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The relationship between initial context memory completeness, updating, and systems consolidation in hippocampus and cortex

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Certificate of original authorship

I, Weitian Sun declare that this thesis, is submitted in fulfilment of the requirement of the requirements for the award of the Doctor of Philosophy, in the school of life sciences at the University of Technology Sydney.

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Statement of the thesis format

This thesis is written as a conventional thesis.

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Abstract

We are forming memories every day. The fate of those memories varies depending on many causes, such as the importance of the memory, the time spent informing that memory, or the emotional state. Therefore, some memories are reliable and long-lasting, but others are inaccurate or short-lived. However, it is largely unknown whether those memories undergo the same development or not. A newly formed memory will undergo a process called memory consolidation, by which a labile memory is fixed and converted into a stabilized memory. Previous studies showed that the memories formed with different learning durations varied in accuracy and neural activity. Therefore, in this study, I further investigated whether those memories undergo the same consolidation process.

To address that question, I used context fear conditioning in mice to investigate how learning durations affect memory consolidation. Different learning durations were achieved by controlling the different amount of time that mice spend in the conditioning context prior to shock, i.e. different PSIs (placement shock interval, PSI). This study focused on the two stages of memory consolidation, synaptic and systems consolidation. Firstly, by disrupting protein synthesis, an indispensable process in synaptic consolidation, I found that the short and long PSI memories underwent synaptic consolidation at the same rate. Secondly, I found HPC inhibition significantly impaired the long PSI memory at recent time points but not the short one, suggesting the long PSI memory was contextual and HPC dependent, but the short PSI one might not be. This result showed that the short and the long PSI memories are significantly different in the HPC dependent consolidation. Thirdly, I investigated whether improving a short

PSI memory by updating affects its following consolidation. I found that an improved short PSI memory was still resistant to HPC inhibition. This result can be interpreted in two ways. Firstly, the original memory was not encoded in the HPC, so the improved memory was not HPC dependent either. Secondly, the original memory was encoded in the HPC and resistant to the HPC inhibition. In this case, memory updating did not render it susceptible to inhibition, and presumably, the following consolidation was not affected. However, the two interpretations cannot be delineated in this study.

Chapter 1: Introduction

1.1 Memory and Memory consolidation

Every important moment in our lives becomes our memory. People have been wondering how memory is formed and consolidated since the history of mankind. In modern neuroscience, the earliest memory research dates back to1900 by Müller and Pilzecker (McGaug et al., 2000; Müller et al, 1900). They found that a newly formed memory requires a certain amount of time to become fixed. Before being fixed, the new memory is susceptible to disruption by a different memory. Thus, they named the memory fixation process memory consolidation (Müller et al, 1900). However, at that time, memory consolidation was still a mystery. Over a century of study, the mechanism of memory consolidation has begun to be revealed.

In order to understand how memory is consolidated in the brain, one needs to understand how memory is formed. Memory refers to the retention of information in the brain that can be retrieved in future when needed (Poo, et al., 2016; Josselyn, et al., 2020). There are two essential features of memory as follows: 1. Memory has to encode information. 2. The encoded information is accessible. The first section will introduce how memory is formed, and the second section will introduce how memory is consolidated across time.

1.1.1 Memory formation

The physical substrate that encodes memory is called engram, which was initially termed by Richard Semon in 1904, (Semon., 1904; Josselyn, et al., 2017). In modern science, the neurons that encode memory are also named as engram neurons

(Poo, et al., 2016; Josselyn, et al., 2020; Rogerson., et al. 2014). According to memory allocation theory, becoming engram neurons is an excitability-based competition (Han, et al., 2007; Rogerson., et al. 2014). Once a new experience becomes a memory, only the high excitability neurons will be allocated to engram neurons (Josselyn, et al., 2020; Rogerson., et al. 2014). The neurons' excitability critically depends on the CREB (cAMP response element-binding protein) expression level. Previous studies have shown that artificially overexpressing CREB levels in neurons would make those neurons more likely to become engram neurons (Han, et al., 2009; Kim, 2014). Compared to non-engram neurons, the high excitability in engram neurons also fires more action potentials in the postsynaptic membrane to respond to the signals from other neurons. The increased action potentials are synapses-specific instead of cell-specific (Abdou., et al., 2018). Thus, even the same engram neuron could form different synaptic connections to encode different memories (Mayford, et al., 2012; Benfenati, et al., 2015).

The previous finding has shown that memory is encoded in synapses, and synapses are the basic unit of memory. Abdou and others showed that different memories could be encoded in the same engram neurons, maintaining their own identity based on a synapses-specific manner (Abdou*, et al*., 2018; Wang, et al., 2020). In that paper, the memories formed with different frequencies of tones were encoded in different synapses from the same engram neurons. Silencing the synapse of one memory did not affect the other. In addition, numerous studies observed dendritic spine changes in training the animal (Hayashi-Takagi, et al., 2015 Lai., et al., 2012; Shehata, et al., 2018; Yang, et al., 2009). In particular, Niu and others showed that ablating the dendrites in the hippocampal CA1 pyramidal neurons caused a spatial memory deficit

(Niu, et al., 2017). Moreover, Frankland and Josselyn's lab also showed that ablating the neurons generated after training caused a loss of the acquired memory (Frankland. et al, 2011). However, other findings showed that memory formation or storage does not require a new synaptic connection. Synaptic connection is only required for retrieving and expressing the memory. In that study, forming learning-induced synaptic connection was prevented in the HPC at learning, and it caused a memory deficit in the test. However, that memory deficit was rescued if those neurons were activated by optogenetics (Tonegawa, et al, 2015). Thus, they found that forming new synapses only made the memory retrievable, but it is not essential for storage. Nevertheless, having a stored but not retrievable memory is not a normal scenario in most studies. Therefore, most previous studies still second that memory formation critically depends on forming new synapses.

1.1.2 LTP and protein synthesis in memory formation

In a recent study, the engram is also defined as a persistent change in the brain that is formed in response to a stimulus. The persistent change is achieved by a fundamental mechanism that underlies memory formation, long-term potentiation (LTP). LTP was initially found as the neurons maintaining their depolarisation for a period after the stimulus disappeared (Hebb., 1950). In memory research, LTP is also characterised as the long-term effect on neuronal communication. When the presynaptic membrane releases neurotransmitters to other neurons as a signal of encoding memory, the neurons that receive the neurotransmitters will be in potentiation for a period of time. During this time, the postsynaptic membrane recruits more receptors, such as NMDA receptors (NMDAR), to respond to the signal. NMDAR is

only required to maintain the LTP shortly after memory formation, which is regulated by protein kinase M-ζ (Borroni., et al., 2000; Sutton, et al., 2006). However, the retention of memory or LTP in remote time does not require NMDAR (Borroni., et al., 2000).

Besides NMDAR, how long an LTP can last critically depends on the phase of the LTP. LTP can be dissected into early LTP (E-LTP or LTP1) and late LTP (L-LTP including LTP 2 and 3) (Blundon, et al., 2008; Raymond, 2007). For LTP1, no protein translation is required in the synapses, but this LTP can only last up to 60 mins. LTP2 can last longer than that but requires local translation in the synapse. LTP3 requires both transcription and translation to permanently stabilise the synapses (Abraham, et al., 2002; Abraham, et al., 1991). L-LTP dependent memory can become a long-term memory that exists for months or years, which requires protein synthesis immediately after memory formation. On the other hand, the E-LTP dependent memory could only exist for a few hours as short-term memory, but no protein synthesis is required in this case. The behavioural study also showed similar results, where if the memory could only a few hours, protein synthesis inhibition failed to inhibit that memory. Nevertheless, for those memories that can last over one day, they can be impaired by protein synthesis inhibitors (Ballarini, et al., 2009). Therefore, in order to maintain the LTP and memory in the long term, protein synthesis is indispensable (Morris, et al., 2006).

1.2 Memory consolidation

Memory is mutable and constantly undergoes development. The development process that transforms a memory from a labile state into a stable state is termed memory consolidation (Müller, & Pilzecker, 1900; Dudai, 2012; Dudai, et al., 2015; McGaugh, 2000). Memory consolidation can be dissected into two stages: synaptic consolidation and systems consolidation (McGaugh, 2000; Winocur, et al., 2011). Synaptic consolidation is the first stage of memory consolidation that rapidly occurs after learning. Systems consolidation, however, is a late-phase and long-term consolidation. It refers to transforming the newly formed memory from the hippocampus (HPC) to the cortex and losing details (Nadel, et al., 2007; Winocur, et al., 2011). Notably, the detailed information still stays in the HPC for a period of time after consolidation (Nadel, et al., 2007; Winocur, et al., 2011). However, the substantial mechanism of systems consolidation is still largely unknown. In this section, I will introduce the theories and mechanisms in detail for synaptic and systems consolidation.

1.2.1 Synaptic consolidation

Synaptic consolidation refers to forming new synapses to encode memories, and it mainly happens in the HPC for episodic memories. The synapses between engram neurons are made from the dendritic spine, which is a protrusion of the cell membrane receiving or releasing neurotransmitters to other neurons (Hayashi-Takagi, 2015). Those synapses are strengthened if the neurons where they grow from becoming engram neurons during learning (Redondo, et al., 2010; Rogerson et al., 2014). Synaptic consolidation is characterized by those synaptic connections being strengthened through forming new dendritic spines and/or enhancing the pre-existing synaptic

connections between engram neurons (Redondo, & Morris, 2010; Rogerson et al., 2014).

1.2.1.1 The synaptic tagging and capture theory

Once a new synaptic connection is established, numerous biochemical processes start to stabilize the newly formed connections. The well-accepted theory that describes forming synaptic connection is called the synaptic tagging and capture theory (STC) (Redondo, & Morris, 2010; Rogerson et al., 2014). According to SCT, there are two steps to stabilize the new formed synaptic connections, which are synaptic tagging and capture.

Firstly, Synaptic tagging is a process that only temporally enlarges the area of the postsynaptic membrane and strengthens the synaptic connection. Once a neuron is allocated to an engram neuron, the postsynaptic membrane will be enlarged by E-LTP induced action potentiation. By doing so, the enlarged and newly formed synapses are tagged. During the E-LTP, the postsynaptic density (PSD), the protein density in the postsynaptic membrane, is modified to make the synapse ready for further stabilization. The scaffolding protein in the postsynaptic membrane, such as PST-95, neuroligin and actin, are critical for maintaining the membrane structure. The modification of PSD alters the structure of the postsynaptic membrane proteins and makes them temporally malleable. The malleable membrane structure allows inserting more neurotransmitter receptors, such as AMPAR, receiving more signalling molecules. If the memory encoding signal is weak and only causes a weak tetanisation in the synapse, then the soma will not synthesizes new proteins to support this membrane enlargement. In this

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case, the enlarged area and modified PSD will go back to the normal states shortly. Subsequently, the new synaptic connection will be diminished, and its corresponding memory will also disappear. This is the fundamental mechanism for short-term memory, which is a memory that only lasts for a short period. However, if there is a strong tetanisation caused L-LTP, the enlarged membrane will be further stabilized by the second process, synaptic capture. The capture refers to the soma sending large molecules to the new synapses to permanently stabilize the enlarged postsynaptic membrane. With a strong tetanisation, L-LTP will initiate transcription and translation in the cell and synthesize plasticity related proteins (PRP). PRP will be captured by the synapses that were tagged in E-LTP. Once the PRPs arrived at the tagged synapses, they will interact with the scaffolding protein to further stabilize and enhance the synaptic connection. So far, numerous PRPs have been found. For instance, protein kinase M-ζ, one of PRPs has been reported to play a key role in maintaining LTP (Sutton. et al., 2006). Another important PRP is CaMK II (Calcium-calmodulin-dependent protein kinase II), which is reported to regulate postsynaptic translation and subsequently enhance synaptic connections (Giese, et al., 2013; Giese, et al., 1998). With the support of PRPs, the enlarged postsynaptic membrane will be stabilized, and the new connections will be permanently strengthened.

In summary, the synaptic connection is strengthened mainly through enlarging the post-synaptic membrane to receive more neurotransmitters released from the presynaptic membrane. This enlargement process critically depends on synthesizing new proteins such as PRPs (Redondo, & Morris, 2010; Rogerson et al., 2014). PRPs are synthesized in the soma and sent to the target spines in order to stabilize and fix the enlarged post-synaptic membrane. This fixation process is important only for a memory

that is meant to be encoded as a long-term memory. Therefore, *de novo* protein synthesis is only indispensable for synaptic consolidation of long-term memory, but not a short-term one. The necessity of protein synthesis in synaptic consolidation has been confirmed by previous research. It has been shown that using a protein synthesis inhibitor to disrupt synaptic consolidation resulted in a failure to form memory (Lattal, & Abel, 2004). However, after the proper establishment of the synaptic connections, i.e. synaptic consolidation is completed, protein synthesis is no longer required for further memory consolidation (Benfenati, & Valtorta, 2015; Nader, et al., 2000; Barrientos, et al., 2002 Touzani, et al., 2007; Bourtchouladze, et al., 1998; Squire, & Barondes, 1974). The tagging and capture mechanism is fundamental to synaptic consolidation. Through this process, the learning-induced synaptic connection will be stabilized in the long term. Once the synaptic connection is established, the hippocampal engram neurons will support the next stage of consolidation, systems consolidation.

1.2.2 Systems consolidation

Systems consolidation is a process by which different regions of the brain collectively interact with each other to reorganize memory in the long term. Unlike synaptic consolidation, the essential cellular or molecular mechanisms of systems consolidation are still largely unknown. Systems consolidation was originally found in seizure patients, most prominently a patient called H.M. (Squire, et al., 2011; Nadel., & Moscovitch, 1997). To treat his seizures, the HPC was resected from his brain. After resecting the HPC, H.M. suffered permeant anterograde amnesia, which is the inability to form new memories, but his old memories were largely intact. This finding raised

the attention regarding old memory could be transferred to different regions of the brain. On the basis of this hypothesis, there two theories, stand model theory (SMT) and multiple trace theory (MTT), have been proposed. Both of the theories have explained how memory is transferred from the HPC to other areas. However, the differences between them will be discussed in this section in detail.

1.2.2.1 SMT and MTT

This first theory regarding systems consolidation is the standard model theory (SMT), which posits that memories are initially encoded in the HPC and transferred to extrahippocampal sites after systems consolidation (Nadel, et al., 1997). Once transferred to the extrahippocampal sites, the HPC is no longer involved in the retrieval of that memory. In the beginning, SMT was supported by many clinical cases. Patients with hippocampal resection suffered from temporally graded anterograde amnesia, which means the brain was unable to form new memories, but the old memories were largely intact (Haist, et al., 2001; Nadel, et al., 2000). However, the increasing evidence showed that the detailed information from the old memories was also lost after brain trauma in the medial temporal lobe, where the HPC is located (Haist, et al., 2001; Nadel, et al., 2000).This finding suggested that the HPC is still involved in retrieving old memories, which did not support SMT. Therefore, Nadel and Moscovitch proposed a new theory about systems consolidation called multiple trace theory (MTT). The MTT posited that memory constantly and passively generates new traces irrespective of the original experience at the online state, such as awake, or an off-line state, such as sleep (Nadel, et al., 1997; Klinzing, et al., 2019). Each new memory trace contains a certain amount of information. The pre-existing information in those traces, such as the

semantic knowledge, is extracted as general information, which is processed separately from that experience-specific information (Moscovitch, et al., 1998; Nadel, et al., 1997). The general information is consolidated in extrahippocampal sites after systems consolidation, and the experience-specific information stays in the HPC (Moscovitch, et al., 1998; Nadel., et al., 1997). At the remote retrieval, the HPC is only reactivated if the experience-specific information needs to be retrieved (Plitt, et al., 2019). Notably, the experience-specific information is not consolidated in extrahippocampal sites such as the PFC, where the remote memory is retrieved from. Thus, the consolidated PFC memory lacks the details of the memory. The process of losing details during systems consolidation is called memory generalization, which becomes the main argument of MTT (Dudai, et al., 2015; Nadel., et al., 1997). In addition, since semantic memory, which is a memory of knowledge, is independent of episodic experience and has no detailed information, it will be encoded in the cortex since its formation (Nadel, et al., 1997). After MTT was proposed, this theory has been supported by numerous studies. One important research supporting MTT is finding the neural circuits where the hippocampal engram neurons support the formation of cortical engram neurons during systems consolidation. Also, after the memory is consolidated in the cortex, the hippocampal engram neurons can still be optogenetically activated (Kitamura, et al., 2017). This suggests that the HPC could still be involved in retrieving old memories under some circumstances.

To conclude, SMT initially posited that memory is transferred from the HPC to the cortex, and no memory is encoded in the HPC after that. By contrast, MTT suggests that the general information in the original memory is extracted and consolidated in the cortex, but the detailed information still remains in the HPC. Albeit the substantial

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mechanism of systems consolidation is still largely unknown, most of the recent studies second the MTT, and found the HPC may have a long-term role in memory retrieval.

1.2.3 Reconsolidation

Memory is transferred from its labile state to its stable state through memory consolidation (Dudai, 2006; Gisquet-Verrier, et al., 2018; Lee, 2009; McKenzie, et al., 2011). However, once the stabilized memory is reactivated, it will temporally become unstable again. Subsequently, the reactivated memory undergoes reconsolidation to become stabilized again (Debiec, et al., 200; Fukushima, et al., 2014; Lee, et al., 2004). Reconsolidation shares many similarities with synaptic consolidation. For instance, both of them stabilize memories, and reconsolidation also takes place in the HPC and requires protein synthesis (Trent., et al., 2014). By contrast, reconsolidation also has its unique features, such as Zif 268 is only required for reconsolidation, but not reconsolidation (Lee, et al., 2004). In addition, BDNF is only found in consolidation but not reconsolidation (Hall., et al, 2001). The similarities and differences at the molecular level raise questions regarding the relationship between memory consolidation and reconsolidation.

An important question one may ask about reconsolidation is why memory becomes labile again after being reactivated. Some findings suggested that reactivating a memory may, at least partially, generate a new memory trace, and that new memory is labile (Alberini., et al. 2011). Other evidence supports that becoming labile is to integrate the new information into the old memory trace (Dudai., & Eisenberg., 2004; Sara., 2000). However, different conclusions are drawn based on different subjects and

different behavioural paradigms. A previous study from our lab showed that updating a poorly formed memory requires reconsolidation but not consolidation in the HPC (Zinn., et al., 2020). In that study, blocking memory consolidation in the HPC at updating did not improve poor memory. However, blocking reconsolidation in the HPC prevented the poor memory from being updated to a well-formed one. This result suggests that there are some substantial differences between reconsolidation and consolidation. Also, updating a poor memory and improving its accuracy implies that memory updating integrates new information into the original memory trace.

In summary, memory reconsolidation has a complex relationship with consolidation. The similarities and differences shared by them raise the question of whether the updated memory is a new memory or a modified old memory. This question depends on a variety of complicated situations. An updated memory can be either retrieved from the original trace or retrieved as a new memory formed by updating.

1.2.4 Identifying the engram neurons

Revealing the engram ensembles of memory has been drawing attention in the last decades. It is unknown how memory is represented in the brain and how neurons are responsible for encoding and retrieving a memory. However, in current research, certain criteria can be used to characterize the engram neurons and distinguish those from non-engram neurons. Having such criteria is critical targeting and manipulating memory in research. Firstly, engram neurons should have higher excitability than non-engram neurons. As introduced before, forming an engram is an excitability based competition, and artificially overexpressing CREB could increase the chance of

becoming engram neurons (Han, et al., 2009; Kim, 2014). Nevertheless, excitability can only predict the chance of becoming engram neurons before memory formation but not identify the engram neurons after learning. Albeit this is one of the fundamental differences between engram and non-engram neurons, it is rarely used in research. Secondly, the connections between engram neurons are more enduring than the ones between non-engram neurons (Druckmann, et al., 2014). According to the synaptic tagging and capture theory, the synaptic connections between engram neurons will be significantly enhanced by learning-induced LTP. Examining the synaptic strength is one of the most direct methods to identify engram neurons. However, engram neuron is only a small part of the entire population, and the synapses between engram neurons are too fragile to examine. Thus, only very a few studies successfully labelled and identified the engram synapses. Thirdly, Engram neurons are activated during learning and re-activated during memory retrieval (Tonegawa, et al., 2015; Kitamura, et al., 2017). This is the most commonly used criteria to identify engram neurons. Compared to previous criteria, this method is much more feasible in research. The engram neurons can be identified by using different markers to label the activated neurons at learning and retrieval. I also used such labelling method in this study, which will be introduced in the method chapter. Fourthly, artificially manipulating engram neurons could alter the corresponding memory expression. This criterion requires using engineering methods, such as optogenetics to control neuronal activity. The advantage of this method is that it can establish the causality link between the engram neurons and memory.

1.3 Context fear conditioning

1.3.1 Classical conditioning and the fundamental learning mechanism

Learning and memory have been studied for over a century in modern neuroscience. There are many methods and subjects that have been used to study how memory is formed through learning. Memory is acquired from a learning event, and one commonly used learning paradigm in the lab is conditioning. Conditioning refers to animals generating specific responses after receiving certain stimuli. The most famous conditioning study was done by Ivan Pavlov with his Pavlov dogs experiment. In that experiment, a dog salivates when presented with food. The presentation of food is called unconditioned stimulus (US), which is a stimulus that naturally produces an unlearned behaviour. This unlearned behaviour, such as salivation, is called unconditioned response (UR). When the food presentation (US) is paired with a neutral stimulus, such as a tone, that neutral stimulus becomes a conditioned stimulus (CS) that associates with the US. Once associated with a US, the CS could elicit a conditioned response (CR). However, the neural circuits underling how CS causes CR/UR were unknown at that time. Until many years later, Eric Kandel found the basic learning mechanism in *Aplysia* by studying its siphon-withdrawal reflex. He found that the sensory signal from the siphon stimulation (US) converges with the sensory signal from touching the tail (CS) at interneurons. This CS-US association subsequently caused gill retraction (CR). Therefore, the fundamental mechanism for learning is establishing the CS-US association.

1.3.2 Context fear conditioning

Since Ivan Pavlov and Eric Kandel, learning and memory research has been increasingly advanced. Among different types of memories, fear memory is a long-lasting and commonly used one in research. This is because remembering the fear source is vital for animals to survive danger in the wild, and most fear memories are

robust and persisting. Thus, fear memory is often used in studying the basic features of memory. Depending on the research question, fear memory is usually associated with different neutral CSs, such as a tone or an environment in the lab (Raybuck, & Lattal, 2014). For those studies that focus on the memory of an experience, fear memory is commonly associated with a neutral environment, which is called context.

Context can be an external environment or an internal state. It is usually defined as a framework where events take place (Nadel., & Willner., 1980). Context is not only the background of an experience, but also involves meaningful and logical connections to all the compounds within it (Nadel., & Willner., 1980; Stark, et al., 2018; Mack, et al., 2018). Context and the events that occur within it are integrated as a unified entity, which is called a conjunctive representation. Rudy and O'Reilly also found evidence to support that context exists in a conjunctive form (Rudy & O'Reilly., 2001). They found that different combinations of the contextual features affect how rats remember the context, suggesting all features from a context are not simply piled together but, instead, interlaced in a conjunctive form. Therefore, a contextual memory is encoded as a complex form that contains the physical environment and intrinsic relationship between objects.

Since contextual memory exists in such a complex form, it is challenging to understand the neural bases of its representation in the brain. A contextual memory usually integrates different sensory inputs as one unified representation and stores this representation as one ensemble. Unlike the memories with single sensory input, such as visual or odour memory, the contextual representation and its neural substrate cannot be decoded currently. In other words, it is unclear how context is constructed and

comprehended in the brain. However, contextual memory research is not prevented by it. Once the subjects receive certain stimuli in a specific context, they will express the corresponding behavioural readouts to that context. In this case, context could associate with the stimuli and become a CS in memory research. By virtue of associating context and a stimulus, the contextual memory can be identified on the basis of the expected outcome from that stimulus. The most commonly used method is called context fear conditioning (CFC). CFC refers to associating a neutral context with an aversive stimulus, such as a foot shock, resulting in generating a fear response to that context. CFC has been used for decades in rodents generating consistent and reliable behavioural readouts.

1.3.3 Behavioural readouts of CFC

Rodents, such as rats or mice, are the most commonly used subjects in CFC, as they show distinctive behavioural readouts at and after conditioning. Those behavioural readouts can be used to assess if they form a contextual fear memory. When rodents receive a shock in the context of conditioning, they will express vigorous movements, such as running or jumping, which is called activity burst. If they form a contextual memory of the conditioning context, they will express a specific defensive behaviour, freezing, when being re-exposed to this context. Freezing is defined as stopping all movements except for those required for breathing (Bevins, et al., 2000; Fanselow, 1980). The freezing level in the memory test can be used to determine whether the animals have a contextual fear memory or not.

When rodents recall a fear memory, they will express a variety of behaviours, such as urinating or increasing blood pressure. Among those behaviours, freezing is the most reliable and observable behaviour for recalling a fear memory. Freezing, as a species-specific defence reaction, is a rapidly acquired response and suppresses many other behaviours in aversive situations (Bolles, 1970; Crawford, et al., 1982). Thus, besides freezing, other observable behaviours are limited after conditioning. Therefore, freezing has been used as the most important criterion for fear memory retrieval for a long time. Numerous studies have also shown that the quality of memory can be reflected in the freezing level at memory tests. Dating back to the last century, it has been reported that rats showed different levels of freezing when they were conditioned with different durations. Especially, when animals fail to form a contextual memory, they will show significant low freezing (Fanselow, 1950; Fabselow, 1990; Kiernan, et al, 1995; Landeira-Fernandez, et al., 2006). Therefore, the freezing level could reflect whether the mice remembered the context or not in this study.

1.3.4 Anatomical structures involved in memory encoding and consolidation

1.3.4.1 Role of the hippocampus (HPC) in context fear memory

The role of the HPC in memory formation has been drawing attention since H.M. had his hippocampal resection surgery. After that, decades of studies have shown that the most important brain region for forming a contextual memory is the HPC (Coelho, et al., 2018; Matus-Amat, 2004; Rozeske., et al, 2014; Teixeira, et al., 2006). Since the late of the 20th century, many studies, such as lesion on the HPC, were performed to examine the role of the HPC in memory formation. In those early studies, lesions in the HPC shorter after CFC caused a memory deficit, but this memory deficit was not

observed if the lesion was made at a remote time point (LeDoux., & Phillip., 1994; Anagnostaras, et al., 1999). Also, complete and partial hippocampal lesions caused the same memory deficit (Lehmann, et al., 2007). Besides lesion, pharmacological inhibition of the HPC also showed similar results. Inhibiting the dorsal HPC (dHPC) with muscimol prior to context exposure prevents the animal from forming a contextual memory (Morawska, et al., 2012; Haubrich, et al., 2016; Jafari-Sabet., et al., 2009). After identifying the role of the HPC in memory formation, the neural circuits and subregions have been further studied.

The fundamental circuit in CFC is converging the contextual sensory information and the conditioning input. At conditioning, all sensory inputs are integrated into the HPC forming a contextual representation as a CS. The shock-induced fear is encoded in the basolateral amygdala (BLA) as a US. Shortly, the shock (US) converged with the contextual sensation (CS) at the central amygdala (CeA) forming the CS-US association (Chaaya, et al., 2018; Rozeske., et al., 2014). When the memory is retrieved, this CS-US association causes a freezing response through the projects from the CeA to the periaqueductal gray (PAG) (Rozeske., et al., 2014). In these circuits, CA1 or the dHPC is responsible for collecting the contextual information and projecting it to other regions. Therefore, CA1 gates the global contextual memory retrieval. However, the detailed information is initially encoded in the dentate gyrus (DG) and CA3. Previous studies showed that inhibiting the DG during conditioning severely impaired contextual memory acquisition (Bernier, et al., 2017; Frankland, et al., 2006). In addition, activating the DG engrams using optogenetics was sufficient to retrieve a contextual memory (Liu., et al. 2012). Further, if the DG engram neurons that encode the conditioning context were activated in a new context, mice could still retrieve the

memory of conditioning context in that new context (Ramierz., et al., 2013). That evidence suggests that the DG is the key region for encoding and retrieving a recent contextual memory.

1.3.4.2 Role of prefrontal cortex (PFC) in memory formation and remote memory retrieval

During systems consolidation, the hippocampal engram neurons support the maturation of the PFC engram neurons, and remote memory becomes PFC dependent eventually (Barry, et al., 2016; Ding., et al., 2008, Kitamura., et al., 2017; Rozeske., et al, 2014; Teixeira, et al., 2006). The most important circuit is the projections between the HPC and the PFC during systems consolidation. The fundamental mechanism by which the PFC regulates contextual memory formation is processing sensory inputs (Gilmartin., et al., 2014). As discussed above, context is encoded in a complex form that contains all sensory inputs. Thus, the PFC, as a higher function centre, processes different sensory inputs and orchestrates the unified representation as a CS during memory formation (Heroux, et al., 2017).

The anterior cingulate cortex (ACC) is one of the key regions in the PFC that is involved in memory formation (Aceti, et al., 2015). The previous study showed that the newly formed memory encodes information in the HPC and the ACC (Kitamura., et al., 2017). However, the ACC engram neurons are immature after learning, which are unable to retrieve any memory at recent time points. Thus, recent memory retrieval is completely dependent on the HPC. During systems consolidation, the HPC engram neurons continuously support the development of the ACC engram neurons, which turns those neurons into "mature" engram neurons that are able to retrieve the encoded

memories. This "maturation" process is based the definition given by a previous study from Tonegawa's laboratory (Kitamura., et al., 2017). That study suggests that the PFC engram cells are immature at the time of learning, meaning they cannot retrieve the memory. With the support of the HPC engram cells during systems consolidation, those immature PFC engram cells become mature and are able to retrieve memories. Therefore, the ACC engram neurons could only retrieve the memory at remote time points (Kitamura., et al., 2017). However, the details are still largely in mystery regarding how the hippocampal engram neurons support the development of the ACC engram neurons.

Besides involving in memory formation, PFC is also the key region for retrieving remote memory, which has been investigated by numerous cellular and behavioural studies. The immediate early genes (IEG) are a group of genes that rapidly respond to cellular stimuli, which are also commonly used as a marker for neural activity. The previous study found that the c-fos and Zif 268 expression, common IEGs, in the PFC showed a significantly higher expression level at the remote time points than the recent time points. (Aceti, et al., 2015; Wiltgen., & Silva., 2004). Besides IEG expression, pharmacological inhibition of the PFC also showed similar results. When muscimol, a GABA receptor agonist used as a neuron inhibitor, was injected into the PFC two days after conditioning, no memory deficit was observed in rats. However, if muscimol was injected 45 days after the conditioning, the remote memory retrieval was significantly impaired (Haubrich., et al., 2016). Therefore, the above evidence suggests that the PFC is only required for remote memory retrieval, but not recent.

1.3.5 Timing effect of CFC

One of the key features of forming contextual fear memory is that it requires time (Kukushkin, et al., 2017). As is mentioned previously, context is represented in a form that integrates all sensory inputs as one unified ensemble. Thus, integrating those sensory information requires time. Previous studies showed that the quality of a contextual memory was subject to how much time the animal spent in context at conditioning (Zinn, et al., 2020). In those studies, there was a critical time period when the animal could explore the context that is between placing the animal in a context and delivering a shock, which is also known as placement-shock interval (PSI). PSI determines the contextual fear level in the memory test. Previous studies showed that if PSI is shorter than 10s, the animals failed to show freezing in a subsequent test (Kiernan, et al., 1995; Landeira-Fernandez, et al., 2006; Lattal, &Abel., 2001). This brief exposure to a context that leads to a failure to generate freezing (or any CR) to that context is called immediate shock deficit (ISD) (Kiernan, et al., 1995; Landeira-Fernandez, et al., 2006; Lattal, &Abel., 2001). Since ISD suggests a failure to associate the context with fear due to a short PSI, it becomes the main feature of contextual fear memory. In other words, a very short PSI should not generate a contextual memory. This is because a very short PSI does not reach the minimum amount of time to form a context representation. However, there is one exception the animals are pre-exposed to the context and have already formed a contextual memory before conditioning. In this case, the animals could associate the existing contextual memory with the shock at conditioning even with a short PSI. Subsequently, the animals could still show contextual fear at the tests. This is called the context preexposure facilitation effect (CPFE). Besides CPFE, ISD can also be alleviated by increasing PSI at conditioning.

The previous study showed that if PSI is longer than 81s, it is sufficient for rats to form a contextual fear memory and showed freezing in the tests (Fanselow, 1990).

Besides the freezing level, PSI could also determine how accurate a contextual memory is. The accuracy of a contextual memory can be tested by how well an animal could discriminate similar contexts. In context discrimination experiments, animals are conditioned in one context and tested in a different one. If the animals formed an accurate memory about the conditioning context, they would distinguish the testing context from the conditioning one and show a low, but not zero, freezing in the tests. Previous studies found that even animals could still form a contextual memory with short PSIs (longer than the minimum requirement), but they showed a poor ability to discriminate similar contexts (Leak, et al., 2021; Zinn, et al., 2020). In those experiments, animals showed equal freezing regardless of being tested in the conditioning context or a different context. Subsequently, they found that poor discrimination ability was improved by increasing PSI (Zinn, et al., 2020). At the long PSI conditioning, animals could identify that the contextual components from the conditioning context were not presented in the testing context, which prevents the animals from retrieving the original contextual memory. Also, it can be interpreted as the animals retrieving the memory but deciding not to fear the context because it does not match what it retrieved from memory. Regardless of which interpretation, these results support that a more detailed context was constructed with a long PSI that allows the animals to discriminate the conditioning context from the testing context. By contrast, with a short PSI, the unique details of the conditioning context were not encoded. When those animals were tested in a different context, they could not distinguish the contextual components between the conditioning context and the testing

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context. Thus, the animals retrieved their contextual memories from conditioning by mistake.

More features of the memories with different PSIs have been revealed in recent studies. Previous studies from our lab showed that PSI could affect the neural activity level in the HPC. In that study, the memories formed with long PSIs showed a high Arc expression in the DG/CA3 and CA1 at conditioning after conditioning (Leak., et al., 2017). This result may imply that long-PSI memories encode more information in the HPC compared to the short-PSI ones. A further study identified the engram size of the memories that were formed with different PSIs. That study suggested that the ability to discriminate between contexts may depend on its engram size. They found that the long PSI memories are more accurate than the short ones. This may be because it has more engram neurons in the DG to encode the contextual representation (Leak. et al, 2021).

Some essential features of the poorly formed contextual memories were also identified in previous studies. The poor memory can be updated to a well-formed memory that is able to discriminate similar contexts in the tests. Besides improvement, a poor memory could also be distorted to a different memory depending on what context is being re-exposed. This contextual memory distortion only happened in short PSI memory but not in long-PSI one. In addition, compared to a long PSI, a short PSI memory is more difficult to extinguish. Further, they also found that updating short PSI memories only initiates memory reconsolidation in the HPC, but not consolidation (Zinn, et al., 2020). This study suggests a variety of features of a poorly formed memory and how this poorly formed memory is different from a well-formed memory.

In summary, the time being spent in forming a contextual memory is critical to the quality of the memory and may also alter the properties of the memory. Conditioning with a very short PSI will cause an ISD, which suggests a failure to form a contextual memory. Slightly increasing PSI would associate the contextual information with the shock and form a poor contextual fear memory, which is unable to discriminate similar contexts. Further increasing PSI will form an accurate memory that only shows freezing in the conditioning contexts but not in a different one. Memories formed with different PSIs showed distinctive features, but it is still unknown how the quality of the initial memory affects the following memory consolidation. Therefore, I studied how memory completeness affects memory consolidation in this project.

1.4 The present study

Memory consolidation has been studied for over a century, and numerous factors that are involved in this process have been well studied. However, how initial learning affects memory consolidation is still largely unknown. This is because most previous studies only used the learning paradigm that generates well-formed and accurate memories. Nevertheless, most memories in everyday life are inaccurate, and these inaccurate memories have been rarely studied.

Many important events in our daily lives depend on our memories, but our memories are not always infallible. Particularly, the information encoded in a poorly formed memory can be unreliable. In this case, such memory might encode incorrect or incomplete information and develop into an inaccurate memory. For humans, an

inaccurate memory of an important event may lead to a catastrophic consequence. For animals, such an inaccurate memory about the predators may put their lives in danger. Thus, it is important to know how an inaccurate memory is different from an accurate one. Knowing such differences will shed light on how different memories are encoded and how to improve inaccurate memory.

Memory starts consolidating since its formation, and a consolidated memory is more stable than a new one. In many scenarios, we have to access our memories while they are still undergoing a consolidation process, such as the systems consolidation in humans may take years. Knowing if an inaccurate memory consolidates similarly to an accurate one could inform us when each memory becomes stable and resistant to disruption. Therefore, we can decide under what circumstances we can trust an inaccurate memory and how to improve it as well.

Memory consolidation has been studied by many methods. Among those methods, the advantage of using CFC is that contextual memory can be formed with different completeness in the lab, which could represent the memories with different accuracy in real life. In the lab, a complete memory can be defined as an accurate memory that allows the animals to discriminate similar contexts. By contrast, the animals formed with an incomplete memory will fail to discriminate similar contexts due to the inaccurate information encoded. Contextual memory completeness can be achieved by controlling the time spent at conditioning, i.e. a PSI. For instance, a long PSI generates a complete memory, *vice versa*. Therefore, I could use CFC and manipulate PSIs to investigate how initial completeness affects memory consolidation in this study. In order to use CFC to study memory consolidation, I have to establish

and validate the set-ups in our lab. This is a task in itself because CFC is sensitive to the experimental environment and minor changes. The same procedures may generate completely different results when conducted with different apparatus.

After validating the basic CFC set-ups, the first consolidation I investigated was synaptic consolidation. Previous studies had shown that hippocampal neural activity and engram size varied when animals were conditioned with different PSIs. That result implies that memories formed with different PSIs may contain different amounts of information. It is largely unknown whether the memories containing different amounts of information consolidate at the same rate or not. Answering such a question could shed light upon how the brain processes memory. Therefore, the first aim of this thesis is to investigate whether PSI affects synaptic consolidation rate. Considering that more engram neurons are found in long PSI memories, and synaptic consolidation mainly involves forming new synapses between engram neurons, the hypothesis is that long PSI memory may consolidate slower than the short PSI one. As introduced previously, synaptic consolidation is characterised by protein synthesis, but protein synthesis is no longer required once synaptic consolidating is completed (Benfenati, & Valtorta, 2015; Barrientos, et al., 2002; Bourtchouladze, et al., 1998; Squire, & Barondes, 1974). Thus, being independent of protein synthesis can be regarded as a sign of finishing synaptic consolidation. By comparing when different memories become protein synthesis independent, I could decide how long it takes to complete their synaptic consolidation. The result is shown and discussed in Chapters 3 and 6.

Following synaptic consolidation, the next process is systems consolidation, which is characterized by converting an HPC dependent memory into an HPC

independent one. There are two approaches to examining systems consolidation. The first approach is to examine memory generalization. Unlike a new memory, the remote and consolidated memory only contains general information with which the animals cannot discriminate similar contexts. In other words, an animal could only discriminate similar contexts before its memory completes systems consolidation, not after. Therefore, based on the context discrimination results, I could know when those memories complete their systems consolidation. However, context discrimination is very sensitive to the basic set-ups. Even though I have tried designing many similar contexts, none of them was discriminable without memory updating. Therefore, I took the second approach, which is to examine the regional dependency of the memory. A contextual memory can only be retrieved from the HPC before completing systems consolidation. After the systems consolidation is completed, the memory will only be retrieved from the PFC. Thus, knowing when memory becomes HPC independent, I could decide its systems consolidation completion time. Since a long PSI memory may contain more information than a short PSI one, I hypothesized that the long PSI memory may take more time to consolidate and become HPC independent than the short one. The results are presented and discussed in Chapters 4 and 6.

If I found PSI affects systems consolidation, it would suggest that the rate of systems consolidation is dependent on initial memory completeness. However, memory completeness can be changed through memory updating. Previous studies showed that re-exposing to the conditioning context could improve an incomplete memory, and that updated memory behaved similarly to a complete one. Therefore, the next aim is to study whether changing the initial completeness by updating affects the following memory consolidation. Since contextual memory undergoes systems consolidation that

involves processing the information encoded in memory, changing that information could be expected to affect the following systems consolidation. The result is presented and discussed in Chapters 5 and 6.

To conclude, this project studies whether or how initial learning duration affects memory consolidation. This study bridged the knowledge gap in the relationship between initial memory formation and its following consolidation.

Chapter 2: Methods

2.1 Subjects

Wild-type C57BL/6J mice (male/female, 9-10 weeks old) obtained from the Australian BioResources facility (Moss Vale, NSW) were housed in groups of 4 per cage. FosCretdT mice were obtained by crossing $Fos^{2A-iCreER/=}$ (TRAP2) mice (Jax #030323) and ROSA26-CAG-stop-floxtdTomato (Ai14) mice (Jax #007914) from The Jackson Laboratory. All mice were kept in the Biological Testing Facility (BTF) of the Garvan Institute of Medical Research (Sydney, NSW) for 1 week for acclimatization after received from BioResources facility. All mice were under a 12h light/dark cycle (7am-7pm) regime in the BTF with food and water provided ad libitum. All procedures in this thesis were approved by the ethics committee at the Garvan Institute of Medical Research, Darlinghurst, NSW, 2010.

2.2 Drug preparation

2.2.1 Anisomycin

Anisomycin from Streptomyces griseolus (Supplier: Sigma-Aldrich, NSW; #A9789) was dissolved in 1M of equimolar hydrochloride acid and diluted in 0.9% saline to make a final concentration of 15 mg/ml (pH=7.4). 0.9% sterile saline (NaCl) was used as vehicle. Anisomycin or vehicle was injected into mice through i.p. injection, 150 mg per kg of body weight.

2.2.2 4-Hydroxytamoxifen (4-OHT)

5 µg of 4-Hydroxytamoxifen (Sigma-Aldrich, H6278-50MG) was dissolved in 500 µl of 100% ethanol and by shaking at 37 °C for 5 mins to make a concentration of

10mg/ml. Once it dissolved, 500 µl of sunflower oil was added to the solution and by shaking at 55 °C for 15mins. After being mixed, the ethanol from the solution was evaporated by vacuum centrifugation to make the final concentration of 10 mg/ml. The final solution was kept in 37 °C water bath until being used.

2.2.3 Paraformaldehyde (PFA) and sucrose solution preparation

40 g of Paraformaldehyde (Sigma-Aldrich, 158127-5G, powder, 95%) was dissolved in 1L of 1X PBS to make a final concentration of 4% with a pH adjusted to 7.2. 30% of sucrose solution was made by dissolving 300g of sucrose (Sigma-Aldrich, S9378-10KG, 99.5%) in 1L of 1X PBS.

2.2.4 0.1M phosphate buffer preparation

Phosphate dibasic (Sigma-Aldrich, S9763-500G) was dissolved in distil water to make 0.1M of sodium phosphate dibasic solution. Sodium phosphate monobasic (Sigma-Aldrich, 7150-250G) was dissolved in distil water to make 0.1M of sodium phosphate monobasic solution. Adding the sodium phosphate monobasic solution to sodium phosphate dibasic solution until the pH of the mixed solution reached 7.2.

2.2.5 Anti-freeze solution preparation

Anti-freeze solution is made from 30% of Ethylene glycol, 15% of sucrose, 1XPBS, and 0.02% sodium azide.

2.3 Experimental Procedures

2.3.1 Context fear conditioning system

Context fear conditioning chambers were purchased from Med Associates Inc (St. Albans, VT 05478, USA). 4 identical context fear conditioning chambers (59.59cm (L) × 71.12cm (W) × 31.75cm (H), Med Associates, Inc. VT, USA). Each chamber consisted of two grey aluminium walls and a plexiglass door facing a video camera. The video camera captures the image through infrared light to allow the recording of mice while in light or dark conditions. A stimulus light on the side wall automatically turns on to indicate the start of the experiment. The photo the normal conditioning context (context A) showed below:
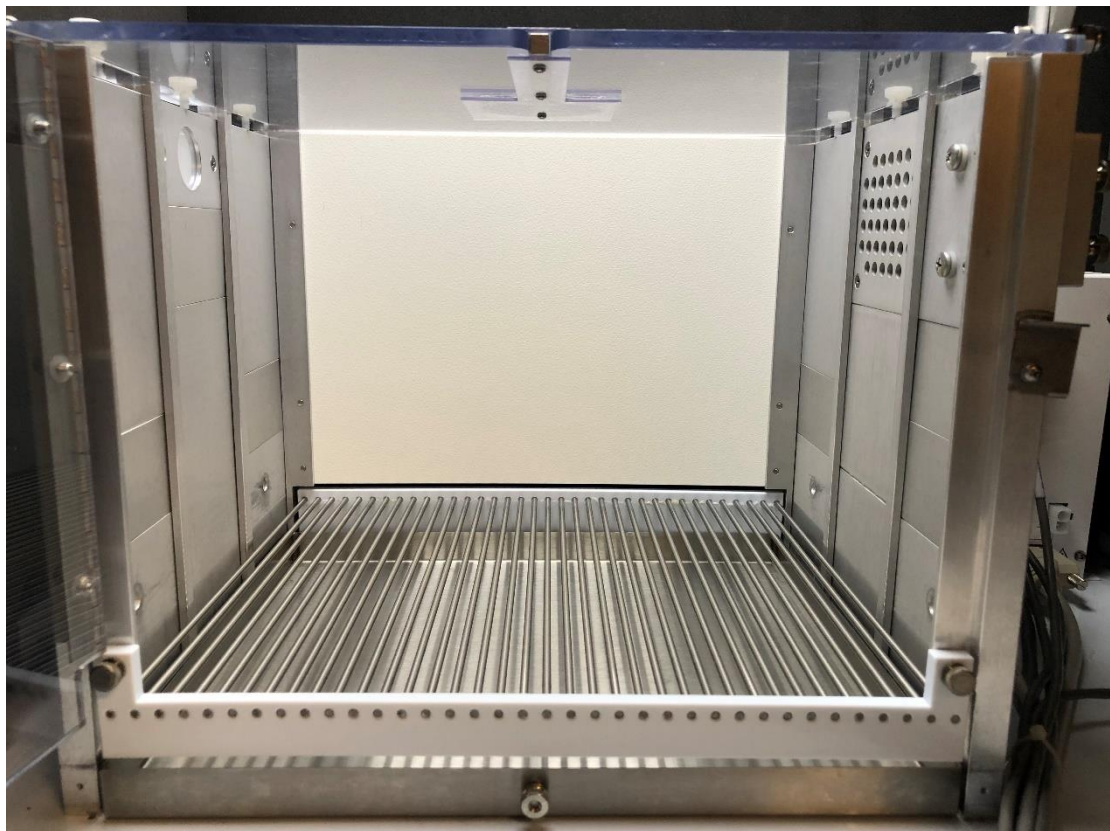
Fig. 1. Normal context (context A)

Besides the normal context, additional accessories were used to design different contexts showed below:
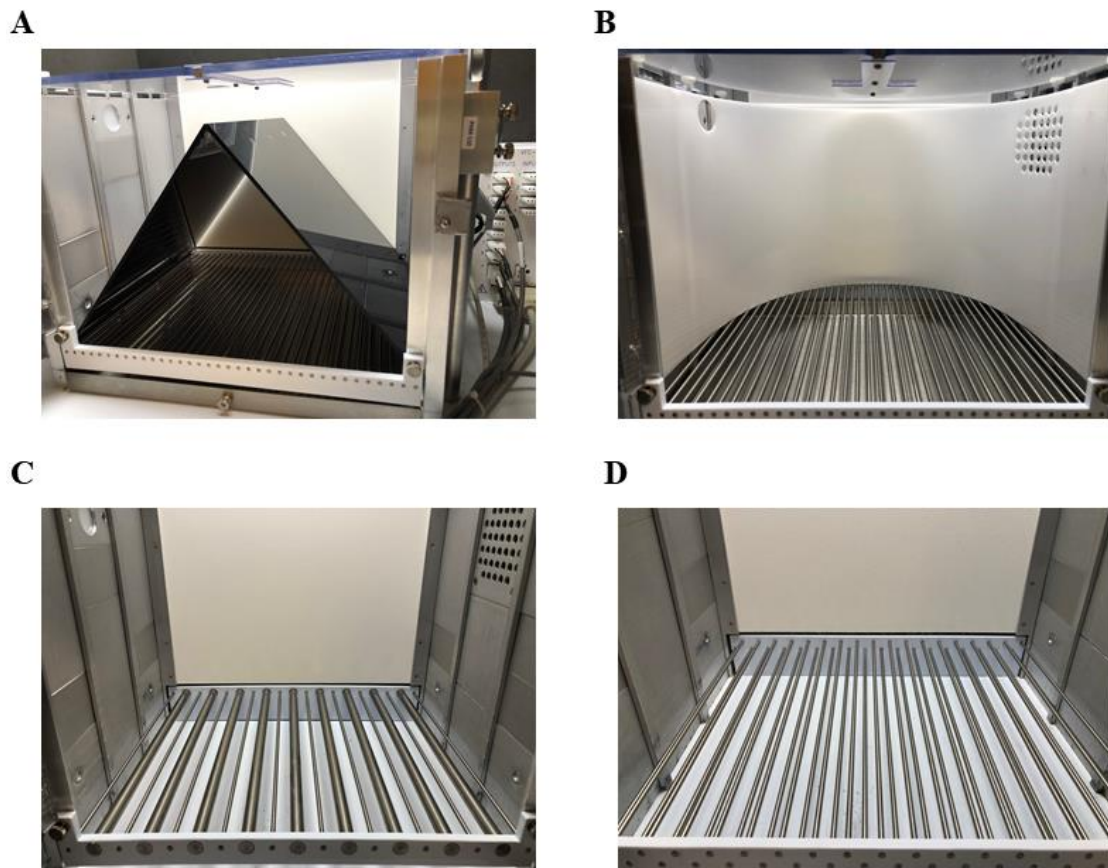
Fig 2. CFC accessories. (**A**) A frame insert. (**B**) white curved wall. (**C**) Thick/thin grid. (**D**) Stagger grid.

2.3.2 Context fear conditioning (CFC) and memory tests

In context fear conditioning, four mice were conditioned and/or tested at the same time. Mice received a single 2s, 1mA foot-shock in context A (except for the context design experiments in chapter five) and were left in the chambers for 30s post-shocking before returning to home cages. The memory tests, lasting 180s, were performed at 24h post-conditioning, unless indicated otherwise. For memory tests, the mice were re-exposed to testing context for 180s without foot-shock. The testing behavioral videos and results were recorded and calculated by "Video Freeze" software (Med associates Inc. SOF-843). The memory test results indicated as the percentage of

freezing (formula showed below), and the freezing was calculated based on the accumulated freezing duration and the total testing duration:

$$Freezing~\% = \frac{accumulated~freezing~duration}{testing~duration} \times 100\%$$

2.3.3 HPC and PFC cannula implantation

All surgical tools were soaked in chlorhexidine overnight before the surgery. Mice (7-8wks old) were anaesthetized by i.p. injection of a mixture of ketamine (150 mg/kg of body weight, Mavlab, Slacks Creek, QLD, Australia) and xylazine (100 mg/kg of body weight, Troy laboratories Pty Ltd, Smithfield, Australia) 20 mins prior to the cannulation surgery. The surgery only commenced if no response was observed from pinching the feet, confirming complete anaesthesia in mice. The hair from the ears to the eyes was carefully shaved to have a clear vision of the top of the head. The head was fixed in the stereotaxic frame (KOPF model 963) through the ear bars and the nose bar. The shaved region was disinfected with ethanol swap before incision. The incision was made with a blade, size 22, on the midline of the head. After incision, the excess tissue underneath the skin was cleaned and removed with hydrogen peroxide (20%). Two holes (one hole per side) were drilled at the target coordinates (DG: AP: -1.9mm, ML: ± 1.5mm, DV: -2.2mm; ACC: AP: +1.9mm, ML: ± 0.15mm, DV: -1.7mm) for cannula guides (PlasticsOne: C235G-0.7/PSC, 26GA for the ACC; #C235G-3.0/PSC, 26GA for the HPC) implantation. The cannula guide was made of two hollow steel tubes and a plastic petal. The steel tubes were placed into the holes with a moveable cannula holder, and the plastic petal merely attached to the skull surface. After being implanted, the petal was fixed onto the skull surface with dental cement, which was subsequently solidified by UV light. Once the cannula guide was fixed, the skin was

sutured up, and betadine was applied to the incision site. The cannula was covered with dust cap (PlasticsOne: 303DC/1), and the mouse was placed to a new home cage with food and water ab libitum for recovery. The conditions of the cannulated mice were closely monitored after surgery. The monitored healthy condition includes unusual activity, alertness, pilo erection, sedation, body posture hunched, inflammation, gripping, eye state, scar state, and cannula falling.

2.3.4 HPC coordinates validation

There were six mice used in dye injection validation. Mice were injected with anesthetics (8.7 mg/ml ketamine and 2mg/ml xylazine) through i.p. injection approximately 15min prior to the surgery. The heads of mice were horizontally fixed in the stereotaxic instrument in a flat position. The coordinates (anterior-posterior (AP), medial-lateral (ML), dorsal-ventral (DV)) of target regions were indicated according to Bregma (AP: 0.00mm, ML: 0.00mm, DV: 0.00mm). Bregma is an anatomical point at which coronal suture is intersected by sagittal suture. All mice were injected with Alexa Fluor 594 Biocytin (supplier: Sigma-Aldrich) into the dorsal CA1 region of the hippocampus (AP: -1.9mm, ML: ±0.35 mm, DV: -1.7mm to Bregma) bilaterally for 2 µL at each side using a Neuros Syringe (25 µL, Model 1702 RN, 33 gauge; supplier: Hamilton, 4970 Energy Way Reno, NV 89502 U.S.A.). The needle was slowly lowered to the target site, where Alexa Fluor 594 was injected for ~2mins and remained for another 10mins before injecting the other side. Brain coronal sections were prepared through cutting on a cryostat followed by staining with DAPI (4',6-Diamidino-2-phenylindole, 0.1% v/v, supplier: Sigma-Aldrich). The images of the coronal sections were acquired through a fluorescent microscope (Leica DM400, supplier: Leica microsystems, 11 Talavera Road, Macquarie Park, 2113, Australia).

AF 594 and AF 488 were injected by different methods but at the same coordinates (AP: -1.9mm, ML: ±1.5mm, DV: -1.7mm). The AF 594 was injected directly into the dHPC in stereotaxic surgery through a Hamilton syringe. 10 mins after

the injection, the brain was collected and fixed in 4% PFA. The mice were in anaesthesia during the entire process. Nevertheless, the AF 488 was injected into cannulated mice while the mice were awake and freely moving. The brains were collected 15mins after cannula injection and fixed in 4% PFA.

2.3.5 Cannula placement validation

Mice were implanted with cannula guides following the procedures listed above, and their post-surgery condition was closely monitored. After seven days of recovery, the cannulated mice were infused with ACSF (Tocris, Cat.NO. 3525). After the infusion, the mice were perfused by 4% PFA, and their brains were collected and proceeded with counterstaining to validate the coordinates of the HPC. In order to identify the placement in different mice, I matched the microscopy images with the brain atlas using ImageJ. Then I marked the placement site in the matched brain atlas.

2.3.6 Drug infusion through cannula

In this study, I infused ACSF (Tocris, Cat.NO. 3525) and muscimol (Tocris, Cat. NO. 0289, Batch NO. 10) into the PFC and the HPC through a cannula, and all mice were awake and freely moving during the infusion. The internals (PlasticOne, C235I/SPC, 33GA) were inserted into the cannula guide for 1 min before the infusion. Both ACSF and muscimol were infused with a PHD ultra-syringe pump (Harvard Apparatus) in a volume of 250nl at a rate of 300nl/min. After the infusion was completed, the internals stayed in the cannula for another 1 min.

2.3.7 Engram neurons labelling

I labelled the neurons that were activated at conditioning and reactivated at. Since contextual memory is initially encoded in the HPC, I specifically identify the contextual engram neurons in the DG. The method I used in this project is based on a FosCretdT system. The transgenic mice TRAP2 (targeted recombination in active populations) contains two transgenic loci as follows:1.Tamoxifen inducible Cre recombinase (CreER). 2.tdTomato as a reporter gene. CreER is driven by the c-fos promoter and synthesises Cre recombinase infused with estrogen receptor 2 (CreERT2) in the cytoplasm. The reporter gene, tdTomato, is prevented from being expressed by the stop sequence regulated by the loxp sites. In the study, the two loxp sites are palindromic repeats that could excise the stop sequence in between at memory formation. Once the neurons receive stimuli and 4-hydroxytamoxytamoxifen (4-OHT) at the same time, the 4-OHT will combine with CreERT2 and enter the nuclei binding the loxp sites. Then the loxp sites will excise the stop sequence to express the reporter gene, tdTomato (Fig. 1) (DeNard., et al., 2019). Thus, injecting 4-OHT at conditioning would label the activated neurons with tdTomato. After the memory test, the activated neurons will be labelled by a c-fos antibody. Therefore, the engram neurons that are activated at conditioning and reactivated retrieval can be identified in this study.
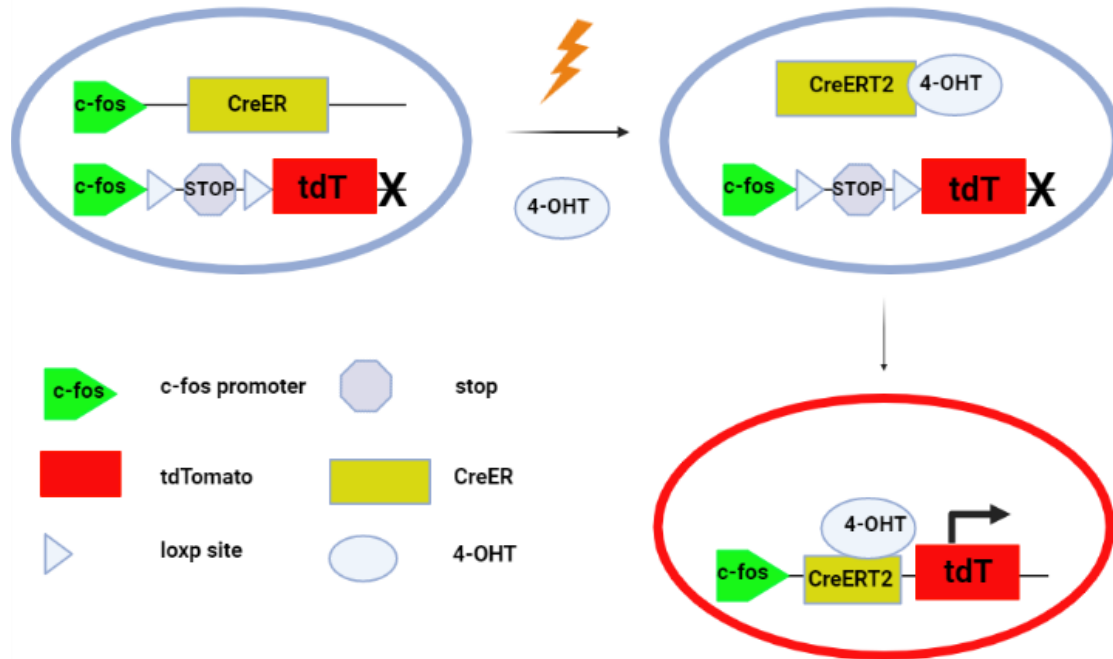
Fig.3. Schematic of fosCretdT system.

The FosCretdT mice were kept in the dark for three days before experiments. 17 mice were allocated to the engram cell labelling group and three control groups. On the experiment day, for the cell labelling group: 5 mice were injected with 4-OHT 30 mins before being conditioned at 30s PSI. For the 4-OHT control group: 4 mice were conditioned at the same PSI without 4-OHT injection. For the shock control group: 4 mice were placed into the conditioning chamber but without receiving foot-shock. For the conditioning control group: 4 mice were injected with 4-OHT in their home cage. All mice were tested seven days after conditioning.

Once the neurons were labelled, activation rate, reactivation, and reactivation rate chance were calculated as follows:

Activation rate $= \frac{tdT+neurons}{DAPI+neurons} \times 100\%$

$$\text{Reactivation rate} = \frac{tdT + c-fos+ \; neurons}{tDT+neurons} \times 100\%$$

$$\text{Reaction rate chance} = \frac{tdT+neurons}{DAPI+neurons} \times \frac{c-fos+neurons}{DAPI+neurons} \times 100\%$$

2.3.8 Perfusion and brain fixation procedure

Before perfusion, mice were anaesthetized by ketamine (150 mg/kg of body weight) and xylazine (100 mg/kg of body weight). No response from pinching the feet confirmed a complete anaesthesia state. After anaesthesia, the mouse was fixed on a pad by stapling the four legs. The chest and ribs were cut open to have a clear vision of the heart. Needle, size 25, stabbed into the left ventricle to infuse 0.9% saline and 4% PFA, and the right atrium was cut open, so the blood and solutions could flow away. 0.9% saline was first perfused through the mice for 2 mins and subsequently switched to 4% PFA solution to perfuse for 10 mins until the body was stiff. After perfusion, the brain was collected and soaked in 4% PFA solution for two days and transferred to 30% sucrose solution for another two days. After two days in sucrose solution, collected brains were embedded in optimal cutting temperature (OCT) and stored in a -80 °C freezer. Once the embedded brains were fully frozen, the brains were sliced at a thickness of 35 μm using a cryostat. The brain slices were stored at room temperature in 1XPBS until being used for counterstaining or immunohistochemistry staining.

2.3.9 Counterstaining and slides preparation

The brain slices were mounted onto gelatin-coated slides and dried at room temperature overnight before counterstaining. The dried brain slices were first washed with distilled water twice for the 30s each time. Then, the slides were immersed in 70%,

80%, and 100% ethanol for 2mins each. Next, the slides were immersed in 0.1% of Cresyl violet (ProSciTech, No. DcW-3) for 1min to stain. After staining, the slides were washed with distilled water, 70%, 80%, and 100% ethanol for the 30s each. After washing, the slides were immersed in 100% xylene for 2mins, and the excess xylene was cleaned from the slides afterwards. Eukitt Quick-hardening mounting medium (Sigma-Aldrich, 03989-500ML) was used as a mounting medium to cover the mounted slices before covering them by a coverslip.

2.3.10 IHC staining and slides preparation

The brain slices were immersed in 1%BSA and by shaking at room temperature for 1 h. Then, the slices were transferred to a c-fos antibody and by shaking at 4 °C for three nights. On the fourth day, the slices were washed with 1X PBS for 30mins before adding a secondary antibody. The secondary antibody, Alex flour 488, was added to the slices and by shaking in the dark at room temperature for 2 hrs. After shaking, the slices were transferred to 0.1M phosphate buffer (PB) and by shaking in the dark for 30 mins. Then, the slices were immersed in DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride, ThermoFisher, D1306) (1:2000) in the dark for 5 mins. Finally, after being stained with DAPI, the slices were washed in 0.1M phosphate buffer in the dark by shaking for 10 mins before mounting. In mounting, fluoromount G (ThermoFisher, 00-4958-02) was used as a mounting medium before being covered by a coverslip.

I collected four brain slides stained with both c-fos primary antibody and AF 488 secondary antibody for each brain. The number of c-fos neurons is the average number from those four slides for each mouse. Besides the four slides, I also added the

fifth slide that was only stained with AF 488 secondary antibody, which is a control slide of the IHC procedure.

2.4 Image taking and data processing

2.4.1 Cell counting

All fluorescent images in this thesis were taken by slide scanner (ZEISS AxioScan. Z1) at UTS. After the images were taken, the number of labelled neurons were counted through ImageJ. For the cell counting in the DG, c-fos neurons were only counted within the DG region stained with DAPI. For the cell counting in the PFC, I matched the microscopy image with the corresponding coronal section in the brain atlas (The Mouse Brain in stereotaxic Coordinate, second edition). After matching, I only counted the labelled neurons in the ACC.

2.4.2 Data analysis and statistics

All the behavioural data analysis were performed using GraphPad Prism 8.

Chapter 3: Whether PSI affects synaptic consolidation

3.1 Introduction

In this chapter, I used context fear conditioning (CFC) to study whether PSI affects synaptic consolidation. Firstly, I validated the CFC paradigms used in our lab, which showed a significant freezing level in mice if being conditioned with a decent PSI. Next, to further optimize the paradigm, I performed the memory retrieval tests using different sex, different testing times, and different set-ups outside the chamber. After the paradigm validation, I studied whether PSI affects synaptic consolidation rate. The previous study has shown that short-PSI memory is less precise than long-PSI memory, which reflects in they have different abilities to discriminate similar contexts (Zinn*, et al*., 2020). Moreover, those memories also recruited different amounts of cell populations during memory formation (Leak, *et al*., 2021). However, it is still unknown whether the memories with different cell populations and different precision undergo the same synaptic consolidation process.

The most important feature of synaptic consolidation is it requires protein synthesis before completing the consolidation (Benfenati, & Valtorta, 2015; Nader, et al., 2000; Barrientos, et al., 2002 Touzani, et al., 2007; Bourtchouladze, et al., 1998; Squire, & Barondes, 1974). Therefore, in this study, I tested the time points when each memory completed its synaptic consolidation by measuring its protein synthesis dependency. In order to disrupt protein synthesis and interrupt synaptic consolidation subsequently, I validated the potency of the protein synthesis inhibitor, anisomycin, used in the research. Anisomycin has been reported to disrupt protein synthesis by

inhibiting the 80S ribosome system (Osorio-Gómez et al., 2019; Wanisch, & Wotijak, 2008; Lattal., et al., 2004). After the potency validation, I tested the protein synthesis dependency at a variety of time points during the synaptic consolidation window. The final result showed that the short and the long PSI memories have the same protein synthesis window during synaptic consolidation.

3.2 Results

3.2.1 CFC validation

In order to use the CFC paradigm to address the questions above, I first started with validating this approach through a series of experiments. The validation experiments consisted of CFC validation for recent/remote memories, testing time points validation, and sex validation. In order to study both synaptic and systems consolidation, the fear memory formed in this CFC paradigm needs to be retrieved at different time points. Thus, the first validation was to test the freezing levels at both recent and remote time points when the mice were conditioned with different PSIs (immediate, 180s and 720s). In the CFC validation experiment, male mice were assigned to three PSI groups (immediate, 180s, 720s). After being introduced to context A, each group of mice received a mild foot shock either immediately, 180s or 720s. As for the control group, mice were exposed to context A for 210s without foot-shock. For recent memory tests, all mice were re-exposed to context A for 180s without foot-shock at 24h post-conditioning. The same mice were retested in context two weeks post-conditioning for remote memory tests. The 180s and 720s PSI memories showed significant freezing compared to the no shock control at both recent and remote tests.

However, the immediate shock group showed freezing at recent tests but not at remote tests suggesting no ISD at recent tests (Fig. 1).
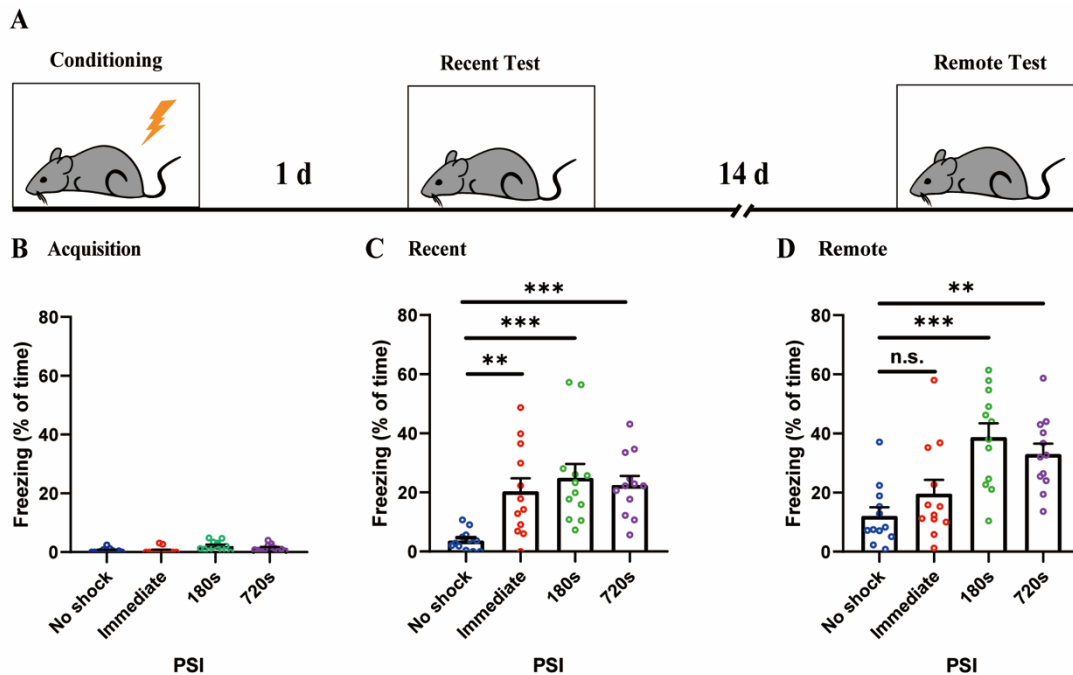
Fig. 1. CFC paradigm validation. (**A**) CFC behavioural paradigm. (**B**) Mice were given 2s, 1mA foot-shock at immediate, 180s, and 720s PSI in context A, and the no shock control was achieved by exposing the mice to context A for 210s without delivering foot-shock. This serves as the baseline freezing levels before receiving foot-shock. (**C**) Mice were tested in the conditioning context at 24h post-conditioning as a recent memory test. (**D**) The same mice groups from (C) were re-tested 14 days post-conditioning as a remote memory test. Bar graph represents the group means ± SEM, N = 12/group. Statistical analysis was performed via 2-way ANOVA. Significant differences from No shock control are denoted with asterisks: **$P<0.005$, ***$P<0.001$, n.s. = not significant.

Next, I explored at which recent time points mice show the highest freezing level. This is because a robust freezing level would be easier to detect the differences following various manipulations including drugs and contexts shifts in future experiments. Thus, I conditioned the mice in context A (180s PSI) on day 0 and tested starting on one of the following days: day 1, day 2, day 3, and day 4 (Fig. 2A). After I

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analysed their freezing levels, no difference was found among the first four testing days (Fig. 2A).

The next validation was performed regarding sex differences. Similar to the CFC paradigm validation, instead of male mice, the females were conditioned with 180s PSI in context A and tested in the same context one day after conditioning. Female mice were conditioned with one of the four PSIs (immediate, 30s, 180s, 480s), and tested 24h post-conditioning. Compared to the males, the females did not show a significant freezing at 180s or 480s PSI (Figure 2B). Thus, I decided to use males for the rest of this project.

Considering that no differences were observed when tested among the first four days after conditioning, I then aimed to generating a more robust fear by improving the chamber's environment. Thus, the last validation I conducted was cubicle designs. Cubicles are the boxes outside of the conditioning chambers covered with soundproof materials used to isolate the chambers from outside. I manipulated the cubicle outside the conditioning chamber to optimize context A. Such context A optimization involved several cubicle designs (A1, A2, A3, A4). All male mice were conditioned in context A's and tested 24h post-conditioning. These designs contain the similar components with context A except for the following modifications:

Context	Light intensity	Internal cubicle cover	Cubicle door
A1	3	N.A.	close
A2	1	N.A.	close
A3	3	White cover*	close
A4	3	White cover*	open

* The original cubicle is black inside, whereas the white cover changed the colour.

However, there was no significant difference among those trials (Fig. 2C). Thus, I used the original context A (A1) as the conditioning context for the subsequent experiments.
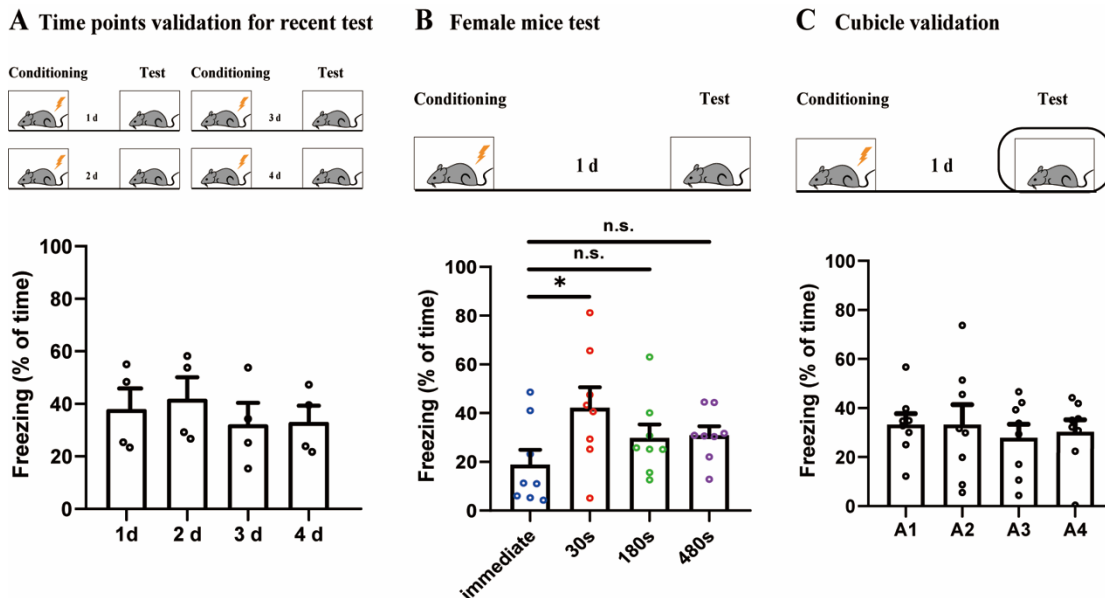
Fig. 2. Validation for the basic set-up. (**A**) Testing time points validation paradigm (upper panel) and results (lower panel). Recent memory tests were performed at 1d, 2d, 3d, and 4d post-conditioning, N = 4/group. No statistical difference was observed among the 4 separate groups via 2-way ANOVA ($P = 0.78$). (**B**) Female mice testing paradigm (upper panel) and results (lower panel). Female mice were tested at 24h post-conditioning following a 180s PSI conditioning, N = 8/group. (**C**) Cubicle validation paradigm (upper panel) and results (lower panel). Mice were conditioned and tested in 4 different cubicle designs (A1-A4), N = 8/group. No statistical difference was observed among all cubicle designs via 2-way ANOVA, $P = 0.77$. Bar graph indicates group mean ± SEM, significance is denoted with asterisks: *$P < 0.01$, n.s. = not significant.

3.2.2 PSI effects on synaptic consolidation rate

To tackle the question of whether PSI affects synaptic consolidation rate, I first need to know the synaptic consolidation ending time points when conditioned at different PSIs. The ending point of synaptic consolidation is characterized by that memory becomes robust to the disruption caused by protein synthesis inhibitor, anisomycin. Thus, I first validated the efficacy of anisomycin through a pilot experiment. In the pilot experiment, Male mice (N=12) were conditioned in context A with 180s PSI. Immediately after the conditioning, each mouse was injected with either anisomycin or 0.9% saline (vehicle) through intraperitoneal (i.p.) injection prior to returning to its home cage. All mice were tested in context A twice at 24h and 14 days post conditioning. Compared to the vehicle group, the anisomycin-treated one showed a significant memory deficit (Fig. 3B), and this deficit persisted even when tested remotely (Fig. 3C).

Following the anisomycin efficacy validation, there were two sets of context fear conditioning experiments. For the first set of the experiments, mice were conditioned at either 30s or 180s after being introduced into context A. After conditioning, each mouse was injected with anisomycin or vehicle at one of the five following time points: immediate (0h), 2h, 4h, 16h, 20h, or 24h post-conditioning. The mice were tested at 48h post-conditioning through re-exposing to context A for 180s without foot-shock (Fig. 3D). At both PSIs, only the immediate injection groups showed a significant memory deficit (Fig. 3E, F). Therefore, I didn't find any differences between the two PSIs in synaptic consolidation. Then to confirm that this was the case across a very wide PSI range, I conditioned the mice with either 30s or 720s PSI and injected them with anisomycin or vehicle at one of the three following

time points: immediate, 1h, or 2h post-conditioning. The mice were tested in context A at 24h post-conditioning (Fig. 3G). Similar to the last results, no difference when injected at 1h or 2h after conditioning (Fig. 3H-J).
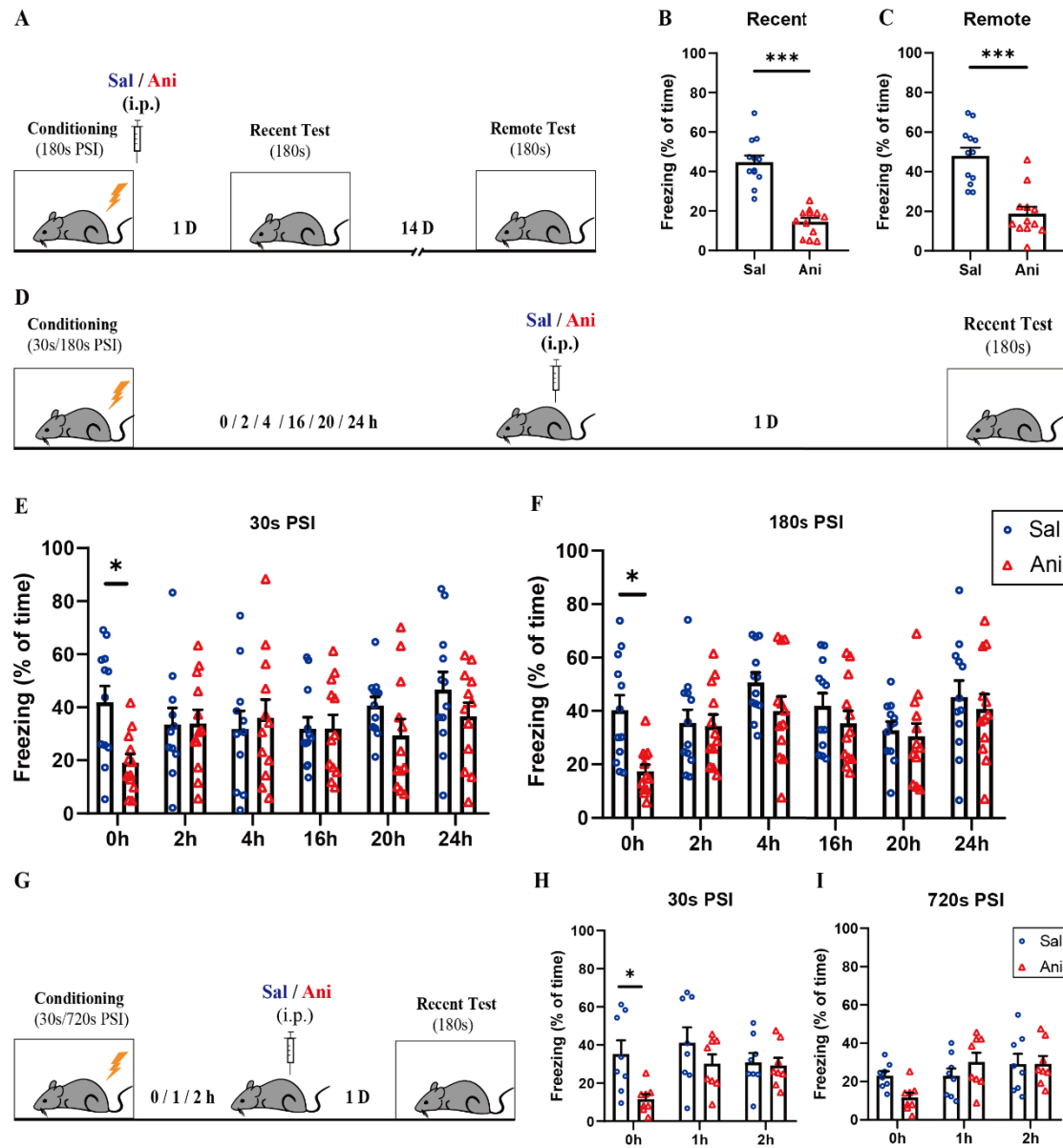
Fig.3. Anisomycin effect on synaptic consolidation. (A) Behavioural paradigm for anisomycin efficacy validation. Ani: anisomycin, Sal: saline. (**B**) Mice were injected with anisomycin or saline immediately after CFC in context A and tested at 24h later for recent memory test, N = 12/group. (**C**) The same group of mice were retested14 days after CFC as remote memory test, N = 12/group. (**D**) Behavioural paradigm for studying anisomycin effect on synaptic consolidation rate. Mice were i.p. injected with

either ANI or VEH at one of the following time points after conditioning: immediate, 2h, 4h, 16h, 20h, 24h. All mice were immediately returned to their home cages after i.p. injection. The recent memory tests were conducted at 2 days post-conditioning. (**E**) Memory tests for 30s PSI after Ani/Sal injections, N = 12/group. (**F**) Memory tests for 180s PSI after Ani/Sal injections, N = 12/group. (**G**) Behavioural paradigm for the prolonged PSI experiments. (**H**) Memory tests for 30s PSI after Ani/Sal injections, N = 8/group. (**I**) Memory tests for 720s PSI after Ani/Sal injections, N = 8/group. Statistical analysis was performed via Mann-Whitney test for (B-C), and via Sidak's multiple comparison test for (E-I). Bar graph represents the group means ± SEM, significance is denoted with asterisks: *P<0.05, ***P<0.001, n.s. = not significant.

3.3 Discussion

It is largely unknown whether poorly formed memory consolidates in the same manner as well-formed memory does. Memory consolidation rate is an important readout that potentially sheds light on the consolidation process from a behavioural perspective. Thus, I investigated the memories formed with different learning durations regarding their consolidation rate. The first aim of my thesis is mainly to address the question of whether the memories formed with different PSIs undergo synaptic consolidation at the same rate. I first aimed to establish a reliable CFC paradigm that generates consistent freezing upon testing the animals. On the basis of this CFC protocol, I proceeded to explore whether PSI, defining the learning duration, affects synaptic consolidation rate.

Context fear conditioning is the main behavioural paradigm used in this project. The freezing level was validated before conducting experiments. The freezing level is defined as how much time the mice spent in the freezing status during the tests. Previously, our lab calculated the freezing duration by manually scoring in the test videos. However, our lab updated the systems when I started this project with a new conditioning chamber and other apparatus. The new system automatically records and

scores the freezing level at the tests. So, the validation trials did not just validate the behavioural paradigm but also the parameters of the auto-recording systems. After having a reliable recording system, I began with context fear conditioning validation.

The first set of experiments validated the basic CFC paradigm used in this study. Conditioned mice showed constant freezing when conditioned with different PSIs (Fig. 1). However, although the CFC paradigm generated consistent freezing across different trials, the immediate shock group also showed freezing at recent tests suggesting no ISD in this paradigm (Fig. 1). No ISD observed at the recent tests implies those memories might not be contextual. This is because forming contextual fear memory requires time. Nevertheless, an alternative interpretation could be that the memory was contextual but that there was no ISD because the mice displayed a non-contextual fear during the recent tests. This could be for various reasons, including associating the shock with mice handling or being scared of a new salient environment. Firstly, since this study aims to compare the memories formed with different learning durations, the mice were not handled in these experiments in order to avoid context preexposure. Thus, the freezing at the recent tests might be because of the lack of handling. Secondly, the stimulus light might affect the memory as well, as that light was turned on during the recording. There is a small unlikely possibility stimulus light might became a discrete CS for the mice where there is a short PSI and further experiments would be needed to entirely rule out the possibility. However, we considered that the stimulus light was turned on all the time during conditioning, which can be regarded as a part of the context. Also, the light was not matched with the shock. Although I cannot fully delineate the possibilities, it is important to note that the ISD did emerge at a remote time. This would

seem to support the latter argument more. It would also be consistent with the previous studies that have used these apparatuses to study CFC.

Following the validations, I tested the parameters (testing time, sex, and cubicle) that may affect the CFC protocol. None of those parameters affected contextual fear memory in the current CFC protocol (Fig. 2). The result suggests the conditioning chamber isolated the outside environment from the chamber, and the freezing response is caused by the context from the inside chamber. However, due to the limited amount of mice used in this project, results from the sex differences experiments were inconclusive. Thus, the sex choice in this study was based on a previous paper published by Fanselow, et al. In that paper, they found male rats showed significantly higher freezing levels at the tests than the females when conditioned at the 20s, 40s and 60s PSI (Fanselow. et al., 2001). Since some of my experiments used 30s PSI, I chose the male mice in this study. However, the results from studying the males are becoming increasingly clear that those results may not apply to females. A further dedicated study of females would be valuable. After finishing the validation experiments, I proceeded investigate whether PSI affects synaptic consolidation rate. Through comparing the finishing time points of each memory, I can subsequently compare their consolidation rate, i.e. completing synaptic consolidation earlier means that memory has a faster consolidation rate. This mentioned, I decided to consider targeting protein synthesis, which is an indispensable step in synaptic consolidation. Being no longer dependent on protein synthesis can be regarded as a sign of completing synaptic consolidation. Thus, the finishing time point of synaptic consolidation can be viewed as when memory becomes resilient to a protein synthesis inhibitor, anisomycin. Based on this fact, we designed aim 1 experiments.

Firstly, I started with validating the efficacy of the anisomycin used in this project (Fig. 3A). The result showed that anisomycin successfully impaired the expression of contextual memory when injected immediately after conditioning and that this impairment was not recovered at recent, nor remote memory tests (Fig. 3B,C). Due to its short half-life (about 2-3 hrs), such long-term memory impairment is not because of the persisted potency of the drug (Wanisch & Wotjak, 2007; Bekinschtein. et al., 2007). Instead, the disruption at the early stage of memory consolidation by anisomycin accounts for such impairment and causes a compromised memory in the future. This result is consistent with the previous findings showing that anisomycin disrupts memory storage instead of memory retrieval (Hardt, et al., 2009).

Next, to investigate whether PSI affects synaptic consolidation rate, I compared the memories formed at short (30s) and long (180s) PSIs. The PSIs chosen in this experiment were based on previous literature in which Zinn, et al has shown that the quality of memory can be varied by conditioning at different PSIs (Zinn, et al., 2020). In that paper, the memory formed at 30s PSI was inaccurate, where mice could not discriminate between two similar contexts, whereas 180s PSI was sufficient to allow them to acquire an accurate memory and reliably discriminate between two similar contexts (Zinn, et al., 2020). In this study, those groups completed synaptic consolidation at the same time (Fig. 3E, F). To further confirm that synaptic consolidation rate was not subject to PSI, I increased the PSI range by using 30s and 720s (Fig. 3 G-I). Also, since the first few hours are the most important time window for synaptic consolidation, I narrowed the injection time interval to 1h. Similar to the previous results, memory deficits can only be observed in the groups that were injected

with anisomycin immediately after conditioning. These results showed that the memories formed with different PSI groups were only dependent on protein synthesis for a short time after conditioning. Altogether, the results suggest that even though PSI can induce different completeness of memories, synaptic consolidation rate is not affected by it. In addition, the fundamental mechanism of synaptic consolidation is forming new synapses between engram neurons (Druckmann, et al., 2014; Abel., et al., 2001). This process is determined by many factors besides *de novo* protein synthesis, such as neuron excitability, CREB expression, and LTP maintenance (Ling, et al., 2002; Yao., et al., 2008). In future, investigating those non-protein syntheses processes could further inform us of whether synaptic consolidation is, in fact, different between PSIs. This would significantly change our conclusion about whether memories of different levels of completeness undergo synaptic consolidation at the same rate or not.

This study, for the first time, investigated the difference between short and long PSI memories regarding their consolidation rate. Regardless of the quality, contextual information is always integrated into the memory and consolidates at a consistent rate. Once the acquired contextual information reaches a certain threshold (30s PSI), it will serve as a conditioned stimulus to associate with unconditioned stimulus and consolidate. Any extra contextual information will not expedite the protein synthesis-dependent part of the consolidation process. This is supported by previous literature and my validation experiments where 30s PSI generated the same freezing levels as the 180s and 720s PSIs. However, the freezing levels were significantly lower if conditioned at 15s PSI (Zinn, et al., 2020). On the surface of it, this could suggest that synaptic consolidation as a whole occurs at the same rate across different PSIs.

In summary, this section has explored whether PSI affects synaptic consolidation rate. The results have shown that memories formed with different PSIs completed the protein synthesis dependent phase of synaptic consolidation at the same rate. In my next aim, I will study the second stage of memory consolidation, systems consolidation, and explore whether it is affected by initial learning.

Chapter 4: Whether PSI affects systems consolidation

4.1 Introduction

In this chapter, I studied whether PSI affects systems consolidation. Systems consolidation is long-term consolidation that transfers hippocampal memory to cortical memory (Frankland., et al., 2005; Frankland., et al., 2006; Restivo, et al., 2009). The two well-accepted systems consolidation theories are the standard model theory (SMT) and the multiple trace theory (MTT). The SMT posits that the information encoded in the HPC is transferred to the PFC during systems consolidation (Corcoran, et al., 2016; Einarsson et al., 2012; Einarsson., et al., 2014). By contrast, the MTT posits that multiple memory traces are generated in the HPC and the PFC extracting the generalized memory instead of transferring the information between the two regions (Nadel, et al., 1997; Nadel., et al, 2000; Sekeres., et al., 2018; Sweegers., et al., 2014). Regardless of the differences between the two theories, both posit that systems consolidation requires interactions between the HPC and the PFC to consolidate memory in the long term (Aceti., et al., 2015; Bian., et al., 2019; Cullen., et al., 2015). Also, memory expression depends on the HPC for a certain amount of time, after which it becomes HPC independent and dependent on the PFC (Barry, et al., 2016; Ding., et al., 2008, Kitamura., et al., 2017; Rozeske., et al, 2014; Teixeira, et al., 2006). By measuring this dependency shift, I could decide whether the memory completed systems consolidation or not.

Contextual memory is encoded and retrieved from the hippocampus before completing systems consolidation (Barry, et al., 2016; Ding., et al., 2008, Kitamura., et

al., 2017). Hence, if the memory retrieval is impaired by hippocampal inhibition, it suggests that the memory is still undergoing systems consolidation and HPC dependent. In this section, I used a GABA receptor agonist, muscimol, to inhibit the HPC and subsequently interrupt the memory retrieval during the systems consolidation window. I found the 720s PSI memory showed its HPC dependence at the beginning of systems consolidation and gradually became HPC independent at remote time points. Surprisingly, I found that the 30s PSI memory was not subject to hippocampal inhibition since the beginning. These findings are discussed in detail in the chapter discussion and general discussion.

4.2 Results

4.2.1 Placement and HPC coordinates validation

To address the question of whether PSI affects systems consolidation rate, I started with identifying where contextual fear memories are located. Firstly, I validated the HPC coordinates used in previous literature by injecting fluorescent dye (Alex Flour 594) (Fig. 1A). The coordinates (AP: -1.9mm, ML:±1.5mm, DV:-1.7mm) reported in previous publications also fit the mice I used in my research (Kitamura., et al., 2017). Next, I also validated cannulated mice placement and injection sites. Another fluorescent dye (Alex Flour 488) was injected into the HPC while the cannulated mice were awake. The image of the injected fluorescent dye confirms that cannula placement is in the DG (Fig. 1B). I also validated the placement of the implanted cannula through the cannula trace in the dHPC, the DG, and the ACC (Fig. 1C-F). The validation trials have shown that our stereotaxic surgery successfully implanted cannulas into the target locations.
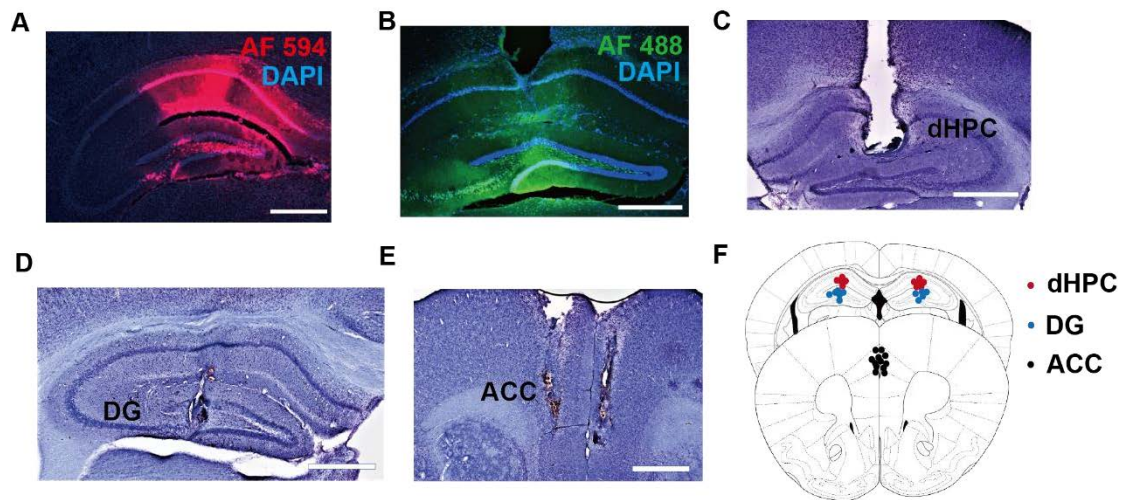
Fig 1. Coordinates and placement validation. (A) Injecting Alex Flour 594 into the dHPC through stereotaxic surgery, scale bar is 700µm. **(B)** Injecting Alex Flour 488 into the DG through a cannula, scale bar is 700µm. **(C)** Cannula placement in the dHPC, scale bar is 700µm. **(D)** Cannula placement in the DG, scale bar is 600 µm. **(E)** representative image of cannula placement in the ACC, scale bar is 800 µm. **(F)** Cannula placement confirmation.

4.2.2 PSI effects on systems consolidation rate

After validating the surgery protocol and cannula placement, I started studying whether the 30s PSI memory and the 720s PSI memory completed systems consolidation at the same time. After the cannulated mice fully recovered from surgery, they were conditioned at 30s or 720s PSI with a vehicle (ACSF) injected into the HPC 15mins prior to the conditioning. The vehicle injection ensured that the mice received the same treatment during the conditioning and the testing session. According to previous literature (Kitamura, et al., 2017), systems consolidation was completed in 14 days in mice. Therefore in my study, the conditioned mice were tested at 2, 9, or 16 days after conditioning, respectively, to cover the systems consolidation time window (Fig. 2A). To test whether the memory is encoded in the HPC at retrieval, I infused muscimol, a GABA receptor agonist used to inhibit neuron activity, into the DG at memory retrieval. This is because the DG is where detailed information is encoded and

responsible for recent memory retrieval (Silva, et al., 2016; Hobin, et al., 2006; Haubrich, et al., 2016). If memory retrieval is impaired by the hippocampal infusion of muscimol, it implies that the memory has not complete the systems consolidation. This is because before systems consolidation is completed, the memory still requires support from the hippocampal neurons to mature the PFC neurons (Kitamura, et al., 2017).

The results showed the 720s PSI memory was HPC dependent at two days after conditioning, but not after nine days, suggesting the long PSI memory gradually became HPC independent at remote time points. However, and surprisingly, I found the 30s PSI memory was not impaired by hippocampal infusion of muscimol even when tested two days after conditioning (Fig. 2B). Being independent of the HPC implies that the 30s PSI memory is either not encoded in the HPC or exists in a highly robust form to hippocampal inhibition. To further confirm that the 30s PSI memory is not affected by hippocampal inhibition, I infused muscimol into the dHPC, which would inhibit the entire HPC at retrieval. Similar to the previous result, the memory was not impaired by dHPC inhibition (Fig. 2E). Both placements were confirmed by cresyl violet staining after the tests (Fig. 2D). In addition, the c-fos expression level, a neural activity marker, was also assessed for the two-day-old memory for the dHPC inhibition group. However, no significant difference was observed between the muscimol and vehicle groups in the DG (Fig. 2 G). All cannula placements have been verified after the experiments (Fig. 3). Therefore, this result showed that the 30s PSI memory retrieval is not affected by hippocampal inhibition. However, it is still unknown whether the memory is encoded in a different location or exists in a robust form.
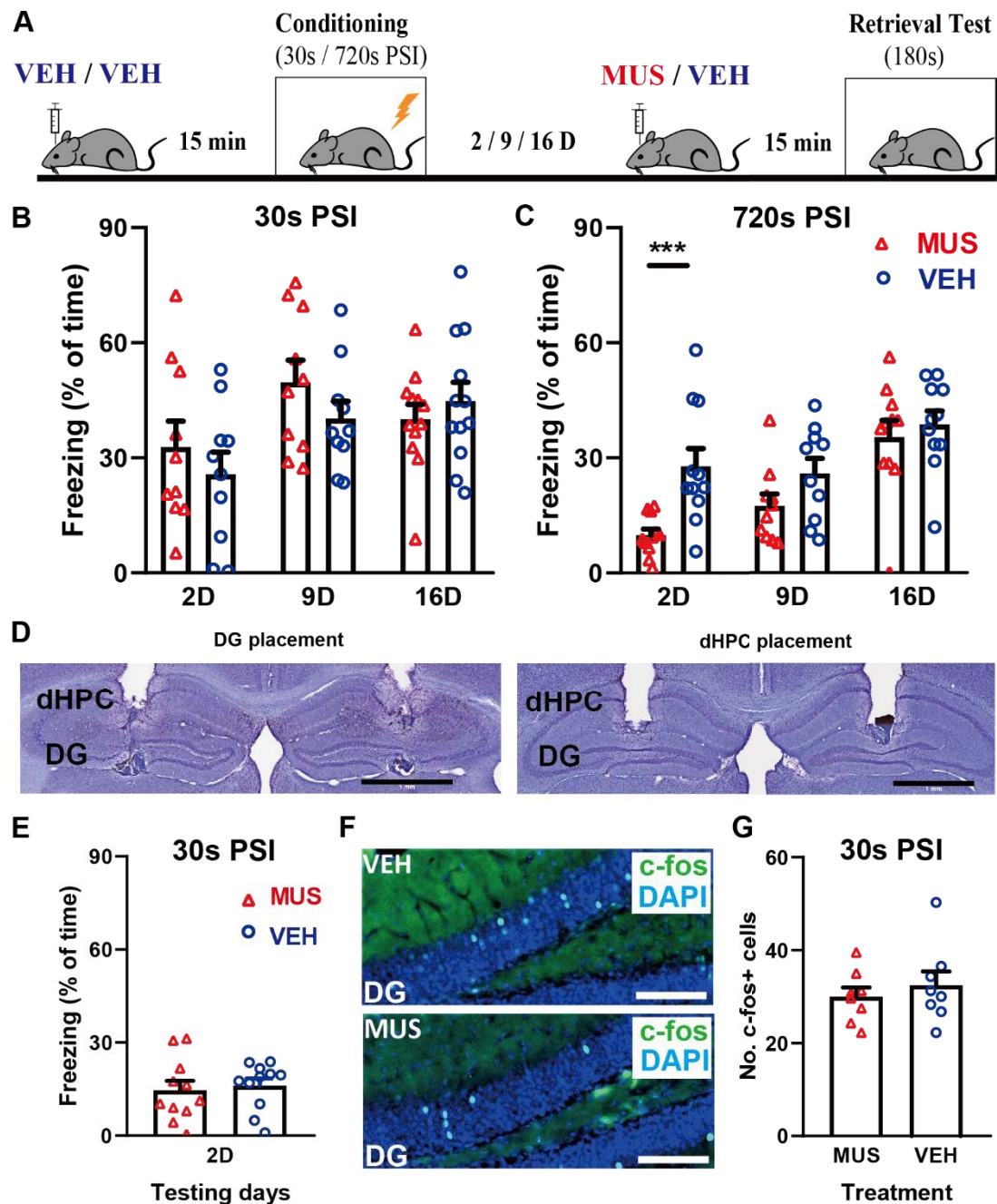
Fig. 2. Inhibition of the DG and the dHPC for the 30s and the 720s PSI memories.
(A) Behavioural schematic. **(B,C)** DG inhibition of the 30s and the 720s PSI memories retrieval at 2, 9, 16-day, respectively, N = 9~12/group. MUS: muscimol, VEH: vehicle (ACSF). **(D)** Representative image showing the DG placement (left panel) and the dHPC placement (right panel). **(E)** Retrieval test after the dHPC being inhibited, N = 11/group. **(F)** Representative image showing expression of c-fos cells in the DG, scale bar is 100 μm. **(G)** The number of c-fos+ cells from the 2-days old memory, N = 8/group. The bar graph represents the group means ± SEM, significance is denoted with asterisks: ***P<0.0001.
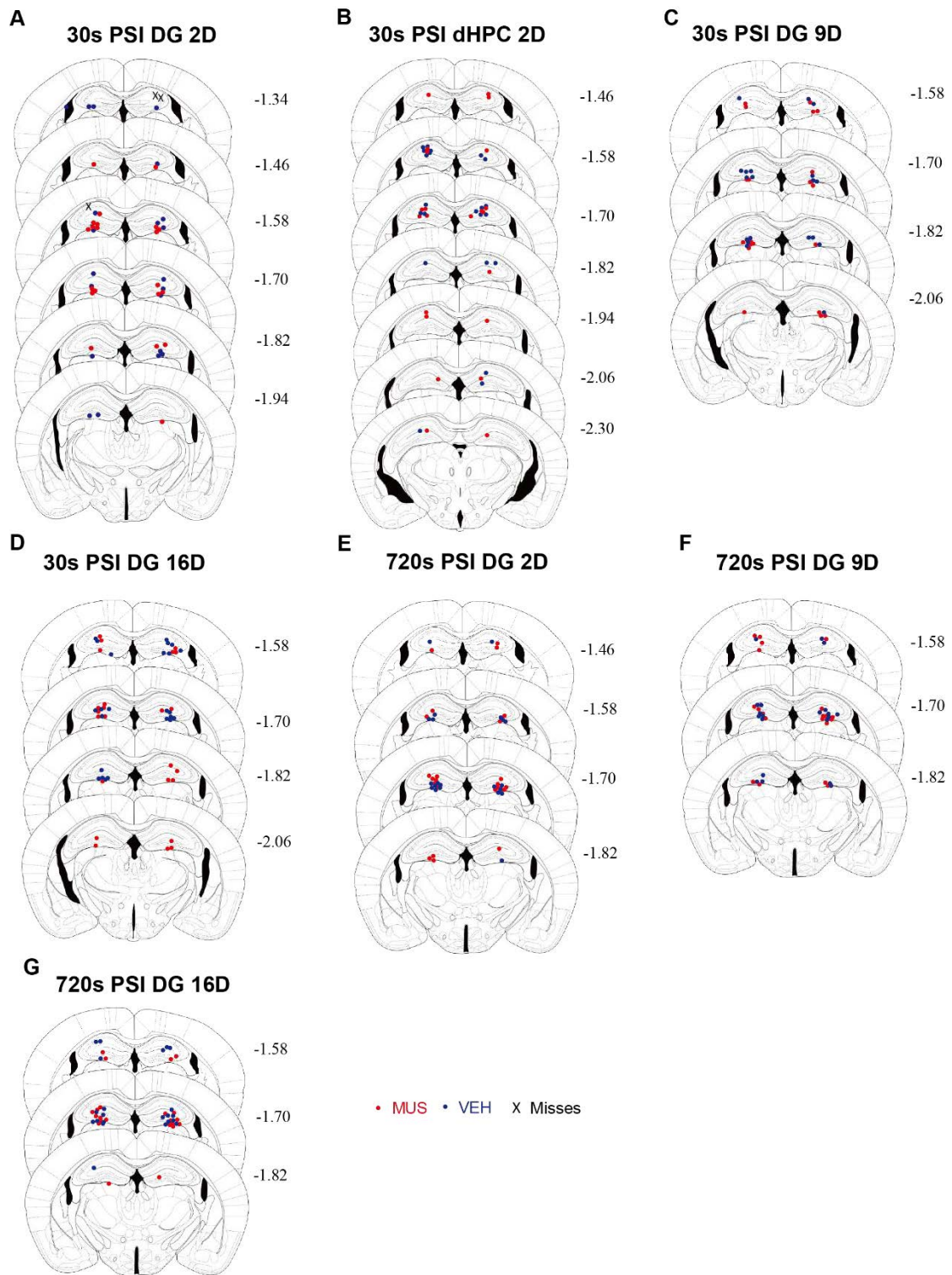
Fig 3. HPC placement verification. Verifying the cannula placement in the HPC for muscimol and vehicle injection sites.

4.2.3 Is the 30s PSI memory encoded in the HPC?

To study whether the 30s PSI memory is encoded in the HPC, the ideal method is to use optogenetics specifically to inhibit its engram neurons in the DG. Then, if the engram inhibition impaired the memory, it could prove that the 30s PSI memory is actually HPC dependent. However, since optogenetics is unavailable in our lab, I have to use a correlative analysis to obtain further information. Therefore, I used FosCretdT mice to label engram neurons in the DG and tried to find if there was a context memory formed in the HPC. FosCretdT mouse is a transgenic line expressing a reporter gene tdtomato driven by a c-fos promoter. When the neurons were activated at conditioning, the c-fos promoter in the cell will also be activated correspondingly. The 4-OHT injected at conditioning will combine the estrogen receptor 2 (ERT2) entering the nucleus (DeNardo, et al., 2018; Leak, et al., 2021). Next, 4-OHT will and ERT2 targeted the loxp site and removed the stop sequence. Once the stop sequence is removed, the reporter gene, tdTomato, will be expressed and permanently label the neurons. Thus, the neurons that are involved in memory formation were labelled by this marker (Fig. 4H). However, due to this fosCre system requires seven days to label neurons, the memory test has to be done in seven days after the conditioning (Fig. 4A). To ensure the labelled neurons are due to the conditioning only, three control experiments were conducted in this trial. In the first control group, the mice were placed in the conditioning chambers after injecting 4-OHT but did not receive the foot-shock (chamber, 4-OHT). In the second control group, the mice stayed in the home cages on the conditioning day with 4-OHT injection, and they were tested on the same day as other groups (home cage, 4-OHT). The third is conditioning at 30s PSI but not receiving 4-OHT (30s PSI, NO OHT). The conditioned groups showed a significantly high freezing level compared to the non-conditioned ones (Fig. 4B). As expected, the

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chamber and conditioned groups expressed a higher tdtomato level compared to the home cage group (Fig. 4C, E). Since all the mice were tested in the chamber on the testing day, they all showed the same c-fos expression level (Fig. 4D).

Based on the amount of neurons were activated at conditioning (tdT neurons) and those that were reactivated at the tests (c-fos neurons), I calculated the reactivation rate by dividing the number of tdT neurons by the number of c-fos neurons. The reactivation rate indicates how many initially activated neurons were reactivated at the tests. Those reactivated neurons were engram neurons that encoded a contextual memory. However, the reactivation rate was not significantly different between the conditioned and home cage group, which may be because of low sample size (Fig. 4F). A more accurate analysis, however, is the reactivation rate / chance. The reactivation rate chance indicates how likely a neuron is to be double labelled due to the learning event as opposed to chance. A value of 1 means the number of double labelled neurons does not differ from the number that would be expected by chance. In this case, it is impossible to determine whether the double labelled neurons are engram neurons or not. However, higher values indicate that an increasing percentage of the neurons cannot simply be double labelled by chance, indicating that may reflect the engram (See the calculation in the method chapter). The reactivation rate chance of the 30s PSI group is significantly higher than the home cage one (Fig. 4G). This result showed that the DG neurons were significantly activated at conditioning and reactivated in the tests, which indicates forming a contextual memory. Altogether, this experiment showed a contextual memory formed when the mice were conditioned with 30s PSI. However, more solid evidence is required to prove that memory would actually cause freezing at tests in future.
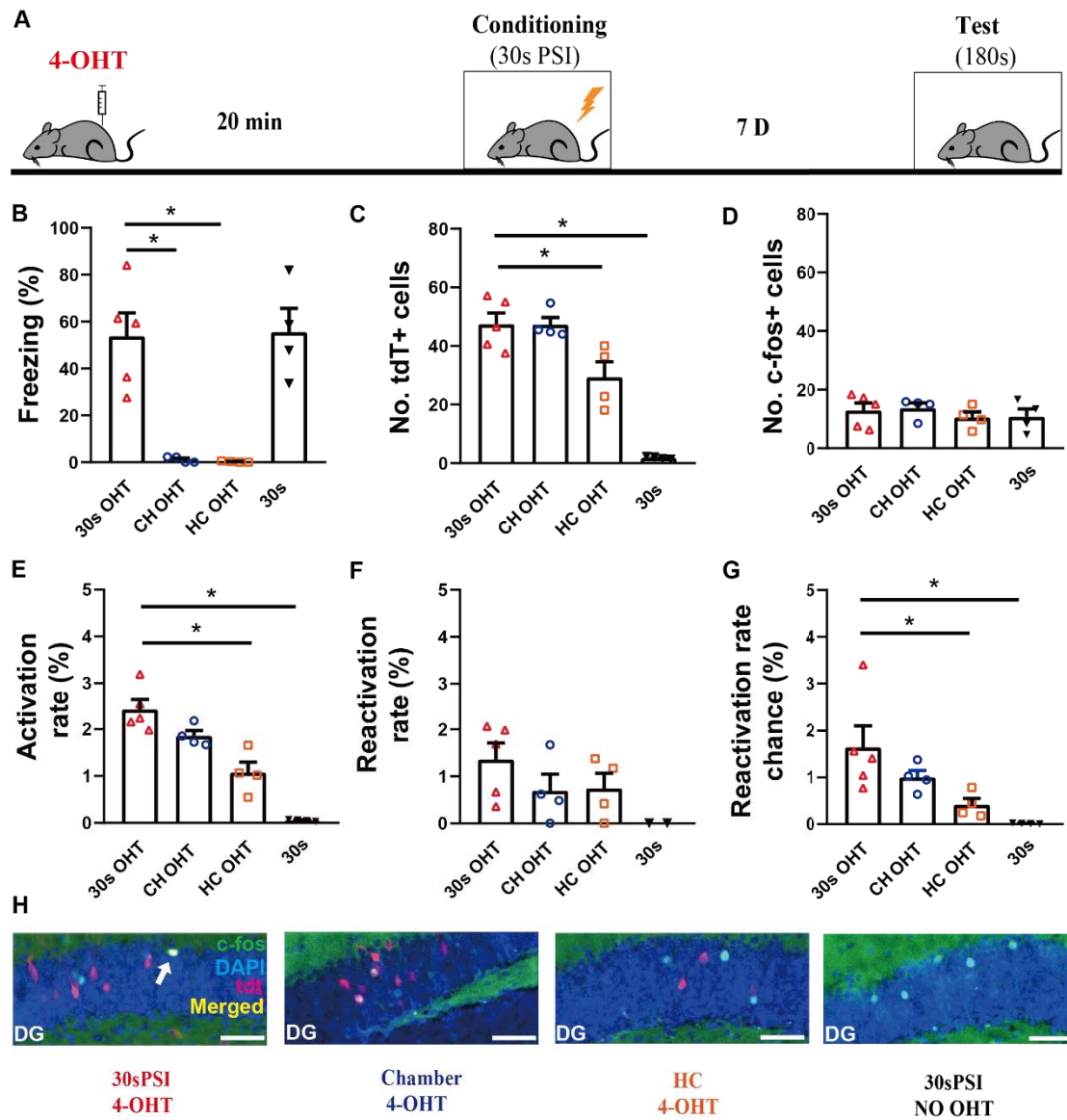
Fig. 4. Engram neurons labelling in the DG. **(A)** Behavioural schematic. **(B)** Freezing level tested on day 7 after conditioning. 30s: 30s PSI conditioning; CH: exposing to the chamber without shock; HC: home cage. **(C)** The number of neurons expressing tdT at conditioning. **(D)** The number of neurons expressing c-fos after test. **(E)** Activation rate at conditioning. **(F)** Reactivation rate after test. **(G)** Reactivation rate chance after test. N = 4~5 /group. The bar graph represents the group means ± SEM, significance is denoted with asterisks: *P<0.05. **(H)** Representative image of labelling the activated and reactivated neurons in the DG. Arrow points at the double labelled neuron, Scale bar, 50μm.

4.2.4 Is the 30s PSI memory encoded in the PFC?

The previous results indicate that the 30s PSI memory is not subject to

hippocampal inhibition, which makes me wonder whether that memory is encoded

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somewhere else. According to a previous publication (Kitamura, et al., 2017), the PFC is also involved in contextual fear memory formation. Thus, the next experiment was to study whether the 30s PSI memory is encoded in the PFC. The rationale of my design is if memory hasn't transferred to a different location, it should be retrieved from where it is encoded. On the basis of this hypothesis, if the 30s PSI memory is encoded in the PFC, inhibiting the PFC should impair its retrieval. After the mice recovered from PFC cannulation surgery, they were conditioned at the 30s or 720s PSI. Two days after the conditioning, they were injected with muscimol or vehicle into the ACC 15mins prior to the test (Fig. 5A). In this experiment, the c-fos expression is significantly lowered by muscimol (Fig. 5C, D), but the freezing level remains no difference between the two treatment groups (Fig. 5B). This result indicates that the 30s PSI memory retrieval is not dependent on the ACC either. All the placements have been verified after the experiments (Fig. 6). Combined with previous results, the 30s PSI memory is not HPC or PFC dependent. This might mean the memory was simply not encoded in either region or encoded in the HPC/PFC but resistant to inhibition by muscimol.
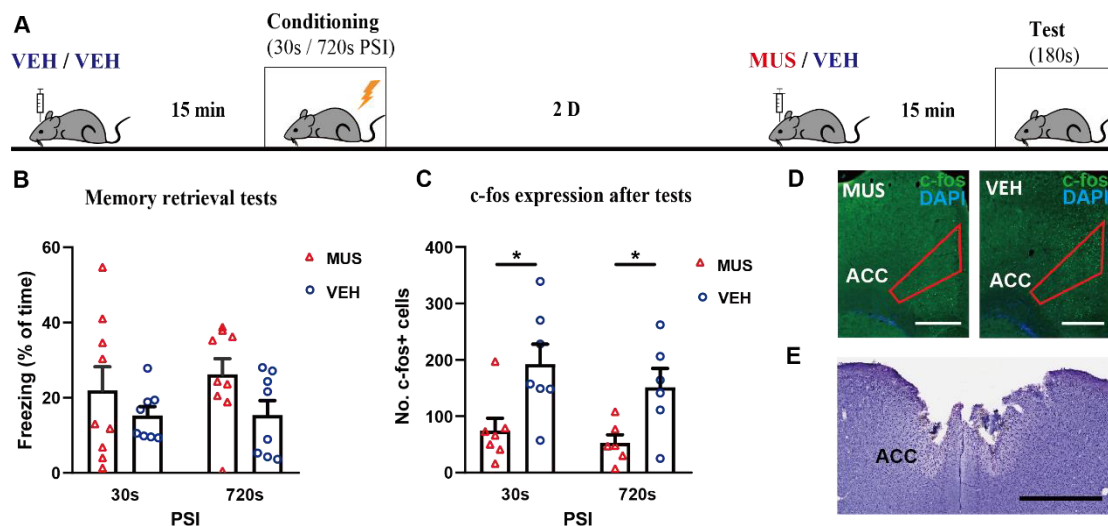
Fig. 5. Memory tests with PFC inhibition. (A) Behavioural schematic. **(B)** Memory retrieval tests of 30s and 720s PSI memories, N = 8~9/group. **(C)** The number of c-fos^{+} cells from the 2-days old memory, N = 5~ 6/group. **(D)** Representative image showing expression of c-fos cells in the ACC. Left panel: 30s PSI memory with vehicle; right

panel: 30sPSI memory with muscimol, scale bar is 500 μm. **(E)** Representative image showing the ACC placement, scale bar is 1mm .The bar graph represents the group means ± SEM, significance is denoted with asterisks: *P<0.05.
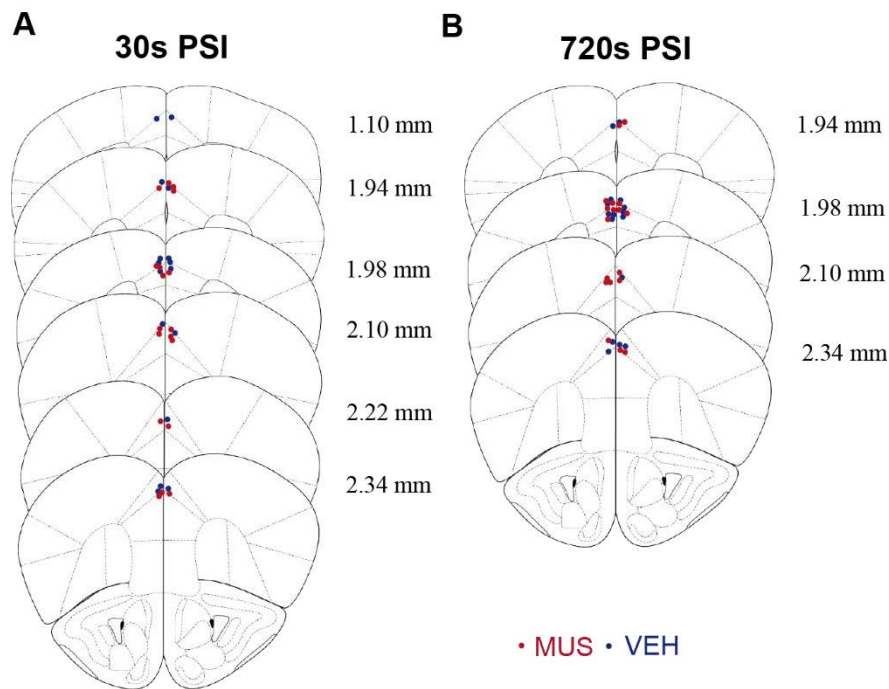
Fig. 6. PFC placement verification. Verifying the cannula placement in the ACC for muscimol and vehicle injection sites.

4.3 Discussion

This chapter studied whether PSI affects systems consolidation as the second aim of my thesis. The mechanism of systems consolidation and the regional dependency shift is still unclear. In order to study the role of the HPC and regional dependency shift during systems consolidation, I first validated the coordinates of the HPC in surgery. The location of the HPC was verified through dye injection and counterstaining (Fig.1). The counterstaining verified where the cannula and internals reached in the HPC, which can be regarded as the injection location. Using different sizes of cannula internals, I was able to inject the dHPC and the DG (Fig. 1 C, D). After the cannula infusion, all placements were verified and labelled in the atlas for all experiments. The dye injection

validation showed that fluorescent dye would diffuse in a wide range and cover the entire HPC, especially through a cannula in freely moving mice (Fig 1. B). AF 594 and AF 488 were injected by different methods but at the same coordinates (AP: -1.9mm, ML: ±1.5mm, DV: -1.7mm). The AF 594 was injected through a syringe when mice were in anaesthesia, and the AF 488 was injected through a cannula in freely moving mice. As the image shown, AF 488 seems diffused slightly wider than AF 594. This may be because the dye diffused faster in the freely moving mice. The ACC placement validation was slightly different from the HPC one. Anatomically, there are three sections from the top layer to the bottom in the PFC as follows: prelimbic cortex (PL), infralimbic cortex (IL), and anterior cingulate cortex (ACC). Only the ACC is the key region for memory formation and remote memory retrieval (Aceti, et al., 2015; Corcoran, et al., 2016; Ding, et al., 2008). Thus, I specifically inhibited the ACC in the subsequent experiments trying to impair memory retrieval.

After validating the surgery and IHC protocol, I began studying the HPC dependency for the short and the long PSI memories during systems consolidation. The results suggest that the short and the long PSI memories underwent different systems consolidation, or the short PSI might not even undergo systems consolidation. The previous study has shown that the main difference between the 30s PSI and 720s PSI memories is they have different abilities to discriminate similar contexts (Zinn, et al., 2020). Since the DG is mainly involved in encoding detailed information and pattern separation (Kitamura, et al., 2017; Struyf, et al., 2015; Sweegers, et al., 2014), it becomes the target region for this study. Hence, I specifically inhibited the DG with muscimol. The results showed that the 720s PSI memory was HPC dependent at recent time points and became HPC independent with the passage of time (Fig. 2B, C).

However, the 30s PSI memory was not affected by hippocampal inhibition at any time points (Fig. 2B). The IHC analysis also showed that when the mice received a single shock at conditioning, the c-fos level was relatively low regardless of being treated with muscimol or vehicle. Even though muscimol is a potent neural inhibitor, it was not able to inhibit all the neurons in the region. Therefore, there wasn't any significant difference between the two treatment groups in the number of c-fos cells observed (Fig. 2G). Surprisingly, the engram labelling experiments showed some contextual memories formed when the mice were conditioned with the 30s PSI, but that contextual memory might not be responsible for the freezing at the tests as HPC inhibition failed to impair the memory retrieval (Fig. 4G). Then, I investigated another region involved in contextual memory consolidation, the PFC. Inhibiting the PFC failed to impair the 30s and the 720s PSI memory, even though the IHC results showed the neural activity was significantly lowered by muscimol (Fig. 5B,C). This result suggests that both short and long PSI memories were not retrieved from the PFC at recent time points, which is consistent with previous findings (Frankland., et al., 2015; Sierra., et al., 2017)

It is still unclear why the 30s PSI memory was not affected by the HPC and the PFC inhibition. The obvious interpretation is that the memory was not contextual or HPC dependent at all. As shown in the validation trial, no ISD suggests that brief exposure to the context might still generate a freezing response at the tests, which was not driven by a contextual memory. A possible explanation is the freezing could be non-associative. Non-associative fear in this study could be caused by placing the mice in an unfamiliar environment but not giving them sufficient time to explore it. As introduced in Chapter 1, forming a contextual memory requires a certain amount of time. However, the minimal amount of time required to form a contextual memory

could vary based on different contexts. Thus, it is unclear whether the 30s was sufficient for the mice to form a contextual memory in those trials. This would also explain why the 30s PSI memory was not HPC dependent. Moreover, since the freezing level between cannulated and non-cannulated mice were similar, I did not specifically conduct a comparison trial to investigate how cannulation affects mouse behaviour. To mitigate such effect, the mice were handled for seven days by performing mock hippocampal injections. However, the hippocampal injection might still cause extra stress to the mice. Lastly, although the engram neurons in the DG suggest a contextual memory was formed, no solid evidence showed that contextual memory was associated with the fear response at the tests. Thus, the freezing at the test might be tied to a cue. This interpretation will be elaborated in the general discussion.

Nevertheless, an alternative interpretation could also account for why the 30s PSI memory was not affected by the HPC inhibition. The previous study showed that the 30s PSI memory requires significantly fewer engram neurons than the 720s one (Leak., et al., 2021). Therefore, the 30s PSI memory engram is too small to be inhibited by muscimol, which also explains the labelled engram neurons in the DG. This possibility will also be further discussed in the general discussion.

Chapter 5: Whether memory updating affects the original memory trace and its following consolidation

5.1 Introduction

In the previous section (chapter four), I showed that the 30s PSI memory was resistant to hippocampal inhibition, but the 720s one was not. Albeit it is still questionable whether the 30s PSI memory is contextual, the previous results showed a significant difference between the two memories two days after conditioning. Since this difference was caused by different learning durations, thus, the following question is whether changing the learning durations affects how memories behave in the following consolidation. In order to prolong the short PSI memory learning duration, I re-exposed the conditioned mice in the conditioning context one day after the conditioning. Such context re-exposing is called memory updating. Previous studies showed that updating the 30s PSI memory by re-exposing the mice to their conditioning context would generate an updated memory that showed similar behavioural readouts as the long PSI one (Zinn, et al., 2020). In this case, the mice formed with a 30s PSI memory could update and improve their original memory. Therefore, in this chapter, I investigated whether updating the 30s PSI memory affects its consolidation or HPC dependence.

In this chapter, the short and the long PSI memories showed the same level of freezing, which indicates that the freezing level cannot be regarded as a criterion for memory updating. Hence, I first need to set up the criterion that could indicate the success of memory updating. The previous studies showed that a successful memory updating could improve a poorly formed memory suggested by improving its ability to

discriminate similar contexts (Zinn, et al., 2020). Thus, I first designed a context that is similar to the conditioning one, so the mice cannot discriminate it from the conditioning context unless they possess a detailed contextual memory. The updating and context discrimination trial showed that the mice formed with 30s PSI memory could only discriminate similar contexts after updating. The memory updating was achieved by re-exposing the mice to the conditioning context for 720s one day after conditioning. After establishing this memory updating paradigm, I conducted experiments studying how memory updating affects its subsequent consolidation. The results showed that the updated 30s PSI memory was still resistant to the HPC inhibition. This result could be interpreted in two ways. Firstly, if the original 30s PSI memory was not originally HPC dependent, re-exposure would not have made the memory that drives behaviour suddenly contextual or HPC dependent. This would then explain why it is still unaffected by hippocampal inhibition. An alternative interpretation is that the 30s PSI memory was contextual and improved by memory updating. In this case, the memory might still be HPC dependent, but it remains resistant to muscimol just like it was prior to updating.

5.2 Results

5.2.1 Context B design experiments

To establish the memory updating paradigm, I started designing a context B that is similar to, but that can be distinguished from context A (See the details of different contexts in table 1-3). Such context B will be designed to study memory generalization in systems consolidation. Memory generalization denotes the ability of animals to differentiate between contexts and is a measure of the detail contained within memory

(Einarsson, et al., 2014; Nadel, et al., 2000). Generalization can therefore be used to track multiple things. Firstly, the degree of initial detail acquisition during learning. The previous study showed that PSI affects the level of discrimination and generation. Secondly, it can be used to track systems consolidation, since information becomes less detailed as memory becomes less HPC dependent and more PFC dependent (Barry, et al., 2016; Ding., et al., 2008, Kitamura., et al., 2017; Rozeske., et al, 2014; Teixeira, et al., 2006). Besides, a context C (an obviously different context from context A) will be needed to set the upper limit of contextual similarity, which the context A memory cannot generalize to. I first validated the pre-existing context B (B1) and C (C1) used in our lab. This result showed that context C1 is discriminable from context A1, whereas no difference between context A1 and B1 has been shown (Fig. 1B). To verify that the indistinguishability between context A1 and B1 was not due to inadequate learning duration, I prolonged the PSI from 180s to 480s. Even with the prolonged PSI, context B1 was still not discriminable from context A1 (Fig. 1G). Since context B1 was not discriminable from context A regardless of the PSIs, I sought to design a new context B that might allow discrimination. To achieve this, I tried to isolate the components that affect freezing. Therefore, I tested which contextual component affects contextual similarity the most. So, I modified each individual component in context A (grids floor, insertion, light intensity, and odour) at the recent memory tests. The single-component modification experiments showed no statistical difference among these trials (Fig. 3 C-F).

Considering that context B1 was indistinguishable from context A1, I subsequently designed three new context B's, namely, B2, B3, B4. I found that B3 and B4 were statistically different from context A1 in this trial, and B4 seems to show a

greater significance than B3 (Fig. 1H). So, I decided to use B4 as context B. In order to use it in systems consolidation study, context B should only be distinguished from context A in two following situations: 1. At recent, but not remote time points. 2. When animals learn the context with adequate learning duration (long PSI). Thus, to confirm that context B4 is only distinguishable from context A1 with long PSI but not short, I conditioned animals with short and long PSIs (30s, 720s). However, inconsistent with the previous trial, this result showed no difference between context B4 and context A1 with both short and long PSIs (Fig. 1I). Considering context B4 has shown some promising results in the previous trial (Fig. 1H), I then tried to slightly modify B4 to make it even more different from context A. In addition, I also tried to create a new context C and to make a contextual similarity gradient between contexts A, B, and C. Thus, I dimed the light intensity in context B4 to create context B5 and built a new testing environment to create context C2. Nevertheless, the difference between contexts A and B5 is still not significant (Fig. 1J).

Insert	Grid	Light intensity	Odour
N.A.	N	1	5 BZ
AF	S	3	5 AC
WC	TT	SL	AO
WCP	PS	NL	PM

*This table only indicates the changed components. The rest set-ups were consistent with context A1.

Table 1. The single component manipulation experiments. The modified contextual components were indicated as following: Light intensity: SI= Stimulus light only, NL= No light, and numbers (1, 3) indicate the intensity of house light. Insert types: AF= A frame insert, WC= white curved wall, WCP= White curved wall with pattern. Grid floor: S=Stagger grid, N= normal grid, TT=thick/thin grid, PS= Plastic floor with stagger grid. Odour material: 5 AC= 5% acetic acid, 5 BZ= 5% benzaldehyde, PM= pepper mint, AO= aniseed oil.

Context	B1	B2	B3	B4	B5	C1	C2
Light intensity	1	SL	SL	SL	1	1	3
Insert	AF	WC	AF	WC	WC	WC	CB
Grid	S	N	TT	TT	TT	TT	N
Odour	10 AC	5 AC	5 AC	5 AC	5 AC	AO	5 AC

Table 2. Context B and C designs. The basic set-up is similar with context A except the changes indicated above. Light intensity: SL= Stimulus light only, and numbers (1, 3) indicate the intensity of house light. Insert types: AF= A frame insert, WC= white curved wall, CB= Carboard box with bedding material. Grid floor: S=Stagger grid, N= normal grid, TT=thick/thin grid. Odour material: 10 AC=10% acetic acid, 5 AC=5% acetic acid, AO=aniseed oil.

Experiment	Conditioning context	PSI	N	Testing context
Single element validation (Fig1. C-F)	A	180s	12	S1/S2/S3/S4
A/B1/C1 validation (Fig1. B)	A	180s	12	A/B1/C1
A/B1 validation (Fig1. G)	A	180/480s	8	A/B1
Context B2/B3/B4 validation (Fig1. H)	A	180s	16	A/B2/B3/B4
Context B4 validation (Fig1. I)	A	30s/720s	8	B4
Context B5/C2 validation (Fig1. J)	A	720s	3	A/B5/C2

Table 3. Summary of context validation experiments. All male mice were conditioned in context A (A1) and tested at 24h post-conditioning.
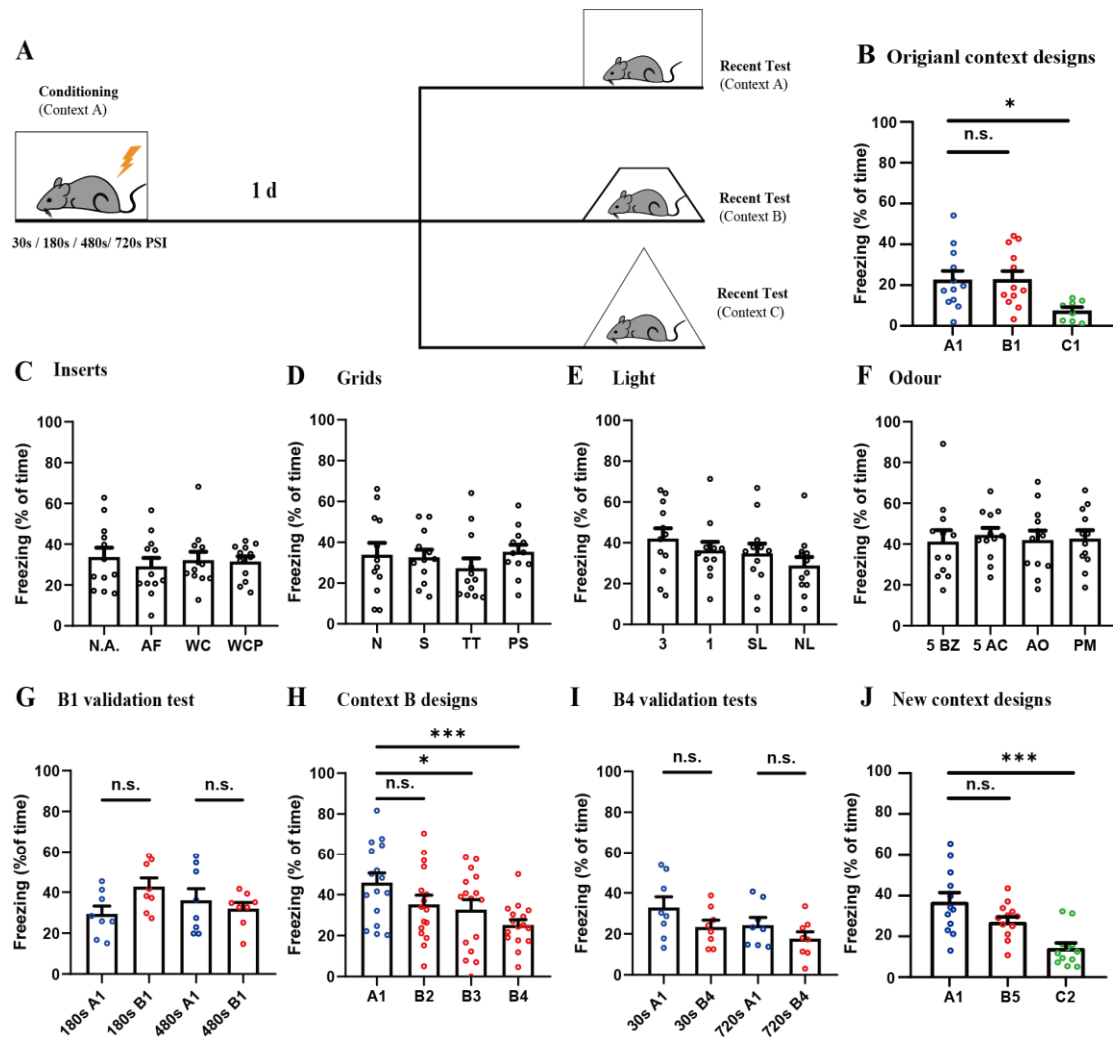
Fig. 1. Different context designs and validation. (**A**) Behavioural paradigm for context design experiments. (**B**) Mice were conditioned in context A1 and tested in either context A1, B1, or C1, N = 12/group for context A1 and B1, N = 8 for context C1. Statistical analysis was performed by running an unpaired t-test. (**C-F**) Single contextual component manipulations. Mice were conditioned in context A1 and tested in the same context except for those changes: inserts, grids, light, and odour. The detailed manipulations are indicated as following: Insert types: AF= A frame insert, WC= white curved wall, WCP= White curved wall with pattern; Grid floor: S=Stagger grid, N= normal grid, TT=thick/thin grid, PS= Plastic floor with stagger grid; Light intensity: SI= Stimulus light only, NL= No light, and numbers (1, 3) indicate the intensity of house light; Odour material: 5 AC= 5% acetic acid, 5 BZ= 5% benzaldehyde, PM= pepper mint, AO= aniseed oil. No statistical difference was observed by changing a single contextual component, N = 12/group. (**G**) Context B1 validation with prolong PSI. All mice were conditioned in context A1 with either 180s or 480s PSI and tested in context A1 or B1 for 180s 1 day after conditioning, N = 8/group. No statistical difference was observed via Sidak's multiple comparison test, $P(180s) = 0.07$; $P(480s) = 0.76$. (**H**) Context B designs regarding their similarity. All mice were conditioned in context A1 for 180s PSI and tested in context B2/B3/B4 for 180s 1 day after conditioning, N = 16/group. Statistical analysis was performed via Dunnett's multiple comparison test. (**I**) Context B4 validation tests with short and long

PSI, N = 8/group. No statistical difference was observed via Sidak's multiple comparison test, *P*(30s) = 0.20; *P*(720s) = 0.44. (**J**) The latest context B (B5) and C (C2) designs with discrimination tests. Only C2 shows significant difference from A1. Statistical analysis was performed via Dunnett's multiple comparisons test. Bar graph represents the group means ± SEM, significance is denoted with asterisks: *P < 0.01, ***P<0.005, n.s. = not significant.

5.2.2 Validation of the memory updating paradigm

The previous section aimed to establish, as the lab previous found, that PSI controls discrimination, thus showing that it affects the degree of initial learning and the degree to which animals can differentiate similar contexts. This could then be used to assess whether memory updating occurs, ultimately allowing me to determine whether updating of poorly formed memories can alter their rate of systems consolidation. However, I was unfortunately not able to establish a context B that would allow me to demonstrate a PSI dependent change in discrimination. Therefore, here I asked whether an updating session could improve discrimination nonetheless. If so, it could still prove some validity to the approach. Thus, I started with validating the memory updating paradigms using context B (B5) from the previous trails. Since context B5 did not show a significantly different freezing level from context A, I tested whether this context is discriminable from context A after updating. Again, when the mice were conditioned at the 30s and 720s PSI, they were unable to discriminate between the two similar contexts (Fig. 2 B). However, the 30s PSI memory mice were able to discriminate the context after re-exposing to the conditioning context one day after the conditioning (Fig. 2A, C). Also, the freezing level in the first 3min of the reexposure showed no significant difference from the tests, suggesting no memory extinction (Fig. 2C). These results indicate that the 30s PSI memory became more

precise after being updated and that context B5 can be discriminated from context A after the updating.
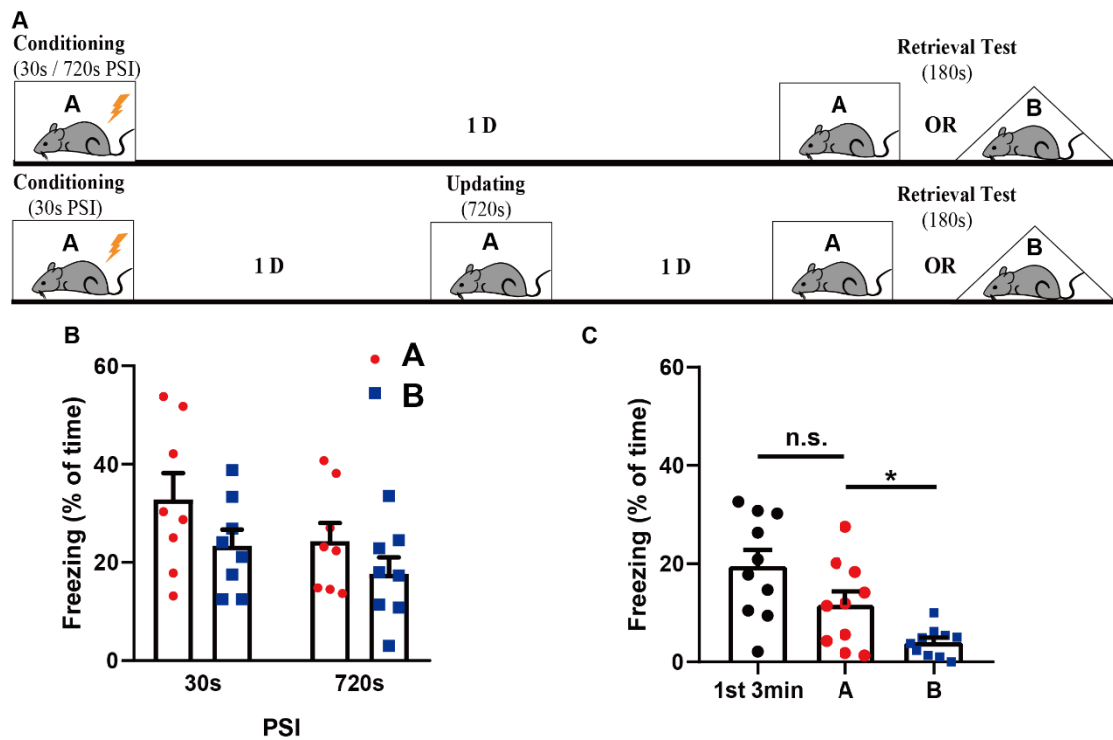
Fig. 2. Verification of updating poorly formed memories. (A) Behavioural schematic. **(B)** Memory retrieval test of 30s and 720s PSI memories without updating, N = 8/group. **(C)** Freezing of the first 3 min in the updating session and the tests after 720s of updating, N = 10/group. The bar graph represents the group means ± SEM, significance is denoted with asterisks: *P<0.05; n.s. indicates no significance.

5.2.3 The 30s PSI memory did not change its form after updating

After validating the updating paradigms, I conducted the experiment to study whether updating the 30s PSI memory will alter its regional dependence in the following consolidation. The mice were conditioned at 30sPSI and re-exposed to the conditioning contexts for 720s one day after the conditioning. Based on the results in Chapter 4, the 720s PSI memory was subject to the HPC inhibition two days after conditioning, but not nine days. Hence, if the short PSI memory behaved similarly to a long PSI memory after updating, I would expect the updated 30s PSI memory is subject

to hippocampal inhibition two days after conditioning. The mice were tested in the conditioning contexts at two or nine days post-conditioning with muscimol or vehicle infused in the HPC (Fig. 3A). Even being updated to a long-PSI memory, The IHC analysis showed the neural activity in the DG was significantly inhibited by muscimol (Fig.3D, E). However, inhibiting the HPC still didn't impair the memory (Fig. 3C). All the cannula placement has been confirmed through cresyl violet staining, and the data with the missed placements have been excluded from the figure (Fig. 4). This result implies that even after updating, the original 30s PSI memory trace remains intact and HPC independent.
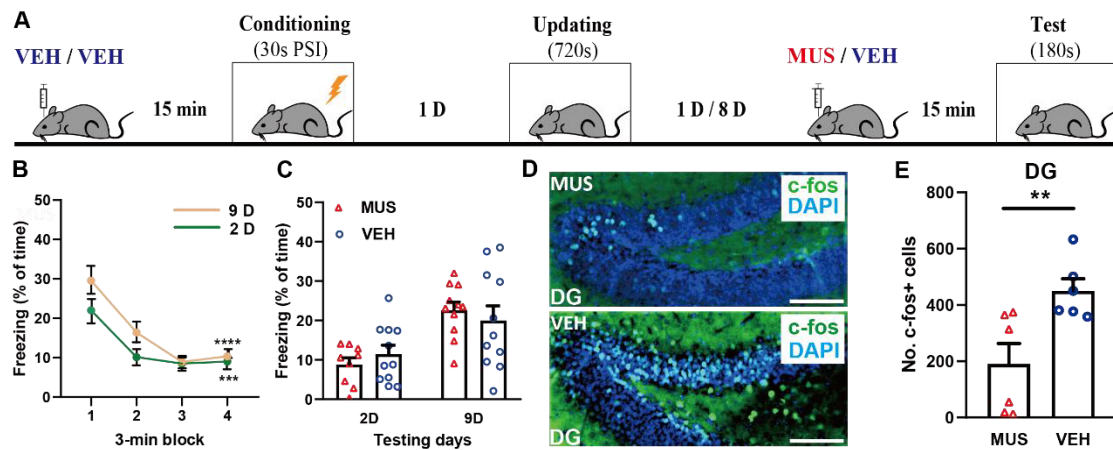
Fig. 3. Hippocampal inhibition on updated memories. (A) Behavioural schematic. **(B)** Memory updating session in 3-min block for 2-days and 9-days old memories, N = 11/group. **(C)** Memory retrieval test of 2-days and 9-days old memories after updating, N = 9~11/group. **(D)** Representative image showing expression of c-fos cells in the DG. Scale bar is 500µm. **(E)** The number of c-fos$^+$ cells from the 2-days old memory, N = 6/group. The bar graph represents the group means ± SEM, significance is denoted with asterisks: **P<0.01, ***P<0.001, ****P<0.0001.
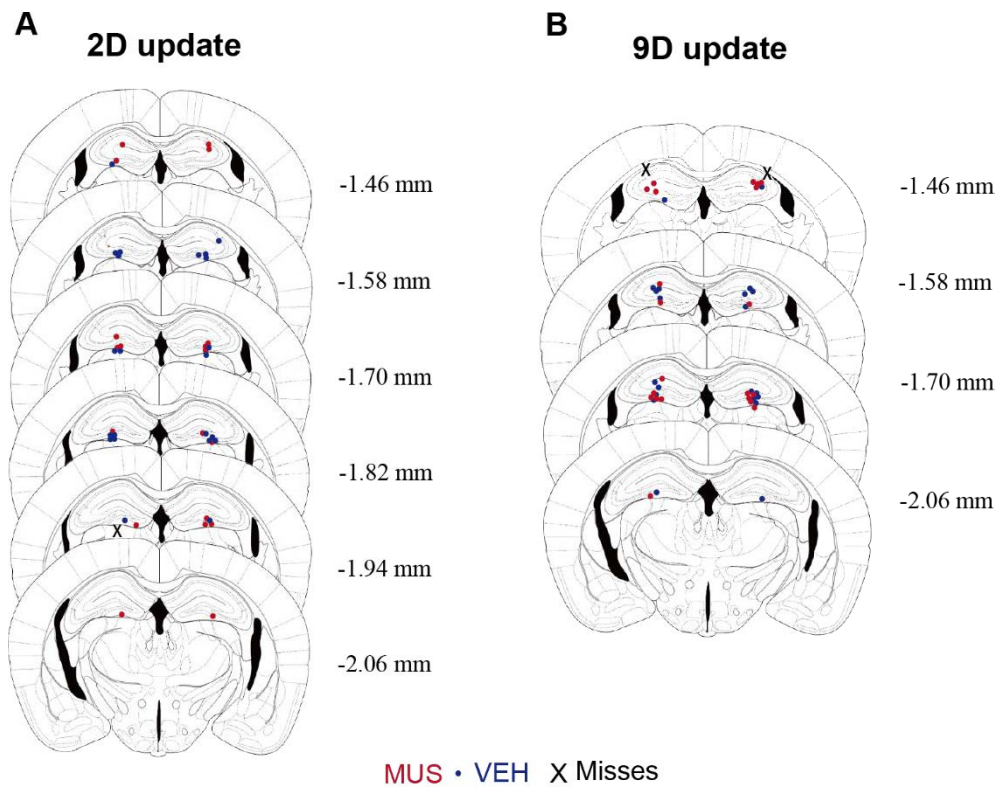
Fig. 4. HPC placement verification. Verifying the cannula placement in the HPC for muscimol and vehicle injection sites.

5.3 Discussion

To study whether memory updating affects the following consolidation or HPC dependence, I first validated the memory updating paradigm used in this project. Memory updating refers to an alteration in memory based on experience subsequent to initial learning. In this project, the criteria for a successful updating are discriminating between two similar contexts. Thus, the first experiment was to design a context B similar to but different from context A. This similar context, context B, should not be discriminated from context A if conditioned with 30s PSI, but it can be discriminated after updating. Contextual discrimination can be regarded in showing different freezing levels in memory tests. However, the challenge in designing the contexts is that it is unclear how the context is presented in a mouse's brain. I could only try a variety of

designs to verify the similarities between contexts. Thus, I designed a set of experiments aimed at creating a context (context B) that is similar but can be discriminated from the conditioning context (context A) when conditioned with a long PSI. Then, this context can be used in a memory update study.

Since mice could not discriminate between the conditioning context and the existing context B (B1) in our lab even after increasing the PSI to 480s (Fig. 1B, G), I started designing a new context B. I found that changing a single context component could not affect the similarity between the contexts (Fig. 1C-F). This suggests that mice integrate the entire environment as a unified contextual representation in the brain instead of using any single cue to associate with fear. However, since only limited components I could change, this result could not completely exclude the possibility that the mice still associated shock with a subtle cue inside the chamber. The following context B designs did not show any difference in freezing, suggesting the mice could not discriminate between them (Fig. 1J). The reason why there was no discrimination might be that the common features in those chambers were too salient to allow discrimination. Such as the size of the chamber or the texture of the walls cannot be changed in context B designs. This interpretation is supported by context C design. Context C (C2) used the bedding material from their home cages and cardboard box, which were significantly different from the steel texture in the conditioning context. And context C2 showed a significant difference from context A in the tests. However, I cannot use context C2 for contextual discrimination because it is a significantly different context, but not a similar one. Unfortunately, due to the total amount of mice is limited in this study, I was unable to counterbalance contexts A, B or C in those trials. Therefore, to be strategic, I only use context A as the conditioning context, and other

contexts as similar or different contexts. The counterbalance trials need to be done as part of future studies.

In those discrimination trials, the mice only had a single session to learn the context and failed to differentiate those contexts. However, if they have a second chance to learn about the contexts with a decent amount of time, the result may differ. The following results showed context B5 is discriminable from context A after reexposure, suggesting that the 30s PSI memory was successfully updated to one that was able to discriminate similar contexts (Fig. 2C). Also, the freezing in the first 3min of the updating session showed no significant difference from the test in context A ($P = 0.1$), suggesting no memory extinction during the updating session (Fig. 2C). This result is inconsistent with the possibility that the low freezing in context B is because of that memory extinguished during updating. However, we consider much more work is needed to investigate and understand the data we have observed in this thesis and that is beyond the scope of this thesis. In addition, this result is consistent with a previous study, where a short PSI memory has to be updated first before undergoing memory extinction (Zinn, et al., 2020). Unlike the updating group, there was no difference in freezing between contexts A and B in the 720s PSI group (Fig. 2B). This result indicates that, without memory updating, mice were unable to discriminate contexts A and B regardless of the learning durations. At first glance, this result is inconsistent with previous studies, where the mice who formed a long PSI memory were able to discriminate between two similar contexts (Leak, et al., 2021; Zinn, et al., 2020). However, the extent to which the mice formed long PSI memories can discriminate similar contexts is unknown. As discussed in Chapter 1, context discrimination is very sensitive to the conditioning environment, and even a small change in the context may

render the entire context perceived differently. Thus, the differences in the contexts may account for being unable to discriminate similar contexts with a single-session learning paradigm, which will be discussed in Chapter 6. Pertaining to why the mice could discriminate the contexts after updating, it can be interpreted in different ways. Firstly, the 30s PSI memory was not originally contextual, and its freezing was caused by non-contextual fear, such as being stressed to the novel environment. Such stress-induced non-contextual fear could be alleviated by re-exposing to the conditioning chambers. Thus, the mice showed low freezing in context B. Alternatively, it can be interpreted as the 30s PSI memory was originally contextual and improved by memory updating. In this case, the updated memory is more accurate than the long PSI one even though they have the same contextual exposure time. It may imply that, with the same total learning durations, having two separated learning sessions could generate a more accurate memory than long-duration single-session learning.

In Chapter 4, I found inhibiting the HPC of the 30s PSI failed to impair memory retrieval. This resulted in one interpretation that the 30s PSI may not be contextual. However, this updating trial might contradict that interpretation and suggest the 30s PSI memory is still contextual. Moreover, if the 30s PSI memory was non-contextual, memory updating would generate a new contextual memory in the HPC that was not associated with any shock. In this case, the freezing in the test was only caused by the initial conditioning. Thus, there should still be no freezing difference when tested in context A or B after updating, which is the opposite of the results. The alternative interpretation is that a cue memory may have formed. This memory would have updated itself and become more accurate through re-exposure. In this case, memory updating improved the cue memory. Additionally, as discussed before, contextual discrimination

is very sensitive to the entire paradigm. In the contextual discrimination trials, all mice were wild-type and not cannulated, but the mice in the HPC inhibition trials were cannulated. Since cannulation is an intensive surgery for mice, it may also contribute to the difference between the trials.

After memory updating, I conducted the recent and remote memory tests on the same group of mice (Fig. 3A). Based on previous results, hippocampal inhibition of the 720s PSI memory, but not the 30s one, caused a memory deficit two days after conditioning. This experiment showed that inhibiting the HPC after updating did not impair memory retrieval, which was similar to the 30s PSI memory but not the 720s one (Fig. 3 C). This result can be interpreted with different possibilities. Firstly, the original 30s PSI memory was not contextual or HPC dependent, and memory updating formed a new contextual memory in the HPC, which was not related to the original one. In this case, hippocampal infusion of muscimol only inhibited the contextual memory formed during updating but did not impair the original one, as that one was not encoded in the HPC. Alternatively, the original 30s PSI memory might be HPC dependent, and it was resistant to the HPC inhibition. Unfortunately, based on the data acquired in this study, the two interpretations cannot be completely delineated but will be discussed in Chapter 6.

Besides the nature of the memory, it is still puzzling why the c-fos labelled cells were dramatically increased after updating. Compared to the c-fos cells in Chapter 4, where the mice were tested without memory updating, the amount of c-fos cells in this experiment was significantly increased. Since all trials underwent the same staining protocol and used the same patch of chemicals, such difference was unlikely caused by

the staining methods. Nevertheless, the low sample size (N = 6) might account for such a dramatic difference between the two trials. Moreover, the experiments in Chapter 4 were finished a few months before the Chapter 5 experiments. The individual difference among those mice might also slightly contribute to the difference. Another possibility is that not all c-fos neurons were responsible for memory retrieval. As a ubiquitous neuron activity marker, some c-fos expression in this study might not necessarily be related to memory at all. An extra step of exposure to the conditioning context (memory updating) and interaction with mice might cause them to be more responsive to the environment. This might also cause a c-fos increase. Besides, memory improvement could be another reason. A previous study found that the mice who were able to discriminate similar contexts had significantly large engram sizes (Leak, et al., 2021). Although both of them expressed the same of c-fos level after the tests, they still showed a high level of c-fos expression even without updating (around 200 in the DG). Altogether, the experiments from Chapter 4 were conducted in a different scenario from this chapter, where the mice, the environment, and the behavioural manipulations were varied to some degree. The compound effects, especially memory improvement and additional context exposure, might cause such a significant difference in c-fos expression. However, the exact reason still remains for further investigation. For instance, I'm unable to explore the physiological changes of the hippocampal neurons after memory formation and updating, which could also account for why those neurons are more active.

In summary, I first validated and established different contexts used in the memory updating paradigm. Secondly, I tested how memory updating affects the following consolidation. It has been debated how memory updating affects the original

memory trace for a long time. I found that discrimination was improved in a poorly formed memory after re-exposure, but the memory was still unaffected by hippocampal inhibition using muscimol. This could be caused by either the original memory was not HPC dependent, or the memory updating paradigm in this study did not affect the original memory in the HPC. The future study will first identify if the short PSI memory is contextual. Based on that, I could decide if memory updating improved the original memory or generated a new contextual memory that is not linked to the original one. Then, I could further study the relationships between the original memory trace and the new trace generated during updating. Understanding how memory updating affects the original memory trace will contribute to understanding how memory is processed in the brain and how old and new memories interact.

Chapter 6: Discussion

6.1 Results summary

In this PhD project, I explored how learning duration affects memory consolidation using context fear conditioning in mice. I first studied how initial learning affects synaptic and systems consolidation. To do that, I modified the PSI, which controls the extent to initial learning. The results showed that the short and the long PSI memories underwent synaptic consolidation at the same rate. However, only the long PSI memory was HPC dependent, the short one was not. Since this difference was caused by different learning durations, I wonder if the short PSI memory can consolidate the same as the long PSI one after being improved by updating. Therefore, I investigated whether updating the short PSI memory could affect its following consolidation.

Firstly, I investigated the synaptic consolidation of both short and long PSI memories. The results showed that the memories formed with different PSIs underwent synaptic consolidation at the same rate. Secondly, to study whether PSI affects systems consolidation, I inhibited the HPC for the 30s and 720s PSI memories during the systems consolidation window trying to disrupt their consolidation. The results showed that the 720s PSI memory was initially HPC dependent and gradually became HPC independent later. However, the 30s PSI memory was not HPC dependent at any time point. Next, I labelled the engram neurons in the DG, which showed that a contextual memory was formed when the mice were conditioned with 30s PSI. However, it is still unclear whether that contextual memory caused the freezing at the tests or not. Next, I inhibited the PFC at the recent memory retrieval tests. Again, the 30s PSI memory was not PFC dependent either. These results suggest that the 30s PSI was either not encoded

in the HPC/PFC, or it exists in a form that was resistant to pharmacological inhibition. Finally, I updated the 30s PSI memory by re-exposing the mice to the conditioning context for 720s. These results showed that, even after memory updating, the 30s PSI memory was still HPC independent, suggesting memory updating did not affect the 30s PSI memory physiochemical makeup.

6.2 The context fear conditioning system is different from the previous work in our lab

There are some differences in behavioural experiments between this study and previous work from our lab. Different from this study, the previous experiments from our lab were conducted with different apparatus. In that study, the conditioning chambers were in a small room without specific soundproof materials to isolate them. The light source was provided by the room light, and the freezing was manually scored. In the manual scoring, each mouse was scored in four seconds blocks. The final freezing was calculated by dividing the freezing blocks by the total blocks in the tests. However, in this study, the conditioning chambers were isolated in the soundproof cubicles, and the light source in the cubicles was provided by stimulus light and house light. The light intensity in this study was not exactly the same as the one used in the previous one. Moreover, the freezing level in my study was automatically scored by the "Video Freeze" software, which automatically calculated the freezing percentage based on the freezing duration and total testing time.

Those differences caused some unexpected results in the validation trials. In the CFC validation experiments, compared to the no shock group, all of the conditioned groups showed significant freezing, including the immediate shock one at the recent

memory tests (Ch 3., Fig. 1C). According to previous studies, forming a contextual memory requires a certain amount of time (Kiernan, et al., 1995; Landeira-Fernandez, et al., 2006; Lattal, &Abel., 2001). Thus, conditioning the animal immediately after exposure to context would cause an immediate shock deficit (ISD), which means low freezing in the test. However, no ISD observed in this validation trial suggests the fear memories at the recent tests were not contextual. Moreover, unlike other groups where the fear memory still persisted two weeks later, the immediate shock group did not show freezing when tested remotely. This result suggests that, in this paradigm, a brief exposure to the context might generate some non-associative fear, but that non-associative fear could not last for long.

The difference in the chambers may also account for the inconsistency in the contextual discrimination tests. The previous work showed when the mice were conditioned with 180s PSI or longer, they were able to discriminate between two similar contexts. However, the mice failed to do so in this study (Ch 5, Fig. 1). Thus, I designed a series of experiments to replicate the results of those different chambers to optimize the basic CFC paradigm (Ch 3, Fig. 2). After testing memories with different times, sex, and cubicle colour, the result did not show any improvement. Thus, the differences in the fundamental set-ups dissociate the findings in the study from the previous ones to some extent.

6.3 Only one anisomycin sensitive time window during synaptic consolidation

My results aligned with previous studies by showing injecting anisomycin during synaptic consolidation impaired memory retrieval at tests (Bourtchouladze, et

al., 1998). Thus, injecting anisomycin at different time points could reveal when the memory no longer requires protein synthesis (Ch3, Fig. 3E, F), and thus, presumably, when the consolidation time window closes after different degrees of initial learning. In this study, immediate after conditioning was the only time when synaptic consolidation was subject to anisomycin. The previous study has shown that there might be two time windows where synaptic consolidation is subject to anisomycin (Bourtchouladze, et al., 1998; Sutton, &Schuman, 2006; Wanisch, & Wotjak, 2008). Other studies showed that the two time windows were caused by the separate steps of memory formation and stabilization (Igaz, et al., 2002). The first protein synthesis step is because of the expression of immediate early genes in response to the general neural activity. The second step is the synthesis of PRP that maintains LTP and memory formation (Igaz, et al., 2002; Kandel, 2001; Bekinschtein, et al., 2010). However, only one anisomycin sensitive time window in my study can be explained by two interpretations. Firstly, Bourtchouladze, et al found that only the single CS-US pairing produced two protein synthesis windows, but the three CS-US pairings only produced one protein synthesis window that was immediately after conditioning. It suggests that only a weak learning paradigm will cause two protein synthesis windows. In this study, Although all mice only received one shock, the shock intensity was stronger than the previous study, which might still generate a stronger learning paradigm. Also, the Chapter 5 results showed the 30s PSI memory was not HPC dependent, suggesting it might be a cue memory with a strong foot shock. One intense foot shock might compensate for three weak shock. Therefore, those trials might be equivalent to the three CS-US pairing trials in the previous paper, which only generated one protein synthesis window. Secondly, if the memories in this study were contextual, then the differences between contextual memory and cue memory may also account for why

only one protein synthesis window was observed. Unlike a context fear memory, a cue conditioning memory is not encoded in the HPC and does not need to integrate the contextual information at encoding. How the brain encodes a contextual representation is mysterious and complicated, as it needs to integrate all sensory inputs into one unified representation. This process may be significantly different from encoding a single auditory cue. Thus, the differences in encoding sensory inputs may also account for the difference in the protein synthesis window.

6.4 Systems consolidation window used in this study

In Chapter 3, I found that all memories were no longer protein synthesis-dependent at one hour after conditioning. Since protein synthesis is one of the key features of synaptic consolidation, this result can be regarded as synaptic consolidation completed within one hour. However, it is noteworthy that synaptic consolidation could still be undergoing even though no protein synthesis is further required. However, to our knowledge, in mice, no contextual memory undergoes synaptic consolidation beyond one day after learning. Additionally, *In vitro* study showed forming new synapses only requires one to two hours, which is the fundamental mechanism for synaptic consolidation (Frey, & Morris, 1997). The previous study also showed the systems consolidation has already started one day after conditioning, suggested by the HPC engram neurons started supporting the maturation of the PFC engram neuron at that time (Kitamura, *et al*., 2017). Therefore, inhibiting the HPC two days after conditioning could examine how well systems consolidation is progressing, as no synaptic consolidation was taking place at this time point. Notably, defining a systems

consolidation time window does not necessarily imply all memories undergo systems consolidation during that time in this study.

6.5 Inhibiting the HPC was unable to interrupt the 30s PSI memory but able to interrupt the 720s PSI one

In Chapter 4, the 720s PSI memory was HPC dependent shortly after conditioning but not after nine days, which behaved as an expected contextual memory. The 30s PSI memory was, however, never HPC or PFC dependent. This is clearly anomalous from the previous findings, but it is important to note that the set-ups were very different. Therefore, the 30s PSI memory here may be different from the ones in previous studies. Yet, inhibiting the HPC did not impair the 30s PSI memory retrieval at any time point is still puzzling and can be interpreted with multiple possibilities.

The obvious interpretation is that the memory was not HPC dependent. The validation trials showed immediate shock did not produce ISD, suggesting a brief exposure to context could still form a fear memory, but that fear memory might not be contextual. Similarly, the 30s PSI in this study might be too short to form a contextual memory, as the memory here might provide relatively less opportunity for animals to process the context. This could have resulted in animals failing to associate the context with shock, and instead to associating the shock with a cue and developing cue-conditioned fear. For instance, the mice handling or transporting cues might overshadow the context and result in a cue memory. Fanselow has reported that the fear in the test was not caused by the shock, but by the shock-related cues (Fanselow., 1980). Therefore, mice handling or transporting might become a shock-related cue for the 30s PSI memory. In addition, Abel and Lattal have reported that the animals associated the

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last elements they encountered with shock at conditioning (Lattal, & Abel., 2001). For the 30s PSI memory, because of the mice only spend little time in the conditioning context, handling might become the last element they encountered. However, for the 720s PSI memory, since the mice had spent a long time in the chamber, the mice dissociated the handling cues and associated the chamber context with shock. Therefore, the 720s PSI memory was contextual and encoded in the HPC, but the 30s PSI was not. Moreover, previous study also showed that a full exploration of the entire context is required for forming a contextual memory. (McHugh., & Tonegawa., 2007). If the mice did not have time to finish exploring the entire context in 30s, they failed to form a contextual memory. In contrast, 720s was sufficient to explore the context and form a proper contextual memory. If this is true, it raises another question to investigate in future, which is how long it take to dissociate from one cue and associate with another one. Besides developing into cue-conditioned fear, the freezing showed in the 30s PSI group might also be non-associative fear, as the mice were introduced into an unfamiliar context. This applies to both cannulated and non-cannulated mice. In addition, although the cannulated mice were handled for seven days before conditioning, injecting through a cannula into the HPC while the animals were awake is still an intensive procedure. Such possible explanations, if proven, would explain why our data is not inconsistent with previous work.

The second interpretation is that the mice actually formed a contextual memory, but that memory failed to associate with shock when being conditioned with 30s PSI. Previous studies found that forming a contextual representation and associating that context with shock are two separate processes (Matus-Amat., et al., 2004; Landeria-Fernandez. et al., 2006). In that research, they pre-exposed the rats to a context one day

before conditioning, and on the next day, the rats received an immediate shock at conditioning. Matus-Amat., et al. found that inhibiting the HPC before context pre-exposure prevents the rats from forming a contextual fear memory at conditioning, which was suggested by the ISD in the subsequent tests. This result showed the HPC is essential for forming a contextual representation. However, inhibiting the HPC immediately after the pre-exposure did not affect contextual memory formation, and no ISD in the subsequent tests. Taken together, these results suggest that the HPC is only required for forming a contextual representation but not for associating that contextual representation to shock. Therefore, they argued that forming a contextual representation is a separate process from associating the contextual representation to shock. This phenomenon is also called unconditional stimulus processing deficit (USPC), which refers to the US is failed to associate with the CS in some circumstances. The USPC may also account for why inhibition of the HPC did not impair the 30s PSI memory. Especially, the labelled engram neurons in the DG in this study suggest a contextual memory was formed, but that contextual memory was not responsible for retrieving the 30s PSI memory. Therefore, in this study, the 30s PSI memory could also fail to associate the contextual memory with shock at conditioning for some unknown reasons.

Nevertheless, given that the 30s PSI was previously shown in our and others' hands in other set-ups that require the HPC, it is not unreasonable to assume that the memory here also depends on the HPC. This is supported by the correlational engram data I obtained, which does suggest that contextual engrams form during the procedure and is recalled on later re-exposure. On this alternate account, the contextual fear memories formed at the short PSI are HPC dependent as previously shown by our lab, but they are resistant to inhibition by muscimol. One possible reason is the 30s PSI

memory engram was too small to be inhibited by muscimol. In Chapter 4, I showed that the muscimol group had the same c-fos expression level as the vehicle group (Ch4, Fig. 2F). This is because the c-fos neurons from both groups were too low to be further inhibited by muscimol. This result implies that only a small amount of neurons were sufficient to retrieve the 30s PSI memory in the DG. This interpretation is supported by previous findings. Compared to the 720s PSI memory, the 30s PSI memory has a smaller engram size and lower neural activity level (Leak., *et al*., 2021; Leak., *et al*., 2017). Therefore, although muscimol is a potent inhibitor, it might not be able to target all neurons in the HPC and, possibly, spare some memory neurons. This could be the third reason why muscimol failed inhibiting the 30s PSI memory retrieval.

In addition, how muscimol inhibits the short PSI memory retrieval is unknown, which may also account for why the 30s PSI memory was resistant to the HPC inhibition. It has been reported by many studies that infusing muscimol into the HPC before testing impairs recent memory retrieval (Haubrich, et al., 2016; Jafari-Sabet., et al., 2009; Misane, et al., 2013; Holt, & Maren, 1999; Nagahara., et al., 1992). This is because the cholinergic and GABAergic fibres in the septohippocampal projections, the projections between the medial septal area and the HPC, play a critical role in the mnemonic functions in the HPC (Nagahara., et al., 1992). Muscimol could inhibit the septohippocampal cholinergic system by increasing the uptake of choline and acetylcholine and subsequently impair memory function (Nagahara., et al., 1992). However, the role of septohippocampal projections in forming a short PSI memory is unknown. Additionally, the difference in neurotransmitter release between short and long PSI memories is also unclear. Therefore, musicmol might cause a different effect

on the short and the long PSI memories. Unfortunately, I was unable to delineate the interpretations listed above in this study.

6.6 The updated 30s PSI memory was still resistant to HPC inhibition

In Chapter 5, I showed updating the 30s PSI memory improved the memory regarding its context-discriminating ability. However, inhibiting the HPC did not impair the updated 30 PSI memory, suggesting the 30s PSI memory is not HPC dependent. At first glance, these two results seem contradictory. Context discrimination is critically contingent on the HPC, and thus, if the updated memory was not encoded in the HPC, the mice would not be able to discriminate the similar contexts. However, these results can be interpreted in the following ways. Firstly, the original 30s PSI memory was not encoded in the HPC as being discussed in the previous section, and it might be a cue memory, which specifically bound to some cues inside the conditioning chamber. Given that updating improved that cue memory, the mice could discriminate the conditioning cues from the testing cues. Therefore, inhibiting the HPC after updating did not affect the memory retrieval as the updated memory was still a cue memory and not HPC dependent. Secondly, the original 30s PSI memory is contextual and encoded in the HPC, but it was resistant to muscimol. In this case, updating improved the original poor memory and allowed the following discrimination. However, since the original memory is resistant to HPC inhibition as discussed before, the updated memory might still be resistant to musicmol. This would mean that hippocampal updating of a poor memory might not fundamentally change its physiochemical makeup. This result might pave the way for understanding how

memory updating affects the original memory trace. However, more evidence is needed in future to delineate the two interpretations.

6.7 Limitations in techniques

6.7.1 Single shock generates low freezing in memory tests

There are several technique limitations in this project. Firstly, low freezing in the tests was problematic for the contextual discrimination experiments. Regardless of the fact that learning duration is closely related to contextual discrimination and engram populations, the freezing level was not significantly affected by it once the PSI was longer than the 30s (Ch3, Fig. 1) (Zinn, et al., 2020). Since I used a single-shock conditioning protocol, the aversive stimulus was weaker than most studies that used multiple shocks. This issue made the context B design experiments become a challenge. When the mice were conditioned in context A and tested in context A or B, the freezing level from context A was not high enough to make it significantly different from the one tested in context B (Ch 5, Fig.1). The low freezing in context A maybe because the single-shock learning paradigm itself could not generate a high freezing level. However, if I used the multiple shock paradigm, it would be difficult to define a PSI because there will be a time interval between the first and the last shock. Thus, PSI could be the time interval between the placement and receiving the first shock or the last shock. However, it is unknown whether the time interval between the first and the last shock itself affects how animals encode context. Therefore, using a multiple shock paradigm may involve extra learning durations with unknown consequences, which need to be tested more thoroughly in the future.

6.7.2 Unable to measure the protein synthesis level in mice

The second limitation is that we did not have the technique to compare the actual protein synthesis level between the anisomycin and vehicle group in mice in the synaptic consolidation experiment. Even though many studies have reported that injecting anisomycin would disrupt synaptic consolidation, no previous study provided direct evidence showing how it happens. The well-accepted interpretation that accounts for the disruption is the synthesis of plasticity-related proteins (PRP) was inhibited. PRP, such as PKM, MAP kinase, are critical for maintaining LTP and regulating local translation in the synapses (Sutton., et al., 2006; Yao, et al., 2008). In fact, some *in vitro* studies even showed that protein synthesis is not required for LTP between cultured neurons (Villers, et al., 2012). However, the LTP in that *in vivo* study might only be the E-LTP, which is only involved in forming a short-term memory that does not require protein synthesis. When it comes to forming a long-term memory, instead of inducing E-LTP, the L-LTP is critical for permanently enhancing the synaptic connections and requires *de novo* protein synthesis. Thus, being independent of anisomycin in that *in vitro* study may not be applicable to *in vivo* memory study. Nevertheless, it still requires further evidence showing why inhibiting protein synthesis in living mice impairs memory consolidation.

6.8 The novel finding of this study

For the first time, this study explored the relationship between the learning duration of memory and its subsequent memory consolidation. Memory consolidation has been studied for over a century, but most studies were based on well-formed memories. However, most memories formed in daily life are not perfect due to the short

learning time. Therefore, this project aims to bridge the gap between lab research and everyday life.

It is largely unknown how the learning duration affects memory consolidation. This study used PSI to form memories with different learning durations. In Chapter 3, I found the short and the long PSI memories were impaired by anisomycin in the same way. This implies no significant difference between them in synaptic consolidation rate. Since synaptic consolidation mainly involves the biochemical process of forming new learning-induced synapses, the initial memory completeness may not critically affect this process. In addition, all mice have received the same shock intensity, which might cause the same neural response. Moreover, both short and long PSI memories were long-term memories in this study, suggesting they had the same learning-induced synaptic strength. Those might account for why different PSI memories are subject to anisomycin in the same manner. Therefore, this study indicates that the memories with different durations of learning undergo the same synaptic consolidation from the behavioural perspective.

Chapter 4 studied how PSI affects systems consolidation. This section focused on long term consolidation and how this consolidation was affected by PSIs. How or whether the initial learning affects systems consolidation has been rarely investigated in previous studies. The substantial mechanisms of systems consolidation are still largely unknown. However, a distinct feature of systems consolidation has been well documented: a contextual memory will be from HPC dependent to PFC dependent, and inhibiting the HPC at recent time points impairs memory retrieval. However, I found the short PSI memory was not HPC or PFC dependent at any time points in this study.

Although a contextual memory was formed in the HPC during conditioning, that contextual memory was not associated with shock. This result suggests that the short and long PSI memories might exist in different forms and be encoded at different locations in this paradigm. Further, increasing PSI at learning could convert a non-contextual fear memory to a contextual one.

Chapter 5 suggests that although memory updating could improve the accuracy of amemory, its fundamental physiochemical makeup might not completely be changed. The previous study showed that re-exposing to the conditioning context integrated new learning into the original memory (Gisquet-Verrier, & Riccio, 2018; Sara, 2000; Dudai, 2004a). After memory updating, a memory temporally became labile in a short period, but the updated memory would soon be consolidated through reconsolidation (Dudai, 2004b; Dudai, 2012). However, the relationship between the updated and the original memory is still unclear. Here, I found the updated 30s PSI memory was still resistant to the HPC inhibition. Regardless of whether the original memory is encoded in the HPC or not, retrieving the updated memory seems still depends on its original physiochemical makeup. This result implies that even being modified by memory updating, the original memory still plays an important role in the following memory retrieval. However, how memory updating interacts with the original memory trace is still unclear. Therefore, such questions will be explored in the future.

6.9 Future direction

6.9.1 Identifying the reason why contextual memory can be dissociated from the conditioning

In this project, I found that the 30s PSI memory was not HPC dependent, but there might be a contextual memory formed during conditioning. Nevertheless, there was no direct evidence showing the causal link between the freezing level at tests and contextual memory. Therefore, the future experiment will aim to study whether that contextual memory caused freezing in the tests. If the contextual memory formed with the 30s PSI actually caused the freezing, then the next question is why inhibiting the HPC failed to impair that memory. Alternatively, if that contextual memory was unrelated to the fear at the tests, the next aim will be to explore under what circumstances a contextual memory would fail to associate with fear in CFC.

To address such questions, firstly, the basic set-ups still need to improve in future. As I showed in the validation trial, no ISD was observed from the basic paradigm suggesting a fear response will present regardless of forming a contextual memory or not. Therefore, future experiments first need to improve the basic set-ups and generate robust ISD.

Secondly, the 30s PSI memory might be a cue memory associated with some conditioning cues inside the chambers. Since cue memory is not encoded in the HPC at all, inhibiting the HPC immediately after conditioning should test that possibility. However, this possibility is less likely because all mice were habituated to the conditioning room (not the conditioning chambers) and being handled for seven consecutive days before conditioning. Thus, all mice should dissociate from the external environment with the shock at conditioning. Moreover, in Chapter 5, when I

changed those single compounds (light, grid floor, inserts, odour) inside the chamber, the results did show any differences among those contexts (Ch 5, Fig. 1C-F). This result suggests that it is also unlikely for the mice to associate a specific cue from inside the chamber. Albeit this possibility is less likely, more solid evidence is still required in future.

Thirdly, the tdTomato and c-Fos labelled neurons indicated those neurons were activated at conditioning and the tests. However, whether those neurons constructed a proper contextual memory is still questionable, and those labelled neurons might not necessarily be engram neurons. Thus, further experiments are required to address this question. As introduced in Chapter 1, optogenetics is one of the commonly used methods of studying engrams. Since context fear memory involves associating the contextual representation to shock at conditioning, inhibiting such representation at tests is expected to alleviate its corresponding fear. Due to optogenetics can precisely and quickly inhibit the reactivated hippocampal neurons, it can examine the causality link between those neurons and their contextual representation. For instance, if inhibiting those engram neurons would also impair the corresponding memory, the causality link between the engram neurons and the contextual fear memory could be established. Otherwise, those reactivated neurons were not responsible for forming a contextual representation.

6.9.2 Exploring memory consolidation in future

In this study, it is questionable whether the short PSI memories were contextual or not, and how memory completeness affects memory consolidation is still not clear.

Thus, the next goal is to establish a more robust contextual conditioning paradigm, whereby memory completeness can be controlled by PSI. In such a paradigm, both short and long PSI memories should be subject to HPC inhibition immediately after conditioning, suggesting both memories are contextual. In addition, PSI should reflect memory completeness. Therefore, the short PSI memory should not be able to discriminate similar contexts, but the long PSI one should. It would suggest that the short and the long PSI memories are different in accuracy and completeness. On the basis of these set-ups, I could further investigate the memory consolidation of the memories with different completeness.

6.9.3 How memory updating affects the original trace

This study showed that the 30s PSI memory was improved regarding its contextual discrimination ability through updating, but the updated memory was still resistant to the HPC inhibition. These results raise questions such as if updating actually improved the contextual representation, if so, then why the updated contextual memory was still not HPC dependent? Alternatively, if the original memory was non-contextual, and re-exposure was long enough to form a new contextual memory, then how such a newly formed contextual memory interact with the original memory? Also, if the original memory was a cue memory, and updating improved that cue memory, then what is the difference between updating a cue memory and updating a contextual memory? This study is unable to reveal how memory updating enhances the original memory and answer such questions. Therefore, the future direction will be exploring the role of the neurons activated during updating, and how those neurons contribute to memory retrieval. To tackle this question, those neurons need to be labelled and

manipulated by optogenetics. Then, only inhibiting those labelled neurons in the tests would inform us of the role they play in memory retrieval.

Another interesting question to address in future is the overlap between the updating neurons and the original memory neurons. Memory updating initiates the memory reconsolidation process. There are many similarities and differences between consolidation and reconsolidation. The relationship between the original memory and the updated memory is also complicated. The main focus of the debate is whether memory updating generates a new memory or updates the old one. Also, distinguishing between the old and new memories is still challenging for current research. Therefore, another future direction would be labelling the neurons that are activated at conditioning and updating. By doing so, to what extent the updating memory overlaps with the original memory trace can be identified.

6.9.4 How the updated memory is different from a well-formed memory

Re-exposing to the conditioning context is a second chance to acquire an accurate memory of that context. However, it is unclear how the updated memory in this second learning differs from the well-formed memory in single-session learning. Especially, animals usually only associate the aversive stimulus with the context they encountered before conditioning, not after. This is also called forward conditioning. By contrast, backward conditioning refers to receiving the shock prior to the context. A recent study showed that backward context conditioning is able to retrieve contextual memory as well (Ressler., et al., 2021). This study suggests that even exposing the context after conditioning could still form a contextual memory that associates the

shock at conditioning. Thus, updating the 30s PSI memory with a 720s rehearsal session should be able to add the contextual information to the original context. This is also consistent with previous papers, where updating an impoverished context memory can make it an enriched context (Zinn., et al., 2020). However, how the memory trace of an updated 30s PSI memory is different from a 720s PSI memory is still in mystery. In future, the difference between the memory trace of a well-formed memory and an updated memory needs to be further investigated.

6.10 Conclusion

I investigated how learning affects memory consolidation in the study. These findings shed light upon studying the relationship between memory consolidation and its initial completeness. The learning duration may not affect the memory during synaptic consolidation, but systems consolidation might be varied by learning durations. Also, updating a poorly formed memory may not substantially change its physiochemical makeup. Although it is unclear whether the short PSI memory is contextual, this study suggests learning durations might affect memory storage location and systems consolidation.

This study also paved the way for further understanding of memory encoding and updating. However, it is still unclear how memory updating interacts with the original memories and modifies them, which will be investigated in future. In conclusion, this study provides behavioural evidence that memory consolidation with different learning durations might vary. Still, a more in-depth study at the molecular and cellular level is required to explain the mechanism in future.

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