How the brain forgets: An investigation into predictions of Active Decay theory

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Table of contents

List of figures

Abstract

Active decay theory outlines a framework where forgetting by memory interference and decay unfold in a neurobiologically plausible way. Each is assumed to principally operate in different brain regions supporting declarative memory. The circuit architecture of the neocortex recruits overlapping content traces which, in periods of synaptic plasticity leaves cortical representations highly vulnerable to interference from perturbations related to ongoing sensory stimulation. Left unchecked, this could lead to states of catastrophic interference and amnesia. Such states are naturally avoided by virtue of the hippocampus, which is assumed to generate orthogonal index representations, that when linked to content elements, provide indirect pattern separation for cortical traces, allowing them to maintain coherence despite representation overlap. Due to its orthogonal coding, interference cannot account for hippocampal forgetting which is instead, suggested to mainly arise from active decay, a constitutive cellular process that gradually reverses synaptic modifications induced by learning and memory. Active decay is predicted to recruit pro-apoptotic processes, without causing cell death, to gradually forget established memory. Here, we directly investigated these predictions of active decay theory in rats.

We used the novel object recognition task, which relies on the hippocampus for long-term memory formation but houses object representations in the perirhinal cortex to explore the role of the hippocampus in stabilising extra-hippocampal traces. We found that inactivating the dorsal hippocampus during object learning lead to amnesia for objects tested 24 hours later. Reducing visual stimulation during the hour after object learning, but not later, prevented amnesia despite hippocampal inactivation. We also found that synaptic plasticity in the hippocampus during, but not directly after object learning is required to prevent interference from sensory stimulation. Finally, we discovered that amnesia for objects resulting from inhibiting protein synthesis in the hippocampus after memory reactivation, i.e., during memory reconsolidation, could be rescued by reducing sensory stimulation after reactivation. Using the neuronal activity marker c-Fos, we found that inactivating the hippocampus prior to object learning increased expression of c-Fos in the perirhinal cortex, which lowered to levels comparable to control animals when sensory stimulation was reduced after learning.

To test the role of pro-apoptotic signalling in active decay, we blocked two components of this pathway – caspase 3 activity and extra-synaptic NMDA receptor activation – in the dorsal hippocampus during a memory retention period which typically leads to natural

forgetting of long-term object location memories. These interventions preserved established object location memories and extended their persistence. Importantly, inhibiting extra-synaptic NMDA receptors in the hippocampus did not prevent the learning of object locations.

While these outcomes provide strong support of active decay theory, they also further our understanding of pathological loss of memory in various neurological conditions.

Résumé

La théorie de la dégradation active décrit un cadre dans lequel l'oubli par interférence et la dégradation de la mémoire se déroulent d'une manière neurobiologiquement plausible. Chacun de ces processus est supposé opérer principalement dans les différentes régions du cerveau qui soutiennent la mémoire déclarative. L'architecture des circuits du néocortex recrute des ensembles de contenus qui se chevauchent, ce qui, en période de plasticité synaptique, rend les représentations corticales très vulnérables aux interférences provenant des perturbations liées à la stimulation sensorielle continue. Si rien n'est fait, cela peut conduire à des états d'interférence catastrophique et d'amnésie. De tels états sont naturellement évités grâce à l'hippocampe, qui est présumé générer des représentations indexées orthogonales. Lorsqu'elles sont liées à des éléments de contenu, elles fournissent une séparation indirecte des motifs pour les traces corticales, ce qui leur permet de conserver leur cohérence malgré le chevauchement des représentations. En raison de son codage orthogonal, l'interférence ne peut expliquer l'oubli dans l'hippocampe, qui proviendrait principalement de la dégradation active, un processus cellulaire constitutif qui inverse progressivement les modifications synaptiques induites par l'apprentissage et la mémoire. La dégradation active recruterait des processus pro-apoptotiques pour oublier progressivement les souvenirs établis, sans toutefois provoquer la mort cellulaire. Ici, nous avons directement étudié ces prédictions de la théorie de la dégradation active chez le rat.

Nous avons utilisé une nouvelle tâche de reconnaissance d'objets, qui repose sur l'hippocampe pour la formation de la mémoire à long terme, mais qui héberge des représentations d'objets dans le cortex périrhinal, afin d'explorer le rôle de l'hippocampe dans la stabilisation des traces extra-hippocampiques. Nous avons constaté que l'inactivation de l'hippocampe dorsal pendant l'apprentissage d'un objet entraînait une amnésie pour les objets testés 24 heures plus tard. La réduction de la stimulation visuelle pendant l'heure qui suit l'apprentissage de l'objet, mais pas plus tard, a empêché l'amnésie malgré l'inactivation de l'hippocampe. Nous avons également découvert que la plasticité synaptique dans l'hippocampe pendant l'apprentissage d'un objet, mais pas directement après, est nécessaire pour prévenir l'interférence de la stimulation sensorielle. Enfin, nous avons découvert que l'amnésie pour les objets résultant de l'inhibition de la synthèse des protéines dans l'hippocampe après la réactivation de la mémoire, c'est-à-dire pendant la reconsolidation de la mémoire, pouvait être corrigé en réduisant la stimulation sensorielle après la réactivation. En utilisant le marqueur

d'activité neuronale c-Fos, nous avons constaté que l'inactivation de l'hippocampe avant l'apprentissage d'un objet augmentait l'expression de c-Fos dans le cortex périrhinal, diminuant à des niveaux comparables à ceux des animaux témoins lorsque la stimulation sensorielle était réduite après l'apprentissage.

Pour tester le rôle de la signalisation pro-apoptotique dans la dégradation active, nous avons bloqué deux composantes de cette voie - l'activité de la caspase 3 et l'activation du récepteur NMDA extra-synaptique - dans l'hippocampe dorsal au cours d'une période de rétention de la mémoire qui conduit généralement à l'oubli naturel des souvenirs de localisation d'objets à long terme. Ces interventions ont permis de préserver les souvenirs de localisation d'objets établis et de prolonger leur persistance. Il est important de noter que l'inhibition des récepteurs NMDA extra-synaptiques dans l'hippocampe n'a pas empêché l'acquisition d'informations spatiales. Bien que ces résultats soutiennent fortement la théorie de la dégradation active, ils permettent également d'améliorer notre compréhension de la perte pathologique de la mémoire dans diverses affections neurologiques.

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My first and most sincere thanks go to Oliver Hardt, who gave me this opportunity and introduced me to the field of memory. His passion, integrity and creativity inspired me to pursue the unorthodox questions while his adamant trust and faith in me gave me the freedom to figure out how to do so in my own way, even when I was hesitant to. The somewhat maverick ideologies he holds were the clearest truth to me, and have undoubtedly shaped my approach to science and life itself. The energetic and playful nature of his supervision has ensured that my time working with him has been nothing short of pure fun – for that, I am truly thankful.

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Contribution to original knowledge

- Inactivating the dorsal hippocampus impairs the formation of long-term object recognition memory and not object encoding or expression.
- Reducing sensory stimulation for 1 hour after object learning prevents amnesia for objects under hippocampal inactivation.
- Object amnesia arising from hippocampal dysfunction is due to excessive interference rather than a lost capacity to consolidate new episodic-like memory.
- Under healthy conditions, the hippocampus prevents excessive neuronal activity which arises in the perirhinal cortex after object learning and normal sensory experience under hippocampal impairment.
- The hippocampus is only required for up to 1 hour after object learning to form new long-term memory for objects.
- Hippocampal plasticity is required during, but not after, object learning to prevent amnesia from excessive interference related to ongoing sensory experience.
- Established object memory can be destabilised with the presentation of a partial cue from a previously seen object.
- The hippocampus supports object recognition reconsolidation by protecting restabilizing traces from interference related to ongoing sensory experience.
- Caspase-3 activity and extrasynaptic NMDAR activation in the dorsal hippocampus promote active decay of long-term memory for object locations.
- Inhibiting extrasynaptic NMDARs in the dorsal hippocampus does not impair learning of object locations.

Contribution of authors

This work originated from the projects conceived by Dr. Oliver Hardt (Chapters 3 & 4) and Dr. Virginia Migues (Chapter 4). The experiments presented here were designed in collaboration with Isabelle Groves and Dr. Oliver Hardt. Karine Gamache translated the abstract. Except for Dr. Oliver Hardt (and those that supplied the nano-particles) all other contributors listed below were trained and supervised by Isabelle Groves.

Chapter 3:

In Chapter 3, bar experiments in Figure 3.5, Isabelle Groves completed all cannulation surgeries, prepared drugs for infusion, ran rats during behavioural testing, administered infusions, manually scored all behaviour and sacrificed rats. For section 3.3 Isabelle Groves perfused rats, processed and sectioned brains for immunohistochemistry, conducted all c-Fos staining and image analysis. Brains were sectioned for placement check by Isabelle Groves. For experiments presented in Figure 3.5, Célia Sciandra assisted Isabelle Groves with cannulation surgeries. Stephanie Donaldson ran the behavioural studies. Stephanie Donaldson and Isabelle Groves scored the behavioural data and jointly sectioned brains for placement. All data was analysed by Isabelle Groves.

Chapter 4:

In Chapter 4 cannulation surgeries were done mostly by Isabelle Groves and also with contributions from Dr. Oliver Hardt for experiment 4.3A. For experiment 4.3D, Célia Sciandra completed most of the surgeries, with assistance from Isabelle Groves. AuM was supplied by Dr Molokanova (NeurANO Bioscience, State, USA). Conantokin peptides and nanoparticles were supplied by Dr. Fiammengo (Unversità di Verona, Italy) Isabelle Groves and Megan Baron Goldwax ran the behavioural experiments, manually scored the data and sacrificed the animals for 4.1A. Experiments 4.1E and 4.2A were fully completed by Isabelle Groves. Kelly Brues and Célia Sciandra ran the behavioural studies for Experiment 4.3A with Célia Sciandra administering the infusions. Isabelle Groves, Kelly Brues and Célia Sciandra manually scored the data for this experiment and sacrificed animals. For Experiment 4.3D. Kelly Brues, Célia Sciandra and Isabelle Groves ran the behaviour, with Isabelle Groves administering the infusions. All data was analysed by Isabelle Groves.

Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid
AMPAR	AMPA receptor
GluA1-AMPAR	AMPA receptor containing GluA1 subunit
GluA2-AMPAR	AMPA receptor containing GluA2 subunit
ANI	Anisomycin
ANOVA	Analysis of variance
AuM	Gold nanoparticle coated with memantine
AuNP-ConR	Gold nanoparticle coated with Conantokin -R
AP5	DL-2-amino-5-phosphonopentanoic acid
Arc	Activity-regulated cytoskeletal-associated protein
CA1	Cornu ammonis 1
CA2	Cornu ammonis 2
CA3	Cornu ammonis 3
CaMKII	Calmodulin-dependent kinase II
ConR	Conantokin -R
Con-F-G	Conantokin -F-G
d	Discrimination index
dHPC	Dorsal hippocampus
MAPK	Mitogen-activated protein kinase
LTP	Long-term potentiation
LTD	Long-term depression
NMDA	*N*-methyl-D-aspartate
NMDAR	NMDA receptor
GluN2A-NMDAR	NMDAR containing GluN2A subunit
GluN2B-NMDAR	NMDAR containing GluN2B subunit
exNMDAR	NMDAR located extrasynaptically
sNMDAR	NMDAR located synaptically
PBS	Phosphate buffered saline
PKA	Protein kinase A
PKCι/λ	Protein kinase C ι/λ
PKMζ	Protein kinase M ζ

PSD	Post-synaptic density
Zif268	Zinc finger-containing transcription factor 268
ZIP	ζ inhibitory peptide

Chapter 1

Introduction and literature review

In a dynamic world, the utility of information is in flux. Memory systems must match this by sustaining information that helps guide future behaviour and promote the survival of an organism while disregarding that which has become irrelevant or even false with time. To forget is therefore a natural operation of a brain in need of adaptable memory and flexible behaviour. Yet, despite over a century of research it remains the most elusive aspect of memory processing. To address this, active decay theory proposed a framework of forgetting that emerges across synapses and brain systems. This account aims to unite long-standing observations in experimental psychology with recent advances in the neuroscience of memory. This thesis investigates whether core predictions of active decay theory uphold in the rat.

1.1. Early studies and theories of forgetting

The empirical study of forgetting began in 1885 when Herman Ebbinghaus published a series of reports detailing the decline of his own memory over time. After learning a list of nonsense syllables (i.e., SEN, HIF) he found that the number he was able to recall dropped rapidly in the hours after learning and then more gradually in the days and weeks following (Fig 1.1). This non-linear pattern of memory loss is now widely known as the forgetting curve, and the question of how and why it occurs has been central to research on memory loss since.

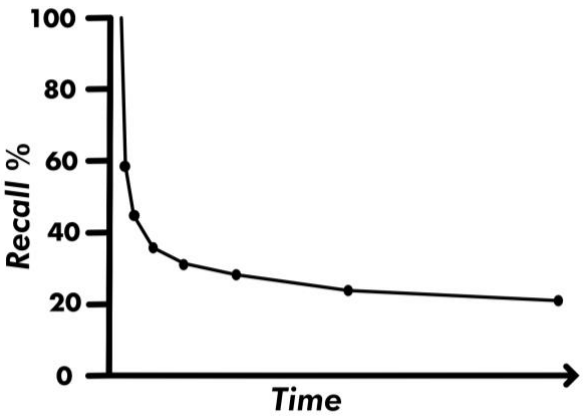


Figure 1.1. Ebbinghaus' forgetting curve. Illustration of the non-linear pattern of memory loss for established memory.

1.1.1. The law of disuse

Ebbinghaus himself suggested that the forgetting curve was explained by the passage of time (Ebbinghaus, 1885), an idea that was later echoed in Edward Thorndike's "law of disuse" (Thorndike, 1913), or, as it became known, "decay theory". This account proposed that without regular recollection memory weakens as a function of time, so that eventually it can no longer be recalled. This intuitively plausible explanation for forgetting had its conceptual predecessors in the well-known analogy likening memory to the fading impressions made onto a wax tablet (Plato, *Theaetetus*). More recently, it has been compared to radioactive decay (Hardt, Nader, & Nadel, 2013) or muscle atrophy (Della Sala, 2010), in that Thorndike's account assumes that memory wastes away in a passive and inevitable manner when not being used.

While decay theory initially gained a strong following, it failed to gain empirical support and in fact, was eventually rejected. In a pivotal study by Jenkins and Dallenbach (1924), participants were taught a list of nonsense syllables which was then followed by either a sleep-filled or a wakeful period of the same duration. Decay theory predicted no differences in later recall performance, as the same amount of time, and therefore forgetting, would have occurred. In stark contrast it was found that memory recall was better after sleep than after being awake, suggesting that forgetting was instead determined by the events that followed learning. Shortly after these findings, a scathing review of decay theory was published in which the account was heavily criticised as a simplistic description of forgetting that lacked any mechanism which could sufficiently explain forgetting (McGeoch, 1932). From there, decay was not directly studied, but occasionally accredited to unexplained memory loss in later studies.

1.1.2. Retroactive inhibition and consolidation

The idea that forgetting arose from the events that follow learning was proposed by Bigham (Münsterberg et al., 1894) shortly after publication of the forgetting curve in 1885. He emphasized that in daily life 'unfilled periods' between learning and recall are not common, and rather, mental activity related to ongoing experience occupies this period. This activity, he argued, is responsible for the loss of previously acquired memories.

Just over a decade later, Müller and Pilzecker (1900) conducted a series of experiments to directly investigate this idea. They studied the impact of interpolating activity (i.e., tasks occurring between learning and recall) on previously learnt pairs of nonsense syllables in healthy participants. Their findings suggested that, whether related to the learned material or not (i.e., learning a second list of syllable pairs or observing landscape paintings), interpolating stimuli consistently diminished later syllable recall in comparison to conditions where no stimuli were presented. Furthermore, interpolating activity immediately after learning impaired recall to a greater extent than at a later time point (e.g., 6 minutes after learning) suggesting a transient vulnerability of newly acquired memories. Thus, Müller and Pilzecker proposed that a time-dependent preservative process was required to store and stabilize, i.e., consolidate, recent memories. If this process were disrupted by mental effort required to attend to interfering stimuli –originally termed retroactive inhibition – forgetting would be the result.

While the preservation-consolidation account became paradigmatic to memory research for the century that followed (McGaugh, 2000; Squire, Genzel, Wixted, & Morris, 2015; Sutherland, Sparks, & Lehmann, 2010) this view was not so readily accepted by early forgetting researchers. Many failed to replicate the temporal gradient of retroactive inhibition, often reporting that similar degrees of forgetting occurred irrespective of when interpolating activity was introduced (Robinson, 1920). In fact, these other early studies suggested that the greater threat to memory integrity was the *similarity* of interpolating activity, and not the timing of mental effort per se (Robinson, 1920; Skaggs, 1925). For example, McGeoch and McDonald (McGeoch & McDonald, 1931) reported greater forgetting in word list recall when the interpolating word list was more semantically related to the original learning, a finding which could not be explained by the preservation-consolidation hypothesis.

As a consequence, the concept of retroactive inhibition in memory consolidation was abandoned as an explanation for forgetting in favour of the notion of retroactive interference; the idea that new learning and accumulation of *similar* content in long-term memory leads to increasingly inefficient memory recall and ultimately forgetting (Dewar, Cowan, & Della Sala, 2007; McGeoch, 1942).

1.1.3. Interference

The retroactive interference account quickly assumed the leading view of forgetting as it could explain most findings, including Ebbinghaus' forgetting curve (McGeoch & Irion, 1952) and Jenkins and Dallenbach's observations on the role of sleep in memory. In the decades that

followed, forgetting research and theory formation therefore focussed almost exclusively on interference.

The gold standard paradigm in early interference studies was the A-B, A-C word list task, an associative verbal learning paradigm where subjects learn different responses to the same cue (i.e., bagel-tyrant, bagel-chair), in sequential learning trials. Later, participants are then asked to recall the word pair (either tyrant or chair) that matched the original cue (bagel). If the participant recalled the later association (bagel-tyrant) the earlier association (bagel-chair) was thought to have suffered forgetting from retroactive interference (Nedergaard, Wallentin, & Lupyan, 2023), while if the earlier association could be recalled but not the later, proactive interference was thought to have occurred. This paradigm formed the foundation for the majority of empirical studies and, importantly, provided a set system for theorists to develop explanations of cause.

The first full mechanistic theory for retroactive interference in the A-B, A-C task was proposed by Irwin and Melton in the 'two-factor theory' (Melton & Irwin, 1940). This account asserted that forgetting arose by (a) unlearning of an original association, i.e., A-B, due to proceeding interpolated learning of a new association, A-C, and (b) response competition at recall from both interpolated learning and spontaneous recovery of the unlearned, original association (Houston, 1968; Melton & Irwin, 1940). Response competition was presumed to produce proactive interference, as existing memories impaired the acquisition of new ones, which at the time was only believed to play a minor role in forgetting. While the two-factor theory itself was accepted hesitantly (Keppel 1968), response competition and unlearning became two influential mechanisms in traditional interference thinking. For example, in later years, the popular competition-response theory stated that forgetting only manifested because of interference at recall, i.e., response-competition (McGeoch, 1942; Mcgovern, 1964). Indeed, nearly all major interference accounts used either one or both processes as mechanistic explanations for everyday forgetting in the form of retroactive or proactive interference (McGeoch, 1942; Mcgovern, 1964; Underwood & Postman, 1960).

In 1957, Underwood pointed out that numerous retroactive interference studies reported different degrees of forgetting across similar, if not identical, protocols. He suggested that these variations could be best explained by the amount of *prior* learning participants were asked to do (Underwood, 1957). In other words, Underwood suggested that proactive interference drove the forgetting effects reported in these findings, rather than retroactive interference, and by extension, was the primary source of everyday forgetting.

From there, retroactive interference was seldom studied in favour of proactive interference, yet this stream of research also eventually seeped away in later decades. A leading view during this time was that proactive interference in studies arose from extra-experimental sources of interference i.e., knowledge of already established word-structures (Keppel, 1968; Underwood & Postman, 1960) Specifically, it was proposed that the known language structures would initially disrupt task acquisition, undergo unlearning and then spontaneously recover at recall causing response competition. At length, empirical evidence for unlearning of existing linguistic knowledge could not be obtained and indeed by this point, unlearning was considered an ill-defined process with no agreement on its precise mechanism (Anderson, 1974; Slamecka, 1966; E Tulving & Madigan, 1970). This, along with mounting concerns that the leading theorists could not fully explain interference phenomena, led to a steady decline of interference theory research in the field of forgetting, and, since the 1970s, investigations on forgetting became rare. This situation began to change following the turn of the century, but in a different light to its initial programme. Contemporary forgetting research has been heavily influenced by learning and memory formation research, which, in stark contrast to forgetting, greatly progressed across the 20th century. To understand modern thinking on forgetting, it is therefore important to sketch the history and major findings of memory research that have led it to the current positions.

1.2. Neurobiology of Memory formation and maintenance

1.2.1. Classifications

Memory emerges in many forms with distinct characteristics, functions and underpinning neurobiology that support encoding, maintenance and expression (Nadel & Hardt, 2010; Squire, 2004). As early as the late 1800s, memory was being partitioned by type, i.e., motor or 'pure' recollected memory (Bergson, Paul, & Palmer, 2004) and by persistence, i.e., primary and immediate or secondary and longer lasting memory (James, 1890). Since then, various memory taxonomies have been proposed that encapsulate these specific aspects in addition to conscious experience and utility (Cohen & Eichenbaum, 1993; Nadel, 1994; Schacter & Tulving, 1994; Tulving, 1983). Most now generally agree on a distinction between declarative and non-declarative memory, where the former describes explicit expression of memory for facts (semantic) and events (episodic), while the latter, implicit expression of priming, non-associative learning, classical conditioning, skills and habits (procedural). Importantly, these memory categories appear to be supported by different brain systems, and in fact, this

taxonomy largely emerged from a set of seminal observations in patients with damages to certain brain areas and animal studies (Squire, Cohen, & Nadel, 1984).

For much of the early twentieth century, memory was thought to be distributed across the brain, rather than centralized to a specific region (Franz, 1912). This idea gained traction following Lashley's work, where lesion mapping across the rat cortex after maze navigation learning, suggested that the size of the lesion, rather than the location, better predicted memory impairment (Lashley, 1924). However, this view drastically shifted in the 1950s following the pioneering work on the late Henry Molaison, or patient H.M. After bilateral removal of the majority his hippocampus (and surrounding cortices) from the medial temporal lobe to treat his epilepsy, H.M. experienced both anterograde amnesia (inability to form new memories for events) and temporally graded retrograde amnesia (loss of past event memories, particularly more recent ones). Nonetheless, he retained the ability to learn and express motor skills and perceptual patterns even though, he had no recollection of the learning sessions beforehand (Scoville & Milner, 1957). These findings revealed a clear anatomical divide of memory in two respects; firstly, between systems processing declarative and non-declarative memory and secondly, between brain regions supporting recent and remote episodic memory.

1.2.2. The hippocampus: neuroanatomy and connections

The seminal work on H.M. brought the hippocampus to the forefront of memory research as a core brain region supporting declarative memory, and in particular, episodic memory. The hippocampus is a highly conserved structure across species, and as such, both human and animal research have been employed to understand its physiology and role in memory.

Anatomical studies have revealed a centralised position of the hippocampus in terms of cortical connectivity, receiving highly processed, multi-modal sensory information from the surrounding perirhinal, entorhinal and parahippocampal cortices, which collectively form the parahippocampal region. In particular, these cortices receive substantial projections from the visual system and broadly convey two major streams of information toward the hippocampus (Felleman & Van Essen, 1991). While the perirhinal cortex is thought to support representations of object and item stimuli ('what"), the parahippocampal cortex primarily relays scene and location information ("where"), which both project onto the entorhinal cortex. The entorhinal cortex then relays these inputs to the hippocampus which itself projects back to it.

The hippocampal formation is made up of the dentate gyrus (DG), subfields of CA1-3 and the Subiculum. The main flow of information in the hippocampus occurs along the trisynaptic pathway, which commences from layer II neurons in the entorhinal cortex projecting to granule cells of the DG via the perforant pathway. Granule cells in DG send axons (mossy fibres) which synapse partly to pyramidal excitatory neurons, and extensively to inhibitory interneurons in the CA3 region. CA3 pyramidal neurons send both associational outputs to other CA3 pyramidal neurons in a recurrent network and to CA1 neurons via the Schaffer collaterals. CA1 neurons then send projections back to layer V of the entorhinal cortex, thus acting as the primary output of hippocampal processing to the extra hippocampal areas. These cortices then project back to neocortical areas, placing the hippocampus at the top of a recurrent, hierarchical network of sensory processing.

1.2.3. Animal models of hippocampal amnesia

Attempts to replicate H.M.'s pattern of amnesia in animal models were initially unpromising. For example, it had been observed that H.M. struggled to complete delayed visual discrimination tasks, where the memory of a previously seen object must be retained. In comparison, monkeys with medial temporal lobe lesions showed no deficit in a similar task and performed as well as control animals, even with longer delays which typically resulted in complete memory loss in clinical patients (Clark & Squire, 2010). While confusing at the time, it was later determined that differences in the involvement of the hippocampus between species can account for these discrepancies. In other words, lesioned monkeys were able to employ incremental, habit-like learning strategies that depend on other structures, such as the basal ganglia, to complete visual discrimination tasks. Thus, combined with the observations of H.M., this furthered the idea of multiple memory systems and that animals can readily employ skill or habit-based learning that does not rely on the medial temporal lobe, even in tasks in which humans do not.

Later development of one-trial learning paradigms for animal models of amnesia were able to reveal a pattern of memory impairment that better resembled the human condition. One of the first demonstrations used the delayed non-matching to sample task in monkeys with lesions to the medial temporal lobe. In this task, animals were presented with two identical objects during a sampling phase, and then after a delay period, were presented with another two objects; one was the already seen one and the other one was novel (Mishkin, 1978). Monkeys were trained to choose the novel object, which relied on recognition memory, a

subdivision of declarative memory where a subject must make a judgement of whether an object was previously encountered. At short delays, i.e., 10 minutes, no differences in performance were observed between medial temporal lobe lesioned and intact animals. Yet, at longer delay (hours), control animals were able to perform the task while lesioned animals failed to identify which object had been previously seen, similar to amnesic patients (Stuart Zola-Morgan, Cohen, & Squire, 1983). Furthermore, the same findings were reported in monkeys with selective hippocampal lesions (Alvarez, Zola-Morgan, & Squire, 1995; Murray & Mishkin, 1998), thus more directly modelling of H.M.'s condition.

When implementing delayed non-matching to sample object recognition tasks in rodents with hippocampal lesions, a less clear phenotype was observed. To assess object memory in rodents requires the novel object recognition paradigm, which relies on a rodent's natural tendency to explore novel stimuli. By comparing exploration times between a novel and a familiar object, one can infer memory for the familiar if less time is spent exploring it in favour of the novel (Ennaceur, 2010). While many early studies reported that lesioned rats showed deficits in completing this task (Broadbent, Gaskin, Squire, & Clark, 2010; Mumby & Pinel, 1994), numerous others did not (Aggleton, Hunt, & Rawlins, 1986; Barker & Warburton, 2011). Instead, a consistent amnesia phenotype was observed in *spatial* discrimination tasks, where lesions lead to the loss of previously acquired spatial discriminations (retrograde amnesia) and the inability to learn new ones (anterograde amnesia) (Cho, Kesner, & Brodale, 1995). Indeed, later work supported the notion that the hippocampus uniquely supported spatial processing, as both lesioned rats and monkeys failed to perform spatial tasks, such as learning the location of food stashes or completing spatial working memory tasks (Aggleton et al., 1986; Clark, Broadbent, & Squire, 2005; Faraji, Lehmann, Metz, & Sutherland, 2008; Morris, Garrud, Rawlins, & O'Keefe, 1982).

Taken together, lesioned animal studies revealed a critical role for the hippocampus in rapid single exposure learning - and in particular spatial memory - thus prompting a number of new theories on hippocampal functioning.

1.2.4. Spatial mapping

While recording from CA1 pyramidal neurons in freely moving rats, O'Keefe and Dostrovsky (1971) found that individual neurons increased their firing rate as an animal moved around a particular region of space. These 'place cells', appeared to chart across a region of space such that different cells uniquely increased their firing rate as an animal moved towards a specific

location, generating individual ‘place fields’ (Muller & Kubie, 1987). Removing external maze cues stopped place cell firing, suggesting that sensory information (e.g., visual) controlled firing of these cells. Across CA1, the constellation of active place cells is distinct between environments, such that some cells active in one arena, or spatial context, became silent in a new (and vice versa). This ‘remapping’ process thus provided neural activity patterns that are discrete between contexts.

These observations, and the spatial deficits seen in animals with damage to the hippocampus, led to the proposal that the hippocampus acts as a cognitive map (O’Keefe & Nadel, 1979a). While Tolman (1948), who introduced this term, first suggested that animals used a mapping system to navigate complex mazes, O’Keefe and Nadel (1978) put forward a greater expansion of this idea, which incorporates physiological and behavioural findings in rodents and clinical observations in humans. Central to their Cognitive Map Theory is the position that animals can rely on a locale or taxon system to navigate in space. The taxon system embodies an egocentric framework where animals incrementally learn relations between itself and set features of an environment (i.e., at the blue door, turn left). In comparison, the locale system supports allocentric spatial navigation, in which automatic and rapid encoding of place representations are organised into a framework that allows an animal to determine directions and distances within a wider environment (i.e., head towards the North Wing). The hippocampus is proposed to uniquely support the locale system, such that when this region is damaged, an animal can only perform simple, taxon-like spatial navigation, but not complex route planning. A clear demonstration of this duality came from rats undergoing the Morris Water Maze task. Here, animals learn to locate a platform in a circular water tank surrounded by external cues. When the platform is raised (i.e., animals can see it), both rats with hippocampal and sham lesions can escape the water, suggesting intact taxon systems. However, when the platform is submerged, and animals have to rely on the external cues to navigate (i.e., the locale system), animals with lesioned hippocampi fail to learn the platforms location (Morris et al., 1982).

Similarly, in humans, patients with damage to the medial temporal lobe are impaired at complex spatial tasks, but can perform maze navigation when following visible egocentric cues (i.e., follow a trail of pegs) (O’Keefe & Nadel, 1979b). A further extension of cognitive map theory is the role of the locale system in general episodic memory formation. Episodic memory is characterised by its grounding of information to a specific time and place, and furthermore, is not incrementally acquired but instantaneously encoded, i.e., in one trial. Thus, the hippocampal locale system is predicted to store information relating to that specific episodes

within a set context, and in its absence, impairs the acquisition of new episodic memory, and gives rise to anterograde amnesia.

1.2.5. Pattern Separation and Pattern Completion

A related, yet alternative, view of hippocampal functioning focuses on its computational properties to coordinate episodic memory formation and retrieval. In early mathematical modelling, David Marr (Marr, 1971b) proposed that auto-associative memory permits the ability to recall a full representation when only a fraction of the original elements are presented, and that such a computation could be achieved by recurrent collaterals. The circuit architecture of CA3, where over 90% of neurons are interconnected, provides the computational infrastructure to perform this type of pattern completion important for memory retrieval (Rolls 1987). Furthermore, the auto-associative network in CA3 has been thought of as an intermediate store for memory (not necessarily spatial), that binds together elements of an episode, rather than the site of spatial computation itself (Treves & Rolls, 1994).

To optimise the capacity and functioning of an auto-association network, each binding element relating to an episode eventually needs to be decorrelated, as otherwise, due to finite storage space, at some point patterns will begin to overlap. Thus, a complementary process of pattern separation is required to orthogonalize overlapping representations in CA3 regions. Given its upstream position to CA3, and circuit structure, the dentate gyrus has been largely attributed to perform this function (Rolls, 2016). The region can influence the patterns of activity in CA3 due to its sparse firing patterns, which synapse onto sparse groups of cells in the CA3, thus reducing representational overlap there. Furthermore, the dentate gyrus, in humans as well as rodents, is a site of continuing neurogenesis in adulthood, such that new granule cells are generated and incorporated into the existing circuitry, which could further promote pattern separation and due to the development of new downstream projection pathways.

1.2.6. Consolidation

Ribot first observed that head trauma patients presented with a peculiar temporal gradient of amnesia: their recently acquired memories were much more likely to perish than more remote ones, which invariably were not affected by the injury (Ribot, 1883). He proposed a temporal memory stabilization process wherein a transient metabolic mechanism exists to provide nutrients to the brain areas engaged in memory formation, and that the disruption of this process

– as during head trauma - will impair long-term retention of memory. Later work revealing H.M.'s sparing of older (remote) but not newer (recent) episodic memory suggested that a time-dependent progression necessary to sustain long-term memory had been disrupted upon hippocampal removal. From this, arose the concept of 'systems consolidation', which attempts to capture a long time-dependent transformation (weeks to years) that reorganizes memory across brain systems. Systems consolidation describes a separate process to the consolidation process put forward by Müller and Pilzecker, which was envisioned to unfold in the hours after learning to initially capture and stabilize memory into long-term representations. To distinguish between the two, this shorter time-scale stabilization is referred to as 'synaptic' or 'cellular' consolidation.

1.2.6.1. Systems consolidation

The acquisition and early maintenance of long-term memory episodic memory depends on the neocortex and hippocampus, yet over time, loses its dependency on the hippocampus and instead relies more on cortical areas (Squire et al., 1984). While the neocortex houses numerous, dispersed neuronal ensembles which collectively support the contents of an event, the hippocampus is believed to bind and index these elements together in a spatial or contextual representation (Nadel & Moscovitch, 1997; Squire et al., 1984; Teyler & DiScenna, 1986). The question of how memory reconfigures across brain regions with time has been tackled by a number of major theoretical positions.

1.2.6.1.1. Standard model

Under the standard theory of systems consolidation, the transition of hippocampal dependent to independent expression of memory is thought to rely on the hippocampus itself. First envisioned by Marr (1970), declarative memory was proposed to be initially stored within the hippocampus, and, through consolidation, gets transferred to neocortical regions for long-term storage. This was later generalised to a more commonly known stance today, that hippocampal-neocortical interactions directly stabilize and incrementally strengthen dispersed cortical connections (Davis & Squire, 1984; Frankland & Bontempi, 2005). Once consolidated, declarative memory - either semantic or episodic - was thus thought to be relatively robust and independent of the hippocampus in both maintenance and expression. By extension, damage to the hippocampus would disrupt this initial storage of acquired memory, giving rise to anterograde amnesia. Furthermore, any ongoing activity patterns in the hippocampus that drove

stabilization of recent memory into remote would be terminated, therefore accounting for the temporally graded nature of retrograde amnesia.

1.2.6.1.2. Complimentary learning systems

Complimentary Learning Systems theory is an alternative, computational explanation for amnesia arising from hippocampal damage which builds off of the standard consolidation framework (McClellend 1995). This account emerged to explain the discrepancies in continual learning between biological systems and early connectionist modelling. Namely, while humans and animals show a lifelong ability to acquire and process new information, artificial networks struggle to do so, such that after learning to complete one task, sequential training of a second task completely overwrites any prior knowledge within a network in what is otherwise known as catastrophic forgetting or interference (French 1999).

To account for this, complimentary learning systems proposed that effective learning relies on two systems; a system for rapid acquisition of detailed information and a slow learning system which maintains shared structure of representations. In the brain, these two systems are thought to rely on the hippocampus and neocortex respectively, such that new information (or episodic memory) is quickly learnt in the hippocampus which is then replayed in an interleaved manner with old (or semantic) information in the neocortex, therefore allowing current and previous learning to occur somewhat simultaneously. Thus, in the case on hippocampal damage, no new information relating to events can be interleaved into the neocortex, therefore leading to states of cortical catastrophic interference and amnesia.

1.2.6.1.3. Multiple trace theory

In the decades that followed its proposal, findings emerged in conflict with the core predictions of the standard model of systems consolidation. While many patients experienced a temporal gradient of retrograde amnesia, numerous other cases reported flat retrograde amnesia, such that the extent of memory loss in patients was equivalent across a given time period. Indeed, the question of how memory could biologically be steadily transferred was neither fully explained nor understood by many within the field, and questions about the utility of consolidation periods spanning months and years arose. Furthermore, functional imaging studies in healthy participants showed that recall of some remote episodic memories stimulated activity in the hippocampus, suggesting its re-engagement to a ‘systems consolidated’ representation. Later case studies of amnesic patients with hippocampal damage showed that

some of these patients were still able to learn factual knowledge, i.e., acquire semantic memory, while episodic memory formation was consistently impaired (Glisky, 1992; Vargha-Khadem et al., 1997). By extension, it had become apparent that episodic memories can be rich in detail, while semantic memories are typically 'gist-like', which, altogether, suggested that the two were not identical in quality nor underlying anatomy. Given these observations, an alternative explanation was proposed by the Multiple Trace Theory as to how the hippocampus co-ordinates systems consolidation (Nadel & Moscovitch, 1997).

Multiple Trace Theory holds that episodic memory, no matter the age, i.e., both recent and remote, requires the hippocampus to provide spatial-contextual detail and the 'vividness' characterizing true episodic remembering, while semantic memory does not. Building on cognitive map theory, multiple trace theory predicts that information pertaining to episodic memory is grounded in both the neocortex and the hippocampus, thus recent memory, which is more hippocampally dependent, will be richer in detail compared to remote memory that can be recalled without the hippocampus. In agreement with the standard view, Multiple Trace Theory also asserts that interactions between the hippocampus and the neocortex influence the stability and persistence of cortical elements. In contrast, the original version of this theory argued that each reactivation of an episodic memory generates a new hippocampal trace, which allows contextually rich information typical for episodic memory to be retained across multiple hippocampal-neocortical connections. Thus, memory that is no longer bound to a context, such as semantic memory, would not require the hippocampus for later expression, and can be fully supported by neocortical regions. Or, in other words, memories expressed without hippocampal contribution lack the typical quality of episodic memories and have the 'flavour' of semantic memories.

1.2.6.2. Synaptic consolidation

Synaptic consolidation refers to a set of transient biological events unfolding after learning to stabilize memory, transforming a fleeting 'impression' into a long-lasting one. The first neurobiological model of this consolidation process emerged with Hebb's dual-trace theory (Hebb, 1949), which stated that reverberating neural activity (cf. perseveration) in cell assemblies represented short-term memory for an experience. If left undisturbed, this patterned activity can induce long-lasting morphological and metabolical changes to synaptic connections (cf. consolidation), such that later, long after it had vanished, the reverberatory activity pattern can be reinstated in order to express long-term memory. Early animal studies

supported this view; delivery of electroconvulsive shocks (Duncan, 1949), and then later protein-synthesis inhibitors in the hours following learning prevented long-term memory formation (Agranoff, Davis, & Brink, 1965; Flexner, Flexner, de La Haba, & Roberts, 1965). To induce changes in synaptic strength between neuronal associations, Hebb predicted that coactivity of presynaptic and postsynaptic neurons occurred during learning ("neurons that fire together, wire together"), foreshadowing the later discovery of long-term potentiation.

1.2.6.2.1. Model of Long-Term Potentiation

While recording from the rabbit hippocampus, Bliss & Lømo (1973) found that tetanic stimulation of the perforant pathway led to lasting, activity-induced, increases in synaptic connectivity in the dentate gyrus, termed Long-Term Potentiation (LTP). Counter to LTP, activity dependent synaptic weakening, or Long-Term Depression (LTD) was later reported, which showed the bidirectionality of synaptic plasticity (Lynch 1977; Dudek & Bear, 1992). Both forms of plasticity have a transient early-phase and a longer-lasting late-phase, which is dependent on protein translation for the transition. In particular, the transformation of early- to late-phase LTP has been likened to the transformation of short-term to long-term memory (Frey & Morris, 1997). Numerous investigations have since reported parallels in the molecular events underlying LTP *in vitro* and with those required for learning and memory formation in animals *in vivo*, thus popularizing this model of synaptic plasticity as describing the neuronal basis of memory (Bliss & Collingridge, 1993; Shepherd & Huganir 2009; Kessels & Mallinow 2009).

1.2.6.2.2. Inducing and encoding

Central to synaptic consolidation is the strengthening of neuronal connections supporting a memory trace, by more efficient functional communication and/or by more stable morphology. The molecular events that stabilize synapses is highly preserved across species, crucial to a variety of memory modalities and is characterized by distinct kinase cascades.

Fast synaptic transmission in the mammalian brain is mediated by α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPARs). These tetrameric protein complexes are composed of varying subunit combinations (GluA1-4), with GluA1/A2, GluA1/GluA1 and GluA2/A3 being the most abundant in the hippocampus (Shi, Hayashi, Esteban, & Malinow, 2001). Increasing the number of AMPARs in the post synaptic density (PSD), or increasing the single channel conductance of these receptors, therefore boosts synaptic transmission. A

critical regulator of AMPAR trafficking and efficacy is the N-methyl-D-aspartate receptor (NMDAR), which together with AMPARs, coordinates plasticity changes within the synapse.

Upon glutamate binding, a sodium influx from AMPARs depolarizes the post synaptic compartment. If the depolarization reaches a threshold, it drives the repulsion of a magnesium block (Mg^{2+}) within NMDARs, which, when glutamate and glycine also bind to these receptors, causes these channels to open allowing an influx of calcium ions (Ca^{2+}) into the post-synapse. As such, NMDARs are thought to act as coincidence detectors for activity from pre-synapse (glutamate) and the post-synapse (depolarization). Calcium entry activates numerous protein kinases including protein Kinase C (PKC), cAMP-dependent protein kinase (PKA) and calcium/calmodulin-dependent kinase II (CaMKII) which themselves promote signaling cascades to encourage synaptic strengthening. For example, CaMKII phosphorylates GluA1 subunits of AMPARs, which (a) increases conductance of already present receptors (Lee, Barbarosie, Kameyama, Bear, & Huganir, 2000) and (b) promotes AMPAR trafficking to the post synapse through the insertion of receptors from vesicles and lateral diffusion from extrasynaptic sites (Malinow, Mainen, & Hayashi, 2000). Furthermore CaMKII, along with PKA, promotes the activity of mitogen activated protein kinase (MAPK) which regulates cell adhesion molecules to encourage synaptic growth and spine remodeling. While not exhaustive, these cascades highlight the means which promote LTP induction (Baltaci, Mogulkoc, & Baltaci, 2019; Collingridge, Kehl, & McLennan, 1983). Importantly, many of these pathways seem to be also critical for initial learning. For example, blocking NMDARs or CaMKII prevents spatial learning in rodents (Buard et al., 2010;Morris et al., 1986), and acquiring sensitization memory in Aplysia depends on increasing the conductance of glutamatergic receptors in the post-synaptic membrane (Asok, Leroy, Rayman, & Kandel, 2019). Furthermore, the time scale in which these molecular events occur is in the range of minutes to hours, which correlates both to the time scale of early-LTP and short-term memory.

1.2.6.2.3. Maintaining synaptic strength and memory

Longer lasting changes in synaptic plasticity rely on *de novo* protein synthesis, protein degradation and gene expression which all unfold in the hours following activity. The kinase cascades critical for induction also activate transcription factors such as the cAMP- responsive element binding protein (CREB), which upregulate the transcription of a number of immediate early genes such as *Arc* and *c-fos*, and ultimately promote the synthesis of proteins critical for stabilizing synaptic plasticity.

Dendritic *de novo* protein synthesis is initiated by synaptic activity and NMDAR activation. In particular, mRNA of the atypical PKC isoform M-zeta (PKMζ) is enriched in dendrites and subsequent glutamatergic releases a translation block, leading to a localized increase of this protein. PKMζ uniquely lacks a regulatory binding domain acting on its catalytic region, therefore upon translation, PKMζ remains constitutively active (Hernandez et al., 2003). In electrophysiological studies, PKMζ levels initially increase 10 minutes after LTP induction which permits the formation of long-lasting LTP (Kelly, Crary, & Sacktor, 2007; Osten, Valsamis, Harris, & Sacktor, 1996). Application of a PKMζ inhibitor, such as zeta inhibitory peptide (ZIP) to hippocampal slice preparations does not prevent LTP induction, but when applied in the hours after induction causes rapid depotentiation (Serrano, Yao, & Sacktor, 2005), suggesting a unique role in the maintenance of synaptic plasticity changes.

Correspondingly, PKMζ plays a crucial role in long-term memory maintenance. Infusions of ZIP following long-term memory establishment leads to memory erasure, in multiple brain regions and corresponding behavioural tasks (Hardt, Migues, Hastings, Wong, & Nader, 2010; Pastalkova et al., 2006; Shema, Sacktor, & Dudai, 2007). Crucially, following ZIP metabolism, erased memories do not spontaneously recover but the ability to encode and maintain new long-term memories is not impaired. This suggests that, similar to *in vitro* synaptic plasticity, PKMζ critically promotes the maintenance of long-term memory *in vivo*. Yet, in an unintuitive set of studies, animals which have the PKMζ gene deleted are able to learn and maintain long-term memories, and infusions of ZIP still induce memory erasure in a similar manner to that of control animals (Lee et al., 2013; Volk, Bachman, Johnson, Yu, & Huganir, 2013). This discrepancy was later accredited to another PKC isoform, PKCι/λ, which similarly is targeted by ZIP, and is proposed to provide compensation in the absence of PKMζ (Tsokas et al., 2016). The readily available substitution for PKMζ suggests that memory maintenance critically relies on the activity of a constituent kinase.

To maintain increases of synaptic connectivity, PKMζ promotes trafficking of GluA2-AMPARs to the post synaptic density and prevents endocytosis of GluA2-AMPARs inserted during memory formation and consolidation (Migues et al., 2010; Yao et al., 2008). During LTP induction, GluA1-AMPARs are inserted into the PSD, which are eventually replaced by GluA2-AMPARs as the potentiation stabilises (Plant et al., 2006). This replacement has been attributed to small calcium entry which is permitted by GluA1-AMPARs, and then stabilised by the replacement of GluA2-AMPARs which are not calcium permeable. Once in the post-synaptic density, GluA2-AMPARs can be internalised in an activity-dependent manner,

through proteins such as BRAG2, which target a tyrosine rich motif on the carboxy tail of the GluA2 subunit (Scholz et al., 2010). Infusing $GluA2_{3Y}$, a small peptide that replicates this motif and competitively prevents internalisation, preserves early LTP and importantly, prevents long LTP erasure from subsequent infusions of ZIP (Migues et al., 2010). Furthermore, $GluA2_{3Y}$ delivery to the amygdala, before ZIP, blocks fear memory erasure from animals, therefore suggesting that PKMζ acts to preserve memory by maintaining changes in synaptic efficacy (Hardt, Nader, & Wang, 2014) .

1.2.7. Reconsolidation

Once consolidated, memory can return to malleable states under certain conditions of recall, such that it is vulnerable to erasure by amnesic agents or can be updated with new knowledge (Hardt, Einarsson, & Nader, 2009; Hupbach, Gomez, Hardt, & Nadel, 2007; Nader, Schafe, & Le Doux, 2000). In order to be retained, these reactivated memory traces must undergo a time-dependent restabilisation process, i.e., they need to re-consolidate.

There are a number of conditions that determine whether memory will destabilise upon reactivation. Firstly, the greater the saliency of initial learning (often referred to as memory strength) the more resistant to destabilization at recall a memory trace will be. For example, in rats, auditory fear memory induced from weak shock conditioning paradigms (i.e., 1 tone-shock pairing) can be disrupted after recall, suggesting its destabilisation, while those acquired with a strong shock protocol (i.e., 10 tone-shock pairings) will not (Josué Haubrich, Bernabo, & Nader, 2020). Secondly, the time elapsed since learning limits the window of memory destabilisation, in that typically memory can be recalled in a labile state in the days after encoding, but not weeks later (Alberini, 2011). Finally, the presence of unexpected stimuli to a familiar situation, otherwise known as prediction error, greatly promotes the likelihood of memory destabilisation. It is generally thought that prediction-error induced memory destabilisation provides an adaptive means for organisms to update existing memory in a changing environment (Hardt et al., 2009).

Initial destabilisation of a memory trace depends on processes that dismantles a synaptic connection, such as protein degradation and the induction of depotentiation-related synaptic plasticity. For example, blocking AMPAR endocytosis or protein degradation in rodents prior to a reactivation session in contextual fear learning prevents memory destabilisation and updating (Ferrara et al., 2019). Furthermore, dopaminergic signalling is a hallmark of prediction error detection in the brain, and it similarly, promotes memory updating. For

instance, blocking the D1/D5 dopaminergic receptors before, but not after, an object recognition reactivation trial in rodents prevents memory destabilisation (Rossato et al., 2015).

Restabilisation most prominently depends on new protein synthesis. A standard protocol for animal reconsolidation blockade experiments (i.e., to illustrate a memory was recalled into a malleable state) is to inhibit protein synthesis by pharmalogical intervention of anisomycin after memory recall, thus inducing amnesia for a previously acquired trace (Alberini, 2011; Nader et al., 2000). A number of molecular events are shared across synaptic consolidation and reconsolidation, such as the activation of NMDARs (Wang, de Oliveira Alvares, & Nader, 2009), CREB-induced gene transcription (Kida et al., 2002), and MAPK activity (Duvarci, Nader, & LeDoux, 2005). That said, there are distinct molecular signatures of reconsolidation such as the unique requirement of the immediate early gene zif268. When zif268 is genetically deleted, an animal's ability to reconsolidate memory is impaired, but initial memory formation and consolidation remain intact (Lee, Everitt, & Thomas, 2004). Therefore, while there is overlap between synaptic consolidation and reconsolidation, these are ultimately discernible processes.

Similar to consolidation, reconsolidation has also been studied in terms of the transient dependence of memories on the hippocampus for recall. A number of reports have investigated this in rats undergoing contextual fear conditioning. Up to a week after conditioning, expression of recent fear memory for a context relies on the hippocampus, but not a month later, at which point the representation for remote memory seems to rely on cortical areas (Frankland et al., 2006). Yet, there is evidence that on occasion, the hippocampus is reengaged during remote memory recall, leaving traces vulnerable to reconsolidation blockade. For instance, one study showed that after a reactivation session (i.e., brief re-exposure to the conditioning context) hippocampal lesions or infusion of a protein synthesis inhibitor into the hippocampus can cause amnesia for remote contextual memory (Debiec, LeDoux, & Nader, 2002). Neither intervention had any effect on remote memory in the absence of a reactivation session, indicating that hippocampal involvement required memory expression. Whether hippocampal re-engagement reflects the replacement of a hippocampal trace lost to decay (Barry & Maguire, 2019b), or the addition/updating of existing trace (Nadel & Moscovitch, 1997) remains unclear.

1.2.8. Summary

Altogether, the fields of learning and memory have uncovered a wealth of knowledge on the neurobiology underlying systems, and synaptic, consolidation of long-term memory. This has led many to reconsider the study of forgetting, as the knowledge of how memory is formed and maintained, may provide insight as to how it is lost.

1.3. Revisiting Interference

In light of the findings from memory research, many revisited the original retroactive interference literature in recent years to determine whether the disrupted consolidation account was appropriately dismissed. In doing so, it's been found that nearly all those that initially could not replicate Müller and Pilzecker's temporal effect of retroactive inhibition, inadvertently failed to facilitate 'interference free' periods after initial learning (Dewar et al., 2007; Wixted, 2004). For example, re-evaluation of McGeoch and MacDonald's (1931) report of material similarity driving retroactive interference, revealed that during the delay period, participants were engaged in other verbal processing. In other words, all subjects would have been exposed to non-experimental interference (i.e., reading a paper) before the intended interpolating activity was introduced, thus equalising the interference timepoints. As such, the existence of a temporal window in which retroactive interference unfolds on stabilizing memory remains unclear. Furthermore, a direct comparison between similar and non-similar interpolating material was never conducted, making any rejections of general mental activity in favour of material specific interference unfounded (Dewar et al., 2007).

1.3.1. Everyday forgetting and retroactive interference

A number of recent studies have attempted to resolve these questions regarding the role of retroactive interference in impaired consolidation. Firstly, to determine whether general mental effort could drive retroactive interference, Dewar *et al.* (2007) conducted a series of experiments in a protocol similar to Müller and Pilzecker's original investigation. Participants were given a verbal list of words, which were to be retained and tested after an 8-minute delay. During the delay period, subjects were assigned to one of six groups; a control, unfilled delay period (i.e., wakeful rest), periods of intentional learning (i.e., visual and auditory learning tasks), periods of exposure to new material without memory requirements (i.e., playing 'spot the difference', solving math equations), or a period with a cognitively demanding task that did not contain new material or memory demands (i.e., piano note detection). Put simply, a variety

of sensory and semantic modalities were presented to participants in the delay period which had differing demands on memory and perceptual processing. When comparing the percentage of correct word list recall, the highest score was found in the group with the period of wakeful rest, consistent with prior work. All other conditions diminished recall, and importantly, no differences were observed between groups. This suggests any form of mental effort following learning suffices to promote interference, i.e., forgetting.

Later studies revealed that these types of interpolated activity after learning not only impaired short-term memory recall, but also disrupted consolidation of traces into long-term storage. For instance, participants asked to learn two stories, one followed by 10 minutes of wakeful rest and another followed by a spot-the-difference game, could better recall the story learnt before a period of wakeful rest when tested 7 days later. The same pattern of memory enhancement was also reported for participants learning 'non-recallable non-words' (Dewar, Alber, Cowan, & Sala, 2014), ruling out the possibility of rehearsal in the delay period driving enhanced memory persistence. By employing the spot-the-difference game to drive forgetting, the authors limited the overlap in modality between interpolated and to-be-retained learning (i.e., verbal and visual), thus reducing retrieval competition from similar material.

Furthermore, post-learning wakeful rest was shown to improve spatial knowledge, where participants had to navigate through a virtual environment (Craig, Dewar, Harris, Della Sala, & Wolbers, 2016). In comparison to those who played a spot the difference game in the same period, a 10-minute period of wakeful rest after learning improved memory accuracy at both recent- (i.e., 30 minutes later) and remote (a week later) time points.

Importantly, similar findings have also been reported in rodents undergoing a spatial memory task (Arkell, Groves, Wood, & Hardt, 2021). In this paradigm, rats are exposed to two identical objects within an open field arena containing distinct spatial cues during a sampling phase in which rats can freely explore the objects. After a delay, one of the objects is moved to a new spatial location in the arena, while the other remains at its old place. Like the previously discussed object recognition paradigms, this task relies on the natural preference of rodents for novelty: if the animal remembers the previous locations of the objects, it should spend more time exploring the object moved to a new place. After sampling, rats are typically returned to their home cage, i.e., a transparent cage, with an open grill roof, containing at least one other cage mate. Put simply, rats are exposed to everyday sensory experience after sampling. Arkell *et al* (2021) observed that rats undergoing a brief sampling session followed by a delay in their home cage, did not prefer to explore the object moved to a novel location 6 hours later. However, when rats were placed individually into a familiar dark box for 1-hour immediately

after sampling, they expressed memory for object locations during the memory test. Furthermore, turning on the light in the dark box or placing another cage mate into it without turning on the light preserved memory. These findings, in line with those from human studies, suggest that retroactive interference disrupting consolidation can arise from various sensory modalities.

1.3.1.1. Impaired synaptic consolidation

One proposed explanation for the above described findings is that the majority of everyday forgetting arises from *non-specific* retroactive interference (i.e., mental activity), which disrupts ongoing consolidation processes in the hippocampus (Wixted, 2004). More specifically, mental activity following learning will detract from a finite supply of hippocampal resources that are needed to consolidate recently acquired memories. In this sense, new memories do not overwrite immediately preceding ones; rather, both compete for a limited set of resources required for consolidation and long-term retention. Therefore, greater mental activity after learning will result in a stronger interference effect, i.e., more pronounced forgetting. Conversely, memory enhancement, or retroactive facilitation, can be achieved by preventing mental activity and input to the hippocampus (i.e., during wakeful rest), allowing more resources to be allocated to consolidation.

Evidence for this position arose from a number of studies exploring the effect of 'artificial learning' (i.e., LTP induction) on 'natural learning' (i.e., animals completing a task). For example, retention of a hippocampus-dependent memory (i.e., a water maze task) is impaired in rats when artificial LTP is induced after learning, an effect which is negated by administration of an NMDAR antagonist to the hippocampus prior to high-frequency stimulation (Brun, Ytterbø, Morris, Moser, & Moser, 2001). Moreover, application of a NMDA receptor antagonist to the hippocampus of rats immediately after learning a hippocampus-dependent task (eight-arm radial maze), extended the persistence of memory for the task i.e., retroactively enhanced spatial memory (Villarreal, Do, Haddad, & Derrick, 2002). Thus, these data prompted the conclusion that LTP induction in the hippocampus, whether induced artificially or alternatively, by new learning, provides a viable source of retroactive interference which theoretically impairs the maintenance of established LTP associated with recently formed memories (Wixted, 2004).

In alignment with the ideas of systems consolidation, a natural extension of the impaired synaptic consolidation account is that damage to the medial temporal lobe, and specifically the

hippocampus, will reduce hippocampal resources required to promote consolidation. In other words, anterograde amnesia arises from the lost capacity to consolidate new long-term memory.

1.3.2. Medial temporal lobe damage and retroactive interference

A corresponding stream of research explored the effect of reducing interference after learning in patients suffering from anterograde amnesia. While the amnesia of those recruited arose by various means (i.e., stroke, brain injury, mild cognitive impairment), all patients experienced forgetting of newly learnt material within minutes.

In these studies, patients were taught either a list of words or a story and then put in into a 10-minute period of either wakeful rest (i.e., sitting in a dark quiet room) or a 10-minute period of psychometric testing (both verbal and non-verbal). Immediately after this period, memory for the learnt material was assessed, and similar to healthy controls, the period of wakeful rest increased recall performance in comparison to those exposed to the filled delays. While performance was still poorer than healthy controls, it suggested that patients were able to maintain memory when everyday interference was controlled for (Cowan, Beschin, & Sala, 2004). A follow up study explored whether introducing interfering stimuli during the period of wakeful rest after learning reversed the reported memory improvements (Dewar, Garcia, Cowan, & Sala, 2009). Across the 10-minute period following learning, an interfering task (picture naming) was introduced at either 3, 6 or 9 minutes, corresponding to early, mid or late intervention respectively. During the test phase, patients showed a graded effect of impaired recall, in that early interfering interventions caused severe forgetting which lessened as the delay between learning and interfering task increased. This set of results showed a clear demonstration of decreasing vulnerability of memory to retroactive interference in patients, or inversely, increasing memory consolidation.

Next, the authors determined whether a brief wakeful rest (again, sitting in dark, quiet room) can boost newly acquired memory into long-term storage (i.e., test their recall at later periods), similar to healthy controls. As such, Alber *et al.* (2014) asked amnesic patients to learn a piece of prose, and immediately after, placed subjects in either a 10-minute period of wakeful rest or playing a spot the difference game. Recall for the prose was tested either 30 minutes, or 7 days later. Patients exposed to the reduced sensory stimulation condition after learning showed drastically improved prose recall at both short and long-term time points in comparison to those exposed the condition of mental activity (Alber, Sala, & Dewar, 2014).

This suggests firstly that anterograde amnesia results from increased susceptibility to retroactive interference, and secondly, merely 10 minutes of reduced interference after encoding allows the formation of new declarative memory.

Similar findings have also been reported in rats with lesions to the hippocampus. For example, Jarrard (1975) reported that rats with lesioned hippocampi were more susceptible to retroactive interference on a single-trial alteration task when placed in a running wheel (i.e., interpolated activity) between the sample and choice period, than those left in their holding cages. More direct evidence that amnesia induced by medial temporal lobe damage is the result of excessive interference disrupting memory stabilization, comes from lesioned rodents undergoing object recognition. Typically, damage to the perirhinal cortex leads to robust object amnesia, in that rats are unable to acquire and retain memory for objects beyond delays of 10 minutes (Warburton & Brown, 2015). Yet, object memory can persist for an hour in lesioned animals if interference is attenuated between sampling and probe trials. Specifically, McTighe et al. (McTighe, Cowell, Winters, Bussey, & Saksida, 2010) found that returning lesioned rats back to their standard home cages during an hour delay period after learning led to the expected impaired recognition memory at test. Yet, placing them in a dark, quiet environment (i.e., low sensory stimulation) during the delay, lead to intact expression of object memory, comparable to control animals. Whether such treatments can also promote longer lasting object recognition memory remains unknown.

These observations which suggest that anterograde amnesia can be greatly reduced when a period of quiescence follows learning does not readily fit with the previously described thinking on memory and forgetting. In fact, they directly clash with a core position of systems consolidation: that amnesia arising from hippocampal or medial-temporal lobe damage reflects a lost capacity to stabilize newly acquired memory into long-term storage. In other words, compromised hippocampal functioning should limit neocortical-hippocampal interactions assumed *necessary* for long-term memory formation. Yet, in stark contrast, amnesic patients as well as animals with damage to these brain regions are able to form new long-term memory when post-learning stimulation is reduced, suggesting that memory consolidation is *preserved*. These results, however, can be explained by the alternative position that hippocampal damage leads to an increased vulnerability to retroactive interference arising from everyday sensory stimulation. Thus, while both explanations argue that amnesia following hippocampal damage reflects impaired memory consolidation, the latter account proposes that the hippocampus protects against interference and therefore promotes consolidation, while the former claims that hippocampal processes directly consolidate memories.

1.3.3. Active Decay Theory

One account which readily explains these findings in amnesic patients is active decay theory. This theory assumes that different modes of forgetting unfold across brain regions, circuit architectures, trace compositions and timescales (Hardt et al., 2013). In particular, active decay theory attempts to place the long-standing observations of interference and decay within a neurobiological framework, where these two modes of forgetting are assumed to naturally collaborate together to drive forgetting of declarative and non-declarative memory. In brief, active decay theory proposes that during periods of wakefulness one of the main sources of forgetting will be interference in brain regions that do not supply circuit mechanisms reducing it, such as pattern separation. During periods of sleep a cellular process of synaptic weakening termed 'active decay' can reverse synaptic modifications supporting established memory, which is assumed to occur in all brain regions. We will explore the proposed mechanisms of active decay in the next section, but will first discuss how the theory accounts for interference driven forgetting.

In accordance with earlier views (Marr, 1971a; McClelland, McNaughton, & O'Reilly, 1995; Squire et al., 1984), active decay theory assumes that most memory traces initially comprise a spatial-contextual representation that depends on the hippocampus and content representations dispersed across neocortical regions. In other words, the hippocampus serves to index and indirectly link the dispersed neocortical content representations (Teyler & DiScenna, 1986). Owing to the different architecture of each region, representations dependent on the neocortex or the hippocampus will be organised differently. In cortical areas, where representations are layered and pattern separation mechanisms are assumed to be absent or little efficient, traces will likely overlap. In comparison, little to no trace overlap is presumed in the hippocampus owing to its robust pattern separation capabilities. This arrangement permits the hippocampus to orthogonally index neocortical traces, allowing them to retain their individuality despite pattern overlap within a shared ensemble, and, more importantly, because these indices bind the dispersed neocortical content representations, the hippocampus provides indirect pattern separation for overlapping representational patterns in neocortical regions.

A core assumption of active decay theory is that the brain is engaged in *continuous and unselective* encoding of experience throughout the day. Under such a regime of 'promiscuous encoding', the brain will have to 'decide' at a later time point whether to retain or forget new memories (Hardt et al., 2013). Given the assumed circuit architectures, the theory

predicts that in the neocortex newly acquired, and still stabilizing, traces will be highly vulnerable to interference from ongoing new encoding of experiences. On the other hand, this type of interference will unlikely occur in the hippocampus. Therefore, during initial encoding the hippocampal trace critically provides indirect pattern separation which *protects* recently acquired memories in neocortex from disruption by interference caused by new learning.

With time, likely promoted by hippocampal-neocortical interactions, (Rudoy, Voss, Westerberg, & Paller, 2009), these weakly connected neocortical representations integrate into the neocortical networks as directly associated ensembles. Thereafter, as proposed by various accounts of systems consolidation (Nadel & Moscovitch, 1997; Squire et al., 1984) representations are able to be retrieved without the hippocampus, yet such memories may lack full contextual detail, which is thought to require contributions from the hippocampus (St-Laurent, Moscovitch, Jadd, & Mcandrews, 2014; St-Laurent, Moscovitch, & McAndrews, 2016; Wiltgen et al., 2010). Decay-like mechanisms will remove hippocampal traces rather quickly (on the order of days and weeks), whilst consolidated neocortical representations may be able to persist for longer (McClelland et al., 1995). Over time, neocortical ensembles can subsequently organize more sparsely, thus reducing future memory representation overlap (Kuhl, Shah, Dubrow, & Wagner, 2010).

This framework makes several distinct predictions. Firstly, given its proposed role in attenuating interference, the hippocampus will always be initially recruited regardless of the type of memory. Secondly, should hippocampal functioning be impaired, initial interference protection for neocortical representations will be compromised, therefore, susceptibility to interference will increase, possibly resulting in a state akin to catastrophic interference. This excessive interference will then manifest as an inability to remember a previous experience, i.e., amnesia. Furthermore, should hippocampal functioning be impaired, cortical representations will stabilize and persist provided *interference* – arising from subsequent encoding – is prevented.

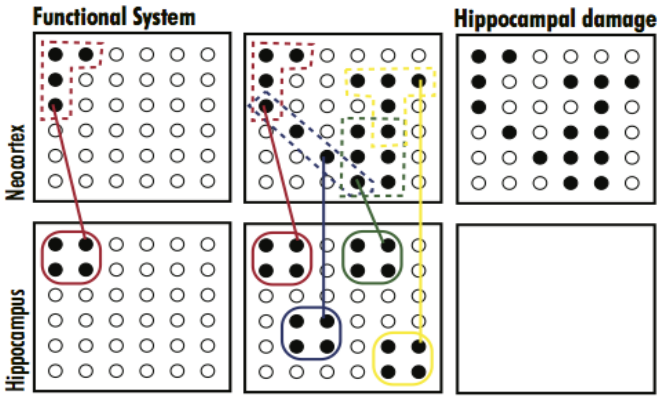


Figure 1.2. Illustration of the predictions of active decay theory. Figure is adapted from (Hardt et al., 2013). Pattern separation in the hippocampus leads to orthogonalized index representations which link to individual neocortical traces that overlap in a shared ensemble. The hippocampus reduces the risk of interference in extra-hippocampal areas by virtue of its own non-overlapping representation, allowing cortical traces to retain their coherence. In the case of hippocampal damage, the hippocampal component is lost, leading to inseparable patterns, which manifests behaviourally as amnesia.

1.4. Modern ideas: Active Decay

Active decay theory holds that while interference predominates in neocortical areas for new and reactivated memories, forgetting of established long-term memories in the hippocampus and other brain areas is to a great extent the result of active decay, an endogenous process that weakens synaptic connections. Similar to early ideas on decay mentioned above, active decay is thought to unfold continuously, i.e., over time, resulting in a steady decline of established long-term memory. Yet, rather than these earlier accounts, this decay process is thought of as a constitutive, regulated process that gradually erases the structural underpinnings of long-term memory, eventually leading to memory loss. The proposal of a neurobiological mechanism responsible for this type of forgetting reflects a crucial shift in modern thinking on forgetting with widespread theoretical implications of the role of forgetting in memory (Hardt et al., 2013; Kraemer & Golding, 1997; Richards & Frankland, 2017).

1.4.1. A glitch or a function?

Traditionally - and occasionally still today - forgetting has been regarded as a deplorable shortcoming of memory. In particular, the human experience of forgetting is often felt as a frustrating limitation or an assault on our persona (Della Sala, 2010) perhaps tapping into a

common aversion to impermanence. This outlook has undoubtedly influenced the idea that forgetting emerges from failures or glitches in a memory system trying to acquire and retain as much of our daily experience as possible. From this, two main conclusions emerge; that forgetting is unwanted, and that it is unintentional.

In opposition to the claim that forgetting is a vice, an often-used case in point is the small number of clinical patients with highly superior autobiographical memories (HSAM). These individuals are able to recall most of their personal life events in extreme detail, yet describe this ability as burdensome and adverse (Mazzoni et al., 2019). Indeed, under normal memory regimes, a number of empirical studies, computational models and theoretical positions have argued that without forgetting, many basic memory functions would not be possible. Firstly, in order for memory to generalize, the specific details regarding the context in which information was learnt must be removed or at least suppressed for that information to be applied to novel situations (Richards and Frankland 2017; Hardt et al 2013). For example, when natural time-dependent forgetting is pharmacologically inhibited in the dorsal hippocampus of rats, animals retain the ability to discriminate between contexts, while control animals generalize a fear response across different contexts (Migues et al., 2016). Thus, forgetting within the hippocampus may drive trace decontextualization such that the contextual details of the conditioning episode are lost, allowing fear memory to generalize to novel situations. This process has been proposed to similarly underlie the transformation of detailed episodic memories to eventually become more semantic with time, such that the specifics of an event are forgotten, and the representation becomes more gist-like (Hardt & Nadel, 2018).

Generalization can also occur while memory is stabilising, and has not yet 'consolidated' into long-term memory. This form differs from the above described generalization, in that the stabilising memory supports new, related learning of the same structure (i.e. the first knowledge is generalized across to second knowledge) and is instead often referred to as transfer learning. This was demonstrated in one study where human participants learnt two modally distinct tasks (i.e., cognitive and procedural) that shared a common abstract structure shortly after one another. Retroactive interference for the task first occurred, but crucially, at the benefit of accelerated learning for the second (Mosha & Robertson, 2016). When the two tasks were learnt across a time point that reduced the effect of forgetting (i.e., separated by two hours), memory for the first task was unimpaired, but the second task took longer to acquire. These findings of 'leaky' memory (Robertson, 2022) suggest that forgetting allows common information to be shared among different contexts or task domains, and subsequently promote transfer learning.

Finally, forgetting can promote the ability to remember or recall specific information accurately. Although initially counterintuitive, adaptive retrieval-induced forgetting has been shown to suppress other, competing representations (i.e., within the same category) that are not retrieved, thus limiting trace competition at recall, allowing for more adaptive response selection. Importantly recent findings in rodents suggest that retrieval-induced forgetting not only inhibits expression of other potentially interfering memory traces but also can actively erase them. For instance, rats exposed to multiple objects in the same context that are then later required to repeatedly retrieve one of the objects, show impaired memory at a later recall of the other objects initially presented. While this phenomenon is initially dependent on inhibitory GABAergic signalling, the inhibition declines with repeated retrieval attempts, suggesting that competing memory traces had been steadily degraded (Bekinschtein, Weisstaub, Gallo, Renner, & Anderson, 2018). These findings can be extended to recent computational work showing that forgetting of older information can improve decision making of agents exploring mazes for reward (Yalnizyan-Carson & Richards, 2022). Taken together, reducing the 'noise' arising from other acquired response options allows for quicker deliberation, and more efficient behaviour.

Given the importance of forgetting in memory processing, the claim that it is based on unintentional process seems ill-posed and, in fact, clashes with increasing evidence that organisms have dedicated and endogenous forgetting processes. For example, in *Drosophila*, specific 'forgetting cells' have been identified which release dopamine to actively drive the active-decay like forgetting of appetitive olfactory memories (Berry, Cervantes-Sandoval, Nicholas, & Davis, 2012). Furthermore, the previously discussed studies of ZIP-induced memory erasure also point towards an active forgetting process. Looked at from another angle, if inhibiting a *constituently active* kinase (most likely PKMζ) leads to memory erasure, there must be a continuous, opposing force that degrades memory traces, which PKMζ counteracts under normal conditions.

1.4.2. Mechanisms of Active Decay

Initial investigations on the molecular events underpinning active decay were guided by findings that PKMζ preserves long-term memory by maintaining GluA2-AMPARs at the post-synaptic density. Given the critical role of GluA2-AMPARs in maintaining memory, activity-dependant endocytosis of these AMPARs seems a likely means of synaptic weakening underlying forgetting. In a direct test of this, after training rats in an object location (non-

associative memory) or a place preference task (associative memory), Migues *et al.* (2016) infused GluA2$_{3Y}$ (which blocks GluA2-AMPAR internalisation as discussed) into the dorsal hippocampus over a time course during which these memories were naturally forgotten. Critically, animals that received GluA2$_{3Y}$ expressed memory for objects and place preference beyond their natural timespan, suggesting that time-dependent forgetting had been blocked (Migues et al., 2016). Correspondingly, infusions of GluA2$_{3Y}$ to hippocampal slices prevented LTP depotentiation (Migues et al., 2016). Expanding on this research, mice lacking the synaptotagmin-3 gene, which encodes an integral membrane protein that mediates activity-dependent internalization from the plasma membrane, failed to exhibit LTP decay, nor could LTD be induced (Awasthi et al., 2019). Furthermore, synaptotagmin-3 null animals showed normal learning rates in spatial tasks, but the persistence for spatial memory far surpassed that of control animals (Awasthi et al., 2019). Taken together, these findings suggest that a natural time-dependent forgetting of long-term memories arises from the steady weakening of synaptic efficacy.

As discussed prior, NMDA receptors regulate AMPAR trafficking at the post synaptic density during synaptic plasticity. Critically, this regulation is bidirectional, as NMDAR activity also drives the removal of AMPARs from the post synaptic density during LTD and depotentiation. *In vitro* studies have shown that blocking NMDARs with AP5 after LTP induction prevents both the decay of established potentiation (Villarreal et al., 2002) and the induction of long-term depression. Importantly, these forms of NMDAR-dependent reduction of synaptic potentiation lead to reduced GluA2-AMPAR levels at post synaptic sites (Migues et al., 2016; Scholz et al., 2010; Unoki et al., 2012)

In agreement, increasing evidence suggests that NMDAR activity similarly regulates forgetting. For instance, rodents trained on either object location or spatial reference tasks in the Morris Water Maze show extended memory expression when NMDAR antagonists (AP5 and CPP) are regularly infused into the dorsal hippocampi during a memory retention interval (Migues 2019; Shinohara & Hata, 2014). Inversely, promoting NMDAR activity *enhances* the rate of forgetting. For instance, infusing D-serine, an NMDAR co-agonist that attaches to the glycine binding site required for receptor opening, into the dorsal hippocampus of rats after object location training impaired memory expression. In this task, animals typically express memory for object locations up to 7 days after training, yet those infused with D-serine showed impaired memory 6 days later (Migues, Wong, Lyu, & Hardt, 2019). Thus, these studies suggest that NMDAR signalling actively drives the forgetting of established memory.

NMDARs are tetrameric complexes composed of two GluN1 subunits and two regulatory subunits made up of various combinations of GluN2 or GluN3 subunits. The most abundant compositions in the brain are the GluN1-GluN2A and GluN1-GluN2B, which regulate synaptic plasticity. While GluN2A subunits are associated with quicker deactivation times, GluN2B-NMDARs show a higher binding affinity to glutamate, slower inactivation and greater calcium influxes (Sobczyk, 2005). It is generally accepted that the degree of calcium influx regulates the direction of synaptic plasticity, where moderate calcium levels promote LTD, while greater levels of calcium induce LTP (Lisman, 1989). Yet despite differences in calcium influx, a clear assignment of either learning or forgetting to specific NMDAR subunits has been difficult to establish (Shipton & Paulsen, 2014).

The ratio of GluN2A to GluN2B NMDARs at the post-synapse is instead believed to determine states of metaplasticity, or how stable/unstable synapses are. Given the greater calcium currents arising from GluN2B-NMDAR activation, these subunits are more likely to determine the probability and direction of synaptic plasticity than GluN2A (Hardt et al., 2014). Although conflicting, there is evidence to support this notion. Animals with genetic overexpression of the GluN2B, but not the GluN2A subunit showed enhanced LTP *in vitro* (Cui et al., 2013; Wang et al., 2009). Inversely, selective blockade of GluN2B-NMDARs attenuates LTP decay and abolishes NMDAR-dependent LTD (Liu et al., 2004).

Importantly, the ratio of GluN2A:GluN2B similarly determines the encoding and loss of memory. For instance, animals with overexpression of GluN2B-subunits, showed enhanced formation of long-term memory for spatial navigation in the Morris water maze (Brim et al., 2013). In comparison, mice that overexpressed the GluN2A-subunits were selectively impaired in long-term memory formation across a variety of tasks, including novel-object recognition, fear condition, and spatial navigation. This suggests that a lower GluN2B:GluN2A ratio limits metaplasticity required for memory, and perhaps, for forgetting. In alignment with this notion are findings that after strong auditory fear conditioning, rats express robust, non-malleable memory that does not reconsolidate after reactivation, which correlates with a reduced expression of GluN2B in the amygdala (Wang et al., 2009) .

Taken together, these studies suggest that the presence and activity of GluN2B-NMDARs may uniquely contribute to active decay of long-term memory. In a direct confirmation of this Migues *et al.* (2019), found that infusion of Ro25-6981, a selective GluN2B subunit antagonist, into the dorsal hippocampus of rats after object location training preserved location memory beyond their natural time span. Due to the lack of GluN2A selective antagonist, it is difficult to rule out the contribution of GluN2A-NMDARs in

forgetting at present. Nonetheless, GluN2B-NMDARs appear to play a critical role in GluA2-AMPAR internalisation which underpins active decay of long-term memory.

Given the discussed studies, two obvious questions arise: (1) if NMDAR subunits determine states related to memory formation and loss, how then do these receptors direct these opposing processes? (2) What signalling cascades are activated by NMDAR signalling that promote AMPAR endocytosis in active decay?

1.4.3. Proapoptotic signalling

NMDARs are located at both synaptic and extra-synaptic sites. Functionally, extra-synaptic NMDA receptors (exNMDARs) are defined as those not recruited during low-frequency synaptic activity (Hardingham & Bading, 2010), or anatomically as those located further than 100 nm away from the post-synaptic density, encompassing the perisynaptic zone and dendritic shaft (Papouin & Oliet, 2014).

Signalling from NMDARs at each location facilitates opposing fates in neuronal health. For instance, synaptic NMDAR (sNMDAR) signalling promotes long-lasting neuroprotection by triggering transcriptional activation of survival genes and repression of cell death genes (Hardingham & Bading, 2010; Zhang et al., 2007). Contrastingly, activation of exNMDARs by glutamate excitotoxicity induces cell death pathways (Choi, 1988; Hardingham & Bading, 2010). CREB shut-off pathways are activated, which block the transcription of a number of genes that support and maintain synaptic structures (Hardingham, Fukunaga, & Bading, 2002), constituent AMPAR trafficking is disrupted, and mitochondrial dysfunction is induced. Specifically, calcium currents through exNMDARs trigger the intrinsic apoptotic pathway, hallmarked by the mitochondrial release of cytochrome c, which then activates the cysteine caspase-9, which subsequently cleaves and activates the key executioner proteins caspase-3 and -7 (Tait & Green, 2010).

Critically, there is increasing evidence that these divergent pathways related to the location of specific NMDAR signalling regulate opposing forms of synaptic plasticity. Stimulation of synaptic, but not extra-synaptic, NMDARs is required to induce LTP whereas selective activation of ex NMDARs triggers LTD by promoting AMPAR internalisation and removal from the synapse (Liu, Yang, & Li, 2013). Specifically, exNMDAR-LTD has been shown to recruit the intrinsic pro-apoptotic pathway, as pharmacological inhibition of caspase-3 or caspase-9 block NMDAR-induced LTD and AMPAR internalisation (Li et al., 2010). Addition of Bcl-xL (an antagonist of pore opening proteins in the mitochondria required for

cytochrome c release) similarly prevented NMDA-induced AMPAR endocytosis and LTD. Taken together, these findings highlight an alternative role of proapoptotic signalling in synaptic plasticity.

The discrepancy between cell death and local synaptic apoptosis appears to depend on the degree of exNMDAR stimulation, and therefore, the degree of down-stream activity (Jiao & Li, 2011). Calcium influxes activate the serine/threonine phosphatases, calcineurin/PP2B, and PP1 which dephosphorylate and translocate the protein Bad (Bcl-2-associated agonist of cell death) to mitochondrial attached BAX (Bcl-2-associated-X-protein), which promotes BAX activation. BAX facilitates pore formation within the mitochondrial membrane, allowing the release of cytochrome *c* to the cytosol. Thus, the level and duration of Bad-BAX interaction, will determine the extent of cytochrome c release and subsequent caspase-3 activation, where extended activity induces cell death, while shorter periods induce LTD without causing cell death.

While debated, there is evidence to suggest GluN2B-NMDARs are preferentially localised to extra-synaptic sites (Paoletti, 2011; Thomas Papouin et al., 2012). Thus, given that active decay is critically regulated by GluN2B-NMDARs, the above described findings suggest that active decay may similarly recruit the intrinsic proapoptotic pathway to promote AMPAR internalisation following extra-synaptic NMDAR signalling.

1.4.4. Other forms of trace erasure

While active decay theory focuses on trace erasure by reducing synaptic efficacy, it is important to note that over the last couple of decades, a number of other neurobiological mechanisms underpinning this form of forgetting have been identified. For example, forgetting has been shown to occur by downscaling spines (Davis & Zhong, 2017), eliminating spines by microglial phagocytosis (Wang et al., 2020), or replacing existing spines with new connections through neurogenesis (Frankland, Kohler, & Josselyn, 2013). However, whether these processes erode the content of memory traces or aspects critical for retrieval of a representation is still being debated, as a number of these processes show specificity to forgetting in hippocampal traces, which, as discussed, are crucial for recall of detailed representations (Hardt et al., 2013; Ryan & Frankland, 2022). Importantly, these various processes are thought to *collectively* contribute to natural memory loss, a vastly difference approach to early forgetting research which looked for a single underlying cause of forgetting.

1.5. Summary and Aims

Despite over a century of investigation it still remains largely unresolved how and why the brain forgets. To address this, active decay theory has proposed a framework where interference and active decay collectively co-ordinate memory loss across brain regions, levels of representation and under distinct timescales. During wakeful periods, stabilising memory is susceptible to interference from every day sensory experience, which predominantly unfolds from overlapping representations in the neocortex. To limit excessive interference and stabilisation disruption, the hippocampus provides indirect pattern separation, by virtue of its own orthogonal traces, to protect the coherence of newly acquired cortical memory despite being embedded in a shared representational ensemble. During periods of sleep, a cellular process of active decay reverses the synaptic modifications induced by memory formation, which steadily erases the structural underpinnings supporting long-term memories. While this process occurs in many regions of the brain, active decay is the predominant forgetting mechanism in the hippocampus, leading to forgetting of long-term memory in a gradual, time-dependent manner. Thus, interference and active decay naturally collaborate to drive forgetting.

Support for the interference-based predictions of active decay theory arises from recent work in anterograde amnesia patients with comprised hippocampal functioning. Typically, these individuals forget new declarative information within minutes, yet, when sensory stimulation is reduced after learning, memory can persist at both short- and long-term time points. While these findings suggest that impaired hippocampal functioning leads to increased interference vulnerability from sensory stimulation, a direct test of this has yet to be carried out.

The cellular events that underpin active decay remain to be fully identified. So far, it has been established that active decay requires NMDAR activation and activity-dependent internalisation of GluA2-AMPARs from the post synaptic density. Yet, what molecular cascades link these two events remains to be determined. Recent findings from electrophysiological studies suggest that NMDAR-dependent LTD recruits the intrinsic proapoptotic pathway. Specifically, exNMDAR signalling promotes cytochrome c release from mitochondria, which activates caspases -9 and -3, that promote AMPAR internalisation. Whether active decay recruits a similar signalling cascade is unknown.

To resolve these open questions, this thesis aims to (a) determine if the hippocampus prevents sensory interference during phases of memory stabilisation; (b) investigate proapoptotic signalling in the active decay of long-term memory.

Firstly, we discuss in chapter 2 the methods used in the experiments described in this thesis and why they allow us to investigate these aims in rats.

In chapter 3 we investigate the role of the hippocampus in stabilising memory into long-term memory. Based on the predictions of active decay theory, we tested the following hypotheses: (1) inactivating the hippocampus around the time of encoding leads to anterograde amnesia; (2) this amnesia arises from excessive interference from sensory stimulation, rather than a lost ability to consolidate new memory; (3) reducing sensory interference after learning rescues amnesia in the face of hippocampal impairment; (4) hippocampal learning is required at encoding to provide interference protection; (5) the hippocampus provide similar protection from sensory interference during reconsolidation.

In chapter 4 we explore the role of proapoptotic signalling in the active decay of long-term memory. To do so, we tested three main hypotheses; (1) Inhibition of caspase-3 after learning prevents time-dependent forgetting; (2) blocking ex NMDARs preserves memory beyond its natural time span; (3) exNMDARs are not required for learning.

Finally, in chapter 5 we discuss the implications of our findings, and importantly, whether they support or challenge active decay theory.

Chapter 2:

Methods

Active decay theory assumes that distinct brain regions supporting declarative memory are more likely to be subject to forgetting by interference or active decay. Rats express memory that models aspects of declarative memory, have a brain homologous in structure and function to humans but allow for more invasive investigations. One well-established memory task that models declarative memory in rodents is object-based recognition. Object paradigms themselves have a rich literature which encompasses the underlying neuroanatomy and circuits supporting object memory formation and discrimination. That said, it remains unclear how object memories are forgotten. To determine whether these paradigms permit our investigations into active decay theory, the main findings and methodological considerations from the field of rodent object memory are discussed in the following chapter.

2.1. Episodic-like memory in rodents

Tulving defined episodic memory as the conscious recollection of events which would require conscious introspection to access it and language to express it (Tulving, 1983). Applying this phenomenological criterion to non-human organisms is subsequently impossible, and so instead, animal research has focused on definitions which focus on other elements of episodic memory. Specifically, memory for events contains information on the content (what), spatial location (where) and temporal occasion (when) that was experienced. From this, a behavioural criterion for *episodic-like memory* has been developed, which is defined as the integration of what-where-when information into a single, flexible representation (Clayton, Yu, & Dickinson, 2003).

Under this content-based approach (Easton, Webster, & Eacott, 2012), a variety of tasks to assess knowledge for what-where-when memory have been developed for animal investigations (Babb & Crystal, 2006; Eichenbaum, 1998). Arguably the most versatile are object-based paradigms, which are a widely considered a good model of human declarative memory. These paradigms can be easily modified to test object identity (what), location

(where), and temporal order (when). These tasks have been adapted across a number of species that express memory for objects, thus, highlighting the ecological validity of object paradigms. The basis of these tasks requires an animal to determine whether an object has been encountered or not before, thus replicating recognition memory, a subdivision of declarative memory.

In humans, recognition memory can be expressed in either explicit recall of a prior experience or by some sense of familiarity toward already encountered stimuli (Tulving, 1985) as revealed by judgements of 'remembering' and 'knowing', respectively (Yonelinas, 2002). While recollection is characterised by the slow recall of contextually-rich and detailed information, familiarity describes quick judgements based on an awareness of prior experience. Some have argued that recollection and familiarity reflect differences in confidence or trace strength along a shared system, yet, increasing evidence also supports a 'dual-process' approach, in that separate systems mediate each expression type (Squire, Wixted, & Clark, 2007; Wixted, 2007; Yonelinas & Parks, 2007). Replicating such characteristics in animals undergoing recognition tasks seems, at first, challenging given the conscious experience of recollection. That said, recollection and familiarity can also be distinguished by distinct memory expression profiles. For instance, in humans each retrieval type has certain receiver operating characteristics (ROCs) which reflect the likelihood of correct recognition (i.e., a comparison of correct hits and false alarms across confidence levels). Under the appropriate behavioural paradigms, similar expression profiles of either familiarity or recollection-based recall in animals can match that of humans (Brown & Aggleton, 2001; Eacott, Easton, & Zinkivskay, 2005; Sauvage, Fortin, Owens, Yonelinas, & Eichenbaum, 2008). For example, rats undergoing an odour-texture recognition task, where an animal can make a quick familiarity-based judgement, or a slower, more deliberated decision across a spectrum of reward amounts, express comparable ROC curves (Sauvage et al., 2008). This therefore suggests that recognition in rats may closely resemble recognition in humans, furthering the case for translations between human and rodent studies of episodic-like recognition memory.

2.2. General procedures for object paradigms

There are a number of object task variants which assess different aspects of episodic-like memory content, processing and persistence. Early versions of object recognition typically used purely visual stimuli, and encouraged animals to make a judgement of recognition by providing a food reward upon choice of a novel object (Aggleton et al., 1986; Mumby, Pinel,

& Wood, 1990). More commonly used today are spontaneous exploration recognition tasks, which exploit the innate preference to novelty of domesticated rats and mice, that drives spontaneous exploratory behaviour toward a novel stimulus (Ennaceur & Delacour, 1988). This task switch has been largely motivated by the wish to avoid the reinforcing nature of rewarded tasks as spontaneous object exploration does not rely on reward or punishment, thus avoids goal-directed behaviour which is highly moderated by reward processing systems. Furthermore, no negative reinforcement is required, making the task less stressful, and therefore reducing the possibility of atrophy to regions supporting memory formation (Maras et al., 2014). Finally, it better models incidental encoding typical for episodic memory in humans, which is automatic and not driven by reinforcement contingencies.

Generally speaking, spontaneous object paradigms are based on three distinct phases: habituation, sampling and probe/test. During initial habitation, animals are familiarised to the test environment or arena, often in the absence of objects. Habituation to the test arena ensures that at during sampling only the objects are novel stimuli, thus providing an important methodological stimulus-control aspect of the procedure. In the most basic configuration of the task, during the sampling phase at least two objects are presented in the familiarised context. After a delay period, animals are returned for a probe trail, in which one object remains unchanged, while the other is somehow modified, constituting the novelty. For example, either a new object is introduced (object recognition), the known object is moved to a novel spatial position (object location), or both (object in place). Total exploration time for both novel and familiar objects is measured, and the difference between the two is used to reveal novelty preference, indicative of memory for the familiar object.

Performance in this basic paradigm depends on a variety of parameters. Firstly, the task arena is typically adjusted between tests of object recognition and location. For instance, object recognition tasks are typically run in an open-field or Y-maze that lacks visual spatial cues and uses consistent odour and textual stimuli across the experimental procedure to limit contextual information (Cohen & Stackman Jr., 2015). In comparison, novel object location paradigms are generally run in an open field with distinct spatial cues to encourage learning of spatial relations between each object and the environmental cues within and surrounding the arena. Secondly, the number and duration of sampling phases can be altered to encourage short-term or long-term object representations, and, likewise, the persistence of such memory can be explored with different delay periods. Thirdly, given the absence of external motivation, great care must be taken when designing these tasks to encourage animals to express spontaneous exploration behaviour. For instance, the objects themselves have been argued to have certain

affordances that determine the amount of natural exploratory behaviour a rat can express toward it (i.e., nose-poking, climbing on top of) (Chemero & Heyser, 2005). As such, the size, colour and texture of objects may modulate judgements of object identity and location.

2.3. Neural correlates of object associated memory

The development of behavioural paradigms in rodents has allowed the investigation into the neurobiological structures support recognition memory. In particular, the question of how the brain processes object identity and location have been the subject of substantial research. From these, the medial temporal lobe has emerged as a critical region for recognition memory which supports distinct modular processing of various aspects.

2.3.1. Novel object recognition

2.3.1.1. Perirhinal Cortex

A consistent impairment in performance for object recognition is observed in rats with lesions to the perirhinal cortex. Damage to this region leads to both amnesia for established object memory and impairs the ability to acquire new long-term object memory under reinforced and spontaneous exploration tasks (Ennaceur, Neave, & Aggleton, 1996; Mumby & Pinel, 1994; Winters, Saksida, & Bussey, 2008). Under short delays (i.e., up to 10 minutes after encoding), many reports suggest lesioned animals can still express object memory, but not thereafter (Norman & Eacott, 2004). These findings are consistent across rodents, primates and humans (Eichenbaum, Yonelinas, & Ranganath, 2007) suggesting the ability to recognise objects is a highly conserved function of the perirhinal cortex.

Later studies which used temporal inactivation methods further supported this claim. For instance, targeted pharmalogical infusions of CNQX, which blocks AMPAR mediated excitatory transmission, to the perirhinal cortex before or after sampling, or before probe, all impair object recognition memory in rats (Winters & Bussey, 2005). These findings suggest that the perirhinal cortex is critical during encoding, consolidation and recall of object memory. A similar pattern of results is observed when AP5, the NMDAR antagonist, is selectively infused into the perirhinal cortex, thus highlighting a mechanistic means of plasticity and learning within this region to encode novel objects (Winters & Bussey, 2005). In agreement with this notion, blocking PKMζ in the perirhinal cortex either 1 or 6 days after learning impairs long-term retention of object memory (Augereau, Migues, & Hardt, 2022), suggesting that the perirhinal cortex acquires and stores representations relating to new objects.

In intact animals, the perirhinal cortex shows elevated neuronal activity in response to the presentation of a novel object. The immediate early gene (IEG) *c-fos* is upregulated following neuronal activity, such that its protein product, c-Fos, is abundant in neurons up to an hour afterwards, providing an indirect maker of neuronal activity (Bisler et al., 2002). Numerous immunohistochemical studies of the perirhinal cortex have reported increased c-Fos expression following novel object exposure (Aggleton & Brown, 2005; Aggleton, Brown, & Albasser, 2012). If c-Fos expression is blocked around the time of object encoding, later object recognition is impaired (i.e., at 24 h), but not shortly after encoding (i.e., 20 min) (Seoane, Tinsley, & Brown, 2012). Taken together, these results suggest a critical role of neuronal activity in the perirhinal cortex in forming long-term object representations.

There is debate as to what the neuronal activity in the perirhinal cortex reflects in object recognition tasks. One position is that this region is critical for *discrimination* of novel from familiar objects, rather than encoding of objects per se. This view arose from early findings that global activity in the perirhinal cortex changed in response to repeated object exposure. For instance, early electrophysiological recordings found that neurons which initially responded to a novel object, tended to decrease their firing upon re-exposure of the same, now familiar object (Zhu, Brown, McCabe, & Aggleton, 1995). In agreement, some studies reported that total levels of c-Fos expression are lower after the presentation of a familiar object compared to a novel in caudal regions of perirhinal cortex (Albasser, Poirier, & Aggleton, 2010). Yet there are also reports of no change in c-Fos expression after initial novel object exposure, and second object presentation (Ameen-Ali et al., 2021), suggesting that measuring the global activity of the perirhinal cortex may not readily indicate the overall novelty (i.e., more activity, more novel) of objects. Indeed, a number of studies have identified perirhinal cells which increase their firing with subsequent object presentation (i.e., as it becomes more familiar), thus suggesting neuronal activity may reflect both relative novelty and familiarity of an object (Ahn, Lee, & Lee, 2019; Xiang & Brown, 1998). As such, a more nuanced version of this position is that novelty and familiarity sensitive cells within the perirhinal cortex support object discrimination (Ameen-Ali et al., 2021; Kinnavane, Amin, Horne, & Aggleton, 2014).

A critical consideration to the notion that the perirhinal cortex encodes novelty and familiarity is the observation that perirhinal cortex lesions do not impair an animal's ability to detect novel stimuli (Olarte-Sánchez, Amin, Warburton, & Aggleton, 2015). This is most commonly inferred by the exploration times during initial encoding, or sampling phases, where animals are presented with two novel stimuli. As novelty drives rodent exploration, an animal that is unable to detect novel stimuli should spend less time exploring novel objects in

comparison to intact animals. However, numerous reports have consistently found no such difference in sampling exploration between intact and lesioned animals (Kinnavane, Amin, Olarte-Sánchez, & Aggleton, 2016). As such, many have emphasised that distinguishing and detecting novelty are different, and the ability to judge which object is new versus old is a markedly different operation.

An alternative position, the perceptual-mnemonic hypothesis, holds that the perirhinal cortex encodes conjunctive representations that reduce overlap of similar items with shared features (Bussey, Saksida, & Murray, 2005). In other words, simple features of an object – such as edges or line orientation –encoded in the visual cortex are largely similar and overlapping between objects, yet are discriminated by the unique and conjunction of those elements which relies on the perirhinal cortex. In support of this notion, this account draws on a study in which rats with lesions to the perirhinal cortex showed increased susceptibility to interference from visual interpolated activity introduced before or after (i.e., proactive, retroactive interference respectively) object encoding (Bartko, Cowell, Winters, Bussey, & Saksida, 2010). Furthermore, it was observed that forgetting was heightened in lesioned animals when interpolated stimulation was more similar (i.e., another object) to the objects shown during sampling, an outcome that is readily predicted and explained by this model. An extension of the perceptual-mnemonic hypothesis is that the delay-dependent impairments seen in object recognition reflect interference-driven forgetting by visual stimulation that normally arises under natural conditions. In confirmation of this, a follow up study found that reducing sensory interference between sampling and recall lengthens the natural life span of object memories in lesioned animals (McTighe et al., 2010).

Taken together, while the exact role of the perirhinal cortex in object recognition is undetermined, there is broad agreement on its critical role for encoding and maintaining representations supporting memory for new objects.

2.3.1.2. The hippocampus

As mentioned in Chapter 1, selective hippocampal lesions produced mixed findings for object recognition in rodents, with some studies reporting impaired (Clark, West, Zola, & Squire, 2001; Clark, Zola, & Squire, 2000) while others spared performance (Bussey, Duck, Muir, & Aggleton, 2000; Forwood, Winters, & Bussey, 2005; Mumby, Gaskin, Glenn, Schramek, & Lehmann, 2002). One source of this discrepancy may reflect the limitations of lesioning studies. For instance, often reported is lesion-induced compensation in animals, suggesting

lesion studies may well reveal sites which host memory representations, but not the function of those areas (Cohen & Stackman Jr., 2015). Instead, using temporal inactivation strategies are arguably more appropriate to understand how a brain region contributes to certain phases of memory processing.

Under this approach, a more coherent pattern of results has been observed in object recognition paradigms. Namely, transient inactivation of the hippocampus around the time of object encoding impairs long-term object recognition memory. For instance, infusion of the GABAergic agonist Muscimol, which effectively shuts down neuronal activity, into the dorsal hippocampus of rats directly before or after sampling impairs object memory tested hours later (Cohen et al., 2013). This finding has been replicated with hippocampal infusions of the protein-synthesis inhibitor anisomycin and selective chemogenetic inactivation of the hippocampal CA1 region immediately after sampling, but not hours later (Rossato et al., 2007; Tuscher, Taxier, Fortress, & Frick, 2018). When hippocampal functioning is impaired during sampling, performance in probe trials administered after a short delay (i.e., 10 minutes later) is unaffected, but not when given after longer delays, similar to the findings in animals with impaired perirhinal cortex functioning (Ásgeirsdóttir, Cohen, & Stackman, 2020; Cohen et al., 2013; Hammond, Tull, & Stackman, 2004). Interesting, one study reported that weak or short exposure to objects during sampling was not impaired after hippocampal inactivation, but under longer training protocols, caused impairments in object recognition memory (Cinalli Jr., Cohen, Guthrie, & Stackman Jr., 2020). Altogether, these findings suggest that in intact animals the hippocampus, under certain conditions, supports initial memory formation.

Furthermore, there is increasing evidence that the hippocampus plays a critical role in object memory reconsolidation. For instance, it has been reported that after training rats with two different objects (i.e., A-B), a brief exposure to a new object pairing (i.e., A-C), rendered memories susceptible to amnesic agents targeting the hippocampus. Specifically, infusion of anisomycin into the dorsal hippocampus after the reactivation with the novel combination (A-C) impaired memory for the original one (A-B), but not after a reactivation session with the familiar objects (A-B) (Rossato et al., 2007), suggesting the presence of novelty critically drives hippocampus dependent object memory updating. Along a similar vein, a separate study found that re-exposing rats to familiar objects but in a novel context also renders memory for objects susceptible to impairment by intra-hippocampal infusion of anisomycin (Winters, Tucci, Jacklin, Reid, & Newsome, 2011). Altogether, these findings suggest that the hippocampus is critical for maintaining object recognition memory in phases of synaptic plasticity.

To explain the described findings, a number of theories on how the hippocampus contributes to object recognition performance have been proposed. Given the established role of the hippocampus in spatial behaviour and cognition discussed in section 1.2.4., many have proposed that this region provides some contextual information for object recognition tasks, that are required for event memory. For instance, such contextual information is thought to support recollection-like retrieval of objects during more complex discrimination tasks (Eacott et al., 2005). Alternatively, under the account focussing on object-feature conjunction, the hippocampus is proposed to provide the top hierarchical position of feature disambiguity, as it allows to discriminate item conjunctions in the perirhinal cortex across different contexts (Cowell, Bussey, & Saksida, 2010).

While the function of the hippocampus in object memories remains unclear, there is a clear consensus for its critical role during the encoding and stabilization periods of new object memory, as well as during the restabilization of reactivated long-term object memory.

2.3.2. Novel Object Location

Considering the spatial nature of object location recognition tasks, it is perhaps not surprising that this task is dependent on the hippocampus. While animals with lesions to the perirhinal cortex show sparing of location memory, damage to the hippocampus consistently disrupts performance in object location memory (Augereau et al., 2022; Mumby et al., 2002; Save, Buhot, Foreman, & Thinus-Blanc, 1992). In agreement, temporal inactivation of the hippocampus by pharmacological, optogenetic and chemogenetic intervention around the time of encoding, consolidation and recall impairs expression of memory for the location of objects (Tuscher et al., 2018). Furthermore, blocking plasticity within the hippocampus during spatial learning also impairs object location memory, suggesting that some acquisition of spatial information related to the task is encoded in the hippocampus (Yamada, Arai, Suenaga, & Ichitani, 2017).

Neuronal activity, as indicated by c-Fos expression, is selectively elevated in hippocampal regions after completing the novel object paradigm (Mendez, Arias, Uceda, & Arias, 2015). Electrical recordings in the CA1 region while animals undergo object recognition and object location tasks revealed distinct changes in place cell field firing (i.e., remapping only under conditions of spatial, and not object identity, change (Lenck-Santini, Rivard, Muller, & Poucet, 2005). Of particular relevance to the experiments reported here, after object location encoding, infusions of pharmalogical inhibitors that target known active decay

processes into the dorsal hippocampus of animals extends memory to persist beyond its natural lifespan (Migues, Wong, Lyu, & Hardt, 2019; Migues et al., 2016). Taken together, these findings provide strong support for a critical role of the hippocampus in encoding and maintaining representations for object locations.

2.3.3. c-Fos expression

The proto-oncogene *c-fos* regulates cell growth, development and death. It is part of the Fos family of proteins that form dimeric compounds with Jun proteins to create the AP-1 transcription factor complex (Morgan Curran 1991). In neurons, the protein product of *c-fos* i.e., c-Fos, is constitutively expressed at low levels under basal conditions, which is drastically and transiently elevated following neuronal activity. After depolarisation, large calcium influxes through NDMARs activate MAPK, which phosphorylates the transcription factors CREB and Elk-1. These translocate to the nucleus to promote transcription of the *c-fos* gene at its promoter. Between 5 to 30 minutes after stimulation, *c-fos* mRNA is detected in neurons, and about 1-2 hours later the c-Fos protein (Greenberg & Ziff, 1984). Thus, this immediate early gene typically has a high signal to low background expression profile across active and inactive neurons, respectively. Immunohistochemical studies have targeted both the mRNA and protein products of *c-fos* following neuronal activity. More often, the c-Fos protein is targeted, as the window to collect a signal is longer, and, given the later emergence of c-Fos, its expression is unlikely to be influenced by experience related to the animal's euthanasia (Jaworski, Kalita, & Knapska, 2018).

Despite its common use as a marker of neuronal activity, very little is known about the function of c-Fos beyond its AP-1 related transcriptional role. Many have proposed a critical contribution to synaptic plasticity, as it is typically expressed in protocols that induce late-, but not early-LTP. Correspondingly, activating NMDARs induces c-Fos mRNA and protein production (Liste, Rozas, Guerra, & Labandeira-Garcia, 1995) and blocking NMDARs, in particular GluN2B-NMDARs (Inta, Trusel, Riva, Sprengel, & Gass, 2009), leads to reduced c-Fos expression. That said, increased c-Fos levels have also been reported following chemical LTD induction (Lindecke et al., 2006), and when c-Fos levels are reduced, LTD can still be induced, but not maintained (Kemp, Tischmeyer, & Manahan-Vaughan, 2013). Furthermore, recent work compared *c-fos* expression after contextual fear conditioning (i.e., a memory forming event) to a random shock (which does not lead to plasticity related to memory formation), and found equal levels of *c-fos* mRNA in the amygdala across both conditions

(Simbriger et al., 2021). Thus c-Fos seems not to be a marker of learning per se, but rather, signals general neuronal activity (Leeyup, 2015).

2.4. Rationale for investigations

Active decay theory predicts that declarative memory is susceptible to forgetting by interference during stabilization phases, and once established, endogenous active decay during sleep periods. While this account proposes that the principal mode of forgetting in the hippocampus is weakening synaptic connections during active decay, it suggests that interference predominantly drives forgetting in neocortical areas, which can be moderated by hippocampal activity during trace stabilisation. Since active decay theory sets out to explain forgetting of 'everyday' episodic memories, object-based paradigms as discussed above, provide an ideal experimental model for testing its predictions in rats.

Firstly, active decay theory predicts that the role of the hippocampus during initial declarative memory formation is to protect extra-hippocampal traces from interference arising from ongoing sensory experience. In order to study this, we therefore need a memory that (1) is episodic-like in nature (2) relies on the hippocampus for initial formation but engages extra-hippocampal areas to process content representations. As discussed above, while long-term storage of object memory appears to critically rely on the perirhinal cortex, the hippocampus is still required during stabilisation phases (i.e., consolidation and reconsolidation). Therefore, the object recognition task provides a good paradigm to explore these predictions of active decay.

Secondly, established long-term memory traces that depend on the hippocampus throughout their lifetime are predicted to be gradually forgotten because a time-dependent process actively removes the synaptic structures supporting memory. This process of active decay has been previously shown to promote the forgetting of hippocampal traces supporting memory for object locations. Therefore, to investigate the molecular mechanisms which underpin active decay of established long-term memory, we will use the object location recognition paradigm.

2.5. Common behavioural methods

Spontaneous exploratory behaviour in rodents can be moderated by several factors related to the internal states of animals, therefore care must be taken to control these sources of variance (Antunes & Biala, 2012). While removing food rewards from these tasks controls for

reinforcement-driven learning and better models incidental learning, it may also reduce the motivation of animals to engage in the task. Therefore, to promote exploratory activity, animals can be food deprived, thus promoting food seeking behaviour (A. Ennaceur, 2010). As such, we kept rats on a maintenance feeding schedule, such that they did not have *ab lithium* access to food, but instead received daily portions of food that sustained their free-feeding adult weight while allowing for small continuous gains in body weight. Motivation to explore objects fluctuates across the light-dark cycle in rodents. Although in rats the period of highest overall activity is during the dark periods of the day, it is common practise to perform experiments during the light phase. Since rats are crepuscular, to capitalize on their period of peak activity, we ran animals early in the light phase, and kept the light intensity in the experimental rooms at a level typical for dawn and dusk, around 15 to 20 lux.

Stress strongly moderates exploratory behaviour. For instance, regimes of chronic stress cause animals to spend less time exploring novel objects (Nelissen, Prickaerts, & Blokland, 2018). Pre-experimental handling can greatly limit stress and improve animal exploration performance (Roy & Chapillon, 2004). Thus, prior to all experiments, animals participated in multiple handling sessions (described in detail in below). Furthermore, our low-light conditions limited the potential stress reaction rodents have to high light intensity (Ishida et al., 2005).

2.5.1. Animal preparations for behavioural experiments.

Animals. We acquired Long-Evans rats weighing between 275 and 300 g (Envigo). Rats lived in groups of up 4 in two-tiered polyethene cages containing sawdust bedding and environmental enrichment (PCV tube). Food (Teklad 2920X, Envigo) was restricted to maintain 85% of free-feeding adult body weight while water was provided *ab libitum*. Animals were kept on a 12 h light cycle (lights were turned on at 07:00 and turned off at 19:00). Experimental procedures were conducted in the light phase between 08:00 and 14:00. All procedures were performed according to the guidelines of the Canadian Council on Animal Care and approved by the McGill University Animal Care Committee.

Surgeries. Rats underwent surgery at an average body weight of approximately 325 g. We induced anaesthesia with isoflurane (4% in O2) in a transparent PVC tube where rats remained for approximately 12 min. We then placed the animals into a stereotaxic frame (Kopf Instruments). We maintained isoflurane levels between 2-3% in O2 during surgery. To target the dorsal hippocampus, we implanted 22-gauge stainless steel cannulas (Plastics

One, Roanoke, VA) pointing 10 degrees away from the midsagittal plane at AP (anterior/posterior) +/- 3.60 mm, ML (medial/lateral) 3.10 mm, DV (dorsal/ventral) 2.40 mm (Paxinos & Watson 2004). We stabilized the cannulas with jeweller screws implanted in the skull and dental cement. We inserted obturators into the guide cannulas in order to seal the opening and prevent blockage. Surgeries lasted between 60 and 75 min. For post-surgery recovery, rats lived alone for 3 days with *ab libitum* access to food and water. Thereafter, they returned to group housing and the previously described maintenance feeding.

Handling during recovery period. Within the animal colony, we placed rats into a large plastic box (W 40 cm, L 80 cm, H 40 cm) which contained about 5 cm of sawdust bedding covering the floor. The box contained five differently sized PVC tubes and four wooden gnawing cubes. Groups of eight animals explored the box together, staying in there for up to 45 min per session, with a minimum of three sessions across five days. During this time, an experimenter would pick up each rat several times in random sequences, placing animals on the lap for a brief period no longer than 15 s), and then put the animal on the arm while walking around with it in the colony room for up to 3 min. In later sessions, rats would have obturators removed, cleaned and replaced. This procedure familiarized the animals to the form of handling they will experience during the experiments.

2.6. Apparatus

2.6.1. Object recognition

Open field arena. We conducted the behavioural studies in an open field arena (W: 60 cm x L: 60 cm x H: 60 cm) made of black laminated wooden panels. To control for spatial cues, the four walls did not have visible marking that could help orientate the animals. The arena floor was covered with about 5 cm of the same sawdust bedding also used in the home cages. The arena was mounted 65 cm above the ground, a camera that recorded the activity of the animals was located 135 cm above the floor of the arena and was termed the 'Escher' arena.

2.6.2. Object location

Open field arena. We used the same apparatus as in our object recognition studies (described above) and, in addition, another open field arena in a different room with the same dimensions (W: 60 cm x L: 60 cm x H: 60 cm), and made of white laminated wooden boards that had the same visual cues attached to the North and South as the other open field. Visual cues were made of laminated paper (21.5 X 28 cm) with black and white rings or stripes (please see

examples below). The additional arena was mounted 10 cm above the ground and a camera was fixed 190 cm above.

2.7. Behavioural Procedures

In line with standard spontaneous exploratory testing, our object recognition paradigm consisted of the phases of habituation, sampling and probe. For habituation, sampling and probe trials, rats were transported from their colony room to the holding room 60 min prior to experimentation. Animals were transferred using holding cages (L 45 cm, W 25 cm, H 20 cm), two rats per cage (from the same home cage). The cages consisted of a clear polyethylene base, metal lid, sawdust bedding, and a water bottle. All protocols commenced with 4 consecutive, daily habituations trials.

Habituation. Eight days after surgery, animals underwent four habituation trials, 1 session per day for four consecutive days. For each habituation trial, we placed a single rat in the empty (i.e., no objects) open field and left the animal to freely explore it for 10 min. We always placed rats into the open field facing a different corner from trial to trial (i.e., NE, NW, SE, SW). The order of starting positions was randomised between and across animals to prevent any bias in positions later occupied by objects. After each trial, the bedding was cleaned of faeces and swirled around to dispel possible scent traces left behind.

2.7.1. Object recognition

Sampling. Sampling trials were administered to animals the day following the last habituation trial. During sampling, animals explored two novel objects placed into the arena. Typically in rats, around 30 seconds of total object exploration is sufficient for animals to acquire long-term object memory (S. J. Cohen & Stackman Jr., 2015). Yet, this method of limiting exploration to the total accumulated time spent exploring is methodologically challenging and difficult to keep consistent across trials, presenting a possible source of variance. As such, we kept the duration of each sampling trial constant, but excluded animals which failed to reach 30 seconds of exploration during the sampling trial. Two identical copies of the same object (A, A') were placed at opposing corners of the open field (i.e., NE-SW or NW-SE) and the rat began the trial in one of the available corners.

Two sets of objects were used across an experiment, such that during sampling half of the animals were exposed to one of the two pairs (i.e., A, A' or B, B'), such that the probe trial was identical for all (i.e., A, B), controlling for any possible unspecific effect of an object that

might make it more attractive to the animals. Objects were selected based on prior pilot studies of preference, such that neither one was inherently more interesting to animals, provoking stronger exploratory activity. Additionally, object assignments and arrangements were counterbalanced between animals. All objects were made of ceramic materials and ranged in height and shape (Fig. 2.1). Identical transparent mason jars were glued with clear silicone to the bottom of objects, and the jars were then screwed into lids anchored to the arena floor. The mason jars slightly protruded above the saw dust bedding layer and could be explored by rats. The objects all had a designated front and middle that generated two symmetrical halves for each. Using this centre point, the objects were placed on the open field such that the centre pointed directly to the middle of the arena floor, therefore when approaching each object, the same scene appeared from the perspective of the approaching animal, and the object itself did not serve to identify cardinal coordinates. At the start of each trial, animals were placed into one of the corners unoccupied by an object and orientated towards the corner. The trial was determined to have begun once the rat turned its head away from the corner. Between trials, the objects were cleaned with 70% ethanol to remove any odour traces from previous animals which may influence object exploration.

Probe. The probe trial was administered after a delay that could vary between experiments. Animals were returned to the arena contained again two objects – another copy of the object used in sampling, and a novel object that the rats had never encountered before. The objects were located at the same positions the objects had during sampling. We counterbalanced the location of the novel and familiar object across the treatment groups. We also counterbalanced the novel and familiar objects across Sampling and Probe. Each trial was 3 minutes long, after which we returned rats to their transport cages. Open field and objects were cleaned between rats as described during sampling.

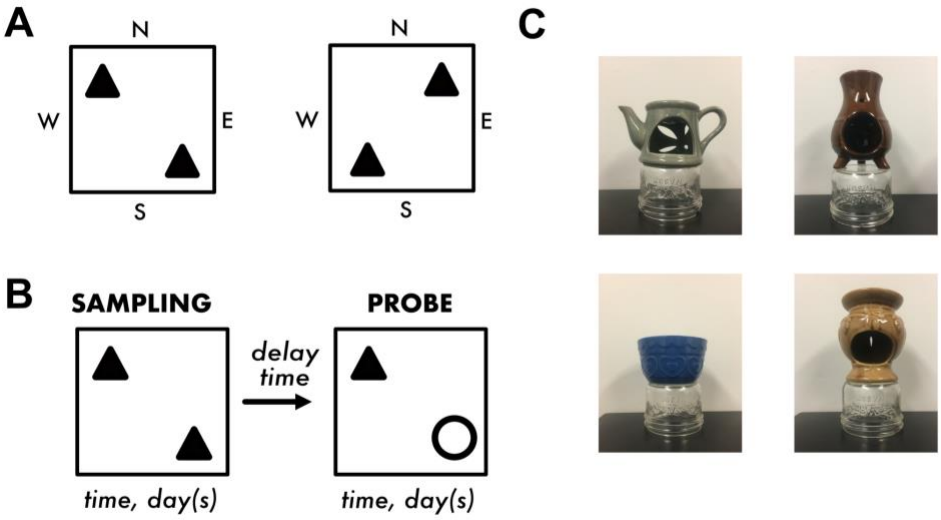


Figure 2.1. Novel object recognition details. ***A*** Example positions for object placement during experiments. ***B*** Schematic diagram for experiments which only includes details regarding sampling, probe and the retention period in between (i.e., habituation information not specified as these trials did not differ between experiments). ***C*** Sample images of objects used.

2.7.2. Object location

Sampling. For object location studies, animals began the sampling phase of these experiments 24 h after the last habituation trial. During sampling trials two sets of objects were used across an experiment, such that animals were exposed to one of the two pairs (i.e., either A, A' or B, B'). The same copy of each object was kept in the same location (i.e., A in NW; A' in SE) across sampling days, as there may be arbitrary marks differing subtly between the object copies that animals could use to identify individual copies of the same object (Ennaceur 2010). Objects were placed in opposing corners of the area and rats alternated starting the sampling trials in one of the two vacant corners across sessions. Animals were allowed to explore objects for a set time period.

Two sets of objects were used across an experiment, such that during sampling half of the animals were exposed to one of the two pairs (i.e., A, A' or B, B'), such that the probe trial was identical for all (i.e., A, B), controlling for any possible unspecific effect of an object that might make it more attractive to the animals. The same object specifications (i.e., object type, arrangement with the arena) were used as described in section 2.7.1. yet fewer variants of

objects were used (Fig. 2.2). As described above (section 2.7.1.), the trial was determined to have begun once the rat turned its head away from the corner. Between trials, the objects were cleaned with 70% ethanol to remove any odour traces from previous animals which may influence object exploration.

Probe. The probe trial was administered after a set delay period that was specified for each experiment (detailed in section 4.3). The open field contained the same two copies of the objects used during sampling. One object remained in its previous location, while the other was moved to a new place. We counterbalanced object placements during sampling and probe. Objects and open field were cleaned as described above

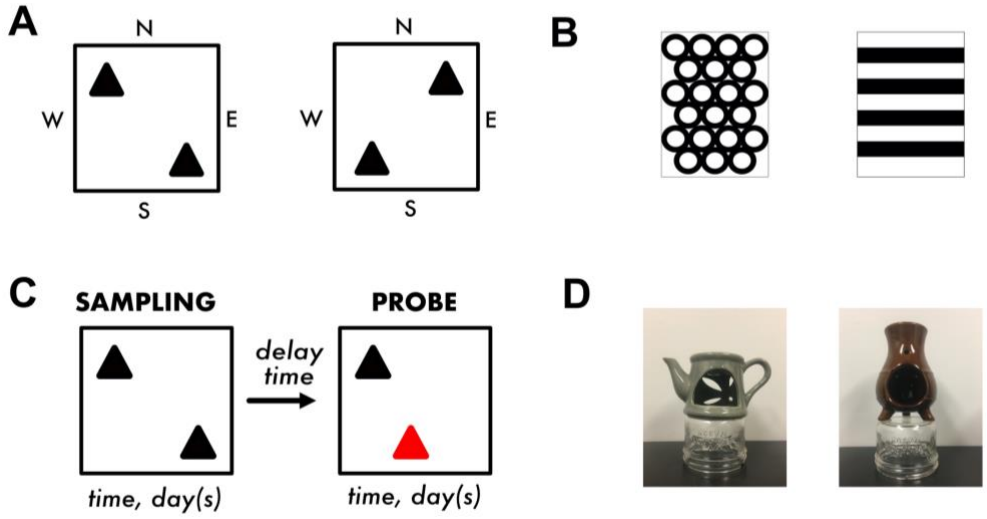


Figure 2.2. Novel object location details. ***A*** Example positions for object placement during sampling phases ***B*** Sample diagrams of visual cue cards attached to walls of the open field arena. ***C*** Schematic diagram for experiments which only includes details regarding sampling, probe and the retention period in between (i.e., habituation information not specified as these trials did not differ between experiments). ***D*** Sample images of objects used.

2.8. Measures and Analysis

Exploration behaviour was manually scored by an experimenter blind to rat identity from the recorded videos. Expression of exploration was defined as the snout of a rat being within 2 cm of an object and orientated at an angle of 45 degrees toward it, a widely used standard in the field (Ennaceur & Delacour, 1988; Mumby et al., 2002). Climbing and resting on an object was not considered exploratory behaviour. Exploration for both sampling and probe trials was

measured, as analysis of behaviour during sampling allows determining an animal's baseline expression of exploratory behaviour.

Novelty preference is typically quantified by either the recognition index (*r*) or the discrimination index (*d*). The recognition index is calculated by dividing the time spent exploring the new object (t_{new}) by the total exploration time ($r = t_{new} / [t_{new} + t_{old}]$), thus generating scores between 0 and 1, where 0.5 is defined as chance level (i.e., both objects are explored the same). Thus, scores significantly above 0.5 suggest novelty preference, those significantly below 0.5 indicate familiarity preference, while those around 0.5 infer no preference. In comparison, *d* is calculated by dividing the difference in exploration time between the new object (t_{new}) and old object (t_{old}), by the total exploration time (see equation 2.1 below). Values can therefore fall between -1.0 and +1.0, where scores significantly below 0 indicate a preference for the familiar object, scores significantly above 0 indicate a preference toward the novel object, and scores around 0, no preference between objects. As such, discrimination index *d*, is more sensitive to the magnitude and direction of novelty preference, and therefore was used in this report.

$$d = \frac{[\,t_{new} - \ t_{old}]}{[t_{new} + \ t_{old}]} \tag{2.1}$$

2.8.1. Object recognition

While some investigations calculate *d* from exploration time across the entire probe trials, we instead calculated *d* across a set period of accumulated object exploration in our object recognition studies. This is because novelty preference tends to wane off as the once novel object becomes more and more familiar during exploration (Ennaceur, 2010). A number of studies agree that in rats, 30 seconds of accumulated object exploration is needed to encode a novel object (Cohen & Stackman Jr., 2015). As the probe trial introduces a new object that an animal will explore and encode, a likely window revealing robust novelty preference is first 30 seconds of accumulated exploration across both objects. As such, we took exploration values for novel and familiar objects after 30 seconds of exploration in the probe trial ($d = [t_{new} - t_{old}]/[30]$), which generally occurred within the first 90-120 seconds of the probe trial. The specific times taken to reach 30s of exploration in the probe trials for each experiment are presented with the novelty preference.

2.8.2. Object location

To determine novelty preference in novel object location, we again used the discrimination index *d*, but calculated *d* across the first minute of the probe trial, rather than after a certain period of accumulated exploration. The rational for separate recognition criteria is largely due to the distinct patterns of exploration that animals express when acquiring memory for space and objects. The former is described as diversive exploration, and can include behaviors such as rearing and thigmotaxic movements to acquire information about the surrounds (Berlyne, 1966), while the latter is termed specific exploration and describes the behaviour an animal expresses toward new objects, such as climbing and sniffing. Given these two modes, it seems that exploration toward a new object is more biased towards interacting with an object, than changes in spatial configuration. In support of this, some evidence suggests that replacing objects in a known location increases exploratory behaviour to a greater extent than moving familiar objects around an environment (Chrzanowska, Modlinska, Goncikowska, & Pisula, 2022). As such, applying the same exploration criteria from our object recognition to object location likely means that we overshoot beyond the window of novelty detection. Therefore, we used a shorter time period to calculate *d* scores in our object location studies.

2.8.3. Statistical considerations

All data was analyzed using Jamovi (Version 2.0.0.0). For statistical tests, type I error level was set to α=0.05. All data-sets were tested for normality with the Shapiro-Wilk normality test, where if not normally distributed (i.e., $p<0.05$), then parametric tests were applied to the data. For the novelty preference data, one-sample *t*-tests were used to determine whether an individual group showed novelty preference by assessing *d* values against $d = 0$. Depending on the number of conditions within each experimental design, either an independent samples *t*-tests (groups of 2) or a One-Way ANOVA *F* test (groups of >2) was used to determine statistical differences between each condition. For exploratory behaviour during the sampling and probe trials, between group comparisons were determined and conducted as described for the novelty preference data. Repeated Measures ANOVAs were used to determine group differences in exploratory behaviour across multiple sampling sessions, either within sampling days, or an interaction between groups and sampling days as well as between groups. If a significant comparison was detected ($p<0.05$) a Tukey's post-hoc comparisons test was

performed for either between the sampling days, groups or the interaction of group and sampling day.

2.9. Histology

After completion of behavioural studies, we euthanized rats with CO_2 and then decapitated them. We removed the brains and fixed them in a solution of 4% paraformaldehyde and 30% sucrose–saline for at least 48 h. Using a cryostat, we collected 50 µm coronal sections across the hippocampus. We used a light microscope (Olympus IX81) to check cannula placements. An experimenter blinded to treatment group determined whether both injector tips were in the dorsal hippocampus, and if this was not the case, we excluded the animal from further analyses.

Chapter 3:

The hippocampus protects stabilizing memory traces from ongoing sensory interference

3.1. Preface

The hippocampus is critical for forming long-term declarative memories. Damage to this area leads to anterograde amnesia, i.e., the inability to form new memories for facts and events; yet, how and why this occurs remains unresolved (Scoville & Milner, 1957; Zola-Morgan, Squire, & Amaral, 1986). As discussed in Chapter 1, recent findings from amnesic patients suggest that memory formation can be restored when post-learning interference is reduced (Alber et al., 2014). By extension, this suggests that in the healthy brain, the hippocampus protects new stabilising traces that are vulnerable to interference from ongoing sensory stimulation. In alignment with this notion, active decay theory (Hardt, Nader, & Nadel, 2013) proposes that without a functioning hippocampus, unchecked neuronal activity will propagate and give rise to states of catastrophic interference in cortical areas, which manifest behaviourally as amnesia. Given the critical role of the hippocampus for both initial stabilisation (i.e., consolidation) and subsequent restabilisation of memory recalled in a malleable state (Rossato et al., 2007; Winters et al., 2011), the hippocampus likely serves a similar function during reconsolidation. Thus, in a broader sense, the hippocampus is predicted to diminish forgetting by retroactive interference on stabilizing (or restabilizing) memory.

Here, we investigated the role of the hippocampus on promoting consolidation and reconsolidation of object recognition memory in rats. As discussed in Chapter 2, prior work suggests that, although object traces themselves are supported by the perirhinal cortex, the initial acquisition of such memory involves also the hippocampus. Thus, object recognition provides a model system for declarative memory that allows us to investigate the contribution of the hippocampus to an extra-hippocampal memory trace.

Based on these observations and deductions, we tested the following hypotheses: (1) inactivating the hippocampus around the time of encoding impairs long-term object memory formation; (2) reducing sensory interference shortly after learning rescues amnesia for objects

in the face of hippocampal impairment; (3) the hippocampus acts to limit excessive neuronal activity in cortical areas under normal sensory experience; (4) hippocampal learning is required during object encoding to provide interference protection; and (5) the hippocampus assumes a similar role of protecting vulnerable memory against sensory interference during object reconsolidation.

3.2. Introduction

Damage to the medial temporal lobe and, particularly the hippocampus, typically results in anterograde amnesia, where patients forget new information within minutes (Scoville & Milner, 1957; Zola-Morgan et al., 1986). In other animals, such as non-human primates and rodents, lesions to the hippocampus similarly disrupt long-term memory formation for events, mirroring the human amnesic syndrome (Clark & Squire, 2010; Stuart Zola-Morgan & Squire, 1985). Yet, after decades of research, it remains unclear why and how hippocampal damage leads to anterograde amnesia.

There is general agreement that anterograde amnesia likely arises from disrupted memory consolidation required for forming long-term memories (McGaugh, 2000). Under healthy conditions, consolidation processes are thought to gradually stabilise new declarative memories over time. Considering that damage to the hippocampus leads to anterograde amnesia, the consolidation of declarative memories seems to involve this region of the brain.

While new memories are vulnerable to various forms of interference, consolidated memories are not (Dudai, 2004; Wixted, 2004). The primary source of interference in real-world settings is ongoing sensory stimulation that naturally follows learning. Indeed, reducing sensory interference immediately after learning promotes long-term memory formation in both humans and rodents, such that newly acquired declarative memory persists for longer than under everyday conditions (Arkell et al., 2021; Dewar et al., 2007). Importantly, even memory in patients with anterograde amnesia following damage to the medial-temporal lobe can benefit from a short period of reduced sensory interference after learning, such that they can recall new information for extended delays, when, under normal circumstances they would normally have forgotten them (Alber et al., 2014; Cowan et al., 2004; Dewar et al., 2009). These findings suggest that damage to the hippocampus challenges the ability to protect new, not yet consolidated memories from potentially interfering ongoing sensory experience that typically follows learning in natural settings.

Continuous encoding in neuronal networks leads to catastrophic interference where new experience overwrites previously acquired knowledge (French, 1999). One method to reduce this effect is to reduce the learning rate for synaptic weights critical to a representation, thus preserving their connectivity, while leaving those deemed non-essential unpreserved (Kirkpatrick et al., 2017). In the brain, it is unknown whether a similar mechanism unfolds, in that neuronal activity related to non-salient or unimportant experience is quickly diminished, thus avoiding the potential disruption of excessive neuronal activity on memory formation. Because long-term memory formation is preserved in amnesic patients with compromised hippocampal functioning when sensory interference is reduced, it seems possible that anterograde amnesia may arise from states analogous to catastrophic interference in artificial neural networks. Therefore, under healthy conditions, the hippocampus may prevent excessive interference by limiting neuronal activity related to sensory experience in brain areas critically supporting memory content.

Numerous theoretical positions (Hardt et al., 2013; McClelland, McNaughton, & O'Reilly, 1995; Teyler & DiScenna, 1986) hold that during the acquisition of new declarative memories, the hippocampus generates indices linking to dispersed neocortical memory components that together form episodic memory. Aside from binding together these distributed neuronal ensembles that, due to limited direct neocortical connections between them could not otherwise be rapidly coherently associated, the hippocampal indices can also protect not-yet consolidated memory representations in discrete neocortical areas from disruption from ongoing sensory experiences. These indices are proposed to identify individual traces in cortical areas despite overlap which would otherwise lead to interference among shared populations. The creation of such an index is predicted to rely on the rapid strengthening of hippocampal synapses, thus pointing toward a critical role of hippocampal plasticity in attenuating interference during initial episodic memory formation.

Furthermore, upon reactivation during recall, consolidated event memory can enter a state of lability, such that it once again becomes susceptible to intrusions by interference (Hupbach et al., 2007), and must undergo a process of restabilisation to persist in long-term memory (Hardt, Einarsson, & Nader, 2009; Nader & Hardt, 2009). A number of studies suggest that the hippocampus becomes reengaged during this period of instability (Rossato et al., 2007; Winters et al., 2011), and that it critically promotes the reconsolidation of destabilized, extra-hippocampal traces. How the hippocampus promotes restabilisation is unclear. Given its potential function in reducing interference during initial consolidation, the hippocampus may

assume a similar role during reconsolidation, protecting restabilising traces from ongoing sensory experience.

Here, we tested these predictions in rats. We used the novel object recognition task because it is an accepted model of human declarative memory. As it the case for humans, damage to the hippocampus of rats impairs the encoding and consolidation of object recognition memory, although the hippocampus is not the site hosting representations underpinning object recognition memory, which instead depends on the perirhinal cortex (Aggleton & Brown, 2005; Augereau et al., 2022; Broadbent et al., 2010; Cohen et al., 2013; Hammond et al., 2004; Outram, Brown, Warburton, & Barker, 2022). To model anterograde amnesia, we inactivated the dorsal hippocampus in rats with GABA-A and B agonists and assessed memory retention with an object recognition task. We found that inactivating the hippocampus both prior to and immediately after object learning impaired novel object recognition tested 24 h after learning. Reducing sensory stimulation immediately, but not 1 h after learning rescued this memory deficit. Reintroducing visual sensory stimulation during the 1 h post-learning period reinstated amnesia for objects in rats with inactivate hippocampi but not in intact animals. Inactivating the hippocampus 1 h after learning under normal sensory conditions did not induce amnesia for objects, suggesting that object recognition memory sufficiently stabilizes within 1 h to persist long-term. In the perirhinal cortex, c-Fos expression was elevated in animals with inactivated hippocampi after object learning and 1 h of normal sensory conditions in comparison to intact animals. Reducing sensory stimulation for 1 h after object learning reduced c-Fos activity, such that animals with inactivated hippocampi had expression levels comparable to those with active hippocampi under normal sensory stimulation. Next, we blocked memory formation in the hippocampus by infusing the NMDA receptor antagonist AP5 prior and after object exposure. Here, preventing hippocampal memory formation before, but not after object learning led to amnesia for objects 24h later, which was absent when we reduced sensory stimulation after learning. Finally, we developed an object reconsolidation paradigm, where 24 h after object encoding animals undergo a reactivation session that destabilizes object memories. Blocking hippocampal reconsolidation by infusing the protein synthesis inhibitor anisomycin after reactivation induced amnesia for animals under normal sensory conditions, but not when sensory stimulation was reduced for 1 h thereafter. Taken together, our findings suggest that the hippocampus protects stabilizing representations in other brain regions by preventing excessive neuronal activity that arises from ongoing sensory experience after learning, thereby promoting their long-term retention in memory.

3.3. Methods

3.3.1. General procedures described in Chapter 2

Animals. As described in section 2.5.1.

Surgeries. As described in section 2.5.1.

Handling during recovery period. As described in section 2.5.1.

Open field arena. As described in section 2.6.1.

Object recognition Habituation. As described in section 2.7.

Object recognition Sampling. As described in section 2.7.1.

Object recognition Probe. As described in section 2.7.1.

Data analysis. As described in section 2.8.1.

Histology. As described in section 2.9.

3.3.2. Specific procedures

Behavioural studies

Drugs and infusions. We based the concentrations and volumes (1 µL per hemisphere) on previous studies (Hardt, Wang, & Nader, 2009; Migues, Wong, Lyu, & Hardt, 2019; Riaz, Schumacher, Sivagurunathan, Van Der Meer, & Ito, 2017). In brief, we dissolved 75 mg muscimol and 75 mg baclofen (B5399, M1523 Sigma-Aldrich, Oakville, Ontario) in tris-phosphate buffer (TBS), calibrated to a pH of 7.1; AP5 (A5282 Sigma-Aldrich, Oakville, Ontario) was diluted at a ratio of 2.5 mg per 1 ml of phosphate buffered saline (PBS) at a pH of 7.2; Anisomycin was dissolved in PBS to a concentration of 125 µg per µl. During behavioral studies, we infused the drugs at a rate of 0.25 µl/min into the dorsal hippocampus with a 28-gauge microinjector connected to a Hamilton (Model 1701N) syringe with polyethylene tubing (Braintree Scientific, Inc.). Microinjectors (Plastics One) protruded 0.5 mm from the cannulas and were sterilized with 70% ethanol before insertion. Injectors remained connected for an additional 120 s after the infusion stopped. The infusions were done in a room separate from either holding or experiment rooms, and occurred either before or after sampling trials.

Black Box. We used eight black plastic boxes (L:18 cm, W:14 cm, H:32 cm) to reduce sensory stimulation. The boxes had a lid that allowed for air flow, and that contained an indirect dimmable LED strip lamp with a diffuser to provide soft, indirect illumination. For some

experiments, we turned on the lights so that the light intensity at the bottom of the box was at 15-18 lux. The floor of each box was covered with about 1 cm layer of sawdust bedding. Animals were assigned to a specific Black Box which remained consistent throughout a given experiment. To prevent animals from falling asleep in the Black Box, every 15-20 minutes an experimenter checked on animals and provided gentle handling (Vecsey et al., 2013).

General procedures for object recognition. Behavioural procedures would begin once rats had returned to their home cage after the 3 days of individual housing following surgery. This comprised three handling sessions across 5 days, 4 days of habituation, 1 day of sampling and then 1 day of probe trial 24 h after sampling. On the third habituation trial animals were given mock infusions either before or after being placed in the arena, depending on the specific protocol. We removed animal's obturators and placed in the microinjectors connected to empty tubing, as described above, and turned on the infusion pumps to familiarise animals to the infusion procedure. When infusions were administered prior to sampling or probe trials, we noted the start time of drug administration and then placed animals into the open field arena for sampling 20m after the start of the infusion, around 10-15m after the end of the infusion. Between infusion and sampling or probe we returned animals to their transport cages. For infusions after sampling, we took animals straight from the open field to the infusion room. Depending on the protocol, we returned animals to their transport cages or the Black Box after infusion.

Specific procedures for Object recognition reconsolidation. Animals that had previously been exposed to the open field arena were used for these studies. To limit the influence of this prior experience, animals were run in the same open field arena as described in section 2.6.1., except that the North and South wall were replaced with white laminated wood panels while the East and West remained black. In line with our object recognition protocol described above, animals first underwent 4 consecutive days of habituations trials, lasting 10 minutes each. The next day, animals participated in a sampling trial as described above, expect that the sampling trial lasted for 10 minutes, which produced statistically significant novelty preference after 48 h (Appendix: Fig. A.3.). The next day, animals participated in a reactivation session (described below), and then a probe trial 24 h later (i.e., 48 h after sampling). The probe trial was conducted as in all other object recognition experiments. For animals exposed to the Black Box after reactivation, we alternated returning animals to the black box and their transport cages

during habituation, such that animals were returned to their transport cages on the first and third habituation trial, and then the Black Box on the second and fourth.

Reactivation. The open field contained 2 mason jars only, i.e., the type of mason jar to which objects were glued during sampling and probe but without the objects, at the same opposing locations that animals had experienced objects during sampling. These jars were to serve as partial reminder cues for the objects present during sampling, without providing another learning trial since the objects themselves were not present. The front of the mason jars were orientated toward the center of the open field (as had been done with objects). Each reactivation trial was 2 minutes long, after which animals were then moved to another room for infusions, and then returned to either their transport cage or designated black box. Between reactivation trials, mason jars were cleaned with 70% alcohol and the bedding was dispersed.

Immunohistochemistry

Perfusion. After completion of a sampling phase, rats were placed for 1 h either into their shared transport cage or separately into the black box depending on the conditions tested detailed in Figure 3.5. Rats were then placed in an induction chamber containing isoflurane until deeply anaesthetised, and then transcardially perfused with saline followed by a 4% paraformaldehyde in 0.1 M phosphate buffer. We then removed brains and post fixed them in paraformaldehyde overnight and then in a solution of 4% paraformaldehyde and 30% sucrose–saline for 48 h. After this, we froze brains by placing them in a beaker containing 2-methylbutane (Fisher, O3551-4) which placed inside a container of dry ice. Once frozen, the brains were wrapped in aluminium foil and submerged in the dry ice before final storage at -80°C.

c-Fos staining. The brains were cut coronally in 40 µm sections in a cryostat and stored in anti-freeze. Sections were washed in 0.2% Triton-X 100 in 0.1 M TBS (TBST) 3 times for 10 min each and then incubated in a mix of TBST and 5% Normal Donkey Serum (NDS; Sigma-Aldrich, Oakville, Ontario) to block for 1 h. The block was then removed and the c-Fos antibody was introduced in a mix of TBST and NDS, in which slices were left overnight. Around 18 h later, sections were washed again in TBST (3 times for 10 min each) and then incubated in a mix of PBS and Alexa-488 secondary antibody for 1 h. Sections were then washed again with PBS 3 times for 10 min each, and then mounted onto slides and immediately cover-slipped with Fluoromount-G with DAPI (Thermo Fischer).

Image capture and c-Fos analysis. We focused on the caudal perirhinal cortex between -4.8 and -6.3 mm along the anterior/posterior axis of the rat brain (Albasser et al., 2010). Images were acquired by fluorescent microscopy (Leica DM 5000 B) with a x5 objective lens. Four images per rat were taken, two from each hemisphere, across the caudal perirhinal. The cell counts of the 4 images was averaged to provide a single data point for each rat. Image analysis was done using imageJ (Schindelin et al., 2012), with images set to threshold of around 20-25 units from the peak intensity while regions of interest were manually identified. Cell-counting was quantified by imageJ. Cells were determined by a particle size of 40- 250 μm^2 (around 8-25 μm mean ferret), and then checked by an experimenter blinded to condition. C-Fos neurons were only counted when clear immunostained nuclei were co-localized with DAPI staining. Counts were then normalized to sample areas by calculating the number of counts/area size to produce density values.

3.4. Results

3.4.1. Inactivating the dorsal hippocampus during sampling impairs long-term memory formation for objects.

We first set out to confirm in rats that the hippocampus is necessary for acquiring long-term object recognition memory in our protocol (Broadbent et al., 2010; Cave & Squire, 1991; S. J. Cohen et al., 2013; Stackman, Cohen, Lora, & Rios, 2016). Using a standard novel object recognition paradigm, we habituated cannulated rats to an empty open field arena for 4 days. The next day, we bilaterally infused into their dorsal hippocampus a cocktail of muscimol and baclofen (MB), GABA-A and -B agonists respectively, to inactivate the hippocampus for about 2-3 h (Allen et al., 2008; Ásgeirsdóttir et al., 2020; Bonnevie et al., 2013; Riaz et al., 2017; Wells et al., 2013), or the inactive vehicle, tris-buffered saline (V). Twenty minutes later, we exposed the rats to two copies of the same object placed in opposing corners in the arena for 5 min during the sampling session, then animals returned to their transport cage. Twenty-four hours later, we returned the animals to the arena for a probe trial, where we had replaced one of the old objects with a novel one. Rats are drawn to novelty and if they have memory for the old object, they should spend more time exploring the novel one (A Ennaceur & Delacour, 1988), so that the object discrimination index *d* should be significantly higher than zero. If memory for the old object is absent, they should explore new and old objects equally long, and *d* will not be significantly different from zero, or what would be expected by chance alone.

We found that *d* was significantly above chance only in rats infused with vehicle (V, $t_{(7)} = 2.381$, $p = 0.049$, Cohen's d = 0.842; MB, $t_{(6)}=-1.467$, $p = 0.193$, Cohen's d = -0.554; Fig. 3.1B). The difference between the two groups was also significant ($t_{(13)} = 2.713$, $p = 0.018$, Cohen's d = 1.4; Fig 3.1B). Total exploration time during initial object learning was the same for both groups ($t_{(13)}=-0.822$, $p = 0.426$; Fig. 3.1C), and both groups took the same amount of time to accumulate 30 s of object exploration during the memory test ($t_{(13)}=-1.569$, $p = 0.141$; Fig. 3.1D), suggesting that group differences in novel object preference during the memory test cannot be attributed to variability in motivation or motility. These results show that compromised hippocampal function during learning leads to anterograde amnesia for objects in a novelty recognition test administered 24 h later, suggesting that the hippocampus is required for the formation of long-term object memories, in agreement with findings in humans and non-human animals (Cohen 2013; Pascalis 2004; Zola 2000; Squire 2007).

We then tested whether the hippocampus was required only during learning or in addition, in the time period afterwards. We therefore repeated the first experiment, but infused MB or V directly after, not before sampling, and testing memory 24 h later (Fig. 3.1E). This led to the same outcome – only rats infused with V express long-term object recognition memory, significantly preferring to explore the novel over the familiar object, while rats that had received MB treated both objects the same (one-sample *t* test*:* V, $t_{(10)}= 3.22$ $p = 0.009$, Cohen's d = 0.97; MB, $t_{(9)}= -0.33$, $p = 0.75$, Cohen's d = -0.11, Fig. 3.1F). Novelty preference was significantly different between the groups (independent sample *t* test: $t_{(18)}= 2.18$, $p = 0.043$, Cohen's d = 0.979, Fig. 3.1F), and there were no differences in exploration behaviour during sampling or probe, so that changes in motivation or motility are unlikely factors explaining these outcomes (independent sample *t* test: sampling, $t_{(18)}= -2.05$, $p = 0.84$, Fig. 3.1G; probe, $t_{(18)}= -1.58$, $p = 0.137$, Fig. 3.1H). In combination with our first set of results, these findings show that activity in the hippocampus is required during and after encoding of objects to form long-term object memories.

We next explored the nature of object amnesia caused by hippocampal inactivity. Infusing MB into the hippocampus prior to learning might have compromised encoding or affected later retrieval, which could provide alternative explanations for our findings. We tested these possibilities in the following experiments. First, we sought to determine whether the hippocampus is required merely for the encoding of object memory, not also stabilization. We trained animals as in our first experiment (Fig. 3.1A), infusing MB or V prior to sampling. This time, however, we tested recognition memory in a Probe trial 10 min after the end of sampling (Fig. 3.1J). We found that both groups preferred to explore the novel object, as *d* was

significantly above chance both for rats infused with V or MB, and the difference between them was not significant (one-sample *t* test*:* MB, $t_{(7)}$= 4.65, p = 0.002, Cohen's d = 1.65; V, $t_{(5)}$= 3.19, p=0.024, Cohen's d = 1.3; Fig. 3.1K; independent sample *t* test: $t_{(12)}$= 0.0229, p = 0.982, Cohen's d = 0.0124, Fig. 3.1K). There were also no differences between the groups in exploratory activity, suggesting that the infusions had similar effects on motivation and motility (sampling: $t_{(12)}$= 0.0188, p = 0.985, Fig. 3.1L; probe: $t_{(12)}$= 0.651, p = 0.527, Fig. 3.1M). These findings show that inactivating the hippocampus prior to sampling does not prevent the encoding of object memory required for novelty recognition.

We then investigated whether the hippocampus is required for expressing long-term object memories. We trained animals as in the previous experiment and tested object memory 24 h after sampling. This time, however, we infused rats with either MB or V twenty minutes prior to the probe session (Fig. 3.1N). We found that both groups expressed significant and equivalent preference for the new object over the familiar one (V, $t_{(7)}$= 4.48, p = 0.003, Cohen's d = 1.58; MB, $t_{(6)}$= 3.02, p =0.023, Cohen's d = 1.14, Fig. 2D; $t_{(13)}$= -0.064, p =0.950, Cohen's d = -0.033, Fig. 3.1O). Both groups spent a similar amount of time exploring objects during sampling ($t_{(13)}$= -0.154, p = 0.880; Fig 3.1P) and probe trial ($t_{(13)}$= 1.27, p = 0.225; Fig. 3.1Q). Thus, inactivating the hippocampus does not affect expressing recognition memory for objects.

3.4.2. Amnesia for objects following dorsal hippocampal inactivation is prevented by reducing sensory stimulation immediately, but not one hour after learning.

Prior work in human patients with anterograde amnesia showed that reducing sensory stimulation after learning promotes long-term declarative memory formation (Alber et al., 2014; Cowan et al., 2004). As such, we next sought to determine whether we would observe a similar effect in object recognition memory in rats with inactivated dorsal hippocampi. To diminish sensory stimulation, we used the black box paradigm (Arkell et al., 2021), wherein animals are placed individually into a familiar dark box (i.e., the Black Box) for 1 h after learning. Our gentle handing procedure was used while animals were in the Black Box to prevent animals falling asleep during this period (see section 3.2.2.). For familiarisation, animals were placed into the same box for 1 h following habituation to the open field for 4 d prior to sampling. The Black Box reduces sensory experience typically present in the transport cage, including visual, olfactory and social stimulation. We therefore repeated our first experiment, infusing rats with either V or MB into the dorsal hippocampus prior to sampling,

and immediately after, placed them into a Black Box for 1 h (Fig. 3.2A). Memory for objects was tested 24 h after sampling, i.e., 23 h after Black Box exposure.

We found that rats infused with MB showed significant novelty preference, in line with inactive vehicle infusions (V: $t_{(4)} = 15.1$, $p < 0.001$, Cohen's d = 6.76; MB: $t_{(5)} = 4.19$, $p = 0.009$, Cohen's d = 1.71, Fig. 3.2B). There were no group differences in novelty preference ($t_{(9)} = 1.32$ $p = 0.221$, Cohen's d = 0.797, Fig 3.2B). Furthermore there were no differences in exploration behaviour during sampling or probe (Sampling: $t_{(9)} = 1.58$, $p = 0.149$, Fig. 3.2C; Probe: $t_{(9)} = 0.502$, $p = 0.627$, Fig. 3.2D). These findings suggest that the hippocampus promotes long-term memory formation by reducing interference arising from sensory stimulation after learning.

We then explored whether reducing sensory stimulation after a 1 h delay following sampling could also rescue object amnesia when the hippocampus is inactivated. We therefore repeated the experiment (Fig. 3.2A), but placed rats after sampling first into their transport cage for 1 h, and then into the Black Box for another hour (Fig. 3.2E). Memory for objects was tested 24 h after sampling, 22 h after Black Box exposure. Only animals that received inactive vehicle infusions displayed a significant preference for the novel object, while animals that received MB explored novel and familiar objects similarly (V: $t_{(9)} = 3.61$, $p = 0.012$, Cohen's d = 0.998; MB: $t_{(10)} = 0.515$, $p = 0.618$, Cohen's d = 0.155; Fig. 3.2F). We found *d* to be significantly different between groups ($t_{(19)} = 2.17$, $p = 0.043$, Cohen's d = 0.946, Fig. 3.2F). Similar to before, there were no differences in exploratory behaviour between groups at sampling or probe (sampling: $t_{(19)} = -0.123$, $p = 0.903$, Fig. 3.2G; probe: $t_{(19)} = -0.123$, $p = 0.903$, Fig. 3.2H). Therefore, one hour of typical sensory stimulation after learning suffices to produce amnesia in object recognition in rats with inactivated hippocampus, suggesting that events critical to promote long-term consolidation take place in the hour immediately following object exploration during sampling.

The Black Box reduces stimulation from a variety of sensory modalities, yet whether a particular modality can promote interference leading to disruption of long-term object memory formation is unknown. Previous work has shown rats rely on visual processing in learning object identities (Zoccolan, Oertelt, DiCarlo, & Cox, 2009), suggesting a potential sensory source vulnerable to interference disruption. We therefore set to determine if introducing visual stimulation in the Black Box would promote forgetting of object memories. To test this, we repeated our first experiment described in Figure 3.2A but placed animals into a familiar light box for 1h after sampling (Fig. 3.2I). The light box was generated by turning on dim lighting on inside the Black Box, allowing animals visual access to the walls and floor.

Similar to the main result from our first experiment, *d* was only significantly above chance for rats infused with V (V: $t_{(5)} = 4.67$, $p = 0.005$, Cohen's d =1.91; MB: $t_{(6)} = 1.35$, $p = 0.225$, Cohen's d = 0.511, Fig. 3.2J) suggesting that those which received MB did not show long-term object memory. Moreover we observed a significant difference in novelty preference between groups ($t_{(11)} = -2.84$, $p = 0.016$, Cohen's d = 1.58, Fig. 3.2J). Time to reach 30s of exploration in the probe trial was significantly different between groups ($t_{(11)} = -3.54$, $p = 0.005$, cohen's d = -1.97, Fig. 3.2L) but total exploration time during both probe and sampling was not (Probe: $t_{(11)} = 2.0$, $p = 0.071$, Table 3.1; Sampling: $t_{(11)} = 0.553$, $p = 0.692$, Table 3.1). These findings suggest that visual stimulation following learning in the absence of a functioning hippocampi can induce amnesia for objects in rats 24 h later.

Given that object amnesia could not be prevented by introducing the Black Box 1 h after normal post-sampling sensory experience, nor when only visual stimulation was provided in the 1 h after sampling, this suggests that under hippocampal inactivation object memory is particularly susceptible to forgetting from sensory stimulation in the hour after learning. We therefore tested whether under healthy conditions the need for the hippocampus to form long-term object memory matched this critical period. To test this, we placed rats in our object recognition paradigm but infused animals 1 h after sampling (Fig. 3.2M). Between sampling and infusion, animals were placed in transport cages as previously described, and prevented from entering restful states associated with replay activity by gentle handling. Both animals infused with MB and inactive vehicle showed significant preference for the novel object 24h after sampling, i.e., 22 h after infusions. (V: $t_{(5)} = 2.83$, $p = 0.037$, Cohen's d = 1.15; MB: $t_{(5)} = 7.45$, $p < 0.001$, Cohen's d = 3.04, Fig. 3.2N). There were no group differences in novelty preference ($t_{(10)} = 0.669$, $p = 0.519$, Cohen's d = 0.386, Fig. 3.2N) or exploration behaviour during probe and sampling (Probe: $t_{(10)} = 0.270$, $p = 0.792$ Fig. 3.2P; Sampling = $t_{(10)} = 1.76$, $p = 0.108$, Fig. 3.2O). This result suggests that having a functional hippocampus, for 1hr after object learning is sufficient to form long-term object memories.

3.4.3. Inactivating hippocampus during sampling leads to excessive neuronal activity in perirhinal cortex which is attenuated when sensory stimulation after sampling is reduced.

Interference during consolidation and reconsolidation is thought to primarily arise from perturbing neuronal activity in a shared population (Hardt et al., 2013; Libby & Buschman, 2021). As discussed in Chapter 2, the immediate early gene (IEG) c-Fos is typically used as an

indirect measure of neuronal activity (Tischmeyer & Grimm, 1999). Its expression is robustly increased in the perirhinal cortex following novel object exposure, which, when blocked, impairs object memory formation (Aggleton & Brown, 2005; Albasser et al., 2010; Seoane et al., 2012). We therefore hypothesized that the amnesia we observed for object memory in animals with inactivated hippocampus may arise from increased neuronal activity, indicated by c-Fos levels, in the perirhinal cortex stemming from sensory stimulation.

To investigate this, we trained rats as in our first experiment, infusing either inactive vehicle or a cocktail of the two GABA agonists muscimol and baclofen prior to object sampling, and then placed animals back into their shared transport cages. After 1 h, animals were perfused and brains were collected, in line with elevated c-Fos expression (Bisler et al., 2002). To control for c-Fos activation related to the infusion procedure or arena exposure we also infused a group of rats with inactive vehicle and placed them in the familiarized open arena without any objects before returning them to their transport cages. We then ran these three conditions i.e., vehicle infusion before an empty open arena; vehicle infusion before sampling with novel objects or MB infusion before sampling with novel objects again, except this time returned animals to the Black Box after sampling (Fig. 3.3C). As the caudal perirhinal shows the greatest neuronal activity in response to novel object exposure (Albasser et al., 2010) we performed immunohistochemical investigations of c-Fos expression selectively in this region.

Overall, we found a significant difference in c-Fos levels across groups (One-way ANOVA: $F_{(5, 6.99)} = 8.37$, $p = 0.007$; Fig 3.3.D.). We observed a general trend of reduced c-Fos expression between animals returned to the Black Box and those exposed to normal sensory experience after sampling. Within each sensory condition, animals exposed to an empty arena show the lowest c-Fos expression, while those with an intact hippocampus exposed to objects showed increased counts, and finally, animals with inactivated hippocampi showed the highest c-Fos levels. Under normal sensory conditions, c-Fos expression was significantly greater in animals that received MB infusions prior to object sampling compared to those that received inactive vehicle (Tukey's: $p = 0.014$) and expressed the highest levels of c-Fos across all groups (mean ± S.E.M.: 137.4 ± 30.14). Out of all conditions, this was the only one which induced amnesia for objects in our behavioural studies (Fig. 3.1B). Placing animals with inactivated hippocampus into the black box after sampling reduced c-Fos levels (Tukey's: $p = 0.003$; Mean ± S.E.M.: 85.8 ± 13.6), suggesting that attenuated neuronal activity may correlate to the absence of amnesia in these animals. Animals spent a similar amount of time in object

exploration across groups (see appendix Fig. A.2.1), indicating any differences in c-Fos expression do not reflect differences in motility or motivation, or an inability to detect novelty. To determine whether c-Fos reflected new object learning or general object experience, we exposed animals two sampling phases (intact hippocampi, normal post-learning sensory experience) with the same objects across two days and collected brains 1 h after the second trial (i.e., when objects should be familiar). We found no significant difference in c-Fos density levels between novel and familiar objects ($t_{(7)} = 1.52$, $p = 0.172$, Cohen's d = 1.02, see appendix, Fig. A.2.2.). Taken together, these results suggest that inactivating the hippocampus prior to object sampling leads to excessive neuronal activity under normal sensory experience, which is reduced when post-sensory stimulation is reduced.

3.4.4. Plasticity in the hippocampus during, but not after sampling is required to form long-term object recognition memories; reducing sensory stimulation after learning rescues this deficit.

So far, our findings suggest that the hippocampal activity critically contributes to forming long-term object recognition memory, but that its role unlikely involves the encoding or retrieval of objects. To further determine this, we next explored whether plasticity typically underpinning learning and memory in the hippocampus plays a role in promoting the formation of long-term memory supporting object recognition.

We repeated our first experiment (Fig. 3.1A), but infused the NMDAR antagonist AP5, which at the used concentration and volume, blocks the receptors for 2-3 h (Steele & Morris, 1999), or its inactive vehicle PBS (V) into the hippocampus 15 min before sampling, and tested memory 24 h later (Fig. 3.4A). We found that rats infused with V expressed, as expected, novel object recognition memory ($t_{(12)} = 4.29$, $p = 0.001$, Cohen's d = 1.24, Fig. 3.4B), while those that had received AP5 did not ($t_{(9)} = -0.049$, $p = 0.962$, Cohen's d = -0.0165, Fig. 3.4B). The difference in novelty exploration preference between the two groups was significant ($t_{(21)} = -2.20$, $p = 0.032$, Cohen's d = -0.964, Fig. 3.4B). There were neither group differences in total exploration time during sampling ($t_{(21)} = -0.819$, $p = 0.422$, Fig. 3.4C) nor in the time taken to reach 30 seconds of accumulated exploration time during the probe trial ($t_{(21)} = 0.636$, $p = 0.532$, Fig. 3.4D), indicating that motility or motivation to interact with the objects cannot account for divergent novelty preference. Thus, NMDAR -dependent plasticity in the hippocampus is required during object encoding for forming long-term memory necessary for novel object recognition.

This finding could indicate that the hippocampus acquires critical knowledge about the identity of objects during encoding, which blocking synaptic plasticity in the hippocampus prevented, resulting in absent long-term object recognition memory. If this were the case, then blocking NMDARs *after* encoding should not lead to a memory deficit the next day. Thus, we trained and tested rats as before, but infused AP5 or V immediately after sampling (Fig. 3.4E). Now, both groups expressed novelty preference (V: $t_{(7)} = 2.93$, $p = 0.022$, Cohen's d = 1.04; AP5: $t_{(6)} = 2.79$, $p = 0.032$, Cohen's d = 1.05; Fig. 3.4F), and to the same extent (independent sample *t*-test: $t_{(13)} = -0.197$, $p = 0.847$, Cohen's d = -0.102; Fig. 3.4F). There were no differences in exploratory activity during sampling ($t_{(13)} = -0.0242$, $p = 0.981$, Fig. 3.4G) or Probe ($t_{(13)} = -0.0227$, $p = 0.982$, Fig. 3.4H). Thus, plasticity depending on NMDAR activation in the dorsal hippocampus during object learning, but not thereafter is required for forming long-term memory supporting novel object recognition.

We then wondered whether, like in the preceding studies, reducing sensory stimulation after learning could rescue the anterograde amnesia that AP5 induced. This outcome would indicate that hippocampal plasticity does not reflect encoding of the identity of objects. We tested this possibility, infusing AP5 prior to sampling and placing rats into the dark Black Box for 1 h immediately thereafter, assessing memory 23 h later (Fig. 3.4I). This time, rats infused with AP5 prior to sampling expressed novelty preference, and as strong as rats in the control group (V: $t_{(7)} = 2.68$, $p = 0.032$, Cohen's d = 0.946; AP5: $t_{(8)} = 2.66$, $p = 0.029$, Cohen's d = 0.885; independent sample *t*-test: $t_{(15)} = 0.579$, $p = 0.571$, Cohen's d = 0.281; Fig. 3.4J). Exploratory time in sampling and probe trials was the same across groups (sampling: $t_{(15)} = -0.426$, $p = 0.676$, Fig 3.4K; probe: $t_{(15)} = 0.385$, $p = 0.706$, Fig. 3.4L). Together with the two preceding studies, this makes it unlikely that the hippocampus acquires knowledge about the objects themselves during object learning.

3.4.5 Reducing sensory interference after reconsolidation blockade in the hippocampus rescues amnesia for objects

A number of reconsolidation studies point toward the involvement of the hippocampus in restabilizing and updating reactivated long-term object recognition memory (Rossato et al., 2007; Winters et al., 2011). Yet, the exact role of the hippocampus during this process remains unclear. We reasoned that since post-reactivation destabilization also leaves traces susceptible to interference in humans (Hupbach et al., 2007), the hippocampus may, similarly to its

contribution to consolidating object memories identified in our preceding experiments, protect restabilising object memories from everyday sensory stimulation during reconsolidation.

To test this, we first developed an object reconsolidation recognition paradigm, in which a single sampling session of 10 minutes induces memory lasting at least 48 h, since animals will be tested 2 days after sampling (see Appendix: Fig A.3). In this protocol, animals were returned to the open field arena 24 h after sampling with only the bases of the two objects were present (i.e., the mason jars), i.e., animals were reminded of the original objects by exposing them to a partial cue, thus reactivating the object memory without providing another learning trial, as in widely used fear-conditioning reconsolidation protocols (Alberini, 2011; Nader et al., 2000). Immediately after reactivation, we infused into the dorsal hippocampus either anisomycin (ANI) or inactive vehicle (V), and memory for objects was tested, as in the preceding experiments, 24 h thereafter (Fig 3.5A).Animals who received V showed significant novelty preference, while those infused with ANI did not (V, $t_{(5)} = 3.73$, $p = 0.014$, Cohen's d = 1.52, Fig 3.5B ANI, $t_{(4)} = 0.0481$, $p = 0.964$, Cohen's d = 0.0215; Fig 3.5B). No significant group differences were observed in novelty preference ($t_{(9)} = -2.03$, $p = 0.073$, Cohen's d = -1.23, Fig 3.5B). There were no group differences in the time taken to accumulate 30 s of object exploration during the probe ($t_{(9)} = -1.10$, $p = 0.302$, Fig 3.5E). Furthermore no group differences were observed in time spent exploring objects during sampling ($t_{(9)} = 1.49$, $p = 0.171$, Fig 3.5C) or the mason jars during reactivation ($t_{(9)} = 0.125$, $p = 0.903$, Fig 3.5D), suggesting any observed differences in novelty preference were not due to impaired expression of exploration behaviour. These results suggest that the hippocampus is needed for the reconsolidation of object recognition memory.

Next, we set out to determine if the amnesia for objects following blockade of reconsolidation in the hippocampal arose from vulnerability to sensory stimulation after reactivation, similar to what we observed for initial object consolidation. We repeated our experiment but this time placed rats into the black box for 1 h after the reactivation trial and infusion of ANI or V (Fig 3.5F). We tested memory for object 23 h after animals had been placed in the black box. We found that both groups of animals expressed significant novelty preference (V, $t_{(7)} = 6.01$, $p < 0.001$, Cohen's d = 2.13; ANI, $t_{(5)} = 4.01$, $p = 0.01$, Cohen's d = 1.64; Fig 3.5G) with no group differences ($t_{(12)} = -0.569$, $p = 0.580$, Cohen's d = -0.307, Fig 3.5G). There were no group difference in exploratory activity during probe (time to reach accumulated 30 s of exploratory activity: $t_{(12)} = 0.027$, $p = 0.979$; Fig 3.5K; total time $t_{(12)} = 0.454$, $p = 0.658$), reactivation ($t_{(12)} = -0.565$, $p = 0.583$; Fig 3.5I) or sampling ($t_{(12)} = 0.974$, $p = 0.349$; Fig 3.5J). Taken together, these results show that hippocampal reconsolidation

blockade leads to amnesia for objects, which can be rescued with 1 h of reduced sensory stimulation following object recognition memory reactivation.

3.5 Figures

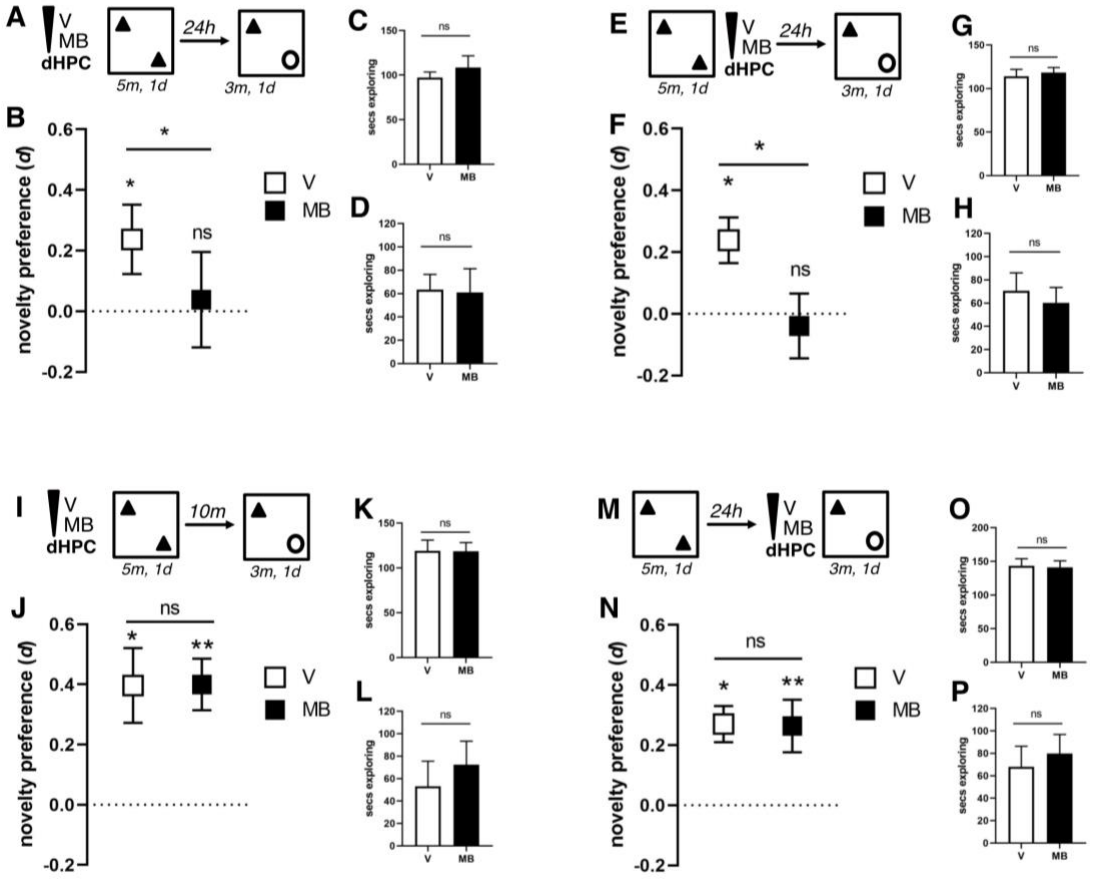


Figure 3.1. The hippocampus promotes the formation of long-term memory for objects. Schematic diagrams for experimental protocols (***A, E, I, M***), novelty preference at 30s accumulated exploration during probe (***B, F, J, N***), total exploration time during sampling (***C, G, K, O***) and time taken to reach 30s of exploration during probe (***D, H, L, P***). ***A-D*** The hippocampus is required during object encoding to form long-term memories for object 24h later. Rats were infused with V (*n*=6) or MB (*n*=7) prior to sampling, and then memory was tested the next day. ***E-H*** Inactivating the hippocampus after object encoding impairs long-term memory for objects 24h later Animals were infused directly after sampling (V *n*=11; MB *n*=10) and then tested for memory the next day. ***I-L*** The hippocampus is not required for object encoding. Animals were infused prior to sampling and tested 10 m thereafter (V *n*=8; MB *n*=6). ***M-P*** Inactivating the hippocampus does not impair object recognition memory recall. 24hr after sampling rats were infused with V (*n*=8) or MB (*n*=8) prior to probe.

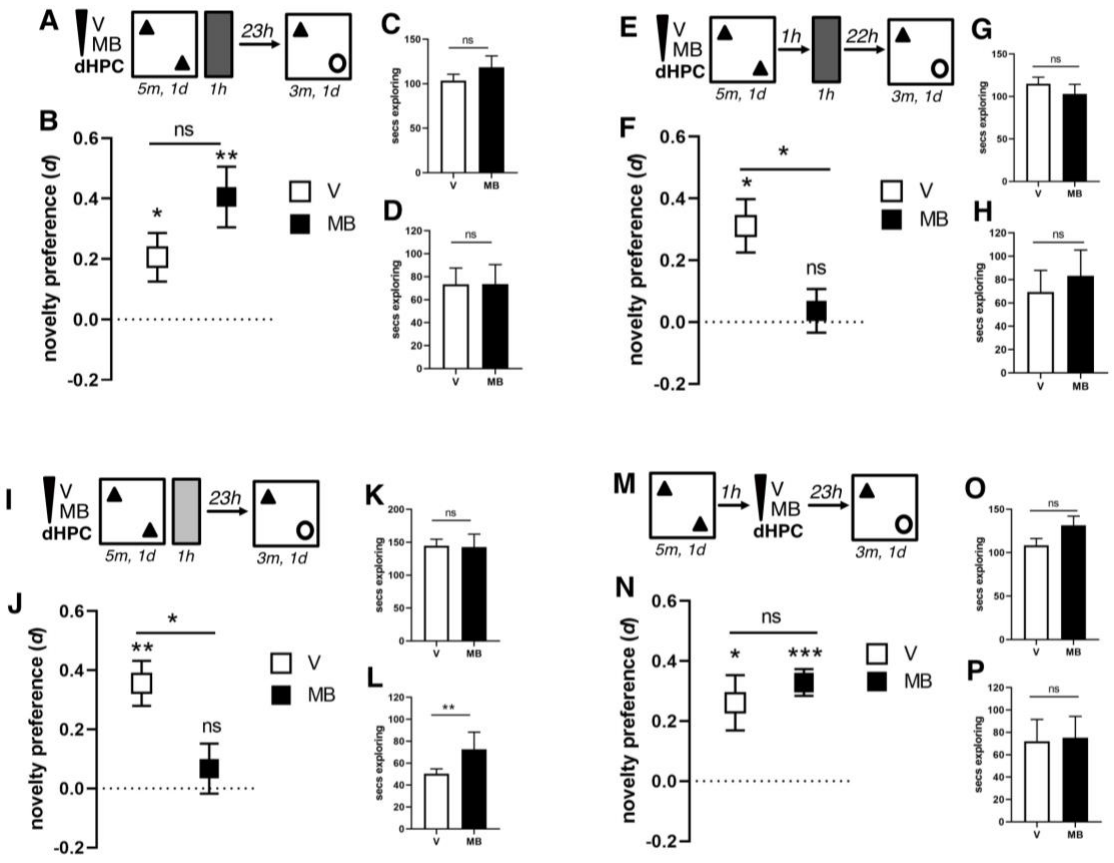


Figure 3.2 Amnesia for objects following dorsal hippocampal inactivation is prevented by reducing sensory stimulation immediately, but not an hour after learning. Schematic diagrams for experimental protocols (***A, E, I, M***), novelty preference at 30s accumulated exploration during probe (***B, F, J, N***), total exploration time during sampling (***C, G, K, O***) and time taken to reach 30s of exploration during probe (***D, H, L, P***). ***A-D*** Reducing sensory interference for 1 h after sampling led animals infused with V (*n*=5) and MB *(n*=6) prior to sampling to express long-term memory for objects. ***E-H*** Reducing sensory interference after 1 hr of normal sensory stimulation does not rescue object amnesia. Animals were infused with either V (*n*=10) or MB *(n*=11) prior to sampling, returned to their home cages for an hour and then placed in the black box for an hour, before a probe trial the next day. ***I-L*** Returning animals to the black box with visual stimuli impaired long-term memory for objects in animal infused with MB (*n*=6), but not V (*n*=6), before sampling. ***M-P*** Inactivating the hippocampus 1 h after sampling and normal sensory experience did not induce amnesia for animals infused with MB (*n*=6) or V (*n*=6).

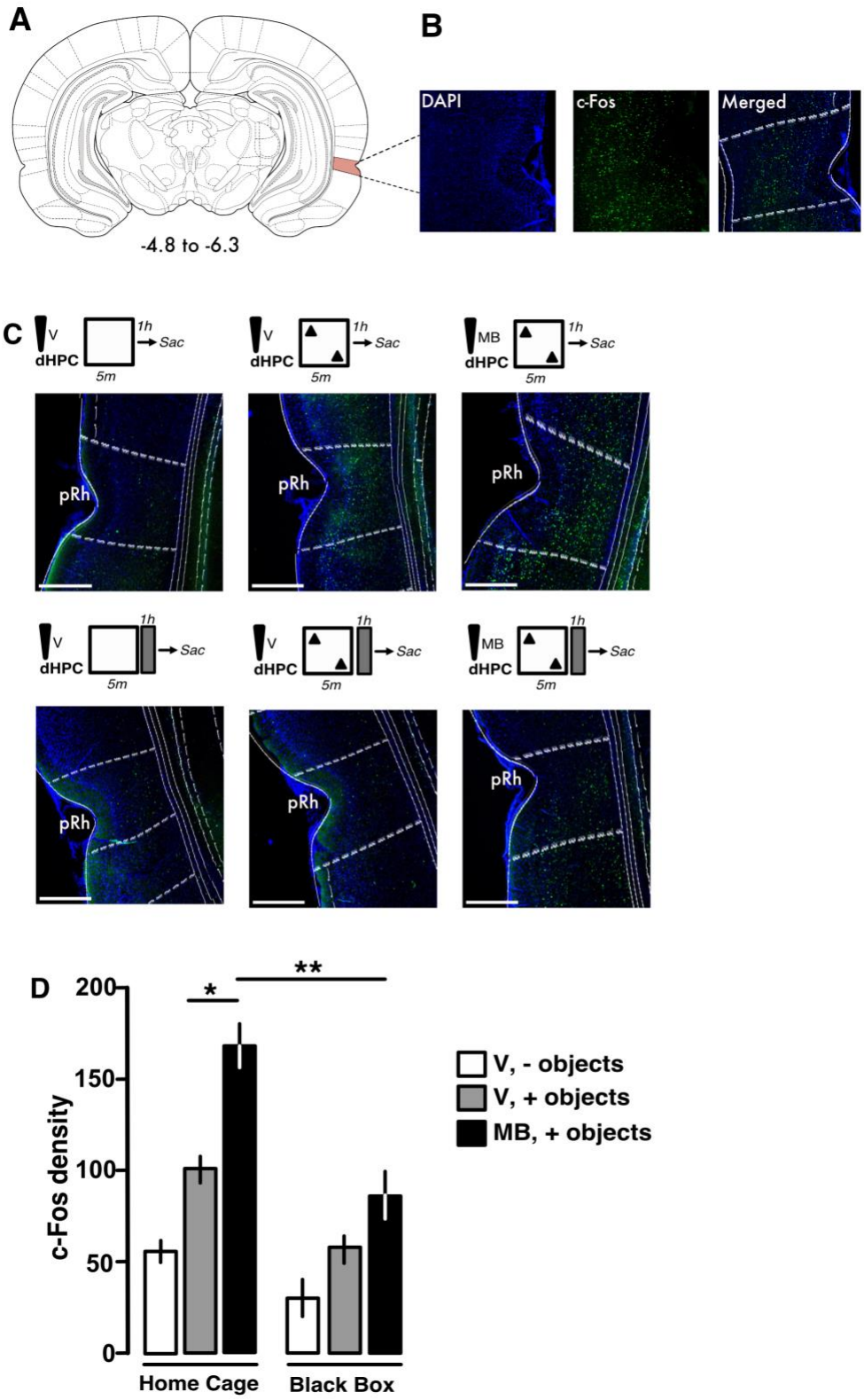


Figure 3.3. Excessive neuronal activity in the perirhinal cortex is reduced in amnesic animals when exposed to reduced sensory stimulation after object encoding. ***A*** Schematic representation of cortical region of interest (-4.8 to -6.3), pictured is -5.4 from bregma. ***B*** Sample images showing split channels for DAPI+, c-Fos+ and merged channels. ***C***

Schematic diagrams of each experimental condition with sample images from the caudal perirhinal cortex (pRh) of merged channels across behavioural conditions, scale bars represent 500μm. Rats were infused with inactive V or MB and then placed into the familiarised open area with and without novel objects for 5 minutes. Animals were either returned to their Transport cages perfused (V, -objects *n*=3; V, + objects *n*=5; MB, + objects *n*=4) or the Black Box perfused (V, -objects *n*=3; V, + objects *n*=4; MB, + objects *n*=4) for 1h, after which they were euthanised. ***D*** c-fos/μm^2 across each condition. Bars represent mean counts, error bars represent s.e.m.

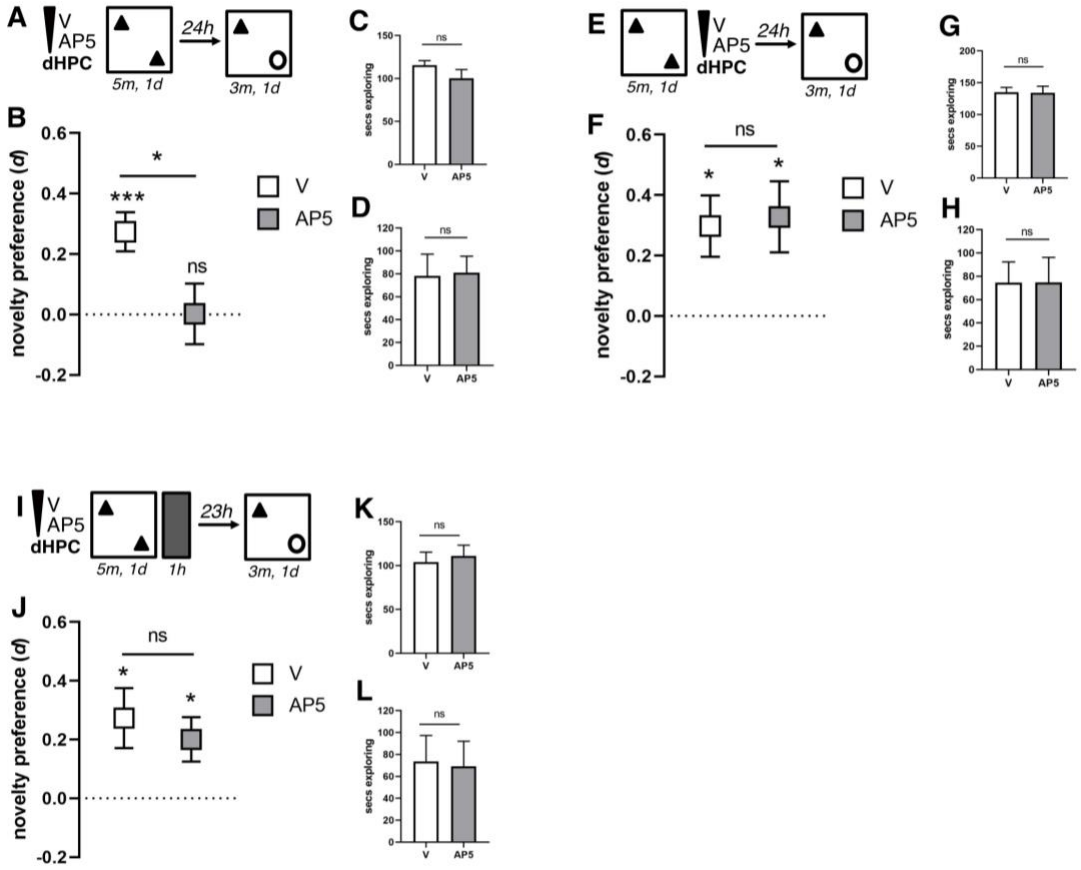


Figure 3.4. Reducing sensory interference after sampling rescues object amnesia induced by inhibited hippocampal plasticity. Schematic diagrams for experimental protocols (***A, E, I***), novelty preference at 30s accumulated exploration during probe (***B, F, J***), total exploration time during sampling (***C, G, K***) and time taken to reach 30s of exploration during probe (***D, H, L***). ***A-D*** Rats were trained and tested as before (Fig 3.1A) and received infusions to the dorsal hippocampus (dHPC) of either V or AP5 prior to sampling. ***B*** Rats that received V infusions prior to sampling preferred to explore the novel object 24h later (*n*= 13) while those that received infusions of AP5 (*n*=10) explored both objects equally. ***C-D*** Blocking hippocampal plasticity after object encoding does not lead to amnesia for objects 24h later. ***E-H*** Rats were trained and tested as before (Fig 3.4A) yet received infusions of either V or AP5 immediately after sampling. ***F*** Both infusion groups of animals expressed preference for the novel object (V: *n*=8; AP5: *n*=7). ***I-L*** Reducing sensory interference after learning rescues amnesia for objects arising from blocking plasticity in the hippocampus during encoding. ***I*** Rats underwent the same training, test and black box exposure as before (Fig 3.4A) yet were infused with either V or AP5 into the dorsal hippocampus prior to

sampling. ***J*** Both infusion groups (V: *n*=8; AP5: *n*=9) showed exploratory preference toward the novel object when memory was tested for 24h after sampling (23h after black box).

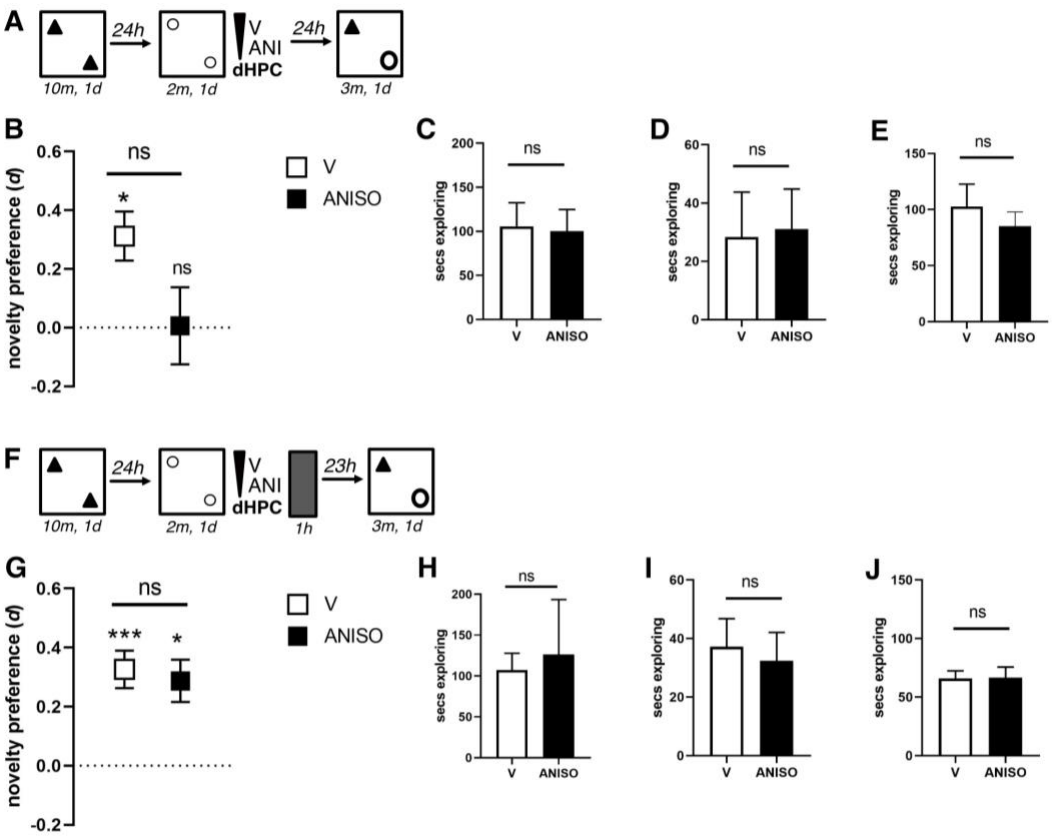


Figure 3.5. Reducing sensory interference after reconsolidation blockade in the hippocampus rescues amnesia for objects. ***A-E*** Inhibiting protein synthesis in the hippocampus after object reactivation induces amnesia for objects tested 24h later under normal sensory conditions. ***A*** Schematic diagram for experimental protocol. ***B*** Novelty preference for rats infused with V (*n*=6) and ANISO (*n*=5) at probe ***C*** Total exploration time during sampling between groups ***D*** Total exploration during reactivation ***E*** Time taken to reach 30 s of exploratory behaviour at probe. ***F-J*** Reducing sensory stimulation after hippocampal reconsolidation blockade leads to long-term memory for objects. ***F*** Schematic diagram for experimental protocol. ***G*** Novelty preference for rats infused with V (*n*=8) and ANISO (*n*=6) at probe ***H*** Total exploration time during sampling between groups ***I*** Total exploration during reactivation ***J*** Time taken to reach 30 s of exploratory behaviour at probe.

Figure	Group	Probe (total time)	Statistical comparisons
3.1A-D	V	63.5 ± 4.37	$t_{(13)}$= -1.57, p = 0.141
	MB	77.2 ± 9.4	
3.1E-H	V	70.6 ± 4.65	$t_{(18)}$= 1.59, p = 0.130
	MB	63.7 ± 4.39	
3.1I-L	V	68.6 ± 7.1	$t_{(12)}$= 0.293, p = 0.774
	MB	71.1 ± 5.11	
3.1M-P	V	79.9 ± 6.64	$t_{(13)}$= -1.24, p = 0.235
	MB	69.2 ± 5.48	
3.2A-D	V	73.4 ± 4.96	$t_{(9)}$= 1.97, p = 0.094
	MB	59.6 ± 5.46	
3.2E-H	V	70.8 ± 4.28	$t_{(19)}$= 1.98, p = 0.063
	MB	60.1 ± 3.41	
3.2I-L	V	85.7 ± 5.82	$t_{(11)}$= 2.00, p = 0.071
	MB	68.6 ± 6.14	
3.2M-P	V	68.5 ± 4.96	$t_{(10)}$= -0.274, p = 0.789
	MB	66.3 ± 6.6	
3.4A-D	V	66.1 ± 3.06	$t_{(24)}$= -1.55, p = 0.133
	AP5	58.4 ± 4.01	
3.4E-H	V	63.4 ± 6.63	$t_{(13)}$= -0.539, p = 0.599
	AP5	69 ± 8.18	
3.4I-L	V	62 ± 4.81	$t_{(15)}$= -0.486, p = 0.627
	AP5	66.7 ± 7.92	
3.5A-E	V	47 ± 1.84	$t_{(9)}$= 0.842, p = 0.422
	ANI	47.3 ± 4.4	
35.F-J	V	61.5 ± 7.15	$t_{(12)}$= -0.454, p = 0.658
	ANI	69.9 ± 7.98	

Table 3.1 Total exploratory time across probe trials for behavioural data. Average total exploratory time ± s.e.m. with statistical comparisons on independent t-tests of behavioural data.

3.6. Discussion

This series of experiments sought to determine whether the hippocampus promotes long-term object memory formation by preventing retroactive sensory interference on stabilizing traces arising from ongoing sensory stimulation after learning. We found that object amnesia induced by hippocampal inactivation was prevented when animals were placed into a familiar dark box (Black Box) immediately after sampling for one hour. This intervention did not prevent memory loss when the Black Box was illuminated, or when exposure to the dark was delayed for one hour. Furthermore, delaying hippocampal inactivation for one hour after sampling did not lead to a memory impairment. These results suggest that hippocampal activity is required for one hour or less immediately following object presentation in order to form long-term object recognition memories and that without this contribution from the hippocampus, visual stimulation will induce amnesia. Exploring neurobiological correlates of these effects, we found that neuronal activity in the perirhinal cortex tends to be higher in animals with hippocampal inactivation exposed to sensory stimulation compared to animals exposed to the Black Box and reduced sensory input, which may indicate vulnerability to increased interference in animals without a functional hippocampus. Furthermore, we found that blocking hippocampal plasticity during, but not after object encoding induced amnesia for objects, which, again, could be prevented by reducing sensory stimulation for 1 h after sampling. Finally, we explored whether amnesia for objects following reconsolidation blockade in the hippocampus after memory retrieval, a well-replicated effect in the literature (Rossato et al., 2007; Winters et al., 2011), could also be attributed to increased vulnerability to sensory stimulation, and found that, indeed, exposing animals to the Black Box after reactivation preserved memory.

Overall, our results suggest that: (1) impaired hippocampal functioning leads to anterograde amnesia arising from excessive interference during memory consolidation; (2) amnesia for objects arises from catastrophic interference in the perirhinal cortex that manifests as excessive neuronal activity; (3) inhibiting hippocampal plasticity during object encoding caused amnesia for objects, which was prevented when sensory stimulation was reduced after learning; (4) the hippocampus promotes trace restabilisation by protecting destabilized traces from interference arising from ongoing sensory experience during memory reconsolidation.

3.6.1. The requirement of the hippocampus to form long-term object recognition memory

Our findings align with numerous reports on the initial requirement of the hippocampus to form long-term object recognition memory in rodents. In agreement with others (Cohen et al., 2013; de Lima, Luft, Roesler, & Schröder, 2006), we find that inactivating the hippocampus before and immediately after sampling impairs long-term object memory formation. Similarly, we also found no difference in novelty preference between animals with an intact and inactivated hippocampus at shorter delays between learning and recall (Hammond et al., 2004), suggesting the hippocampus is not required for initial encoding but rather, for consolidation of long-term memory.

In comparison, while there have been some reports that inactivating the hippocampus prior to probe impairs object recognition memory expression (Cinalli Jr. et al., 2020; S. J. Cohen et al., 2013; Hammond et al., 2004), we found no such effect (Fig 3.1.). One possible explanation for this discrepancy is that 'stronger' object memories rely on the hippocampus for maintenance and expression while weaker ones do not (Cinalli Jr. et al., 2020). Therefore, inactivating the hippocampus prior to recall will only impair recall of 'strong' object memories. While, the term memory strength is terminologically vague, as it can refer to greater memory persistence, robustness (i.e., resistance to post-recall destabilization), number of details encoded, and behavioural change following experience, here, it is determined by the time spent exploring an object in a context. Therefore in agreement with this interpretation, our sampling sessions were typically only 5 minutes long while studies which reported impaired object recall with hippocampal inactivation used longer individual sampling sessions and/or multiple trials across days (Cinalli Jr. et al., 2020; Cohen et al., 2013; Stackman et al., 2016). Yet, an alternative explanation may lie in variations in protocols moderating the difficulty to discriminate objects. As discussed in Chapter 2, animals can make quick judgements of resemblance (i.e., akin to familiarity in humans) or more deliberated comparisons (akin to recollection). Altering the task demands can favour one recollection type over the other, such that objects that are more dissimilar are easily discriminated, while those that share features require more deliberation (Bartko et al., 2010). While the latter critically depends on the hippocampus, the former does not (Cowell et al., 2010). The objects used in our studies are typically contrasting in shade (i.e., light and dark), are placed in a position such that animals can fully explore all parts of the objects (i.e., not pushed against the wall), and allow animals to express a range of naturalistic exploratory behaviours (i.e., nose poking, climbing, sniffing, touching, etc.). In comparison, the studies reporting hippocampal dependence of object

discrimination place objects right in the corner of the open field arena, such that animals cannot explore the back of objects (Cinalli Jr. et al., 2020; Cohen et al., 2013; Stackman et al., 2016). As such, our paradigm may favour a more familiarity based-judgment, whereas the highlighted studies may require a more deliberated recollection, and therefore, remain dependent on the hippocampus during novelty recognition.

Another alternative explanation for our observed amnesia is that animals formed a state-dependent object memory, such that memory can only be recalled under the same drug/state to the one also experienced during learning. Prior work has reported that contextual fear memories are typically encoded in a state-dependant manner when drugs that target GABA-A receptors - such as those used in this report - (Berger & Stein, 1969), and in particular extrasynaptic GABA-A receptors (Jovasevic et al., 2015) are infused into the hippocampus. This is thought to be achieved by a number of ways. At the cellular level there is evidence that distinct molecular pathways are activated by exGABA-A signalling (Jovasevic et al., 2015) which may sub serve some cellular trace (i.e., upregulated PKCβII, mir-33). Alternatively, exGABAergic signalling generates distinct oscillatory patterns in the hippocampus (i.e., shifting away from naturally occurring oscillation), which if reinstated during recall, may favour hippocampal-cortical activity which promotes the reactivation of patterns initially acquired in such network dynamics (Meyer et al., 2017). While it remains unclear whether such an interaction unfolds for object recognition memory, there are a number of unique features of contextual fear memory which may favour its hippocampal state-dependent expression over object recognition memory. Firstly, contextual fear memory expression becomes hippocampal dependent over a much longer period than object recognition memory (Frankland et al., 2006) and thus the hippocampal-cortical-interactions induced by GABA agonism at learning may influence fear memory to a greater extent than object memory. Secondly, fear learning is a salient and stressful event which is associated with distinct neuromodulator signalling, such increased noradrenaline, which has been shown to enhance plasticity at GABAergic synapses (Inoue et al., 2013). In comparison, object learning is fairly benign, and more critically dependent on the perirhinal cortex for its expression.

Taken together, this series of results suggest that object amnesia induced from hippocampal inactivation around the time of encoding selectively impairs object consolidation, and not encoding or recall.

3.6.2. The hippocampus protects stabilizing object traces from sensory interference

Critically, rats that received pre-sampling intra-hippocampal infusions of Muscimol and Baclofen expressed long-term object memory when placed in the Black Box for 1 h after sampling. In other words, reducing sensory stimulation immediately after sampling rescued object amnesia, as animals with inactivated hippocampi were able to form long-term memory for objects. Our results extend previously discussed investigations in patients with anterograde amnesia. To briefly reiterate, the ability of these patients to acquire new, long-term declarative memories is greatly boosted when they are placed in a dark, quiet room for 10 minutes after encoding (Alber et al., 2014). These findings, along with those presented here, suggest that amnesia from damage to the medial temporal lobe arises from increased vulnerability to sensory interference, rather than a loss of the capacity to consolidate memories, i.e., of some basic forms of plasticity. This conclusion does not readily fit with current theories of systems consolidation, the implications of which will be discussed fully in Chapter 5.

During sleep, memory systems disengage with sensory perception which could otherwise drive interference on stabilizing memory traces. Indeed, under healthy conditions, post-learning sleep improves memory retention compared to a period of wakefulness as first shown in Jenkins and Dallenbach's (1924) classic study. This finding has been replicated in rats, where animals that undergo a 2 h period of sleep immediately after object sampling show memory retention for objects when tested 3 weeks later, while those kept awake during the same post-learning time period did not (Sawangjit et al., 2018). Yet, recent work reported that intra-hippocampal infusions of Muscimol and Baclofen immediately after object sampling and just before a 2 h sleep period impaired later recall of object memory, thus leading the authors to conclude that the hippocampus is required during sleep to promote long-term memory consolidation for objects (Sawangjit et al., 2018). Given our results, why then, do the rats in the above study not show improved memory under hippocampal inactivation and in a reduced sensory experience condition (i.e., sleep)? This may be more directly answered by observations that rats do not immediately fall asleep after object exploration, and the 'sleep-box' used in the described study contained some visual stimulation. Thus, an alternative explanation for these findings is that rats with an inactivated hippocampus showed later amnesia for objects because of an increased vulnerability to sensory stimulation which drove catastrophic interference before the animal fell asleep and not because of an impaired sleep dependent consolidation process.

When we introduced the Black Box 1 h after learning i.e., after an hour of typical sensory experience, animals infused with MB prior to learning did not express novelty

preference 24 h later. Put simply, we were unable to rescue object amnesia after an hour of sensory stimulation. We then set out to determine whether a particular sensory modality could drive amnesia during this period. The perirhinal cortex sits at the top of the ventral visual stream, and, as mentioned, rats use visual information to discriminate objects (Zoccolan, 2015). Furthermore, prior work has shown that animals with lesions to the perirhinal cortex are more susceptible to visual interference when performing an object recognition task (Bartko et al., 2010), suggesting that visual stimulation greatly influences object representations dependent on this area. We found that after sampling, placing rats with an inactivated hippocampus into an illuminated Black Box lead to object amnesia the next day. As such, visual stimulation alone in the hour after sampling sufficiently impaired object memory formation in amnesic animals.

As discussed in Chapter 1, prior work with intact animals found that object location memory in rats similarly suffers from interference by post-learning visual stimulation (Arkell et al., 2021). Furthermore, introducing a known cage mate into the Black Box also impaired memory for object locations, suggesting tactile, olfactory and social stimuli can similarly drive retroactive interference. These findings are in line with related human investigations (discussed in Chapter 1) supporting the idea that general sensory information converges onto a central consolidation function. Given its anatomical positioning, requirement for long-term memory formation and here observed role in sensory processing, the hippocampus seems the strongest candidate for such a function. From our findings, it remains an open question as to whether there is stimulus specificity for inducing amnesia in our rodents, since we only explored the impact of visual stimuli. As such, to address this, future studies will need to be conducted where after object sampling, animals with hippocampal dysfunction are returned to the Black Box to be exposed to varying sensory stimuli (i.e., audio, odour, etc.) that may not directly relate to object learning.

We found that inactivating the hippocampus immediately, but not 1 h, after learning lead to amnesia for long-term object memory. Initial memory formation is generally thought to last up to 4-6 h (McGaugh, 2000), which is assumed to reflect the period of time that cellular events underlying synaptic consolidation unfolds. While the hippocampus is not the primary host site for object memories, it plays a time-dependent role in forming long-term object memory. For instance, previous work has shown that inactivating the hippocampus immediately, but not 6 h after sampling impairs long-term object recognition memory (de Lima et al., 2006). In comparison, our results suggest that the critical window for hippocampal involvement is much shorter, and that object traces are sufficiently stabilised 1 h after encoding, such that they are no longer susceptible to amnesia from hippocampal inactivation.

Together with the above described results we demonstrate that the hippocampus promotes object memory formation by protecting stabilizing traces in extra-hippocampal sites, like the perirhinal cortex, from interference arising from ongoing sensory interference.

3.6.3. The nature of object amnesia

Retroactive interference to the point of amnesia has been previously observed in artificial neuronal networks, as an effect known as catastrophic interference. Despite a number of positions suggesting catastrophic interference also unfolds in biological systems (Hardt et al., 2013; McClelland et al., 1995), it is unclear how this manifests neuronally. Our immunohistological investigations in the perirhinal cortex of rats attempt to address this.

From our c-Fos data, a few trends can be observed which merit discussion. Firstly, across all behavioural protocols, reducing sensory interference after arena and/or object exposure lead to decreased c-Fos expression in the perirhinal cortex. Therefore, sensory experience itself seems to promotes c-Fos expression, which in agreement with the previously discussed literature (section 2.3.3.) furthers the notion that c-Fos activity is not unique to cells supporting a memory trace. A number of other immediate early genes are upregulated upon neuronal activity, which although largely label the same cells, show distinct patterns within a neuronal population. In particular, Arc expression is upregulated following neuronal activity, and, unlike c-Fos, sustains transcription for many hours following neuronal activity (Jaeger et al., 2018). *In vitro* investigations suggest that Arc expression correlates strongly to neurons that show increased activity, to a greater extent than c-Fos (Jiang & VanDongen, 2021). Along a similar vein, it has been proposed that Arc expression better reflects the activity of cells which are recruited to support a memory trace (Cazzulino, Martinez, Tomm, & Denny, 2016; Sethumadhavan, Strauch, Hoang, & Manahan-Vaughan, 2022). As such, regarding our investigations, we can at best infer that c-Fos reflects general neuronal activity, rather than confidently asserting memory trace identity.

Contrary to prior work, we did not observe reduced c-Fos expression (see appendix: Fig. A.2.2) after repeated object presentation (Albasser et al., 2010; Zhu et al., 1995). This could be explained by the comparably short sampling sessions in our paradigm (i.e., 5 minutes), such that animals are still learning about the objects during second sampling. Yet, while no significant differences in exploratory behaviour between first and second sampling were observed, there was a trend of reduced exploratory behaviour for animals during second sampling, which may suggest that animals continued to familiarise to the objects (see appendix:

A.2.2). Alternatively, a number of studies have shown that there are cells in the perirhinal cortex that respond selectively to either novelty or familiarity (Ahn et al., 2019; Xiang & Brown, 1998). Thus, rather than purely reflecting the novelty of an object, c-Fos activity may also signal some familiarity effects. In agreement with this, a recent study found that while a *fully* familiar object may reduce c-Fos expression, objects that are *relatively* familiar were associated with no differences in c-Fos expression between initial learning and sequential object presentations (Ameen-Ali et al., 2021). Thus, the lack of reduced c-Fos signalling we observed may not reflect continued novelty and learning, but a lack of maximum familiarity.

The highest levels of perirhinal c-Fos was seen in amnesic animals exposed to everyday sensory stimuli. Placing animals with inactivated hippocampi into the Black Box after sampling, which rescued behavioural amnesia, lowered c-Fos density counts to levels comparable to intact animals under normal sensory experience. Together, this suggests that amnesia for objects arises from excessive neuronal activity, and that under healthy conditions, the hippocampus acts to limit it. The short temporal window that our c-Fos studies capture indicates that the hippocampus exerts this function in a rapid and highly co-ordinated manner after learning.

One possible explanation for this may arise from recent work investigating the spiking profiles of hippocampal and perirhinal neurons in rats undergoing a rewarded novel object discrimination task. Ahn *et al.* (2019) found that repeated presentation of a novel object reduced spiking in subsets of excitatory neurons in both perirhinal cortex and hippocampus. Specifically, those that fired out of phase with local oscillatory rhythms (theta in the hippocampus, gamma in the perirhinal cortex) were reduced by 'phase-pruning' while neurons with persistent firing across presentations where 'phase-locked'. Thus, phase pruning may act to enhance the signal to noise ratio of perirhinal firing where neurons spiking in relation to irrelevant ongoing sensory information are suppressed while those spiking in relation to object relevant information are enhanced. At present, it is unclear how these oscillatory patterns interact with one another and from where they originate. While the hippocampus exerts prominent oscillatory rhythms, which could influence the perirhinal cortex through direct or indirect (via the entorhinal cortex) pathways (Fiorilli et al., 2021), there may also be regulation from other projecting regions.

3.6.4. Hippocampal learning in object encoding

Blocking plasticity in the hippocampus prior to sampling also induced amnesia for objects 24 h later. This aligns with prior findings showing impaired recognition memory tested 3 h after object learning with pre-sampling infusions of NMDAR antagonists (Baker & Kim, 2002; de Lima, Laranja, Bromberg, Roesler, & Schröder, 2005). Importantly, we found that, in comparison to Muscimol/Baclofen, infusing AP5 into the dorsal hippocampus immediately after sampling did not lead to object amnesia. While GABA agonists attenuate neuronal firing in the hippocampus, NMDAR inhibition impairs the molecular processes which lead to alterations in synaptic efficacy and leave fast synaptic transmission intact (Collingridge et al., 1983). Thus, our findings suggest that plasticity-related processes initiated during sampling are critical for long-term object memory formation, such that infusing AP5 immediately afterwards was not able to induce amnesia.

Placing animals under reduced sensory stimulation for 1 h after object learning prevented amnesia in animals infused with AP5 prior to sampling. A number of theoretical positions hold that the hippocampus encodes some content related to spatial object relations. For instance, in the Perceptual-Mnemonic/Feature Conjunction discussed in chapter 2, the hippocampus is proposed to encode some aspect of context, which allows to discriminate between highly similar objects, by virtue of the context in which they were seen. In other words, the hippocampus sits at the highest position among the conjunctive representation hierarchy (Cowell et al., 2010). Our findings do not readily agree with this interpretation, because, if hippocampal learning is required to discriminate objects, then regardless of post-sampling experience, animals infused with AP5 prior to sampling should show amnesia for objects. In comparison, we find that animals with inhibited hippocampal plasticity express novelty preference for objects tested 24 h later when post-learning sensory stimulation is reduced, indicating long-term memory formation for objects. Therefore, our results suggest that hippocampal learning during encoding is required to support the *function* of interference protection, rather than encoding object *content* per se.

3.7.5. The role of the hippocampus in object reconsolidation

When investigating the role of the hippocampus in object memory restabilisation we, like others (Rossato et al., 2007; Winters et al., 2011), found a critical role of this brain region in reconsolidation. Prior work suggests that the engagement of the hippocampus during object reconsolidation depends on the presence of novelty during the reactivation session. For

instance, a number of object reconsolidation studies expose animals to two objects in an open field during an initial sampling session (i.e., A-B) and then during a reactivation session, re-expose animals to one of the familiar objects with a new object (i.e., A-C). Post-retrieval blocking of cellular restabilisation in the hippocampus (i.e., infusion of protein synthesis inhibitor or Zif286 antisense) leads to amnesia, not only for the objects just seen (i.e., A-C) but also for the familiar object that was previously learnt in association (i.e -B) (Gonzalez, Rossato, Radiske, Pádua Reis, & Cammarota, 2019; Rossato et al., 2007), suggesting the initial trace had destabilized, and was therefore susceptible to amnesic agents.

Alternatively, novelty may arise from modifications to the context in which objects are presented. For instance, a study by Winters *et al* (2011) exposed animals to two copies of the same object (i.e., A-A') in a Y-maze during sampling, then, during a reactivation session presented a novel and familiar object together (A-B) and tested memory for the original memory 24 h later (i.e., A-C). While post-retrieval infusions of anisomycin to the perirhinal cortex led to amnesia (suggesting trace destabilization), infusions targeting the hippocampus did not. However, when the reactivation session re-exposed animals to already known objects and instead changed the texture of the floor in the arena, post-retrieval infusions of anisomycin to the hippocampus lead to amnesia for objects. Taken together with the above described findings, these suggest novelty is needed to induce object destabilization and hippocampal re-engagement to restabilize object traces.

Here, we developed a novel object reconsolidation paradigm, which is hippocampal-dependent and is triggered by only presenting a partial cue (i.e., the mason jars which objects are attached to). Prediction error i.e., when expectancy and reality are mismatched, is a boundary condition for reconsolidation, which typically is induced in object paradigms by presenting a new object with an already seen one. A critical limitation with introducing novel objects or contextual information to induce trace destabilization is that it becomes difficult to discern consolidation of the novel stimuli and reconsolidation of the known object. Furthermore, it also remains unclear whether presentation of the original object during reactivation lead to new learning and consolidation instead of merely retrieval and reconsolidation. As discussed in chapter 1, although there is overlap in the molecular events underlying these processes, there are distinct signatures of each, suggesting separable processes. While our paradigm does not expose animals again to the original objects or new ones, there may be some element of learning concerning the object base (i.e., the mason jars), as animals will have not been able to explore the tops of these, which were previously occupied by objects. To better determine whether our post-reactivation interventions are impairing

consolidation or reconsolidation, we will need to repeat our experiment in Fig 3.5A, except during the reactivation trial, re-expose animals to objects already seen in sampling. Post-retrieval infusions of anisomycin should not impair memory for objects, if they were fully consolidated from sampling, as no prediction error is likely to be present. This control experiment will allow us to establish whether this paradigm more accurately investigates object reconsolidation processes.

We found that intra-hippocampal infusions of anisomycin after reactivation trials led to amnesia for objects when rats were exposed to normal sensory stimuli thereafter, but not when sensory stimulation was reduced for 1 h post destabilization. This suggests that a role of the hippocampus during restabilisation is to protect destabilized memories from interference arising from ongoing sensory stimulation. These results mirror those we observed in our experiments on initial memory consolidation and further the notion that the hippocampus is not required to encode the content of objects themselves.

One possible limitation in these results is the use of anisomycin to induce reconsolidation blockade. Although the gold-standard for reconsolidation studies has been to infuse anisomycin post-retrieval, this drug has a number of off-target affects in addition to preventing protein synthesis. For example, recent *in vitro* work suggests that anisomycin drastically disrupts neuronal firing patterns in the hippocampus (Scavuzzo et al., 2019), thus achieving a similar effect to the GABA antagonism we used in earlier consolidation studies. As such the drug does not allow us to attribute the impairments observed to potential effects on protein synthesis alone. Although this is not of relevance for our studies, future investigations could use either GABA agonists or plasticity inhibitors during post-retrieval infusions targeting the hippocampus.

Overall the main aim of these experiments was to determine whether damage to the hippocampus leads to amnesia for object due to excessive interference, as predicted by active decay theory. In agreement, we found that reducing sensory stimulation after learning rescued object amnesia in animals with inactive hippocampi, and in those with blocked hippocampal learning. Object amnesia after hippocampal inactivation corresponds to states of catastrophic interference in the perirhinal cortex, as indicated by excessive neuronal activity. Finally, reducing sensory interference after reconsolidation blockade in the hippocampus rescued amnesia for reactivated memory. We will discuss the relevance and impact of the results for active decay theory in Chapter 5.

Chapter 4:

Proapoptotic signalling in active decay of long-term memories.

4.1. Preface

Endogenous forgetting mechanisms can cause long-term memory loss. One such process is active decay, which continuously acts to reverse the synaptic modifications induced by learning and memory formation (Hardt et al., 2013). So far, our group has established that active decay is initiated by NMDAR activation – in, particular, those containing GluN2B subunits – and ultimately leads to the internalisation of GluA2-AMPARs from the post synaptic density (Migues, Wong, Lyu, & Hardt, 2019; Migues et al., 2016). Yet two key issues remain unresolved. Firstly, given that NMDAR signalling is also critical for learning, how are opposing outcomes of NMDAR activation – learning vs forgetting – implemented at the cellular level? Secondly, what molecular events are initiated following NMDAR signalling that promote GluA2-AMPAR endocytosis?

As discussed in Chapter 1, one possible signalling cascade that active decay may recruit is the intrinsic proapoptotic pathway. This signalling cascade is characterised by the mitochondrial release of cytochrome c, which cleaves the proapoptotic proteins caspases -9 and -3, which then go on to drive GluA2-AMPAR internalisation (Sheng & Ertürk, 2014). Findings in electrophysiological studies have shown that during NMDAR-induced long-term depression, this pathway is recruited to internalise AMPARs (Li et al., 2010). Considering the opposing roles of extrasynaptic NMDARs in cell death and synaptic NMDARS in cell survival, it raises the possibility that the same functional division also holds for forgetting (i.e., active decay) and memory formation, with extrasynaptic NMDARs promoting the former and synaptic NMDARs the latter. Here, we investigate this possibility.

Active decay is well-documented for the hippocampus, therefore we used novel object location recognition which relies on the hippocampus for the learning, maintenance and expression in the rat. We tested the following predictions: (1) Active decay of long-term object location memories recruits proapoptotic signalling; (2) extra-synaptic NMDRs are needed to initiate active decay of long-term memory for object locations; and (3) learning for object locations requires synaptic but not extrasynaptic NMDARs.

4.2. Introduction

Trace decay is an old, but largely ignored explanation for time-dependent forgetting. The notion arose soon after Ebbinghaus published the pioneering forgetting curve (1885), which described rapid memory loss in the hours after learning, followed by a more gradual decline in the days and weeks thereafter. Some decades later, Thorndike formalised the idea of memory decay in the 'Law of Disuse' (Thorndike, 1913) which stated that without regular recall, memory would gradually be lost as a function of time. Yet time itself was rejected as an agent of forgetting as it ignores other external forces that correlate with longevity, a criticism which trace decay has since struggled to overcome (McGeoch, 1932; Ricker, Vergauwe, & Cowan, 2016). While later attempts were made to provide explanations for passive trace decay by neuronal fatigue (Ricker et al., 2016) or general protein turnover, an alternative view emerged that gradual memory loss may reflect an independent and dedicated process (Squire et al., 1984). In direct confirmation of this, recent work has uncovered a cellular process underpinning memory loss that over time, reverses the synaptic modifications induced by learning and memory formation, termed, *active decay* (Hardt et al., 2013; Migues et al., 2019, 2016).

The persistence of long-term memory is positively correlated with the expression of GluA2-AMPAR at post-synaptic sites, which needs to be continuously sustained for memory to persist (Migues et al., 2010). Active decay interferes with this maintenance mechanism by driving the activity-dependent removal of GluA2-AMPARs from the post synaptic density. As such, pharmacological blockade of this internalization process prevents the natural time-dependent forgetting of long-term memory (Migues et al., 2016) and similarly, genetic deletion of a vesicle regulatory protein that promotes AMPAR internalization, also preserves long-term memory beyond its natural life span (Awasthi et al., 2019). AMPAR trafficking from the post-synapse is promoted by the activity of NMDARs, a process which also appears to unfold during active decay of long-term memory. Inhibiting NMDARs during a memory retention interval similarly extends memory expression beyond its typical timespan, while promoting their activation during the same period accelerates forgetting of established long-term memory (Migues et al., 2019). Yet, there exists an even larger body of evidence highlighting the critical requirement of NMDAR activation during memory formation and synaptic strengthening (Martin, Grimwood, & Morris, 2000), thus it remains unclear how NMDAR signalling in active decay leads to AMPAR internalisation, and subsequent memory loss.

NMDAR-induced AMPAR internalization also occurs during long-term depression (LTD), a division of synaptic plasticity that leads to synaptic weakening. Recent electrophysiological studies have found that this form of LTD is dependent on the intrinsic proapoptotic pathway (Li et al., 2010), where NMDAR signalling induces cytochrome c release from the mitochondria, which then activates the proteases caspases-9 and -3 that then go on to promote AMPAR endocytosis. This proapoptotic pathway is initiated by NMDARs located extrasynaptically (exNMDARs), and not synaptically (sNMDARs) which instead promote neuroprotective cascades (Hardingham & Bading, 2010), thus highlighting the critical dependence of NMDAR location for regulating opposing fates of neuronal health. These findings raise the question of whether a similar dynamic unfolds in synaptic plasticity, in that exNMDARs promote synaptic weakening through the intrinsic proapoptotic pathway, while sNMDARs are critical for cellular processes underlying learning, thus suggesting that distinct forms of synaptic plasticity supporting opposing memory processes are elicited depending on NMDAR location. Some support for this notion comes from observations that sNMDARs uniquely promote potentiation processes during LTP, which also underpin memory formation (Lu et al., 2001). Taken together, this would offer an explanation why drugs that block NMDAR activation at all locations can impair both memory formation and endogenous memory loss.

The absence of highly selective drugs to target exNMDARs has prevented *in vivo* investigations into their role in learning and memory. Over the last few years, a number of nano-engineered NMDAR antagonists have been developed that specifically inhibit NMDA receptors at extrasynaptic sites *in vitro*. These compounds are typically composed of numerous NMDAR antagonist molecules bound to a single gold particle core, which together form a compound whose diameter is greater (35-50 nM) than that of the synaptic cleft (~20 nM). One such compound is AuM (Savchenko, Braun, & Molokanova, 2016), a nanoparticle comprised of around 50 memantine molecules attached to an inert gold particle. Memantine is a well-characterised NMDAR antagonist, which has been shown to impair spatial learning in rats when administered around the time of learning (Creeley, Wozniak, Labruyere, Taylor, & Olney, 2006). Alternatively, AuNP-ConR (Valente et al., 2020) is a compound in which Conantokin-R (ConR), a small peptide extracted from the venom of a marine snail, is bound to a gold particle. ConR is a non-selective NMDAR antagonist that binds to both GluN2A- and GluN2B –NMDARs. While ConR has yet to be tested in *in vivo,* its conjugated nanoparticle (AuNP-ConR) blocks exNMDARs induced currents in electrophysiological studies (Valente 2020) in a manner consistent with AuM.

Here we tested the hypothesis that active decay recruits proapoptotic signalling to induce forgetting of long-term memory. We investigated in rats whether inhibiting caspase-3 and extrasynaptic NMDARs in the dorsal hippocampus during a memory retention period could preserve long-term memory for object locations beyond their natural timespan. Furthermore, we explored whether blocking extrasynaptic NMDARs affects learning.

4.3. Methods

4.3.1. General procedures described in Chapter 2

Animals. As described in section 2.5.1.

Surgeries. As described in section 2.5.1.

Handling during recovery period. As described in section 2.5.1.

Open field arena. As described in section 2.6.2.

Object location Habituation. As described in section 2.7.

Object location Sampling. As described in section 2.7.2.

Object location Probe. As described in section 2.7.2.

Data analysis. As described in section 2.8.2.

Histology. As described in section 2.9.

4.3.2. Specific procedures

Behavioural studies

Drugs and infusions All animals received bilateral infusions targeting the dorsal hippocampus. To inhibit caspase-3 activity we infused z-DEVD-fmk (Sigma-Aldrich, Oakville, Ontario) at a concentration of 6.8 nmol per 1µl based on previous findings (Dash, Blum, & Moore, 2000). z-DEVD-fmk was dissolved in 75 µl of dimethyl sulfoxide (DMSO). A 34 µl stock was diluted in 66 µl phosphate buffered saline (PBS), bringing the solution to a pH of 7.24. DMSO diluted in PBS was used for as inactive vehicle for a control group (V). Animals received 1 µl of either V or z-DEVD-fmk per hemisphere at a rate of 0.25 µL/min. To target extrasynaptic NMDA receptors we used two different compounds; AuM and AuNP-ConR. AuM was supplied by Dr Molokanova (NeurANO Bioscience, State, USA) and set to a concentration of 50 nM per 1 µL, made by diluting 2.84 µL AuM in 22.16 µL PBS (pH adjusted to 7.24). Animals received 1 µl of AuM or PBS as inactive vehicle per hemisphere at a rate of 0.15 µL/min. For studies with the conantokin peptides and nanoparticles, all drugs were supplied by Dr. Fiammengo (Unversità di Verona, Italy) and set to a pH of 7.2. We used ConantokinG-F (ConG-F, 13.8

nM in PBS) as a control as it is an inactive peptide and does not target NMDARs. We also used the non-selective NMDAR antagonist Conantokin-R (ConR), set to a concentration of 15 nM in PBS, and the extra-synaptic selective NMDAR antagonist Gold-Conantokin-R (AuNP-ConR) at a concentration of 13.8 nM in PBS. Animals received 2 µl of either ConG-F, ConR or AuNP-ConR per hemisphere at a rate of 0.4 µL/min. We infused drugs into the dorsal hippocampus with a 28-gauge microinjector connected to a Hamilton (Model 1701N) syringe with polyethylene tubing (Braintree Scientific, Inc.). Microinjectors (Plastics One) protruded 0.5 mm from the cannulas and were sterilized with 70% ethanol before insertion. Injectors remained connected for an additional 90 s after the infusion stopped. Depending on the protocol, the infusions were done either in the colony room, the experimental holding room or a separate experiment rooms (detailed below).

General procedures for novel object location recognition. Behavioural procedures began once rats had returned to their home cage after the 3 days of single housing following surgery. There were three handling sessions across 5 days, 4 days of habituation before the sampling phase which began 24 h after the last habituation trial. Variations to total time in each sampling phase, or the number of sampling trials administered across days are described in the specific procedures below. When infusions were administered prior to sampling, animals received mock infusions before the third habituation trial. We removed animal's obturators and placed in the microinjectors connected to empty tubing, as described above, and turned on the infusion pumps to familiarise animals to the infusion procedure. During the infusions, we noted the start time of drug administration and then placed animals into the open field arena for sampling 15m after the start of the infusion, around 10m after the end of the infusion. Between infusion and sampling or probe we returned animals to their transport cages. Animals completed a probe trial at the end of the experimental protocol, the specifics of the delay period are described below.

Specific procedures for novel object location recognition.

Experiment 4.1A We first determined the time window in which object location memories are forgotten in rats undergoing our object location paradigm. After habituation trials, animals underwent 3 consecutive days of sampling trials. Each lasted 10 min and animals were returned to their shared transport cages for 1 h after each trial. Animals then returned to the arena after a delay period of 1 day, 3 days, 5 days or 7 days after sampling for a probe trial.

Experiment 4.1E This experiment investigated the role of caspase-3 during a memory retention interval for our object location paradigm. Habituation and sampling protocol were as in experiment 4.1A. Based on the results from experiment 4.1A, the probe trial was administered 7 d after the last sampling trial. During the retention period, animals received twice daily (AM & PM) infusions of either z-DEVD-fmk or inactive vehicle (DMSO in PBS) for 5 days across days 2-6 of the 7-day retention period where animals still are able to express significant novelty preference for the novel object location. The infusions were administered in the animal colony room, as described above.

Experiment 4.2A Here we used the nano-particle AuM to explore the role of extrasynaptic NMDAR signalling during a memory retention period of extended object location paradigm. Seven consecutive days of sampling followed 4 days of habituation. Each sampling trial took 10 min. Prior work from our group has established that this training protocol raises memory for object location that is forgotten between 7 and 10 d after sampling (Migues et al., 2019). We infused animals 24 hr after the last sampling trial for 2 days (AM & PM) in the colony room, thus a total of 4 infusion sessions. The short infusion protocol for this experiment was chosen as AuM can persist actively for extended periods (i.e., is not degraded or inactivated within hours).

Experiment 4.3A This experiment determined whether intra-hippocampal infusions of ConR prior to object location learning impaired short-term memory for object locations. After each habituation trial, animals were placed into a dark box (Black Box: detailed in section 2.3.2) for 5 min after being retrieved from the open field arena. Around 20 min prior to the sampling trial, animals received infusions of either ConR or PBS in a room different from the experimental room. During sampling, animals were placed in the open field for 20 min where two identical copies of an object were placed in opposing corners. At the end of sampling, rats were placed into the black box for 5 min and then returned to the open field for a probe trial of 3 min.

Experiment 4.3E Here, we investigated the role of ConR and AuNP-ConR during object location learning in a long-term memory paradigm. After 4 days of habituation, there were 5 consecutive days of sampling, with each sampling trial lasting 5 min. Around 20 min prior to each sampling trial, animals received infusions of either ConR, AuNP-ConR or ConG-F.

Infusions were conducted in the animal holding room. The probe trial was 24 h after the last sampling session.

4.4. Results

4.4.1. Inhibiting caspase-3 in the dorsal hippocampus during the memory retention interval preserves long-term memory for object location

First, we set out to establish the timeline of natural forgetting for object location memories in a 3-day sampling paradigm (Fig 4.1A). After habituation, we exposed cannulated animals to 3 consecutive days of sampling, each trial lasting for 10 min. Memory for object location was tested at delays of 1, 3, 5 and 7 days after the last sampling trial. Figure 1B shows that animals although there was not a significant difference in novelty preference between retention days ($F_{(3,15.5)}$=1.38, p=0.286; Fig 4.1B), animals did express significant preference for the object at the novel location 1-, 3- and 5-days after training (1d: $t_{(7)}$=3.12, p=0.017, Cohen's d = 1.1, 3d: $t_{(7)}$=2.48, p=0.042, Cohen's d = 0.876 5d: $t_{(7)}$=3.1, p=0.017, Cohen's d = 1.1) but not 7-days later (7d: $t_{(7)}$=1.05, p=0.327), suggesting that object location memory loss had occurred with a 7 day retention period. There was no significant difference in total exploration time at 60s during the probe trial ($F_{(3,15)}$=2.1, p=0.143; Fig 4.1C) suggesting similar motivation and motor capabilities across groups. There was a main effect of sampling day on exploratory activity across retention groups as revealed by repeated-measures ANOVA ($F_{(2,56)=}$ 20.845, p<0.001 , η^2_p = 0.445; see Table A .1 for *post hoc* test outcomes), suggesting animals familiarised to objects and their locations over the course of sampling. There was a significant main effect of retention group on exploratory activity (Fig 4.1D, $F_{(3,26)}$=3.30, p=0.036 η^2_p = 0.276; see Table A .2 for *post hoc* test outcomes), and the interaction between retention group and sampling was significant (F <1). Overall, we can conclude that in this paradigm, memory for object locations lasts for at least 5 days, but is forgotten by 7 days after sampling.

Next, we set out to investigate whether this time-dependent forgetting of object location memory was driven by proapoptotic signalling. Caspase-3 is a key component in the proapoptotic signalling that promotes AMPAR internalisation during long-term depression (Li et al., 2010). We therefore reasoned that caspase-3 may also be involved in the natural forgetting of long-term memory (Migues et al., 2016). To test this, we infused the capase-3 inhibitor z-DEVD-fmk into the dorsal hippocampus of animals during a 7 d memory retention interval following sampling, and tested memory for object locations 7 d after sampling, at a time when animals no longer express these memories (Fig 4.1E). Animals infused with DMSO

diluted in PBS served as our controls (V). Twice daily infusions (AM & PM) were administered starting 48 h after the last sampling trial (5 d of infusions in total). Memory for object locations was tested the following day after the final infusion. Animals infused with z-DEVD-fmk showed a significant preference for the object at the novel location while those infused with inactive vehicle did not (V: $t_{(5)}$=-0.777, p=0.472, Cohen's d = -0.317; z-DEVD-fmk: $t_{(3)}$=3.95, p=0.029, Cohen's d = 1.97, Fig. 4.1F). The difference in novelty preference between groups was significant ($t_{(8)}$=2.97, p=0.018, Cohen's d = 1.92; Fig. 4.1F). There were no significant differences in exploratory behaviour during the probe trial ($t_{(8)}$<1, Fig. 4.1G) suggesting differences in object exploration are not due to changes in motivation or motility. Exploratory activity was different across sampling trials, ($F_{(2,16)=}$ 36.16, p<0.001, η^2_p = 0.819; Fig. 4.1H) suggesting that animals familiarised to the objects and object locations over the course of sampling. Post hoc tests revealed that animals explored objects more during the first sampling trial compared to the trials on day 2 and 3 (see Table A.3 for *post hoc* test outcomes). There were no significant differences between the groups (F<1) and there was no significant interaction (F<1). Taken together, these results suggest that caspase-3 is critical for the natural forgetting of long-term memory for object locations in the hippocampus.

4.4.2. Blocking exNMDAR activity prevents the natural time-dependent forgetting of long-term memory for object locations

Activation of the intrinsic proapoptotic pathway is associated with NMDARs located extrasynaptically (Hardingham & Bading, 2010). Therefore, we tested whether blocking these receptors during a retention period would allow object location memories to persist beyond their natural lifespan. To inhibit exNMDARs we used the gold nanoparticle AuM, which uses memantine to block NMDAR currents. Given the long persistence of this drug (Savchenko et al., 2016), we sought to determine whether a small number of infusions could promote memory persistence over a longer delay period. As such, we trained animals on a previously established object location paradigm in which rats undergo 7 d of sampling, and typically forget object locations between 7 and 10 days (Migues et al., 2019). Two days after the final sampling session, we infused animals twice per day (AM & PM) for two consecutive days (i.e., 4 sessions of infusions) and tested memory for objects 10 d later (i.e., 14 d after last sampling trial). Due to unforeseen circumstances[1], the data presented here are that of animals infused with AuM

[1] Our QNAP lab server was successfully attacked by QLocker Ransomware in 2020 – a significant amount of our data was lost.

compared to animals undergoing a separate yet identical protocol infused with PBS during the retention period (Fig 4.2A). This dataset only comprises exploration scores for sampling day 1 and sampling day 7.

We found that only animals infused with AuM showed significant novel location preferences (AuM $t_{(5)}$= 4.04, p=0.01, Cohen's d = 1.65; V $t_{(4)}$= 0.927, p=0.406, Cohen's d = 0.415; Fig 4.2B). The difference in novelty preference between groups was significant ($t_{(9)}$= 2.55, p=0.031, Cohen's d = 1.545; Fig 4.2B). Exploration time was the same for both groups during the probe trial ($t_{(9)}$= -2.0, p=0.076; Fig 4.2C). Exploratory activity decreased from the first to the last sampling trial ($F_{(1, 9)}$ = 25.914, p<0.001, η^2_p = 0.742; Fig 4.2D), and at the same extent for both groups (F<1; interaction term: F<1). These findings making it unlikely that the differences observed in novelty preference were due to variability in expression of exploratory behaviour. Our results suggest that selective inhibition of exNMDARs prevents the time-dependent active-decay of long-term memory for objects.

4.4.3. Blocking extrasynaptic NMDA receptors does not impair forming long-term object location memories

While our studies with AuM point toward a selective role in exNMDARs for forgetting, the long half-life of the drug makes it difficult to isolate its effects on different memory phases, i.e., on learning, consolidation, forgetting, and retrieval. As such we decided to use an alternative compound to address this question, namely AuNP-ConR, which uses Conantokin-R (ConR), a peptide that is naturally degraded after 2-4 hours (Blandl, Warder, Prorok, & Castellino, 2000; Valente et al., 2020). The ConR peptide has not been used *in vivo* before, therefore, we first set out to determine if it affected behaviour in a manner consistent with other NMDAR antagonists. As discussed in Chapter 1, spatial learning and memory critically rely on the hippocampus i.e., infusing the well-established NMDAR antagonist AP5 prior to object sampling impairs memory for object locations tested 20 min later (Yamada et al., 2017). We therefore set out to determine whether infusing ConR prior to object sampling would similarly impair novelty preference for a new location in a short-term memory test. We exposed rats to a single 20-min long sampling trial, and then administered the probe trial 5 min thereafter (Fig. 4.3A).

We found that while animals infused with inactive vehicle showed significant novelty preference, indicating memory for object location ($t_{(5)}$ = 7.3, p < 0.001, Cohen's d = 2.98, Fig 4.3B), those infused with ConR did not ($t_{(5)}$= 1.79, p = 0.133, Fig 4.3B). The difference in

novelty preferences was significant between groups (independent sample *t*-test: $t_{(10)} = 3.75$, $p = 0.004$, Cohen's d = 2.16, Fig 4.3B). There was no significant difference in exploration activity during sampling ($t_{)} < 1$, Fig 4.3D) or Probe ($t<1$, Fig 4.3C), suggesting that our infusions of ConR did not impair motility or motivation. Our results suggest that ConR acts in a manner consistent with other well-established NMDAR antagonists that prevent long-term memory formation.

Given the short time delay between sampling and probe trials in our previous experiment, we cannot determine whether NMDAR antagonism by ConR impaired encoding or recall of object location memory. We therefore sought to isolate these processes by using a long-term object paradigm, where animals receive 5 consecutive days of a daily sampling trial, each lasting 5 minutes. We infused animals with either ConR, AuNP-ConR or ConG-F (the inactive peptide, serving as control) around twenty minutes prior to each sampling trial and tested memory for object locations 24 h after the final trial (Fig. 4.3E).

We found a significant group difference in novelty preference ($F_{(2, 11.2)} = 5.84$, $p=0.018$, Fig. 4.3F). Post-hoc comparisons (Tukey's) revealed a significant difference only between ConG-F and ConR ($p = 0.06$) groups and not between other group comparisons (ConG-F v AuNP-ConR $p = 0.945$, ConR v AuNP-ConR $p = 0.057$). Animals infused with ConG-F and AuNP-ConR expressed novel location preference significantly above chance, while those infused with ConR did not (ConG-F: $t_{(7)}=2.95$, $p=0.021$, Cohen's d = 1.04; ConR: $t_{(6)}=0.0891$, $p=0.932$, Cohen's d = 0.034; AuNP-ConR: $t_{(4)}=5.08$, $p=0.007$, Cohen's d = 2.27, Fig 4.3F). There were no differences in exploration time between the groups during the probe trial, although we observed a trend ($F_{(2, 10.7)} = 3.07$, $p=0.088$, Fig. 4.3G). Animals reduced exploratory across the sampling trials ($F_{(4,68)} = 28.21$, $p<0.001$, $\eta^2_p = 0.624$, Fig. 4.3H; see Table A.4 for *post hoc* comparisons), and differently between groups ($F_{(2,17)} = 3.73$, $p=0.045$, $\eta^2_p = 0.305$). Post hoc comparisons determined that this effect was driven by the difference between groups ConG-F and AuNP-ConR ($p = 0.04$), with the latter exploring objects for a mean of 15.14 seconds longer than the former across all sampling trials combined (other comparisons; ConR v AuNP-ConR $p = 0.133$; ConG-F v ConR $p = 0.802$; Table A.5). Although visual inspection of the data suggest that the strongest difference in exploratory activity between these groups emerged on the fourth day of Sampling (AuNP-ConR explored objects for a mean of 35 seconds more than ConG-F, and a mean of 30 seconds more than ConR, the interaction was not significant ($F_{(8,68)} = 1.19$, $p=0.318$, $\eta^2_p = 0.123$, Fig. 4.3H). Taken together, these results

show that ConR impaired learning of object locations, while AuNP-ConR did not, suggesting exNMDARs are not required for spatial learning.

4.5. Figures

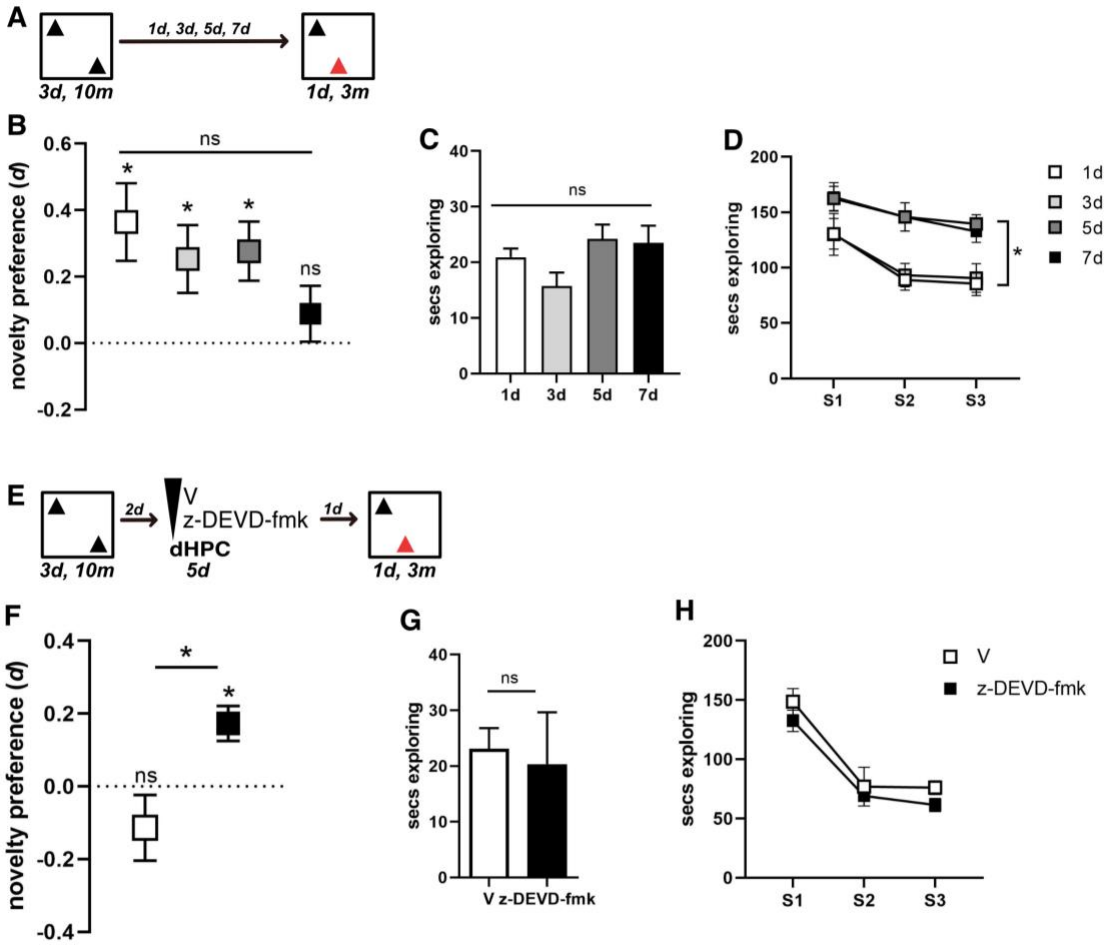


Figure 4.1. Inhibiting caspase-3 during memory retention in active decay of long-term memories for object location. ***A-D*** Long-term memory for object locations is naturally forgotten by 7 days. ***A*** Schematic diagram for novel object location protocol. ***B*** Novelty preference for rats tested 1d (*n*=8), 3d (*n*=8), 5d (*n*=8) and 7d (*n*=8) after final sampling trials. ***C*** Exploration time after 60s for each retention group during probe. ***D*** Total exploration time across sampling days for each retention group. ***E-H*** Inhibition of caspase-3 during the memory retention interval prevent active decay of long-term memory for object locations. ***E*** Schematic diagram for novel object location protocol. ***F*** Rats infused with z-DEVD-fmk (*n*=4) had *d* values significantly above chance while those infused with inactive vehicle (*n*=6) did not. ***H*** Exploration time after 60s for each retention group during probe. ***I*** Total exploration time across sampling days for each retention group.

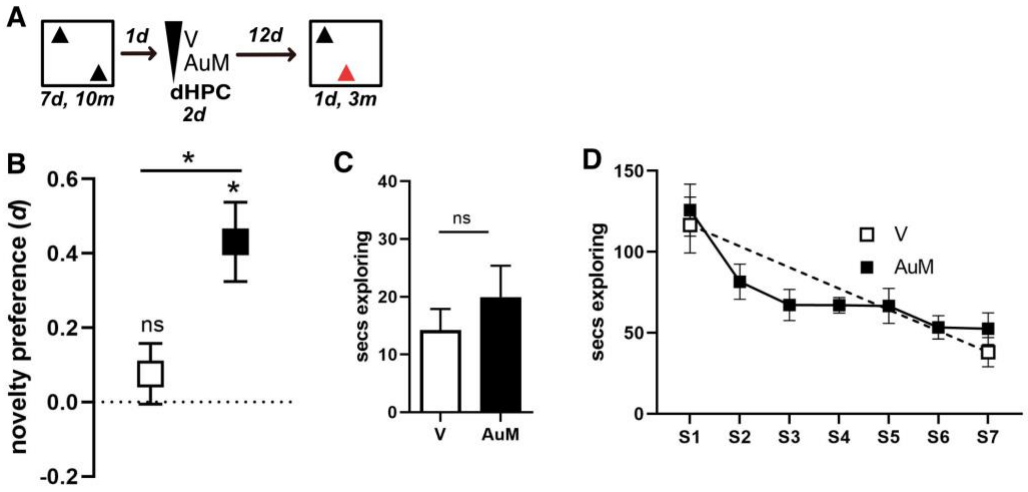


Fig 4.2 Blocking exNMDAR activity prevents the natural time-dependent forgetting of long-term memory for object locations. ***A-D*** Inhibiting exNMDARs during the memory retention interval prevent active decay of long-term memory for object locations. ***A*** Schematic diagram for novel object location protocol. ***B*** Rats infused with AuM (n=6) had d values significantly above chance while those infused with inactive vehicle (n=5) did not. ***C*** Exploration time after 60s for each retention group during probe. ***D*** Total exploration time across sampling days for AuM group, exploration for sampling day 1 and 7 only for V infused group.

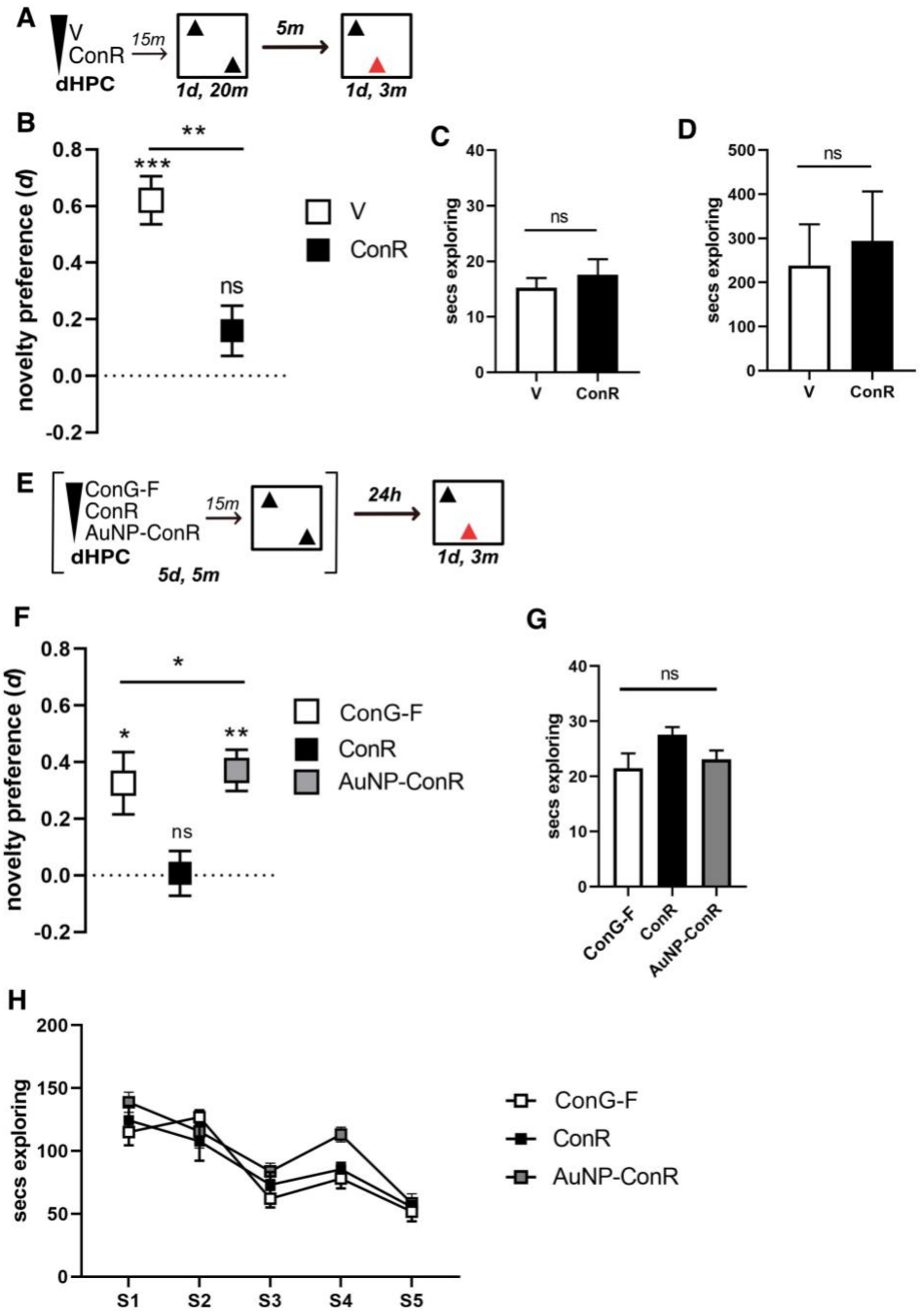


Figure 4.3 Blocking extrasynaptic NMDA receptors does not impair forming long-term object location memories ***A-D*** Inhibition of NMDARs with ConR impairs short-term memory for object locations. ***A*** Schematic diagram for short-term novel object location protocol. ***B*** Rats infused with inactive V (*n*=6) had *d* values significantly above chance while those infused with ConR (*n*=6) did not. ***C*** Exploration time after 60s for each retention group during probe. ***D*** Total exploration time across sampling for each infused group. ***E-H*** Blocking all NMDARs with ConR but not selectively extrasynaptic NMDARs with AuNP-ConR impairs object location learning. ***E*** Schematic diagram for novel object location

protocol. ***F*** Novelty preference for rats infused with ConG-F (*n*=8), ConR (*n*=7) and AuNP-ConR (*n*=5). ***H*** Exploration time after 60s for each retention group during probe. ***I*** Total exploration time across sampling days for each infusion group.

4.6. Discussion

Here we tested whether the loss of long-term object location memories involves pro-apoptotic signalling in the dorsal hippocampus. We found that inhibiting the pro-apoptotic capase-3 in the dorsal hippocampus of rats during a memory retention period extended memory for object locations beyond their natural lifespan. A similar result was observed when we selectively inhibited extrasynaptic NMDARs during the memory retention interval, which are upstream of the intrinsic pro-apoptotic signalling that leads to caspase 3 activation. We found that blocking exNMDARs did not affect acquiring location memories, while blocking both synaptic and extra-synaptic NMDARs did. Taken together, our results suggest that (1) proapoptotic signalling drives natural time-dependent forgetting, and (2) NMDARs have different roles in memory processes depending on their synaptic location.

4.6.1. Caspase-3 drives active decay of long-term memory

Inhibiting caspase-3 in the hippocampus of rats during a memory retention period prevented the time-dependent forgetting of object location memory. Our findings align with recent work investigating the role of GSK-3β, a downstream target of caspase-3 activity, in the natural decay of long-term memory. Specifically, caspase-3 disinhibits GSK-3β by cleaving its regulatory protein Akt. Inhibition of GSK-3β impairs NMDAR-induced AMPAR internalisation in LTD (Du et al., 2010), and prevents LTP decay (Yi et al., 2018), thus suggesting that it promotes AMPAR internalisation during synaptic weakening. In alignment with this, inhibiting GSK-3β during a memory retention period in mice sustains memory for object locations beyond that of control animals (Yi et al., 2018). Jointly with our data, these findings demonstrate a critical role for proapoptotic signalling in the time-dependent forgetting of established memory and supports the prediction that active decay recruits proapoptotic signalling.

While our results reveal a novel role for caspase-3 in forgetting during active decay in rats, a number of investigations have explored its role during initial memory formation. Acquiring new patterns of synaptic connectivity relies on both the rapid strengthening and the weakening of synaptic weights, in which caspase-3 seems critical for the latter. For instance, mice with a genetic deletion of caspase-3 express elevated levels of GluA1- and GluA2-AMPARs following learning (Lo et al., 2015), suggesting impaired weakening of synaptic strength. In song birds, caspase-3 is found in synapses bound to its regulatory protein XIAP, which is released after novel song learning, leading to a rapid increase of caspase-3 expression

after 10 minutes (Huesmann & Clayton, 2006). Inhibition of caspase-3 during this novel song learning impairs song habituation. Correspondingly, impairing caspase-3 immediately after place avoidance learning in rats impairs later expression of these spatial memories (Dash et al., 2000), suggesting caspase-3 also critically supports memory formation. Given the short temporal window, capsase-3 activation during memory formation aligns with the notion that a fast-clutch like mechanism drives caspase-3 during learning, while during active decay, its activation may rely on a slower, direct drive of the full intrinsic proapoptotic pathway (Mukherjee & Williams, 2017). A direct test of this has yet to be carried out, yet, altogether, these findings highlight a critical role for caspase-3 in various phases of memory processing.

One limitation of our results is the use of z-DEVD-fmk, which in addition to targeting capase-3, also reduces the activity of caspases -6, -7, -8 and -10. To selectively impair capase-3 activity, future work could employ shRNA knockdown strategies, which would selectively block the translation of caspase-3, and thus reduce off-target effects.

4.6.2. Active decay is triggered by exNMDARs

Selective inhibition of exNMDARs prevented the time-dependent forgetting of long-term memory for object locations. This extends prior work showing that inhibition of both sNMDARs and exNMDARs by AP5 in the hippocampus preserves spatial memory (Migues et al., 2019; Shinohara & Hata, 2014). Furthermore, our results align with the finding that selective inhibition of GluN2B-containing NMDARs attenuates active decay, which, as discussed in Chapter 1, are strongly expressed at extrasynaptic sites (Paoletti, Bellone, & Zhou, 2013).

While our data suggest that exNMDAR signalling and caspase-3 activity converge on a common pathway to promote active decay, there is an alternative explanation for our results. Namely, NMDAR-dependent AMPAR internalisation has also been reported to occur via a separate capase-2 dependent pathway during LTD (Xu et al., 2019). Here, NMDAR currents activate caspase-2, an apoptosis initiator protease, which inhibits the mTORC2 pathway. Inhibition of mTORC2 signalling releases GSK3β from its inhibition by Akt, thus allowing GSK3β to promote AMPAR internalisation (Xu et al., 2019). Mice with a genetic deletion of caspase-2 are impaired at reversal learning in the Morris water maze and express contextual fear memory far longer than control animals. Therefore, it will be critical for future studies to determine whether a caspase-3 dependent pathway is driving active decay after exNMDAR activation. One possible experiment to address this question would be to enhance NMDARs

with an agonist, such as D-Serine, after capase-3 or caspase-2 activity has been blocked during a memory retention period. Our lab has previously shown that D-Serine accelerates the natural decay of long-term object location memories, thus, if this treatment slows down memory loss, it would provide support for the idea that NMDAR and caspase-3 signalling are causally linked during active decay of long-term memory.

4.6.3. Synaptic vs extra-synaptic NMDARs in memory processing.

The long persistence of AuM limits our ability to study its effect on distinct memory phases to distinguish effects of encoding from those on active forgetting and memory recall. We therefore addressed this question with a different nanoparticle, which uses ConR as an antagonist that is more rapidly metabolized i.e., around 2-4 hours *in vitro* (Blandl et al., 2000). This approach allowed us to demonstrate that the synaptic location of NMDARs determines how they influence memory processes, in that synaptic NMDARs are critical for learning, while extrasynaptic NMDARs drive forgetting.

How do neurons control the activation of these populations of NMDARs that exert opposing effects on synaptic plasticity? One possible explanation may lie in the circadian regulation of astrocytes, glial cells that have processes that encapsulate synapses to regulate synaptic activity (Perea, Navarrete, & Araque, 2009). During awake periods, astrocytic processes encase synapses, localising glutamatergic signalling to the synaptic cleft, while during sleep phases, these astrocytic processes retract and allow glutamatergic spill-over to extrasynaptic regions (Bellesi, de Vivo, Tononi, & Cirelli, 2015). As such, astrocytes reduce or prevent activation of extrasynaptic NMDAR during periods of activity and promote their activation during periods of sleep, which aligns with an assumption of active decay theory prediction that this type of forgetting process predominantly unfolds during the sleep (Hardt et al., 2013). Alternatively, astrocytes have been shown to directly activate NMDARs by release of glutamate (Park et al., 2013), yet whether such a mechanism occurs exclusively for exNMDARs remains to be determined.

The use of gold nanoparticles in basic research and clinical practise has drastically risen in recent years, yet there are many unresolved questions as to how these compounds interact with biological systems which remain to be addressed. For instance, when injecting these compounds into the blood stream, a number of proteins bind to and cover the surface of some nanoparticles, which modifies the original surface chemistry and changes the biological properties of the compound (Ma, Hong, & Ding, 2020). Although the nanoparticles used in this

report employ coating constructs (i.e., thiol PEG), which prevent such protein interactions and aggregate formation (Savchenko et al., 2016; Valente et al., 2020), a critical control for our experiments may be to infuse an inactive gold particle (i.e. AuNP-Con-F-G), to ensure the effects reported are not solely due to the presence nanoparticles.

Chapter 5

General Discussion

5.1. Summary

Active decay theory provides a theoretical framework suggesting biologically plausible mechanisms for two major forms of natural memory loss; interference and decay. Regarding interference, the theory proposes that content representations in neocortical areas are at a high risk of overlapping, a limitation of the circuit architecture which facilities this form of forgetting. Due to poor pattern separation capacities, representations in these areas, notably newly acquired ones that have not yet stabilized, are therefore at risk of perturbation by ongoing sensory stimulation of the same modality, a process that can, eventually, lead to states of catastrophic interference, resulting in amnesia. Unlike neocortical networks, the circuit architecture of the hippocampus provides highly efficient pattern separation, generating orthogonalized traces even for highly similar input. When event-like, 'declarative'-type memories are formed, they depend on an index representation in the hippocampus linking to content representations in neocortical areas. This way, the hippocampus indirectly provides pattern separation for neocortical areas, allowing these representations to retain coherence and protecting them from interference arising from ongoing sensory stimulation during states of instability. Because of its effective pattern separation, forgetting in terms of interference is unlikely in the hippocampus, and active decay is instead the major cause of natural memory loss in this region, while this process is one among other sources of forgetting in other brain areas. Active decay is assumed to predominantly occur during sleep, when new experiences are not encoded, and it reverses synaptic modifications induced by learning and memory, reducing synaptic efficacy, a process that is suggested to recruit pro-apoptotic signalling. This thesis aimed to test and further explore these two central predictions of active decay theory.

Chapter 3 explored interference within the framework of active decay theory to determine whether one role of the hippocampus is protecting stabilizing object memories in the perirhinal cortex of rats from interference arising from ongoing sensory stimulation. We found that inactivating the dorsal hippocampus immediately before and after object encoding lead to amnesia, which arose from an increased susceptibility to interference, stemming from continued sensory experience as reducing sensory experience after learning lead to long-term

memory for objects in the absence of a functioning hippocampus. The susceptibility of new object memories to interference lasted no more than one hour. We observed that inactivating the hippocampus in these experiments lead to higher levels of neuronal activity in the perirhinal cortex when animals were exposed to everyday sensory experience after object learning, compared to when post-learning stimulation was reduced. The levels of neuronal activity reported under reduced sensory stimulation were comparable to those in animals with an intact hippocampus under normal experience. Correspondingly, these two conditions lead to long-term memory formation for objects in our behavioural studies, which, in line with the predictions of active decay theory, suggest that amnesia for objects arose from a state of catastrophic interference in the perirhinal cortex. To prevent excessive activity in extra-hippocampal areas, active decay theory assumes that hippocampal learning of a new index occurs synchronously with cortical learning related to memory content which indirectly provides interference protection during initial acquisition. When exploring this prediction, we found that blocking plasticity in the hippocampus during object encoding induced amnesia under normal, but not reduced sensory stimulation, suggesting hippocampal learning is indeed required to protect stabilising object traces from interference. Given that the hippocampus is critical for object memory reconsolidation, we finally tested whether it also performed the role of interference protection against ongoing sensory experience during trace restabilisation. We found that limiting sensory stimulation after hippocampal reconsolidation blockade prevented amnesia that arose under normal sensory experience, mirroring our findings in initial object consolidation. From these investigations we conclude that the role of the hippocampus is not to directly consolidate or reconsolidate memory, but rather to protect stabilizing memory from interference arising from ongoing sensory experience during these phases of heightened synaptic plasticity. Thus, amnesia arising from hippocampal dysfunction is due to excessive interference and not from a lost capacity to consolidate new memory.

In chapter four, we investigated predictions about cellular pathways underpinning active decay. We found that inhibiting the pro-apoptotic caspase-3 as well as blocking the activity of extrasynaptic NMDARs in the dorsal hippocampus of rats during a memory retention period prevented the natural time-dependent memory loss for object locations. Notably, blocking extrasynaptic NMDAR receptors, while preventing active decay, did not affect learning of object locations. Therefore, we conclude that proapoptotic signalling drives the time-dependent forgetting of hippocampal memories, and that NMDARs contribute differently to memory formation and memory loss depending on their location.

5.2. Theoretical implications

5.2.1. The nature of anterograde amnesia

The importance of the hippocampus for memory was discovered with patient H.M., who could no longer form new long-term declarative memories following bilateral removal of his hippocampus. Leading explanations for this anterograde amnesia are associated with the idea of systems consolidation, according to which the hippocampus plays a time-limited role in declarative memory, gradually supporting the integration of newly formed memories into long-term memory in neocortical areas, a process that takes weeks to months in rodents and potentially years in humans. When this consolidation process is interrupted, anterograde amnesia can arise. As detailed in Chapter 1, according to the 'standard' model of systems consolidation, over time and, without affecting the quality of the memory itself, hippocampal activity (e.g., replay) promotes the stabilisation of neocortical components while the hippocampal memory component is lost. Expanding on this notion, complimentary learning systems theory specifies that newly acquired information in the hippocampus is replayed in an interleaved manner with existing cortical knowledge, thus integrating new information into the neocortex (i.e., the hippocampus actively 'teaches' the neocortex) to avoid states of cortical catastrophic interference. In contrast, multiple-trace theory asserts that for true episodic memories, the hippocampal component is never lost, as it is necessary to provide the unique "flavour" of these vivid memories of events. If it is lost for a particular memory, which can happen naturally or because of damage to the hippocampus, then the memory will become factual, or "semanticized", decoupled of the time and place where it was originally encoded. Taken together, all these accounts rely on the common assumption that some activity originating from the hippocampus interacts with the neocortex to actively drive long-term memory formation. A logical extension of this position is that should the hippocampus be damaged, this operation will cease, giving rise to anterograde amnesia by virtue of a lost ability to consolidate new declarative memory.

In Chapter 3 we provide direct evidence against this idea. We found that inactivating the hippocampus of rats before object sampling lead to amnesia for objects when animals were exposed to normal sensory experience after learning – an outcome expected by these positions –, but this effect is attenuated when post-learning sensory stimulation was reduced for 1 hour – an outcome these theories do not anticipate. In other words, rats with an inactivated hippocampus were able to form long-term memory for objects provided sensory interference was reduced. As mentioned in Chapter 3, this result extends findings in human patients

suffering from anterograde amnesia, where 10 minutes of quiescence after story allowed memory for the prose to persist long-term (Alber et al., 2014). Therefore, we conclude that anterograde amnesia following hippocampal damage arises from excessive interference which disrupts consolidation processes in extra-hippocampal areas.

The idea that amnesia arises from an "undue prominence of interference phenomena" was first proposed in Warrington & Weiskrantz's (1970) retrieval-failure hypothesis of amnesia. Here, patients were predicted to learn and consolidate memories normally but medial temporal lobe damage impaired the ability to selectively recall an appropriate memory, instead retrieving too much information, which gave rise to excessive trace-competition and interference-induced amnesia. Our results do not readily support a retrieval-based mode of interference, as animals expressed novelty preference when the hippocampus was inactivated prior to recall. Furthermore, we found that reducing sensory stimulation immediately after, but not 1 hour later rescued amnesia for objects in animals with an inactivated hippocampus, suggesting a distinct temporal window after learning during which amnesia emerges.

While our findings struggle to align with the discussed positions on amnesia, they are readily predicted by active decay theory. Under this framework, hippocampal damage gives rise to states of catastrophic interference in the cortex, as continual, uncoordinated encoding in this region disrupts just previously encoded memories. Should cortical activity be attenuated after learning (i.e., by reducing ongoing sensory stimulation), active decay theory assumes that new memory traces can stabilize into long-term memory because their local (synaptic) consolidation is not perturbated by ongoing sensory inputs. Thus, amnesia from a dysfunctional hippocampus, arises because the stabilization process for newly acquired memory is exceptionally vulnerable to disruption from ongoing experience.

It is important to note here that while complimentary learning systems also states that amnesia is due to catastrophic interference, this account differs from active decay theory in the mechanism by which the hippocampus is predicted to prevent this under healthy conditions. This will be explored in the next section.

5.2.1. The role of the hippocampus in long-term memory formation

If amnesia arising from hippocampal damage is not due to a lost ability to consolidate, what then, is the role of the hippocampus in long-term memory formation under normal conditions? The series of results presented in chapter 3 suggest that the hippocampus protects stabilizing

object traces from sensory interference related to ongoing experience. We found that reducing sensory stimulation during phases of synaptic plasticity in the hippocampus, i.e., before object learning and after retrieval, prevents amnesia, preserving long-term memory for objects. Taken together, this suggests a general function of the hippocampus in somehow "managing" the "natural" effects of sensory stimulation during states of object trace instability.

We found that during learning, inactivating the hippocampus before object presentation led to excessive levels of neuronal activity in the perirhinal cortex under a situation of normal sensory experience. Yet, when sensory stimulation was reduced, the levels of neuronal activity were comparable to those in intact animals with normal sensory experience. From this, it seems that the hippocampus acts to preserve object memory by constraining neuronal activity related to ongoing sensory stimulation in the perirhinal cortex, thus preventing states of overexcitation. These findings cannot be explained by complimentary learning systems theory, which assumes the hippocampus prevents cortical catastrophic interference by replaying newly acquired information (stored in the hippocampus) in an interleaved manner with already acquired information housed in the neocortex. Given that hippocampal inactivation would prevent such a process, and considering the short temporal window that our immunohistochemical studies capture (i.e., around 5 min after learning), interleaved training cannot account for the differences we see in neuronal activity as well as in behaviour. Instead, our findings suggest that the hippocampus provides a different function to promote long-term memory formation in that it prevents excessive interference, as active decay theory suggests. As discussed in section 3.6., one possible mechanism may be that the hippocampus drives phase-pruning in the perirhinal cortex, where neuronal activity related to everyday sensory stimulation is reduced under distinct oscillatory regimes (Ahn et al., 2019). Yet, little is known about this process and what role it may play in memory formation. An important question that future studies should address is therefore how the hippocampus limits neuronal excitability in real time.

We also explored the role of plasticity in the hippocampus during object memory formation. We found that blocking plasticity prior to sampling also caused amnesia for objects when there was normal post-learning sensory stimulation, but when it was reduced. These findings, together with our other results, strongly suggest that hippocampal learning is not required to encode information about the objects, furthering the case against the notion that events are initially encoded in the hippocampus before they are transferred into the neocortex. As such, at least in the case of object memory, whose *content representation* is dependent on the perirhinal cortex, our results suggest that the hippocampus encodes a form of "auxiliary" representation that may not represent any content at all but rather serves the mechanism

promoting memory consolidation in extra-hippocampal areas. Like other accounts, active decay theory assumes that the content of declarative memory is supported by neocortical areas, while the hippocampus encodes a representation that acts to "bind" together sparse and distributed cortical elements. As such, it is unclear whether reducing sensory interference after "locale" or allocentric spatial learning i.e., acquiring a memory that exclusively depends on the hippocampus for expression, would also permit long-term memory formation in the absence of a functioning hippocampus.

The results presented here do not contradict findings that hippocampal activity over longer periods of time promotes long-term memory formation. While we show that it is *possible* to form long-term event-like memory in the absence of a hippocampus, obviously organisms do not pause and stop attending to ongoing sensory information to allow recent memory traces to stabilize sufficiently before moving on. Yet, if the hippocampus is only required for a short time after learning for these types of declarative-style memories (i.e., at least up to an hour as reported here), what then, might it contribute when it would remain engaged for longer periods, days and weeks, as some theories and studies suggest? Here we can only offer a few speculative answers.

Firstly, the persistence of a hippocampal trace may promote the longevity of extra-hippocampal memory. Support for this comes from a recent study which artificially prevented forgetting in hippocampal pyramidal neurons during an object recognition task. Rac-1 is a Rho-GTPase that modulates actin polymerisation and weakens synapses by dissembling the cytoskeleton supporting spine morphology, which drives the steady forgetting of memory in active decay (Davis & Zhong, 2017). Interestingly, constitutive inhibition of Rac-1 in hippocampal pyramidal cells of mice extended object memory persistence beyond that of intact animals (Liu et al., 2016). Reintroducing animals to the same open field arena but with two new objects 22 hours after sampling and 2 hours prior to a probe trial to assess memory for the original objects, impaired memory for the original objects in intact animals but not with those under hippocampal Rac-1 inhibition. From these findings, the authors concluded that blocking hippocampal Rac-1 prevented forgetting from retroactive interference. Yet, this particular paradigm makes it difficult to assess whether the interfering objects drove retro- or pro-active interference as impairments could be related to recall of the previously seen objects (retroactive interference) or learning (which drives expression of novelty preference) of the new objects, respectively. Certainly, if retroactive interference did drive this result, it was likely due to trace competition as second learning of objects occurred after the initial stabilization time-window during which long-term memory formation is sensitive to this form of interference, as we have

studied in this thesis. That said, these findings demonstrate that extending the lifespan of the hippocampal trace promotes object memory (i.e., a cortically based memory) persistence and minimizes memory loss from task-related interference.

Secondly, there is increasing evidence that the presence of a hippocampal trace in a network of neuronal ensembles supporting a memory correlates with the ability to update this memory. For instance, recent work found that when animals are conditioned to a tone under a weak training protocol (i.e., 1 tone-shock pairing), recalling the fear memory induces a malleable state allowing for memory updating, whereas a stronger training protocol (10 pairings) leads to rigid memories that cannot be updated (Haubrich, Bernabo, & Nader, 2020). Analysis of the brain networks which support recall of these two memory types one day after training revealed a stark contrast in the connectivity patterns. While the amygdala functionally interacted with the hippocampus in 'weak' conditioning, the hippocampus was almost entirely disconnected from the amygdala under 'strong' fear conditioning (Haubrich & Nader, 2023). This suggests that without a hippocampal trace, the ability to update a memory after initial formation may be compromised.

Finally, there is large body of literature highlighting the beneficial role of hippocampal activity during sleep in long-term memory formation. Hippocampal replay i.e., the reactivation of neuronal sequences previously active during event learning, occurs within distinct bursts of sharp-wave ripple activity which is temporally correlated with cortical sleep spindles which are another form of coordinated activity burst (Goto & Hayashi, 2023; Siapas & Wilson, 1998), that are thought to actively strengthen the synaptic connections underlying recently acquired memory (Klinzing, Niethard, & Born, 2019). Indeed, when sharp-wave ripples are selectively blocked during a 1 hour sleep period after learning, animals show impaired spatial memory acquisition over two weeks of daily training (Girardeau, Benchenane, Wiener, Buzsáki, & Zugaro, 2009), lending support to the proposal that sleep-dependent hippocampal replay may sub serve the neural process that drives systems consolidation (Wilson & McNaughton, 1994). As discussed in section 3.6., some data used to support this claim was presented in rats forming long-term object memories, whose content representations are housed in extra-hippocampal areas (the perirhinal cortex). To briefly reiterate, this study reported that inactivating the hippocampus immediately after object learning, and just before a period of sleep, greatly reduced the duration of hippocampal sleep spindles and impaired long-term memory for objects that was otherwise present in intact animals (Sawangjit et al., 2018). While taken as evidence for the critical role of hippocampal activity during sleep to boost extra-hippocampal representations, our data provide an alternative explanation. Namely, that sleep permits states

of reduced sensory stimulation which permit long-term memory formation and therefore hippocampal replay may not be *necessary* to consolidate new memory. Instead replay may be related to the persistence of these memories i.e., the longevity of object recognition memory as described above could be due to an increased proportion of hippocampal replay events. Alternatively, emerging animal studies have highlighted the importance of hippocampal activity during sleep for inferential learning, where organisms are able to form links between events and knowledge acquired on separate occasions (Barron et al., 2020). Thus, hippocampal replay appears critical for offline processing of already established memory that allows organisms to perform flexible behaviour (Barron et al., 2020).

5.2.2. The persistence of hippocampal traces

The observation that recalling both recent and remote memory can recruit the hippocampus raises the question of whether declarative memory ever becomes fully hippocampal independent. As discussed previously (section **1.2.6.1.3.**), multiple trace theory asserts that episodic memory is always reliant on the hippocampus for its expression, and that each time such a memory is retrieved (or reactivated), a new, additional hippocampal trace is generated, which is retained indefinitely. Thus, over time, hippocampal traces are multiplied, which, together, permit the recollection of detailed episodic memory (Nadel & Moscovitch, 1997). Yet, an alternative view was put forward by Barry and Maguire (2019). In agreement with multiple trace theory, they argue that the hippocampus critically supports episodic memory recall, regardless of age. In contrast, their position assumes that all traces within the hippocampus are eventually lost, such that its reengagement generates a new individual trace, which then, over time, again is lost. To support their claim, they draw from recent findings in transgenic mouse lines in which the neurons initially recruited during the formation of hippocampal memory are labelled. In one such study, it was found that the population of active cells in initial contextual fear conditioning drastically decreased during memory recall weeks later (Attardo et al., 2018; Barry & Maguire, 2019a). In other words, re-exposure to the same environment could not reproduce the same pattern of activity in the hippocampus at a later time, suggesting that a stable long-lasting trace does not reside there to support long-term episodic-like memory.

Our investigations reported in chapter 4 into the active decay of hippocampal memory does not readily support the stance that all traces in the hippocampus are preserved. That said, neither can we conclude that there may exist some hippocampal traces or ensembles of

connections that are never lost, either by virtue of being continually reused or by some yet to be determined resistance to forgetting processes. It seems, however, that the persistence of hippocampal memory traces may be overestimated in various theories of systems consolidation (Hardt & Sossin, 2020).

5.2.3. Learning and forgetting in synapses

NMDA receptors have traditionally been regarded as the critical "association detector" underlying learning and memory formation. While well supported, an emerging stream of research has highlighted an additional role for these receptors in driving forgetting (Migues, Wong, Lyu, & Hardt, 2019; Shinohara & Hata, 2014). One proposed hypothesis as to how these receptors can regulate opposing memory processes is that specific subunit compositions of NMDARs distinctly favour each type of synaptic plasticity. Yet, investigations exploring this notion have failed to establish such a relationship (Hardt et al., 2014), and instead suggested that NMDAR subunit type co-ordinated states of metaplasticity (for both forgetting and learning), in that the ratio of GluN2B to GluN2A determines whether a synaptic connection is prone to alterations (i.e. by learning or active decay) or relatively resistant to it. Thus, the question of how NMDARs differentially drive learning or forgetting has remained largely unresolved.

In chapter 4, we provide evidence that the synaptic location of NMDARs determines whether they support learning or forgetting. Namely, synaptic NMDARs promote learning while extrasynaptic NDMARs promote active decay. As discussion previously, the activity of NMDARs at these different locations may be critically regulated by circadian changes of astrocyte wrapping around synaptic connections. This speculative mechanism to regulate active decay would support the prediction that this forgetting process primarily unfolds during periods of sleep, as suggested by active decay theory.

5.2.5. Future Directions

A number of key experiments would greatly add to the work presented here. Firstly, our investigations in chapter 3 revealed a novel role of the hippocampus during long-term memory formation, but many of the parameters of the effect have yet to be detailed. For instance, while our results suggest that at least 1 hr of reduced post-learning sensor stimulation is sufficient to allow object memories to stabilize into long-term memory under hippocampal inactivation, complimentary data in humans has shown that only 10 minutes of wakeful rest boosts long-

term memory formation in amnesic patients (Alber et al., 2014). Thus, it may be the case that the critical time window in which recently acquired object memories dependent on the hippocampus for protection from interference related to sensory experience is much shorter than the hour investigated here. As such future investigations could explore whether shorter time periods within the Black Box (i.e., 30 or 10 mins) after object learning also lead to long-term memory formation for objects under hippocampal inactivation. Conversely, inactivating the hippocampus at similar time points (i.e., 10 or 30 mins after learning) would also further our understanding on the temporal window in which the hippocampus promotes initial long-term memory formation.

Secondly, while the data presented in chapter 4 provides new insights on the neurobiology of active decay, this line of the work needs considerable expansion. For example, we still have yet to determine whether blocking extra synaptic receptors with AuNP-ConR similarly prevents active decay of long-term memory for object locations, replicating our results with AuM. Furthermore, while we showed evidence for the contribution of caspase-3 in the active decay of long-term memory for object locations, a number of other components of our proposed proapoptotic pathway have yet to determined. For instance, futures studies should explore the role of caspase-9 or cytochrome c in active decay to further the case that this pathway critically underlies time-dependent forgetting. As discussed in section 4.6., a causal experiment to determine whether exNMDARs signalling in active decay directly activates the discussed proapoptotic signalling is also essential to rule out whether other potential signalling pathways (i.e., caspase-2 related) may be driving AMPAR internalisation. Finally, while neocortical spines are more stable and persistent than those found in the hippocampus (Yang, Pan, & Gan, 2009) previous studies have shown that active decay unfolds in the infralimbic cortex (Migues et al., 2016), which at least, suggests the process is not exclusive to the hippocampus, but has yet to be fully investigated.

5.3. Active Decay theory

The results reported in this thesis support central predictions of active decay theory. As discussed previously, investigations on memory interference in Chapter 3 confirmed the prediction that the hippocampus protects stabilizing memory from sensory stimulation, thus avoiding states of catastrophic interference in cortical areas. While the data we report make a solid case for object recognition memory, it remains to be seen whether the hippocampus

assumes a similar role in other forms of memory, other behavioural paradigms, and other cortical areas.

While active decay theory presents a model of how the brain avoids states of catastrophic interference that cause amnesia, it does not explain how everyday retroactive interference unfolds. In Chapter 1, we described one explanation, where impaired consolidation can lead to retroactive interference, such that mental activity relating to ongoing experience detracts hippocampal resources that could have otherwise been allocated to hippocampus-dependent memory consolidation. This account critically assumes that the hippocampus drives consolidation - which clashes with our findings – and suggests that the hippocampus *itself* is the site of interference, which seems unlikely given its sparse representations, pattern separation abilities, and our data. Instead our findings suggest that the hippocampus regulates retroactive interference in neocortical areas, but a full mechanism to explain this process has yet to be developed.

According to active decay theory, active decay occurs *exclusively* during periods of sleep. It is standard practice in animal research to conduct behavioural experiments in light phases, which is the inactive phase of rats during which they spend most of their time asleep. In keeping with the majority of published findings in our area of memory research, we inhibited active decay processes by pharmalogical intervention during this period of the circadian rhythm of our rats. Yet it is unclear how such pharmalogical intervention would affect time-dependent forgetting if delivered during the dark phases in which rats are mostly active. Future studies should address this critical question.

5.4 Memory pathologies: impaired formation or excessive forgetting?

Memory pathologies have been generally assumed to arise from impairments in memory formation or maintenance. As such, therapeutic interventions have been designed aiming to enhance these processes; yet, such strategies have had limited success. Alternatively, our findings suggest that dysregulated forgetting mechanisms may also contribute to these pathologies.

In chapter 3 we provide direct evidence that amnesia arising from hippocampal dysfunction is the result of excessive interference rather than a lost capacity for consolidation. Reducing sensory interference after new learning has already been shown to greatly benefit amnesic patients (Alber et al., 2014; Cowan et al., 2004; Dewar et al., 2009), thus highlighting a possible therapeutic approach that incorporates these findings. For example, encouraging

patients with mild cognitive impairment to actively reduce the amount of sensory stimulation right after they acquire an important information (e.g., where they put the house key), might be a simple yet effective way to reduce the amount of memory loss arising from excessive interference.

A number of neurodegenerative diseases are characterized by late-onset dementia, i.e., the development of severe memory loss and the inability to form new episodic memory. Alzheimer's disease is the leading cause of dementia, which arises from a variety of genetic, environmental and age-dependent causes. A hallmark of this disease is the accumulation of amyloid-beta (Aβ) plaques and neurofibrillary tangles, yet whether these proteins are causal or secondary effects remains debated (Hampel et al., 2021). Of relevance here, a number of emerging findings suggest much of the underlying neurobiology of Alzheimer's disease also critically regulate natural time-dependent forgetting. For instance, under certain protocols of LTD, Aβ is recruited to drive AMPAR internalization (Hsieh et al., 2006), thus suggesting it may serve roles in endogenous synaptic weakening. In alignment with this idea, infusing an anti-Aβ antibody, which prevents Aβ aggregation and neutralizes its toxicity, into healthy mice during a memory retention period extended object recognition memory expression beyond that of control animals (Lee, Kim, & Choi, 2018). Taken together, these findings highlight the potential overlap between neurobiological mechanisms underlying healthy and pathological forms of forgetting.

Could dysregulated active decay contribute to neurodegenerative diseases such as Alzheimer's disease? In support of this, aberrant caspase-3 activity has been reported in mouse models. Namely, non-apoptotic caspase-3 activity was elevated in hippocampal dendrites at the onset of memory decline, which could be attenuated with pharmacological inhibition of caspase-3, improving spatial memory persistence (D'Amelio et al., 2011). Similarly, inhibition of caspase-9 activity rescued memory deficits in a mouse model of familial dementia (Tamayev, Akpan, Arancio, Troy, & D'Adamio, 2012), thus suggesting active decay related pro-apoptotic signaling contributes to the progression of pathological, age-related memory decline. More directly, recent work reported that blocking GluA2-AMPAR endocytosis during a memory retention period, which is a method to prevent active decay, improved spatial memory retention in an Alzheimer's mouse model (Dong et al., 2015). Thus, unveiling the molecular underpinnings of active decay and exploring how their dysregulation could contribute to neurodegenerative diseases may provide novel therapeutic targets for treating memory pathologies.

5.5 Methodological considerations

While many of the individual limitations to each experiment were discussed in their respective chapters, the one overarching limitation across all our results is the exclusive use of male rats. At present, empirical evidence for sex differences in forgetting is generally lacking, yet there is emerging work that suggests such differences in memory processing.

Regarding sex differences in object-based tasks, such as those used here, there are mixed reports, with some suggesting the presence and some the absence of differences between the sexes in exploration behaviour. For instance, a number of object recognition studies show that male and female rodents express similar levels of total exploration and discrimination scores (Becegato & Silva, 2022), yet, there are also reports of female rodents showing longer retention of object memories (Sutcliffe, Marshall, & Neill, 2007), and similar levels of novelty preference despite reduced exploration time at learning (Wooden, Spinetta, Nguyen, O'Leary, & Leasure, 2021). Given that we find that object memories are susceptible to interference-based forgetting, findings showing improved object recognition in females could suggest that they may be more resistant to interference. This hypothesis was explored in recent work with a paradigm where, after initial object sampling, mice were exposed to two new objects 22 hours later, and then tested for memory for the original objects 2 hours thereafter (i.e., 24 h after initial encoding). While introducing new objects impaired recall for the previously explored objects in males, this was not seen in female mice (Rinaudo et al., 2022). Pharmacological blockade of α estrogen receptors in female mice prior to exposure of the interfering objects impaired novelty performance in the memory test, expressing exploration patterns similar to males, thus suggesting that the effect was mediated by estrogen. As mentioned above, it is difficult to assess whether pro- or retroactive interference drives the impaired performance for novel object preference in this paradigm; however, these findings raise the possibility that sex-differences may exist in forgetting for retroactive interference.

While it is commonly believed that males outperform females in spatial tasks (Jonasson, 2005), there is no strong evidence supporting this claim for rodents in long-term object location paradigms (Becegato & Silva, 2022). As such, in this paradigm it is not obvious whether there are sex differences in the underlying neurobiology of active decay. Recent findings, however, have demonstrated sex differences in the molecular underpinnings of fear learning and consolidation, despite similar behavioural performance. For example, after auditory fear conditioning, infusion of a neurokinin 3 receptor inhibitor to the amygdala led to

opposing effects of fear memory impairment and enhancement in male and female mice, respectively (Florido et al., 2021). This effect was found to be regulated by hormonal signalling, in that testosterone allowed for infusion-sensitive effects, while estradial did not. Thus, whether active decay unfolds in sex-specific molecular pathways remains an open question. Suggestive of such a phenomenon are finding that females rats express greater levels of the GluN2B receptor in the hippocampus (Wang et al., 2015), which seems critical for active decay in male rats (Migues et al., 2019). Furthermore, in a novel mouse model with a fluorescent reporter for activity of caspase-3 (which our results suggest could be involved in active decay), it was observed that there were differences in the degree of caspase-3 signalling in the amygdala between sexes in response to stressful experiences, in that females showed greater levels of caspase 3 activity than males. Therefore, exploring sex-differences in natural time-dependent forgetting may provide unique molecular signatures of active decay for male and female organisms.

5.6. Concluding remarks

Active decay theory suggests how different forms of forgetting could be explained and explored within an overarching framework. This thesis provides a set of data supporting this theory, confirming core predictions about the nature of memory interference and active decay. Many of our findings fail to align with some of the leading theoretical positions in the fields of both forgetting and memory formation. Future investigations into active decay theory will not only further our understanding on how the brain loses memory, but also, on the dynamic nature of memory itself.

Appendix

A.1. Supplementary data for behavioural data presented in Chapter 3

A.1.1. Placement graphs for behavioural data in Figures 3.1-3.2

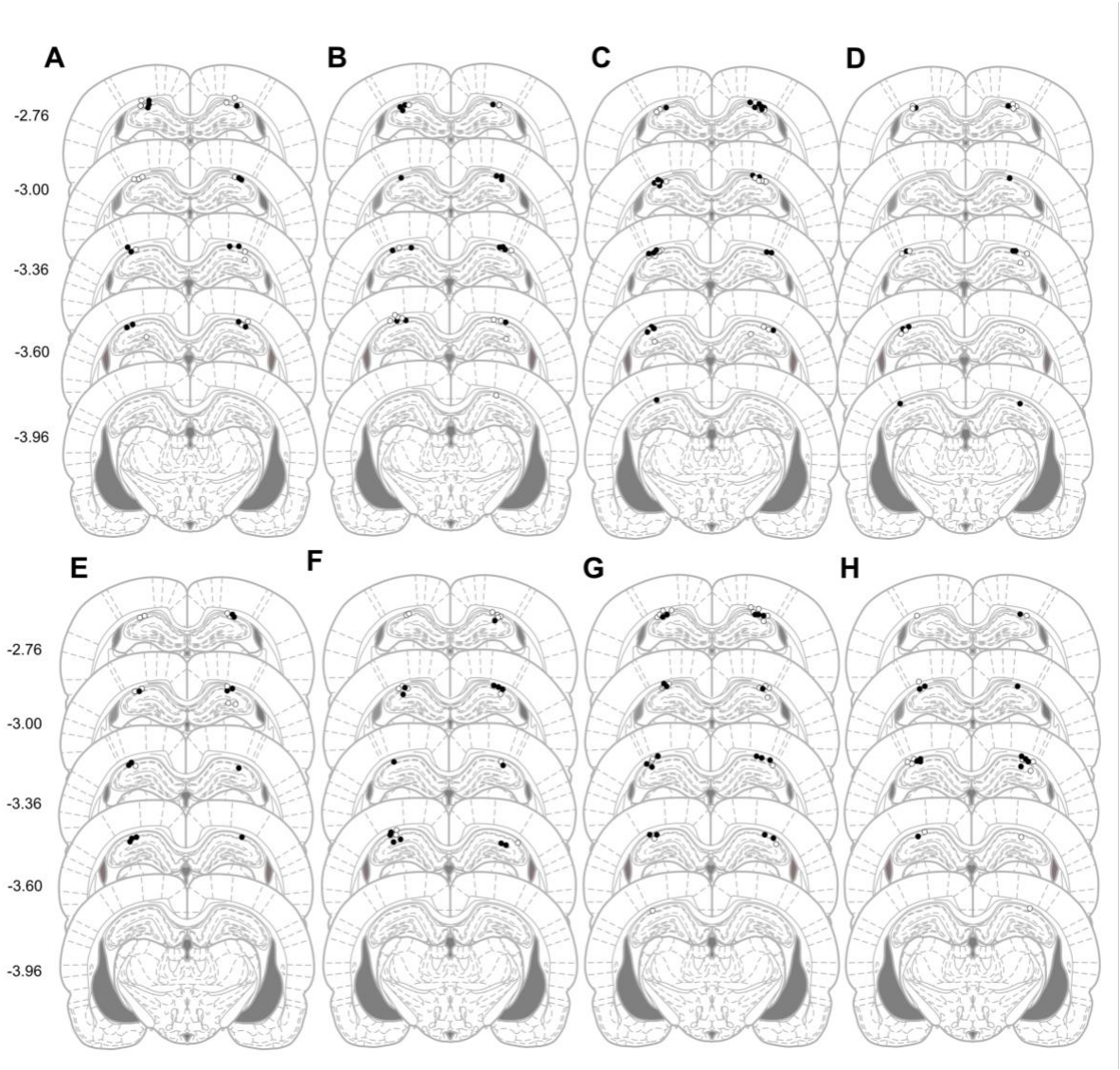


Figure A.1.1 Placement graphs for behavioural data in Figures 3.1-3.2 Across all graphs, white circles: V and black circles: MB ***A*** Figures 3.1A-C ***B*** Figures 3.1D-G ***C*** Figures 3.1H-K ***D*** Figures 3.1L-P. ***D*** Figures 3.5F-J ***E*** Figures 3.2A-C ***F*** Figures 3.2D-G ***G*** Figures 3.2H-K ***H*** Figures 3.2L-P.

A.1.2 Placement graphs for behavioural data in Figures 3.4-3.5

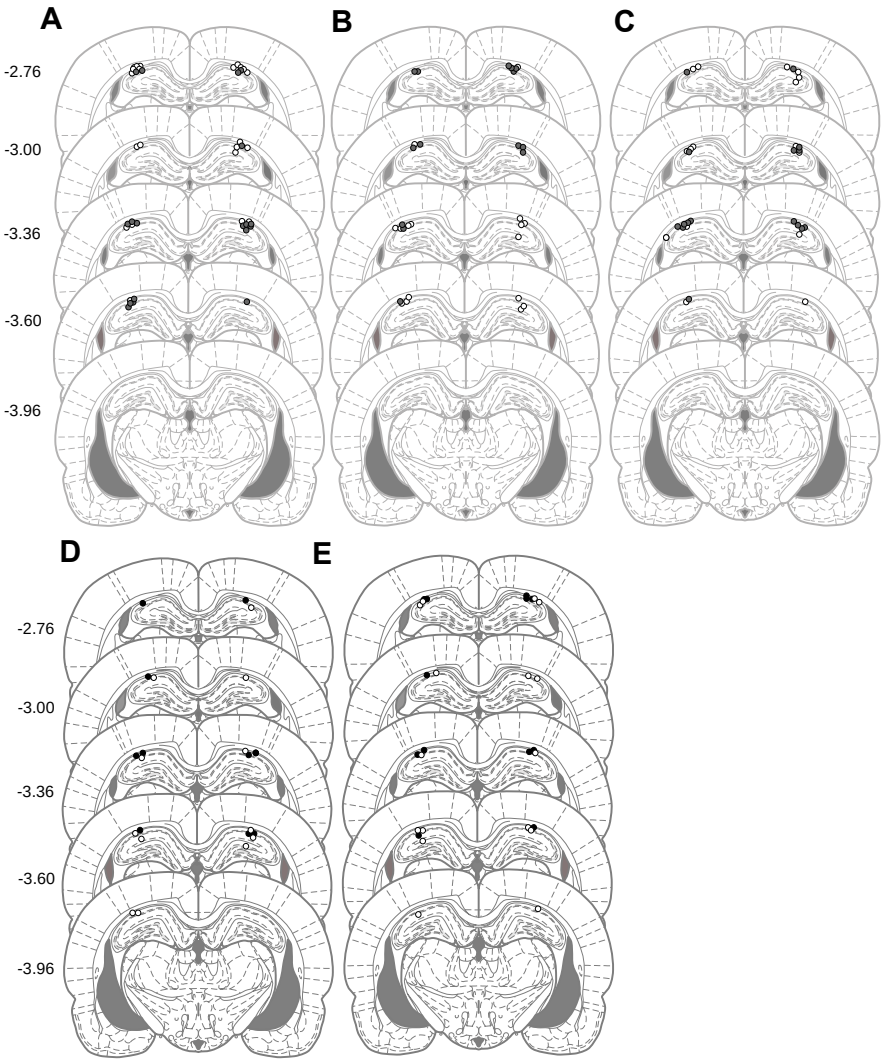


Figure A.1.2 Placement graphs for behavioural data in Figures 3.4-3.5 ***A*** Figures 3.4A-C (white circles: V; grey circles: AP5). ***B*** Figures 3.4D-G (white circles: V; grey circles: AP5). ***C*** Figures 3.4H-K (white circles: V; grey circles: AP5). ***D*** Figures 3.5A-E (white circles: V; black circles: ANI). ***D*** Figures 3.5F-J (white circles: V; black circles: ANI).

A.1.3. Rats express long-term memory for object after 48h with 10 m, but not 5m, long sampling sessions.

When designing our object reconsolidation paradigm, we found that our standardized protocol for object recognition with 5 minutes of sampling did not express significantly novelty preference ($t_{(7)}$= 0.611, p =0.56, Cohen's d = 0.216) when animals were placed in the black box for 1hr after 5 min sampling and tested for object memory 48h later (A.1.3.B.). We therefore tested whether increasing the sampling time would allow memory for objects to persist until 48hr, even when exposed to everyday sensory experience after sampling. Under this protocol, animals expressed d values significantly above chance ($t_{(7)}$= 4.31, p =0.004, Cohen's d = 1.52), indicating memory for objects.

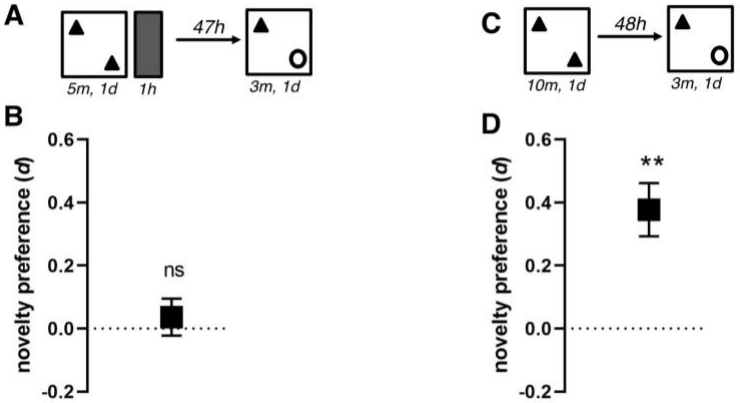
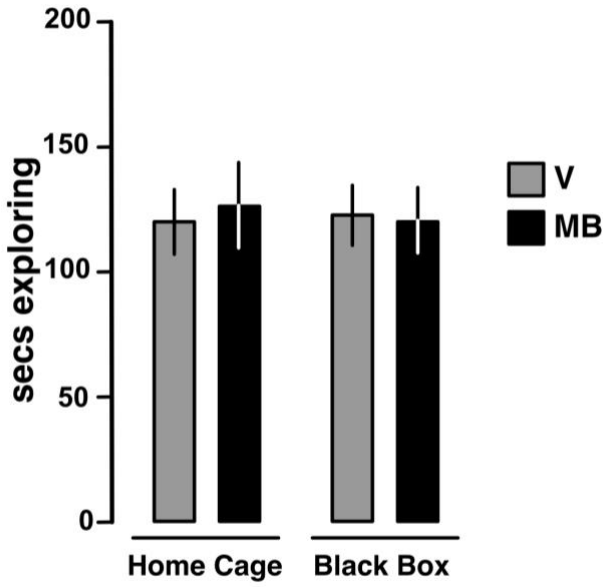


Figure A.1.3. Rats express long-term memory for object after 48h with 10 m, but not 5m, long sampling sessions. ***A-B*** Rats exposed to 5m sampling sessions and 1 hr of the black box do not express memory for objects 48hrs later. ***A*** Schematic diagram for experiment. ***B*** Average *d* values for rats (*n*=8), error bars represent sem. ***C-D*** Rats exposed to 10 m sampling sessions and normal post-learning sensory experience expressed memory for objects 48 h later. ***A*** Schematic diagram for experiment. ***B*** Average *d* values for rats (*n*=8), error bars represent sem.

A.2. Supplementary data for c-Fos studies in Chapter 3

A.2.1. Exploratory behaviour for immunohistochemical studies

To determine whether any changes in c-Fos expression reflect differences in object exploration, we compared total exploratory behaviour across each condition. We no found significant group effects (One-way ANOVA: $F_{(3, 7.06)} = 0.0256$, $p = 0.994$).



A.2.1. Exploratory behaviour for immunohistochemical studies. Exploration time for animals that were either returned to their home cages perfused (V *n*=5; MB *n*=4) or the black box (V *n*=4; MB *n*=4) for 1hr before perfusion. Bars represents average exploration time, error bars represent s.e.m.

A.2.2. Levels of c-Fos expression do not alter after presentation of a novel or familiar object in the perirhinal cortex

A number of positions hold that rather than purely encoding object representations themselves, the perirhinal cortex conveys information relating to novelty (see chapter 2 for discussion). To determine whether c-Fos expression in the perirhinal cortex reflected a novelty signal, we also ran a control group in which animals were euthanised after a second sampling session, in which they exposed to objects previously seen (i.e., familiar objects). Here we compared the familiar group to data previously presented (Fig 3.5.3) and discussed in chapter 3. We found no significant difference in c-Fos densities between groups ($t_{(7)}$= 01.52, p =0.172), nor in total exploration time across sampling sessions (One-way ANOVA: $F_{(2, 6.47)} = 3.39$, $p = 0.098$).

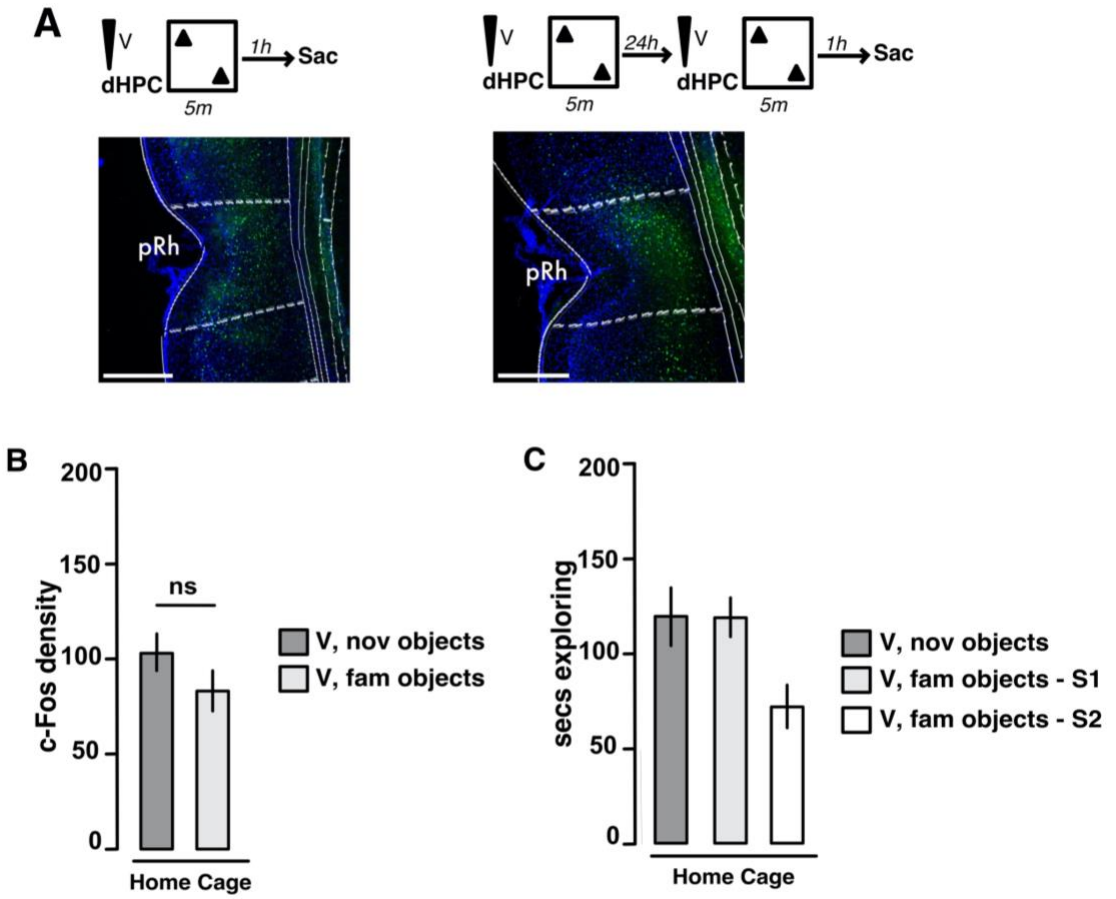


Figure A.2.2. Levels of c-Fos expression do not alter after presentation of a novel or familiar object in the perirhinal cortex. ***A*** Schematic diagram of each experimental condition with sample images from the caudal perirhinal cortex (Prh) with sample images from the caudal perirhinal cortex (Prh) of merged channels across behavioural conditions, scale bars represent 500μm. Rats were infused with inactive V and then placed into the familiarised open area with novel (V, nov objects) or familiar (V, fam objects) objects for 5 minutes. Animals were either returned to their home cages perfused (V, nov objects *n*=5; V, fam objects *n*=4) for 1h, after which they were euthanised. ***C*** c-fos/μm^2 across each condition. Sample images are shown in (Supplementary Figures). Bars represent mean counts, error bars represent s.e.m. ***D*** Exploratory behaviour across sampling session for novel objects and the first (V, fam objects – S1) and second (V, fam objects -S2) sampling session for the familiar objects group.

A.3. Supplementary data for behavioural data presented in Chapter 4

A.3.1. Placement graphs for behavioural data in Figures 4.1 and 4.3.

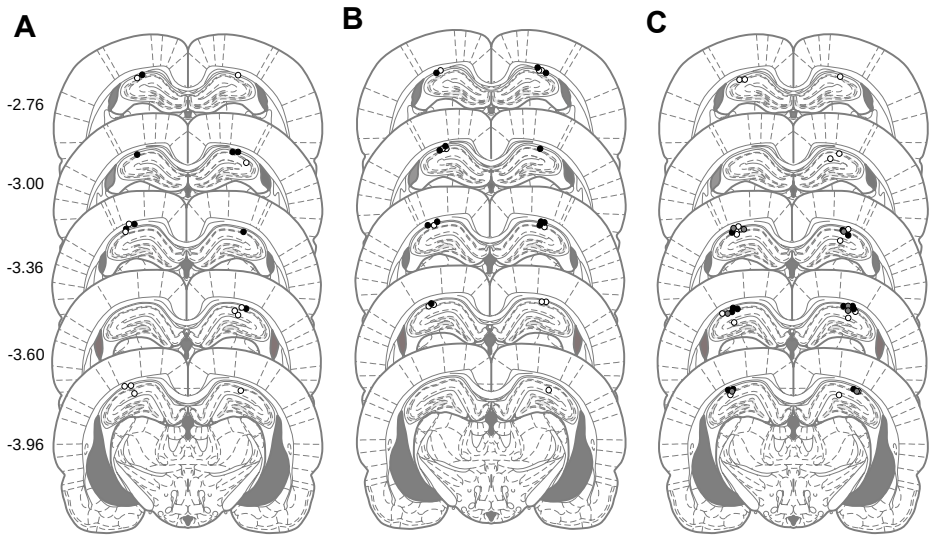


Figure A.3.1. Chapter 4: Cannula placement graphs for behavioural data. ***A*** Figures 4.5.1E-H (white circles: V; black circles: z-DEVD-fmk). ***B*** Figures 4.5.3A-D (white circles: V; black circles: ConR). ***C*** Figures 4.5.3E-H (white circles: V; black circles: ConR; grey circles: AuNP-ConR).

A.3.2. Post hoc analysis for exploration behaviour.

Comparison						
Sampling Day	Sampling Day	Mean Difference	SE	df	t	p_{tukey}
S1	S2	33.0	8.44	26.0	3.91	0.002
	S3	46.3	7.18	26.0	6.45	<.001
S3	S3	13.3	6.40	26.0	2.08	0.113

Table A.1 Post hoc analysis for exploratory behaviour in Fig 4.1D, comparisons within subjects between sampling days.

Comparison						
Retention Group	Retention Group	Mean Difference	SE	df	t	p_{tukey}
1d	3d	-2.97	14.7	26.0	-0.202	0.997
	5d	-43.34	15.2	26.0	-2.945	0.040
	7d	-14.92	15.2	26.0	-0.979	0.762
3d	5d	-40.37	15.2	26.0	-2.650	0.061
	7d	-11.95	15.2	26.0	-0.784	0.861
5d	7d	28.42	15.7	26.0	1.806	0.293

Table A.2 Post hoc analysis for exploratory behaviour Fig 4.1D, comparisons between subjects in each retention groups.

Comparison						
Sampling Day	Sampling Day	Mean Difference	SE	df	t	p_{tukey}
S1	S2	69.11	8.97	8.0	7.706	<.001
	S3	71.16	8.37	8.0	8.505	<.001
S3	S3	2.06	11.04	8.0	0.186	0.981

Table A.3 Post hoc analysis for exploratory behaviour in Fig 4.1I, comparisons within subjects between sampling days.

Comparison						
Sampling Day	Sampling Day	Mean Difference	SE	df	t	p_{tukey}
S1	S2	9.37	11.32	17.0	0.828	0.918
	S3	50.92	6.61	17.0	7.709	<.001
	S4	34.66	7.19	17.0	4.823	0.001
	S5	70.92	7.30	17.0	9.715	<.001
S2	S3	41.55	9.28	17.0	4.479	0.003
	S4	25.29	7.77	17.0	3.255	0.033
	S5	61.55	8.88	17.0	6.934	<.001
S3	S4	-16.26	6.01	17.0	-2.703	0.095
	S5	20.00	5.40	17.0	3.706	0.013
S4	S5	36.26	6.08	17.0	5.960	<.001

Table A.4 Post hoc analysis for exploratory behaviour in Fig 4.3H, comparisons within subjects between sampling days.

Comparison						
Infusion Group	Infusion Group	Mean Difference	SE	df	t	p_{tukey}
AuNP-ConR	ConFG	15.14	5.66	17.0	2.675	0.040
	ConR	11.87	5.81	17.0	2.041	0.133
ConFG	ConR	-3.27	5.14	17.0	-0.637	0.802

Table A.5 Post hoc analysis for exploratory behaviour Fig 4.3H, comparisons between subjects in each infusion group.

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