figure legend). After perfusions, postfixing, cutting, and staining of the brains (Chapter 2, Methods, Histology), the hippocampus and areas surrounding it were analysed. There was little sparing of the hippocampus. Most of the sparing in the hippocampus occurred in the most ventral portion and spared mostly the CA1/subiculum border. Figure 5.1 shows reconstructions (left) from the lesioned animals and shows the largest (orange+blue shading) and smallest (blue shading) lesion size. There was minimal extra-hippocampal damage.

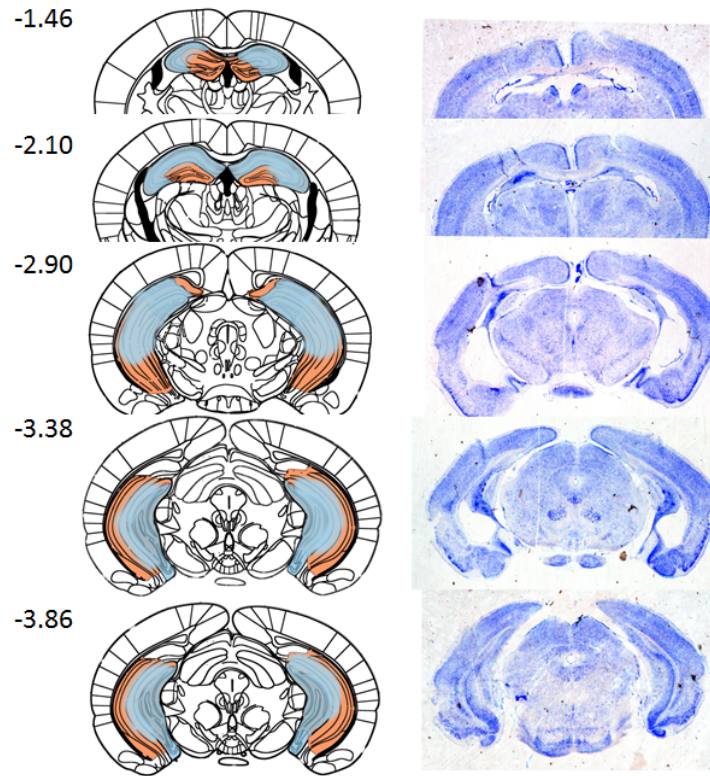


Figure 5.1 Reconstructions and photomicrographs throughout the typical lesioned hippocampus made with NMDA excitotoxin from the ambiguous reward (second cohort) experimental mice. The A-P coordinates given to the left of the schematics are approximate coordinates of the sections. The specific lesioning sites were (A-P from bregma: -1.7, -2.3, -2.8, and -3.1). Orange shading represents largest lesion size. Blue shading represents smallest lesion size. Photomicrographs are through the approximate coordinates given for the reconstructions from a representative lesioned mouse.

To confirm that food restricting the treated transgenic TK mice did not affect the administered amount of valganciclovir, and thus the elimination of newly dividing cells, stereological counting throughout the hippocampus was performed on doublecortin (DCX)-stained neurons. Throughout the hippocampus, virtually all of the young neurons were eliminated (Figure 5.2, A) (one-tailed Man-Whitney nonparametric test,  $U < 1$ ,  $p = 0.0048$ , *a priori* assumption that TK mice would show very few young neurons and equal variance and distribution were not assumed). WT mice treated with valganciclovir showed high levels of doublecortin-positive cells ( $n = 4$ ) compared to treated TK mice ( $n = 6$ ). Representative images from the dorsal dentate gyrus are shown in Figure 5.2 (B) and doublecortin-positive cells in WT mice that were treated are easily visible (brown dots) in the subgranular zone, while none are visible in TK mice that were treated with valganciclovir (B).

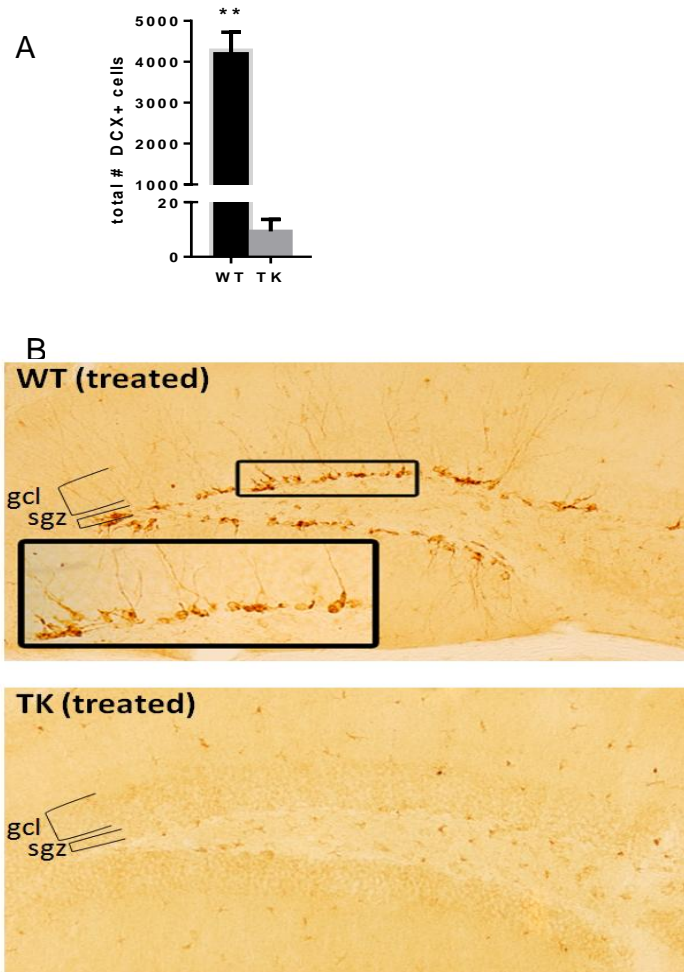


Figure 5.2 A: Stereological estimates of doublecortin (DCX)-positive cells were counted throughout the hippocampus. These cells were virtually eliminated from the dentate gyrus in treated TK mice, as compared to their treated WT littermates, even on food restriction. B: Example bright-field microscopy images revealed an abundance of doublecortin-positive neurons within the subgranular zone of treated WT mice (middle) and very few (none in this representative image) doublecortin-positive cells across the treated TK mice (bottom). gcl = granule cell layer; sgz = subgranular zone. \*\*  $p = 0.0048$ .

### **Expt. 1a: Visual Discrimination**

In order to examine if hippocampal- ( $n = 6$ ) and sham-lesioned ( $n = 6$ ) mice could perform a simple visual discrimination task, one patterned (i.e. uniform gray or black/white striped) goal arm was associated with a reward and the other arm never contained a reward. A three-way mixed ANOVA revealed that there was a significant block effect ( $F_{6, 48} = 12.9, p < 0.0001$ ), and, surprisingly, a main effect of lesion ( $F_{1, 8} = 15.02, p = 0.005$ ), with a block x lesion interaction ( $F_{6, 48} = 6.22, p < 0.0001$ ) (Figure 5.3, A). This reflected a transient impairment in the hippocampal lesioned mice during the early training trials. There were no other significant effects or interactions (effect of high-reward arm pattern:  $F_{1, 8} < 1$ ; lesion x arm pattern interaction: ( $F_{1, 8} = 1.4, p = 0.27$ ; block x arm pattern interaction:  $F_{6, 48} < 1$ ; block x lesion x arm pattern:  $F_{6, 48} < 1$ ). Shams exhibited high levels of performance from the very beginning of their free choice trials. Mice with hippocampal lesions started at above chance levels and gradually rose to the high choice performance displayed by the sham mice. Sham animals performed significantly above the hippocampal animals in the first block ( $p = 0.004$ ) and there was a trend on the second and third blocks ( $p = 0.06$  and  $0.08$ , respectively), as determined by Bonferroni post-hoc analyses, and then both groups continued to display a high level of choice performance throughout the rest of testing.

The difference seen between hippocampal- and sham-lesioned mice on the early trials may be attributable to the large number of forced-

trials given before and during the free-choice trials. Hippocampal lesions produce an inability to suppress the prepotent response of running down a runway (e.g. running to get a reward having been trained like this during the habituation phase), and lesioned mice may pay less attention to novel stimuli introduced onto the runway (e.g. Raphaelson et al. 1965). This may have interfered with the initial encoding or attention paid to the arm contingencies. Sham mice, on the other hand, despite having these forced trials, possibly attended to the stimuli better, and therefore learnt the contingencies quicker. Nevertheless, the hippocampal-lesioned mice did quickly rise to the level of sham mice and were certainly capable of acquiring the task and reaching the same asymptotic level of performance as the controls.

### **Expt. 1b: Ambiguous Reward Discrimination**

In order to examine whether competing reward associations (linked to different probabilities of reward) would differentially affect mice with and without hippocampal lesions, mice were tested on a similar non-spatial T-maze task with differing reward probabilities in each goal arm. One of the arms was associated with 100% (high) probability ('HP arm choice') of reward and the other arm was associated with the same reward but on only 50% of trials. A different subset of hippocampal- ( $n = 6$ ) and sham-lesioned ( $n = 6$ ) mice were used for this experiment. A three-way mixed ANOVA revealed that there was a main effect of block ( $F_{6, 48} = 4.3, p = 0.001$ ) and main effect of lesion ( $F_{1, 8} = 9.8, p = 0.01$ ) which reflected a

robust and lasting impairment in the hippocampal lesioned mice (Figure 5.3, B). There was also an effect of the high-probability arm pattern ( $F_{1,8} = 13.9, p = 0.006$ ) in which animals that were allocated the uniform grey arm as the high-probability of reward arm were quicker to show a preference in arm-choice behaviour than animals which had the black/white striped arm as the HP arm. No other significant interactions were found (arm pattern x lesion:  $F_{1,8} < 1$ ; arm pattern x block:  $F_{6,48} < 1$ ; block x lesion:  $F_{6,48} < 1$ ; arm pattern x block x lesion:  $F_{6,48} < 1$ ).

Further analysis of the data from the simple visual discrimination and the ambiguous reward discrimination tasks taken together was conducted using four-way mixed ANOVA. This revealed a main effect of block ( $F_{6,96} = 12.2, p < 0.0001$ ) and of lesion ( $F_{1,16} = 17.7, p = 0.001$ ), and an interaction between block and lesion ( $F_{6,96} = 2.9, p = 0.04$ ). Additionally, there were overall main effects of the type of task performed (“group”; visual discrimination vs. ambiguous reward discrimination) ( $F_{1,16} = 46.03, p < 0.0001$ ) and the arm pattern (that was either associated with high-magnitude or high-probability reward arm) ( $F_{1,16} = 13.2, p = 0.002$ ), with an interaction between these two variables ( $F_{1,16} = 11.8, p = 0.003$ ). There were no other interactions that were significant (group x lesion:  $F_{1,16} = 3.0, p = 0.1$ ; arm pattern x lesion:  $F_{1,16} = 1.5, p = 0.2$ ; group x lesion x arm pattern:  $F_{1,16} < 1$ ; block x group:  $F_{6,96} = 1.4, p = 0.2$ ; block x arm pattern:  $F_{6,96} < 1$ ; block x group x arm pattern:  $F_{6,96} < 1$ ).

lesion:  $F_{6, 96} = 2.1$ ,  $p = 0.1$ ; block x arm pattern x lesion:  $F_{6, 96} = 1.2$ ,  $p = 0.07$ ; block x group x arm pattern x lesion:  $F_{6, 96} < 1$ ).

Next, TK mice were tested to see if an effect of ablating this population would produce similar results as lesioning the entire hippocampus. We chose to look at performance only on the ambiguous version of the task, because hippocampal lesions had an effect. Furthermore, a central question of the thesis was to examine the role of new neurons in responding to ambiguous or uncertain cues. Transgenic TK mice ( $n = 8$ ) and WT littermates ( $n = 5$ ) were given the same ambiguous reward discrimination task on the non-spatial T-maze task as described above. Unlike the hippocampal-lesioned mice who showed a significant lesion effect, these mice did not show any hint of a deficit between WT and TK mice on the reward probability task as compared to their wildtype littermates (Figure 5.3, C). There was no main effect of genotype (TK vs. WT;  $F_{1, 11} < 1$ ) but a significant effect of block ( $F_{9, 99} = 2.6$ ,  $p = 0.01$ ), signifying that both groups did in fact learn the task to some capacity. There was no genotype x block interaction ( $F_{9, 99} < 1$ ).

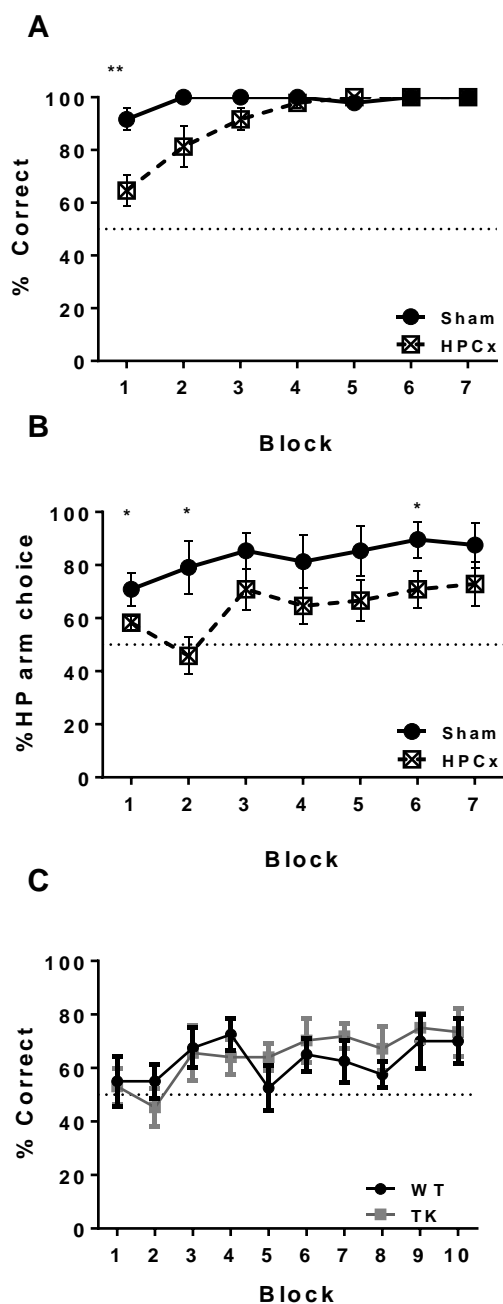


Figure 5.3 Hippocampal lesions, but not ablation of new neurons in TK mice, affect the ability to discriminate between uncertain and certain reward. A: on the simple visual discrimination task, hippocampal-lesioned animals showed a lower percentage correct than shams early on in the

task only, choosing the certain reward arm less often during the first block as compared to sham-lesioned animals. B: In the ambiguous reward discrimination task, hippocampal-lesioned mice consistently showed a lower percentage of high-probability arm ('%HP arm choice') entries compared to sham-lesioned animals, who more frequently chose the reliable arm (100% of trials rewarded) over the ambiguous arm (50% of trials rewarded). C: Mice with and without adult neurogenesis did not differ in their performance levels in the ambiguous reward discrimination task when choosing the high-probability arm ('%HP arm choice'). Note: The last block in each panel was done with post-choice baiting. Dotted grey line represents chance level. Bonferroni post-hoc tests: \*  $p < 0.05$ ; \*\*  $p < 0.01$ . All asterisks represent significant differences compared to their control group from posthoc tests (shams in the top and middle panels; WTs in the bottom panel) in the same block.

## **Experiment II: Reward Magnitude Discrimination**

### **Histology**

Lesions throughout the hippocampus were made with *N*-methyl-d-aspartic acid (NMDA) excitotoxin using the coordinates from Table 2.1, first cohort. The same histological staining and analysis were done on these brains as done in Chapter 2 (Chapter 2, Methods, Histology). There

was little sparing of hippocampal tissue that did not extend beyond hippocampal formation. The sparing from the hippocampal lesions were mainly confined to the ventral CA1/subiculum. Figure 5.4 shows reconstructions (left) from the hippocampus and shows the smallest in orange and largest (blue+orange) lesion size. Some dorsal tissue sparing was evident but cellular lamination was irregular or destroyed completely, thereby rendering it an effective lesion.

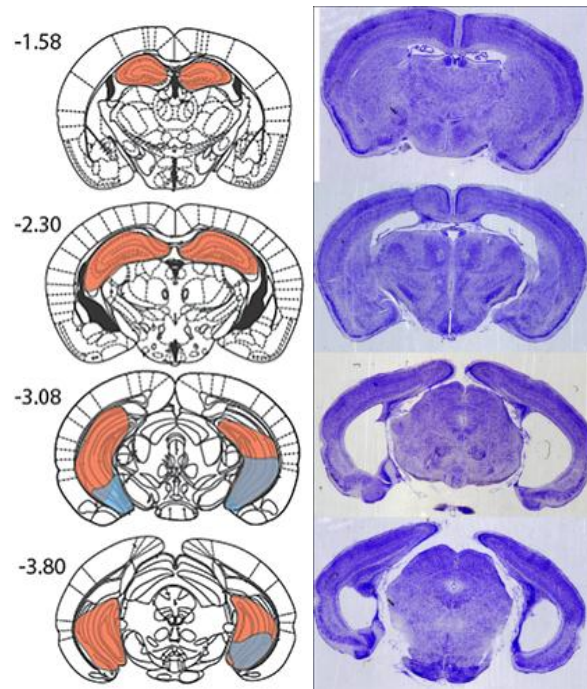


Figure 5.4 Reconstructions and photomicrographs throughout the lesioned hippocampus made with NMDA excitotoxin from the visual discrimination task (Expt. 2a). The A-P coordinates given to the left of the schematics are near coordinates of the lesioning sites from bregma that approximately correspond to the surgical interaural coordinates used for surgery. The specific lesioning sites were (A-P coordinates used the interaural line: +0.7, +1.0, +1.5, +2.1). Orange shading represents smallest lesion size. The orange and blue shading combined represents the largest lesion size. Photomicrographs are through the approximate coordinates given for the reconstructions.

### **Expt. 2a: Visual Discrimination**

Hippocampal-lesioned mice ( $n = 8$ ) and sham mice ( $n = 11$ ) were tested in an alternative version of the simple visual discrimination with fewer forced trials. This visual discrimination is similar to the one mentioned in Expt. 1a but, instead of receiving forced-choice trials, these mice were trained with all free-choice trials (10 free-choice trials per day). One arm was always rewarded while the other arm was never rewarded (arm patterns were the same as used in the previous experiments; black/white striped or a uniform grey arm, and were counterbalanced across mice). Hippocampal-lesioned animals were highly similar to their sham-operated counterparts in acquiring this task (lesion effect:  $F_{1, 17} < 1$ ) (Figure 5.5, A). Both groups started out at chance levels of performance and showed a gradual improvement in successfully associating one arm with reward as training progressed (session effect:  $F_{7, 119} = 30.02$ ,  $p < 0.001$ ), but no interaction between session and lesion appeared ( $F_{7, 119} = 1.45$ ,  $p > 0.2$ ), showing that both hippocampal-lesioned and sham mice learnt the task at a similar rate.

### **Expt. 2b: Reward Magnitude Discrimination**

To examine how competing rewards of varying magnitudes influence choice behaviour, we trained hippocampal-lesioned mice ( $n = 11$ ) and sham mice ( $n = 11$ ) to associate one of the arms with a high

reward (0.25 ml) and the other with less (0.05 ml) of the reward. Mice should be able to learn to discriminate these reward-size differences and accurately choose the high-reward (HR) arm as training progresses. Overall, hippocampal-lesioned mice were substantially impaired and made fewer correct entries into the high-reward arm as compared to shams (lesion effect:  $F_{1, 20} = 12.92$ ,  $p = 0.002$ ) but both groups did show improvement in choice behaviour over time (block effect:  $F_{5, 100} = 17.18$ ,  $p < 0.001$ ). There was a significant overall interaction between lesion and block ( $F_{5, 100} = 4.077$ ,  $p = 0.007$ ). While it is evident in Figure 5.5 (B) that both groups started out at chance levels of performance, Bonferroni post-hoc tests revealed that sham mice differed significantly from hippocampal-lesioned mice by the 3<sup>rd</sup> block ( $p = 0.003$ ) and this deficit was also present in the 5<sup>th</sup> block ( $p = 0.0002$ ).

We next wanted to see if ablating adult neurogenesis would produce similar results on the discrimination between two different reward magnitudes in WT ( $n = 8$ ) and TK mice ( $n = 8$ ) (Figure 5.5, C). There was no effect of genotype ( $F_{1, 14} < 1$ ) but an overall effect of block ( $F_{5, 70} = 3.9$ ,  $p = 0.004$ ), with no genotype x block interaction ( $F_{5, 70} = 1.8$ ,  $p = 0.1$ ). It is noted, however, that TK mice during the first block did perform numerically better than the WT mice (although this was not significant according to Bonferroni post-hoc tests). However, an independent-sample  $t$ -test (two-tailed) revealed that TK mice did, indeed, perform significantly better on the first block of training ( $t_{14} = 2.8$ ,  $p = 0.01$ ). It is not readily apparent why

this difference occurred but it was transient and both groups demonstrated equivalent performance thereafter. Furthermore, TK mice did significantly increase their performance from the second to the sixth block ( $p = 0.01$ ). Importantly, during the critical block (the last block) where a reward was given only after the choice was made (i.e. post-choice baiting), WT and TK mice did not differ.

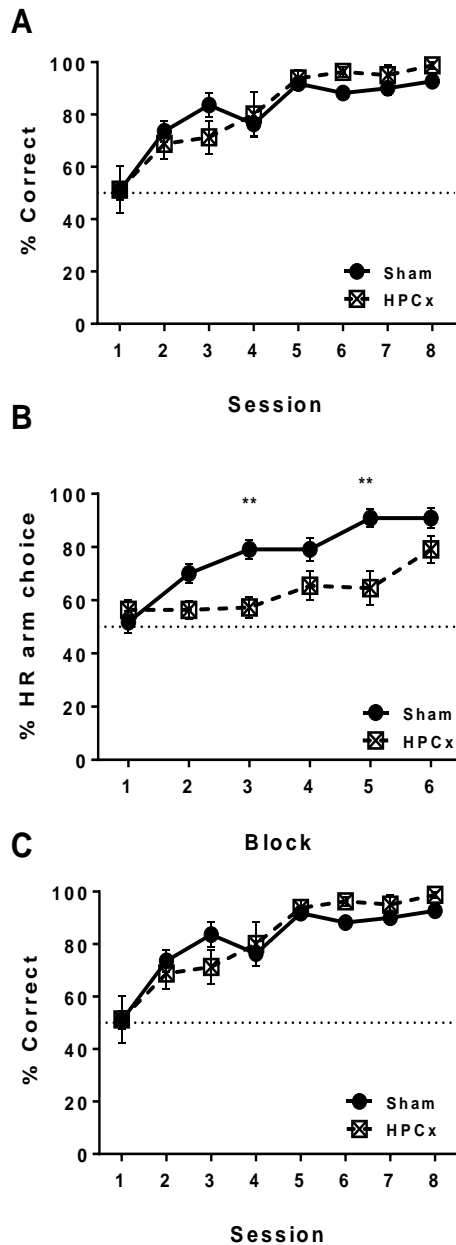


Figure 5.5 Hippocampal lesions, but not ablation of new neurons, affect the ability to discriminate between different reward magnitudes. A: panel represents the simple visual discrimination task, showing that the hippocampal-lesioned and sham lesioned mice did not differ in their percent correct performance across sessions. B: In the reward magnitude

discrimination version of the task, hippocampal-lesioned mice consistently performed below the level of sham-lesioned animals in the percentage of high-reward arm entries (%HR arm choice). C: mice with and without adult neurogenesis did not differ in their performance levels in choosing the high-reward arm (percentage of high-reward arm entries; %HR arm choices) during the reward magnitude discrimination task. Note: The label 'Session' on the x-axis represents 10 free-choice trials each day, whereas 'Block' represents 2 consecutive, daily sessions (20 free-choice trials). The last session or blocks in the panels were from post-choice baiting trials in order to rule out any odour cues that may have guided choice behaviour. Dotted horizontal grey line represents chance performance. Bonferroni post-hoc: \*\*  $p < 0.01$ .

## 5.6 Discussion

### **Summary of Results**

In this chapter, control mice showed a clear preference for a certain reward, or continuously reinforced arm, compared to an arm that was only partially reinforced with a reward. Additionally, control animals showed a discrimination preference for a high-magnitude reward compared to a low-magnitude rewarded arm. Mice with selective ablation of adult

neurogenesis also showed these preferences and did not differ compared to their wildtype control mice. Conversely, mice with hippocampal lesions showed a moderate impairment for choosing an arm that was continuously reinforced over an arm that was partially reinforced. In addition, lesioned mice were impaired at discriminating between a high- and a low-magnitude reward. Control visual discrimination experiments on the hippocampal- and sham-lesioned mice curiously showed a difference in visual discrimination performance that depended on how they were initially trained on the task (possibly relating to the numbers of forced trials used). Importantly, despite early differences in visual discrimination performance, hippocampal-lesioned animals rapidly rose to the high, asymptotic performance level of sham animals. TK mice, as compared to their wildtype littermates, did not show a difference in either experiment, with both genotypes showing equal learning.

### **The Hippocampus and Conflicting Goals**

Here, hippocampal-lesioned mice showed a decrease in preference for choosing (i) the certain rewarded arm and (ii) the high magnitude rewarded arm, as compared to shams. Conversely, mice with ablated adult neurogenesis did not show this decreased preference, as compared to their wildtype littermates. It is maybe not entirely surprising that large hippocampal lesions produce altered behavioural responses whereas only

eliminating a small population of cells, in only one region of the hippocampus, does not produce these same altered responses. In previous chapters, however, it was shown that TK mice were impaired on aversive fear conditioning that included a partially reinforced cue. There was no deficit with the partially reinforced cue in these appetitive experiments. This may be due to the saliency of the aversive vs. appetitive tasks (discussed more in General Discussion, Chapter 6), or to a fundamental difference between appetitive and aversive tasks, and the likely very different levels of stress associated with both paradigms.

However, what underlies this altered choice behaviour in the hippocampal-lesioned mice? The hippocampus has been suggested to play a role in detecting conflicting information related to concurrent goals and/or possible response choices (Gray & McNaughton 2000). In the present non-spatial T-maze tasks, a conflict is generated when the animal is at the decision point on the maze and conflicting goals, and their responses, are generated (i.e. approaching either goal arm will potentially lead to reward). At the decision point, the mouse has to be able to inhibit its tendency to run down an arm in order to optimize its decisions to achieve the highest reward possible.

In order to discriminate successfully between reward sizes or reward probabilities, the animal must engage in investigative behaviours. Recently, Bett and colleagues (2014) found that hippocampal-lesioned rats pre-operatively trained on a modified delayed discounting task

showed similar rates of adjusting their waiting-time behaviour for a high reward but showed more investigative behaviour, suggesting a diminished capacity to detect sliding reward size shifts. The mice used in this thesis's experiments were given training on a reward magnitude task post-operatively but with no delay in obtaining either the high or low magnitude reward; this provided a simple test of the animals' ability to choose between two different reward size magnitudes. The hippocampal-lesioned mice showed a decreased preference for the high-reward arm as compared to sham mice. Bett and colleagues' finding that hippocampal-lesioned rats showed higher amounts of investigative behaviour and less sensitivity to changing reward sizes during the early part of the study may contribute to the continual revisiting of the low-magnitude arm seen in the reward magnitude discrimination experiment presented in this chapter.

In a similar study related to the ambiguous reward discrimination experiment presented in this chapter, Rawlins and colleagues used a Y-maze task with one arm being partially reinforced with a 25% chance of reward as compared to a fully reinforced arm and compared sham- and hippocampal-lesioned rats. They found that, when no delay was imposed, all rats preferred the fully reinforced arm; however, when a delay was imposed on that arm, hippocampal-lesioned rats tended to prefer (at least at the later stages of the experiment) the immediate, partially reinforced arm. Shams, by contrast, stuck to choosing the fully reinforced arm even with a delay, suggesting that the lesioned animals were more sensitive to

temporal delay (Rawlins et al. 1985). In this chapter, hippocampal-lesioned mice chose the partially reinforced arm more frequently than their sham counterparts did even when no such temporal delay existed. This finding suggests that it may be the conflict caused by the uncertainty of reward probability that necessitates the mouse to keep investigating its choices. This, then, would result in a lower preference for the fully reinforced goal arm, in addition to the intolerance to temporal delay displayed by hippocampal-lesioned animals.

These two different sets of experiments in this thesis (reward magnitude discrimination and ambiguous reward discrimination) introduce elements of goal response conflict based on different parameters (i.e. reward size and reward probability), and a type of pattern separation may be required in order to parse apart or disambiguate between these competing goal response tendencies. The conflicting response tendencies, based on these different parameters, may cause the mouse to have more "doubt"; as Simonov (1974) says, "the hippocampus [is] an organ of hesitation and doubt". It has been suggested that the hippocampus is needed to flexibly recombine elements from memory in order for an animal to make predictions about goal outcomes (Buckner 2010, Johnson & Redish 2007). If this ability to recombine elements from memory in order to make predictions about outcomes or goals is hindered due to the presence of conflicting goals (thereby creating interference), more direct investigation of goals may be required. This direct

investigation or sampling of goal choices would eventually be replaced by other less direct methods of sampling, such as vicarious trial-and-error behaviour and predicting the outcome or path to a specific goal (e.g. Amsel 1993, Bett et al. 2014, Johnson & Redish 2007, Ólafsdóttir et al. 2015, Pfeiffer & Foster 2013, Tolman 1938). If the hippocampus does mediate these "predictive" investigative behaviours, destruction of this region would impair these goal predictions and, indeed, result in "hesitation and doubt" related to goal responses.

### **Goals and Pattern Separation**

In this chapter, we did not detect a deficit (or even a trend) toward impairment on the reward-magnitude or reward-certainty/-uncertainty discrimination tasks in mice with ablated adult neurogenesis, as we did for hippocampal-lesioned mice. This may be due to several reasons, chiefly because ablating new neurons is only a fraction of ablating the entire hippocampus. While the dentate gyrus is the main gateway into the hippocampus proper, there do exist separate routes (see Chapter 1, Introduction, Anatomy), and, of note, the entire dentate was not ablated in TK mice.

The dentate gyrus, and in particular adult-born neurons, has been reported to mediate pattern separation, or the disambiguation of overlapping inputs into the dentate gyrus. These overlapping inputs may

be sensory related (Aimone et al. 2011, Clelland et al. 2009) but may equally well be overlapping goal responses or the memories that support these goals (Bannerman et al. 2012, 2014), and inhibitory mechanisms are needed to suppress these overlapping (prepotent) responses. Though new neurons have been proposed to mediate at least some types of pattern separation, there is some evidence that suggests rodents with ablated adult neurogenesis can perform quite well in tasks that require at least sensory-related pattern separation (e.g. Groves et al. 2013, Piatti et al. 2014 but see Clelland et al. 2009, Creer et al. 2010, Sahay et al. 2011). In this chapter, the overlapping memory/goal inputs based on reward magnitude and ambiguous reward discrimination experiences appear not to rely on adult-born neurons, at least in these specific non-spatial tasks. This does not rule out the fact that animals probably do use some sort of pattern separation mechanism in order to solve these discrimination tasks. It may be that the brain has many pattern separators and the adult-born neurons here are not necessary for this type of reward-based separation, which relies instead on the mature dentate gyrus granule cells, other hippocampal subfields, or on extrahippocampal regions. Indeed, mature granule cells may be capable of pattern separating out these discriminations because the differences in reward sizes are large enough (3 vs. 1 pellet in Bett study and 0.25 vs. 0.05 ml of liquid reward in this chapter's study) such that the very fine discrimination, which the new neurons may perform, is not necessary.

### **Visual Discrimination: Free- vs. Forced-Choice Trials**

Related to the investigative behaviour mentioned above, an interesting and unexpected set of results emerged relating to the training technique involved in the simple visual discrimination control experiments that are typically not reliant on the hippocampus (Murray & Ridley 1999). In the experiment where mice had to discriminate between the two patterned goal arms for reward (and there was no reward at all in the other goal arm; Expt. 2a), all mice were run with a high proportion of free-choice trials and thus likely relied predominantly on trial-and-error sampling. Both sham- and hippocampal-lesioned mice started at chance performance levels and gradually increased their choice preference, with no differences between groups. In the control visual discrimination (Expt. 1a) experiment for the ambiguous reward discrimination task (fully vs. partially reinforced), mice were tested in such a way that they received multiple forced trials before being exposed to free-choice trials, in order to experience all different combinations in a counterbalanced manner. Using this methodology, sham mice displayed very high discrimination by the end of the first block (16 forced-choice trials and 8 free-choice trials), whereas hippocampal-lesioned mice were above chance level but not nearly to the degree of the shams.

This set of results raises the question relating to how sham- and hippocampal-lesioned mice may pay attention/encode more of the arm-reward association based upon the way they are initially trained (with free-choice or forced trials). For example, once animals are trained to run down a runway to retrieve a reward, rats with hippocampal lesions pay less attention to an unexpected "distracter" cue that is introduced onto the maze, whereas normal animals will slow their running speeds and may even stop to investigate (Raphelson et al. 1965, Wickelgren & Isaacson 1963). Additionally, rats with hippocampal lesions fail to orient toward a novel sound when they are focused on performing a prepotent response (i.e. drinking water) (Hendrickson et al. 1969). In this chapter, mice were originally habituated to the T-maze by introducing rewards at the end of the goal arms, thereby effectively training the mice to run down arms to retrieve a reward. This may have established a prepotent response similar to the running response in the above Raphelson et al. and Wickelgren and Isaacson et al. experiments.

In this chapter's visual discrimination experiment when there are a high proportion of forced trials (Expt. 1a), it may be that, during these forced-choice trials, mice with hippocampal lesions are relying on the prepotent response and haphazardly running to retrieve a reward without paying much attention to the arm-reward association. Sham mice obviously display the same running behaviour during these forced-choice trials but may also be attending to the environment more and (even

incidentally) learning the arm-reward associations. During training on the visual discrimination experiment for the reward magnitude discrimination (Expt. 2a) where mice received all free-choice trials, hippocampal and sham mice did not differ in their initial and subsequent choice performance behaviour; both groups started out at chance levels and gradually reached high performance levels. Because the majority of trials were free-choice trials, sham mice may be participating in more investigative behaviours (e.g. vicarious trial-and-error sampling and direct investigative behaviour of the arm) and this is why they show (chance) levels comparable to hippocampal-lesioned mice to begin with. As acquisition progresses, both sham- and hippocampal-lesioned mice attend to the arm patterns throughout acquisition and hence they acquire the task at similar rates. It is difficult, if not tortuous, to compare across tasks because it is likely that if mice received forced-choice trials before free-choice trials, different psychological processes and mechanisms are likely dictating how they are attending to and/or encoding the association, and indeed the environment. This will have effects that alter the way the animal solves the task when given a choice to decide between arms, especially during the early stages.

### **Other Factors to Consider**

Other underlying factors may contribute to the findings presented here. Although direct comparisons between the two strains of mice were

not performed (black C57/Bl6 versus albino CD1), one can see that the main difference between sham mice (from the hippocampal lesion experiment) and wildtype mice (from the GFAP-TK experiment) is that performance levels are lower in the CD1 mice, even by the sixth block of testing. The general lack of a deficit detected in the TK mice relative to their WT's may reflect a ceiling effect in performance levels. Although in the experiments (Expt. 1b and 2b) there was no hint of any separation between the WT and TK mice, it is possible that the CD1 background prevented any genotype difference from being observed by limiting the performance levels that could be attained by the WT controls. The strain differences may be related to motivational differences (CD1 mice tended to weigh 50-100% more), or sex differences (sham mice in Expt. 1b and 2b were female; WT mice in these experiments were males). Thus, there are many possibilities that could account for the differences between these groups of control mice that may not be related to the task demands. Alternatively, these task differences may in fact underlie the differences in learning rates. It is difficult to pull these issues apart and further experiments would be necessary to pinpoint the source of these differences.

## **Conclusion**

In this set of experiments, it is demonstrated that the hippocampus is needed for reward-based decision making involving response conflict, whether it be reward magnitude discrimination or reward ambiguity discrimination. In both scenarios, conflict is generated by introducing more than one goal representation and response tendency. In addition, hippocampal-lesioned mice are less able to show a discriminative preference early on if trained with a high proportion of forced-choice trials, which may tap into their natural tendency to keep engaging in pre-potent responses and an inability to attend to novel cues and to inhibit these response tendencies until further training changes the associative arm-reward strength. We did not find a deficit in these tasks in mice with ablated adult neurogenesis, as compared to their wildtype littermates. This may reflect that these new neurons are less important in these particular tasks and that the mature granule cells are able to pattern separate memories or goals.

Nevertheless, these decision-making tasks suggest an important role for the hippocampus in these non-spatial tasks, which are at odds with a pure spatial/navigation system interpretation of the hippocampus (e.g. O'Keefe 1976, O'Keefe & Nadel 1978). It is likely that the hippocampus, while also encoding spatial information, is processing other task-relevant information that goes into detecting and resolving conflicting goals.

# 6 GENERAL DISCUSSION

## 6.2 Overview

Throughout this thesis different approaches have been taken to tackle two common questions that have arisen in the hippocampal and adult neurogenesis literature. First, how does the hippocampus and dentate gyrus handle conflicting memories that support goal-related behaviour? Second, how do memory aspects of the hippocampus align with the emotional aspects known to rely upon the hippocampus and adult-born neurons?

The experiments in this thesis provide for a clearer understanding about the role that the hippocampus and adult-born neurons play in the behavioural response toward ambiguous cues related to aversive and appetitive situations where conflicting associative memories or goals arise. Mice with ablated adult neurogenesis or with hippocampal lesions showed diminished defensive behavioural responses to a cue that was ambiguously predictive of an aversive event. Conversely, in appetitive decision-making tasks involving ambiguity relating to reward probability or reward magnitude conflict, mice with hippocampal lesions tended not to show a clear choice preference for reward certainty or high-reward magnitude unlike sham controls, but these same results were not present in mice with ablated adult neurogenesis. In this final chapter, the results from all the experiments are summarised and their relation to the literature is discussed. Additionally, this thesis ends with the possibility that adult neurogenesis, and the hippocampus as an entire structure, may be better understood in

terms of its role in prediction related to goals and how this may bring together the memory and emotion aspects of these two fields of study.

### 6.3 Ambiguity Related to Aversive Outcomes

The central themes of this thesis were to examine the role that the hippocampus and adult neurogenesis play in the behavioural responses toward cues that are ambiguous predictors about specific situations involving threat or reward. Previous research suggests that the hippocampus and adult-born neurons within the dentate gyrus are involved in the detection, and possibly the disambiguation, of conflicting or overlapping goal information (see Aimone et al. 2011, Bannerman et al. 2014, Gray & McNaughton 2000), especially when conflicting memories exist that support the representations of these different goals.

It was first attempted in Chapter 2 to probe this question by designing a within-subjects experiment where mice with hippocampal or sham lesions were exposed to aversive cues that either reliably or ambiguously signalled shock. In that experiment, it was found that the hippocampal-lesioned mice displayed a decrease in freezing behaviour toward an ambiguous cue (CSa) that predicted shock outcome 20% of the time while 80% of the time signalling non-shock. These results could reflect a learning deficit toward the ambiguous cue but there was not a global learning deficit, because normal responding occurred toward the

fully predictive (reliable) cue in the hippocampal lesioned animals, as compared to their sham counterparts.

This was further developed by targeting adult-born neurons in the dentate gyrus and switching to a more specific between-subjects design that allowed for less cue-cue interference effects and avoided possible "emotional reactivity spreading" to the other predictor cues from previously encountered ones. For example, the impact of experiencing the ambiguous cue during the same conditioning session may affect the emotional responding toward the reliable cue. The between-subjects and within-subjects designs address potentially two separate questions: one would be the "emotional spreading", or how one cue impacts the *meaning* of how the animal encodes the subsequent cue(s); the second focuses more on one specific cue's (either an ambiguous or reliable cue) outcome, specifically whether this one type of cue predicts an aversive outcome all the time or only some of the time.

The results of these experiments (Figure 3.3, Figure 3.4, Figure 3.5, Figure 3.8, Figure 3.9 ) suggest that adult-born neurons are involved in mediating the current defensive responding (freezing and startle) when either the cue was a light or a tone (two separate experiments) that ambiguously predicted an aversive event. During extinction of the reliable cue responding, unlike in the hippocampal study, we did not see any difference between the wildtype and TK mice (Figure 3.3, C,D).

This suggests that, at least in the between-subjects paradigm, these adult neurogenesis-deficit mice do not have a global impairment in new learning. Like with the hippocampal mice, extinction may represent a distinct form of 'ambiguity' or uncertainty as the extinction process progresses.

The decreased freezing toward the ambiguous cue during the fear memory recall (test) day was similar to results from a study involving silencing granule cells in the dentate gyrus, which produced decreased freezing toward the ambiguous, but not the reliable, predictor cue (Tsetsenis et al. 2007). In addition, using a similar design as presented in Chapter 2, serotonin transporter overexpressing mice also showed a freezing deficit toward the ambiguous, but not a reliable, cue (McHugh et al. 2015), although this overexpression was not specific to the hippocampus. These results, nevertheless, point to an important role of granule cells within the dentate gyrus in processing these types of cues and possibly a wider role for serotonin in dealing with ambiguity/uncertainty.

The deficit in defensive behavioural responding toward the ambiguous cue and the involvement of adult-born granule cells was further bolstered by the study of levels of the immediate-early gene *c-fos*'s encoding for the Fos protein (Figure 3.11). This revealed that the dentate granule cells and CA3 pyramidal cells in TK mice showed less Fos expression during conditioning with the ambiguous predictor cue only, as compared to wildtype mice in

the same conditions; the Fos expression levels did not differ between genotypes after experiencing reliable cue conditioning. Importantly, there was no difference in the total freezing levels across the third conditioning day which was just prior to sacrificing the mice and which could potentially have been driving the Fos signal by itself (Figure 3.10, A). This suggests that a difference in general locomotor activity levels or freezing behaviour cannot solely explain these differences in Fos expression levels.

Specifically regarding the CA3 pyramidal cells, using this same GFAP-TK model to eliminate adult-generated neurons, atrophy of CA3 dendrites was observed, increased glutamate levels in this region were detected, and, using elevation-induced stress, increased Fos expression was found in the dentate gyrus and CA3 in these GFAP-TK mice compared to wildtype littermates (Schloesser et al. 2014). These authors suggested that adult-born granule cells, possibly in conjunction with mature granule cells, prevent excitotoxic effects on CA3 from the heightened glutamate levels, and that the ablation of these adult-born granule cells provides a state of disinhibition on CA3. Compared to Chapter 3's Fos finding (Figure 3.10, C, D), the nature of the stressor (elevated platform vs. ambiguous or reliable fear conditioning) would appear to have different effects on downstream CA3 activity in TK mice, and there is not necessarily a general effect on CA3 expression, suggesting that a general stress effect is not sufficient to explain differential Fos expression levels. It may be that the

elevated platform stress, during which the mouse is placed on a small platform elevated high off the ground and cannot move without potentially falling, is acting more like an immediate or phobic threat, and is more like the reliable cue fear conditioning. This immediate threat may enhance Fos expression in the hippocampus.

While targeting and elimination of different cohorts of adult-born neurons throughout different maturation stages cannot be achieved with the GFAP-TK model, it is possible to examine the contribution of different adult-born neuron ages on CA3 activity levels in a very precise manner using other mouse models. The tamoxifen nestin-Cre mouse model, as described in the Introduction of this thesis, allows for 'tagging' neurons and later elimination of these 'tagged' neurons. Using this model, Restivo et al. (2015) found that adult-born neurons reach their peak connectivity with CA3 interneurons at four weeks of age when these new neurons are most excitable and plastic, which then would inhibit the CA3 region (Restivo et al. 2015). Additionally, optogenetic stimulation of six- to eight-week old adult-born neurons did not elevate Fos expression levels in the CA3 interneurons, whereas the same stimulation of the four-week-old neurons produced elevated levels within CA3 interneurons.

The results in this thesis, in addition with the results described above (Restivo et al. 2015, Schloesser et al. 2009), suggest that there is a complex interplay between the age of

adult-born neurons, dentate and CA3 Fos expression (effective connectivity), and the type of stress experienced. It would be of value to see, by use of the tamoxifen nestin-Cre model, how different ages of the adult-born neurons would affect the deficit that is seen in the ambiguous cue fear-conditioning task. Additionally, it would be valuable to see how this correlates with Fos expression within the dentate and CA3. Because four-week-old neurons in adulthood display enhanced excitability and can produce Fos expression in CA3 interneurons, it may be that ablation of this age of new neurons would be sufficient to produce the effects that we see in this thesis as compared to the complete ablation of new neurons. Conversely, it may be that the elimination of all neurons born after dentate gyrus perinatal development is necessary to achieve these behavioural and Fos results. Another aspect not examined in the Chapter 3 results that would be valuable to know is if these Fos-expressing neurons within the dentate and particularly the CA3 subfield are interneurons ( $GAD67^+$ ) and precisely what the temporal specificity afforded by optogenetic activation/inactivation of this population would tell us.

## 6.4 Aversive Outcomes and Future Responding

Defensive responding to current ambiguous predictors of threat is necessary to ensure safety and survival in the present

moment. The ambiguous predictors, however, also allow for adaptation to future situations that might resemble these prior experiences. This adaptability could then ensure well-being for the longer term. In the beginning of Chapter 4, baseline anxiety differences between the genotypes were investigated using ethological tests of anxiety. Experimentally naive TK mice and their wildtype littermates were tested in the elevated-plus maze and, in another naive cohort, in the light/dark exploration box (Figure 4.2). For the elevated-plus maze, there were no genotype (TK vs. WT) differences detected. Mice did not differ in the percentage of time spent in the open, anxiogenic arms; they did not differ in their locomotor or exploratory behaviour; and they showed no differences in the total number of faecal boli on the maze. Similar measures were examined in the light/dark exploration box with no differences between genotypes detected.

In a new cohort of mice that were fear conditioned with either reliable or ambiguous predictor cues (identical to the fear conditioning protocol in Chapter 3), there was no difference in elevated-plus maze anxiety-like behaviour but a difference in the novelty-suppressed feeding task was found. In the novelty-suppressed feeding test, under baseline conditions where no prior fear conditioning had occurred (i.e. no shocks were received), wildtypes and mice with ablated adult neurogenesis did not show any differences in latency to eat a familiar food in a novel environment (Figure 4.4, left). After fear conditioning using a

reliable cue, wildtype mice still did not show a difference in anxiodepressive-like behaviour (latency to feed) compared to non-shocked wildtypes. If the WT mice were exposed to fear conditioning using ambiguous cues, however, they modulated their behaviour relative to baseline controls and those that experienced reliable fear conditioning, showing increased latency to start eating the food. When it came to TK mice, these mice showed uniform increases in their latencies to eat after experiencing either the reliable or ambiguous fear conditioning relative to their baseline controls. Thus, compared to their wildtype counterparts who modulated their behaviour based on prior fear conditioning with reliable or ambiguous cues, TK mice either showed increased or decreased anxiodepressive-like behaviour (latency to feed) respectively (Figure 4.4, right).

These results are consistent with other studies showing that previous stress (unsignalled/unpredictable shock) can affect (normally decrease) future defensive behaviours, such as locomotion, sniffing, rearing, attention, and grooming in novel environments (Daviu et al. 2010, Katz & Baldrighi 1982, Katz et al. 1981, Koolhaas et al. 1990, van Dijken et al. 1992b, 1993). These decreased defensive behaviours did not attenuate by two weeks post-stress (van Dijken et al. 1992b) and hypolocomotion did not extinguish over exposure to multiple different environments (Daviu et al. 2010). Additionally, exposure to shocks increased glucocorticoid receptors by 129% within the hippocampus, as

compared to controls, 14 days after shock exposure (van Dijken et al. 1993), further confirming that having a stress history can produce long-lasting consequences on not only behaviour but also on stress hormone receptors within the hippocampus. While in this thesis we did not measure glucocorticoid receptor numbers within the hippocampus as in the van Dijken et al (1993) study, it would be valuable to know whether these increased after reliable and ambiguous conditioning. Additional neuro-endocrine measures would also be valuable, as well. In the van Dijken et al (1993) study, they found a change in ACTH levels days after unpredictable shock but not in corticosterone levels days after the shock, despite seeing corticosterone elevation 60 minutes after shock as compared to controls. While we did not observe a difference between corticosterone levels 30 minutes after reliable and ambiguous fear conditioning (albeit both groups showed an increase relative to mice that went through only two days of reliable fear conditioning/training), it may be necessary, if looking at the kinetics of corticosterone recovery, to perform a more systematic time-point analysis after fear conditioning. This would require a large cohort of mice (WT and TK mice) at various time points. Nevertheless, the results of van Dijken et al., suggest that ACTH may be a more sensitive measure to examine in future studies after reliable or ambiguous fear conditioning. This is because the adrenal cortex production of corticosterone may be modulated by other factors such as splanchnic nerve activity and ACTH, which is one stimulatory hormones for corticosterone

production, is secreted from the anterior pituitary (see van Dijken, 1993).

One recent study that ablated adult neurogenesis found that when these ablated mice are given prior contextual fear conditioning (tones were played but never reinforced; the shock was random, i.e. unpredictable), they showed an increase in freezing toward the tone in a new environment (a non-associative effect) (Seo et al. 2015). Additionally, these authors also showed that prior delayed fear conditioning decreased time in the centre of the open field and less time in the open arms of the elevated-plus maze (Seo et al. 2015).

Another study found no experience-dependent elevated-plus maze behavioural changes in mice when the dentate gyrus was manipulated (Ramirez et al. 2015), similar to our null finding with the elevated-plus maze and in the previous study examining lasting effects of shock (Daviu et al. 2010). The null experience-dependent effects in the elevated-plus maze suggest that this test may not be sensitive enough to pick up reliable differences, possibly owing to the clear difference in the anxiogenic boundary zones (see Chapter 4's discussion). However, the results from those studies and this thesis suggest that experience-induced changes in exploratory activity can be seen in open field-like tests (i.e. novelty-suppressed feeding).

To investigate the role of experience-induced glucocorticoid rises in a new cohort of mice (Chapter 4), circulating

corticosterone was clamped at low levels by adrenalectomy and by giving a low-dose of corticosterone replacement in their drinking water (Figure 4.5, A). Mice were then fear conditioned and tested in the novelty-suppressed feeding task. The main difference here was that wildtype mice lost this adaptive responding after receiving ambiguous fear conditioning, showing similar latencies to start eating as those that received reliable fear conditioning. TK mice, however, appeared to be unaffected by the adrenalectomy and still showed a uniform increase in latencies, irrespective of prior conditioning type (Figure 4.5, B). These results demonstrate that adult neurogenesis, in conjunction with glucocorticoids, may allow for the modulation of anxiodepressive-like behavioural responding, presumably reflecting adaptive behaviour, in future situations if mice previously experience fear conditioning with ambiguous predictors about the shock.

The results from Chapter 3 and 4 are largely in agreement with the previous literature, namely that unpredictable (or unsignalled) shocks can produce long-lasting defensive behavioural effects in exploratory-related tasks as discussed above. The important element that extends this line of research is that this thesis (Chapter 4) demonstrates that these adaptive behavioural changes seen in future situations after unpredictable (or ambiguous) threats are mediated by adult-born neurons in conjunction with glucocorticoids.

## 6.5 Ambiguity Related to Appetitive Outcomes

Up until this point, the adult-born neurons and the hippocampus as a whole have been discussed in the sense of their necessity for appropriate defensive responding toward cues that ambiguously predict an aversive outcome and the impact on future (adaptive) behaviour. Animals lacking these new neurons, and indeed the hippocampus, can perform this freezing task if the cue reliably, but not ambiguously, predicts a shock. Is the hippocampus and adult-generated neurons, however, only necessary for the processing of ambiguous cues related to aversive outcomes or also appetitive outcomes?

In a set of experiments (Chapter 6), it was shown that the hippocampus as a whole, but not adult-generated neurons necessarily, is involved in decision-making processes using two different non-spatial T-maze tasks that predict various reward-related outcomes. Specifically, when mice are trained on a non-spatial T-maze task where one arm signals reliable reward (continuous reinforcement) and the other signals reward only 50% of the time (partial reinforcement), mice with hippocampal lesions showed less of a preference for the always-rewarded arm over the arm that ambiguously predicts reward presence compared to sham controls (Figure 5.3, B). TK mice, however, do not differ in this preference behaviour compared to their wildtype littermates when tested for the same number of blocks as the hippocampal- and sham-lesioned mice (Figure 5.3, C). In a related T-maze task

during which one arm signalled a high amount of reward and the other a lower amount, hippocampal-lesioned mice were also slower to show a preference for the high-reward arm over the low-reward arm (

Figure 5.5, B). This was not the case for TK mice, which did not differ from their wildtype littermates (

Figure 5.5, C).

These behaviours are reminiscent of studies performed on reward-based decision-making tasks in slightly different paradigms. Rawlins and colleagues (1985) found that sham- and hippocampal-lesioned rats preferred a temporally non-delayed reward that was always present in one arm compared to a reward that was only present 25% of the time. When a temporal delay was imposed on the always-rewarded arm, however, hippocampal rats switched their behavioural choice preference to the arm that allowed immediate access but only a 25% chance of being rewarded, while sham animals persisted in waiting for the always-rewarded but delayed arm (Rawlins et al. 1985).

Similarly, dorsal, ventral, and complete hippocampal-lesioned rats, trained pre-operatively, preferred immediate access to a low-magnitude reward if, in the opposite arm, access to a high-magnitude reward was delayed (Mariano et al. 2009, McHugh et al. 2008). If equal delays were imposed on both arms though, both lesioned and sham animals preferred the high-magnitude reward, suggesting that all animals had intact

associative memories of the goals; but hippocampal-lesioned animals were more impulsive (displayed more intolerance to temporal delays) than their sham controls. This was extended by Bett and colleagues (2014) by showing that hippocampal-lesioned rats in a similar, yet continuously adjusting, delay-discounting task showed less vicarious trial-and-error behaviour on delays of 2- and 10-s. These rats also showed more investigative behaviour when the delay was adjusted and these animals showed a lower indifference point, suggesting that these animals are less responsive to increasing reward magnitude changes. A general impulsiveness in hippocampal lesioned animals has been shown in other tasks where responding must be suppressed or continual responding does not lead to reward (Abela et al. 2013, Chudasama et al. 2003).

These findings, in conjunction with the findings of Chapter 5, suggest that it may be the conflicting goals, or response tendencies, that interfere with their choice performance. Specifically, in Chapter 5, mice received their lesions before any training began, whereas the above studies all examined pre-operatively trained rats. Setting aside species differences, the time of surgery before or after training occurred may be of importance in determining how these lesioned animals initially form associations and may thus affect the inhibition of pre-potent responses or tendencies to run for a reward. For example, if trained after lesioning, these animals may not be able to sort out

conflicting signals of reward associations upon receiving this initial formation. Pre-operative training may have allowed mice to acquire the reward-arm associations comprehensively or some sort of general strategy, causing less interference initially in post-operative performance. The hippocampus's involvement at this stage, then, may be only parsing apart the competition of the associations stored elsewhere in the brain.

The results in this thesis suggest that the hippocampus may be necessary to separate the retrieval of conflicting associative memories or goal responses. It would be quite valuable to know if these animals will ever be able to show preferential behaviour after extensive training on these tasks in Chapter 5. Even if these mice have a perfect memory, for example if they were trained pre-operatively (Mariano et al. 2009, McHugh et al. 2008), a deficit in aspects of decision-making, such as those related to executive control or emotion regulation or behavioural inhibition, could continue to produce these behavioural impairments. The dissociation may suggest that mature granule cells, or other regions of the hippocampus, are necessary for aspects of successful executive decision-making.

## 6.6 Pattern Separation and Ambiguity

An idea that has gained prominence, first relating to the dentate gyrus and then extending to adult-born dentate granule

cells, is that of pattern separation of overlapping inputs. Starting with the computational modelling work, Marr and others suggested that, in conjunction with CA3 recurrent collaterals and dentate axons, the CA3 is well positioned for forming memory representations. Subsequent modelling suggested that there needed to be a means of separating out dentate inputs from the entorhinal cortex in order to increase the signal-to-noise ratio. This increased signal-to-noise ratio would allow for finer inputs onto CA3 and would construct more accurate memories with less interference (see Aimone et al. 2011, Hopfield 1982, Marr 1971). This computational process has been extended to the behavioural realm where discrimination needs to occur between similar places or events in order to orthogonalise outputs (Clelland et al. 2009, Groves et al. 2013, Kesner 2007, Leutgeb et al. 2007, Nakashiba et al. 2012, Sahay et al. 2011).

For example, related to this thesis, contextual fear conditioning tasks have frequently been used to show impairments in discriminating between a previously shocked environment and a neutral environment. The closer these environments are in their appearance, rodents with impaired dentate gyrus function, and specifically adult neurogenesis, show lower freezing behaviour (Leutgeb et al. 2007, Nakashiba et al. 2012). These lower freezing behaviours supposedly reflect a decreased ability in discriminating these two contexts based on sensory cues (i.e. if they look more similar, the more difficulty the

animal has at discriminating the contexts) (Kheirbek et al. 2012, Leutgeb et al. 2007, Nakashiba et al. 2012). However, as mentioned previously, the context may be acting as an ambiguous cue itself, prompting retrieval of a shock and non-shock associative memory, like that seen in this thesis with a discrete cue (Chapters 2 and 3).

While some pattern separation tasks have focused on discriminating spatial or sensory inputs, like those above (e.g. Clelland et al. 2009, c.f. Groves et al. 2013), the results of this thesis point to the fact that the inputs may be more related to memories or goals. Specifically, the use of an ambiguous cue (a cue that signals shock and non-shock) may represent pattern separation at the level of the memory, and is certainly hard to reconcile with a pattern separating process strictly related to sensory or spatial inputs.

Ambiguity as used here refers to the underlying associative structure being 'ambiguous', such that one particular stimulus primes both a CS+ and CS- simultaneously, leading to conflict between what the single cue means. The design of the experiments in Chapter 3 follow this definition, particularly those related to fear conditioning with one specific cue. It is relying solely on the learnt association. Upon hearing this tone and retrieving the memories, the animal may need to bias how it treats the cue. If the cue is paired with an aversive situation, the animal may be more likely to associate it with a negative outcome and

give less weighting to its positive association, like the wildtype animals appear to do in Chapters 2 and 3.

Similarly, this ambiguity as it relates to goals also extends to the appetitive experiments performed in Chapter 6. The goal arms of the T-maze represent distinct visual stimuli that signal some variation of reward. In one arm, the uncertainty of reward presence means that that arm is an ambiguous predictor of reward. In the case of reward magnitude differences, a conflict arises between the two goal arms prompting conflicting goal responses. Both present a slight variation when it comes to conflicting properties (arm-reward uncertainty or arm-reward magnitude differences) but both types of conflict appear to rely on the hippocampus, but not adult-born neurons in particular, to parse apart conflicting goal responses.

Another issue relating to ambiguity and conflict arises upon closer inspection of the extinction data presented in Chapter 3 (Figure 3.3, C, D). There was no difference in extinguishing the freezing response to the reliable predictor cue. Extending this thinking, one might expect the extinguishing tone (now not paired with shock) to induce its own form of ambiguity/uncertainty and produce conflict with its now-older association with the shock. This may relate, however, to a distinct form of conflict or ambiguity related to the formation of different associative structures. Pavlov suggested that, as in the original formation of conditioning that establishes a new association, so too does presenting that

stimulus with its consequence. He concluded that, among other more contemporary studies, that the original knowledge is not erased but is only inhibited by the new extinction learning, representing a new associative memory structure that inhibits access or excitation of the older one (Rescorla 2003). This suggests that it may be something about the initial formation of conflicting associative structures when formed simultaneously that produces the type of conflict that engages the granule cells, and not when associations are potentially formed separately, with the extinction association acting to inhibit the original CS. When a non-conflicting cue-shock relationship is first encoded, as is the case with the reliable cue, further extinction represents new encoding with potentially a different associative structure that *inhibits* the original association. This may mean that extrahippocampal structures, such as the medial prefrontal cortex and amygdala connectivity (e.g. Bukalo et al. 2015, Morgan et al. 1993), are sufficient to learn this new extinction (forming an inhibitory association) and separate out this type of "extinction conflict". Adult-born granule cells, on the other hand, may mediate the conflicting association memories if initially formed in parallel or when finer distinctions need to be made.

## 6.7 Ambiguity and Arousal

Gray and McNaughton (2001) suggested that the hippocampus is a series of logical gates that allow for filtering of information to the subiculum for the comparison of the "conflict value". Specifically, the dentate-CA3 logical gate is proposed to pass along novel information but not familiar information, unless the familiar stimuli (such as the tones in the set of experiments here) have been conditioned to have importance based on arousal signals. These arousal signals may be provided by the septum's cholinergic signalling or by aminergic signals with valence-related information provided by the amygdala (Gray & McNaughton 2000, pg 248-252). These may be important factors in the dissociation we see regarding adult neurogenesis effects in fear conditioning which are absent in appetitive tasks. The decreased level of Fos expression in TK mice (Chapter 3) during ambiguous fear conditioning may give insights into how this decreased activity is sustained within the hippocampal network. This may reflect that the hippocampus is receiving less valence-like information from the amygdala (Felix-Ortiz et al. 2013, Tye et al. 2011) and/or that the recurrent collaterals within the CA3 and the back-projections to the dentate are not being engaged as strongly in TK mice. Subsequently, less information is being sent out of the hippocampus to the amygdala and other extra-hippocampal structures that again, in turn, feedback into the hippocampus,

thereby sustaining decreased activity levels in the amygdalo-hippocampal and intra-hippocampal recursive loops.

The appetitive task's ambiguity, on the other hand, may represent an even more diffuse threat to the animal's survival, whereas a potential footshock is likely to be of more urgent and direct importance to the animal's survival. While it is difficult to make direct comparisons between the two kinds of experiments, some characteristics are shared between the aversive and appetitive ambiguous experiments. For example, like that seen in the fear conditioning studies (Chapters 2 and 3), wildtype mice treat the ambiguous cue like a pure CS+ (the reliable cue). In appetitive experiments (Chapter 6), the ambiguous reward arm is not treated like the pure CS+ (always rewarded or high-reward magnitude arm), based on performance measures. Although speculative, it may be that the dissociation we see in fear conditioning versus appetitive tasks with regard to adult neurogenesis may relate to this saliency issue as mentioned above. Specifically, in the fear conditioning experiments, a tone-shock association was likely more arousing than an arm-reward association. Because a shock is an immediate threat, the association may have been 'tagged' as more important and thus the amount of information and/or inputs into the dentate may have been increased. Increasing the inputs would enhance the probability of engaging the new neurons, resulting in finer scrutiny of the associative structure. This finer inspection and parsing apart

by new, young neurons may be due to their increased excitability and plasticity (Nakashiba et al. 2012, Snyder et al. 2001).

Moreover, these young neurons have been proposed to mediate the inhibitory/excitatory coordination in the dentate and aid in memory discrimination when conflict is introduced (Park et al. 2015).

## 6.8 Prediction and Goals

A more recent idea that is gaining traction seems to fit well with the observed data presented throughout this thesis. One theory set forth by some investigators suggests that the hippocampus is involved in predicting outcomes and, at least in humans, imagination (Buckner 2010, Mullally & Maguire 2013). The role of the hippocampus may be to enhance decision-making and well-being through improving predictions about goals based on memories. This idea has the potential to unite the hippocampus's seemingly separate roles in memory and emotion.

Many, if not all, guided behaviours rely on predictions about goals that rely on some sort of previous learning and memory, just as many every-day predictions and decision-making rely on previously learnt associations. The hippocampus may be primarily concerned with the flexible recombination of associative memories of stimuli or situations and their outcomes in order to predict future outcomes in novel situations in order to optimise behaviour.

Specifically, this thesis points to the dentate gyrus as a key mediator in disambiguating overlapping associative memories that predict conflicting goal outcomes, and in the ability to adapt appropriately for future situations in an uncertain environment.

Some evidence exists that supports this idea, especially from hippocampal place cell recordings as well as human imaging studies. The existence of these place cells have long been assumed to reflect spatial maps stored in the hippocampus to guide navigation (O'Keefe & Nadel 1978). As animals move along a runway, place cells fire in a sequence that can be observed during rest or sleep, leading to an interpretation that this 'replay' of these sequences reflect consolidation of the experience/memory (Carr et al. 2011). Recent findings have also found that novel firing sequences recorded during rest can be observed several minutes later when an animal is traversing a novel runway track, reflecting a 'preplay' of events not-yet experienced (Dragoi & Tonegawa 2011). This 'preplay' may reflect sequences that are used later to support encoding of related novel experiences to those of a similar past experience (i.e. 'replay' recordings).

More direct evidence for the role of the hippocampus in prediction comes from decision-making tasks, such as those on a T-maze where a choice needs to be made between two arm choices. When rats are tested in a multiple-choice T-maze task, place cell ensembles appear to "flow" down each arm, often ahead of the animal's choice behaviour and current position on the

maze (Johnson & Redish 2007), possibly reflecting that the animal may be considering the outcome of choosing each arm if they were to follow that path. This is also suggested by other findings showing that place cell sequences are more prominent during the early stages of learning and predict immediate future behaviour in an open field where multiple start and goal locations exist (Pfeiffer & Foster 2013).

These neural findings are suggestive of what Tolman (1938) observed in his studies, namely that animals use vicarious trial-and-error (VTE) sampling initially to make decisions. VTE behaviour is where the animal looks back and forth between choices or goals, especially during difficult discriminations, during the early stages of learning, and especially when there is a negative consequence of choosing incorrectly (i.e. such as on an apparatus where there is a gap that must be jumped to return to the choice point) (Tolman 1938). These VTE behaviours are correlated with faster learning and, conversely, damage to the hippocampus decreases these behaviours and impairs learning on tasks in which VTE is normally displayed (Bett et al. 2012, Hu & Amsel 1995, Tolman 1938). This VTE behaviour may reflect hippocampus-dependent predictions and evaluations of possible future outcomes arising from particular choices or responses that occur in animals during uncertain or ambiguous situations. This VTE behaviour may be related to the rearing and other risk-assessment behaviours often observed in rodents in novel open

arenas or other anxiogenic situations, which also depend on the integrity of the hippocampus (Blanchard et al. 2011, Lever et al. 2006).

This view of the hippocampus brings together aspects of several other views of hippocampal function discussed in the Introduction. What this theory fails to provide for is how it may incorporate aspects of emotion. However, studies of emotion in rodents performed many years ago suggested a strong link between prediction and emotion. In fact, Simonov proposed a specific role for the hippocampus in bridging these two seemingly distinct domains (Simonov 1974). In Simonov's 1991 "need-information theory of emotions," it states that emotions are determined by a need to know and by the prediction of the likelihood of the animal's satisfaction. For example, a low probability of achieving a goal can lead to negative emotions (e.g. frustration), while a high or increased probability of goal achievement can lead to positive emotion (Simonov 1991). Specifically, he states that the role of the hippocampus in predicting satisfaction is in directing attention toward low-probability events, or those that are unlikely to occur. He writes that, "[it] is not possible to speak of a general memory defect" but instead that their behaviour "is no longer complicated by predictions of events unlikely to happen in a given situation (Simonov 1974, pg. 35) so that they act like "living automata without hesitation and doubt" (pg. 106).

Emotions, however, need not be generated by certain predictions. They may also feed into predictions in the form of cognitive bias. Predictions and decisions are not made in isolation; they are often influenced, or biased, by the emotional state of the animal. Stress, anxiety, and depression elicit a negative interpretation bias that affects how people interpret ambiguous threat cues, and tend to augment remembrance of negative, as opposed to positive, events or details (Clark & Beck 2010) and likewise imagination of future events (Hach et al. 2014, King et al. 2011). Similar results are obtained using animal models of these states. For example, in a recent study rats were trained with one tone and a lever that signalled a food reward and a second tone with a different lever that stopped the onset of a shock. At test, an intermediate tone (based on frequency) was played. Control rats, upon hearing the intermediate tone, pressed the lever associated with delivery of a reward. Rats genetically bred to show learned helplessness, who displayed normal learning during training, pressed the lever associated with preventing a shock when the intermediate tone was played. Similar results were obtained with rats that were treated with corticosterone and the noradrenaline reuptake inhibitor reboxetine (Enkel et al. 2010). In another study, mice lacking that serotonin 1A receptor show normal behavioural responses toward a cue that predicts a shock but show increased freezing (i.e. anxiety- or fear-like behaviour) toward a cue that ambiguously predicted a shock. When dentate gyrus granule cells were silenced in these mice, this effect was eliminated such that

these animals now showed normal anxiety- or fear-like behaviour compared to their wildtype controls toward the ambiguous cue (Tsetsenis et al. 2007).

These above studies, in addition to the ones in this thesis, suggest a specific role for the dentate granule cells in disambiguating overlapping associative memories or goals and, possibly, the hippocampus serving to emotionally bias predictions about certain outcomes. Different portions of the hippocampus could potentially be involved in the cognitive and emotional aspects of predictions; for example, the dorsal region may play a role in the cognitive aspects of predictions while the ventral portion may play more of a role in the emotional biasing. This emotional bias may influence the future type of memories retrieved (e.g. the associations) by biasing the animal's judgements about the probability of each outcome of a given action, reminiscent of Simonov's "need-information theory" about selecting low-probability events based upon predictions.

As has been suggested, there are several different steps in the generation of predictions and it is not clear at what point exactly the hippocampus becomes involved. The hippocampus may participate in selecting relevant knowledge from which to form the predictions, flexibly recombining this knowledge in order to predict how to achieve a goal, and/or disambiguating the goals to make the optimal choice based on various factors relevant to the animal (e.g. motivation). Disambiguating the goals, or the

memories that support the goals, seems closely aligned with Gray & McNaughton's (2000) idea that the hippocampal formation functions to detect and resolve goal conflicts. If emotions bias predictions and, as Simonov suggested, predictions can generate emotions, then emotions and predictions appear to form a loop whereby predictions of failure, for example, could lead to negative emotions. These negative emotions could then further negatively bias other predictions, and so on. If the hippocampus, especially the dentate gyrus, is important for disambiguating goal responses or the memories that support these goal responses by biasing memories that are directly related to the individual rodent that aid in survival, this could provide a possible link between the hippocampus, memory, and anxio-depressive states.

## 6.9 Conclusion

The hippocampus is one of the most studied structures within the brain and has shown itself to be involved in a range of cognitive and emotional functions. With its well-known role in spatial and episodic memory, the hippocampus is also connected to stress-related regions and contributes to the behavioural stress response. These views, however, have remained relatively separate within the literature. One feature of the hippocampus that possibly underpins these functions is the detection and resolution of conflicting goal-related information. Specifically, the dentate

gyrus may serve to disambiguate these overlapping, and therefore conflicting, goal representations. The set of behavioural impairments that this thesis lays out are hard to reconcile with an inability to disambiguate or parse apart overlapping sensory, spatial, or contextual signals that the dentate gyrus receives. It adds an additional element that suggests that the incoming signals to the dentate gyrus are related to memories that support goal representations and this clarity that the dentate provides allows for accurate predictions to be made.

Specifically, this thesis has provided information on the role of the hippocampus in three major ways. First, it provides evidence that the hippocampus and adult-born dentate gyrus granule cells are needed to disambiguate between memories associated with non-spatial, discrete cues. Specifically, it aids in the disambiguation of memories for discrete cues that have been simultaneously conditioned with the presence and absence of shock, presumably reflecting parallel formation of these associative memories. Second, it addresses an important issue as to how this disambiguation of associative memories, such as the ability to predict accurately when an aversive outcome will occur, aids in future stress responding in novel situations. The adult-born neurons, in conjunction with glucocorticoids, appear to promote adaptation, or the modulation of defensive behaviours, based on the cue's prior predictability about upcoming aversive events. Third, this thesis provides evidence that the hippocampus, but not

necessarily adult-born neurons, is involved in appetitive-based decision-making tasks that involve conflicting reward-associated cues. Specifically, the hippocampus, but not adult-born neurons, is necessary when two or more competing goals are introduced, where one goal is an ambiguous predictor about the presence of reward or the competition between reward-magnitude associations.

In sum, these results suggest that ambiguity or uncertainty is a critical feature that engages the hippocampus and is necessary to bias future behaviour. This biasing of behaviour may allow optimal decisions to be made about appetitive or aversive environments that aid in the overall well-being of the animal. Furthermore, the literature mentioned above and results of this thesis suggest that rates of adult neurogenesis do not correlate directly with levels of anxiodepressive-like behaviours, but instead with the ability to adapt the level of these behaviors according to the stressful environment.

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