Trinity College Dublin



Gone or Misplaced?: Investigation of Memory Engrams Across Development

Sarah Power

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Supervisor: Dr. Tomás Ryan

Declaration

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2021

Author Contribution

The author conducted all the work in this thesis independently except for the following contributions.

Chapter 7: Dr Maurizio Pezzoli performed electrophysiology recordings and analyses.

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Abstract

All humans experience amnesia. While about 30% of adults who live to be over 65 will suffer from dementia, 100% of the human population experiences infantile amnesia, which is the loss of memories formed in early childhood (before 2–4 years). Although behavioural neuroscientists have already demonstrated that rodents display infantile amnesia, little is known about the basic neurobiology of infantile amnesia and further its effect on memory engrams. This thesis probes the question of how memories are stored in the brain throughout development by integrating recently developed engram labelling technology with rodent models of infantile amnesia. This thesis aims to characterize infantile amnesia in the mouse using multiple behavioural paradigms. It develops a genetic strategy for labelling infant engram cells in a persistent way that can be maintained across the lifespan. It then uses this strategy as a platform to optogenetically interrogate the functionality of infant engram cells in adulthood. It investigates the degree of information content that is retained after infantile amnesia for various kinds of memory. The mechanisms underlying infantile amnesia are investigated and potential methodologies for the permanent reinstatement of infant engrams are probed. Lastly, approaches for preventing infantile amnesia are explored by inducing immunological brain states that may allow infant memories to survive IA.

Abbreviations

4-OHT	4-Hydroxytamoxifen
AMG	Amygdala
AP	Anterior-posterior
CFC	Contextual fear conditioning
ChR2	Channelrhodopsin-2
CS	Conditioned stimulus
d	day
DG	Dentate gyrus
DV	Dorsal-ventral
E	Engram
EYFP	Enhanced yellow fluorescent protein
HPRA	Health products regulatory authority
IA	Infantile amnesia
IP	Intraperitoneal
KA	Kainic acid
MIA	Maternal immune activation
ML	Mediolateral
mPFC	Medial prefrontal cortex
n	number
NE	Non-engram
NOR	Novel object recognition
NS	No shock
P	Postnatal
PAG	Periaqueductal gray
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
RSC	Retrosplenial cortex
S	Shock
US	Unconditioned stimulus

w	week
Wt	Wild-type

Chapter 1: Introduction

1.1 Memory and memory loss

The ability to take in information from one's surroundings, store it and then retrieve it later when necessary, is fundamental to everyday life. Through the process of learning, we acquire new information about our surroundings, while memory is the storage of such information. In the face of an ever-changing environment, learning and memory are crucial for survival and shaping who we are. Long-term memory can be fundamentally divided into the two broad categories of explicit memory 'knowing what' and implicit memory 'knowing how' (Voss, 2008, Thompson, 1996). Implicit memory, also referred to as unconscious memory, does not require active retrieval of memories, such as knowing how to play a musical instrument (Squire, 2015). Explicit memory draws from recalling personal experiences (episodic) as well as factual (semantic) information and is governed by communication between the hippocampus, the amygdala, and the neocortex (Ben-Yakov, 2015). Although both are of high importance, the two memory systems are thought to work independently and have distinct fundamentals of operation (Yang, 2012).

Scientists have been trying to understand how memories are formed and retrieved in the brain for more than a century. Our basic understanding of memory has substantially been driven by cases of patients with memory loss (Allen, 2018). One of the most informative cases was that of the patient Henry Molaison (H.M.) who in 1953 had surgical resection of portions of the medial temporal lobes, including the hippocampus, removed to alleviate his debilitating epilepsy (Augustinack, 2014, Milner, 1968, Scoville, 1957). At this time, scientists did not know the implications and had little knowledge of the importance, of the hippocampus. Without his hippocampi, H.M. suffered from severe memory problems, becoming incapable of forming new long-term memories and recalling prior memories (Scoville, 1957). One of the most important outcomes from the case of H.M. was that removing the hippocampus only affected explicit memory. After H.M. was described, efforts began to establish models of the condition in animal models

(Mishkin, 1978). Since H.M. several studies in humans, non-primates and rodents have solidified the crucial role the hippocampus plays in the formation of episodic memories.

Memory is prone to error. Forgetting and amnesia are two severities of memory loss. Forgetting is the natural loss of memory but until about a decade ago, scientists believed forgetting was a passive process, a glitch in the system. It was thought that forgetting was caused by an accidental erasure of memories from the brain. Rather, forgetting appears to be an active process crucial to brain function (Davis and Zhong, 2017) (Hardt et al., 2009, Ryan, 2021). Natural forgetting gives us flexibility, allowing us to forget unnecessary pieces of information so we can retain more relevant information (Nørby, 2015). Amnesia is a severe form of forgetting where the retrievability of information that was once successful encoded is negligible. When we speak about amnesia, we typically refer to the maladaptive loss of long-term memories following a known cause of brain injury (neurological amnesia) or psychological trauma (psychogenic amnesia). We can further categorise amnesia by the timing of encoding the information. Anterograde amnesia refers to impairments in memory formation while retrograde amnesia occurs when memories are lost after the onset of injury or trauma. Regardless both forms of memory loss are valuable tools for studying memory.

But where does it all go wrong? Failure of memory function can occur at three points of the memory process; memory encoding, memory storage, or memory retrieval. The information must be changed to a usable form which occurs through the process of memory encoding. If memory loss occurs before the actual learning process finishes, it is referred to as memory encoding failure. In such a case, the information has not likely been encoded in the brain. Once the information has been successfully encoded, mnemonic information is maintained through the plasticity of neuronal ensembles in the brain. Memory cannot be recalled if it simply hasn't been encoded in the first place. The storage of memory in the brain involves persistent structural and physiological plasticity between cells representing the mnemonic information (engram cells) (Tonegawa et al., 2015b). Failure in the formation of the connectivity pattern between engram cells can cause memory storage deficits (Ryan, 2015). However, sometimes information is stored in the brain but remains inaccessible. The process of memory

retrieval allows for the reactivation of mnemonic representations that have been stored in the brain (Ben-Yakov, 2015). In this case, the information is present but cannot be accessed by natural cues. Dysfunction anywhere along the memory process can result in memory loss. One way of studying memory is by dissociating correlates from memory itself. Can we rule out what things are important for memory storage but might not be important for memory encoding and recall and *vice versa*? One way of doing this is by studying different kinds of amnesia. If a memory survives a case of amnesia, then we can rule out that whatever is going wrong in the type of amnesia as being a potential way of storing the fundamental memory, we can attribute it to recall (Ryan, 2015, Roy et al., 2016, Perusini et al., 2017). Thus, we can pull apart the memory and get closer to the fundamental biology of memory.

1.2 Infantile Amnesia

Infantile amnesia is the most pervasive form of amnesia (Madsen and Kim, 2016). Adult humans retain language and habits initially acquired before the age of three and are typically able to recall details from as early as age three, however, autobiographical memory before this age is rarely recalled (Rovee-Collier, 1999). Infantile amnesia refers to the rapid forgetting of memories formed early in development; a largely neglected phenomenon that seemingly affects 100% of the human population. This faster rate of forgetting observed in youth is characterised by two phases, near-complete absence of events that occur before the age of three years, and spotty recall for events that occur between the ages of three and six to seven years old. The earliest research on the phenomenon was published in the late 19th century (Miles, 1895). A group of adults were surveyed on their childhood experiences where they were asked to think about the earliest event they could remember and what age they were at the time of the event. This, among subsequent surveys (Henri and Henri, 1895, Henri and Henri, 1896), led to one of the most robust findings describing the phenomenon we now refer to as infantile amnesia.

The term infantile amnesia was first coined by Sigmund Freud who attributed this early memory loss to repression (Freud, 1899). He proposed that due to their heavily charged

psychosexual content, early experiences are actively forgotten. However, no empirical support for Freud's explanation of infantile amnesia has been provided to date. Human and cognitive psychologists have explained infantile amnesia as a result of the absence of language, suggesting that the rapid fading of autobiographical memories in young children is due to a lack of ability to encode and express these events (Harley and Reese, 1999). Others put forward the idea that young children are incapable of storing memories as autobiographical events due to a lack of a sense of self (Perner and Ruffman, 1995). Although several alternative explanations have been proposed, none account for all available data. The dispute that arises with these explanations is that infantile amnesia is not a uniquely human phenomenon (Arnold, 1997, Campbell and Spear, 1972). Other mammals that are incapable of language, and less affected by psychosexual content, exhibit infantile amnesia (Campbell and Campbell, 1962). Why the psychological explanations of why infantile amnesia exist are very important to consider, it's also to note that this is conserved with rodents and therefore infantile amnesia emerged 600 million years ago at least if not further, showing that this phenomenon is at least as evolutionarily old as mammals and requires basic neurobiological explanations.

The first empirical demonstration of infantile amnesia in rodents was carried out by Campbell and Campbell (Campbell and Campbell, 1962) who employed an active avoidance test to examine the memory retention of rats from five different age groups ranging from P18-P100. Animals were conditioned to avoid a shock-paired compartment using a black-white shuttle box. When the animals were tested immediately after training, no age-related difference in retention was found. However, after 7 days rats trained during infancy (P18) exhibited nearly complete forgetting, while rats trained as adults (P100) retained perfect retention even up to 42 days after training. Infantile amnesia has since been replicated in different species and across several different conditioning protocols.

1.2.1 Hypothesis of the neural mechanisms behind infantile amnesia

Although the exact neural mechanisms involved in infantile amnesia are unknown, several hypotheses have been proposed to explain this rapid forgetting exhibited throughout development. Studies proposed that autobiographical events that occur within the first few years of life are not stored due to the immaturity of the hippocampus, a region of the brain important for episodic memory (Bauer, 2006) (Moscovitch, 1985). In humans, the hippocampus is thought not to be fully developed until around 3-5 years of age, coinciding temporally with the offset of infantile amnesia (Insausti, 2010). While synapse density in the hippocampus shows adult-like numbers by 6 months of age, synapse pruning does not reach adult levels until 4-5 years of age (Insausti, 2010, Eckenhoff, 1991). This developmental hypothesis was supported by an experiment carried out by Albani et al who demonstrated that hippocampal excitatory synaptic transmission, necessary for adult-like memory, does not begin to mature until the third postnatal week in the rat (Albani et al., 2014). Another study proposed that infantile amnesia can be explained by the developmental changes that occur during the retention interval (Spear, 1978). Spear suggested that infantile forgetting can be accounted for by an inability of the animal to perceive similarities between conditioning and testing environment due to their growth in body size. In a subsequent study, Fiegley and Spear disproved this explanation when they trained infant rats in an avoidance task using an apparatus measured to scale with the animal's size (Feigley and Spear, 1970). They demonstrated that regardless of whether infant rats were trained in scaled or normal size boxes they still exhibit rapid forgetting when compared to adults.

In the subgranular zone of the dentate gyrus (DG), new neurons are continuously generated and synaptically integrated into hippocampal circuits beyond development and into adulthood (Deng, 2010). The effects of increasing and decreasing this postnatal neurogenesis in infant mice were examined in early studies carried out by Campbell and Spear (Campbell and Spear, 1972). This research formed the foundation of a more recent theory of infantile amnesia, referred to as the neurogenic hypothesis, which was first proposed by Josselyn and Frankland (Josselyn and Frankland, 2012). They suggested that the high rate of hippocampal neurogenesis during development may destabilize memory representation or replace existing synaptic connections resulting in the

forgetting of infantile memories. They then found that increasing hippocampal neurogenesis in adult mice, by administration of neurogenic drugs or voluntary wheel running, caused forgetting of the contextual fear memory (Akers, 2014). Similarly, they demonstrated that pharmacologically inhibiting hippocampal neurogenesis in infant mice mollified forgetting. From these results, they proposed an inverse relationship between hippocampal neurogenesis and forgetting of a contextual fear memory. Although both neurogenesis and forgetting are high during the infantile amnesia period, the relationship may simply be correlational.

An alternative hypothesis posits that infantile amnesia is not due to a failure in memory encoding or storage, but rather reflects a failure in memory retrieval. The retrieval hypothesis suggests that early developmental memories are not lost, but somehow their expression is inhibited or suppressed. Indicative evidence for this theory has been put forward using reinstatement and reactivation behavioural paradigms in both rodents (Campbell and Jaynes, 1966, Silvestri et al., 1970, Spear and Parsons, 1976, Campbell and Randall, 1976, Miller et al., 1991, Kim and Richardson, 2007) and humans (Davis and Rovee-Collier, 1983). However, it is equally possible that reinstatement and reactivation paradigms may not be reinstating the memory but rather encoding a similar, new memory engram. The possibility that infantile amnesia is due to an encoding deficit has also been dismissed due to research carried out on children as young as 24 months who demonstrate an ability to recount memories for specific episodes in detail up to weeks later (Nelson, 1993, Bauer, 2000, Bauer, 2007, Hudson, 1992). Thus, despite a century of research, the underlying cause of infantile amnesia has for a long time been shrouded in ambiguity.

1.3 Studying infantile amnesia

1.3.1 The memory engram

By understanding the basics of the nature of memory processes it will allow for the facilitation and treatment of human memory disorders. The central question to address

is how is memory encoded, stored, and retrieved in the brain as information? The term engram was first coined by Richard Semon in the early 20th century to describe the neural substrate responsible for memory (Semon, 1925). The term was not popularized until the American scientist, Karl Spencer Lashley, searched for the engram through a series of experiments where he trained rats to search for food in a spatial maze task. He then ablated different areas of the cortex, hypothesising if a region of the cortex held the engram, the rats would no longer be able to remember the task. From this set of experiments, he concluded that the engram is not localized to a particular region of the cortex, or brain, but rather distributed throughout the brain (Orback, 1998). Semon and Lashley paved the way for advancing theories on how memory is stored in the brain as information.

Due to recent advances in the neuroscience of memory, we now know that during learning a sparse number of cells (engram cells) are recruited (Tonegawa et al., 2015a). Immediately after encoding the process of memory consolidation enables the stabilization of the new connections between engram cells producing what we refer to as the engram (Semon, 1925). These engram cells, connected in a particular pattern, can be considered to contribute to the memory engram for that specific memory. What makes an engram an engram is a particular code in the change of the brain structure that accounts for a particular memory. Engram studies attempt to decipher this code by employing a range of experimental techniques (Liu et al., 2012, Redondo et al., 2014).

1.3.2 The development of engram technology

To rigorously study the memory engram several conditions must be met. A functional behavioural paradigm that can produce an output that demonstrates learning and retrieval of a memory is required. It is important to note that with engram biology we are not just looking at the animal's ability to engage in memory recall, but rather we are looking at how a particular memory is stored in the brain. When studying different types of memory, we need to tag cells activated by a particular experience (engram cells) in the brain. Thus, we need a genetic strategy that allows for this. Until recently it has not been possible to identify engrams amidst the complexity of the mammalian brain. But

by hitchhiking onto immediate early genes (genes that switch on only when a brain cell is active) Reijimers et al. devised a strategy to label specific cells in the mouse brain that store a given memory (Reijmers et al., 2007). By combining elements of the tetracycline-transactivator (tTA) system with the *c-fos* promoter, they developed a transgenic mouse line (TetTag mouse) that allows for the tagging of active neurons during a given period. In effect, they asked the brain to demonstrate where a memory is located. This approach has now widely been used to label engram ensembles in the brain.

Reijjmers and colleagues contributed a major advancement in the memory field, demonstrating it is possible to label putative engram cells in the brain (Reijmers et al., 2007). In parallel, Josselyn and colleagues asked whether it was possible to delete engram cells in rodents (Han et al., 2009, Han, 2007). The researchers randomly overexpressed the transcription activator cAMP response element-binding protein (CREB) in a neuronal population in the lateral amygdala (LA) as a way of biasing the cells to be allocated to a subsequently formed engram. Indeed, they found that the expression of CREB in these neurons made them more like to be recruited to the engram during cued fear conditioning. In a subsequent study the authors demonstrated that if these selectively ablate these tagged engram cells using diphtheria toxin-based methods, it interfered with fear memory recall (Han et al., 2009). Thus, the authors demonstrated it was possible to label and manipulate tagged engram ensembles in the amygdala.

The development of optogenetics for engram manipulation, which was first introduced by Deisseroth and colleagues, has transformed neuroscience. The researchers utilised optical technology and genetic engineering to develop an elegant approach that allows for the excitation or inhibition of specific neurons within the brain (Fig. 1) (Boyden, 2005) (Zhang, 2006, Deisseroth, 2006). Boyden et al. expressed light-activated ion channels on neurons within the brain. They then delivered light of a certain wavelength to these ion channels causing them to open and the cell to fire. To allow for expression within the brain, a viral vector was injected into the target brain area. The cells then begin to express the light-activated channels on their cell membrane. To allow for cell-specific

labelling, a promoter-specific to the cell type is used. Thus, optogenetics allows for the manipulation of target cell types within the brain.

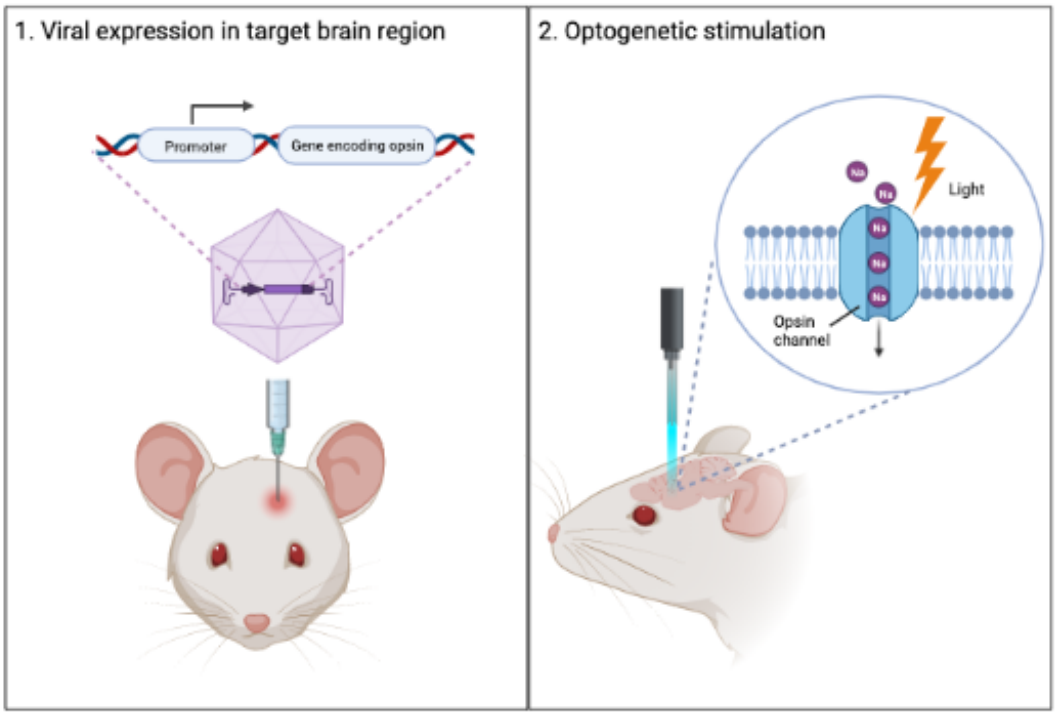


Figure 1.1. Schematic diagram of engram labelling and optogenetics. Image created using Biorender.com.

Engram technology is based on the experimental fusion of engram labelling and optogenetics or chemogenetics (Deisseroth, 2006, Zhang, 2006, Reijmers et al., 2007, Boyden, 2005). The expression of IEGs, such as *c-fos* or *arc*, is a marker of neuronal activity (Minatohara, 2016). Thus, the promoters of IEGs can be co-opted to tag neurons that are active during a given learning experience with an exogenous target protein-coupled with light-induced excitatory optogenetic receptors (Liu et al., 2012). We can then directly optogenetically stimulate these tagged neurons with the light of a certain wavelength (Liu et al., 2012).

One of the first approaches that combined immediate early gene labelling and optogenetics to study memory employed the c-Fos tTA transgenic mice with the AAV-TRE-ChR2-EYFP virus (Liu et al., 2012). In c-Fos tTA transgenic mice, the promoter of the

IEG *c-fos* is directly coupled to the tetracycline transactivator (tTA). tTA is a key component of the Dox system, allowing for the inducible expression of a gene of interest. In this system, tTA is prevented from binding to its target tetracycline-responsive element (TRE) site when DOX is present. When DOX is removed, tTA binds to TRE driving ChR2-EYFP expression in neuronally active cells. Through the delivery of light, it is possible to directly activate these tagged engram ensembles. Using this system, Liu and colleagues labelled engram ensembles, activated by a contextual fear conditioning experience, in the DG of mice (Liu et al., 2012). When animals were placed in a neutral context (where they had not been shocked), optogenetic activation of these labelled engram ensembles resulted in freezing behaviour. Further, the authors demonstrated that optogenetic activation of cells labelled for a neutral context (one they had not been shocked in) did not evoke freezing. This proved that optogenetic reactivation of the engram was specific and controlled. Thus, it is possible to label, manipulate, and study specific memory engram cells in the rodent brain.

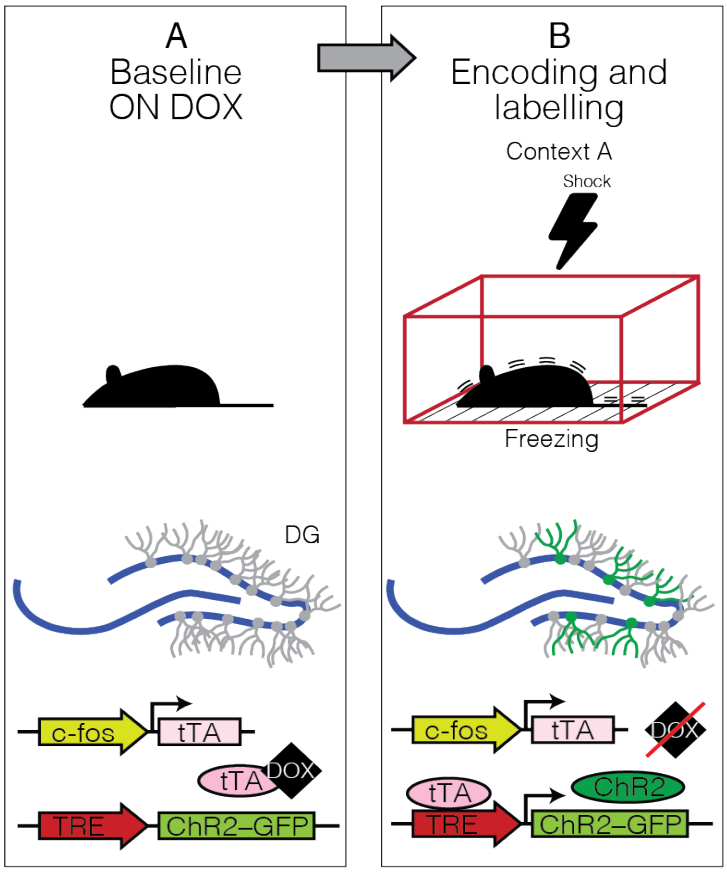


Figure 1.2. Representative image of tTA engram labelling system in mice. Image adapted from Ortega-de San Luis and Ryan 2018.

1.3.3 Manipulating the engram

When engram cells are tagged with light-induced excitatory or inhibitory optogenetic receptors (Kim et al., 2017), it is possible to manipulate the engrams through the delivery of light (Liu et al., 2012). This technology has been applied to numerous questions at the heart of memory and memory loss, advancing our understanding of how memory is stored in the brain. Further, it has allowed for the development and application of other engram labelling systems (Tonegawa et al., 2015a). Using an engram labelling strategy based on the promoter of IEG *Arc* and Cre recombinase, Denny and colleagues were able to label neuronal populations in the DG or CA3 of the hippocampus during encoding of a contextual fear memory (Denny et al., 2014). Inhibition of these cells using optogenetics resulted in impairment of memory recall. These results were mirrored in another study that found that inhibition of labelled CA1 engram cells

resulted in memory impairments (Tanaka et al., 2014). This technology has also been applied to the investigation of false and genuine memories (Ramirez et al., 2013). Ramirez and colleagues labelled a purely contextual engram (context A) in the DG. The following day, they optogenetically reactivated the engram in a different context (context B), while simultaneously delivering foot shocks. When the animals were placed back in context A, despite never having received foot shocks in this context, they demonstrated freezing. Here, animals display a false fear memory for context A. Further, this false fear memory could be reactivated using optogenetics in a third context (context C) (Josselyn, 2020, Tonegawa et al., 2015a).

1.3.4 Applying engram technology to infantile amnesia

The development of novel technologies such as optogenetics has revolutionized the way memory can be studied, allowing us to test long-standing but unproven theories of memory storage. Ryan et al. were able to show that amnesia due to disrupted memory consolidation or reconsolidation in adults (induced by acute post-training inhibition of protein synthesis by the drug anisomycin) is a deficit of memory retrieval (Ryan, 2015). Though the memory appeared to be lost due to amnesia, direct activation of engram cells caused its retrieval. This proved that the memory is not only maintained but that it can be acutely rescued using optogenetics. Thus, engram cells retain a memory after amnesia due to disrupted (re)consolidation (Ryan, 2015). Following this, one study used engram technology to ostensibly erase a fear engram from the network without interfering with other memories interconnected with the same engram ensembles (Abdou, 2018). The authors disrupted the synaptic connectivity, resulting in apparently irretrievable memory loss. Engram technology has also been applied to cases of amnesia in mouse models of early Alzheimer's disease (Roy et al., 2016) (Perusini et al., 2017). Using the engram tagging approach in mouse models of Alzheimer's disease, these studies demonstrated that the connectivity pattern of memory engram survives this form of amnesia. They showed that optogenetic stimulation of the engram was sufficient to rescue the memory, demonstrating this form of amnesia is also due to a memory retrieval deficit. In all cases, memory is preserved through specific connectivity

patterns in the brain, which can be reactivated by direct intervention through optogenetic stimulation of engram cells (Redondo et al., 2014).

A recent study employed optogenetic strategies to paradigms of infantile amnesia to probe the question of whether it is possible to rescue lost infantile memories in adulthood (Guskjolen et al., 2018). Guskjolen et al used engram labelling to directly reactivate neuronal ensembles in the hippocampus that were activated during fear learning during infancy to induce memory recovery at remote time points. These studies suggest that there must be a neural representation of the memory acquired early in development even though is it not being behaviourally expressed. One may propose that the original engram connectivity patterns are not necessarily lost but rather become weakened during development, making it incapable of getting the behavioural readout by natural cues. Direct stimulation of the engram may reinstate these weakened connections resulting in the acute rescue of the lost memory. Taken together these findings demonstrate that infantile amnesia is due to a retrieval deficit, rather than a problem in memory encoding (Guskjolen et al., 2018).

1.4 Possible mechanisms causing infantile amnesia

The most widely studied behavioural paradigms applied to infantile amnesia are conditioning paradigms such as contextual fear conditioning and active avoidance (Madsen and Kim, 2016). Several studies have found that engram cells, active at the time of fear memory acquisition are reactivated during recall of the memory in numerous different brain regions including the hippocampus (Tayler et al., 2013) (Deng et al., 2013), the amygdala (Reijmers et al., 2007) and the prefrontal cortex (Zelikowsky et al., 2014). The hippocampus, a region of the brain essential for learning about the context in which conditioning occurs, is a late-maturing structure in both rodents and humans (Semple, 2013). Although the hippocampus undergoes crucial physiological changes during the postnatal period, it is thought to be relatively mature by P17, reaching functionally maturity at around P24 (Reznikov, 1991, Farooq, 2019). By P17 rodents are capable of learning and retaining contextual spatial and episodic information, albeit for a short time. (Pugh, 1996, Foster and Burman, 2010). Although

the exact age at which developing rodents forget has yet to be determined, hippocampal-dependent memories are more consistently retained at P21 (Rudy, 1987, Blair et al., 2013, Kraemer, 1995). Therefore, we can postulate that whatever is driving infantile amnesia is taking effect between P17 and P21. Since infant memories can be reactivated using optogenetics we know that whatever is causing infantile amnesia is occurring to a consolidated memory (Guskjolen et al., 2018). Although many molecular players have been suggested to be involved, the root cause of infantile amnesia is widely unknown (Alberini and Travaglia, 2017).

1.4.1 Evidence of a memory trace

Intracellular signalling cascades involving glutamatergic activation of N-methyl-d-aspartate receptors (NMDARs) are crucial for contextual fear conditioned learning in both infant and adult rodents (Quinn, 2005) (Ewald and Cline, 2009). NMDA receptors are widely expressed in many areas of the brain including the hippocampus (Ewald and Cline, 2009). In adults during learning, once the information has been acquired, subsequent learning for a similar task no longer requires NMDAR activation but rather mGluR activation (Crestani et al., 2019). Here, prior experience alters plasticity such that new memories can be formed in the absence of NMDAR activation. A switch occurs between acquisition (NMDAR-dependent) and reacquisition (NMDAR-independent). Li and Richardson set out to determine whether this switch also occurs in the case of infantile amnesia (Li and Richardson, 2013). They demonstrated that relearning of a forgotten infantile memory is NMDAR-independent, even if relearning occurs in adulthood. In adult rats, manipulations using anisomycin treatment do not result in this transition to NMDAR-independent reacquisition as the original memory trace appears to be permanently altered (Hardt et al., 2009). These studies indicate that relearning a forgotten infantile memory is not an entirely novel experience and that memories acquired early in development can impact the neurobiology of later learning, even if they are not behaviourally expressed.

1.4.2 The GluN2A/GluN2B switch

In the dorsal hippocampus the expression ratio of the NMDAR subunits GluN2A and GluN2B, significantly changes with learning during infancy (P17), an effect that is not witnessed during adolescence (P24) (Travaglia et al., 2016a). Both NMDAR containing subunits GluN2A and GluN2B are important for NMDAR function in neurons (Shipton, 2014). GluN2B expression is more predominant early in development, where GluN2A expression is upregulated during the second postnatal week (Monyer et al., 1994, Sans et al., 2000) (Ewald and Cline, 2009). This developmental switch accelerates the kinetics of NMDAR-mediated excitatory postsynaptic currents (EPSCs) while decreasing the ability of the synapse to undergo potentiation. Interestingly, this GluN2B/2A switch is a crucial mechanism involved in the rapid maturation of excitatory synapses in response to experience in sensory development (Gambrill and Barria, 2011, Quinlan et al., 1999). The authors propose that like sensory systems, the hippocampal system matures and develops in an experience-driven manner by switching NMDAR subunit composition. Around the same time this NMDAR subunit switch is occurring, synaptogenesis is also occurring in the rodent hippocampus (Gambrill and Barria, 2011).

A study carried out in CA1 pyramidal cells demonstrated that the ratio of GluN2A/GluN2B at a synapse controls the rate of synapse formation and maturation (Gray, 2011). Early expression of GluN2A decreases the number of synapses and blocks the addition of new dendritic spines, while overexpression of GluN2B increases dendritic spine motility (Gray, 2011). Thus, GluN2A acts as a stabilizing force in the synapse whereas GluN2B is required more for structural changes (Gambrill and Barria, 2011, Ryan, 2012, Frank, 2016). Further, they demonstrated the importance of the C termini of GluN2 subunits, particularly GluN2B, for its ability to interact with CaMKII (Gray, 2011). CAMKII levels are low in early postnatal development and increased rapidly over the first 2 weeks of life (Huang et al., 2005, Kelly, 1985). This interaction appears to be important for structural plasticity in particular the formation of new spines and synaptic connections and the incorporation of AMPARs (Mckay, 2018). When the switch occurs and GluN2B containing receptors are replaced with GuN2A containing receptors there is an increase in structural and functional stability (Frank, 2016). Interestingly, inhibition of the induction of excitatory synapse markers that are upregulated following learning

in infants impairs AMPAR response maturation and memory formation (Bessieres et al., 2019). These results indicate the importance of excitatory synapse formation/maturation in the formation of infantile memories. This switch in the ratio of GluN2A/GluN2B may explain the rapid forgetting that occurs during development. Is it possible that the upregulation of GluN2A early in development is reducing the ability of the engram to undergo potentiation rendering the engram inaccessible by natural cues?

1.4.3 Silent synapses

Silent synapses refer to an excitatory glutamatergic synapse whose postsynaptic membrane holds only NMDARs without the presence of AMPARs (Kerchner, 2008). Due to this neurotransmission does not produce a physiological response in the receiving neuron. It is possible to activate silent synapses by receptor trafficking of AMPARs into the postsynaptic membrane (Liao, 2001). During the development trajectory, synapses at individual neurons change in number and may be functionally silent at different time points during development. Early in postnatal development the hippocampus, as well as other brain regions, have a significant number of excitatory synapses that have NMDARs but lack AMPARs. As development progresses synapses acquire AMPARs, while there is little to no change in the NMDAR number (Petralia, 1999). Thus, AMPAR to NMDAR mediated transmission is initially low but increases over the course of early development. Importantly, early in development when GABA acts as an excitatory transmitter, depolarization of GABAergic synapses can release the magnesium block from NMDARs allowing for synaptic transmission (Leinekugel, 1997). It is plausible that early in development NMDAR mediates synaptic transmission with the help of depolarization from GABA, but when the GABA switch occurs NMDARs are no longer functional, and the synapse is rendered ‘silent’. The uptake of AMPARs is then required for further neurotransmission but only engram cells of functional relevance are activated. This hypothesis would mean the memory engram is still present in the case of infantile amnesia, but it is left inaccessible by natural cues. Although this mechanism is consistent with the recall deficit hypothesis, it may not be the only potential explanation for this form of forgetting.

1.4.4 Immaturity of the mPFC

In addition to the hippocampus, the medial prefrontal cortex (mPFC) plays a critical role in memory consolidation and memory reconsolidation (Ye, 2017). The mPFC matures later in development in both humans and rodents and is compromised in neurodevelopmental disorders including Autism Spectrum Disorder (ASD) and Schizophrenia (Klune, 2021) (Schubert, 2015). In rodents the cortical layers of the mPFC develop rapidly postnatally until P20, reaching full adult thickness around P24 (Van Eden, 1985) (Klune, 2021). In adults, the mPFC regulates fear through its subregions the prelimbic and infralimbic cortices where typically, the prelimbic cortex has a heavy involvement in fear expression, while the infralimbic cortex is more involved in fear inhibition (Quirk, 2008, Sotres-Bayon, 2010, Riga, 2014). Early in development, it appears this circuitry differs compared to its mature adult state. For example, Li and colleagues demonstrated through temporary inhibition of the prelimbic cortex, that juvenile (P25) rats rely on the prelimbic cortex to produce a fear response, while infants (P18) do not (Li, 2012). Moreover, the levels of most synaptic and neuronal plasticity proteins in the mPFC undergo significant developmental changes (Jia et al., 2018). These include an increase in proteins that mark synapse maturation and synaptogenesis (GADs, synaptophysin and PSD95) and a decrease in markers of cell activation (IEGs, phospho-CREB, phospho-CamKIIα, phosphor-ERK^{1,2})(Jia et al., 2018). Furthermore, there are significant changes in structural markers (MBP, Nogo receptor), indicating that structural connectivity of the mPFC increases over time. Taken together it appears that although the brain is highly active in early development, maturation of the mPFC is occurring in a temporal window of P17-P24. These studies support the developmental hypothesis, that infantile amnesia is caused by immaturity of certain brain regions, such as the mPFC, at this point in development.

1.4.5 The amygdala

It has become increasingly recognized that memories are not solely dependent on the hippocampal function but rather involve several distinct and interacting brain circuits (Alison, 2013b). Cognitive processes such as learning and memory can be heavily influenced by emotion (Tyng, 2017). If an event elicits an emotional response, it is likely

that the information acquired is important for survival and thus the event is more likely to be remembered. The amygdala is an integrative structure for emotion and behaviour and plays a pivotal role in memory (Schafe, 2005). The amygdala has direct connections with the hippocampus (Yang, 2017). In emotional situations, these two brain regions interact to translate the importance of a memory. Amygdala neuron physiology is dynamic during development and does not begin to stabilize until P28 (Moryś, 1998, Berdel, 1997b, Dávila, 2008). Particularly neurons in the basolateral amygdala undergo great physiological changes between P7 and P21 (Ehrlich et al., 2012) including a threefold increase in the number of synaptic contacts (Moryś, 1998). Although the total number of neurons is mature at P7, the basolateral amygdala continues to increase in volume until P14 (Berdel, 1997b). Further, a lot of neuronal and synaptic proteins in the basolateral amygdala undergo significant developmental changes between P17 and P24 (Bessieres et al., 2019) coinciding with the temporal window of infantile amnesia. Like the mPFC, the amygdala is undergoing developmental changes during the time infantile amnesia is occurring. Although infantile amnesia causes forgetting of non-emotion memories too, the amygdala plays an important role in attachment learning and stress during development.

1.4.6 Stress hormones

Animal model studies have shown that the amygdala modulates the consolidation of hippocampal-dependent memories (Phelps, 2004). This effect is carried out through the activation of adrenergic receptors by stress hormones (Lalumiere, 2017). Stress hormones, such as corticosteroids and catecholamines, are critical in modulating hippocampal brain regions involved in the mnemonic process (Kim, 2015). Previous studies in animals and humans have demonstrated a particular sensitivity of early childhood to stress (Smith, 2020). However, the literature can be somewhat confusing when examining the effects of stress on memory. Mild stressors at the time of memory encoding are associated with improvements in memory retention, while severe stress is associated with memory impairment. Exposure to an early stressful experience, such as maternal separation, results in longer retention of fear memories (Callaghan and Richardson, 2012, Quinn et al., 2014) as well as faster context generalization (Elliott and

Richardson, 2018) and the skewed onset of puberty in both male and female infant rats (Cowan and Richardson, 2018). Interestingly, these stress-induced behavioural alterations can not only be inherited by offspring, but it is also possible to restore the age-appropriate profile of infantile amnesia in rats by treating this generational stress with probiotics (Callaghan et al., 2016). It is thought that early threatening experiences can lead to an early transition into adult-like memory retention (Cowan and Richardson, 2019, Peng et al., 2019). Joels and colleagues put forward a theory suggesting that stress will increase focused attention and improve memory retrieval when the stressor is (i) experienced in the context and around the time of the occurring event or (ii) when stress response hormones or transmitters are released on the same circuit as those activated by the occurring event (Joels et al., 2006).

The neurotransmitter and stress hormone epinephrine is known to facilitate memory consolidation in adult rats and also may play a process in infantile amnesia (Mcgaugh, 2013). The positive effect of epinephrine on infant memory retention has been demonstrated by the administration of epinephrine post-training (Gold et al., 1982) pre-reinstatement (Haroutunian and Riccio, 1977) or pre-test (Flint et al., 2007) for a passive-avoidance task. However, in the Haroutunian and Ricco study training occurred at P21, an age at which it is though hippocampal-dependent memories are more consistently retained (Rudy, 1987, Blair et al., 2013, Kraemer, 1995). Therefore, it is difficult to apply these studies to the scope of infantile amnesia. The role of norepinephrine has yet to be studied in the case of infantile amnesia. Although it is unlikely that epinephrine exerts a direct effect on the brain since it does not readily cross the blood-brain barrier (Axelrod et al., 1959), an experiment carried out by Miyashita and Williams demonstrated that epinephrine administrated systemically may induce norepinephrine release in the hippocampus by indirectly activating neurons in the locus coeruleus (Miyashita and Williams, 2004). Is stress at the time of learning in infants driving the maturation of fear circuity to an adult-like state or is stress only affecting the memory engram being encoded at the time?

1.5 Possible functions of infantile amnesia

1.5.1 Evolution of infantile amnesia

A central question that needs to be addressed is what the purpose of infantile amnesia is, why and how did it evolve. Is infantile amnesia simply a bug of evolution or is it a feature of a complex mammalian brain? If infantile amnesia is a bug caused by development, then it holds no functional significance. However, infantile amnesia may have evolved as a protective feature and that like, natural forgetting it is an adaptive trait to enhance survival (Ryan and Frankland, 2022). Interestingly, precocial rodents, such as guinea pigs, who are relatively neurologically mature at birth, do not experience infantile amnesia (Campbell, 1974, Akers, 2014). Campbell and colleagues first investigated whether this relative maturity at birth had any association with their ability to form long term memories (Campbell, 1974). Using a motivating place discrimination task, the researchers tested memory retention in rats and guinea pigs who were 15-20 days old at the time of training. Both rats and guinea pigs showed memory retention when tested the following day. However, when tested 2 weeks after training, only the guinea pigs showed memory retention for the discrimination task. Further, they demonstrated that 5-day old guinea pigs showed similar memory retention to 100-day old guinea pigs when tested up to 75 days after training. This study was further supported by Akers and colleagues who also demonstrated that precocial mammals such as guinea pigs and dengue show adult-like memory retention from a young age (Akers, 2014). Thus, it appears that precocial mammals do not experience infantile amnesia. These studies give crucial understanding to the function infantile amnesia has evolved for. Has infantile amnesia evolved in altricial mammals as a protective mechanism due to the lack of maturity at birth?

1.5.2 Paradoxical attachment memories

To support nipple attachment and huddling, rodent pups must learn to approach and prefer maternal odour (Raineki, 2010). Rodent pups younger than P10 are said to be in a sensitive period where they will demonstrate odour preference for an odour that has been paired with a shock, an effect that is not witnessed after P10 (Moriceau et al.,

2006, Upton, 2010). If these paradoxical attachment memories are not forgotten the rat pup may not survive on its own. Similarly in humans, certain experiences related to crawling are no longer of positive valence once they can walk. It is plausible that memories formed early in development are forgotten because they are no longer relevant later in life. This is particularly true in infancy where one is continuously learning about the world around them and trying to learn schemas that will allow them to survive. Our early childhood memories may be forgotten because they are no longer adaptive and have been overwritten by newer experiences that are more relevant to our current needs.

1.5.3 The GABA switch

The postnatal brain requires a finely tuned balance between excitation and inhibition to correctly shape neuronal circuits. This balance is retained by the neurotransmitters glutamate and GABA via feedback and feed-forward inhibition (Peerboom, 2021). Interestingly early in development GABA, which generates inhibition in the mature brain, acts as a depolarizing transmitter (Ganguly, 2001). The GABA switch in the transmission is progressive and permanent and has been proposed to underlie many neurodevelopmental disorders (Ramamoorthi, 2011). This shift from depolarizing to hyperpolarizing starts after birth and is complete in rodents by the end of the 1st to 2nd postnatal week (Valeeva et al., 2013). This flip in signalling is caused by a reduction in the intracellular chloride concentration that occurs in the 2nd week of development (Rivera and Pirvola, 1999, Sipila, 2006). The expression of KCC2 substantially increases while NKCC1 expression decreases resulting in the reduced intracellular chloride concentration. Early expression of KCC2 can prematurely convert GABA action from excitatory to inhibitory (Cancedda et al., 2007). Callaghan and colleagues demonstrated that stressful experiences such as maternal separation can prevent forgetting due to infantile amnesia (Callaghan and Richardson, 2012). The researchers exposed infant rats to maternal separation between P2-14. Infant rats were then trained at P17 and tested for memory recall at 1-55 days later. Maternally separated infants showed memory retention up to 30 days after training. Interestingly, maternal separation during this period delays the GABA switch by inhibiting KCC2 expression (Furukawa et al., 2017).

The functionality of GABA signalling is switched to reduce neuronal firing. In humans, the GABA switch occurs over a longer period. For example, in the cortex, the GABA functional switch, reflected by a substantial rise in KCC2 expression, occurs during the 1st postnatal year (Dzhala et al., 2005). This switch in the functionality of GABA signalling may play a part in the mechanism underlying infantile amnesia. It is plausible that when GABA switches from an excitatory to an inhibitory state that the engram is becomes inhibited. Thus, synaptic firing may not allow retrieval of the engram by natural cues.

1.6 Autism

Autism, which is 4-5 times more prevalent in boys than girls, is a prevalent neurodevelopmental disorder that is commonly associated with behaviours such as difficulty with social interactions and repetitive behaviour (Werling, 2013, Losh, 2008). Evidence suggests that a key contributor to the development of ASD is a naturally occurring impairment in GABAergic transmission (Gao, 2015). Animal and human studies have revealed important dysfunctions in GABAergic signalling in models of autism (Cellot, 2014). Rett syndrome mouse models of autism have shown an altered excitatory/inhibitory balance as well as depressed GABAergic synaptic transmission during the infantile period (Dani et al., 2005, Jin et al., 2013). Further, a decreased number of GABAergic interneurons have been demonstrated in multiple mouse models of autism (Gogolla et al., 2009, Sgado et al., 2013, Selby, 2007). Profiling data from mice and humans have demonstrated the expression of ASD related genes in GABAergic interneurons (Xu et al., 2014). In humans, postmortem analysis of brain tissues from individuals affected by ASD shows a significant reduction of the receptors $GABA_A$ and $GABA_B$. One brain region affected is the hippocampus which shows a reduction of the GABAergic receptor system in high binding regions (Blatt, 2001). Further, postmortem analysis has shown that the hippocampal formation there is an increased expression of GABAergic interneurons expressing calcium-binding proteins (Lawrence et al., 2010).

Interestingly participants on the autism spectrum remember autobiographical memories from a significantly earlier age in childhood (2.9 years) when compared to control groups (3.76 years) (Zamoscik et al., 2016). This study implemented an online

questionnaire to assess the recall of early childhood memories of people on the autism spectrum and non-autistic people. They compared the recall of their earliest autobiographical episodic and semantic memories. They found no difference in the earliest age of recall of semantic events between groups. However, when asked about their earliest autobiographical memories, participants with high-function autism seemed to remember events from an earlier age compared to the control group. It appears there are other reports in which some individuals with high-functioning autism seem to be capable of recalling personal events from a very young age (Mukhopadhyay, 2000, Brauns, 2002, Lyons, 2005). However, studies of a personal recall are limiting and can be easily skewed or misremembered. Thus, the investigation of infantile amnesia in models of autism requires more scientific investigation.

1.6.1 Maternal immune activation

Many abnormalities in the brain structures and systems have been studied in individuals with ASD (Ha, 2015). There is much debate on the aetiology of autism. Although ASD is highly heritable, most cases are idiopathic (Courchesne et al., 2020). However, there is consensus in the autism research community that both environmental and genetic factors can contribute to the development of ASD (Korvatska et al., 2002). Recent studies have brought to light the increased frequency of ASD phenotypes in children from mothers that were exposed to viral infection during pregnancy (Ashwood et al., 2006, Patterson, 2009, Atladottir et al., 2010). This hypothesis suggests that immune activation during pregnancy at a period critical for brain development is correlated with the development of ASD. This maternal immune activation (MIA) hypothesis has also been tested in rodent models (Lammert, 2019). Several studies have demonstrated that activation of the immune system during pregnancy through viral mimics results in changes in the brain and behaviour in the offspring that parallels the features of human cases of ASD (Choi et al., 2016, Kalish et al., 2021, Reed et al., 2020, Shin Yim et al., 2017). Rodent offspring from such MIA models exhibit classical ASD phenotypic behaviour such as repetitive behaviour and deficits in social behaviour (Choi et al., 2016, Zuckerman, 2005). Thus, MIA provides a rodent model of autism.

The interleukin-17 (IL-17) family consists of pro-inflammatory cystine knot cytokines that play a role in numerous immune regulatory functions (Pappu, 2011). Of the six IL-17 family members (A-F), interleukin-17a (IL-17a,) is the most widely studied cytokine. IL-17a is produced by T helper 17 (Th17) cells (Xu, 2010). Playing an important role in host defence, Th17 cells can be produced by several populations of immune cells (Ouyang, 2008). Under normal conditions, IL-17a promotes inflammation through the production of chemokines and pro-inflammatory cytokines (Jin, 2013). Studies in humans have detected IL-17a in the serum of a subset of children with autism (Suzuki et al., 2011, Al-Ayadhi, 2012). Interestingly children with ASD have shown improved behaviour and social skills during fever. The authors found that IL-17a was the key determinant for the reversal of ASD symptoms (Mutlu, 2021).

With the use of genetic mutants and blocking antibodies in mice, Choi and colleagues demonstrated that the cytokine IL-17a is required for MIA-induced ASD phenotypes in offspring (Choi et al., 2016). Administration of viral mimics such as polyinosinic: polycytidylic acid (poly I: C) to rodents provokes the production of the predominant cytokine IL-17a in maternal T helper 17 (Th17) cells. Induction of IL-6, caused by the mimic infection, stimulated the differentiation of these Th17 cells, causing the increase in IL-17a secretion (Choi et al., 2016, Smith et al., 2007). IL-17a then translocates across the placenta barrier where it acts on IL-17a receptor-expressing cells in the developing foetus, causing an abnormal cortical phenotype and ASD-like behaviours in the offspring (Fig. 3) (Choi et al., 2016).

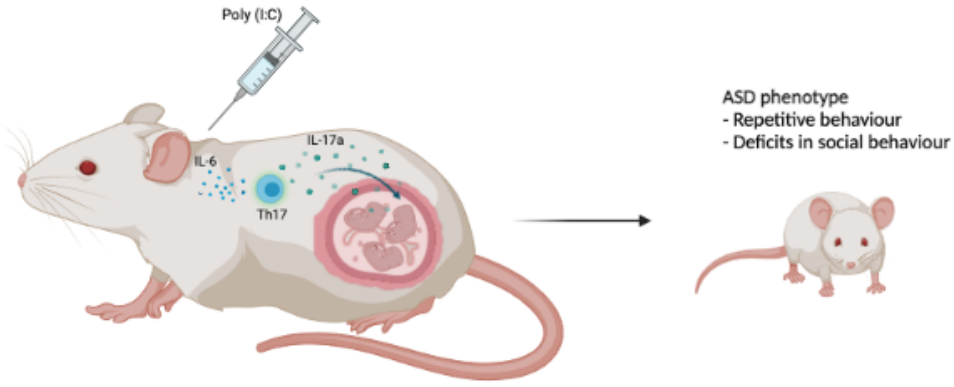


Figure 1.3. Schematic diagram of maternal immune activation in rodents. Image created using Biorender.com.

Despite the recent progress in understanding the disruptive effect of MIA, there remains limited understanding of how MIA alters the neurobiological substrates that underlie the abnormal phenotypes seen in offspring. Although multiple brain regions in the offspring of MIA models are affected, one study suggests that the social deficits associated with MIA offspring are due in part to hypothalamic pituitary adrenal axis dysregulation in the ventral hippocampus (Zhao et al., 2021). Poly(I: C) offspring rats have indeed exhibited smaller hippocampal, striatum and prefrontal cortex volumes and larger ventricular volumes (Piontkewitz et al., 2011). It is clear that, along with other regions, the hippocampus is an area that is affected by MIA. Another study investigated the relationship between neuronal activity in the anterior cingulate cortex (ACC) and social deficits seen in MIA models (Okamoto et al., 2018). By locally injecting clonazepam, the GABBAA receptor enhancer, into the ACC of MIA offspring they were able to rescue social deficits seen in the offspring of MIA models. The authors suggest that GABAergic malfunction in the AAC is critical and leads to social deficits in MIA models. GABAergic and glutamatergic abnormalities have also been seen in the hippocampus of adult offspring from dams who received MIA during gestation (Meyer, 2008). Another possibility is that MIA models may be influencing the expression of the GABAergic migratory factor, Arx, which is critical to the healthy neurodevelopment of PV interneurons. This in turn results in GABAergic dysfunction (Nakamura et al., 2019).

1.7 Research Objectives

The development of memory engram technology has revolutionised the way memory can be studied in rodent models. This integrative thesis employs inter-disciplinary methods to directly probe the importance of engram cell connectivity for memory retrieval, storage, and encoding. The outcomes will help directly inform a novel neurobiological model of memory engram storage across development. Through different behavioural paradigms, this thesis will investigate the degree of memory retention of infant mice both in context and complexity. This research will test the hypothesis that putative engram cells are sufficient for the recall of a forgotten infantile memory and that the latent memory does exist in a reactivatable state. Thus, demonstrating that infantile amnesia is due to a retrieval deficit, rather than a problem in memory encoding or storage. Further, this thesis will probe where it is possible to permanently reinstate a 'lost' infantile memory through optogenetic interventions. Finally, this research will probe the mechanisms causing infantile amnesia and explore possible models in which infantile amnesia is prevented.

Chapter 2: Materials and Methods

2.1 Subjects

2.1.1 Husbandry

All mice were socially housed in numbers 2-5 littermates, on a 12 h light/dark cycle with access to food and water *ad libitum*. The day of birth was designated postnatal day 0 (P0). Infant (P17) mice were housed with parents until the time of weaning (P21), where they were group-housed by the same sex. For delayed weaning experiments, mice were housed with parents until P26. For environmental enrichment protocols, mice were housed in a large transparent cage 60 x 60 x 30 cm with nesting material, a running wheel, plastic tubes, and a plastic house. Enrichment items were substituted weekly for novelty. For behavioural experiments, all mice used were male unless stated otherwise. All mice were 7 weeks old before undergoing surgery. Post-operation mice were allowed to recover for a minimum of 2 weeks in their home cage before experimentation. All procedures relating to mouse care and treatment conformed with the health produces regulatory authority (HPRA) Ireland guidelines.

2.1.2 Mouse lines

Two mouse lines were used for experimental purposes. To evaluate natural forgetting the wild-type mouse line C57BL/6J was used. For engram labelling and optogenetic reactivation protocols, the Ai32-Fos-TRAP mouse line was used. The Ai32-Fos-TRAP mice were generated by crossing Fos-iCre (Guenthner, 2013, Allen, 2017) mice with Ai32(RCL-ChR2(H134R)/EYFP) (Madisen et al., 2012, Madisen et al., 2010) and selecting offspring carrying both the CreETR and EYFP transgenes. Mouse genotypes were determined by sending ear clip DNA samples to Tranetyx. Compound heterozygous mutants for CreETR and EYFP were used for all experiments.

2.2 Engram labelling

2.2.1 4-OHT-dependent expression of ChR2-EYFP in Ai32-Fos-TRAP mice

4-OHT was dissolved at 20 mg/mL in ethanol by shaking at 37^{o}C for 15 min. The dissolved 4-OHT was then stored at –20 ^{o}C for up to several weeks or used immediately. For use, the dissolved 4-OHT was mixed with Chen Oil (of 4 parts sunflower seed oil and 1 part castor oil) at a concentration of 10 mg/mL by shaking at room temperature for 15 min. The ethanol was then evaporated by vacuum centrifugation. The final 10 mg/ml 4-OHT solution was injected intraperitoneally (2 h post-training) the same day at a dose of 50 mg/kg. Mice were sacrificed and immunohistochemically analysed.

2.2.2 Activity-dependent expression of ChR2-EYFP in Ai32-Fos-TRAP mice

Kainic acid (Sigma-Aldrich) was dissolved in deionised H2O (5mg/mL) at room temperature, aliquoted and stored at -20 ^{o}C. Mice (P17) were injected intraperitoneally with 20 mg/kg kainic acid followed by intraperitoneal injection of 4-OHT 2 h post-kainic acid injection. Mice were sacrificed and immunohistochemically analysed.

2.3 Behaviour

2.3.1 Behavioural assays

All behavioural experiments were conducted during the facility's light cycle of the day (07:00 am to 19:00 pm). All behavioural subjects were individually habituated to handling by the investigator for 3 min on 3 consecutive days. Handling took place in the breeding room. Immediately before each handling session mice were transported in separate cages to and from the vicinity of the experimental room to habituate them to the journey. Mice were transported to and from the experimental room in separate Perspex cages. All experimental groups were counterbalanced. All experiments were conducted in accordance with HPRA Ireland guidelines.

2.3.2 Contextual fear conditioning

Three distinct contexts were used for contextual fear conditioning (CFC). Context A was a 31 x 24 x 21 cm Med Associates chamber with a removable grid floor (bars 3.2 mm diameter, spaced 7.9 mm apart), opaque triangular ceiling, and scented with 0.25 % benzaldehyde. Context B chambers were 29 x 25 x 22 cm Coulbourne Instruments chambers with Perspex white floors, bright white lighting, and scented with 1 % acetic acid. All conditioning sessions were conducted in Context A. Each session was 330 s in duration with three 0.75 mA shocks of 2 s duration delivered at 150 s, 210 s, and 270 s. Mice were removed from the conditioning chamber 60 s following the final footshock and returned to their homecage. All recall or context specificity tests were 180 s in duration. Testing conditions were identical to training conditioning except that no shocks were presented. Up to 4 mice were run simultaneously in the 4 identical chambers. Floors of chambers were cleaned with Trigene before and between runs.

2.3.3 Contextual pre-exposure

For context pre-exposure mice were exposed to Context A for 10 min. Mice were then removed from the chamber and placed back in their homecage. To update the contextual engram with shock information mice were placed back in Context A where an immediate foot shock (1mA, 2s) was delivered. Mice were removed from the chamber 1 min after the shock and placed back in their homecage. Up to 4 mice were run simultaneously in the 4 identical chambers except for during the update when one mouse was run at a time. All recall or context specificity tests were 180 s in duration. Floors of chambers were cleaned with Trigene before and between runs.

2.3.4 Novel object recognition

Each subject was allowed to habituate to the rectangular testing arena 30 x 20 x 30 cm for 10 min on the day before object acquisition. Infant mice were allowed an extra habituation day where the whole litter was habituated at the same time to reduce stress levels. During object acquisition, two identical objects were positioned on adjacent walls of the arena. Mice were allowed to explore both objects freely for a 10 min period before being placed back in their homecage. On testing, one familiar object was replaced with a novel object and each subject was introduced for a 5 min exploration

period. Video recordings were made to allow for manual scoring of object exploration. Object exploration was defined as the time when the subject's nose came within a 2 cm radius of the object. In between each trial, the testing arena and objects were cleaned with Trigene.

2.3.5 Novel object in context recognition

The object/context mismatch discrimination task was carried out in a rectangular testing arena 30 x 20 x 30 cm with differing wall cues. Mice were habituated twice to both Context A and Context B for 10 min across four sessions. In two separate acquisition sessions, subjects were allowed to explore Context A – Object A pairing and Context B – Object B pairing for 10 min. In the testing session, mice were placed in either Context A or Context B for 5 min with an object/context mismatch. Excluding the testing day, subjects received two sessions per day. The first session began between 09:00-11:00 hr and the second session began between 16:00-18:00 hr. Object exploration was defined as the time when the subject's nose came within a 2 cm radius of the object. In between each subject, the testing arena and objects were cleaned with Trigene.

2.3.6 Barnes maze

Training consisted of five trials with a 10 min limit per trial. At the start of each trial, the mouse was placed in a black PVC start chamber located in the centre of the maze apparatus. After a 15 sec period, the start chamber was removed, and the latency and distance travelled to enter the escape tunnel were recorded. Subjects were placed back in their home cage and allowed 20 min in between each trial. The position of the escape tunnel remained in a fixed location relative to spatial cues in the room. For each probe trial, the escape tunnel was removed from the apparatus. Each mouse was allowed 5 min to freely explore the four quadrants of the maze before being removed and placed back in their home cage. The latency and distance travelled before reaching the location of the removed escape tunnel, along with the time spent in each quadrant were recorded. The apparatus and escape tunnel were cleaned with Trigene after each trial.

2.3.7 Three-chamber social interaction

Mice were habituated to the chamber with two empty holders for 10 min. The next day, mice were placed in the middle of the chamber and allowed to explore the three chambers for a period of 5 min. During this testing period, a social object (novel mouse) was contained in one holder in one chamber and an inanimate object (lego blocks) was contained in a holder in the other chamber. Object exploration and distance travelled were tracked using ANY-maze video tracking software.

2.3.8 Marble burying

A large testing arena 60 x 60 x 30 cm was filled 5 cm deep with wood chipping bedding, lightly tamped down to make an even flat surface. A consistent pattern of 20 identical glass marbles (15mm diameter) was evenly placed (4 cm apart) on the surface of the wood chip bedding. Mice were left alone in the testing arena for 30 min. A picture was taken before and after the test for analysis. A marble was considered buried if 2/3rds of the depth of the marble was buried.

2.3.9 Analysis and statistics

All behavioural experiments were analysed blind to the experimental group. All videos were randomized before manual scoring. Behavioural performance was recorded by a digital video camera. Videos were manually scored individually, and investigators were blind to the experimental condition and test day during all manual scoring. Data analysis and statistics were conducted using GraphPad Prism 6.00 (GraphPad software). Unpaired Student's t-tests were used for independent group comparisons. Paired Student's t-tests were used to assess differences within groups. ANOVA followed by a Bonferroni *post hoc* test was used to determine conditions that were significant from each other where appropriate. Results are displayed as mean with SEM and deemed significant when $P^* < 0.05$.

2.4 Immunohistochemistry

2.4.1 Immunostaining

Mice were dispatched by overdose with 50 μl sodium-pentobarbital and perfused transcardially with phosphate-buffered saline (PBS), followed by 4 % paraformaldehyde (PFA) in PBS. Extracted brains were kept in 4 % PFA at 4 ^{o}C overnight and stored in PBS. 50 μm coronal slices were cut using a vibratome and collected in PBS. Slices were washed in PBS-Triton X-100 (PBS-T) 0.2% followed by a 1 h blocking in PBS-T with 10% normal goat serum at room temperature before being incubated with the primary antibody at 4 ^{o}C overnight. Slices were washed in PBS-T 0.1 % followed by a 1 h incubation with the secondary antibody before undergoing another round of washing using PBS-T 0.1%. Vectashield DAPI was used to mount the slices onto superfrost slides before visualization using an Olympus Bx%1 upright microscope. For cell counting experiments an additional incubation with DAPI in PBS (1:1000) was carried out to label nuclei and images were acquired using Leica SP8 gated stimulated emission depletion (STED) nanoscope. All images were taken at 40 X magnification.

2.4.2 Cell counting

To measure the extent to which populations of active cells overlap between the exposure to the same or different contexts, the number of EYFP and c-Fos immunoreactive neurons in the dentate gyrus (DG), amygdala (AMG), periaqueductal gray (PAG) and retrosplenial cortex (RSC) were counted as indicated below. All animals were sacrificed 45 min post-assay or optical stimulation for immunohistochemical analyses. Four coronal slices were taken from each animal (N=4, n=4). For optogenetic stimulation experiments, only slices with accurate implant location sites were used for counting. Sections were imaged on a Leica SP8 gated stimulated emission depletion (STED) nanoscope at a magnification of 40X. The area of interest was manually identified and the area of each region of interest was calculated using Fiji. To calculate the total number of DAPI cells in the DG, the average diameter of a sample of EYFP positive cells in the DG was taken from each animal and the area of the cell was calculated. The total number of cells was estimated as the area of the DG divided by the area of the cell. For the AMG, PAG and RSC, the number of DAPI cells in 3 randomly selected regions of

interest were counted and used along with the total area of the region to determine the total number of DAPI cells. The total number of c-Fos positive and EYFP positive cells were manually identified and counted for each region using Adobe Photoshop CC 2018. To calculate the percentage of cells expressing EYFP in each region, the total number of EYFP positive cells was divided by the total number of DAPI-positive cells for each area. To calculate the percentage of cells expressing c-Fos in each region, the total number of c-Fos positive cells was divided by the total number of DAPI-positive cells for each area. To quantify engram reactivation, the number of EYFP and c-Fos positive cells were quantified as a percentage of total EYFP cells.

2.5 Stereotactic Surgery

2.5.1 Stereotactic optical fibre implant

Mice were anaesthetized using 500mg kg^{-1} avertin. Each animal underwent bilateral craniotomies using a 0.5 mm diameter drill bit at -2.0 mm anteroposterior (AP), $\pm$ 1.3 mm mediolateral (ML) for DG injections. A bilateral patch cord optical fibre implant (200 μm core diameter) was lowered above the injection site (-1.9 mm DV). A layer of adhesive cement was applied to secure the optical fibre implant to the skull. Dental cement was applied to secure a protective cap to the implant and close the surgical site. Each animal received meloxicam analgesic (0.075 mL/5g) via subcutaneous injection and remained on a heating pad until fully recovered from anaesthesia. All mice were allowed to recover for two weeks before any subsequent experiments. All fibre sites were verified histologically.

2.6 Optogenetic Stimulation

2.6.1 Optogenetic engram reactivation

Optogenetic engram reactivation was carried out in Context C, a 31 x 24 x 21 cm Med Associates chamber with a white Perspex floor and curved insert, a dim light level and scented with 0.25 % acetophenone. The optical fibre implant was connected to a 45 nm

laser diode fibre light source (Doric LDFLS 450/080). Habituation sessions lasted for 12 min. During testing, the 12 min session was divided into four 3 min epochs split into two light-off and two light-on epochs. During the light on epochs, blue light stimulation (20 Hz) with a pulse width of 15 ms was delivered through the optical fibre implant for the entire 3 min duration. At the end of the 12 min session, the mouse was immediately detached from the laser and returned to its homecage.

2.6.2 Artificial updating

Artificial updating of a contextual memory was carried out in 29 x 25 x 22 cm Coulbourne Instruments chambers with removable grid floors, bright white lighting, and scented with 1 % acetic acid. The optical fibre implant was connected to a 45 nm laser diode fibre light source (Doric LDFLS 450/080). The 7 min session consisted of a 2 min acclimatisation period followed by 5 min of blue light stimulation (20Hz) with a pulse width of 15 ms delivered through the optical fibre implant for the entire 5 min duration. Three shocks, 0.75 mA of 2 s duration, were delivered at minutes 4, 5 and 6 of the session. At the end of the 7 min session, the mouse was immediately detached from the laser and returned to its homecage.

2.7 Electrophysiology

2.7.1 Slice preparation

Animals were anaesthetized, and brains were collected and dissected in oxygenated (95% O_2, 5% CO_2) cold cutting artificial cerebral-spinal fluid solution (in mM): 213 Sucrose, 2.5 KCl, 10 $MgCl{\cdot}6H_2O$, 1.25 NaH_2PO_4, 0.5 $CaCl_2{\cdot}H_2O$, 25 Glucose, 25 $NaHCO_3$. Coronal sections of 300µm thick, containing dorsal hippocampus were obtained with a Vibratom. Slices were collected and let to recover at room temperature for 1 h. During the recordings, the temperature was maintained and controlled at 32°C (Temperature controller IIV, Luigs & Neumann) in oxygenated (95% O_2, 5% CO_2) artificial cerebral-spinal fluid solution (in mM): 125 NaCl, 2.5 KCl, 1 $MgCl{\cdot}6H_2O$, 1.25 NaH_2PO_4, 2 $CaCl_2{\cdot}H_2O$, 25 Glucose, 25 $NaHCO_3$.

2.7.2 Ex vivo electrophysiology

Whole cell recordings in voltage clamp mode were performed under the guidance of a motorized infracontrast DGC microscope (LNScope, Luigs & Neumann) with a water immersion 40X objective, and a CCD ICX205 camera (INFINITY 2-1R). To identify the presence of ChR2-EYFP in neurons a X-Cite XLED1 lamp was used for excitation and a U-M49002 fluorescence EYFP filter cube (Olympus). The recording Ag/AgCl electrodes were fabricated with borosilicate glass pipettes (P1000, Sutter Instrument) of 7 to 10Mohms resistance and filled with the following intracellular solution (in mM): 117 Cs-Methanesulfonate, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 4 Mg-ATP, 0.3 Na-GTP, 5 TEA-Cl, 10 QX314-Br, 0.1 spermine, and mannitol to adjust osmolarity to 290mOsm, pH 7.2. The signal was amplified with a Multiclamp 700B dual amplifier (Molecular Devices), filtered at 2 kHz, digitized (20 kHz), and acquired through an ADC/DAC data acquisition interface (ITC-18, InstruTECH) by using a custom-made software running on Igor Pro (Wavemetrics). Spontaneous postsynaptic currents (sPSC) were recorded in each neuron at -80mV (sEPSC) and 0mV (sIPSC) for 40s or more on each condition.

2.7.3 Signal analysis

For automated event detection, the recordings were processed with NeuroMatic (Rothman and Silver 2018) for Igor Pro (Wavemetrics). Spontaneous excitatory postsynaptic currents (sEPSCs) were detected at -80mV, while the inhibitory postsynaptic currents (sIPSCs) were detected at 0mV.

2.8 Dendritic Morphology

All Z-stack images were taken at 40X using a Leica SP8 scanning confocal microscope and Leica Application Suite X software (Las X). Imaris (Oxford Instruments) software (version 9.5) was used for the analysis of dendritic parameters of engram cells. Image processing filters (Median and Gaussian filters) were applied to images for maximum clarity. The Neurofilament Tracer add-on enabled semi-automatic tracing of individual dendritic branches of each neuron.

2.9 Maternal Immune Activation

Mice were mated overnight for one night. Females were weighed and checked for seminal plugs, noted as E0.5. Pregnant dams were injected SC with a single dose of Poly(I: C) (Sigma-Aldrich) at 20mg/kg, rmIL-17A (Immunotools) at 50ug/kg or control PBS on E12.5.

Chapter 3: Behavioural Characterisation of Wild-type Mice

Infants show clear learning from a young age but rapidly forget episodic memories formed early in life (Campbell and Campbell, 1962, Hayne and Jack, 2011). This phenomenon, termed infantile amnesia, is one of the most common forms of amnesia. In rodents, infantile amnesia can be witnessed between the postnatal days 17 (P17) to 25 (P25) (Campbell and Campbell, 1962). This chapter focuses on characterising the nature of infantile amnesia in wild-type mice. Behaviour is expanded from contextual fear conditioning to look at both spatial and object memory.

3. 1 Fear Contextual Memory

Learning to identify and predict danger is important for survival across species. Pavlovian fear conditioning relies on learned defensive behaviour to mitigate and anticipate threats in the environment (Pavlov, 1927, Rescorla, 1967). This conserved fear allows us to study associative learning across species. Although it is unknown whether non-human animals experience fear there are common behavioural readouts that reflect a state of fear in rodents such as freezing or escape behaviour (Adolphs, 2013).

3.1.1 Pavlovian Fear Conditioning

Pavlovian fear conditioning involves associating a neutral conditioned stimulus (CS) with an aversive unconditioned stimulus (US) (Pavlov, 1927, Rescorla, 1967). In the following experiments, fear conditioning involves a CS composed of a learned multi-modal context (including specific contextual features, lighting, and olfactory cues) while the US usually consists of foot shocks. When the US is delivered at the same time as exposure to the CS, an association between the CS and the US is formed. For learning to occur, the conditioned response must occur in proximity or very soon after the stimulus is presented. Thereafter, conditioned mice will display conditioned freezing behaviour whenever exposed to the CS or elements of the CS (Rescorla, 1967). In the case of contextual fear conditioning freezing in response to the trained CS is a function of memory (Wehner, 2004). Pavlovian fear conditioning is a robust behavioural paradigm

translated across species (Hartley, 2010, LaBar, 1998, Kindt, 2013). While in rodent studies the unconditioned stimulus usually involves footshocks, this is substituted in human paradigms with a stimulus such as pictures of angry facial expressions in humans (Wieser, 2014).

In fear conditioning, neurocircuitry involving the hippocampus, amygdala and cortical areas enables context-dependent behaviour (Tovote, 2015). During fear conditioning, the hippocampus encodes the contextual representation of the conditioned stimulus (Chang, 2017). While the amygdala, important in encoding, storing, and retrieving associations between the context and stimulus, helps encode the CS and US association (Kim, 2020). Beyond the hippocampus and amygdala, cortical regions including, but not limited to, the medial prefrontal cortex, parahippocampal cortices and the retrosplenial cortex also have an involvement in contextual fear conditioning (Rozeske, 2015, Robinson, 2012).

3.1.2 Adult mice retain memory for contextual fear conditioning

Retention for fear memory was first investigated using contextual fear conditioning in adult wild-type mice. Adult cohorts were trained at P63 and tested for fear memory recall 1 or 8 days post-training (Fig. 3.1 A). At both time points, the experimental shock group showed significantly more freezing than the no shock control (Fig. 3.1 B, C). Consistent with the literature adult mice show robust memory retention for a fear memory after one week.

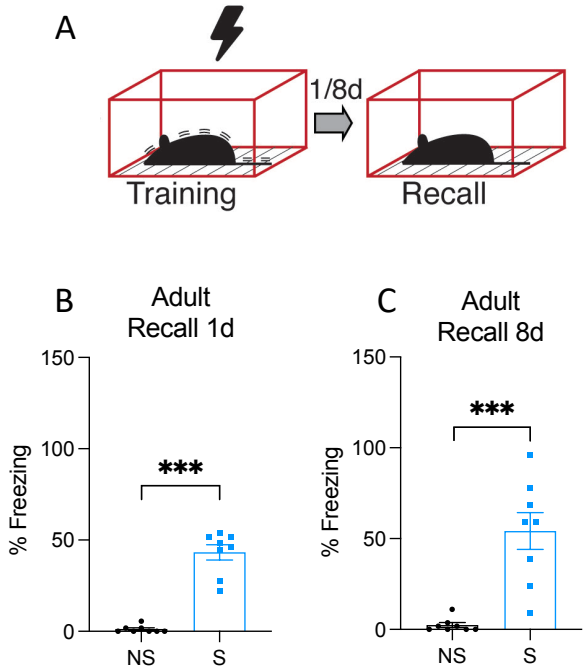


Figure 3.1. Adult C57BL/6J wild-type mice show memory retention for a fear memory 1 and 8 days after training. (A) Schematic diagram of behavioural paradigm for contextual fear conditioning in adult C57BL/6J mice. The black lightning symbol represents foot shocks. (B, C) Freezing levels of adult C57BL/6J wild-type mice when test 1 day (B) or 8 days (C) after training. N=8. No shock (NS), shock (S). Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; ***P<0.001.

3.1.3 Infant mice show infantile amnesia for a contextual fear memory at P25

To characterize this form of amnesia, a range of behavioural paradigms were applied to infant mice (P17) and their behaviour was compared to adult mice (P63). To investigate the time constraint of memory retention in infants, a cohort of infant wild-type mice were trained by contextual fear conditioning at P17 (Fig. 3.2 A). As a control, another cohort of infant mice were trained at the same time but without administering footshocks (no shock group). Using freezing as a measurement of memory, memory recall was tested 1 (Test 1), 8 (Test 3) and 17 (Test 4) days later. In addition, context specificity was tested at P18 in a neutral context B. The experimental shock group froze significantly more than the no shock control when tested 1 day later (Fig. 3.2 B).

However, when tested 8 or 17 days after training the level of freezing dropped to floor levels and no significant difference was seen between groups. No difference was seen between groups in the level of freezing in context B, demonstrating infant mice show context specificity for a fear memory. These results demonstrate that infant mice demonstrate forgetting as early as one week after training.

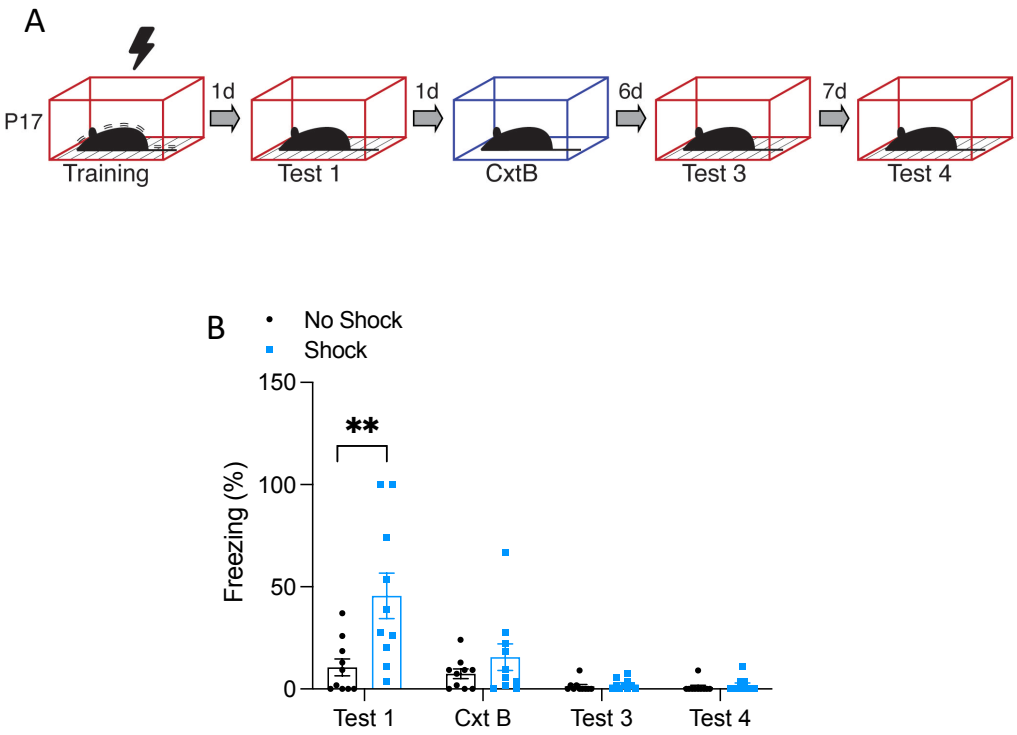


Figure 3.2. Infant C57BL/6J wild-type mice show infantile amnesia for a contextual fear memory 8 days after training. (A) Representative diagram of behavioural paradigm for contextual fear conditioning in infant C57BL/6J mice. The black lightning symbol represents foot shocks. (B) Freezing levels of infant C57BL/6J wild-type mice when tested for recall 1 (Test 1), 8 (Test 3) and 17 (Test 4) days after training. N=10. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; **P < 0.01.

To ensure that the low level of freezing observed 8 days after training was not merely due to extinction learning from exposure to the context in the absence of US presentation during testing, two cohorts of infants were trained at P17 with contextual

fear conditioning and tested for memory recall either 1 or 8 days after training (Fig. 3.3 A). Here, all groups only receive one recall test to eliminate the risk of context exposure extinction. Infants tested for memory recall a day following training showed a significantly high level of freezing compared to the no shock control group (Fig. 3.3 B). While the group tested 8 days after training showed similar levels of freezing to the no shock controls (Fig. 3.3 C). This result further supports that infant mice show rapid forgetting of fear memory a week later. Taken together the above results conclude that infant mice demonstrate forgetting as early as one week after training, while adult mice show continuous memory retention (Fig. 3.3 D).

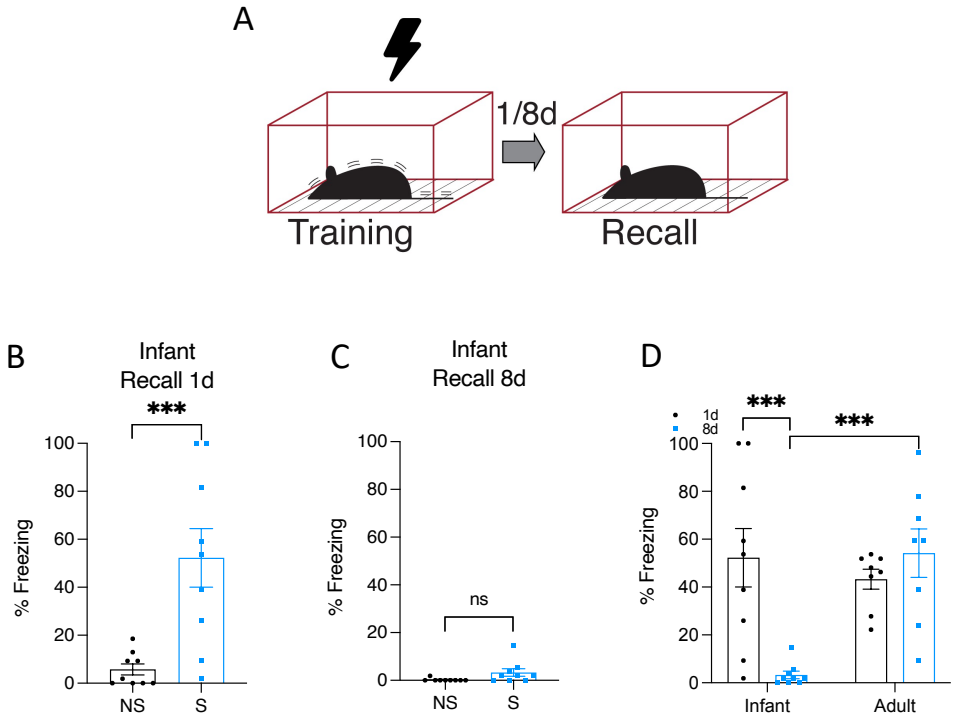


Figure 3.3. Forgetting seen in infant mice 8 days after the training is not due to extinction from exposure to context. (A) Schematic diagram of the behavioural paradigm of contextual fear conditioning in C57BL/6J infant mice at P17. The black lightning symbol represents foot shocks. (B) Freezing levels of infant C57BL/6J wild-type mice when test 1 day (B) or 8 days (C) after training. (D) Age group condition interaction for freezing levels during recall 1 or 8 d after training. N=9. No shock (NS), shock (S). Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests or two-way ANOVA Bonferroni; ***P < 0.001.

3.1.3 Infant mice retain a contextual fear memory for 3 days

It is well established that infant rodents demonstrate infantile amnesia; however, the exact time point at which forgetting occurs is still up for debate. This project demonstrates that infant mice retain a contextual fear memory for a day but rapidly forget a week later. To further investigate the point at which forgetting occurs due to infantile amnesia, a cohort of wild-type infant mice were trained using contextual fear conditioning at P17 and tested for memory recall 3 days later (Fig. 3.4 A). Mice shocked at P17 showed significantly more freezing than the control no shock group during recall (Fig. 3.4 B). Crucially, infant mice trained at P17 and tested 3 days later at P20 showed the same level of freezing to groups tested 1 day later at P18 (Fig. 3.4 C). Infant mice, trained at P17, retain a contextual fear memory at P20.

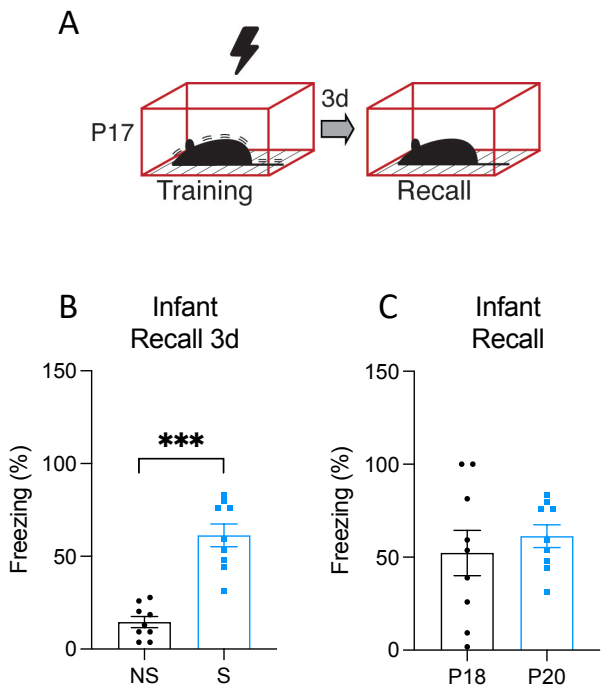


Figure 3.4. Infant C57BL/6J wild-type mice show memory recall 3 days after training. (A) Schematic diagram of behavioural paradigm for testing memory recall at 3 days. The black lightning symbol represents foot shocks. (B) Freezing levels of infant C57BL/6J wild-type mice when tested 3 days after training. (C) Freezing levels of infant C57BL/6J wild-type mice when tested for memory recall at P18 or P20. N=9. No shock (NS), shock (S). Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; ***P < 0.001.

3.2 Contextual Memory

3.2.1 Infant mice can form purely contextual memories

To satisfy the crucial criterion of information specificity, it needed to be investigated whether DG cells, activated previously during context exploration (forming a purely contextual memory), can subsequently serve as a functional conditioned stimulus in a contextual fear conditioning paradigm when artificially reactivated during the delivery of a US. To demonstrate infantile amnesia for an artificially updated context, wild-type mice were pre-exposed to context A at P17 (Fig. 3.5 A). The following day, animals received an immediate shock procedure (where mice receive one shock after 10 secs and are then removed). Immediate shock 'updates' a purely contextual memory with shock information, resulting in freezing to the context thereafter, but this only occurs if a contextual memory exists (Fanselow, 2000).

Infant (P17) mice that were pre-exposed to context A before receiving a shock update showed significant levels of freezing compared to their control counterparts, who were not pre-exposed to context A when tested 1 d later (Fig. 3.5 B). When exposed to neutral context B the control group showed a level of freezing that equated to exposure to context A (Fig. 3.5 B). Thus, they demonstrated an inability to specify between context A and context B. When tested for memory recall at P63 neither group showed memory retention for the context. Here we see that infant mice can form a purely contextual memory, which can be updated with shock information and recalled in infancy but is forgotten due to infantile amnesia. Without this pre-exposure to the context, infant mice do not show context specificity between context A and context B.

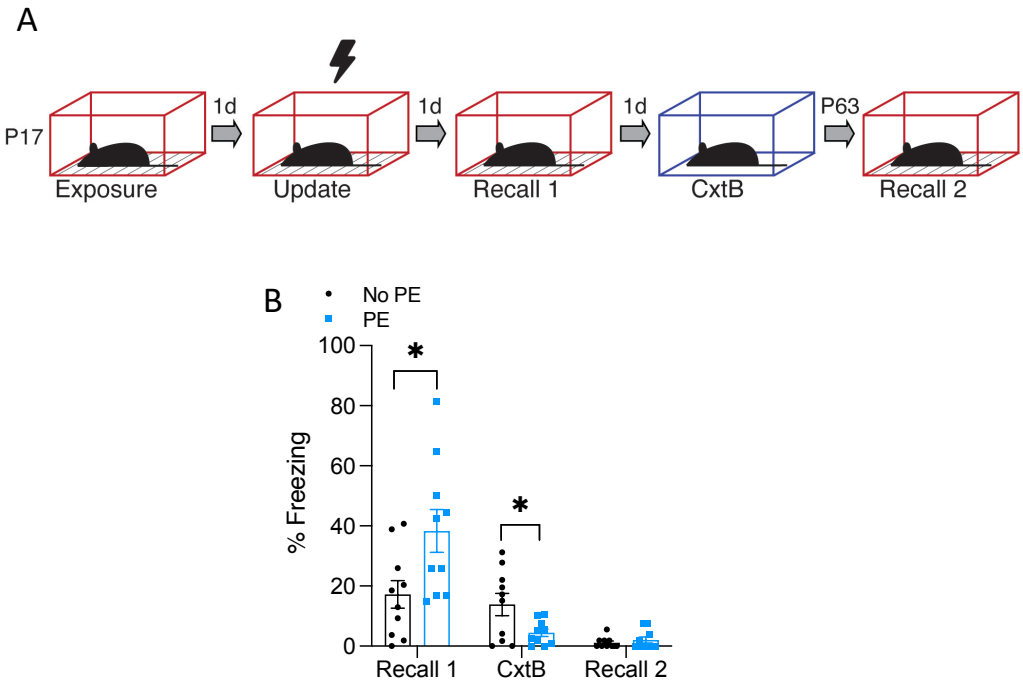


Figure 3.5. Infant C57BL/6J wild-type mice can form context-specific memories. (A) Schematic diagram of behavioural updating paradigm in infant C57BL/6J wild-type mice. The black lightning symbol represents foot shocks. (B) Freezing levels of infant C57BL/6J wild-type mice during recall tests at P19 (Recall 1), P20 (CxtB) and P63 (Recall 2). N=10. Pre-exposure (PE). Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; *P < 0.05.

3.2.2 Infant mice rapidly forget purely contextual memories

To investigate whether context exposure in infancy can act as a conditioning stimulus in adulthood the behavioural paradigm in Fig. 3.6 A was employed. Animals were pre-exposed to context A at P17 but did not receive a shock update until P63 (Fig. 3.6 A). Animals were then tested for recall and context specifiction in the following days. Adult mice that were exposed to context A during infancy (P17) and received a shock update at P63 show similar levels of freezing to mice that did not receive context A exposure when tested 1 d later (Fig. 3.6 B). Similarly, both the pre-exposure and no pre-exposure groups showed the same level of freezing to neutral context B. These results demonstrate that exposure to context A in infancy has no behavioural effect in

adulthood as the contextual memory has been forgotten due to infantile amnesia. Pre-exposure to a context in infancy does not serve as a functional conditioned stimulus when artificially reactivation during the delivery of a US is carried out in adulthood. Thus, infant mice form context-specific memories that are forgotten due to infantile amnesia.

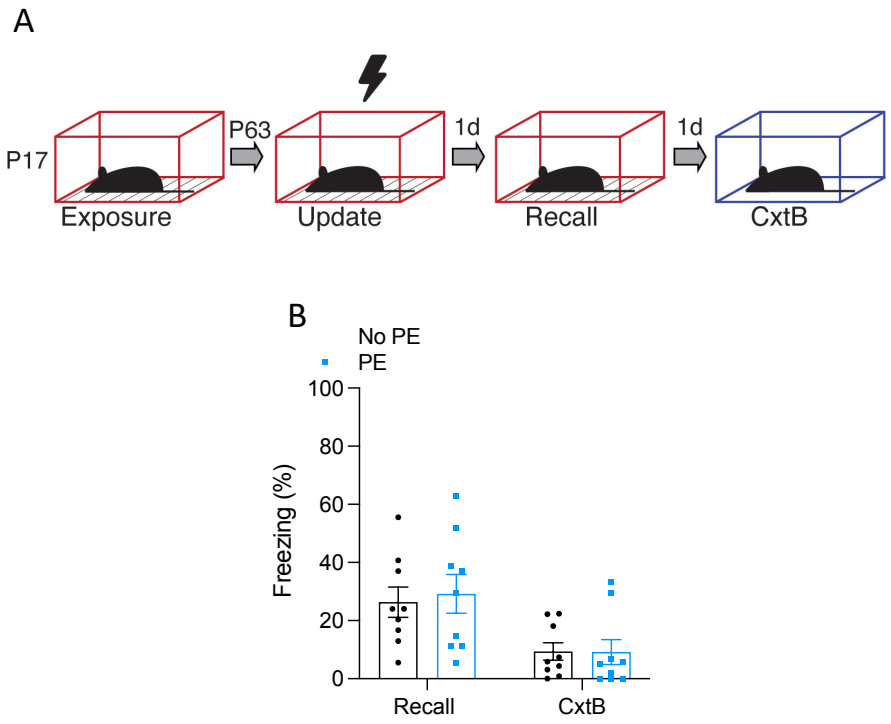


Figure 3.6. Infant C57BL/6J wild-type mice forget context-specific memories. (A) Representative diagram of behavioural paradigm. The black lightning symbol represents foot shocks. (B) Freezing levels of adult C57BL/6J wild-type mice during recall tests at P64 (Recall) and P65 (CxtB). N=9 Pre-exposure (PE). Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; *P < 0.05.

3.3 Object Memory

Rodent models of infantile amnesia have heavily relied on Pavlovian fear conditioning and place avoidance paradigms (Madsen and Kim, 2016). Although contextual fear conditioning is a robust and informative paradigm, it is also limited in its behavioural response (freezing) and the control of the stimulus. Using different paradigms of

learning allows us to assess the detail of memory context more comprehensibly in the case of infantile amnesia.

3.3.1 Novel object recognition

The novel object recognition task is a behavioural paradigm that closely resembles those applied to studying human cognition (Antunes, 2012). This makes it a superior task for translational relevance than some other rodent memory tasks. Due to its simplicity as a visual recall task, it can be widely applied to various species allowing for a cross-species analysis. Carrying out novel object recognition relies on a variety of brain regions. During memory formation of a novel object, memory consolidation of the spatial and contextual features of the objects is hippocampal-dependent, with particular involvement of the dorsal hippocampus (Albasser, 2012, Goulart, 2010). During object recognition, the perirhinal cortex is involved for short term recognition, while the hippocampus is more involved for long term recognition (Reger, 2009). The perirhinal cortex is thought to send information gathered from visual and olfactory cues to the hippocampus (Albasser, 2011), playing an important role in judging the prior occurrence of the object. Studies in humans and rodents have also demonstrated a role for the prefrontal cortex in recognition memory (Warburton, 2015).

3.3.2 Infant mice fail to retain a memory for a novel object

To test the effect of infantile amnesia on other paradigms of learning, this ontogenetic forgetting was investigated on a novel object recognition task in wild-type mice (Fig. 3.7 A). Novel object recognition is based on the tendency of rodents to spend more time exploring a novel object when presented with both a novel and a familiar object. The amount of time taken to explore the new object provides us with an index of recognition memory. Animals were habituated to the testing arena the day before acquisition. During object acquisition, both the adult (P42) and infant (P17) groups were presented with two of the same objects. Both groups spent an equal amount of time with both objects (Fig. 3.7 B, E). The following day, the mice were placed back into the arena with two different kinds of objects, one familiar and one novel. As expected, both groups spent more time exploring the novel object (Fig. 3.7 C, F). However, when the mice were tested again 8 days later, only the adult group spent more time exploring the novel

object (Fig. 3.7 D, G). These results indicate that infant mice also demonstrate rapid forgetting for novel object recognition tasks, following the developmental trend like that of other forms of episodic memory.

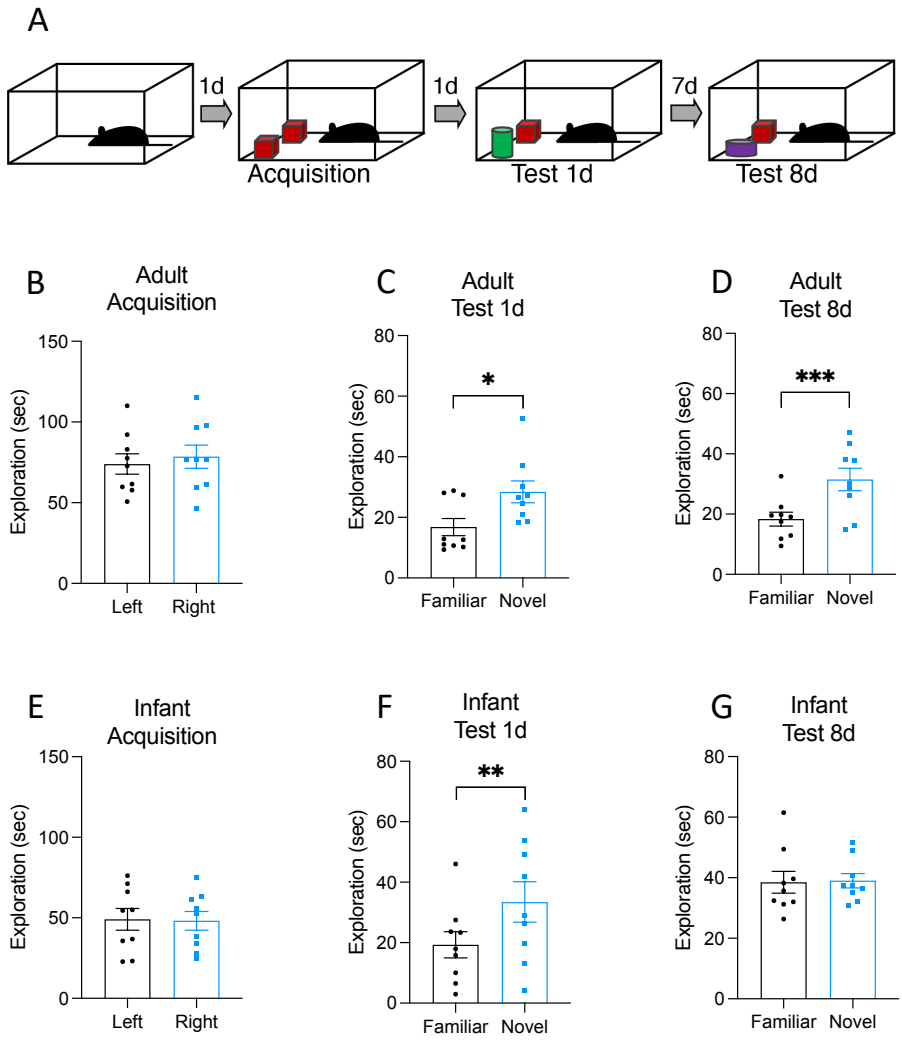


Figure 3.7. Infant C57BL/6J wild-type mice show forgetting for a novel object recognition task. (A) Representative diagram of behavioural paradigm for novel object recognition in C57BL/6J wild-type mice. (B-G) Time spent exploring both objects. (B, E) Acquisition session. (C, F) Novel object recognition test 1 day after acquisition. (D, G) Novel object recognition test 8 days after acquisition. N=9 Data presented as mean ± SEM. Statistical comparison was performed by using paired t-tests; *P < 0.05, **P < 0.01, ***P < 0.001.

3.4 Object in Context Memory

In everyday life, we spontaneously form associations between objects and the environments in which they are encountered. These associations depend on cognitive processes such as object recognition and the ability to recognize the context in which the object was encountered. Object in context memory is not a unitary process. This form of memory required a neural circuit between the hippocampus, medial prefrontal cortex and perirhinal cortex (Aggleton, 2020). The hippocampus is thought to play a key role in the formation of an association between an object and the context (Alison, 2013a). In addition to the hippocampus, the medial prefrontal cortex is involved in object spatial location and object location associations (Barker, 2020). While the perirhinal cortex encodes information about the object (Barker, 2020). These brain regions function together to form objects in memory (Newcombe, 1977, Ngo, 2019, Nomura et al., 2019, Ramsaran et al., 2016, Travaglia, 2018).

3.4.1 Object/context mismatch task

The object/context mismatch discrimination task builds on the animals' natural tendency to explore novel environments and objects. Animals are exposed to two different contexts context A (black) and context B (blue) (Fig. 3.8 A). During acquisition 1, animals are exposed to context A which houses a unique pair of identical objects. During acquisition 2 animals are exposed to context B which houses a different pair of identical objects. After a delay, animals are re-exposed to one of the contexts, this time with one object that was congruent with that context and one that was not. Animals should be able to distinguish between which of the two familiar objects is in a different context. Due to the time constraints of the infantile period, animals received two sessions a day except for on the testing day.

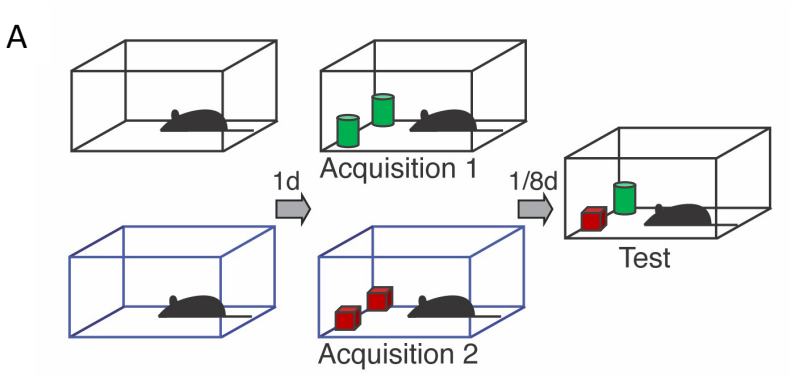


Figure 3.8. Schematic diagram of object/context mismatch task. (A) Representative diagram of behavioural paradigm for object/context mismatch task in C57BL/6J wild-type mice.

3.4.2 Adult mice show memory retention for an object in context memory

Adult mice spent an equal amount of time exploring both identical objects during each acquisition session (Fig. 3.9 A, D, B, E). When tested for an object in context memory, adult mice showed high exploration of the mismatched object both at 1 and 8 days after acquisition (Fig. 3.9 C, F). Thus, adult mice can successfully resolve the object/context mismatch task demonstrating memory retention of an object in context.

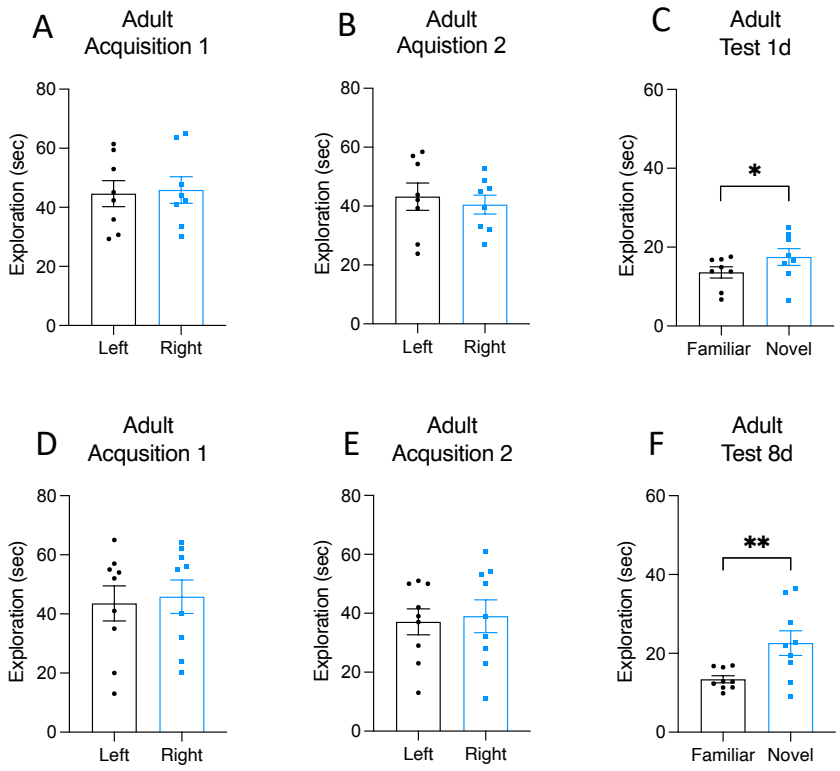


Figure 3.9. Adult C57BL/6J wild-type mice retain a memory for an object in context. (A, D) Time spent exploring both identical objects in context A. (B, E) Time spent exploring each identical object in context B. (C, F) Time spent exploring the familiar and novel object in context A 1 day (C) and 8 days (F) after acquisition. N=9. Data presented as mean ± SEM. Statistical comparison was performed by using paired t-tests; *P < 0.05, **P < 0.01.

3.4 Infant mice show do not retain a memory for an object in context

During each round of acquisition, infant mice spent an equal amount of time with both identical objects (Fig. 3.10 A, B, D, E). Infant mice were able to successfully resolve the object/context mismatch when tested a day later, which is demonstrated by the high exploration time of the mismatched object (Fig. 3.10 C). Congruent with this form of forgetting, when animals were tested 8 days later, infant mice were unable to resolve the object/context mismatch (Fig. 3.10 F). Infant mice show infantile amnesia for an object in context memory.

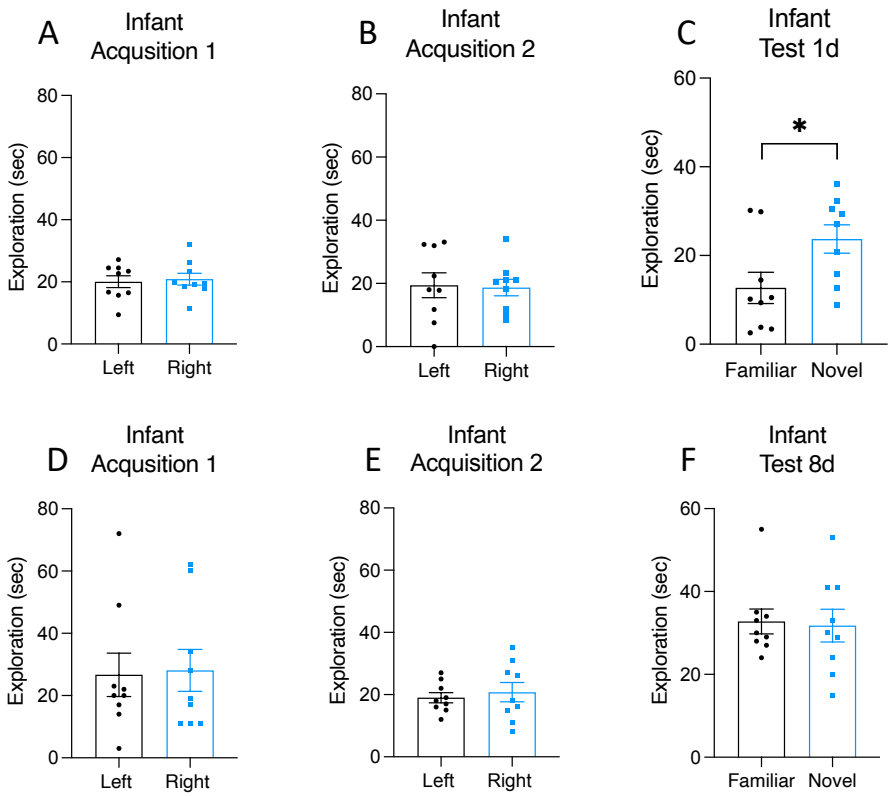


Figure 3.10. Infant C57BL/6J wild-type mice are unable to carry out the object/mismatch task 8 days after acquisition. (A, D) Time spent exploring both identical objects in context A. (B, E) Time spent exploring each identical object in context B. (C, F) Time spent exploring the familiar and novel object in context A 1 day (C) and 8 days (F) after acquisition. N=9. Data presented as mean ± SEM. Statistical comparison was performed by using paired t-tests; *P < 0.05.

3.5 Spatial Memory

Animals rely on spatial memory to effectively navigate through their ever-changing environment, allowing them to successfully find shelter, food and avoid danger. If spatial memory is disrupted, we are incapable of forming representations of our environment resulting in disorientation. Paradigms that probe spatial memory are easily translatable to humans due to their straightforward nature. Spatial memory in rodents can be examined through a battery of tasks including, but not limited to, the Morris water maze

and the T-maze (Vorhees, 2014). While in humans spatial reference memory is commonly assessed using virtual reality environments (Smith, 2019).

Spatial learning heavily involves the hippocampus. As well as encoding contextual information of the environment, the hippocampus contains place cells that fire in relation to the position of the animal in space (Eichenbaum, 1999). The interplay between the hippocampus and prefrontal cortex supports the acquisition of spatial memory reference (Preston, 2013). The hippocampus is thought to gather information about the animal's current location in the environment while the prefrontal cortex takes this information from the hippocampus and uses it to optimize a navigation route. The cortical circuit for spatial memory includes regions such as the retrosplenial, entorhinal and parietal cortex. These regions play important roles in learning and navigation during a spatial task. Like the hippocampus, the retrosplenial cortex contains place cells for the encoding of the environment (Mao, 2017). While the entorhinal cortex contains grid cells that generate virtual maps through hexagonally arranged firing fields (Ginosar, 2021).

3.5.1 The Barnes Maze

The Barnes Maze is a behavioural task typically used to probe the spatial location memory in rodents (Gawel, 2019). The Barnes maze is considered to be less stressful than the Morris water maze as well as more translatable to humans. Rodents are placed in an open circular arena containing several potential escape holes at the boundary of the arena (Fig. 3.11). Such behavioural tasks are typically used to probe spatial location memory in rodents by playing on the dislike of rodents for bright illuminated areas and favouring dark confined spaces. During the trials, only one location provides an escape. Using contextual cues and their surrounding environment, the animals' task is to learn the location of the escape hole relevant to visual cues. After a few trials rodents typically remember the location of the escape hole and will go directly to its location when placed on the Barnes maze. Memory retention for the location of the escape hole can be quantified during a probe test where the escape hole is blocked. By analysing the latency and distance travelled to first find the location of the blocked escape hole, as well as

looking at the mean distribution of time spent in each zone of the Barnes maze, it is possible to assess the ability to form and retrieve location-context memories.

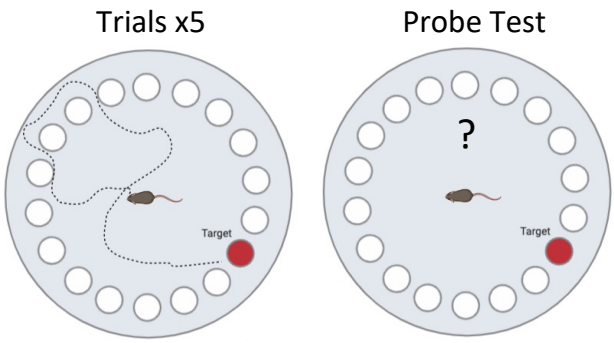


Figure 3.11. Barnes maze behavioural paradigm. (A) Representative image of behavioural paradigm for the Barnes maze. Image created using Biorender.com.

3.5.2 Adult mice show memory retention for a spatial memory

Using a cued version of the Barnes maze task assay, memory for a spatial location was probed in both infant and adult mice. First, cohorts of adult (P63) wild-type mice received 5 training trials on the Barnes maze. Both cohorts sufficiently learnt the location of the escape hole, demonstrated by a gradual decrease in the latency and distance travelled to reach the escape hole (Fig. 3.12 A, B, E, F). Memory retention for the location of the escape hole was tested either 1 or 8 days after training (Fig. 3.12 C, D, G, H). Regardless of whether the probe test was carried out 1 or 8 days after training, adult mice spent more time around the location of the blocked escape hole (Fig. 3.12 C, D, G, H). Further, animals showed no difference in the latency or distance travelled to first reach the blocked escape hole during the probe test at 1 or 8 days (Fig. 3.12 I, J). Thus, adult mice were able to remember the location of the escape hole regardless of whether they were tested 1 or 8 days later.

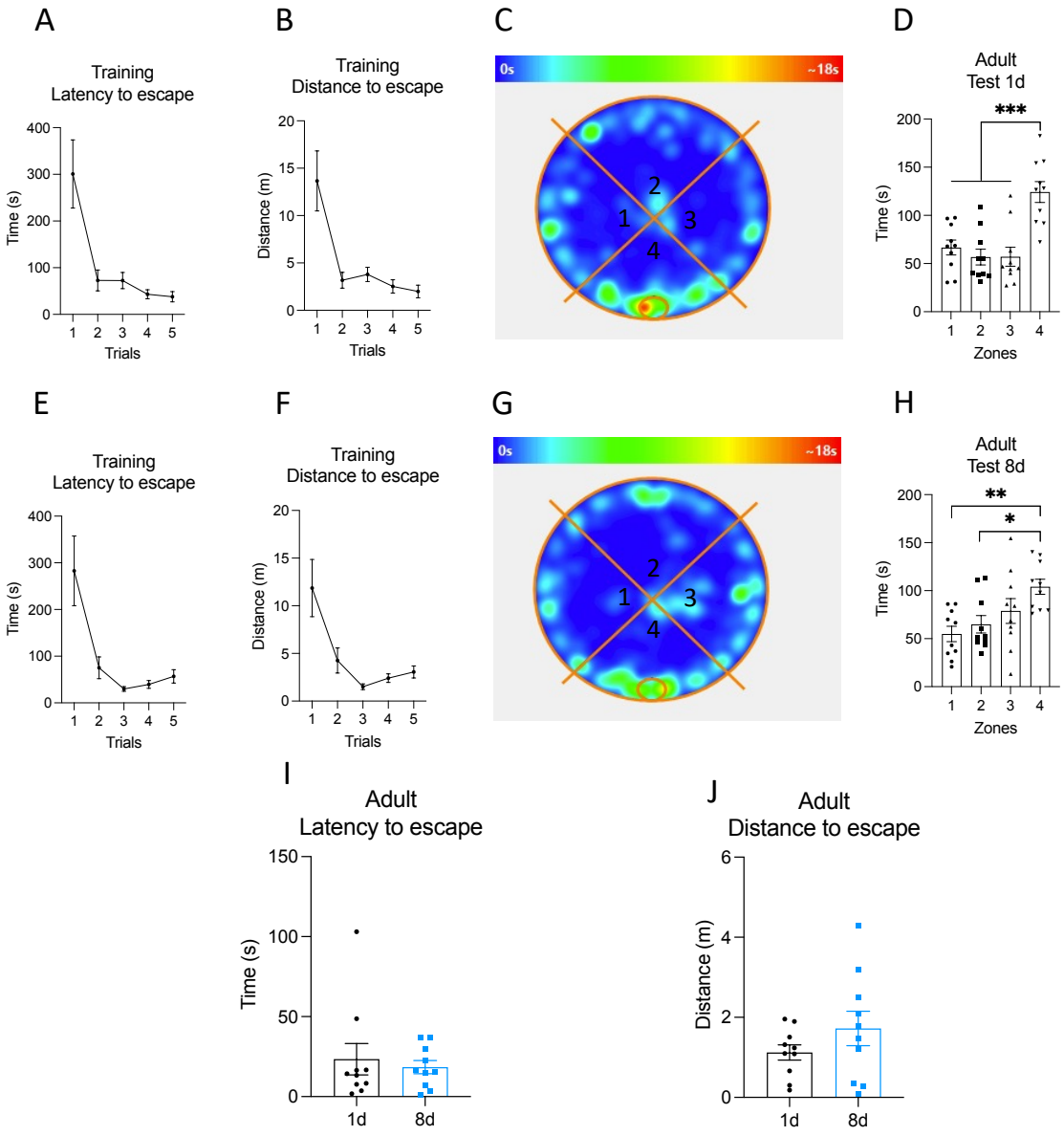


Figure 3.12. Adult C57BL/6J wild-type mice retain the memory for the location of the escape hole in the Barnes maze. (A, E) Latency before entering the escape hole during each trial. (B, F) Distance travelled before entering the escape hole for each trial. (C, G) Heat map of the mean time spent in each area of the Barnes maze during the probe trial. (D, H) Mean time spent in each zone of the Barnes maze during the probe trial. (I) Comparison of the latency to first reach the blocked escape hole during the probe trial. (J) Total distance travelled before reaching the location of the blocked escape hole during the probe trial. N=10 Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests or one-way ANOVA Bonferroni; *P < 0.05, **P < 0.01, ***P < 0.001.

3.5.3 Infant mice rapidly forget spatial memories

To compare memory retention between infant and adult mice for a spatial navigation task, next cohorts of infant (P17) mice were trained on the Barnes maze. A gradual decrease in the latency and distance travelled to reach the escape hole during training demonstrated that infant mice learnt the location of the escape hole relative to contextual cues (Fig. 3.13 A, B, E, F). Memory recall for the location of the escape hole was tested 1 or 8 days after training (Fig. 3.13 C, D. G, H). By looking at the time spent in the zone where the escae hole was located, it was possible to measure spatial memory retention. Infant mice were able to remember the location of the escape hole when tested a day later (Fig. 3.13 C, D). As hypothesised, infant mice were unable to retain this information a week later (Fig. 3.13 G, H). Further, it took infant mice longer to first reach the blocked escape hole during the probe test at 8 days compared to a day later (Fig. 3.13 I, J). Infant mice retain information for the location of the escape hole the day following training, but this information is forgotten 8 days after training.

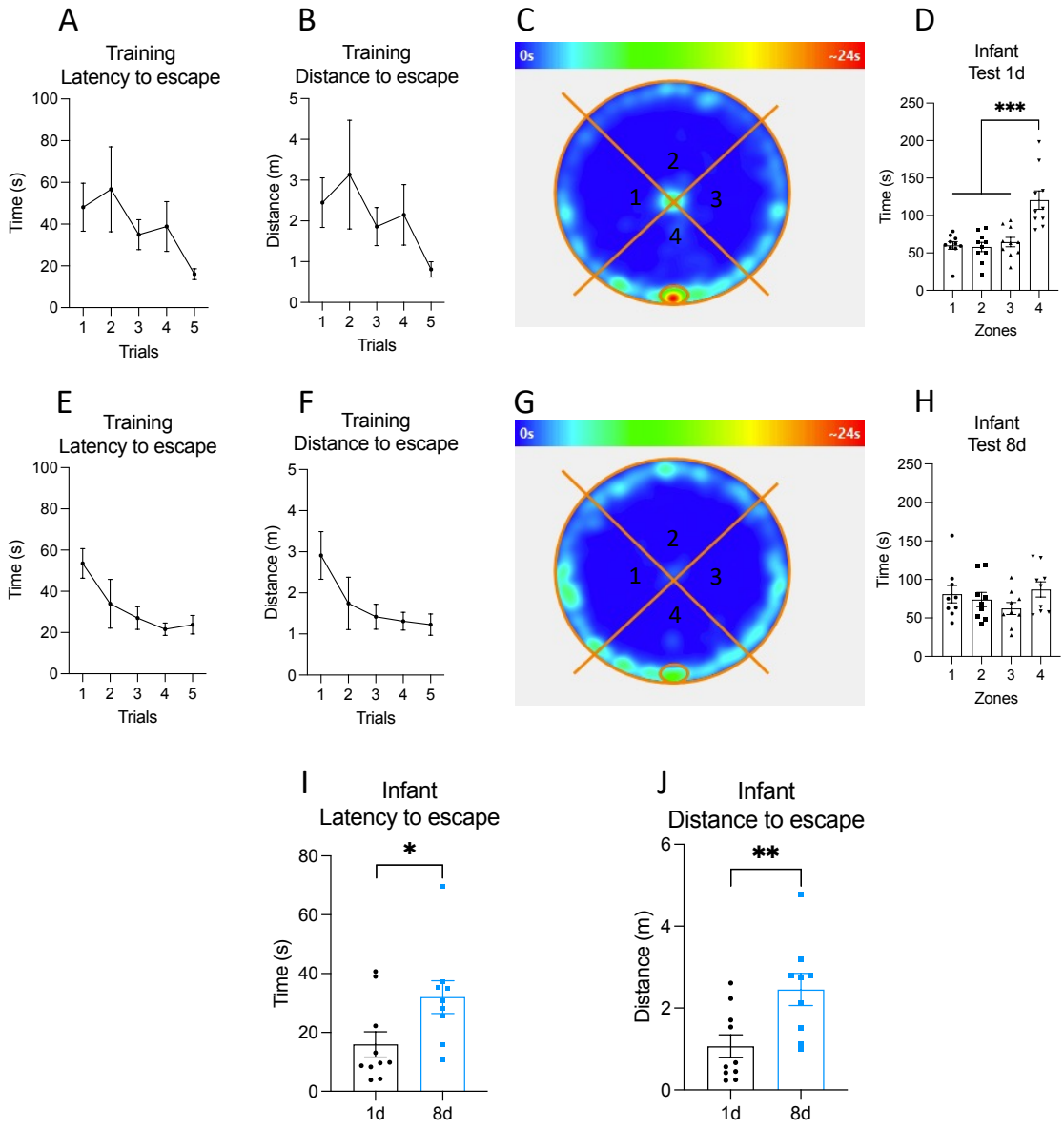


Figure 3.13. Infant C57BL/6J wild-type mice forget the location of the escape hole in the Barnes maze 8 days after training. (A, E) Latency before entering the escape hole during each trial. (B, F) Distance travelled before entering the escape hole for each trial. Results for probe trial carried out 1 day n=9 (C, D) or 8 days n=9 (G, H) after training. (C, G) Heat map of the mean time spent in each area of the Barnes maze during the probe trial. (D, H) Mean time spent in each zone of the Barnes maze during the probe trial. (I) Comparison of the latency to first reach the blocked escape hole during the probe trial. (J) Total distance travelled before reaching the location of the blocked escape hole during the probe trial. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests or one-way ANOVA Bonferroni; *P < 0.05.

3.5.4 Infant mice show different navigational search strategies on the Barnes maze during recall at 1 and 8 days.

Another method of assessing spatial learning is by looking at changes in navigational strategies taken by the animal during the task. Based on previous studies navigational strategies were divided into three classifications random, procedural, and contextual (Berdugo-Vega et al., 2020, Garthe et al., 2009, Garthe et al., 2016). The Barnes maze was divided into distinct zones to calculate the amount of time spent in each area (Fig. 14). The average time and distance of each navigational path to the location of the escape hole were used for the classification process. When the animal is searching at random, they demonstrate navigational search strategies referred to as local and global searching. Here they span a wide area of the maze and do not show the direction of their search. If the animal is moving in circles at the periphery of the maze (chaining) they are demonstrating a more procedural search strategy. When mice display a greater use of contextual strategies it is reflected in their search strategies. When contextual strategies are used, the search path is shorter and more direct to the goal.

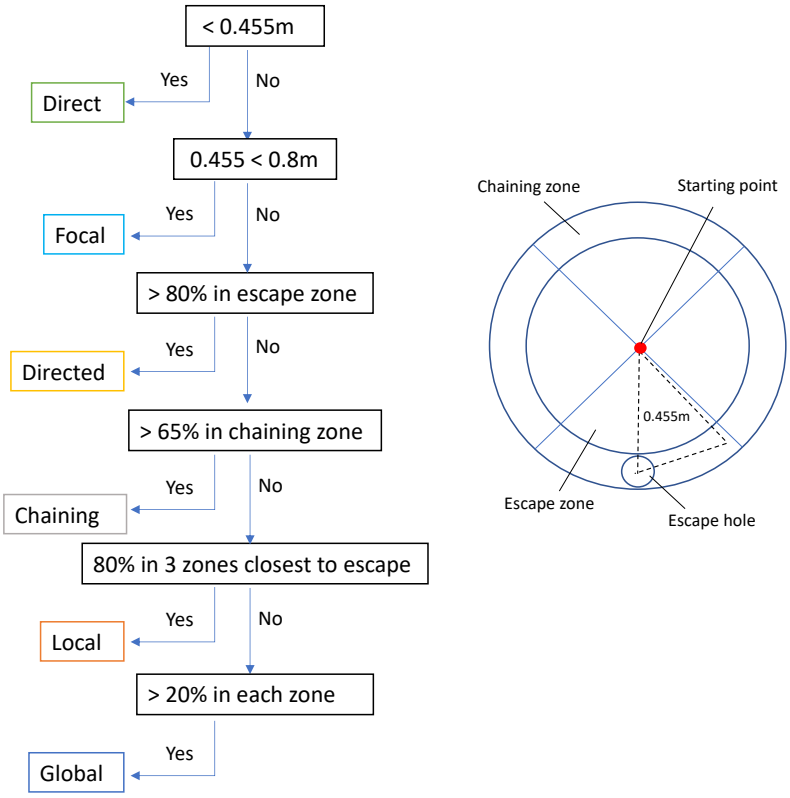


Figure 3.14. Classification of navigational search strategies. Determination of variables used for classification of navigation search strategies during the Barnes maze. Strategies are colour coded.

To investigate the changes in navigational strategies for both infant and adult mice, the search escape strategy trajectory path from the starting point to the first entry into where the escape hole was located was examined during the probe test 1 and 8 days after training. (Fig. 3.15 A). Infant mice predominantly used contextual strategies when tested for spatial memory on the Barnes maze a day after training (Fig. 3.15 B). However, during the 8-day probe trial infant mice show a switch in navigational strategy. Interestingly, infant mice exclusively navigate with procedural and random strategies when tested 8 days after training. This switch in navigational search strategies reflects forgetting of the location of the escape platform. In contrast, adult mice show similar search patterns both 1 and 8 days after training (Fig. 3.15 C). These results further support that infant mice retain spatial information 1 but not 8 days after training, while adults show memory retention at both time points.

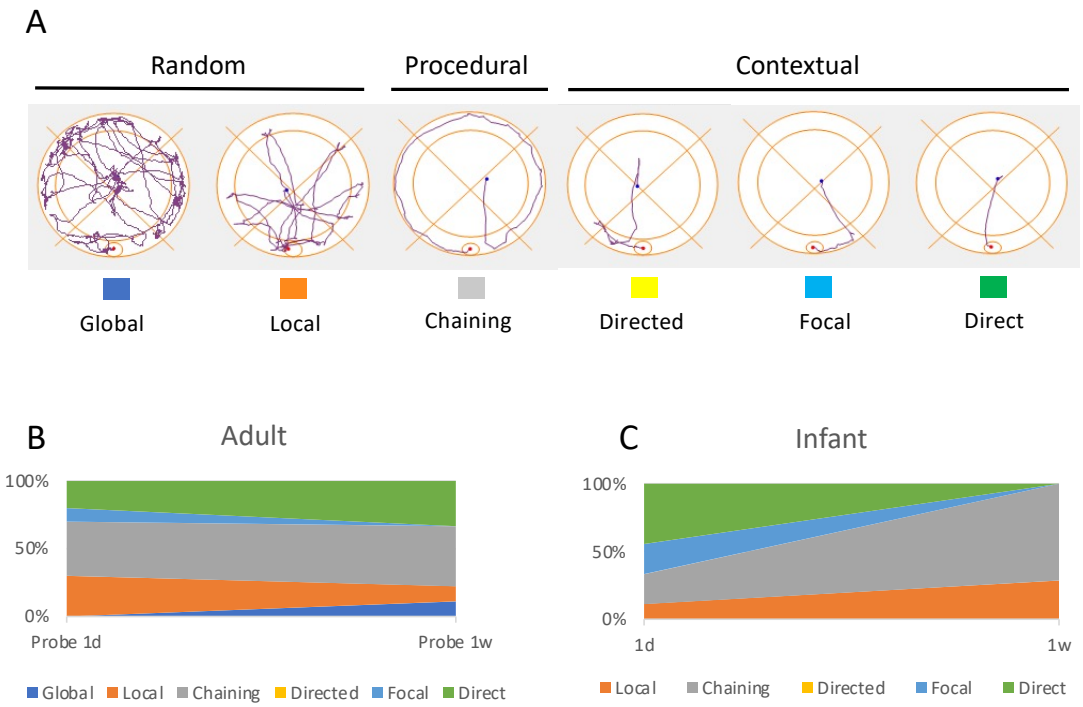


Figure 3.15. C57BL/6J infant mice demonstrate a change in navigational search strategies when tested 8 days after training on the Barnes maze. (A) Representative examples of different navigational search strategies on the Barnes maze. Search strategies are colour coded. (B) Comparison of navigational search strategies of adult C57Bl/6J mice during a probe test 1 and 8 days after training on the Barnes maze. (C) Comparison of navigational search strategies of infant C57Bl/6J mice during a probe test 1 and 8 days after training on the Barnes maze. N=9-10.

3.6 Summary

This chapter focused on characterising infantile amnesia in wild-type mice. Although many assume otherwise, infant rodents display the ability to carry out a range of complex behaviours. However, unlike adults, they rapidly forget. The behavioural paradigms described in this chapter produce a memory that endures 1 day but is rapidly forgotten in the period of 8 days in infant wild-type mice. This effect is not seen in adult mice, which demonstrate robust memory retention across all time points. Infant mice are capable of forming contextual, spatial and object memories at this age. However, these memories are rapidly forgotten due to infantile amnesia. Although an exact time

point in which infant memories are rapidly forgotten has not been reported, these results demonstrated that a fear memory is retained until a day before weaning (P20). We now know that whatever is causing infantile amnesia has not taken full effect by P20. Thus, forgetting is occurring sometime between P20-P25. Whether this is a gradual memory decline or a flooring effect remains to be deciphered.

Chapter 4: Labelling memory engrams in infant mice

Although wild-type behaviour is paramount for understanding the basis of infantile amnesia, it is limiting. By basing research solely on wild-type behaviour we cannot fully scrutinize the complexity of this form of amnesia. It renders us unable to investigate the molecular mechanisms that are driving this developmental forgetting. To answer these more complex questions and to give us greater insight wild-type behaviour must be integrated with recently developed technology such as engram labelling technology.

4.1 Engram labelling strategy

Memory engram technology, which allows the tagging and *in vivo* manipulation of specific engram cells, has revolutionised the way memory can be studied in rodent models (Tonegawa et al., 2015a). Through the application of engram labelling and optogenetics, it is now known that sparse populations of hippocampal neurons that were active during a defined learning experience are both sufficient and necessary for retrieval of specific contextual memories (Liu et al., 2012, Ramirez et al., 2013, Ryan, 2015). Optogenetic reactivation of contextual engram cells in the DG, labelled for a fear memory, is sufficient for memory retrieval (Liu et al., 2012). This project investigates how specific memories are stored throughout development via the plasticity of engram cell connectivity first by looking at the learning phase of infantile amnesia and, following its success, looking at methods of reinstatement. All these goals involve the labelling of engram cells in the mouse brain *in vivo*. Labelling engram cells means genetically tagging a sparse population of brain cells of a given brain region (hippocampus, parahippocampal regions and amygdala) that will be used for labelling to be induced by targeted windows of neuronal activity, and for the manipulation of specific target genes in these subpopulations of cells.

Current engram labelling strategies typically employ viruses or both viruses and transgenic mice (Liu et al., 2012, Josselyn, 2020). However, these methods are not ideal when dealing with development. Due to the nature of working with infant mice, this

project required an engram labelling strategy that allowed for the permanent labelling of engram cells. An engram labelling strategy was required that would allow for the labelling of memories in infanthood, and further, allow for the investigation of the nature of the engram in adulthood. Although engram labelling strategies that use a combination of transgenic mice and viruses are useful for achieving permanent engram labelling, stereotactic surgery in infant mice is too invasive. To overcome these obstacles, this project developed an engram labelling strategy using a double transgenic mouse line which allows for permanent labelling throughout the brain (Madisen et al., 2010, Madisen et al., 2012, Guenthner, 2013).

The developed engram labelling strategy involves iCre-based recombination of a floxed transgene in transgenic mice (Fig. 1 A). The FosTRAP transgenic line expresses an inducible *c-fos* promoter that can be switched on by intraperitoneal (IP) injection of the metabolite of tamoxifen, 4-hydroxytamoxifen (4-OHT) (Guenthner, 2013, Allen, 2017). Transgenic Ai32 mice express an improved channelrhodopsin-2/EYFP fusion protein following exposure to Cre recombinase (Madisen et al., 2012, Madisen et al., 2010). By crossing Fos-iCre with Ai32 mice it is possible to produce a stable transgenic line positive for both Cre and ChR2-EYFP. The Fos-iCre line contains the *c-fos* promoter, and thus injection of 4-OHT activates Cre recombinase and allows it to enter the cell's nucleus, allowing iCre to recombine the loxP sites and remove the stop codon. This results in the expression of ChR2/EYFP fusion protein, driven by the CAG promoter in Cre-expressing tissues. The CAG promoter is a hybrid construct consisting of the cytomegalovirus (CMV) enhancer fused to the chicken beta-actin promoter and is used to drive high gene expression in mammalian expression vectors. Thus, cells that are active 2 hours before IP injection will be labelled with ChR2-EYFP (Guenthner, 2013). Such transgenic strategies for engram labelling allow for permanent labelling throughout the brain.

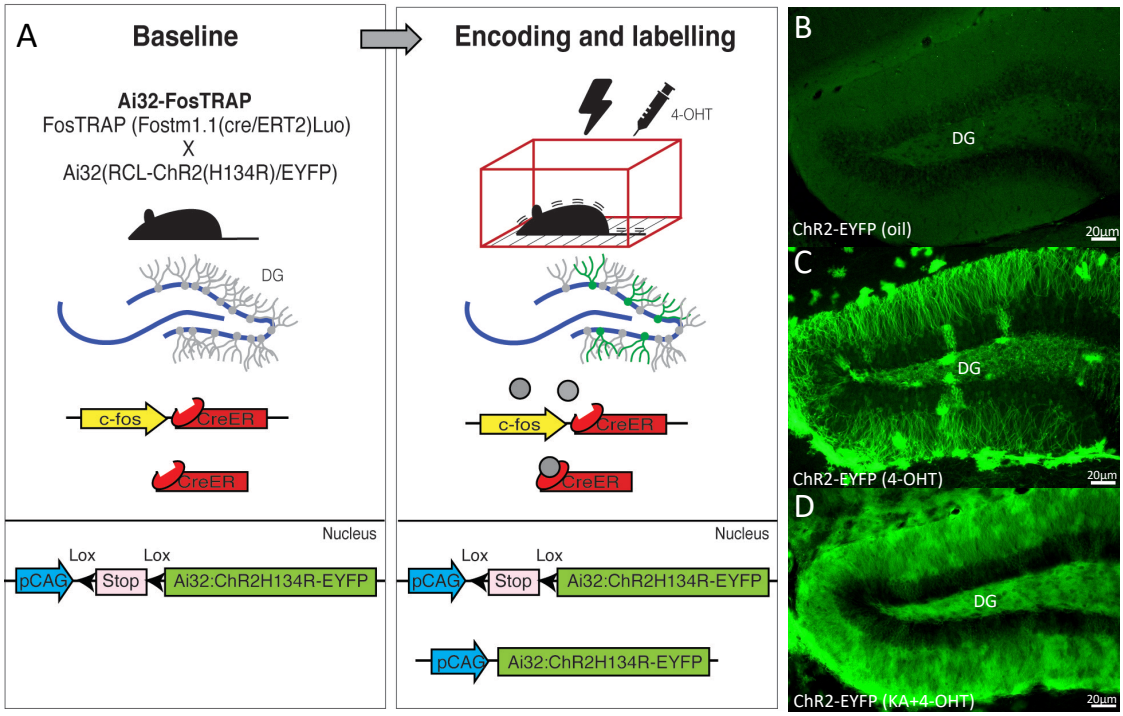


Figure 4.1. Engram labelling system in Ai32-FosTRAP mice. (A) Strategy for labelling of hippocampal engram cells with ChR2-EYFP in Ai32-FosTRAP mice. IP injection of 4-OHT drives ChR2-EYFP expression, labelling activated cells (green) throughout the brain. (B-D) Representative image showing the expression of ChR2-EYFP (green) in an Ai32-FosTRAP P17 mouse injected IP with (B) Chen oil 2 h after CFC, (C) 4-OHT 2 h after CFC, (D) 4-OHT 2 h post-Kainic acid (KA) IP injection.

4.2 Characterisation of engram labelling strategy in infant mice

To ensure this engram labelling strategy could be applied to infant mice, EYFP labelling in the DG was characterised by injecting 4-OHT into Ai32-FosTRAP mice at P17. Ai32-FosTRAP mice injected IP with 4-OHT (Fig. 4.1 C), but not oil (Fig. 4.1 B), showed ChR2-EYFP positive cells. This demonstrates that the engram labelling strategy is 4-OHT dependent. To determine whether labelling in this system was activity-dependent, Ai32-FosTRAP mice were injected IP with Kainic acid followed by 4-OHT 2 h post kainic acid injection (Fig. 4.1 C). Kainic acid causes epileptogenesis, causing a burst of electrical activity between neurons. Kainic acid paired with 4-OHT demonstrated ubiquitous

labelling throughout the DG confirming that the applied engram labelling strategy is activity-dependent.

To apply this engram labelling strategy to investigating the memory engram both before infantile amnesia had occurred and in adulthood, this engram labelling strategy needed (1) to demonstrate sufficient labelling before infantile amnesia occurred and (2) to be sufficient to maintain labelling into adulthood. To determine the earliest point in which ChR2-EYFP labelling in engram cells was visible in our system, Ai32-FosTRAP mice were labelled with 4-OHT at P17 and sacrificed either 1, 2 or 3 days later. The earliest point in which ChR2-EYFP was expressed in this model was 3 days post-injection (Fig. 4.2 A). To ensure labelling would be maintained into adulthood, a cohort of Ai32-FosTRAP mice were injected with 4-OHT at P17 and sacrificed at P63. Staining for ChR2-EYFP at P63 showed sufficient engram labelling for an infant memory labelled at P17 (Fig. 4.2 B). These results demonstrate that engram labelling in Ai32-FosTRAP mice is expressed after 3 days and allows for permanent labelling throughout the brain (Fig. 4.2 C).

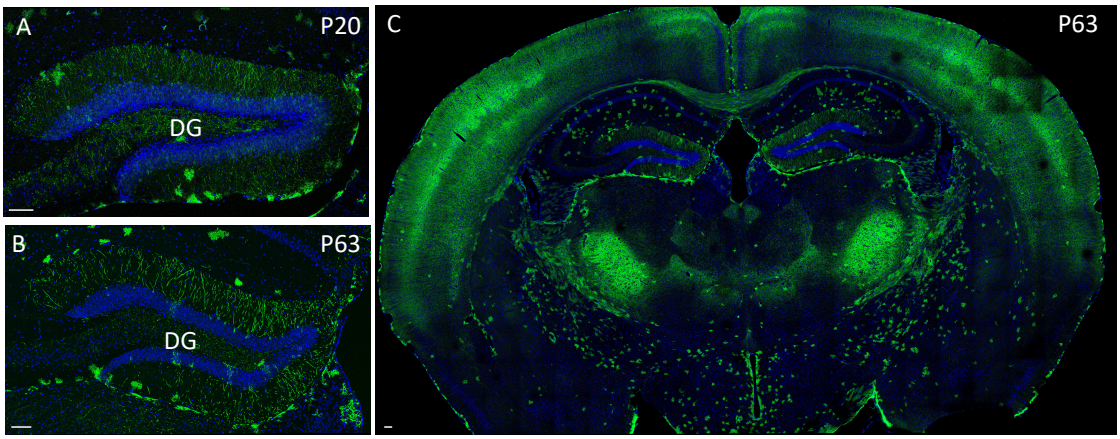


Figure 4.2. Infant and adult Ai32-FosTRAP mice show engram labelling after 4-OHT injection. (A) Labelling of hippocampal engram cells with ChR2-EYFP in Ai32-FosTRAP mice 3 days after IP injection of 4-OHT. (B) Representative image showing the expression of ChR2-EYFP in a Ai32-FosTRAP P63 mouse for engram cells labelled at P17. (C) Whole-brain image of engram labelling system in Ai32-FosTRAP mice. Scale bar 100 μm.

4.3 Efficiency of engram labelling strategy for infant and adult labelled memories in different brain regions

To compare the efficiency of the Ai32-FosTRAP engram labelling system in infant and adult mice, the engram for a home cage, contextual or contextual fear memory was labelled in infant (P17) and adult (P42) mice (Fig. 4.3). The level of engram labelling was then quantified in adulthood (P63) for both groups. The percentage of ChR2-EYFP positive cells were quantified as a percentage of the total DAPI cells in the DG, AMG, RSC and the PAG gray at P63 for both infant labelled and adult labelled engrams.

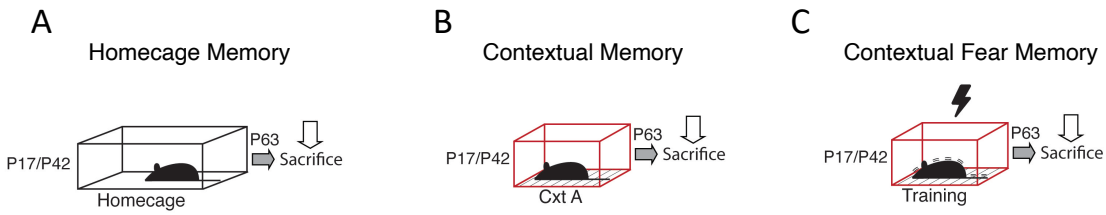


Figure 4.3. Behavioural paradigm for engram labelling in infant and adult Ai32-FosTRAP mice. (A-C) Schematic diagram of behavioural paradigm for engram labelling of a homecage (A), contextual (B), and contextual fear (C) memory in Ai32-FosTRAP mice. The black lightning symbol indicates foot shocks. The needle symbol indicates 4-OHT IP injection 2 h post-training.

4.3.1 The Dentate Gyrus

The dentate gyrus (DG) is a well-studied structure involved in hippocampal memory formation (Hainmueller and Bartos, 2020). Situated in the hippocampus, the DG is involved in a range of mnemonic functions including formation of episodic memories, spatial orientation and navigation, pattern separation and novelty detection (Milivojevic, 2013). The DG combines or differentiates between different information input to facilitate the accurate encoding of memories and retrieval (Hainmueller and Bartos, 2020). As well as receiving cortical input from the entorhinal cortex, the DG also receives intrahippocampal input from CA3 pyramidal cells and mossy cells which predominantly recruit disynaptic inhibition (Basu, 2015). Both inputs provide

information about memory contents. In the last decade, a combination of optogenetic and engram labelling techniques have allowed for the artificial memory recall by optogenetic stimulation of engram cells in the DG (Liu et al., 2012).

Due to the important role the DG plays in the mnemonic process, the level of engram labelling in Ai32-FosTRAP was quantified for a homecage, contextual and contextual fear memory (Fig. 4.4). As expected, there was significantly more ChR2-EYFP positive cells in the DG for context and contextual fear conditioning exposure when compared to the homecage control (Fig 4.4 B, C). This result was true for both an adult (Fig. 4.4 B) and infant (Fig. 4.4 C) labelled memory. These results demonstrate that when exposed to a context, there is more engram activation in the DG of Ai32-FosTRAP mice at the time of learning.

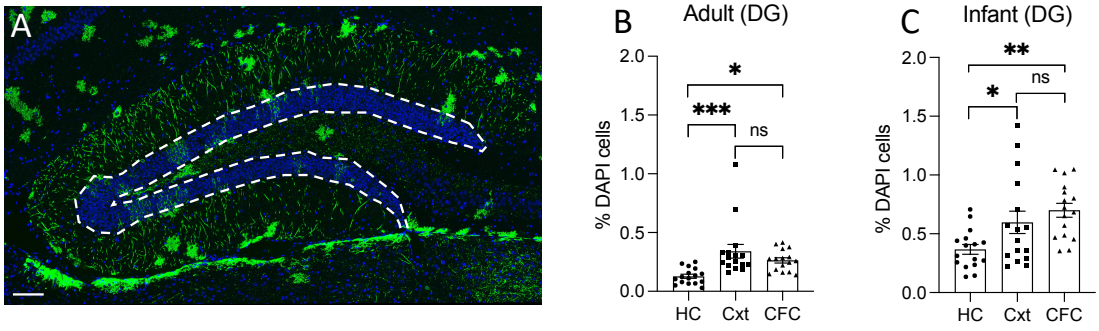


Figure 4.4. Infant and adult Ai32-FosTRAP mice demonstrate increased engram labelling in the DG for a contextual and contextual fear memory. (A) Representative image of the area counted for the DG. (B, C) Percentage of ChR2-EYFP positive DAPI cells for a home cage (HC), contextual (Cxt) and contextual fear memory (CFC) in the DG at P63. (B) Percentage of ChR2-EYFP positive DAPI cells for an engram labelled at P42 (adult). (C) Percentage of ChR2-EYFP positive DAPI cells for an engram labelled at P17 (infant). N=4, n=4. Scale bar 100 μm. Data presented as mean ± SEM. Statistical comparison was performed by using one-way ANOVA Bonferroni; *P < 0.05, **P < 0.01, ***P < 0.001.

4.3.2 The Amygdala

The amygdala (AMG) is another important brain region involved in episodic memory (Hartley, 2010) . The AMG functions by attaching emotional significance during the encoding of a memory. Memories of high emotional valence are usually difficult to forget, suggesting that the AMG and its interactions with the hippocampus are important for the stability of a memory (Phelps, 2004). The AMG is also a key player in the formation of fearful memories (LaBar, 1998). During fear conditioning, a neutral conditioned stimulus (CS) such as a context or tone, is paired with an aversive unconditioned stimulus (US) such as a footshock, where eventually presentation of the CS alone elicits a fear response. The circuitry underlying fear conditioning has been widely studied. In the lateral nucleus of the amygdala (LA), the pathways processing the CS and US converge initiating synaptic plasticity, eliciting an association between the CS and US. Thus, re-exposure to the CS is sufficient to retrieve the associative memory from the LA. Artificial activation of neurons labelled in the LA (for a fear memory) in a novel context is sufficient to elicit recall of a fear memory (Kim, 2014, Yiu, 2014). The hippocampus also interacts with the basolateral amygdala (BLA) to modulate the consolidation of episodic memory. Projections from the ventral hippocampus to the BLA are involved in the eliciting of fear behaviour by a conditioned context and is necessary for the fear memory retrieval (Xu, 2016).

Due to the importance of both regions of the AMG for episodic memory, this project quantified both the BLA and LA (Fig. 4.5 A). There was no significant difference in CHR2-EYFP positive cells in the AMG between a homecage or context labelled memory for either the adult (Fig. 4.5 B) or infant (Fig. 4.5 C) group. However, in both groups, exposure to contextual fear conditioning showed significantly more ChR2-EYFP positive cells in the AMG (Fig. 4.5 B, C). Due to the key role the AMG places in fear memory, higher activation of engram cells in the AMG during contextual fear conditioning, compared to the homecage or context exposure was expected.

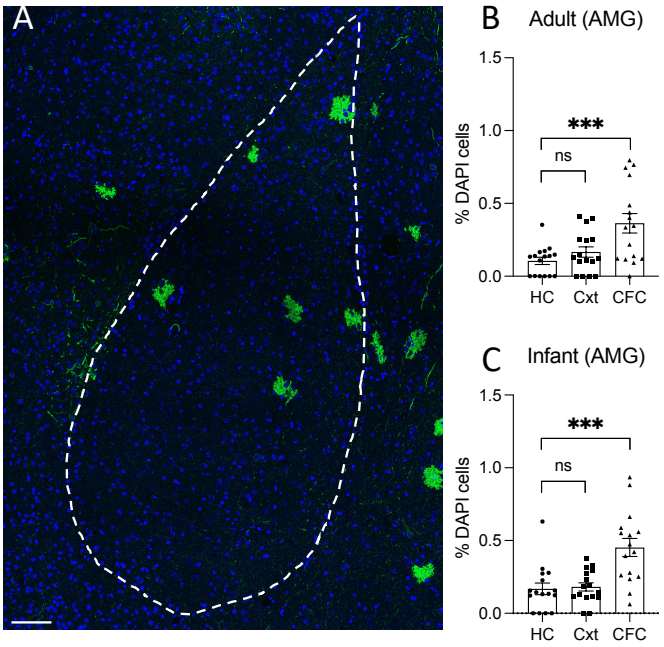


Figure 4.5. Infant and adult Ai32-FosTRAP mice show increased engram labelling in the AMG for a contextual fear memory. (A) Representative image of the area counted for the AMG. (B, C) Percentage of ChR2-EYFP positive DAPI cells for a home cage (HC), contextual (Cxt) and contextual fear memory (CFC) in the AMG at P63. (B) Percentage of ChR2-EYFP positive DAPI cells in the AMG for an engram labelled at P42 (adult). (C) Percentage of ChR2-EYFP positive DAPI cells in the AMG for an engram labelled at P17 (infant). N=4, n=4. Scale bar 100 μm. Data presented as mean ± SEM. Statistical comparison was performed by using one-way ANOVA Bonferroni; *P < 0.05, **P < 0.01, ***P < 0.001.

4.3.3 The Retrosplenial Cortex

Episodic memories are thought to be stored within neuronal networks within the brain. Recently, the retrosplenial cortex (RSC) has emerged as a key player in the underpinning network involved in episodic memory (Vann et al., 2009). Interestingly, the RSC is one of the largest cortical regions in rodents. In both rodents and humans, the RSCis closely interlinked with the hippocampus, an important brain region in episodic memory. The RSC is thought to be important in spatial memory (Immediate early gene), while lesions

in the RSC have demonstrated impairment in spatial memory in rodents (Vann and Aggleton, 2004, Vann et al., 2009). Although the function of the RSC is not limited to spatial memory, it has also been shown to be involved in the processing of contextual information in fear memory (Lukoyanov and Lukoyanova, 2006, Kwapis, 2015). Further, optogenetic reactivation of RSC engram ensembles for a recent context fear conditioning memory is sufficient to elicit behavioural outcomes like that observed during natural memory recall (Cowansage et al., 2014).

The RSC has important involvement in the episodic memory process. Therefore, the level of engram labelling in Ai32-FosTRAP mice was quantified for an adult and infant encoded memory (Fig. 4.6). For an adult memory, there were significantly more ChR2-EYFP positive cells labelled in the RSC for a contextual and fear memory when compared to a homecage memory (Fig. 4.6 B). In contrast, for an infant memory, no difference was seen in the number of ChR2-EYFP positive cells for a homecage, contextual or fear memory (Fig. 4.B C). The RSC plays a role in the encoding of spatial and contextual information (Vann, 2009).

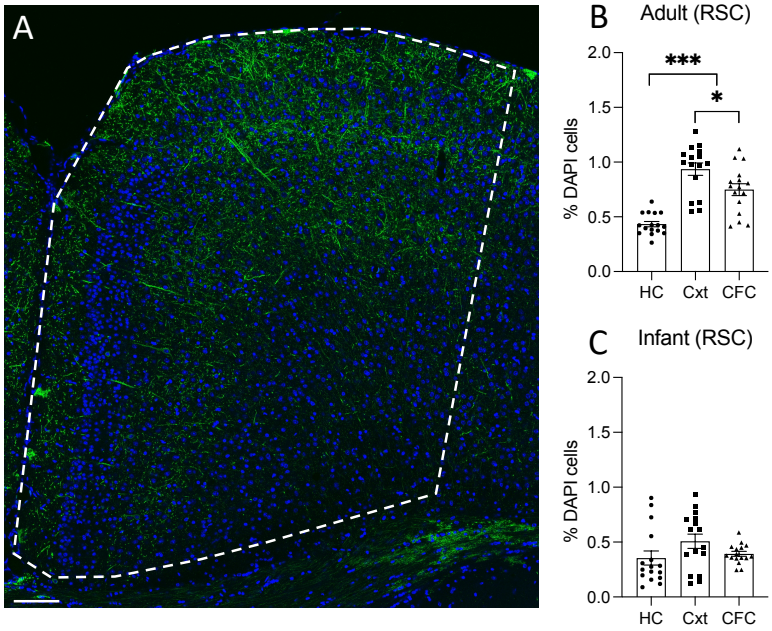


Figure 4.6. Adult Ai32-FosTRAP mice show a higher level of engram labelling in the RSC for a contextual and contextual fear memory. (A) Representative image of the area counted for the RSC. (B, C) Percentage of ChR2-EYFP positive DAPI cells for a home cage (HC), contextual (Cxt) and contextual fear memory (CFC) in the RSC at P63. (B) Percentage of ChR2-EYFP positive DAPI cells for an engram labelled at P42 (adult). (C) Percentage of ChR2-EYFP positive DAPI cells for an engram labelled at P17 (infant). N=4, n=4. Scale bar 100 μm. Data presented as mean ± SEM. Statistical comparison was performed by using one-way ANOVA Bonferroni; *P < 0.05, **P < 0.01, ***P < 0.001.

4.3.4 The Periaqueductal Gray

In the wild, survival depends on the rapid execution of appropriate fight or flight responses, yet the brain circuitry underlying these responses is not fully understood. Located in the area of gray matter found in the midbrain, the periaqueductal gray (PAG) is a complex area that is widely understudied. Interconnected with various other brain regions, the PAG is thought to participate in response to threats aiding in defensive behaviours such as flight, freezing and avoidance (Back and Carobrez, 2018). Optogenetic activation of labelled dorsal PAG neurons induces defensive behaviour in mice including freezing, avoidance and flight (Deng et al., 2016). Further, the PAG has bidirectional projections to the central nucleus of the AMG which is thought to play a

role in fear conditioning (Penzo et al., 2014). It is thought that information associated with a stressor is integrated through the PAG to produce an appropriate survival response.

The amount of engram labelling for a homecage, contextual or contextual fear memory in Ai32-FosTRAP mice was quantified in the PAG. Surprisingly, no difference was seen in the number of ChR2-EYFP positive cells in the PAG for labelling of a homecage, contextual or fear memory in either an adult (Fig. 4.7 B) or infant (Fig. 4.7 C) labelled memory.

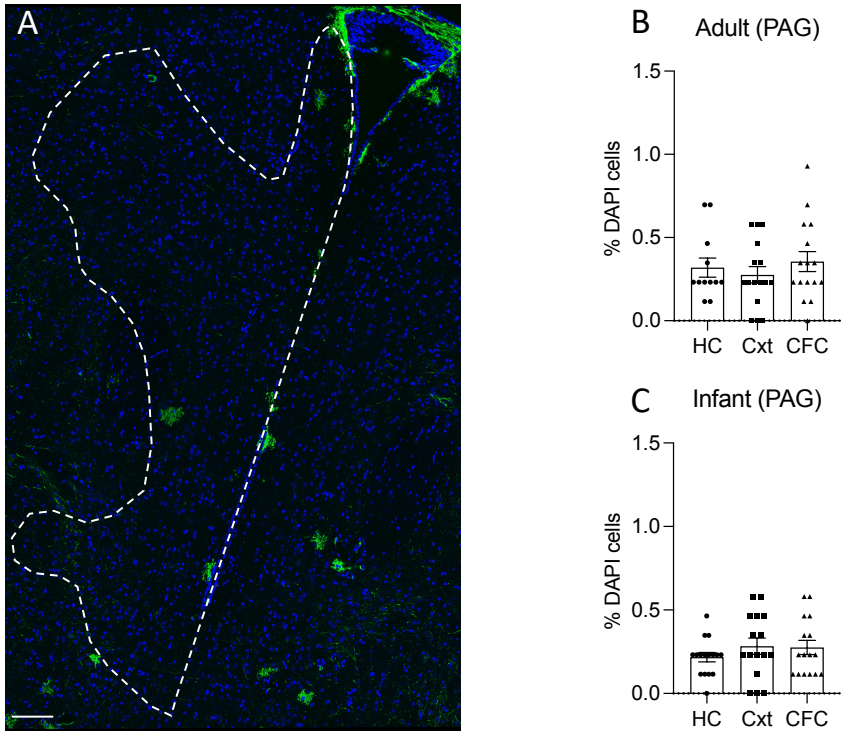


Figure 4.7. Infant and adult Ai32-FosTRAP mice show no difference in engram labelling in the PAG. (A) Representative image of the area counted for the PAG. (B, C) Percentage of ChR2-EYFP positive DAPI cells for a home cage, contextual and contextual fear memory in the PAG at P63 (B) Percentage of ChR2-EYFP positive DAPI cells for an engram labelled at P42 (adult). (C) Percentage of ChR2-EYFP positive DAPI cells for an engram labelled at P17 (infant). N=3-4, n=4. Scale bar 100 μm. Data presented as mean ± SEM. Statistical comparison was performed by using one-way ANOVA Bonferroni; *P < 0.05, **P < 0.01, ***P < 0.001.

4.4 Natural recall of a contextual fear memory before and after the infantile amnesia period

As seen in chapter 4, infant mice recall a contextual fear memory 3 days after training (P20) but rapidly forget by P25. To examine whether these behavioural results are reflected at the cellular engram level the next set of experiments investigated whether engram cells active at the time of memory encoding are reactivated during recall of that memory. Cellular activation of engram cells was examined before (infant recall) and after the infantile period (adult recall) in multiple brain regions.

Infant Ai32-FosTRAP mice were trained using contextual fear conditioning at P17 (Fig. 4.8). The memory engram was labelled for this experience by injection of 4-OHT 2 h after training. Half of the cohort was tested for memory recall at either P20 (before infantile amnesia) or P63 (after infantile amnesia) and sacrificed 45 min after. The other half of the cohort did not receive a memory recall test (no recall) and were sacrificed from their homecage at P20 or P63. Engram cells activated at the time of encoding were identified by ChR2-EYFP expression while cells activated during the recall test or homecage were identified by endogenous c-Fos expression. The level of overlap between engram cells activated at the time of memory encoding and engram cells activated during memory recall was quantified for the dentate gyrus (DG), amygdala (AMG), retrosplenial cortex (RSC) or the periaqueductal gray (PAG).



Figure 4.8. Behavioural paradigm for natural recall of a contextual fear memory in Ai32-FosTRAP mice. Behavioural paradigm for contextual fear conditioning in Ai32-FosTRAP mice. The black lightning symbol indicates foot shocks. The needle symbol indicates 4-OHT IP injection 2 h post-training.

4.4.1 c-Fos activation

Natural recall of a contextual fear memory at P20 (infant recall) resulted in a significant increase in c-Fos positive cells in the DG and the AMG (Fig. 4.9 A, B). This difference was not seen in other brain regions such as the RSC (Fig. 4.9 C) or the PAG (Fig. 4.9 D). While natural recall at P63 (adult recall) resulted in an increase in c-Fos positive cells in the DG and the RSC (Fig. 4.9 E, G) but not the AMG or PAG (Fig. 4.9 F, H). Exposure to the training context before (P20) or after infantile amnesia (P63) resulted in an increase in cell activation in the DG. However, an increase in cell activation in the AMG was only seen during recall before the infantile period.

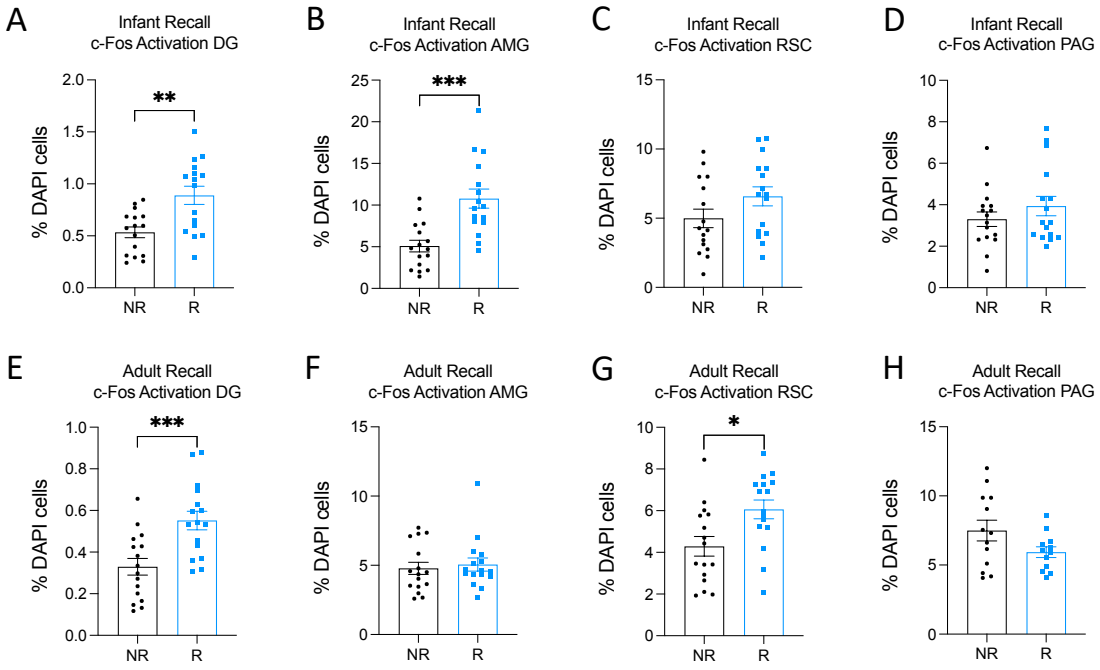


Figure 4.9. Infant Ai32-FosTRAP mice show increased cell activation in the DG and AMG during natural recall of a contextual fear memory at P20. (A-D) c-Fos activation after optogenetic stimulation of engram cells for a memory labelled in adulthood (P42). (E-H) c-Fos activation after optogenetic stimulation of engram cells for an infant labelled (P17) memory. (A, E) Percentage of c-Fos positive DAPI cells in the DG (A, E), AMG (B, F), RSC (C, G) and the PAG (D, H). N=3-4, n=4. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; **P < 0.01, ***P < 0.001.

4.4.2 Engram labelling

During contextual fear conditioning at P17 an equal number of ChR2-EYFP positive cells were recruited to the engram in the DG, AMG, RSC and PAG for both the recall and no recall group (Fig. 4.10). Exposure to a recall test does not alter the quantify of engram cells labelled during training.

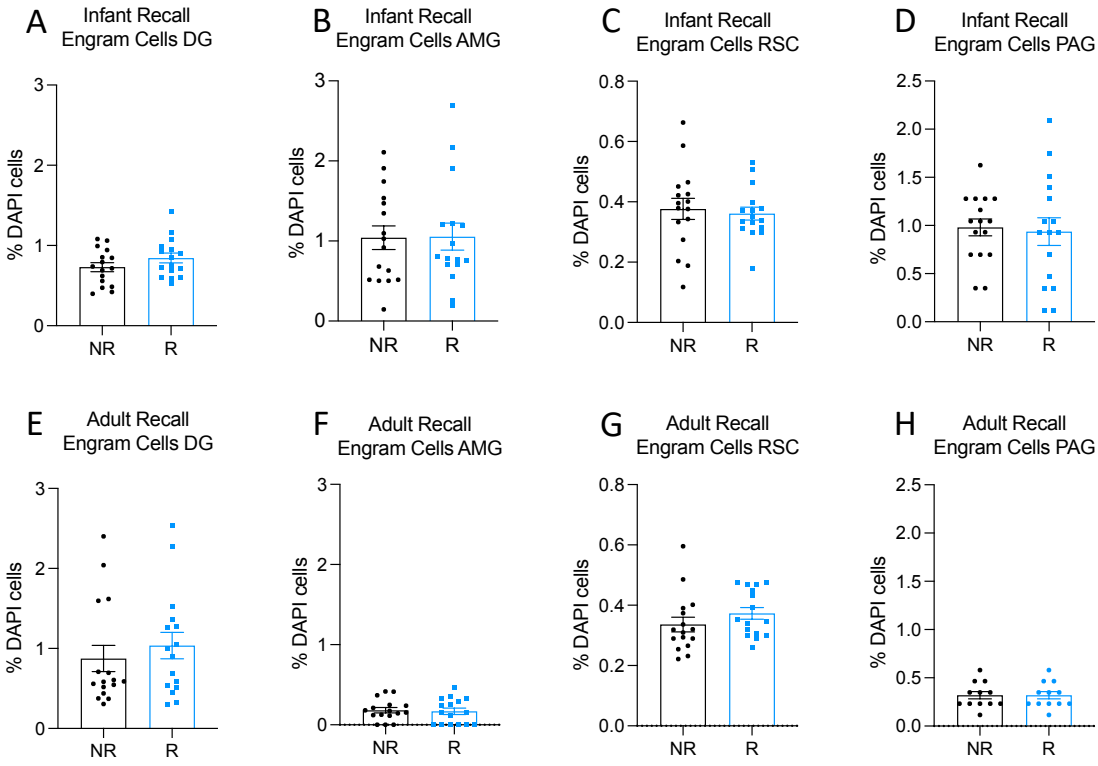


Figure 4.10. ChR2-EYFP labelling of engram ensembles for an infant encoded memory. (A-H) Percentage of ChR2-EYFP positive DAPI cells labelled during contextual fear conditioning at P17. (E-H) (A, E) Percentage of ChR2-EYFP positive DAPI cells in the DG (A, E), AMG (B, F), RSC (C, G) and the PAG (D, H). N=3-4, n=4. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; *P < 0.05.

4.4.3 Engram reactivation

Exposure to a recall test at P20 resulted in an increase in the reactivation (c-Fos positive) of engram cells labelled during training (ChR2-EYFP positive) in the DG, AMG and RSC (Fig. 4.11 A-C). However, if the recall test occurred at P60, no difference was seen in the level of engram reactivation between the recall and no recall groups (Fig. 4.11 E-H).

These results demonstrate that engram ensembles in the DG, AMG and RSC during training at 17 are reactivated during a natural recall test but only if the test occurs before the infantile amnesia period, not after.

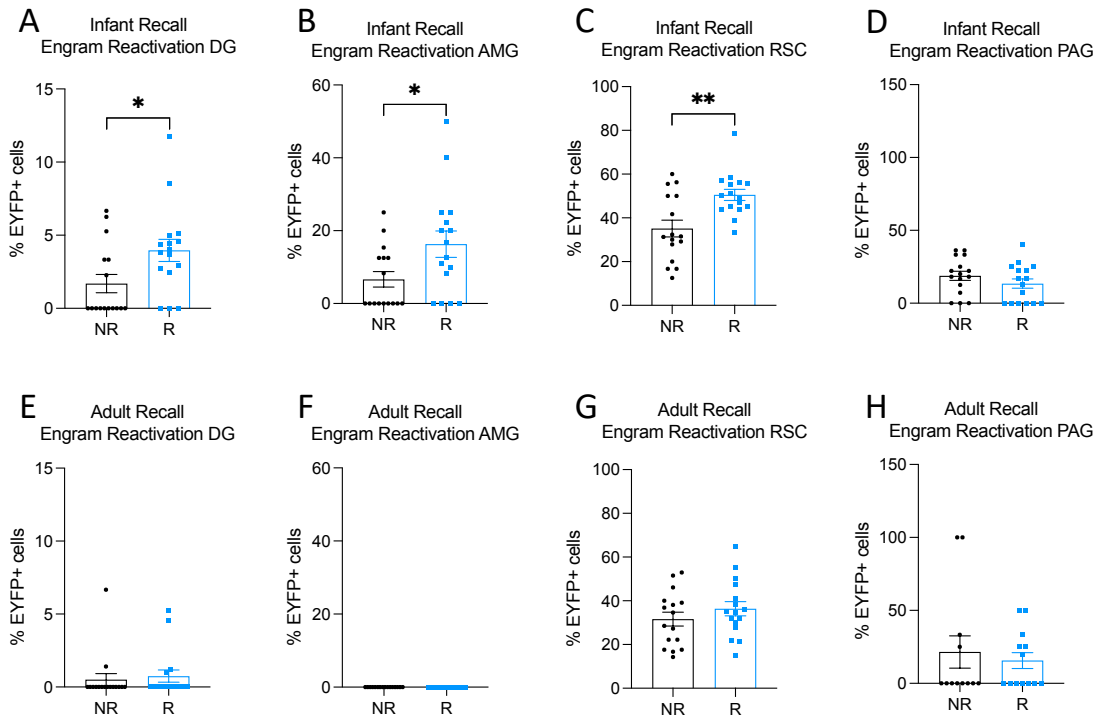


Figure 4.11. ChR2-EYFP reactivation is higher in the DG, AMG and RSC during natural recall of a contextual fear memory at P20 but not at P63. (A-D) Percentage of c-Fos positive ChR2-EYFP cells during natural recall at P20. (E-H) Percentage of c-Fos positive ChR2-EYFP cells during natural recall at P63. (A, E) Percentage of c-Fos positive ChR2-EYFP cells in the DG (A, E), AMG (B, F), RSC (C, G) and the PAG (D, H). N=3-4, n=4. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; *P < 0.05, **P < 0.01.

4.5 Summary

This chapter developed and characterised an engram labelling strategy that allowed for whole-brain permanent labelling of the engram. Although the Ai32-FosTRAP mouse line is both activity and 4-OHT dependent, it requires 3 days post 4-OHT injection for ChR2-EYFP expression in cells. Regardless of whether the engram was encoded in infancy (P17) or adulthood (P42), Ai32-FosTRAP mice show increased engram labelling in the DG for

the AMG for a contextual fear memory. When a purely contextual or contextual fear conditioning memory was encoded in adulthood, Ai32-FosTRAP mice demonstrate increased engram labelling in the RSC. This effect was not seen when the engram was labelled in infancy. Despite the role the PAG is thought to play in threat behaviours, no significant difference was seen in the level of engram labelling in Ai32-FosTRAP mice. During natural recall of a contextual fear memory, significant engram reactivation only occurs when the recall test is carried out before the onset of infantile amnesia, providing further evidence that infant mice show memory retention at 3 days but rapidly forget.

Chapter 5: Optogenetic rescue of infant memories

Amnesia is the loss of memory, characterized by the absence of learned behaviour but the underlying cause of any given type of amnesia remains ambiguous. By assessing amnesia solely on the behavioural or cognitive performance of a subject it is not possible to discriminate between the possibility that learned information has been truly lost from the brain (a memory storage deficit) and the alternative possibility that the memory is still present in the brain but is not fully accessible (a memory retrieval deficit). If a memory survives a case of amnesia, then we can begin to rule out whatever is going wrong in that type of amnesia. Behavioural tests that evaluate the ability of an animal to recognize a previously presented stimulus constitute the core of animal models of human amnesia. Engram labelling technology allows us to take a closer look at the engram cells involved in driving this behaviour. This chapter focuses on investigating whether infant memories are permanently lost from the brain and, if they are still present, can we bring the memory back using optogenetics.

5.1 Optogenetic stimulation of engram ensembles in the DG are sufficient to induce acute recovery of infant memories

It has previously been shown that that in cases of amnesia contextual fear memory recall can be induced by light reactivation of engram cells in the DG (Ryan, 2015, Roy et al., 2016, Perusini et al., 2017, Guskjolen et al., 2018). To investigate whether this applies to a 'lost' infantile memory this chapter crucially tested whether optogenetic stimulation of ChR2-EYFP expressing neurons in the DG, labelled during encoding of a fear memory in infancy, was sufficient for recall of that fear memory in adulthood. Engram cells for context A were labelled in Ai32-FosTRAP mice at P17 (Fig. 5.1 A). Although both cohorts were placed in context A, only the experimental group received foot shocks. Memory recall was then tested 1 (P18) and 8 (P25) days later, as well as a context specificity test at P19 (context B). An optical fibre targeting the DG was implanted to both cohorts at P49 to allow for optogenetic stimulation of the labelled memory engram. At P63 mice were placed in novel context C where they were tethered

through their implants to a lightweight optogenetic patch cord that enables the light of a particular wavelength (473 nm) to be delivered to the brain. Blue light delivered to the DG activates ChR2-EYFP expressing neurons (neurons labelled during encoding of the fear memory) resulting in freezing behaviour. If the memory engrams retain information following infantile amnesia, then when these cells are reactivated by blue light in a context different from the original one used for the conditioning, these animals display freezing behaviour. Using freezing as a measurement of memory, these results give evidence of fear memory recall.

When tested for natural recall 1 day after contextual fear conditioning, Ai32-FosTRAP infant mice freeze significantly more than the no shock control cohort (Fig. 5.1 B). When tested again 8 days later at P25, both groups show similarly low levels of freezing. During optogenetic stimulation at P63, both groups showed background levels of freezing (10%) during the light-off epochs (Fig. 5.1 C, D). However, the experimental group showed significantly higher levels of freezing during the light-on epochs. This light-induced freezing was not observed for animals that underwent the same behaviour paradigm but did not receive any footshocks at P17. Optogenetic stimulation of DG engram cells in adulthood was sufficient to acutely rescue a 'lost' infantile memory (demonstrated by freezing during light-on epochs). These results demonstrate that the infant engram survives the infantile period even though the memory has been forgotten.

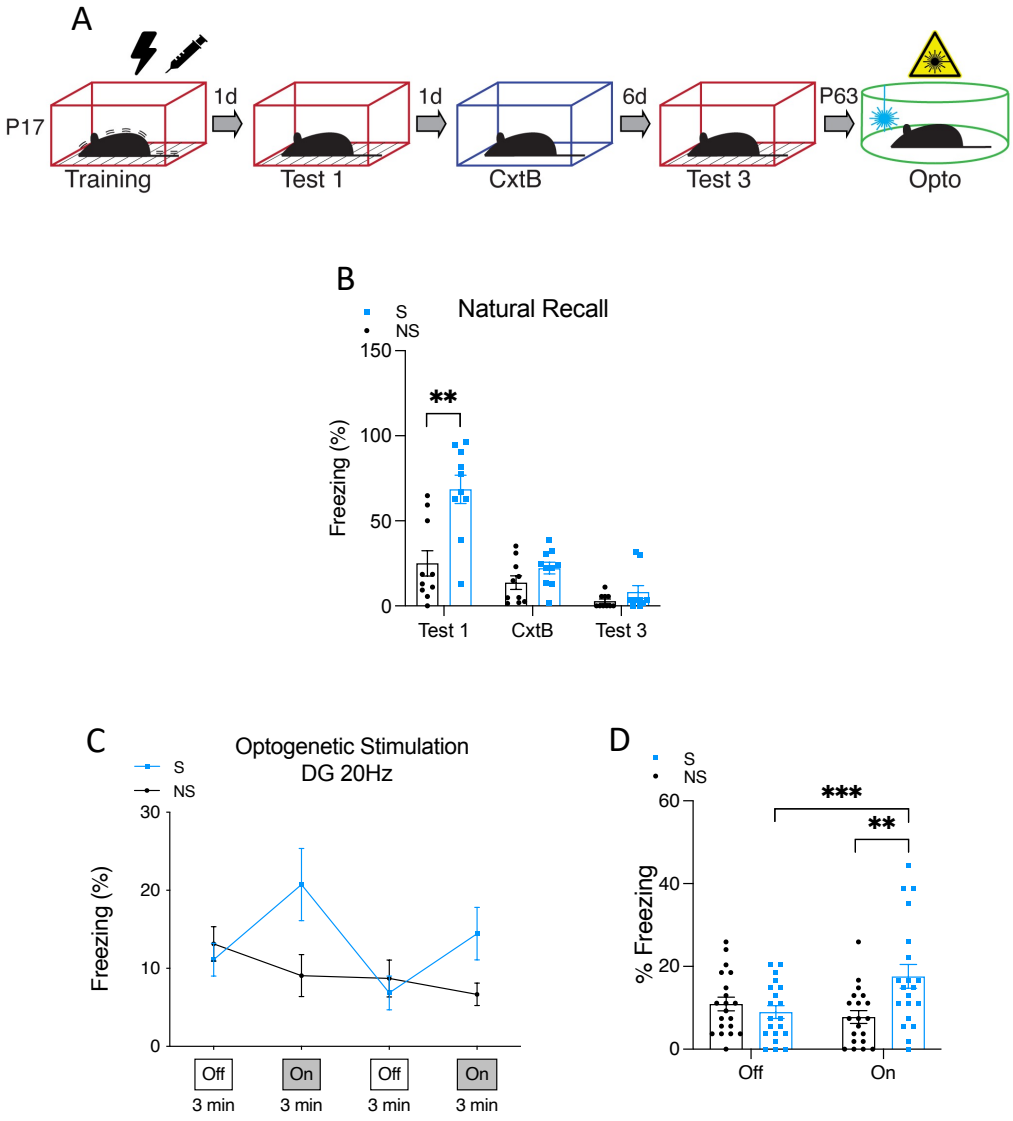


Figure 5.1. Optogenetic stimulation of engram ensembles in the DG is sufficient to induce acute recovery of an infant fear memory. (A) Training and testing scheme for optogenetic stimulation (20Hz) of engram-bearing cells in the DG. The black lightning symbol and the blue spike symbol indicate foot shocks and blue light delivery, respectively. The needle symbol indicates 4-OHT IP injection 2 h post-training. (B) Freezing levels for natural recall at Test 1 (P18), CxtB (P19) and Test 3 (P25). (C, D) Memory recall in context C (engram activation) with light-off and light-on epochs. (D) Averaged freezing for the two light-off and light-on insets. N=10. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired or paired t-tests; **P < 0.01, ***P < 0.001.

As a context control, whether optogenetic stimulation of ChR2-EYFP positive cells in the DG for a neutral context would result in the same freezing behaviour seen in Fig 5.1 was tested. The memory engram for a neutral context B was labelled in a cohort of Ai32FosTRAP mice at P17 (Fig. 5.2 A). The next day, the group were fear conditioned in context A. The animals were then tested for fear recall 1 and 8 days later. Animals showed memory retention when tested 1 but not 8 days after training (Fig. 5.2 B). When DG cells activated during exposure to context B were reactivated with light at P63 in novel context C, the animals showed no significant difference in the level of freezing between the light-off and light-on epochs (Fig. 5.2 C, D). These results demonstrate that DG optogenetic stimulation of a labelled infantile fear memory in adulthood results in freezing behaviour. While stimulation of a neutral memory, labelled in infancy, does not result in freezing behaviour. Here we show that 'lost' infantile memories can be acutely recalled by optogenetic stimulation.

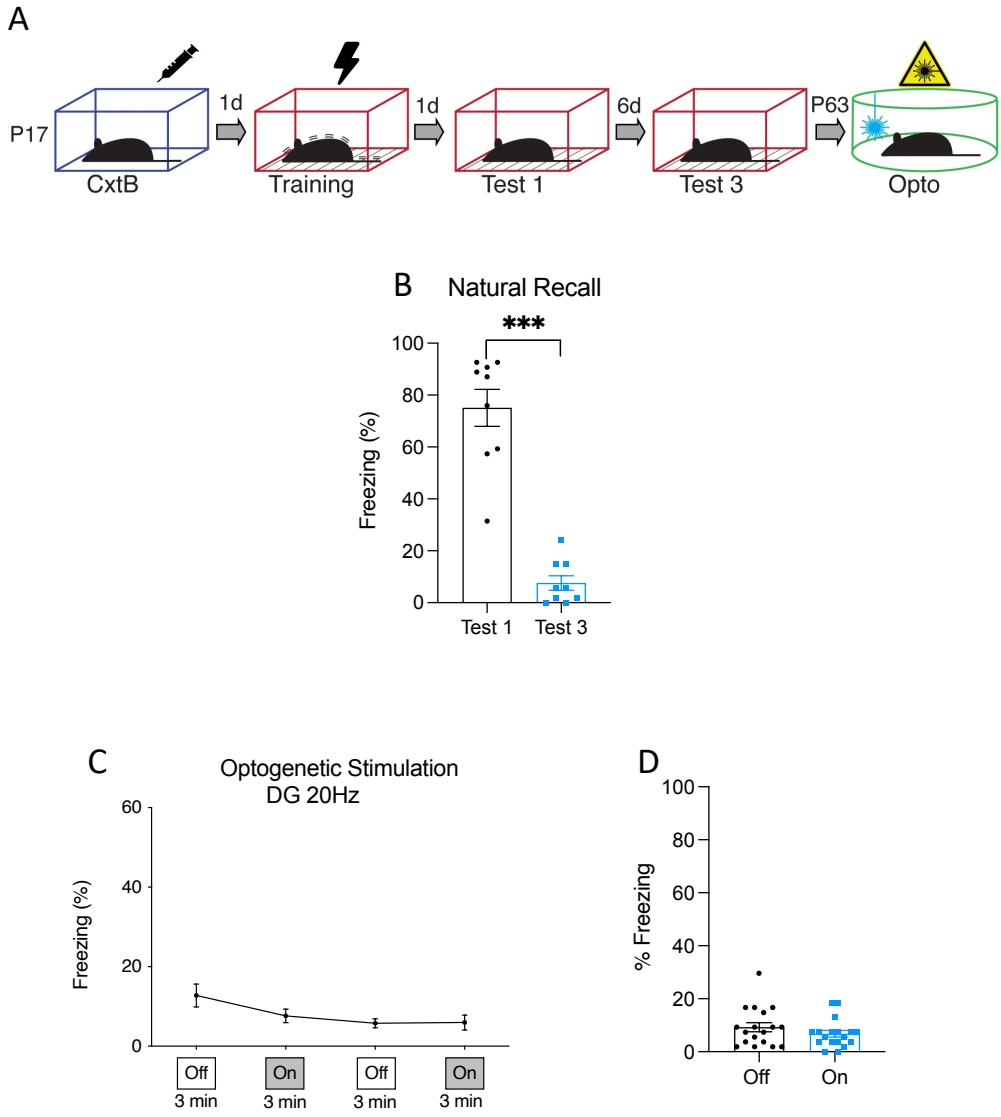


Figure 5.2. Optogenetic stimulation of engram ensembles for a neutral context in the DG does not induce freezing. (A) Training and testing scheme for optogenetic stimulation (20Hz) of engram-bearing cells in the DG. The black lightning symbol and the blue spike symbol indicate foot shocks and blue light delivery, respectively. The needle symbol indicates 4-OHT IP injection 2 h post context exposure. (B) Freezing level for natural recall in context A. (C, D) Memory recall in context C (engram activation) with light-off and light-on epochs. (D) Averaged freezing for the two light-off and light-on insets. N=9. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; *P < 0.05.

5.2 Optogenetic stimulation of engram ensembles in the CA1 are sufficient to induce acute recovery of an infant encoded contextual fear memory

To investigate whether it was possible to optogenetically rescue a 'lost' infantile memory by targeting another brain region, other than the DG, a cohort of infant Ai32FosTRAP were trained identically to Fig.1. Engram cells for context A were labelled in Ai32-FosTRAP mice at P17 (Fig. 5.3 A). Memory recall was then tested 1 and 8 days following training, as well as a context specificity test at P19. At P49, animals were implanted with an optical fibre targeting the CA1. Animals were placed in novel context C at P63 where they were tethered through their implants to an optogenetic patch cord enabling the delivery of blue light to the CA1. Both experimental groups received two days of the optogenetic stimulation protocol. On P63 animals received optogenetic stimulation of engram ensembles in the CA1 at 20 Hz. The following day mice were placed back into context C and received optogenetic stimulation at 4 Hz.

Mice that received foot shocks at P17 showed a higher level of freezing than animals that did not receive foot shocks when tested a day after training at P18 (Fig. 5.3 B). When tested 8 days after training at P25 both the shock and control no shock group showed similarly low levels of freezing. During optogenetic stimulation at 20Hz at P63, both groups showed similar levels of freezing during the light-off epochs (Fig. 5.3 C, D). Although the shock group froze significantly more than the no shock group during the light-on epochs, when the light-off and light-on epochs were compared for the shock group, there was no difference in the level of freezing (Fig. 5.3 D). Previous studies have demonstrated that optogenetic manipulation of engram cells in the CA1 requires optogenetic manipulation at 4 Hz, rather than 20 Hz (Ramirez et al., 2013, Ryan, 2015, Tanaka et al., 2014). Since 20 Hz did not seem sufficient to induce artificial activation of engram bearing cells in the CA1, animals were next tested with optogenetic stimulation at 4 Hz. Both groups showed background levels of freezing (5%) during the light-off epochs (Fig. 5.3 E, F). However, the experimental group showed significantly higher levels of freezing during the light-on epochs. This light-induced freezing was not observed for animals in the no shock control group. Thus, optogenetic stimulation, at 4 Hz but not 20 Hz, of CA1 engram cells were sufficient to acutely rescue a 'lost' infantile

memory (demonstrated by freezing during light-on epochs). These results demonstrate that acute optogenetic rescue of infant labelled engrams is not specific to the DG.

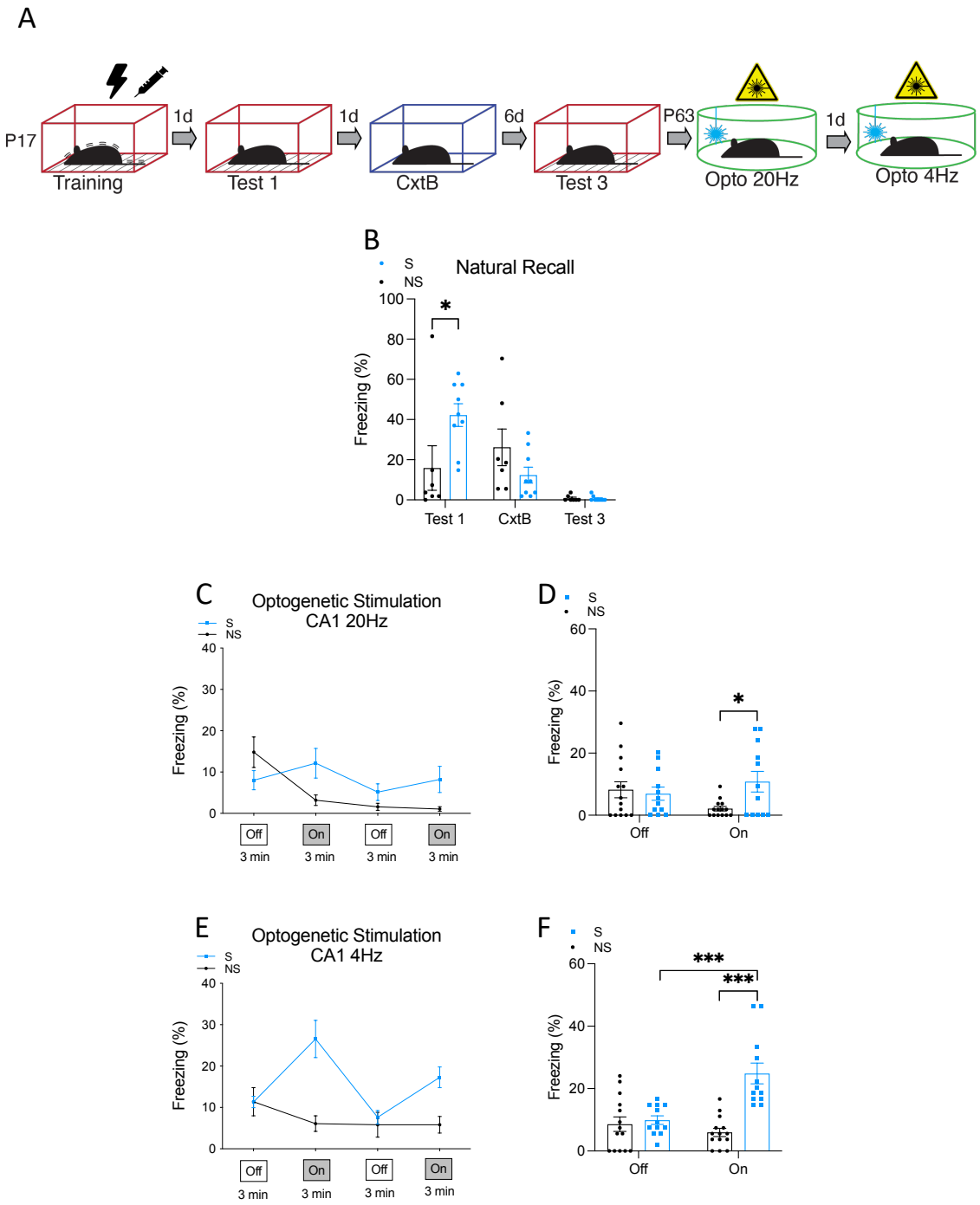


Figure 5.3. Optogenetic stimulation at 4 Hz of infant encoded engram ensembles in the CA1 induces freezing. (A) Training and testing scheme for optogenetic stimulation (20Hz) of engram-bearing cells in the CA1. The black lightning symbol indicates foot

shocks, and the blue spike symbol indicates blue light delivery. The needle symbol indicates 4-OHT IP injection 2 h post contextual fear conditioning. (B) Freezing levels during natural recall tests. (C, E) Memory recall in context C (engram activation) with light-off and light-on epochs during. (D, F) Averaged freezing for the two light-off and light-on insets. (C, D) Freezing levels during optogenetic stimulation at 20Hz in the CA1. (E, F) Freezing levels during optogenetic stimulation at 4 Hz in the CA1. N=7-9. Data presented as mean ± SEM. Statistical comparison was performed by using paired and unpaired t-tests; *P < 0.05, **P < 0.01, ***P < 0.001.

5.3 Acute optogenetic rescue of infant memory is not specific to contextual fear conditioning

Since optogenetic stimulation of infant labelled engram ensembles for a contextual fear memory was sufficient to induce acute memory recall in adulthood, this thesis next investigated whether it was possible to optogenetically rescue other forms of 'lost' infantile memories. To expand the behaviour, the transgenic line Ai32FosTRAP was combined with other behavioural tasks characterised in chapter 3.

5.3.1 Acute rescue of a spatial memory for the Barnes maze

By labelling the engram ensembles for the location of the escape platform in the Barnes maze, it was investigated whether DG optogenetic stimulation of the engram in adulthood results in the location of the escape platform. Ai32FosTRAP were trained on the Barnes maze at P17 (Fig. 5.4 A). Infant mice were injected with 4-OHT 2 h following the 5th and final trial on the Barnes maze. Since by the 5th trial the mice have learnt the location at the platform, demonstrated by their latency and distance travelled to locate the platform, the memory engram for the 5th trial was labelled. An optical fibre targeting the DG was implanted in all animals at P49 to allow for optogenetic stimulation of the labelled memory engram. At P63 animals were tethered through their implants to the optogenetic patch cord. The experimental light group received 3 min of optogenetic stimulation at 20 Hz while the no shock control group did not receive any light stimulation. Both groups were detached from the patch cord and placed on the Barnes

maze for a 5 min probe test. Any-maze software allowed for the time and location spent in each zone to be tracked.

The experimental light group spent significantly more time in the zone where the escape hole should be when compared to zone 2 and 3 (Fig. 5.4 H). Although there was no difference seen in the total time seen between zone 4 and zone 1, heat map analysis of the mean time spent in each location showed a concentration around the location of where the blocked escape hole was (Fig. 5.4 H). No difference was seen in the total time spent between zones for the no-light control group (Fig. 5.4 F, G). Optogenetic stimulation of ChR2-EYFP expressing neurons in the DG (neurons labelled during encoding of the Barnes maze memory) allowed for recall of the location of the escape hole in the Barnes maze. Thus, optogenetic stimulation of an infant memory, other than contextual fear conditioning, acutely rescues the memory.

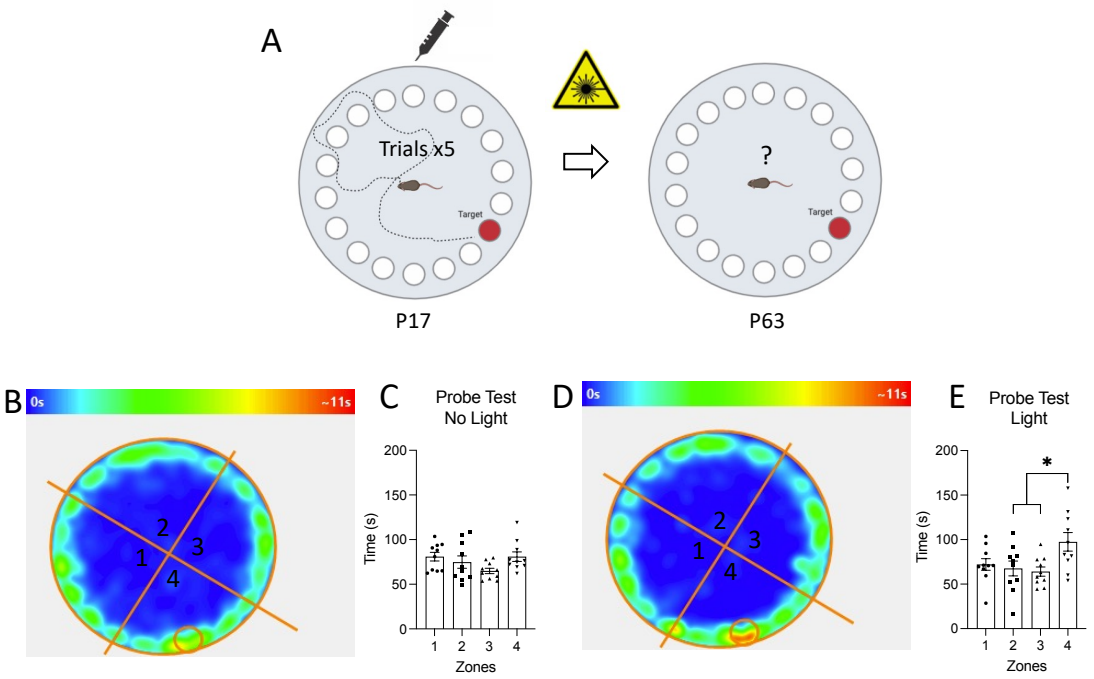


Figure 5.4. Ai32-TRAP mice find the location of the blocked escape hole in a Barnes maze task after optogenetic stimulation of the infant engram ensemble. (A) Schematic diagram of the behavioural procedure for optogenetic stimulation in the Barnes maze. The yellow triangle symbol indicates optogenetic stimulation. (B, D) Heat map of the mean time spent in each area of the Barnes maze during the probe trial at P63. (C, E) Mean time spent in each zone of the Barnes maze during the probe trial. N=10. Data presented as mean ± SEM. Statistical comparison was performed by using one-way ANOVA Bonferroni; *P < 0.05.

5.3.2 Optogenetic simulation of engram ensembles for an object memory

For optogenetic stimulation in a novel object recognition paradigm, infant Ai32-FosTRAP mice were habituated to the testing arena at P16 (Fig. 5.5. A). The following day animals were placed back in the arena with two identical objects for an acquisition session. 4-OHT was injected 2 h after to label the memory engram for the two identical objects. At P42 mice were implanted with an optical fibre targeting the DG. Directly before being placed in the testing arena for novel object recognition at P63, mice received 3 min of blue light stimulation at 20 Hz. Animals were then unplugged from the patch cord and

placed into the testing arena with two different objects (one familiar and one novel). The no light control cohort underwent the same paradigm but did not receive blue light stimulation. Animals were allowed to explore the two objects for 5 min before being removed from the arena.

During acquisition both the light and no light cohorts spent equal amount of time with both objects (Fig. 5.5. B, D). On the test day, animals that did not receive light stimulation prior to the test spent the same amount of time exploring both the novel and familiar object (Fig. 5.5 C). However, animals that did receive optogenetic stimulation of an object memory prior to the novel object recognition test spent significantly more time with the novel object (Fig. 5.5 E).

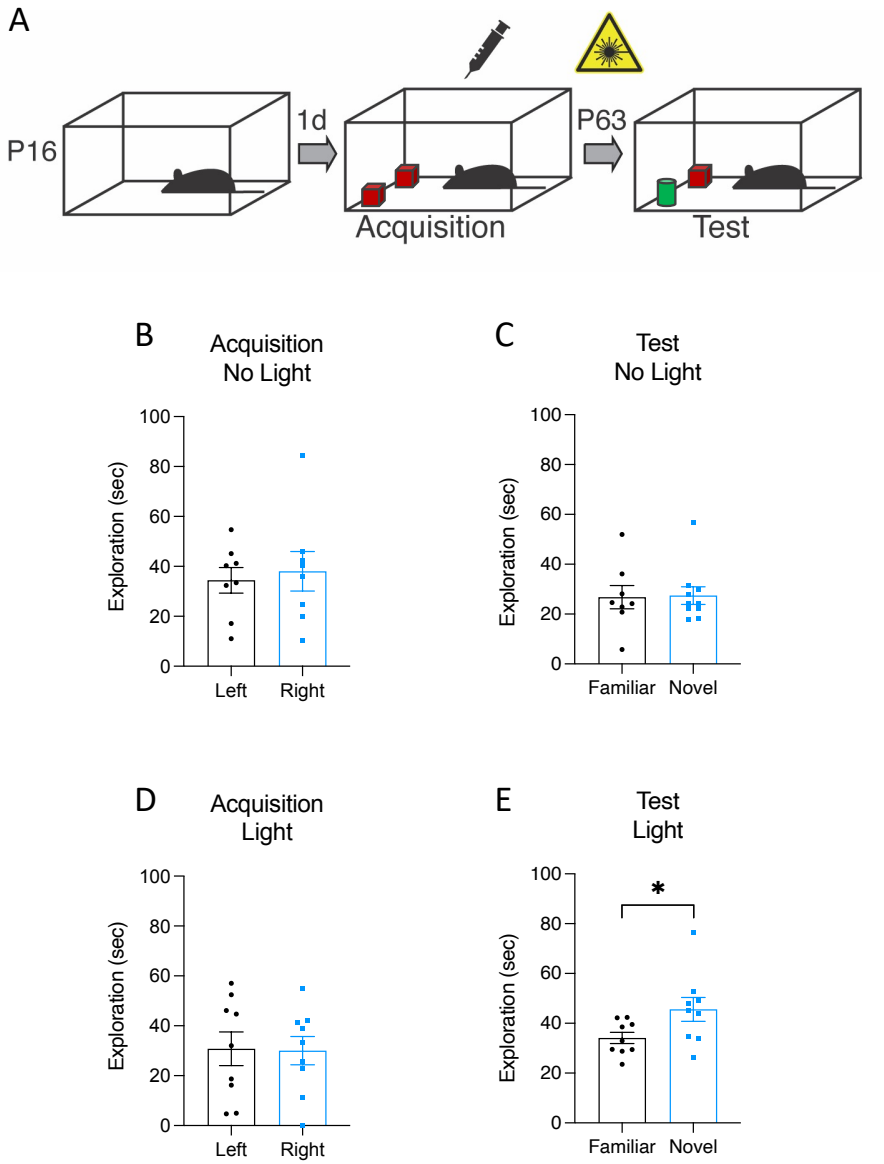


Figure 5.5. Optogenetic stimulation of infant labelled engram ensembles for an object memory in adulthood. (A) Schematic diagram of the behavioural procedure for optogenetic stimulation in a novel object recognition paradigm. The black lightning symbol and the yellow triangle symbol indicate foot shocks and optogenetic stimulation, respectively. The needle symbol indicates 4-OHT IP injection 2 h post the acquisition session. (B, D) Exploration time (sec) during acquisition session at P17. (C, E) Exploration time (sec) during object test at P63. N=9-10. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; *P < 0.05.

5.4 The engram connectivity pattern survives infantile amnesia and can be reactivated using optogenetics

Although optogenetic stimulation of a forgotten infant memory in adulthood resulted in a behavioural response, it is unclear how the information survives. It is thought that each memory has a distinct engram connectivity pattern, where each component of the engram contributes to the overall memory complex (Redondo et al., 2014, Ryan, 2015). The functional connectivity between engram cells can be investigated by looking at engram cell reactivation following optogenetic stimulation of upstream engram cells (Ryan, 2015, Redondo et al., 2014, Guskjolen et al., 2018). These studies suggest that for each engram, a persistent engram connectivity pattern forms across relevant brain regions. These studies provide supporting evidence that information is encoded through synaptic plasticity across engram cells. To investigate whether the functional connectivity between engram cells survived in the case of infantile amnesia, the histological analysis of engram reactivation after optogenetic stimulation in the DG was analysed. Further, whether the connectivity pattern was the same for a fear memory that was encoded before or after the infantile period was investigated.

As described in Chapter 4 the Ai32-FosTRAP engram labelling system allows for permanent, whole-brain labelling making it a valuable system for looking at long-range connectivity patterns. The engram connectivity pattern at P63 for an optogenetically reactivated engram was compared for a fear memory labelled at P17 (before infantile amnesia) or P29 (after infantile amnesia) (Fig. 5.6). The adolescent age P29 was chosen to account for any effects of development on the engram. Like adult mice, adolescent mice retain a fear memory (Akers, 2012). At the time of memory encoding, animals were either exposed to context A with or without receiving a shock. This allowed for the comparison between engram activation of a neutral contextual memory (no shock group) or a contextual fear memory (shock group). The level of overlap between engram cells activated at the time of memory encoding and engram cells activated during light reactivation was quantified for the DG, AMG, RSC or the PAG. Engram cells activated at the time of encoding were identified by ChR2-EYFP expression while cells activated at the time of light reactivation were identified by endogenous c-Fos expression. This

analysis was carried out for a fear memory (shock group) and a neutral memory (no shock group) for both age groups.

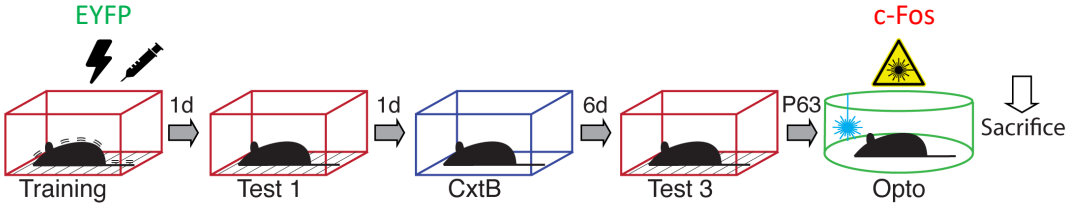


Figure 5.6. Optogenetic stimulation of engram ensembles in the DG. Training and testing scheme for optogenetic stimulation (20Hz) of engram-bearing cells in the DG for cell counting. The black lightning symbol and the blue spike symbol indicate foot shocks and blue light delivery, respectively. The needle symbol indicates 4-OHT IP injection 2 h post-training.

5.4.1 c-Fos activation

Optogenetic reactivation of a fear memory at P63 resulted in a significant increase in c-Fos positive cells in the AMG, regardless of whether the memory was encoded at P17 (infant memory) or P29 (adult memory) (Fig. 5.7 E, R). This difference was not seen in other brain regions such as the DG (Fig. 5.7 B, O), RSC (Fig. 5.7 K, X) or the PAG (Fig. 5.7 H, U). Since the AMG is involved in the emotional component of a memory, an increase in c-Fos positive cells during reactivation of a fear memory was expected. The DG and the RSC play an important role in processing contextual information. When comparing the experimental shock group with the control no shock group, both groups hold the same contextual information. Thus, reactivation of both a fear and neutral memory result in similar levels of c-Fos positive cells.

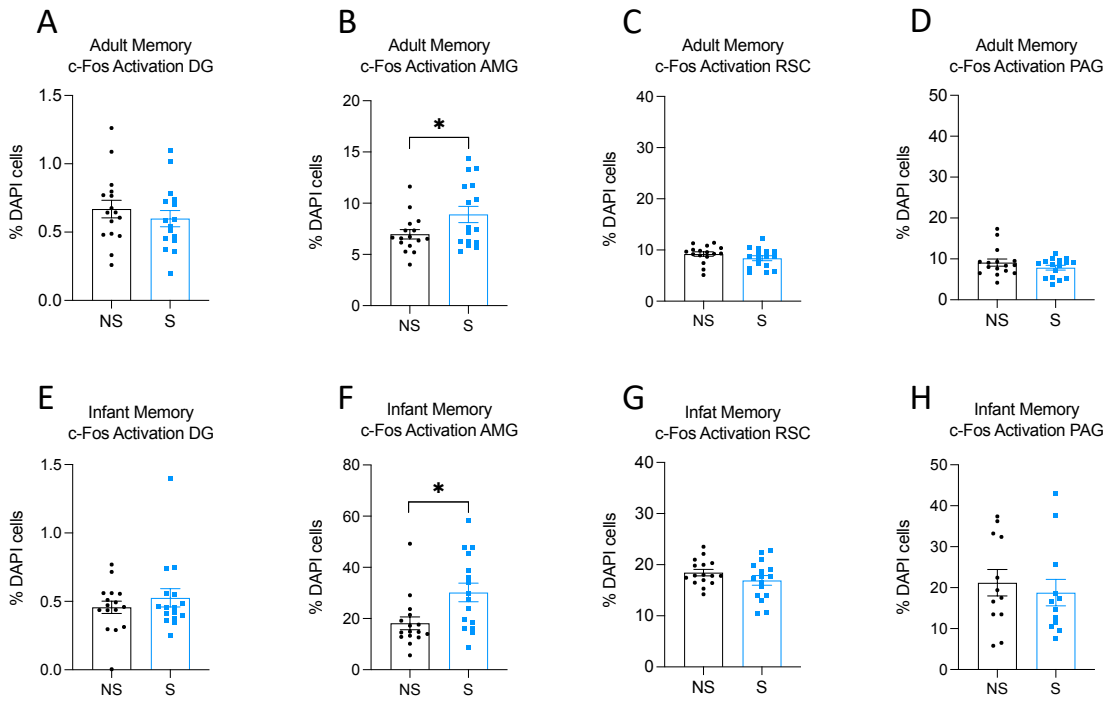


Figure 5.7. c-Fos activation after optogenetic stimulation of engram ensembles in the DG. (A-D) c-Fos activation at P63 after optogenetic stimulation of engram cells for a memory labelled in adulthood (P42). (E-H) c-Fos activation at P63 after optogenetic stimulation of engram cells for an infant labelled (P17) memory. (A, E) Percentage of c-Fos positive DAPI cells in the DG (A, E), AMG (B, F), RSC (C, G) and the PAG (D, H). N=3-4, n=4. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; *P < 0.05.

5.4.2 Engram labelling

During memory encoding, or engram labelling, at P17 or P29 an equal number of ChR2-EYFP positive cells were recruited to the engram in the DG (Fig. 5.8 C, P), RSC (Fig. 5.8 L, Y) and PAG (Fig. 5.8 I, V) for both the shock and control group. However, the size of the AMG component of the engram was significantly greater for the shock group in comparison to the no shock control (Fig. 5.8 F, S). As mentioned previously, these differences in engram labelling are reflective of the role each brain region plays in the mnemonic process. These results demonstrate that for an infant or adolescent labelled

memory, the engram cell connectivity is conserved across multiple brain regions into adulthood.

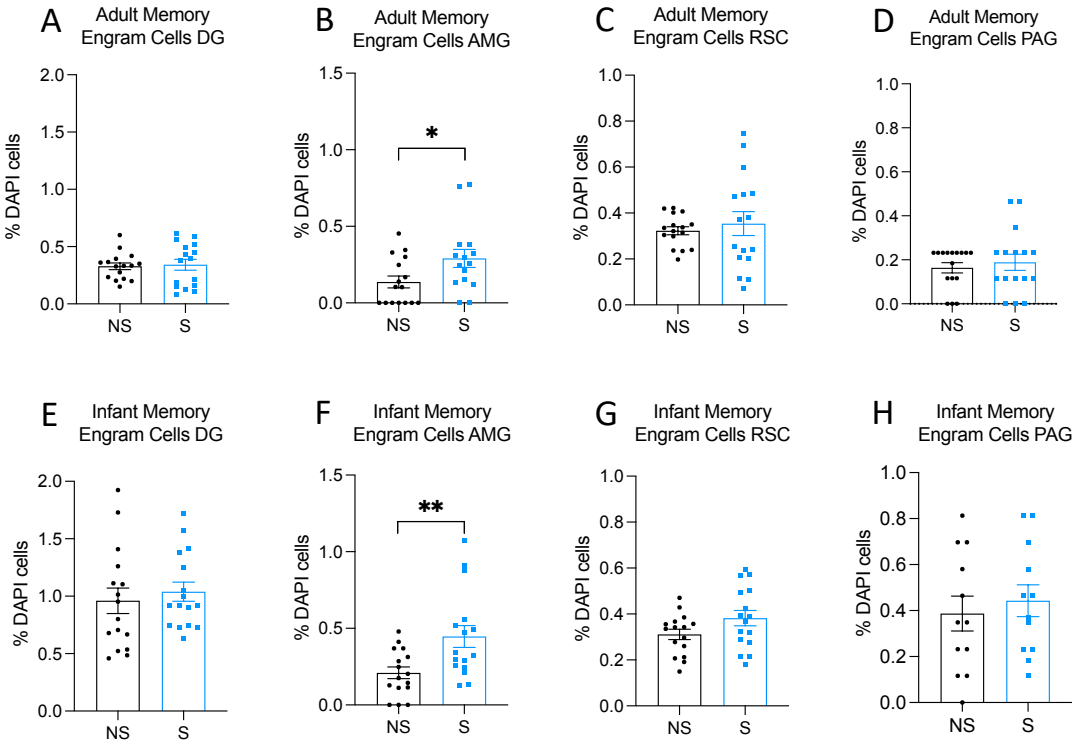


Figure 5.8. ChR2-EYFP labelling of engram ensembles for an adult and infant encoded memory. (A-D) Percentage of ChR2-EYFP positive DAPI cells at P63 after optogenetic stimulation of a memory labelled in adulthood (P42). (E-H) Percentage of ChR2-EYFP positive DAPI cells at P63 for a memory labelled in an infant labelled (P17) memory. (A, E) Percentage of ChR2-EYFP positive DAPI cells in the DG (A, E), AMG (B, F), RSC (C, G) and the PAG (D, H). N=3-4, n=4. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; *P < 0.05, **P < 0.01.

5.4.3 Engram reactivation

The number of cells labelled during encoding (ChR2-EYFP positive) that were reactivated after optogenetic stimulation (c-Fos positive) did not differ between experimental groups in the DG (Fig. 5.9 D, Q), AMG (Fig. 5.9 G, T), RSC (Fig. 5.9 M, Z) or the PAG (Fig. 5.9 J, W). Taken together these results demonstrate that the connectivity pattern of the

engram survives infantile amnesia and that DG optogenetic stimulation of a lost infantile memory is sufficient to reactivate the connectivity pattern. Further, encoding of a memory before (P17) or after (P29) the infantile amnesia period results in a similar engram connectivity pattern.

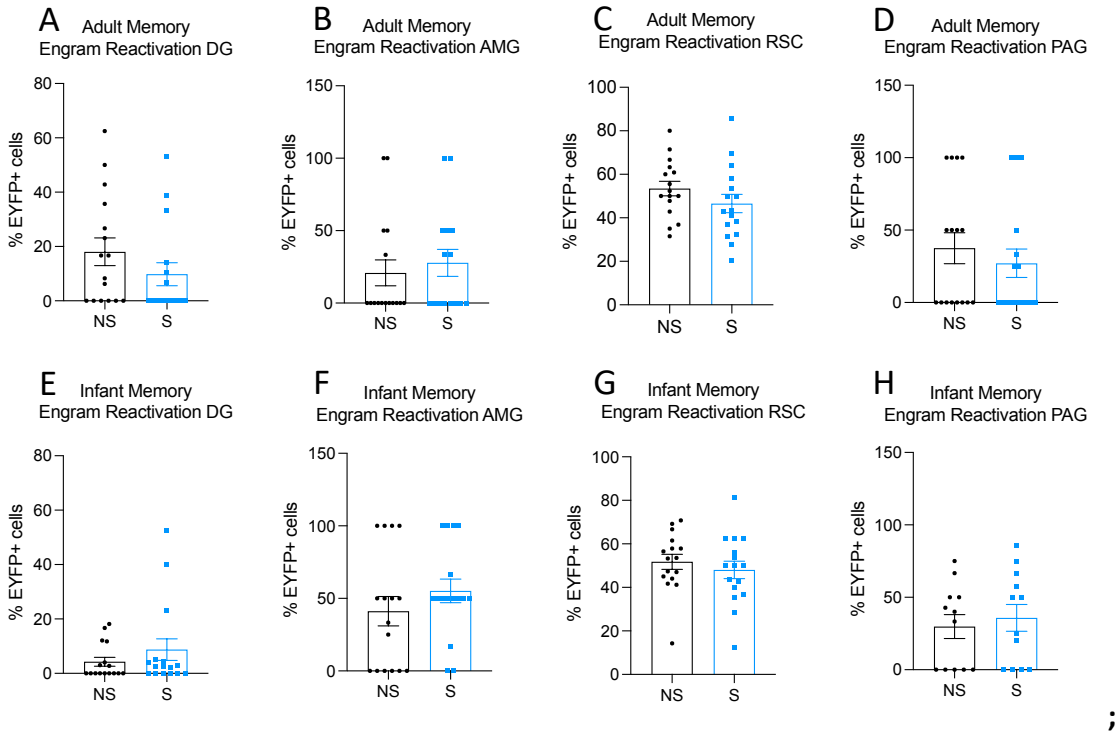


Figure 5.9. Reactivation of ChR2-EYFP labelled engram ensembles after optogenetic stimulation. (A-D) Percentage of c-Fos positive ChR2-EYFP cells at P63 after optogenetic stimulation of a memory labelled in adulthood (P42). (E-H) Percentage of c-Fos positive ChR2-EYFP cells at P63 after optogenetic stimulation of an infant labelled (P17) memory. (A, E) Percentage of c-Fos positive ChR2-EYFP cells in the DG (A, E), AMG (B, F), RSC (C, G) and the PAG (D, H). N=3-4, n=4. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; *P < 0.05.

5.5 Summary

These results provide further supporting evidence that the memory engram survives infantile amnesia. In line with a previous study, reactivation of a 'lost' infantile memory in adulthood acutely rescues the behaviour (Guskjolen et al., 2018). This result provides

supporting evidence that infantile amnesia is due to a retrieval deficit. This also holds for optogenetic stimulation of the memory engram in other brain regions such as the CA1 and other forms of behaviour such as object recognition and spatial navigation tasks like the Barnes maze. Further, the engram connectivity pattern is maintained in adulthood, where optogenetic reactivation in the DG reactivates the engram in multiple brain regions including the AMG, RSC or the PAG.

Chapter 6: Permanent reinstatement of infant memories

Since optogenetic stimulation results in only the acute rescue of the memory, to permanently retrieve a 'lost' infantile memory, this chapter looked at artificially updating the infant memory engram using optogenetics. These experiments allow for the investigation of false and genuine memories at the memory engram level but more importantly test whether a seemingly lost contextual memory can be permanently reinstated through updating with shock information.

6.1 Permanent reinstatement of a 'lost' infantile memory

As demonstrated in Chapter 3, infant mice can form purely contextual memories that can be updated with shock information. Taking advantage of this, Ai32FosTRAP mice were trained at infancy (P17) or adulthood (P42) with pure context exposure (context A) for 10 minutes (no shock) (Fig. 6.1 A). Injection of 4-OHT 2 h post-training resulted in labelling of neurons active at the time of encoding. Both cohorts of mice were exposed to context B 2 days later. Animals were implanted with an optical fibre implant targeting the DG at P49. At P63 mice were placed in a distinct context C, while optically reactivating the DG cells labelled in context A, and crucially while administering footshocks. In the following days, the animals' fear memory was tested in either the original context A or neutral context B (Fig. 6.1 B, C). If the light-reactivated cells labelled in context A can produce a functional CS during fear conditioning in context B, then the animals should express a false fear memory by freezing in context A, but not in context B.

As previously demonstrated, animals who received optogenetic stimulation of DG engram cells for an adult encoded context A memory during fear conditioning, froze significantly higher than control animals when re-exposed to context A (Fig. 6.1 B) (Ramirez et al., 2013, Ryan, 2015). However, this project demonstrated for the first time that this is also the case for optogenetic stimulation of DG engram cells for a 'lost' infant contextual memory. When engram cells for context A, encoded at P17, were optogenetically stimulated in the DG at P63, while simultaneously delivering footshocks,

animals subsequently froze significantly more when exposed to context A (Fig. 6.1 C). This increased freezing was not due to generalisation since the control group, which did not receive optogenetic stimulation, did not show a similar effect. This paradigm resulted in the encoding of artificial associative fear memory to the CS that was not naturally available at the time of the US delivery allowing permanent reinstatement of the 'lost' context infantile memory engram.

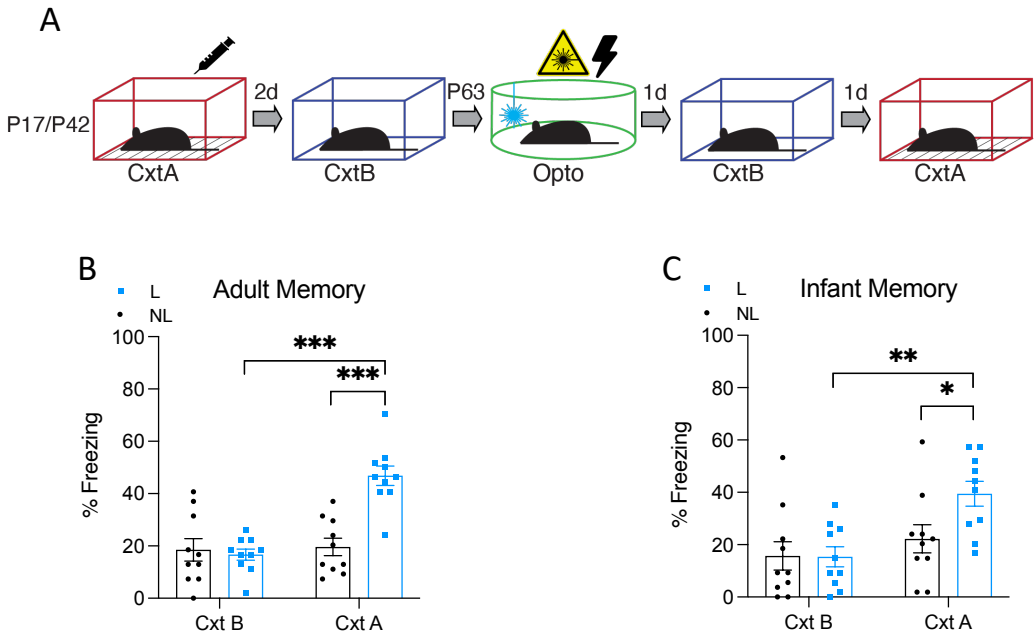


Figure 6.1. Reinstatement of an infant memory restores access to the engram ensembles in the DG. (A) Training and testing scheme for reinstatement paradigm. The black lightning symbol indicates foot shocks and the blue spike symbol represents blue light delivery. The needle symbol indicates 4-OHT IP injection 2 h post context exposure. (B, C) Freezing levels for memory recall in context B and context A. N=10. Data presented as mean ± SEM. Statistical comparison was performed by using paired or unpaired t-tests; *P < 0.05, **P < 0.01, ***P < 0.001.

6.2 Reinstatement of an infant contextual engram restores access to the engram

If the engram is permanently reinstated, this should be reflected at a cellular level in contextual engram bearing cells. To investigate whether access to the original engram was restored the level of overlap between engram cells activated at the time of encoding (infancy) and activated at the time of recall in context A (post reinstatement) were quantified in the DG. Engram cells activated at the time of encoding were identified by ChR2-EYFP expression. While cells activated at the time of recall were identified by endogenous c-Fos expression. Both the light and control no light group showed similar levels of c-Fos positive cells after exposure to context A (Fig. 6.2 B). Further, the level engram labelling for a contextual memory equated between groups (Fig. 6.2 C). However, there was an increased level of overlap of c-Fos positive and ChR2-EYFP positive cells in the light group in comparison to the no light control group (Fig. 6.2 D). This histological result demonstrates that engram cells active at the time of encoding (ChR2-EYFP positive) were reactivated (c-Fos positive) during adult exposure to context A. The control no light group did not show similar reactivation of the engram in the DG. Thus, permanent reinstatement of a 'lost' contextual infant memory restores natural access to the engram.

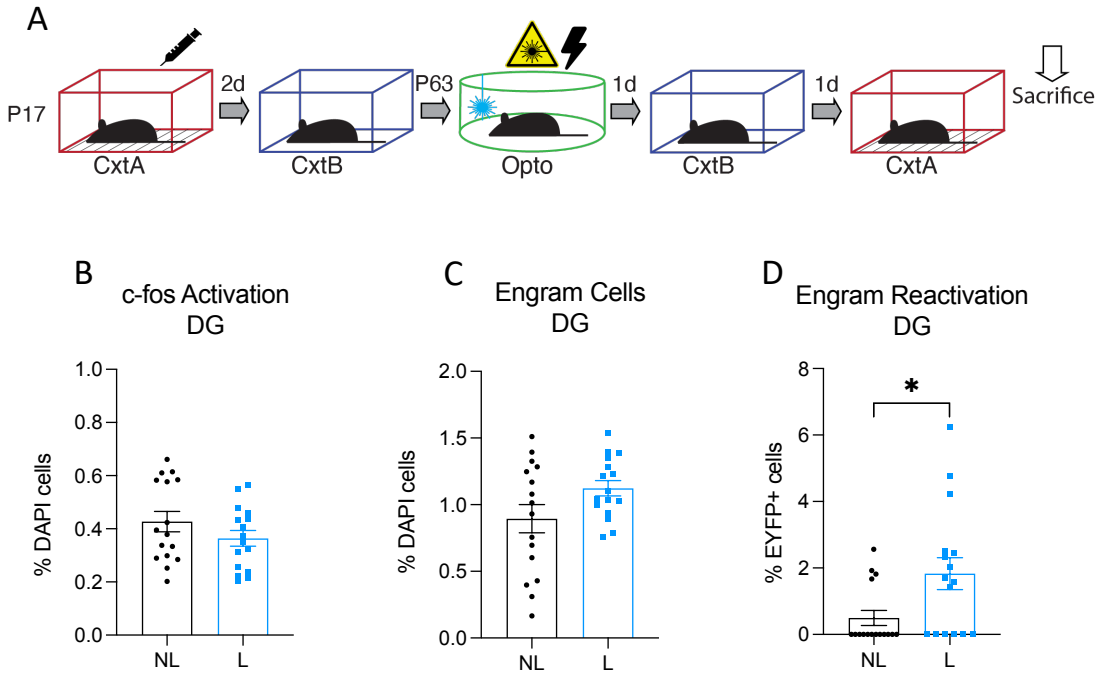


Figure 6.2. Reinstatement of an infant memory restores access to the engram ensembles in the DG. (A) Training and testing scheme for reinstatement paradigm. The black lightning symbol and the blue spike symbol indicate foot shocks and blue light delivery, respectively. The needle symbol indicates 4-OHT IP injection 2 h post context exposure. N=3-4, n=4. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; *P < 0.05, **P < 0.01.

6.3 Reinstatement behavioural paradigm does not rescue object/mismatch task

To investigate whether an updating paradigm can reinstate infant memories in other behavioural tasks, this project applied the object/mismatch paradigm characterised in chapter 3 with optogenetic stimulation of the DG. The engram for context A (black) was labelled at P17 in Ai32-FosTRAP mice (Fig. 6.3 A). Animals were implanted with an optical fibre targeting the DG at P49. At P63 mice were exposed to context B (blue) followed by context C (red) a day later. The following day animals were placed in context B with two identical objects (objects X) for 10 mins (acquisition 1). During acquisition 1 half of the cohort received a 5 min light on epoch followed by a 5 min light off epoch. The no light control cohort did not receive optogenetic stimulation. Here, the memory engram for context A is being stimulated while exploring objects X. Hypothetically optogenetic

stimulation of context A while exploring a set of objects should 'update' the object memory with contextual information of context A. Thus, an association would form between context A and objects X. The next day animals are placed into context C for 10 mins with a second identical pair of objects (objects Y) without receiving optogenetic stimulation (acquisition 2). Here animals, form an association between context C and object Y. Animals are then tested for object/mismatch in context A the following day with one object from each pair. If optogenetic stimulation during acquisition 1 has successfully updated context A with object X, then animals should spend more time exploring the out of place object (object Y).

Both the light and no light control groups spent equal amounts of time with both identical objects during acquisition 1 (Fig. 6.3 B, E) and acquisition 2 (Fig. 6.3 C, F). When tested for object/mismatch in context A both groups spent the same amount of time exploring the novel (out of place) and familiar (matched with context). These results demonstrate that optogenetics reinstatement paradigms of an infant labelled engram do not rescue an object/mismatch task in adulthood.

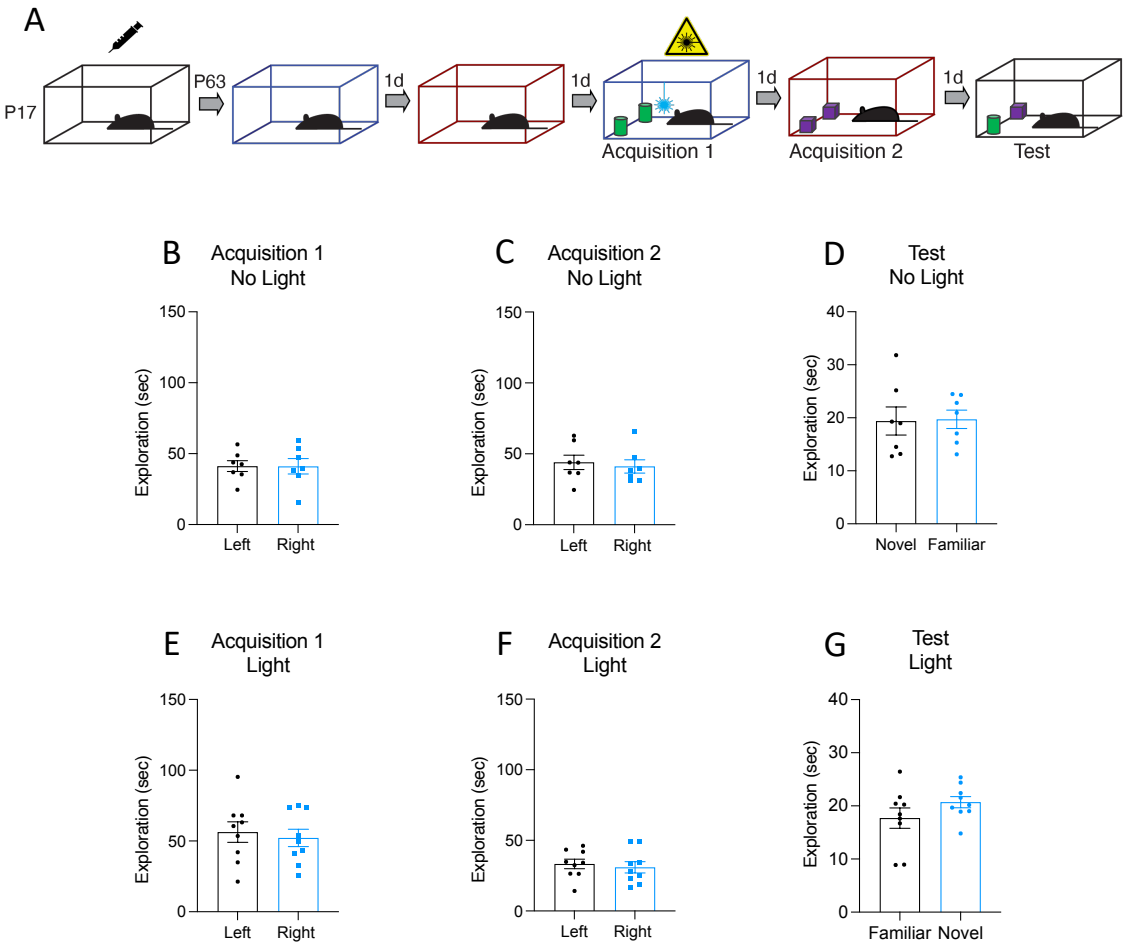


Figure 6.3. Behavioural updating paradigms do not rescue an infant object context memory. (A) Training and testing scheme for object/mismatch reinstatement paradigm. The black lightning symbol and the blue spike symbol indicate foot shocks and blue light delivery, respectively. The needle symbol indicates 4-OHT IP injection 2 h post context exposure. N=7-9. Data presented as mean ± SEM. Statistical comparison was performed by using paired t-tests; *P < 0.05.

6.4 Summary

Importantly, this chapter demonstrates that 'lost' infant memories can be permanently reinstated in adulthood. Behavioural updating paradigms using shock as a negative reinforcer permanently reinstated a 'lost' infant contextual memory. However, this did not hold for behavioural updating of an infant contextual memory with object information. This experimental result opens huge opportunities for investigating the

molecular mechanisms involved in reversing infantile amnesia. By comparing the engram pre and post reinstatement it may be possible to identify key molecular players or structural components involved in restoring access to lost infantile memories.

Chapter 7: Neurobiological Correlates

Although it is a well-documented phenomenon, there have been surprisingly few advancements in our understanding of the underlying mechanisms causing infantile amnesia. Valuable information and insight can be gathered by looking at neurobiological correlates of engram cells at different stages of development or pre and post infantile amnesia. In terms of neurophysiology, two important determinants of neuronal function are neuronal morphology and the expression of ion channels and receptors within the neuronal membranes. Understanding engram cell function necessitates experimental investigation of their electrophysiological properties (Ryan, 2015). One extremely useful technique is the patch clamp recording, which allows for the investigation of the electrical properties of the membrane (Sakmann, 1995). This project used electrophysiological techniques such as patch-clamping for analysis of the biophysical mechanisms or neural computation in engram and non-engram cells throughout development. Strong links have been demonstrated between neuronal morphology and functional characteristics such as neuronal firing patterns. Therefore, this project employed dendritic morphology analytic techniques such as Sholl analysis to investigate whether infantile amnesia had any effect on the dendritic morphology of engram cells.

7.1 Spontaneous activity of DG granule cells

The investigation of electrophysiological properties such as spontaneous excitatory postsynaptic currents (sEPSC) and spontaneous inhibitory postsynaptic currents (sIPSCs) is important for the investigation of transsynaptic connectivity between engram cells (Ryan, 2015). Spontaneous synaptic currents are generated in the absence of experimental stimulation, by action potential-dependent and independent release of neurotransmitters. This spontaneous activity may occur due to true miniature events or to the spiking activity of adjacent neurons.

In cases where amnesia is induced by protein synthesis inhibition, the upregulation in the frequency of sEPSC events in engram cells survives (Ryan, 2015). To investigate

whether this was also the case for infantile amnesia, the memory engram for a fear memory was labelled using contextual fear conditioning in infant (P14) and adult (P42) Ai32-FosTRAP mice (Fig. 7.1 A, B). The infant age of P14 was initially chosen to allow for the comparison between engram cells pre (P20) and post (P25) infantile amnesia. If the infant engram was labelled at P17, ChR2-EYFP expression was not sufficient for engram identification for whole-cell patching at P20. However, due to the time limits of this thesis, this analysis could not be carried out. To ensure sufficient labelling for the identification of engram cells while patching, animals received two 4-OHT injections, one directly after CFC and one two hours later. Electrophysiological screenings of synaptic input were recorded at P49 (Fig. 7.1 C, D). Engram cells were identified by ChR2-EYFP expression in hippocampal DG cells in hippocampal brain slices. Whole-cell patch voltage clamp was used to record the amplitude and frequency of spontaneous excitatory and inhibitory currents from infant and adult labelled engram and non-engram cells.

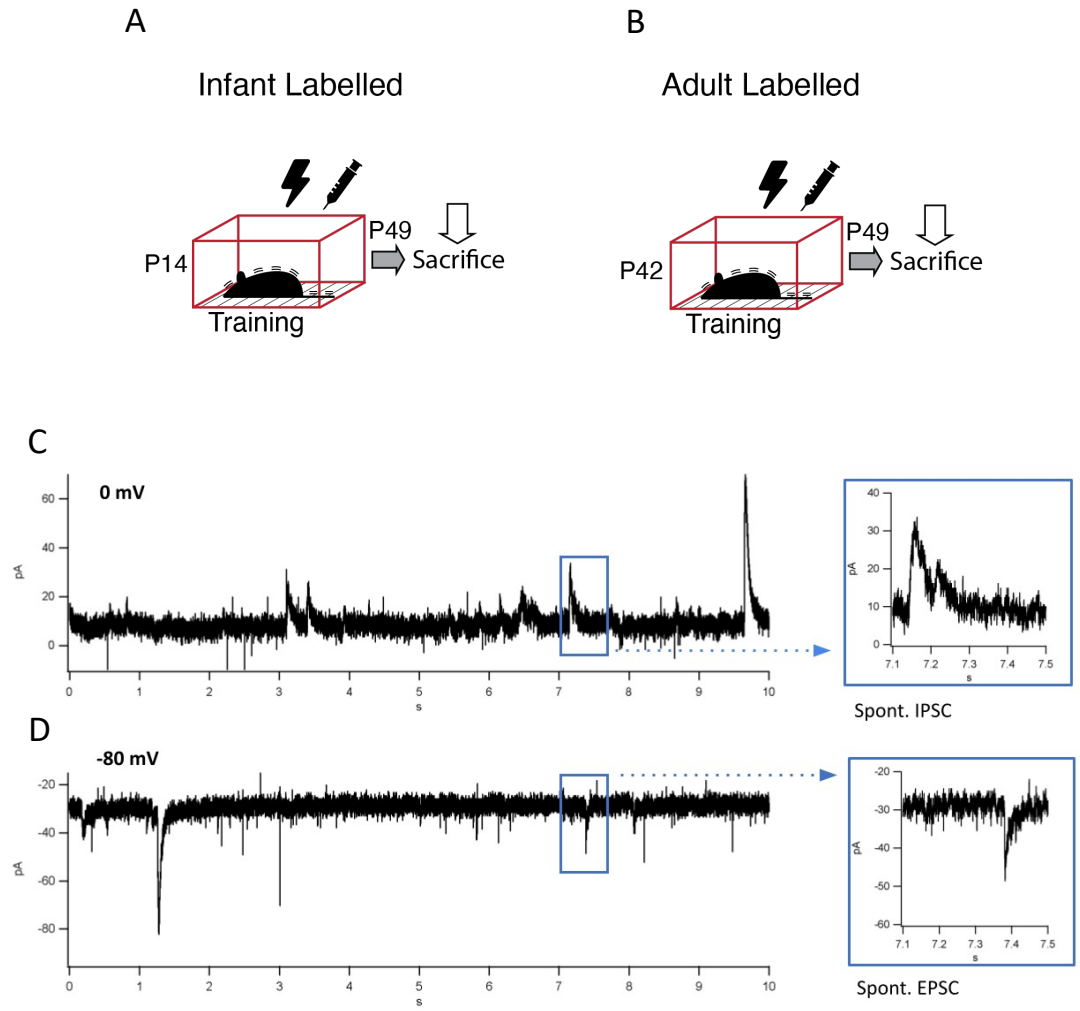


Figure 7.1. Whole-cell patch recordings of sEPSC and sIPSC in infant and adult encoded engram and non-engram cells. (A, B) Schematic diagrams of the behavioural protocol for engram labelling in infant P14 (A) and adult P42 (B) Ai32-FosTRAP mice. The black lightning symbol and the needle symbol indicate foot shocks and 4-OHT IP injection 2 h post-training, respectively. (C,D) Whole-cell patch voltage clamp 10 sec recordings. (C) Example trace of sIPSC at 0 mV. (D) Example trace of sEPSC recorded at -80 mV.

7.1.1 Spontaneous excitatory and inhibitory postsynaptic currents.

The amplitude of sEPSCs in engram cells labelled in infancy (P14) or adulthood (P42) did not differ from non-engram cells during electrophysiology recording at P49 (Fig. 7.2 A). However, adult labelled engram cells demonstrated a higher frequency of sEPSCs compared to non-engram cells (Fig. 7.2 B). This engram cell specific difference in the

frequency sEPSCs was not seen in between infant labelled engram and non-engram cells (Fig. 7.2 B). Adult non-engram cells had a significantly lower frequency of sEPSCs compared to the infant labelled group (Fig. 7.2 B). Whereas no difference was seen in the frequency of sEPSCs between infant and adult labelled engram cells at P49 (Fig. 7.2 B). Analysis of sIPSCs in engram and non-engram cells revealed no difference in the amplitude or frequency of spontaneous synaptic input (Fig. 7.3). Regardless of whether sIPSCs were recorded in infant or adult labelled engram cells, no difference was seen between groups (Fig 7.3 A, B). Further, no difference was seen between engram and non-engram cells within both groups (Fig. 7.3. A, B).

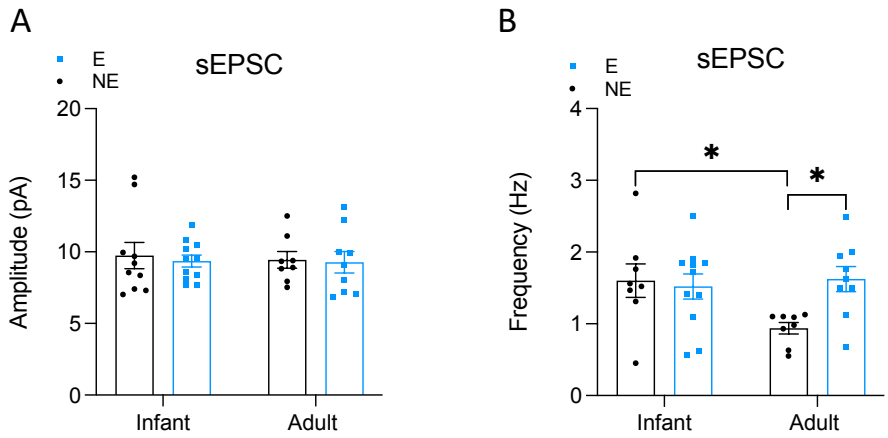


Figure 7.2. Adult encoded engram cells demonstrate a higher frequency of sEPSCs than non-engram cells. (A, B) Amplitude (pA) and frequency (Hz) of sEPSCs recorded during whole-cell patch voltage clamp of Ai32-FosTRAP mice at P49. (A) Comparison of the amplitude of sEPSCs in infant (P14) and adult (P42) labelled non-engram (NE) and engram (E) cells. (B) Comparison of the frequency (Hz) of sEPSCs in non-engram (E) and engram (F) cells labelled during infancy (P14) or adulthood (P42). N=8-11. Data presented as mean ± SEM. Statistical comparison was performed by using a two-way ANOVA Bonferroni; *P < 0.05, *P < 0.01.

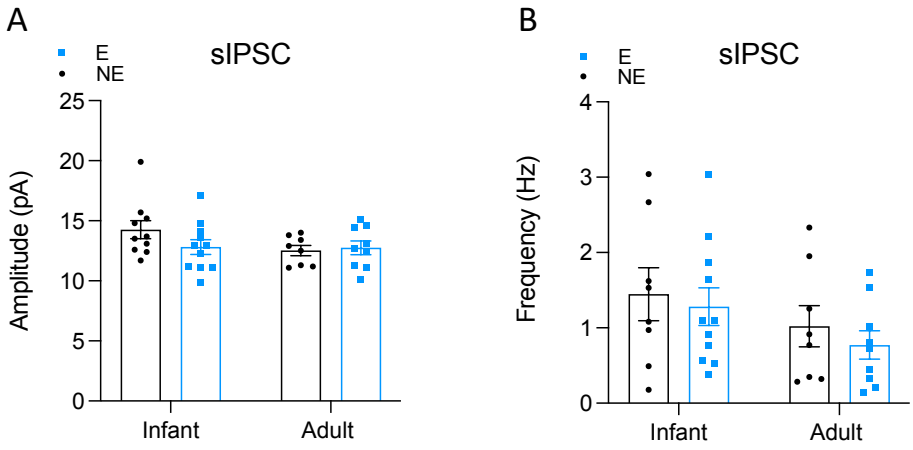


Figure 7.3. Adult and infant encoded engram cells demonstrate similar properties of sIPSCs. (A, B) Amplitude (pA) and Frequency (Hz) of sIPSCs recorded during whole-cell patch voltage clamp of Ai32-FosTRAP mice at P49. (A) Amplitude of sIPSCs in non-engram (NE) and engram (E) cells encoded during infancy (P14) or adulthood (P42). (B) Frequency (Hz) of sIPSCs in non-engram (NE) and engram (E) cells encoded in infancy (P14) or adulthood (P42). N=8-11. Data presented as mean ± SEM. Statistical comparison was performed by using a two-way ANOVA Bonferroni; *P < 0.05.

7.1.2 Ratio of spontaneous excitatory to inhibitory postsynaptic current

The ratio of the amplitude of sEPSCs to sIPSCs did not differ between engram and non-engram cells regardless of whether they were labelled during infancy (P14) or adulthood (P42) (Fig. 7.4 A). However, an upregulation of the ratio of the frequency of sEPSCs to sIPSCs was seen in engram cells compared to non-engram cells labelled in adults (Fig. 7.4 B). This difference was not seen in infant labelled engram cells, where the frequency ratio of sEPSCs to sIPSCs did not differ between engram and non-engram cells (Fig. 7.4 B). When the frequency ratio of sEPSCs to sIPSCs was compared between infant labelled and adult labelled engram cells was compared there was a significant shift in the ratio of excitatory to inhibitory synaptic input in adult labelled engram cells (Fig. 7.4 B). Engram cells labelled in adulthood show greater imbalances in the frequency ratio of sEPSCs to sIPSCs which correlates with memory retrieval. In the future, a complete characterization of electrophysiological parameters could allow for the identification of

physiological parameters that correlate with forgetting, and which (if any) correlate with memory retention.

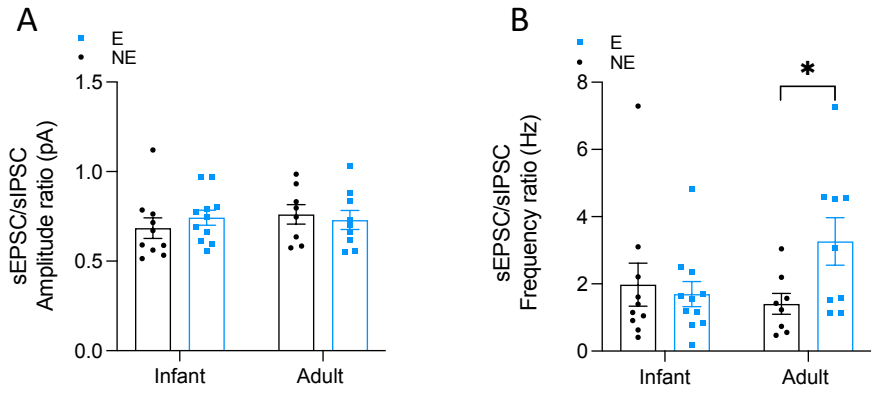


Figure 7.4. Adult encoded engram cells have a higher sEPSC/sIPSC frequency ratio compared to non-engram cells. (A) Amplitude ratio of sEPSC/sIPSCs (pA) of infant (P14) and adult (P42) encoded engram (E) and non-engram (NE) cells at P42. (B) Frequency ratio of sEPSC/sIPSCs (Hz) in engram (E) and non-engram (NE) cells at P42. N=8-11. Data presented as mean ± SEM. Statistical comparison was performed by using a two-way ANOVA Bonferroni; *P < 0.05.

7.2 Changes in dendritic morphology of infant labelled engram cells

Memories persist long after the induction and degradation of molecules involved in the event. Therefore, studies tend to look closer at more long-term changes such as synaptic plasticity. It is thought that during memory formation, activity-dependent synaptic plasticity is induced at the required synapses. This form of plasticity is required for both encoding and storage of the memory engram. Dendritic morphogenesis includes the forming of dendrite arbours and spines and the development of dendritic branches. Dendritic spine density is considerably higher in engram cells compared to non-engram cells (Ryan, 2015). Moreover, there is an increase in synaptic density at the interface of engram cell interactions, such as dendritic spine volume and diameter compared to non-engram cells (Choi, 2018). When studying engram plasticity, studies tend to mostly focus on dendritic spine count while few, focus on dendritic morphology. Dendritic

morphogenesis includes the forming of dendrite arbours and dendritic branches. Dendritic morphology is an important determinant for neuronal function where its alteration can be used as a useful correlation for connection establishment between engram cells. Information is received into the neuron through dendrites through a tightly regulated process. Dendritic morphology and arborization influence and regulates communication (via synaptic input) with other neurons. Dendritic morphology is not fixed, where alterations can result in changes to neural circuitry. Studies have shown that maze training can alter dendritic branching (Greenough, 1979). Further neurons in the DG that are active during spatial tasks demonstrate more complex dendritic trees when compared to silent neurons (Diamantaki, 2016). Disruption to dendritic morphology in the case of certain disorders including schizophrenia and autism highlights its importance for the overall function of neuronal networks (Kulkarni and Firestein, 2012).

This project investigated whether the dendritic morphology of engram cells altered for a recalled fear memory before and after infantile amnesia. To look at the dendritic arbour of memory engram cells in the DG, Ai32-ForTRAP mice were trained with contextual fear conditioning at P17 (Fig. 7.5 A). Animals were tested for recall either 3 or 8 days after training and sacrificed 45 min after. The no recall control cohort did not receive a recall test and were sacrificed from their home cage. Using confocal imagining and Imaris software, a 3D representation of the dendritic tree was obtained of ChR2-EYFP positive cells in the DG for each recall condition. The somatodendritic compartment of each engram cell was reconstructed for morphological analysis (Fig. 7.5 B). Morphological metrics such as the total dendritic length (outer radius), the number of primary dendrites and the branching patterns (Sholl analysis) were analysed.

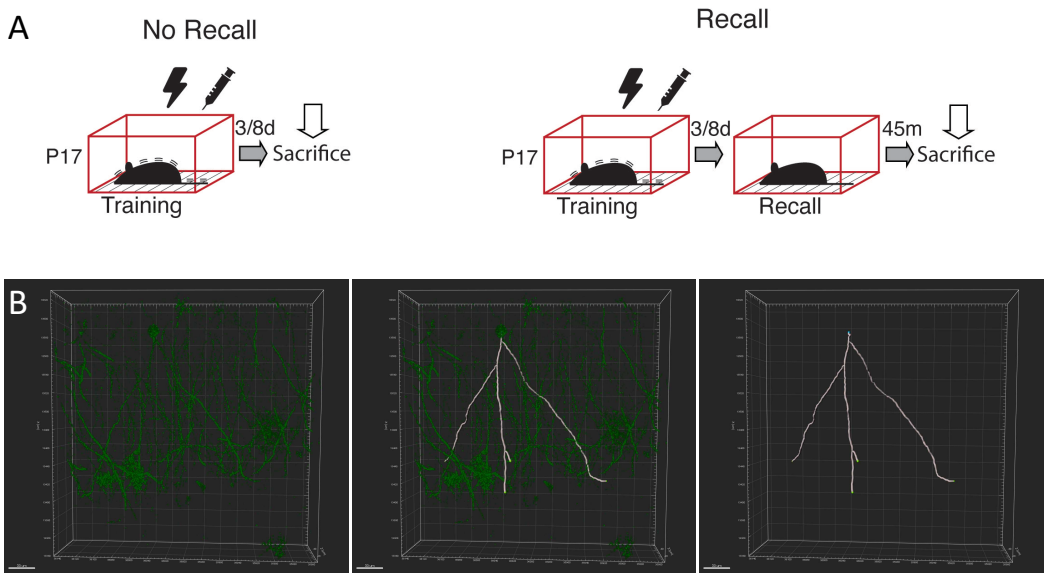


Figure 7.5. Behavioural paradigm for looking at dendritic morphology of engram cells. (A) Schematic diagram of behavioural procedure. (B) 3D representative image of the dendritic tree of an engram cell obtained using Imaris software.

7.2.1 Sholl analysis

Synaptic activity increases the rate of addition of dendritic branches changing the dendritic arbour (Rajan and Cline 1998). Sholl analysis allows us to look at changes to the whole dendritic arbour and is widely used as a method of evaluating the morphology of neurons. By counting the number of dendritic intersections at fixed distances from the soma centre we can deduce the branching patterns of neurons and look at distributions of synaptic contacts. Sholl analysis allows us to gather a structural one-dimensional representation of the complex three-dimensional dendrite. The branching pattern of dendrites is directly associated with the number and distribution of synaptic inputs that neurons are receiving. Sholl analysis provides a useful method to determine the 'connectivity zone' of the neuron, that is how big is the region where synapses are possible. Moreover, it provides us with information about dendritic complexity. If a dendrite has more intersections, this suggests a high dendritic complexity. Altering the morphology of dendritic branching, without affecting the total length of the dendritic tree, has shown to shift firing patterns from busting to non-bursting or vice versa (Van Elburg, 2010).

For both the recall and no recall groups Sholl analysis showed considerably morphological overlap of engram cells at 3 and 8 days (Fig. 7.6 A, B). Dendritic branching of engram cells in the no recall groups was less extensive than engram cells in the recall group at both 3 and 8 days (Fig. 7.6 C, D). Thus, recall significantly increased dendritic branching of engram cells at both 3 and 8 days.

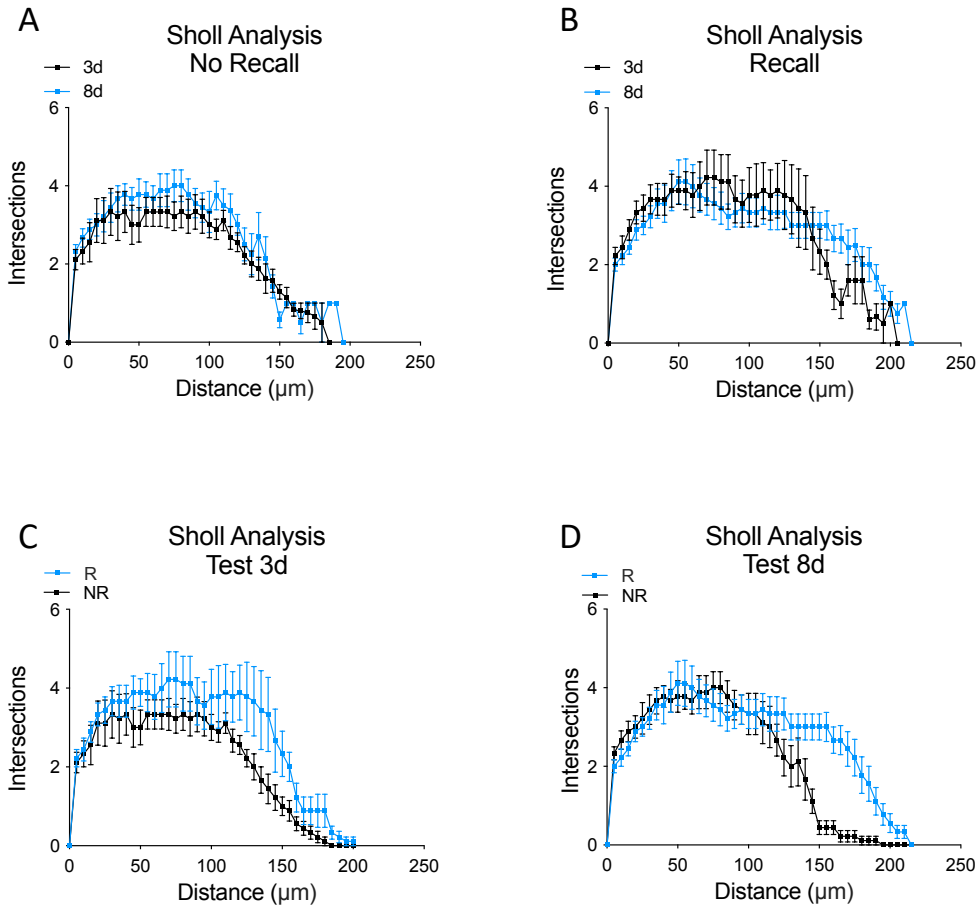


Figure 7.6. Sholl analysis of engram cells pre and post infantile amnesia. (A-D) Sholl analysis of the dendritic arbour of engram cells. N=3, n=3. Data presented as mean ± SEM.

7.2.2 Outer radius

The outer radius reached by each dendritic tree can give greater insight into the branching patterns of the dendrite. Changes in the dendritic size of the dendrite have been shown to influence the bursting of the cell as well as the number of spikes per burst (Van Elburg, 2010). The outer radius of engram cells was quantified for cohorts exposed to a recall and no recall session, 3 and 8 days after training of fear memory (Fig. 7.7). No difference was seen in the outer radius of the dendritic trees of engram cells in the no recall groups at 3 and 8 days (Fig. 7.7 A). A higher expansion of the outer radius of the dendritic trees of engram cells was seen at 8 days for the recall group when compared to 3 days (Fig. 7.7 B). The outer radius remained the same between engram cells of the recall and no recall group at 3 days (Fig. 7.7 C). However, at 8 days, the outer radius of engram cells in the recall group had a significantly larger outer radius (Fig. 7.7 D).

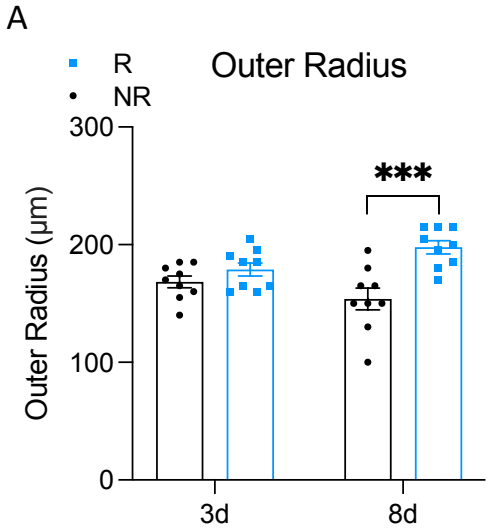


Figure 7.7. The outer radius of the dendritic tree is greater in engram cells after recall at P25. (A) Outer radius (µm) of the dendritic tree of engram cells labelled at P17 after a recall test at P20 (3d) or P25 (8d). N=3, n=3. Data presented as mean ± SEM. Statistical comparison was performed by using a two-way ANOVA Bonferroni; ***P < 0.001.

7.2.3 Primary Dendritic Length

Other morphological correlates such as primary dendritic length provide further information about the synaptic activity of the engram cell. The shortening or

lengthening of the primary dendrite has been shown to change the firing of pyramidal cells from bursting to tonically fire (Van Elburg, 2010). The first or primary dendrites branching from the soma of engram cells labelled at P17 for a fear memory was quantified at P20 (before infantile amnesia) and P25 (after infantile amnesia) (Fig. 7.8). The average primary dendritic length of engram cells labelled for a fear memory at P17 was increased at 8 days compared to 3 days (Fig. 7.8 B). Recall of the fear memory did not seem to affect the primary dendritic length of engram cells 3 or 8 days after training (Fig. 7.8 C, D). Primary dendritic length is generally inversely correlated with the position in the granule layer which the neuron occupies. Thus, neurons in the inner section of the granule layer will have longer primary dendrites than those in the outer layer.

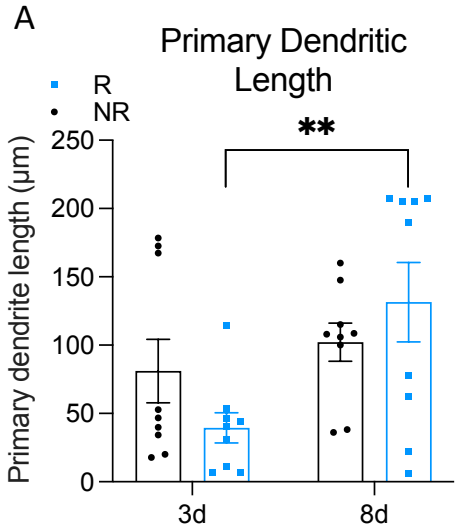


Figure 7.8. Primary dendritic length of engram cells increases between P20 and P25 after recall. (A) Primary dendritic length (µm) of engram cells encoded at P17 after recall at P20 (3d) or P25 (8d). N=3, n=3. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; **P < 0.01.

7.3 Summary

Studying the physiology and morphology of engram cells following amnesia can give greater insights into what is happening at a functional level. From an electrophysiological point, engram cells encoded in adulthood show an increase in the frequency ratio of excitatory and inhibitory neurotransmission relative to non-engram

cells. Further, adult encoded engram cells trended to have an increased frequency in the ratio of excitatory and inhibitory neurotransmission compared to infant encoded engram cells. While there was no difference in synaptic input on to engram and non-engram cells that were encoded in infancy. The dendritic branching structure is thought to have a direct influence on neuronal firing activity (Van Elburg, 2010). Dendritic morphological analysis demonstrated an increase in the dendritic branching of engram cells after memory retrieval at 3 and 8 days after training. Recall of a contextual fear memory affected the outer radius and primary dendritic length of the dendritic tree of memory engram cells 8 days after training. These results demonstrate changes in synaptic firing and dendritic morphology that may correlate with memory retrieval.

Chapter 8: Preventing Infantile Amnesia

8.1 Environmental influences an infantile amnesia

8.1.1 Environmental Enrichment

It is clear that the environment has a strong influence on learning and memory and that during postnatal development there are periods in which the developing brain has heightened sensitivity to environmental influences, but it is unknown whether these influences are strong enough to affect infantile amnesia. One way of deciphering how environmental changes drive alterations to the structure and function of the brain across development is by looking at different environmental paradigms such as environmental enrichment. Exposure of rodents to an enriched environment has been shown to have lasting generalizing effects on behaviour including learning and memory (Frick, 2003). It is thought that these positive effects are mediated by the induction of synaptic plasticity (Eckert, 2013).

To try and combat the rapid forgetting seen in infancy, synaptic plasticity was induced through environmental enrichment at different stages of development (Fig. 1). Mice were exposed to environmental enrichment from different time points. The control group were not exposed to environmental enrichment and remained in a standard home cage. The second group were exposed to environmental enrichment from birth and remained in environmental enrichment, continuing after weaning. The third group who were reared in a standard homecage were placed in environmental enrichment from weaning at P21. The final experimental group were reared in a standard home cage until P16 (1 day before training) when they were placed in environmental enrichment. All groups were trained at P17 using contextual fear conditioning and tested for memory recall 1 and 8 days later (Fig. 8.1 A). Memory recall was tested 1 day (Fig. 8.1 B) and 8 days (Fig. 8.1 D) later as well as an additional recall test in adulthood (P63) (Fig. 8.1 E). When comparing each environmental enrichment condition against the control group,

no significant difference was seen in the level of freezing (Fig. 8.1 B-E). These results demonstrate that environmental enrichment does not rescue forgetting due to infantile amnesia.

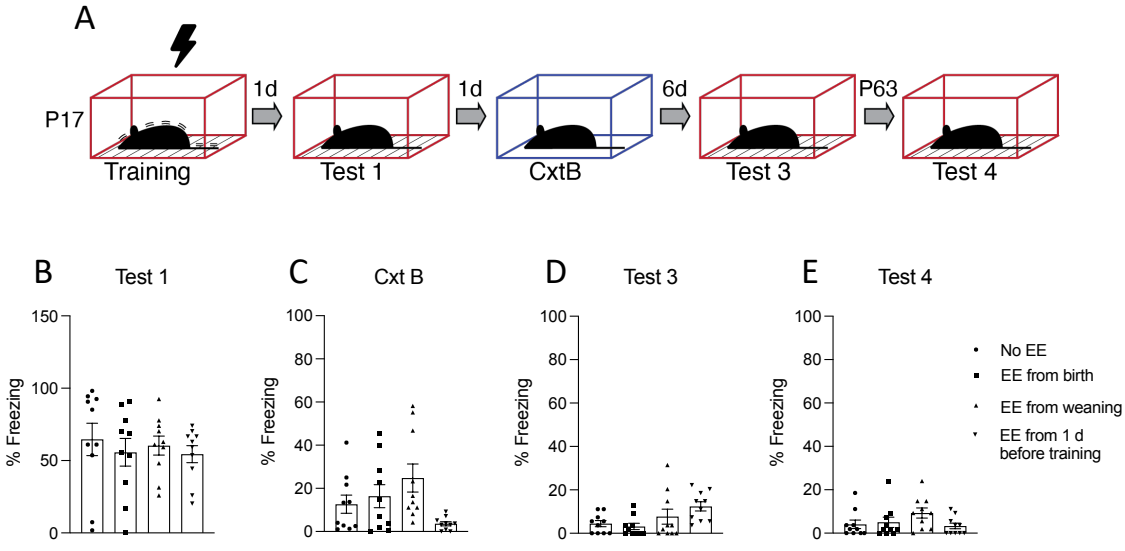


Figure 8.1. Environmental enrichment does not affect infantile amnesia. (A) Schematic diagram of the behavioural procedure for contextual fear conditioning in infant C57BL/6J mice. (B, D, E) Freezing levels during recall test 1 day (B), 8 days (D) after training and at P63 (E). (C) Freezing levels during context specificity test in context B. N=10. Data presented as mean ± SEM. Statistical comparison was performed by using one way ANOVA Bonferroni; *P < 0.05.

8.1.2 Delayed weaning

The world altricial is derived from a Latin word, meaning 'to nurse, to rear, or to nourish'. In other words, during the early stages of development, the infantile period, altricial species are deemed 'helpless'. To survive, they must spend their first period of life being taken care of. They must learn important associations for survival such that a particular scent is associated with their mother and thus feeding. When the offspring become old enough, where a caregiver is no longer essential, the offspring must switch strategy, to that of independence. To become independent, they must either suppress or forget how

they have learnt to survive previously and must now learn a new independent way of survival. For example, if rodents like humans depend on their mother for food in their later years of life they would not survive. Is it a coincidence that this stage of development also coincides with the offset of infantile amnesia? Interestingly, precocial mammals such as the guinea pig and dengue who are relatively mature and independent from a young age do not experience infantile amnesia (Campbell, 1974, Akers, 2014). Thus, it appears that there is something occurring in the altricial brain, that is not present in the precocial brain, causing infantile amnesia.

When you look at the stage of development in which infantile amnesia occurs one significant occurrence is weaning. By delaying the time of weaning in rodents we investigated whether the change in environment (or lack thereof) would have any effect on memory retention during development. To examined whether prolonging the infant's environment from a dependent to an independent state would increase memory retention during the infantile amnesia period, two cohorts of mice were subjected to contextual fear conditioning at P17 and tested for memory recall 1 and 8 days later (Fig. 8.2 A). Experimental mice were left with the parents in the breeding cage and weaned at P26, while control mice were weaned at the standard weaning age P21. There was no significant effect of delayed weaning on memory retention in infant mice (Fig. 8.2 B-E). This set of experiments interestingly concluded that whatever is driving infantile amnesia is not determined by the time of weaning. The lack of effect witnessed in these experiments suggests that these changes in the environment are not underlying infantile amnesia but rather the cause is genetically determined.

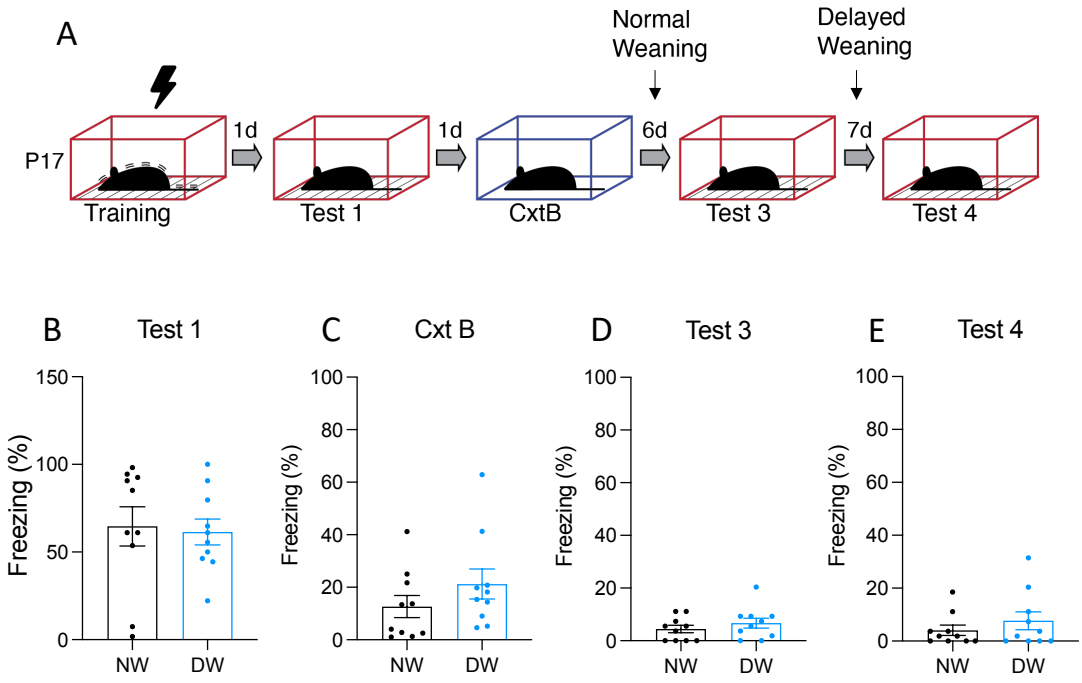


Figure 8.2. The time of weaning does not affect infantile amnesia. (A) Schematic diagram of behavioural paradigm for contextual fear conditioning in C57BL/6J infant mice. The black lightning symbol represents footshocks. (B, D, E) Freezing levels during recall test 1 (B), 8 (D) and 15 (E) days after training. (C) Freezing levels during context specificity test in context B. N=10. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; *P < 0.05.

8.2 The effect of maternal immune activation on memory retention in infant mice

The functional switch in GABA transmission seen early in development occurs within the infantile period (Valeeva et al., 2013). It is plausible that infantile amnesia may be a complication or aftermath of this change in inhibitory/excitatory neurotransmission. In some models of neurodevelopmental disorders, the timing of the functional switch in GABA from a depolarizing to a hyperpolarizing transmitter is delayed (Corradini, 2018, Fernandez et al., 2019). Autism spectrum disorder (ASD) is one neurodevelopmental disorder that has been widely associated with imbalances in GABA signalling (Gao, 2015). It has been shown that maternal immune activation (MIA) induced ASD can be modelled in rodents by the injection of viral mimics such as Poly (I: C) during critical time points of neurodevelopment (Choi et al., 2016, Lammert, 2019). To investigate whether

the GABA functional switch could impact infantile amnesia, memory retention was tested across the development of MIA offspring. A unique phenomenon was discovered in which male, but not female offspring from MIA models do not experience infantile amnesia.

8.2.1 Maternal Poly(I:C) or IL-17a offspring show ASD-like behaviour phenotypes

Offspring from MIA dams develop many of the defining features of ASD including repetitive behaviour and deficits in social behaviour (Choi et al., 2016, Kalish et al., 2021, Reed et al., 2020, Shin Yim et al., 2017). The viral mimic Poly(I:C) or the cytokine IL-17a were used to stimulate maternal immune activation in pregnant dams at embryonic day 12.5. To ensure the applied methods of MIA were functioning, MIA offspring were tested for social impairment in the three-chamber sociability task and repetitive behaviour using the marble burying task. Their behaviour was then compared to control offspring that were prenatally treated with PBS.

8.2.1.1 Sociability

The three-chamber sociability task asses general sociability (Kaidanovich-Beilin, 2011). Rodents generally prefer to spend more time with another rodent over an inanimate object. By taking advance of this, the three-chamber social interaction test allows for the identification of rodents with deficits in sociability. Testing occurred in a three-chambered box with openings allowing free movement between chambers (Fig. 8.3). After habituation to the empty chamber, the subject encounters a contained novel mouse in one chamber and a contained inanimate object in another chamber. The middle chamber remains empty, allowing for a neutral space. By analysing the time spent in each chamber allows for the quantification of deficits in social behaviour.



Figure 8.3. Representative diagram of 3-chamber apparatus. Schematic diagram of behavioural apparatus for the 3-chamber social test. Image created with Biorender.com.

Pregnant dams were injected with Poly(I:C), IL-17a or PBS Ctrl at E12.5. Both male and female adult offspring from all three conditions were tested for deficits in social interaction (Fig. 8.4). Any-maze allowed for the quantification of time spent in each chamber. Control male and female offspring spent equal amounts of time between both the novel mouse and object chamber (Fig. 8.4 C-F) . Although no difference was seen for Poly(I:C) and IL-17a female offspring (Fig. 8.4 I, J, M, N), male Poly(I:C) and IL-17a offspring spent significantly more time in the chamber containing the object compared to the chamber with the novel mouse (Fig. 8.4 G, K). This result was reflected in the heat maps generated of the mean time spent in each chamber (Fig. 8.4 H, L). These results are not due to differences in activity during the test as the mean total distance travelled did not vary between groups (Fig. 8.4 A, B). Here we see that male, but not female, offspring from MIA by Poly(I:C) or IL-17a show deficits in social behaviour.

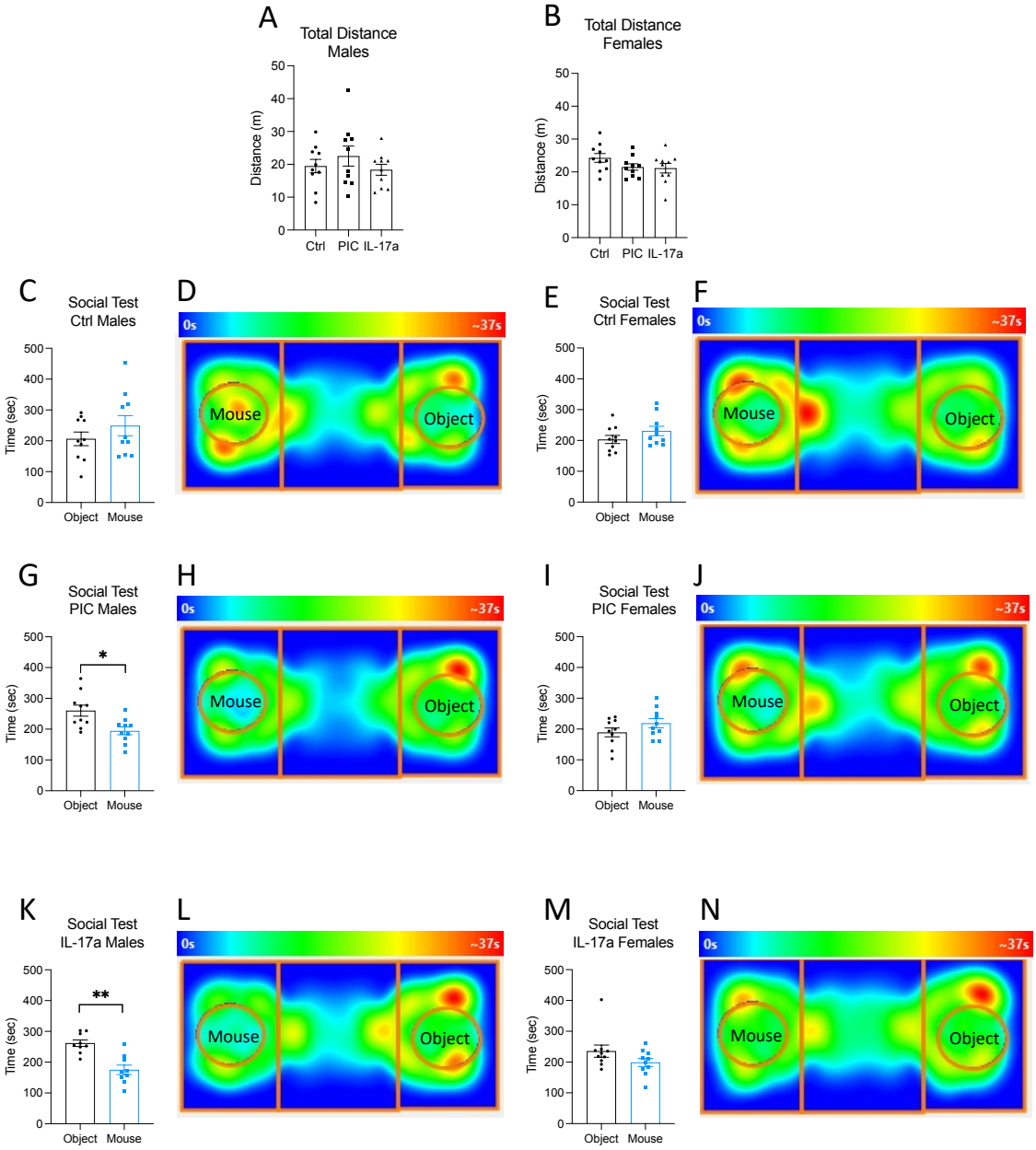


Figure 8.4. MIA male offspring show deficits in social behaviour. (A, B) Total distance travelled during the 3-chamber social test. (C, E, G, I, K, M) Total time spent in the chamber containing the mouse or object. (D, F, H, J, L, N) Heat map of mean total time spent in the 3-chamber arena. N=9-10. Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests; *P < 0.05, **P < 0.01.

8.2.1.2 Repetitive Behaviour

Another common phenotype of ASD is the repeated expression of meaningless behaviours such as repetitive tapping or rocking. One behavioural test that has been developed for evaluating these repetitive behaviours is marble burying (Angoa-Pérez, 2013). Increased digging behaviour is commonly witnessed when mice are placed in a novel cage. If an animal has OCD-like symptoms they tend to engage in a higher degree of repetitive behaviour, such as digging. Increased digging typically results in a higher percentage of marbles buried. This target behaviour occurs spontaneously in mice and is therefore widely used for assessing repetitive behaviours associated with ASD. In this test, mice are individually placed in a large novel cage with 20 glass marbles arranged on clean bedding for 30 min. The main data outcome is the number of marbles buried, where a greater number of marbles buried indicates repetitive behaviour (Fig. 8.5 A-C). No difference was seen in the number of marbles buried by female offspring from either three MIA conditions (Fig. 8.5 E), while male offspring prenatally treated with poly(I:C) or IL-17a showed enhanced marble burying (Fig. 8.5 D). As shown in previous studies, male offspring from pregnant dams injected with Poly(I:C) or IL-17a show ASD-like behaviour phenotypes such as repetitive behaviour and social deficits.

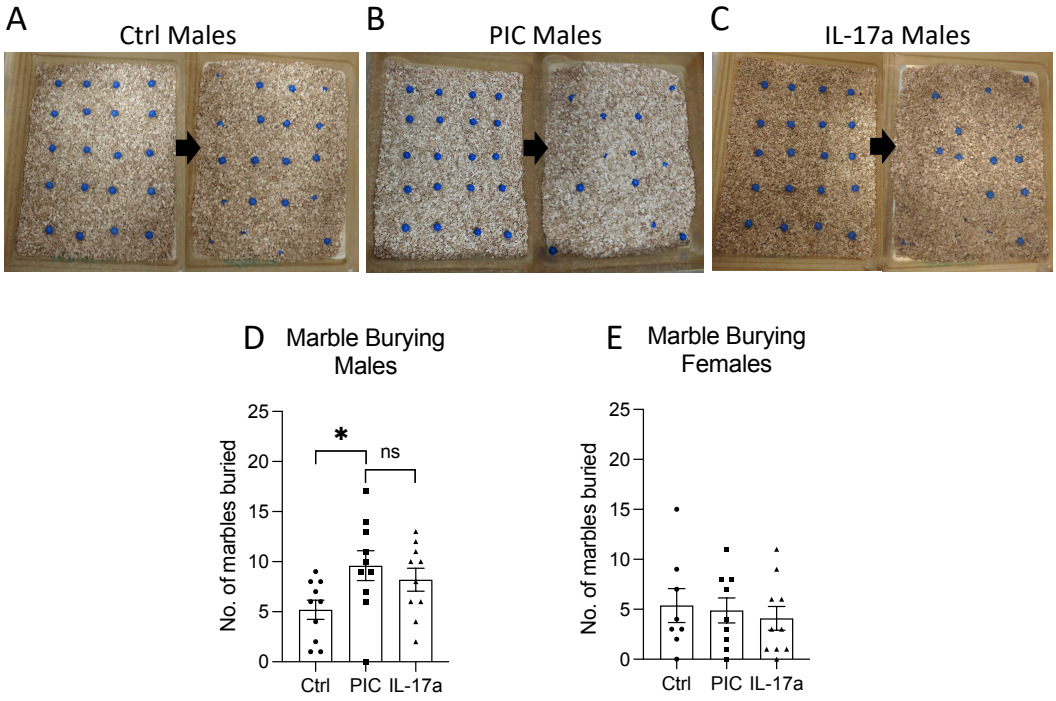


Figure 8.5. MIA male offspring show repetitive behaviour. (A-C) Representative images of marble-burying task for male offspring from pregnant dams injected with control PBS (A), Poly (I:C) (B) or IL-17a (C). (D) Number of marbles buried by male offspring. (E) Number of marbles buried by female offspring. N=10. Data presented as mean ± SEM. Statistical comparison was performed by using one-way ANOVA Bonferroni; *P < 0.05.

8.2.2 Infant male offspring from MIA models do not experience infantile amnesia for a contextual fear memory

To test if MIA causes any effect on memory retention, offspring from dams injected with Poly (I:C), IL-17a or PBS were trained using contextual fear condition at P17 (Fig. 8.6 A). When tested the following day infant offspring from all three MIA conditions show similar levels of freezing between groups, irrelevant of sex (Fig. 8.6 B, E). Interestingly, infant male offspring, but not females, from pregnant dams injected with a single dose of Poly(I:C) or IL-17a during embryonic development showed memory retention like that of adult mice when tested 8 days after training (Fig. 8.6 C, F). Control male cohorts that were not shocked, but were exposed to the context, at P17 did not show similar levels of freezing when tested 8 days after training, implying the level of freezing in the

experimental groups was not by chance (Fig. 8.6 D). Male, but not female, offspring from pregnant dams treated with Poly(I:C) or IL-17a do not experience infantile amnesia for a contextual fear memory.

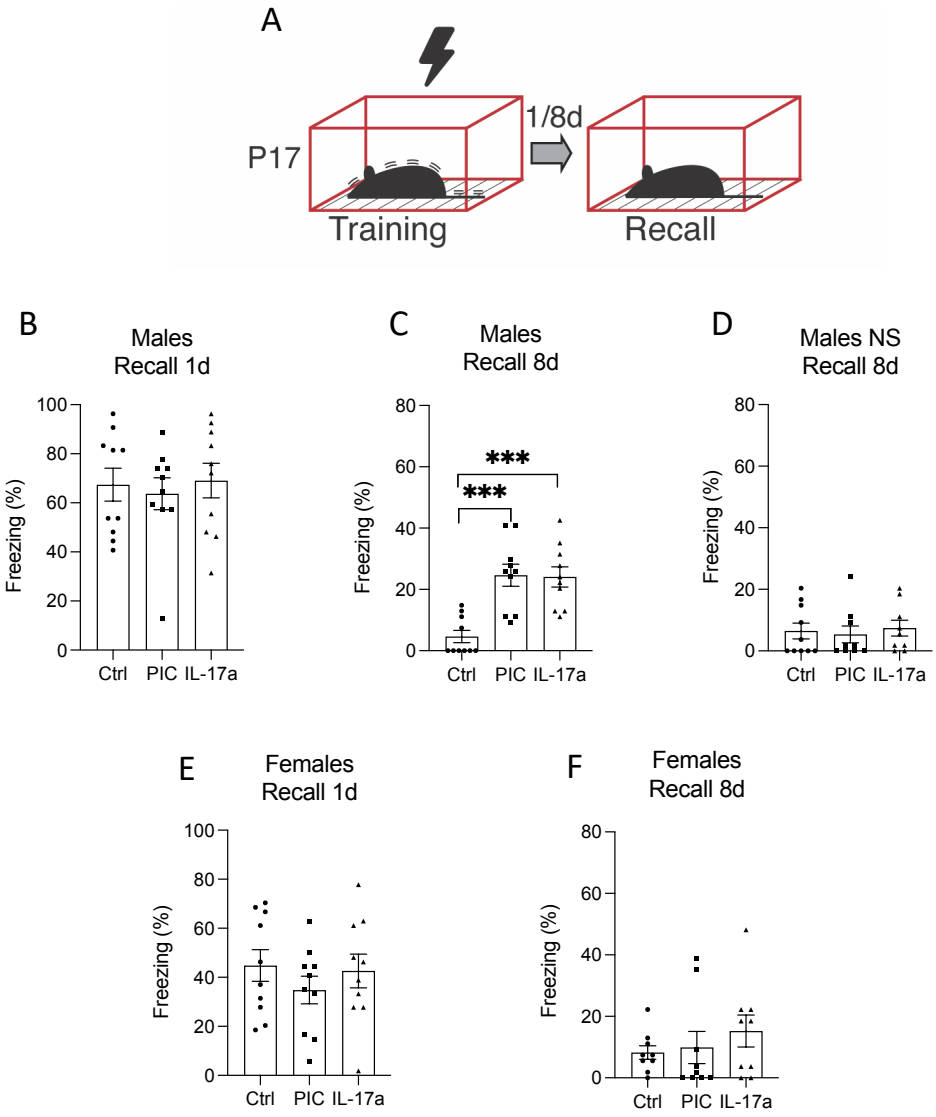


Figure 8.6. MIA male offspring show adult-like memory retention for a contextual fear memory. (A) Schematic diagram of behavioural paradigm for contextual fear conditioning in C57BL/6J infant (P17) MIA offspring. (B, C) Freezing levels of infant male offspring during a recall test 1 day (B) or 8 days (C) after training. (D) Freezing levels of infant male offspring for a no shock control group. (E, F) Freezing levels of infant female offspring during a recall test 1 day (E) or 8 days (F) after training. N=9-10. Data presented as mean ± SEM. Statistical comparison was performed by using one-way ANOVA Bonferroni; **P < 0.01, ***P < 0.001.

8.2.3 Male offspring from MIA models maintain the ability to retrieve an object memory

To investigate whether this phenomenon also held for other forms of learning, MIA offspring were tested for object memory retention using a novel object recognition paradigm (Fig. 8.7 A). MIA offspring from Poly (I: C), IL-17a or PBS treated dams were first habituated to the testing arena at P16. The following day they were exposed to two identical objects during an acquisition session at P17. MIA offspring were then tested for novel object recognition when exposed to a familiar and novel object 8 days later. During acquisition, all cohorts spent an equal amount of time exploring both identical objects (Fig. 8.7 B, C, F, G, J, K). When tested for object recognition, female offspring from either MIA by Poly(I:C), IL-17a or the control PBS group did not show object memory retention for the familiar object (Fig. 8.7 E, I, M). Male offspring from MIA by Poly(I:C) or IL-17a, but not PBS, spend significantly more time with a novel object when tested eight days after training (Fig. 8.7 D, H, L). Like contextual fear memory, male offspring from Poly(I:C) or IL-17a injected dams do not show infantile amnesia for an object memory.

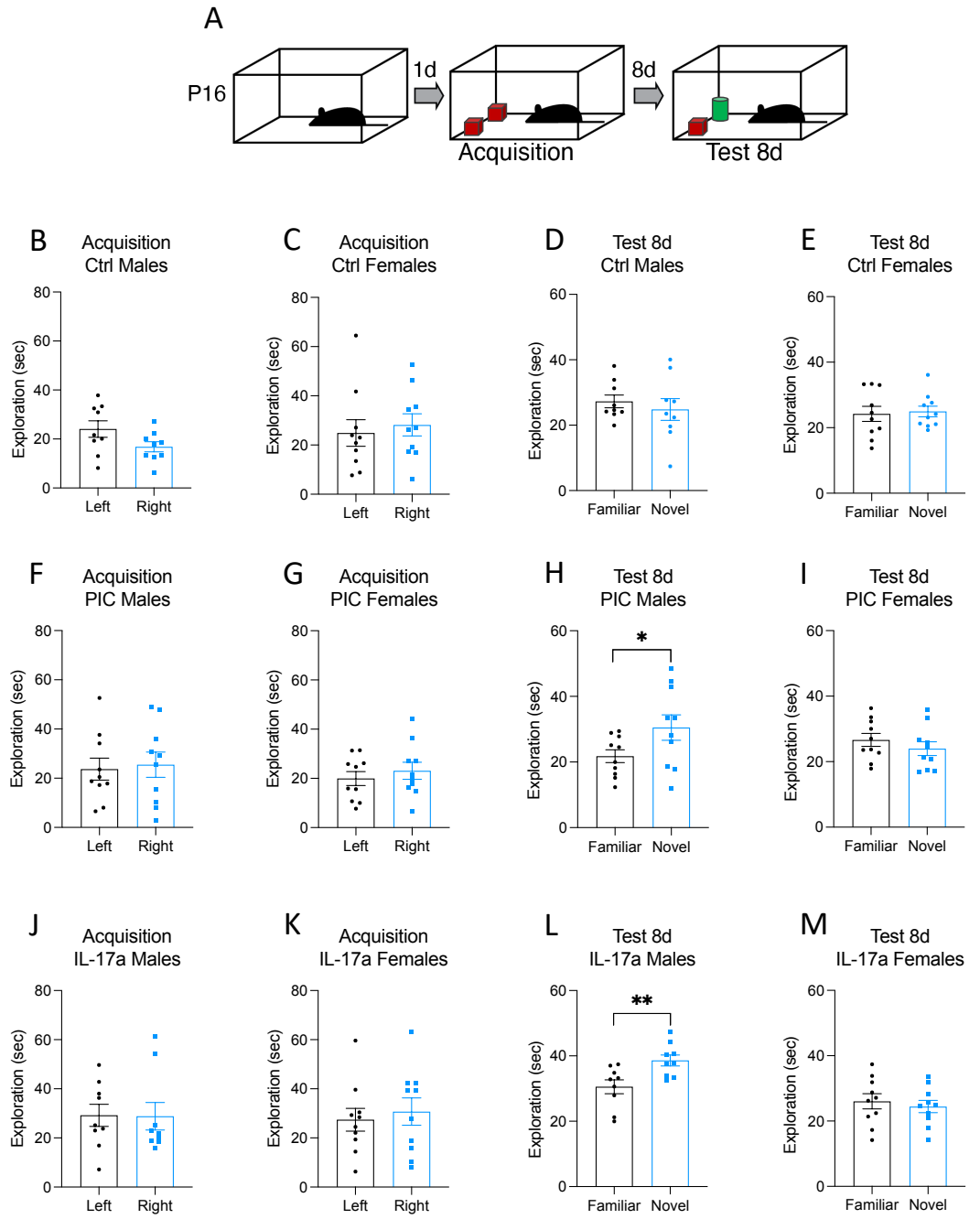


Figure 8.7. MIA male offspring show adult-like object memory. (A) Representative image of novel object recognition task for C57BL/6J offspring from pregnant dams injected with control PBS, Poly (I: C) or IL-17a. (B, C, F, G, J, K) Exploration during acquisition session at P17. (D, E, H, I, L, M) Exploration during test 8 days after acquisition. N=9-10. Data presented as mean ± SEM. Statistical comparison was performed by using a paired t-test; *P < 0.05, **P < 0.01.

8.2.4 Male offspring from MIA models and all females preserve a spatial memory for a Barnes maze task

Although MIA male offspring demonstrate memory retention for a fear and object memory, it remained to be eluded whether memory retention was also seen for a spatial memory. MIA offspring from pregnant dams injected with Poly(I:C), IL-17a or PBS were trained on the Barnes maze at P17. Animals were tested for memory recall of the location of the escape hole 8 days after training. Redolent with other behavioural paradigms in this project, male offspring from MIA models do not show infantile amnesia for a spatial navigation task (Fig. 8.8-8.10). Surprisingly, females from every MIA by Poly(I:C), IL-17a and PBS condition showed memory retention for the location of the escape hole when tested 8 days after training (Fig. 8.8-8.10). Since this result was also witnessed in the control group it indicates that the effect in females is not being caused by MIA but rather is sex-specific.

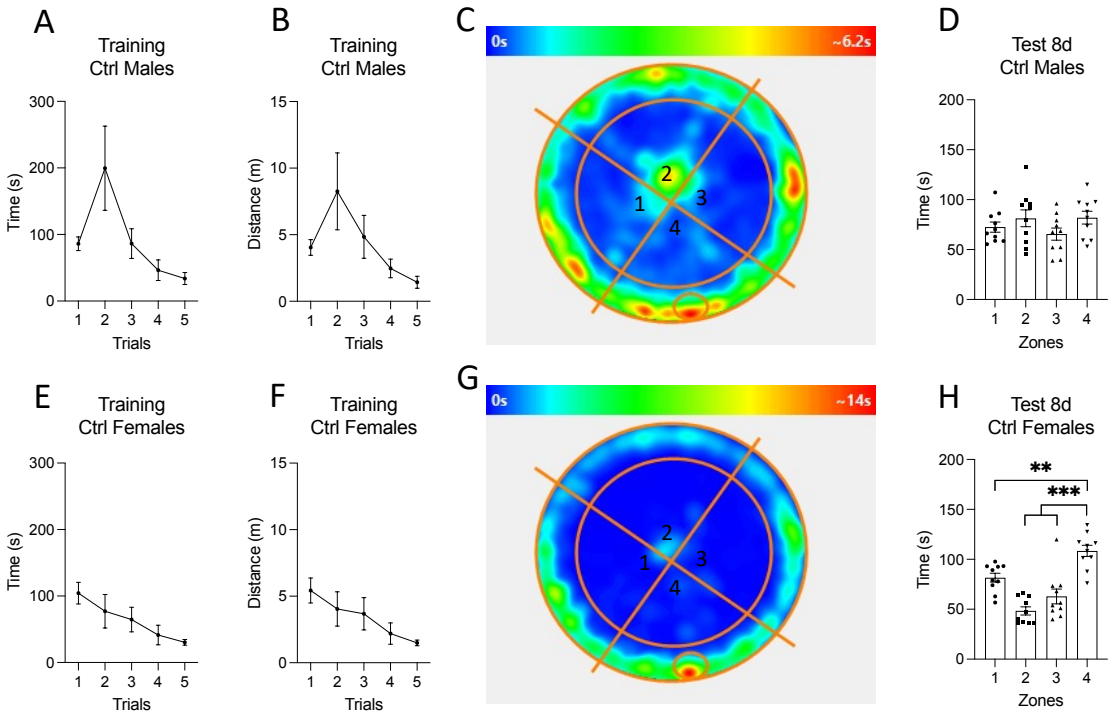


Figure 8.8. Female, but not male, infant mice show memory retention for the location of the escape hole on a Barnes maze 8 days after training. (A, E) Latency before entering the escape hole during each trial. (B, F) Distance travelled before entering the escape hole for each trial. (C, G) Heat map of the mean time spent in each area of the Barnes maze during the probe trial. (D, H) Mean time spent in each zone of the Barnes maze during the probe trial. N=9 Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests or one-way ANOVA Bonferroni; *P < 0.05, **P < 0.01, ***P < 0.001.

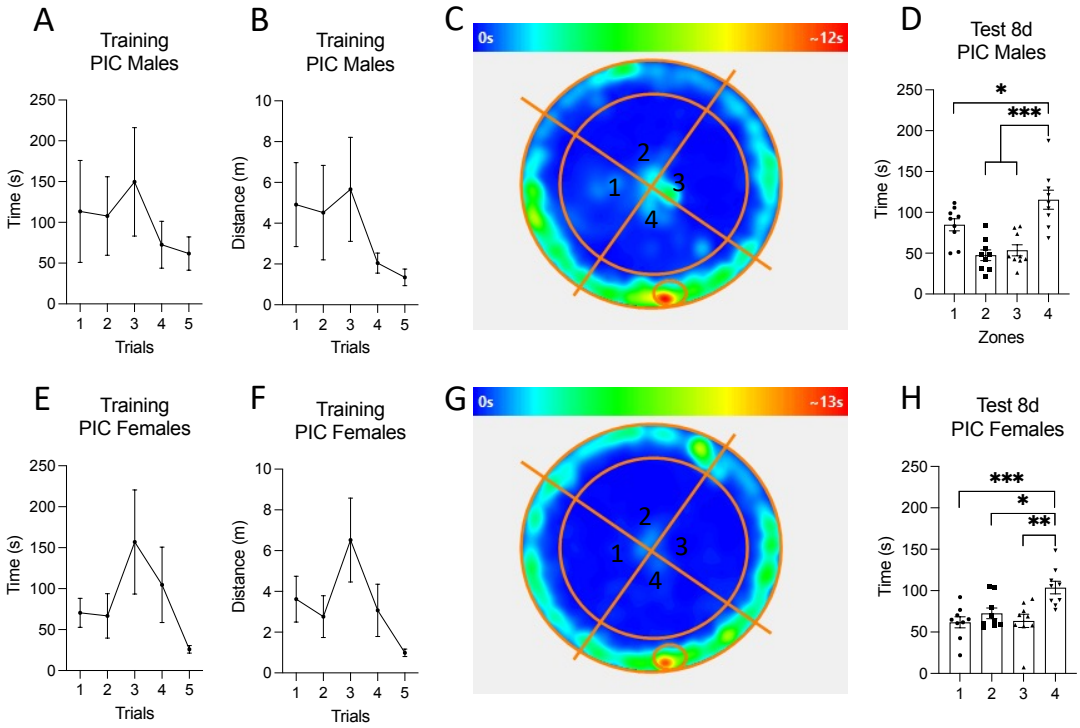


Figure 8.9. Poly (I:C) MIA male and female offspring show memory retention for the location of the escape hole on a Barnes maze 8 days after training. (A, E) Latency before entering the escape hole during each trial. (B, F) Distance travelled before entering the escape hole for each trial. (C, G) Heat map of the mean time spent in each area of the Barnes maze during the probe trial. (D, H) Mean time spent in each zone of the Barnes maze during the probe trial. N=9 Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests or one-way ANOVA Bonferroni; *P < 0.05, **P < 0.01, ***P < 0.001.

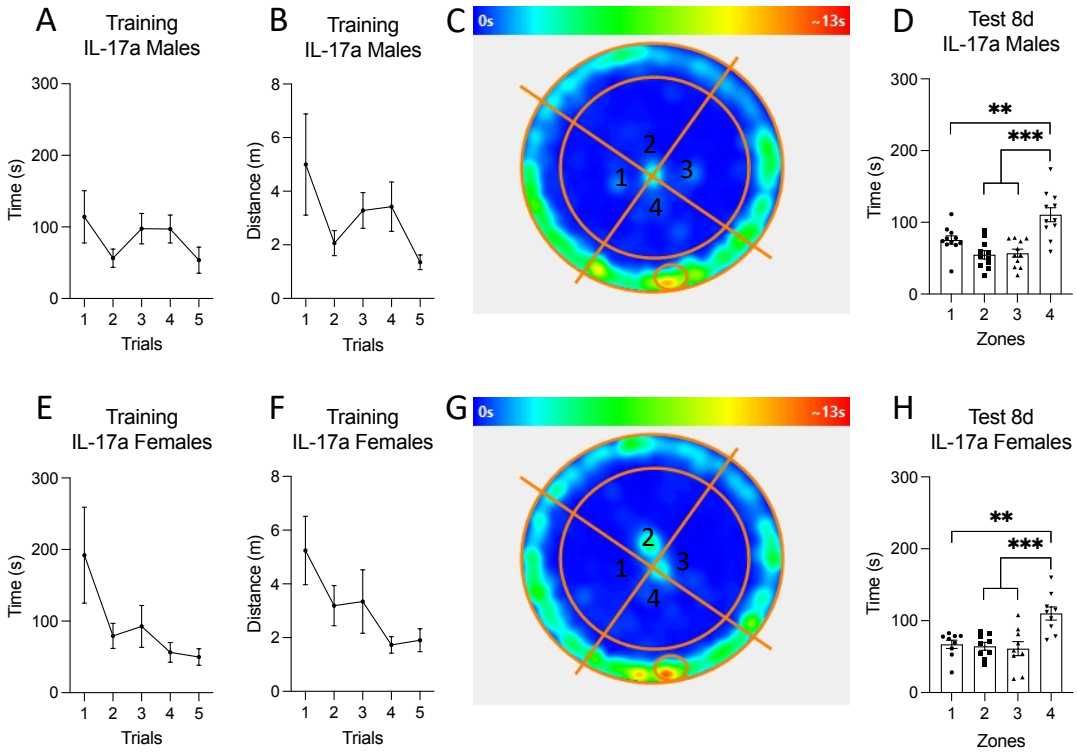


Figure 8.10. IL-17a MIA male and female offspring remember the location of the escape hole on a Barnes maze 8 days after training. (A, E) Latency before entering the escape hole during each trial. (B, F) Distance travelled before entering the escape hole for each trial. (C, G) Heat map of the mean time spent in each area of the Barnes maze during the probe trial. (D, H) Mean time spent in each zone of the Barnes maze during the probe trial for male (D) and female (H) infant offspring. N=9-11 Data presented as mean ± SEM. Statistical comparison was performed by using unpaired t-tests or one-way ANOVA Bonferroni; *P < 0.05, **P < 0.01, ***P < 0.001.

8.3 Summary

This chapter indicates that infantile amnesia is most plausibly predisposed, independent of environmental effects such as environmental enrichment and weaning. Whatever this predisposed effect is, it appears to be absent in the case of male offspring from MIA models. MIA by Poly(I: C) or IL-17a is causing an effect in male offspring that prevents infantile amnesia for a fear, object, and spatial memory. Unexpectedly, infantile amnesia does not affect female infants for a spatial navigation task. In the infantile amnesia literature, studies are more frequently carried out in male rodents, with very

few, if any in female rodents. This project is the first to knowledge that has investigated sex differences in infantile amnesia for a spatial task.

Chapter 9: General Discussion

Memory is one of the most important and widely studied facets of cognition, yet little is known about how memory develops across a lifespan. Despite childhood having such a profound effect on the rest of our lives, many of us do not give a second thought as to why we rapidly forget events that occur early in development. Despite its near impact at foundational stages of mammalian development, the phenomenon of infantile amnesia has received scant attention from the neuroscience community. To help advance our understanding, this thesis aimed to probe the questions of 'what' (nature), 'how' (mechanism) and 'why' (adaptive function) (Krakauer et al., 2017). Specifically, the goal here was to ask, what is causing infantile amnesia? That is, is it due to a storage or retrieval deficit. How is it rendering these memories inaccessible, what are the mechanisms causing infantile amnesia? Why is infantile amnesia occurring, what is the fundamental value of this form of forgetting? Infanthood is one of the most crucial periods of learning, and by understanding infantile amnesia we can potentially design improved educational strategies to enhance informational content and maximize the retention of fundamental knowledge during the earliest years of life.

Over the past 40 years, the mouse has become one of the most widely used animal models in scientific research (Hickman, 2017). Infantile amnesia, although a robust phenomenon, has mainly been studied in rats (Madsen and Kim, 2016). This project is among a handful of studies that have investigated the realms of infantile amnesia in mice. Regardless of the experimental model, the conclusion is unanimous, infant rodents rapidly forget memories formed in early development (Campbell and Spear, 1972). This was demonstrated in chapter 3 where infant mice exhibit forgetting of a fear, object, and spatial memory as soon as a week after training.

Although mouse models are an essential tool for studying behaviour, they are not necessarily the most accessible model organism in the case of studying infantile amnesia the time window in which infancy occurs can be a limiting factor for experimental tasks. Such short periods prevent the application of tasks such as those based on operant

conditioning, which requires multiple training days spread across weeks. Moreover, due to the natural anxious state of the infant mouse, their baseline levels of freezing are relatively high compared to adults. Therefore, it is of importance to expand behaviour beyond contextual fear conditioning tasks. During tasks like the Barnes maze, this natural anxious state acts as a motivational reinforcement to complete the task (Pitts, 2018).

9.1 Bringing the memory back

Until recently an outstanding question remained regarding whether infantile amnesia is caused by a memory storage or retrieval deficit. As previously stated, if the memory engram survives a case of amnesia, then we can rule out a problem in memory storage. One way of investigating if the engram is still present and/or functional in the brain is by combining methods of engram biology with optogenetics (Liu et al., 2012). Before the introduction of engram biology and optogenetics, it was commonly assumed that in cases of amnesia, such as Alzheimer's disease, those memories were permanently lost from the brain. We now know thanks to informative studies that these memories are not only still present in the brain but are recoverable (Ryan, 2015, Roy et al., 2016, Perusini et al., 2017, Ortega-De San Luis, 2018). Moreover, whatever is causing these forms of amnesia is not destroying the engram cells. This paved an important path in the literature for what was happening at a fundamental level to the engram in cases of amnesia.

If this is the case with other forms of amnesia, then it is plausible that infantile amnesia is also due to a retrieval deficit. The employed optogenetic strategy allowed us to probe the question of whether it is possible to rescue lost infantile memories in adulthood. Direct optical activation of hippocampal cells that were active during CFC during the infantile period elicits freezing behaviour, an observation that was not seen during the light OFF epochs. This suggests that direct activation of a subset of cells involved in the formation of a forgotten infantile memory is sufficient to induce the behavioural expression of that memory. Thus, memories formed early in development survive infantile amnesia. These memories are not only still present in the brain in adulthood,

but they can be artificially reactivated to acutely rescue the behaviour. Demonstrating that our earliest childhood memories have not been erased, we are just incapable of accessing them when adults. These results are consistent with a recent study that demonstrated the optogenetic stimulation of tagged DG neurons recovered lost infant memories up to 3 months following training (Guskjolen et al., 2018). As the engram survives this form of amnesia, both studies support the hypothesis that infantile amnesia is due to a retrieval deficit as opposed to a deficit in memory encoding or storage.

Artificial activation of memory engrams in a range of different brain regions has advanced our knowledge of how memory is stored in the brain (Tonegawa et al., 2015a). Currently, the acute optogenetic rescue of an infantile memory has only been demonstrated twice, first by Guskjolen and colleagues and now in this project (Guskjolen et al., 2018). Both paradigms focus on optogenetic stimulation of engram cells in the DG for a contextual fear memory. However, Guskjolen and colleagues employed the engram labelling strategy Arc-TRAP as opposed to the Ai32-FosTRAP strategy (characterised in chapter 4). To further divulge into the mechanics behind infantile amnesia it is necessary to expand the behaviour by developing new assays other than contextual fear recognition and focusing on other brain regions other than the DG.

The functional engram survives infantile amnesia demonstrated by the survival of the connectivity pattern. Although optogenetic stimulation is targeting the DG, engram reactivation is seen beyond the DG in other brain regions including the AMG, RSC and the PAG. This is consistent with the study carried out by Guskjolen and colleagues who characterised engram reactivation in hippocampal and cortical brain regions following acute memory recovery (Guskjolen et al., 2018).

The engram labelling strategy developed in chapter 2 allows for permanent whole-brain labelling. Thus, it is possible to look at the whole brain connectome and identify other brain regions of relevance. The CA1 region is part of the homogenous network of the hippocampus involved in episodic memory (Bartsch, 2011). In chapter 5, optogenetic

stimulation in the CA1 of engram ensembles labelled for a contextual fear memory in infancy, induced freezing in adult mice. This effect was only witnessed when optogenetic stimulation was carried out at 4 Hz as opposed to the conventional 20 Hz. This is in line with a previous study that optogenetic manipulation of engram cells in the CA1 at the same parameters as the DG (20 Hz) was not sufficient to create a false memory (Ramirez et al., 2013). Following this study, it has been shown that engram cells in the CA1 require optogenetic manipulation at 4 Hz (Ryan, 2015, Tanaka et al., 2014). This thesis demonstrated for the first time that optogenetic stimulation of engram cells in a brain region other than the DG was sufficient to acutely rescue the infant memory.

Spatial and object tasks in rodents are extremely beneficial for the development of parallel object/context and maze learning assays in human behaviour experiments (Vorhees, 2014). These forms of behavioural tasks are not dependent on fear alone, they involve the retainment of more complex content such as the shape or place of an object or spatial/navigational cues that allow them to solve a maze. From this project, we see that infant mice can perform and acutely retain an object, object-context, and spatial navigation tasks. However, these tasks are forgotten as rapidly as contextual memories. In chapter 5, acute optogenetic rescue for a 'lost' infant memory was expanded further than contextual fear conditioning by looking at other types of memories such as spatial memory in the Barnes maze and object memory in a novel object reconditioning task. Optogenetic stimulation in the DG for a memory engram for the Barnes maze (labelled in infancy) was sufficient to acutely rescue the ability to perform the task in adulthood. By activating the engram labelled for the training of the task, adult mice successfully showed memory for the location of the escape hole. This project is the first to demonstrate acute rescue of a 'lost' infantile memory for behaviour other than contextual fear conditioning.

The entorhinal cortex, also involved in learning and memory, forms synapses with the DG and is particularly involved in object memory (Hainmueller, 2020). Nothing is currently known about infantile engram reactivation in object memory making it a prime candidate for future work. Crucially, it can be tested whether optogenetic stimulation of engram cells in the entorhinal cortex, labelled during object acquisition in infancy, will

be sufficient to rescue their preference for a novel object memory when tested a week later. The application of engram technology to paradigms of object learning will allow for the investigation of what is happening in other regions of the brain, as well as the hippocampus, during infantile amnesia.

9.2 Neurobiological causes of infantile amnesia

What can infantile amnesia tell us about the engram? Firstly, in chapter 4 histological analysis of engram reactivation during natural recall informs us that during memory recall, a subset of the engram ensemble is reactivated across multiple brain regions. However, this is only the case if the recall event occurs before the onset of infantile amnesia. This supports behavioural analysis, which demonstrates that infant mice show memory retention before, but not after, infantile amnesia.

Chapter 4 used histological analysis to measure the functional connectivity between engram cells following optogenetic reactivation of upstream engram cells. Optogenetic stimulation of DG engram cells preferentially activated the AMG, RSC and PAG engram cells that were originally labelled in the same experience. Thus, the persistent connectivity pattern of engram cells survives infantile amnesia (Guskjolen et al., 2018). This suggests that in the case of infantile amnesia, the information may be stored in the connectivity between engram cells and is mediated by synaptic wiring.

If the connectivity pattern survives this form of amnesia, then what is driving the inaccessibility? It has been suggested that accessibility to the engram can be correlated with synaptic weight changes (Tonegawa et al., 2015b, Ryan, 2021). Following learning, engram cells show engram-specific changes in the potentiated synaptic input (Ryan, 2015). These learning-induced changes in synaptic strength of the engram are reversed in cases of amnesia induced by protein synthesis inhibition (Ryan, 2015, Ortega-De San Luis, 2018). Consistent with this, this thesis demonstrated synaptic changes in the frequency ratio of sEPSCs and sIPSCs between adult encoded engram and non-engram cells. Engram and non-engram cells, encoded during infancy, showed a similar frequency ratio of sEPSCs and sIPSCs in adulthood. Moreover, the frequency of spontaneous

synaptic input onto infant engram cells (post infantile amnesia) reflected that of adult non-engram cells. Interestingly, adult encoded engrams showed an increased frequency ratio of sEPSCs and sIPSCs to engram cells encoded in infancy. In another study using a conditioned suppression behavioural paradigm, researchers demonstrated that they could reversibly shift the accessibility and inaccessibility state of the engram by strengthening or weakening the synaptic weights (Nabavi, 2014). In chapter 5, this thesis demonstrated the reinstatement of a 'lost' infantile contextual engram by pairing optogenetic reactivation of the engram with shock. This crucial experiment restored accessibility to the engram. It is possible that in this case, optogenetic stimulation is strengthening synaptic inputs onto the engram reversing infantile amnesia and permanently reinstating the memory. If changes in the strength of synaptic weight are modulating accessibility to the engram, then the question arises how is the mnemonic information retained in infantile amnesia?

The engram labelling strategy developed and characterised in chapter 4 has proven a useful tool for investigating infantile amnesia at an engram level. However, one limitation of the Ai32-FosTRAP mouse line is that it is effectively a knock in/knock mouse line (Guenthner et al., 2013). In this model, the natural *c-fos* promoter has been knocked out, and an artificial version of the *c-fos* promoter is knocked in. This alone can affect the level and quality of c-Fos expression across multiple brain regions. Recently a new Cre recombinase mouse line has been developed (Allen, 2017). Unlike the FosTRAP1 line, FosTRAP2 takes advantage of the naturally occurring *c-fos* promoter, meaning it is not a knock in/knock out mouse model.

The combination of engram labelling and optogenetics has proved a great tool for investigating the neural networks in which memory is stored in the brain (Tonegawa et al., 2015a). However, one limitation of these techniques alone is that they fail to provide insight into what is happening to the engram at a molecular level. This thesis characterised an engram labelling system that allows for permanent labelling throughout the brain. By employing this engram labelling strategy with methods of flow cytometry it may be possible to separate engram and non-engram cells labelled before and after infantile amnesia. The segregation of engram and non-engram cells could lead

to further analysis at a transcriptional or epigenetic level through RNA or ATAC sequencing.

9.3 Reversing infantile amnesia

We now know that infantile amnesia is due, in part, to a retrieval deficit. The main issue that still needed to be addressed was that of retrievability, that is, how to bring the memory permanently back. While optogenetic engram stimulation in adult mice successfully retrieves the tagged infant memory engrams, the effect is not permanent. Infant mice rapidly forget contextual memories. Another way of looking at memory retrieval is by behaviourally trying to update the engram in a targeted way using reinstatement paradigms (Ramirez et al., 2013). To permanently reinstate an infant 'lost' engram plasticity must be induced to render the engram ensemble assessable to natural perceptual stimuli associated with the learning episode. By pairing optogenetic stimulation of contextual engram cells with natural updating of the target engram cells with new information (shock) it was possible to permanently reinstate infant engrams in adult mice. Artificial updating of the memory engram restored natural access to the target engram, reversing infantile amnesia. Further, reinstatement returned accessibility to the engram which was demonstrated by natural cell counting. This project extends previous findings and is the first to knowledge that demonstrates permanent reinstatement of a contextual infantile memory. Behavioural updating paradigms can now be applied to other behavioural tasks such as novel object recognition and the Barnes maze to see if it is possible to permanently reinstate other forms of infant memory. Moreover, it may be possible to permanently reinstate a 'lost' contextual memory by optogenetically stimulating other brain regions such as the CA1 or the AMG. Understanding what happens to early life memories will provide central insights into brain development and educational psychology. Moreover, the ability to potentially retrieve seemingly lost memories from early childhood would have profound implications for society.

This project has identified a model in which infantile amnesia has been reversed and natural access has been restored to the memory engram. One limitation of this

paradigm is that it still employs artificial stimulation of the engram. However, this paradigm creates a huge opportunity for the identification of proximal mechanisms for memory access on engram cells. Comparing a reinstated and non-reinstated memory at the engram level and identifying neurobiological correlates (such as those described in chapter 7) may give greater insight into what mechanisms are driving infantile amnesia and how they are reversed. It may be possible to identify changes in synaptic input that are specific to reinstatement and accessibility of the engram (Ryan, 2015). As discussed in the introduction, if inaccessibility to the engram is being driven by silent synapses, then changes may be detected at the level of the AMPA/NMDA ratio. Silent synapses can be converted into active ones by activity-dependent processes. Moreover, changes in excitatory postsynaptic current (EPSC) or inhibitory postsynaptic current (IPSC) balance may suggest accessibility to the engram might be related to the functional shift in GABA transmission early in development. Other physiological correlates such as dendritic spine density could be investigated. The identification of correlates, such as those suggested, involved in rescuing, or promoting retrieval of the engram may be used for the development of non-invasive adult interventions.

9.4 Preventing infantile amnesia

Several hypotheses have been put forward to explain the paradox of infantile amnesia, but few studies have successfully prevented this form of forgetting. Early-life exposure to a stressor such as maternal separation, prevents infant forgetting up to 30 days after training (Callaghan and Richardson, 2012). Manipulations activating brain-derived neurotrophic factor (BDNF) or metabotropic glutamate receptor 5 (mGluR5) induce early expression of the GluN2A/Glun2B switch leading to earlier onset of infantile amnesia (Travaglia et al., 2016a). While enhancing neurogenesis in precocial mammals such as guinea pigs or dengue causes an altricial-like phenotype of forgetting (Akers, 2014). To prevent this form of forgetting we must first hypothesise what is causing this form of forgetting. We must then use this information to predict and develop models or interventions.

During postnatal development, GABA, which acts as an inhibitory neurotransmitter in the mature brain, causes synaptic excitation (Peerboom, 2021). During the second

postnatal week, the function of GABA switches from that of excitatory to inhibitory (Valeeva et al., 2013). This thesis hypothesised that this switch in GABAergic transmission may underlie or contribute to the rapid forgetting caused by infantile amnesia. As demonstrated in chapter 3 the engram is accessible for at least 3 days after learning at P17. Thus, it is possible to reactivate the engram by natural cues. At this point, the connectivity pattern has formed between engram cells. If the GABAergic switch causes a change in the ratio of inhibitory and excitatory synaptic input, it may render the engram inaccessible. To investigate the GABAergic switch hypothesis, this project searched for mouse models in which the GABAergic signalling switch is delayed. It is widely suggested that impairments in GABAergic transmission are a key contributor to the development of some forms of ASD (Pizzarelli and Cherubini, 2011). These studies point to increased synaptic excitability driven by a reduction in GABAergic signalling in cases of ASD. Alterations in the developmental switch in GABA polarity have been previously reported in mouse models of autism and Fragile X (Tyzio, 2014, He et al., 2014). Interestingly, the GABA switch is delayed in MIA offspring, where GABA persists in being excitatory during a developmental period when it is classically inhibitory (Corradini, 2018). Moreover, it has been shown that at P14-P15 GABA remains depolarizing, producing excitatory action, in the hippocampus of offspring from MIA models of autism (Fernandez et al., 2019).

Recent research has demonstrated that MIA in pregnant dams produces offspring that demonstrate autistic phenotypic behaviours (Choi et al., 2016, Ashwood et al., 2006, Patterson, 2009, Atladottir et al., 2010). Recently it has been shown that this behaviour is commonly elusively seen in the male offspring (Haida et al., 2019, Xuan, 2014, Kalish et al., 2021). Although the root cause for sex differences seen in ASD prevalence is unknown, the prevalence ratio for ASD is usually cited as for every 4 males 1 female is diagnosed (Loomes, 2017). This project produced the novel finding that male, but not female, offspring from MIA in pregnant dams do not experience infantile amnesia. We now know that the mechanism of infantile amnesia is a reversible process that is modulated in MIA models of autism. This novel finding is useful because it allows us to try and decipher both the mechanisms underlying and the functions of infantile amnesia. Like precocial animals, is the MIA male brain structured to not have infantile

amnesia or is just preventing infantile amnesia from happening? These questions can now be answered by looking at different correlates that are allowing access to the memory.

Studies attempting to unravel the mechanisms induced by MIA have identified pathophysiological mechanisms in various brain regions including the hippocampus (Wolff and Bilkey, 2015, Giovanoli et al., 2016) and prefrontal cortex (Richetto et al., 2014, Paylor et al., 2016). In the DG of the hippocampus, adult female MIA offspring show specific changes in excitatory synaptic transmission in granule cells (Hui, 2020). Female offspring show an increased amplitude of mEPSC, while the frequency was decreased. These results could suggest a loss of excitatory synapses resulting in an increased conductance at surviving contacts. A change in the inhibitory and excitatory balance may explain why memories formed early in development are rapidly forgotten. Further, it may explain why male, but not female, MIA offspring, do not experience infantile amnesia. This thesis provides models that can now be applied to answer such hypotheses.

Due to the prevailing evidence of dysfunction in GABA signalling in models of autism, an interesting avenue for future work would be to look at the inhibitory drive on engram cells in male MIA offspring. This could be carried out by looking at electrophysiological correlates, such as excitatory/inhibitory EPSCs in engram cells. Histological analysis of GABAergic interneurons in the DG such as parvalbumin (PV) or somatostatin (SST) could allow for the quantification of inhibitory synaptic contacts on engram cells (Stefanelli et al., 2016, Guo, 2018). These forms of analysis would allow for the investigation of whether the GABA switch plays a pivotal role in engram accessibility in the case of MIA male offspring. If the GABA switch is delayed in male MIA offspring, one would expect to see a decrease in inhibitory drive on engram cells. Comparing engram cells in MIA male offspring, with engram cells in non-MIA offspring post infantile amnesia may give greater insight into the mechanisms preventing infantile amnesia.

Bumetanide is an FDA-approved agent that restores the inhibitory action of GABA (Ben-Ari and Cherubini, 2022). Oral administration of this NKCC1 chloride importer antagonist has been shown to attenuate the severity of ASD symptoms in children (Lemonnier et al., 2012, Dai et al., 2021, Zhang et al., 2020). Future experiments combining bumetanide with MIA models may allow for the reversal of this newly found phenomenon. Further, using bumetanide to interfere with depolarizing GABA early in development has been shown to prolong the critical period of plasticity in the visual cortical circuit (Deidda et al., 2015). It is plausible that administration of bumetanide early in development may early expression of the GABA switch and in turn an earlier occurrence of infantile amnesia. Thus, bumetanide is a prime candidate for investigating the role of the GABA switch in this form of forgetting.

Microglia play an important role in shaping brain circuits in the developing brain by eliminating weaker synapses (Miyamoto, 2013). Another interesting route for future work could be to look at the effect microglia is playing on the synapses between engram cells. Inhibition of microglial phagocytosis in the adult hippocampus has been shown to prevent forgetting (Wang, 2020). Interestingly, in cases of MIA, female offspring show increased synaptic contact and increased phagosomal activity in the DG compared to male offspring (Hui, 2020, Hui, 2018). Further, this study suggests that male offspring may recruit different microglia subsets. Thus, is it possible that reduced phagocytosis in the hippocampus in MIA male offspring is preventing this rapid forgetting seen during development? It is unlikely that microglia-mediated elimination of engram synapses is causing the inaccessibility in the case of infantile amnesia since we know the engram connectivity pattern survives. However, microglia may affect the stability of synaptic connections between engram cells. One caveat of this line of research is that microglia have multiple functions during development. Thus, we need to separate what changes are related to engram accessibility and what is simply an output of development.

Another potential key player preventing forgetting in the case of infantile amnesia is the Rho GTPase small G protein Rac1. Rac1 has been demonstrated to regulate various forms of forgetting in both mice and *Drosophila* (Berry and Davis, 2014, Davis and Zhong, 2017). Rac1 inhibition in the hippocampus has been shown to inhibit forgetting while

Rac1 activation accelerated forgetting (Liu et al., 2016, Jiang et al., 2016). Further, it has been shown that inhibition of Rac1-dependent forgetting alleviates amnesia in mouse models of Alzheimer's disease (Wu et al., 2019). In *Drosophila*, forgetting-dependent behavioural inflexibility is a common phenotype for autism-risk genes. One study investigated whether Rac1 activation is the convergent molecular target for these autism-risk genes (Dong et al., 2016). Dong and colleagues used aversive conditioning and its associated reversal-learning task to investigate the functions of five autism-susceptibility genes. All the autism-risk gene mutants studied failed to trigger the behaviour-dependent activation of Rac1. Acute down-regulation of these five autism-risk genes at the adult stage led to impaired behavioural flexibility with reduced reversal learning and resistant old memory. This study suggests that an inability to activate Rac1-dependent forgetting is a common mechanism for multiple autism-risk genes. This inability to activate Rac1-dependent forgetting may be preventing infantile amnesia from occurring in the case of MIA male offspring. Future studies combining Rac1 activators with MIA models may allow for further investigation of the role of Rac1 plays in infantile amnesia.

One caveat of these results is that it cannot be concluded whether the effect of MIA on infantile amnesia is strictly related to the autistic phenotype. It is unknown whether this effect is replicated in genetic autistic models or if it is caused by the immunological cascade during MIA at this stage in embryonic development. The prenatal period is a critical development window. Deciphering how changes due to MIA at this time affects structure, function and behaviour output is important. It is well established that Poly (I: C) causes an immune response resulting in IL-17a signalling but whether IL-17a signalling is the root cause of the effects witnessed on infantile amnesia remains to be eluded. The application of transgenic models such as IL-17a KO or IL-17R KO lines paired with MIA will allow for the investigation of whether the effect of Poly (I: C) and IL-17a on infantile amnesia are driven by IL-17a or whether it is an off-target effect.

9.5 Sex differences in infantile amnesia

Studying sex differences in learning and memory has become increasingly recognized as essential to understanding the brain. Due to the prevalence of autism in males, it was crucial to compare the behaviour of both males and females for MIA models. Unexpectedly, all female mice did not show infantile amnesia exclusively for a spatial navigation task. Females showed memory retention for the location of the escape hole when tested 8 days after training on the Barnes maze task. This was not exclusive to females from pregnant dams injected with Poly(I: C) or IL-17a as female offspring from the control group also retained a memory for the Barnes maze. This sex-specific effect on memory retention was specific to the Barnes maze as infant female mice demonstrate forgetting for both a contextual fear and object memory. It appears that there is something different driving mechanistically in the male and female brain that is preventing forgetting in females for the spatial Barnes maze task.

Interestingly, studies in both rodents and humans have shown that during spatial navigation tasks, females and males may rely on two different learning strategies (Hawley et al., 2012, Andersen et al., 2012). Place strategy, more commonly used in men, has high hippocampal engagement, while cue strategy, more prominently employed by females, engages the striatum (McDonald, 19993, Iaria, 2003). In rodents, females tend to preferentially use cued strategies when ovarian hormones such as estradiol are low and place strategy during high levels of estradiol (Korol et al., 2004, Yagi, 2017). These studies suggest that there is a sex difference in the preference of learning strategy for a navigational task that relies on different brain regions during training which may contribute to the performance seen for spatial navigation tasks. Estradiol concentrations in female mice are negligible in the first postnatal week (Dutta et al., 2014). In C57BL mice serum estradiol concentrations increases between P26 and P29 (Ahima, 1997). In this project spatial navigational training occurs at P17 with recall test at P25, both time points at which estradiol is low. This difference in applied learning strategy with the engagement of the striatum may prevent forgetting. Testing infant females on other spatial navigation tasks such as the Morris Water Maze, radial arm maze and looking at search strategy trajectories will give greater insight. Although studies have demonstrated a difference in learning strategies for spatial navigation tasks in children, it is unknown whether female children also do not show infantile amnesia

for similar tasks as the Barnes maze. In humans, estradiol is low until it begins to rise around 8 years of age, as they approach puberty (Becker and Hesse, 2020). Human studies involving memory retention tests for behavioural tasks mirrored on the Barnes maze will provide further insight into whether this is a cross-species effect.

9.6 Relevance of infantile amnesia

Infantile amnesia is due to a retrieval deficit and has a reversible mechanism. What remains to be eluded is the value of this form of forgetting? Due to the lack of effect of environmental factors on infantile amnesia demonstrated in chapter 6, this form of forgetting is likely being driven by a predetermined switch. If this is the case, then we know that firstly the switch is reversible and secondly there is something that is occurring in the brain of male offspring from MIA that is preventing or compromising the switch. Alternatively, the brain of MIA male offspring may be more like the ancestorial precocial brain, where the switch does not occur. Why must memories be switched off at this time of development?

As described earlier, precocial animals do not demonstrate infantile amnesia (Campbell, 1974, Akers, 2014). Altricial animals may have evolved a geneticised switch that shuts down memories at that point and time during development. If this is the case, why did the switch evolve? An interesting hypothesis put forward is that the more intelligent humans become, the longer they need to look after their young outside the womb (Piantadosi, 2016). If a baby spent more time in the womb to reach full development, there would be difficulties encountered during birth. Thus, they must develop outside the womb. Since the offspring are helpless, the ones whose parents are intelligent enough to protect them survive. The authors propose that through this process evolution selects for intelligence. We may have once been like precocial mammals born mature enough to survive without a caregiver but through evolution and time, we began to need more time outside the womb to develop, resulting in the evolution of infantile amnesia. It is also plausible that the geneticised switch causing infantile amnesia is also present in precocial mammals. However, since the precocial mammals are born relatively mature the time point in which the switch is flicked could occur prenatally.

The use of precocial and altricial animals is a valuable tool that can be applied to deciphering the function of infantile amnesia.

Infants learn by interacting with their environment and others in their environment. As demonstrated in chapter 6 it appears environmental factors, such as weaning, or an enriched environment do not affect infantile amnesia. Although a change in the presence of the caregiver (delayed weaning) did not alter the rate of forgetting, it does not necessarily mean that the caregiver does not play an important role in deciphering the function of infantile amnesia. Children learn about their caregivers through the process of attachment learning (Sullivan, 2011). Attachment learning is crucial for the survival of the offspring. It ensures they remain close to the caregiver for protection and feeding. The process of attachment learning starts prenatally and continues through early development (Moriceau et al., 2010). The brain circuitry responsible for attachment learning is biologically predetermined to support robust learning, meaning offspring attach to a caregiver regardless of the level of care received. Like children, rodents demonstrate attachment learning to a caregiver. However, since eye-opening does not occur until the second postnatal week, rodents learn about their caregiver completely through smell. O'Sullivan and colleagues suggest that during a fearful event the presence of the caregiver is sufficient to suppress AMG activity in the offspring (Moriceau, 2006). It appeared that stress hormones were not released if the offspring were still living in the nest or if the caregiver was present. Thus, the researchers proposed the presence of a maternal off switch which is reversely switched when the offspring is away from the nest and caregiver. However, when the danger is the caregiver, the AMG fear circuit is still suppressed. Is it plausible that infantile amnesia developed as a protective mechanism to erase attachment learning against neglectful caregivers?

9.7 Translation to Humans

Rodent studies aim to further our knowledge of what is going on in the human brain. One major obstacle with optogenetic technology is that it is very invasive, making it difficult to translate to human cases. Electroencephalography (EEG) is a non-invasive

technique routinely used in both humans and rodents (Maheshwari, 2020, Srinivasan, 2012). Using this technique in behavioural paradigms of infantile amnesia, it may be possible to identify and compare engram-specific EEG signatures in the mouse and human brain. These signatures can then be used to inform the development of non-invasive interventions, which when paired with the correct behavioural paradigm, may allow for the retrieval of infant memories in humans. This approach would allow for a dynamic interaction between human and rodent studies.

This project identified two models in which infantile amnesia has been prevented. Firstly, chapter 6 demonstrates a case in which infantile amnesia has been reversed. Here we have an infant encoded engram that has been reinstated and is now functional and accessible. Secondly, in chapter 8 we see that something in the male ASD brain is preventing infantile amnesia. By comparing both mouse models in which infantile amnesia has been prevented at an engram level, it may give greater insight into what is driving the inaccessibility of the engram. This may lead to the identification of key molecular players in memory retrieval that can be used for the potential development of cross-species biomarkers of memory recall. These biomarkers may then be used for the development of potential drug treatments allowing for non-invasive interactions in both rodents and humans.

9.8 Summary

But where does this leave us? It is widely accepted that infant rodents forget episodic memories as early as a week after training (Campbell and Spear, 1972). This thesis demonstrated that infant mice display rapid forgetting for contextual, object and spatial memories. Although the hippocampus is an important brain structure in the mnemic process, changes are occurring across multiple brain regions. We now know that the engram survives infantile amnesia, enough to allow for acute optogenetic rescue. Further informs us that infantile amnesia is due to a retrieval deficit, rather than a problem in memory encoding or storage. Permanent reinstatement of a 'lost' infantile memory showed that infantile amnesia is a reversible process. Many possible key molecular players may be involved however because we are dealing with development,

we need to separate what changes are unrelated to infantile amnesia and merely an output of the development trajectory. One way of doing this is by looking at different types of neurodevelopment disorders and how the engram is affected. This thesis put forward a novel phenomenon in which male, but not female, offspring from MIA dams do not experience infantile amnesia.

Understanding a natural process like infantile amnesia will provide us with greater insight into how memory develops across a lifespan. What remains to be eluded is why are these memories that are formed so early in development inaccessible in adulthood? Importantly, what are the exact neurobiological mechanisms preventing this irretrievability and what is driving them? These questions can now be answered by employing contemporary approaches that are becoming increasingly accessible in developmental research. This project led to multiple conceptual advances that will hopefully impact the foundations of our understanding of human infant memory and memory across the lifespan. The paucity of infantile memories in the early stages of development is an interesting phenomenon worthy of the attention of future research.

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