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Thesis

THE CHANGING CELLULAR REPRESENTATION OF A CONTEXTUAL FEAR MEMORY IN REGION CA1 OF THE MOUSE HIPPOCAMPUS

by

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I would like to dedicate this work to my grandfather Hector Urrutia, for his endearing faith in me as a scientist from day one. Thank you to my parents Maria and Todd Odom for creating the opportunity for me to dive fully into science.

I would like to recognize the contributions of PhD candidate Amy Monasterio in the conceptual development and execution of all experiments. This work is not mine, but ours.

Finally, I would like to thank Dr. Steve Ramirez for the opportunity to pursue a senior project and for his endlessly hopeful, positive attitude.

Abstract

Memory formation, consolidation and retrieval depend on the hippocampus and region CA1 which plays a critical role in integrating spatial and contextual information. During memory encoding the connections within an active cell ensemble are strengthened and these strengthened connections are believed to constitute the physical basis of memory, the engram. With time, the memory trace redistributes from the hippocampus to cortical regions according to the model of systems consolidation.

The cellular representation of memory is dynamic, yet memory requires stability to retain accurate information. The purpose of this project is to characterize representational drift, the change in cellular representation over time, of a contextual fear memory in CA1 of the mouse hippocampus. To accomplish this, we apply Tet-Tag-mCherry to label cells active in CA1 during the acquisition of a contextual fear memory and identify c-Fos + cells active during recall tests at 2, 4, 7, 14 and 28 days. Co-labelled mCherry and c-Fos+ cells represent reactivation of the initial memory trace.

Our results demonstrate that the initial representation of the contextual fear memory in CA1 is stably reactivated across all time points and that the behavioral expression of fear memory is sufficiently retained up to 28 days. A reduction in c-Fos+ cells per area is observed at late (7, 14 and 28 days) recall compared to early recall (2 days), consistent with the systems consolidation postulate of reduced engagement of the hippocampus in remote memory retrieval. Finally, a decrease in total number of reactivated cells normalized to area is observed from 2 day recall to 4, 7, 14, and 28 day recall. Together, the results suggest that the representation of the contextual fear memory in CA1 becomes more efficient over time. Further validation of the mCherry reporter is required to confirm these interpretations. Representational drift in the hippocampus is a critical mechanism of memory which may facilitate the efficient consolidation and continual updating of memory.

Introduction

The hippocampus plays a critical role in the encoding and retrieval of episodic memory, which consists of the spatiotemporal trajectory of a past event or experience. It includes patterns of spatial and contextual information which allows temporal segmentation of past events. Episodic memory is hippocampal-dependent during encoding and early retrieval, yet the role of the hippocampus in long-term episodic memory storage and retrieval remains unclear. An episodic memory has been suggested to be encoded by cells which are highly active during a given experience. Synaptic plasticity mechanisms modify the connections between the cells in an activity dependent manner to form the postulated memory engram. The engram hypothesis is validated by optogenetic experiments which tag and manipulate cells to demonstrate the sufficiency and necessity of the engram in memory retrieval (Liu et al 2012, Denny et al 2014). Engram studies have largely tested hippocampal representations of memory and only partially describe the brain-wide mechanisms of memory encoding, consolidation, and storage.

Systems consolidation describes the translation of memory from the hippocampus to cortical regions for long-term storage. The shift in memory representation from the hippocampus to the cortex occurs incrementally, and eventually the memory can become hippocampal independent. The cellular representation of memory is dynamic and continuously updating. Stochastic neural activity modifies the cellular connectivity implicated in memory in a non-specific manner, while recall-driven reactivation strengthens the connectivity in a specific manner. The changing synaptic and cellular representation of memory is characterized as representational drift.

Synaptic plasticity mechanisms are implicated in the encoding, consolidation and representational drift of memory. Transient synaptic plasticity mechanisms facilitate the encoding of a memory by creating an immediate trace of connectivity between highly active cells. Persistent synaptic plasticity mechanisms create a more stable and long lasting representation of the memory. However, there is a limit

to the longevity of synaptic modifications which leads to the plasticity versus stability dilemma when considering synaptic and cellular connectivity as the physical substrate for memory. Representational drift may protect memory against the temporary nature of synaptic structure by continually updating the biophysical trace of memory.

1.1 The Role of the Hippocampus in Memory

The neuroscience of memory stands upon the shoulders of Henry Molaison (H.M.). On September 1st, 1953, neurosurgeon William Beecher Scoville removed H.M.'s medial temporal lobe, including his hippocampus, with the intention of treating epilepsy. From this moment forward, H.M. lived in a "permanent present tense." (Corkin, 2013). Professor Hasselmo met H.M. at Massachusetts General Hospital during his visit for an anatomical MRI scan and recounts the 68-year old H.M. as having "the personality [of a] 27-year old man" (Hasselmo, 2012). H.M retained his youthful personality because the removal of his hippocampus led him to develop anterograde amnesia, an inability to form new memories. Fortunately, H.M. retained self-identity and the ability to recall memories from his youth. Nurses reported that H.M. would be startled by his reflection in the mirror because his internal image of himself did not update as he aged. The development of anterograde amnesia demonstrates the necessity of the hippocampus in episodic memory formation. H.M. also developed graded retrograde amnesia, an inability to recall memories of past experiences which occurred near the time of surgery. The development of graded retrograde amnesia implicates the hippocampus in the processing of recent memories. H.M. 's life provides the foundation for exploring the hippocampus as the memory center of the brain, especially in the encoding and early consolidation of memory.

The specific effect of hippocampal damage on memory identifies the hippocampus as an important subregion of the medial temporal lobe. Many primate and rodent studies have validated the necessary role of the hippocampus in episodic memory. In one such study, hippocampal lesions, but not amygdala lesions, impaired a monkey's memory performance on memory tasks sensitive to human amnesia (Zola et al 1991). Chemical lesions of the hippocampus impaired rodent performance on memory

tasks requiring recall of spatial and contextual information (Jarrard 1993). Pharmacological inhibition of the hippocampus during memory recall tests impairs retrieval of contextual fear memory at recent time points (Deisseroth et al 2011). The hippocampal lesion and inhibition induced memory impairments implicate the hippocampus in the processing and consolidation of contextual information used in episodic memory. Modern optogenetic inhibition studies further demonstrate the necessary recruitment of specific hippocampal regions in episodic memory encoding, consolidation, and retrieval (Deisseroth et al 2011; Goshen 2014; Liu et al 2012).

1.2 Hippocampal Anatomy: The Trisynaptic Circuit

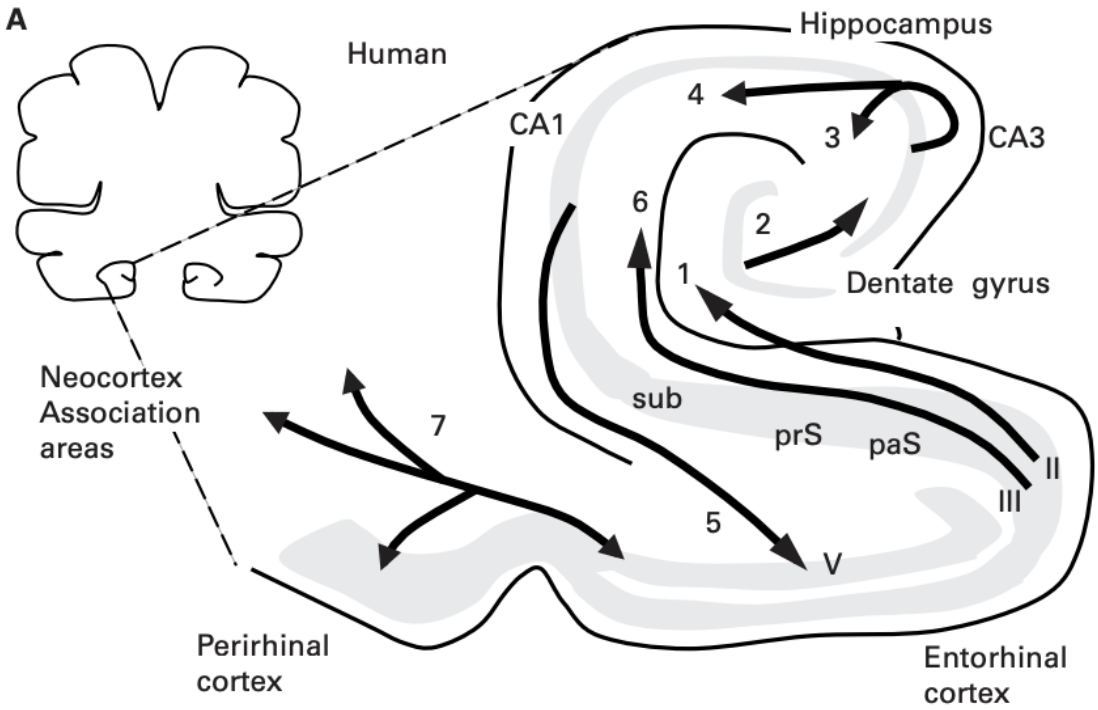

Figure A: Hippocampal anatomy and connectivity.
Source: How We Remember: Brain Mechanisms of Episodic Memory Hasselmo 2012 Figure 5.1 A.

Santiago Ramón y Cajal, a neuroscientist renowned for his drawings of neurons, first identified the hippocampal trisynaptic circuit which consists of CA1, CA3 and the dentate gyrus (DG). Within the medial temporal lobe, the hippocampus exchanges input and output with the entorhinal cortex

(ETC). The following description of hippocampal anatomy is adapted from Hasselmo BOOK Chapter 5 to contextualize the anatomical significance of region CA1. Emphasis is placed on the projection pathways within the hippocampal trisynaptic circuit. (1) The ETC layer II sends input via perforant path fibers to the DG. (2) DG mossy fibers project to CA3 pyramidal cells. (3) Excitatory recurrent connections within CA3 pyramidal cells. (4) CA3 pyramidal cell Schaffer collaterals send input to CA1 pyramidal cells. (5) CA1 sends excitatory feedback to the subiculum and deep layers of ETC. (6) ETC layer III projects to CA1. (7) The ETC and neocortex project bidirectionally.

1.3 The Role of CA1 in Memory

The region CA1 is uniquely positioned to act as a comparator of input from CA3 and the ETC in memory discrimination (Hasselmo, 2012). First, the recurrent connectivity within CA3 allows the region to take a sub-input pattern and generate full memory patterns - a process termed "pattern completion." The pattern completion in region CA3 functions in an auto-associative manner, recruiting many related memories which may share the same sub-input pattern. Thus, CA3 plays a general role in memory retrieval and another mechanism is required for memory specificity. The ETC provides contextual input from sensory cortices to both CA1 and CA3. In CA3 the medial and lateral ETC inputs are mixed and generate a strong merging of spatiotemporal information. The laminar segregation of medial and lateral ETC input is a distinct feature of CA1. CA1 compares the input from CA3 and ETC, and generates a signal only when the input patterns match. Biophysically, matching is the constructive interference of CA3 and ETC induced depolarization of CA1 pyramidal cells to reach their activation threshold. It is postulated that CA1 plays a critical role in reducing redundancy in memory encoding and retrieval by stopping the propagation of non-specific memory patterns generated by CA3.

The context-specificity of CA1 is utilized in the study of contextual fear memory. To demonstrate the context-specificity of CA1 Tayler et al fear conditioned mice in context A, tested one half in context A, and tested the other half in a distinct context B (2013). The cells tagged during fear conditioning were significantly reactivated above chance in the matching train A - test A context, but not in the distinct train

A - test B context. As I write in the Boston Public Library and occasionally walk to find the water fountain or the courtyard, I can't help but wonder about the context-specific activity of my CA1. Especially when my brain fails to remember the opening at the end of the room with tiered bookshelves that leads to the next room which connects to the children's space with the functional water fountain.

1.4 Synaptic Plasticity Mechanisms of Memory: LTP and LTD

Hebbian plasticity provides the framework for learning and memory as activity-driven synaptic modifications. Hebb's rule postulates that cells which are coactive will strengthen their connectivity and that cells that are independently active will weaken their connectivity (Hebb, 1949). Carla Shatz, a Stanford professor of biology and neurobiology, coined the common phrase: "cells that fire together, wire together." Hebbian plasticity is primarily mediated by long-term potentiation (LTP) and long-term depression (LTD). Long-term potentiation describes the increased synaptic transmissibility between two neurons which were strongly coactive (Bliss and Lomo, 1973). Conversely, long-term depression describes the decreased synaptic transmissibility between neurons which were disjointly active. LTP and LTD are mediated by changes in postsynaptic receptor density, namely of AMPA and NMDA receptors, as well as postsynaptic excitability. Both AMPA and NMDA receptors are stimulated by the excitatory neurotransmitter glutamate, yet AMPA receptor (AMPAr) sodium channels are more easily opened. NMDA receptors (NMDAr), in addition to requiring glutamatergic binding, are voltage-gated by a magnesium block. Thus, AMPAr driven depolarization of a neuron can facilitate the voltage activation of NMDA receptors channels which allow the influx of both sodium and calcium. Calcium induces a cascade of cellular effects such as phosphorylation of AMPAr at the postsynaptic membrane and promoting the trafficking of AMPAr to the postsynaptic membrane - all effects which increase the excitability of the postsynaptic neuron. NMDA receptor dependent LTP and LTD in the excitatory hippocampal pyramidal neurons is believed to construct the neural trace of memory (Sumi and Harada, 2020). Studies show that blocking NMDAr during fear conditioning impaired the ability of the mouse to develop a conditioned fear response (Nabavi and Fox et al, 2014; Ext Data Fig 6). The impairment

demonstrates that NMDAr-driven LTP is required for the formation of an associative fear memory. The mechanisms regulating the induction of LTP versus LTD are not fully clear, but competition between exocytosis and endocytosis in AMPAr trafficking likely determines the relative dominance of LTP or LTD (Sumi and Harada, 2020). The identification of molecular mechanisms contributing to LTP and LTD helps to bridge the gap between molecular, cellular and cognitive neuroscience.

In "Engineering a memory with LTP and LTD," Nabavi and Fox address the link between synaptic plasticity and memory from a synaptic, systems and cognitive perspective (2014). From a systems perspective, the researchers identify the auditory cortex, medial geniculate nucleus and lateral amygdala as functional brain regions implicated in forming an associative tone-fear memory. The auditory cortex processes auditory input while the medial geniculate nucleus relays auditory information. The amygdala is implicated in, and necessary for, the expression of fear. The synaptic substrate of neural connectivity contributing to the associative fear memory was isolated and manipulated by a novel optogenetic approach. Traditionally, to induce an associative fear memory mice are fear conditioned with a foot shock, the unconditioned stimulus (US), and a tone, the conditioned stimulus (CS). With incredible ingenuity, Nabavi and Fox replace the tone during fear conditioning with optogenetic stimulation of the specific synaptic projections from the auditory cortex and medial geniculate nucleus at the lateral amygdala. Behavioral data validate that the optogenetic stimulation of auditory neural input successfully replaces the tone in forming an auditory-fear associative memory. Additionally, co-presentation of the optogenetic-audio input and foot shock induce LTP at the specific synaptic sites as evidenced by a more than 2-fold increase in the AMPAr to NMDAr ratio contribution to synaptic current. The observed LTP further validates the neural engineering model to induce and manipulate associative fear memory.

The researchers apply optical LTP and LTD strategies to see the positive and negative contribution of these mechanisms to memory encoding and accessibility (Nabavi and Fox et al 2014). Following paired optogenetic-auditory stimulus and shock, the mice demonstrated learning in a recall test two days later. Presumably, the relevant neural circuitry was potentiated (LTP) allowing for successful recall. Next, an optical LTD protocol was applied and mice demonstrated a significant impairment in

retrieval during a recall test one day later. Then, an optical LTP protocol was administered and mice tested one day later demonstrated recovery and successful recall of the associative fear memory. Successive presentations of optical LTP and LTD protocols in the same mice repeatedly produced the effect of LTD inactivating the memory and LTP recovering the memory. The results demonstrate that the synapses involved in the associative fear memory are capable of memory retention even under several rounds of bidirectional synaptic plasticity. Bidirectional synaptic plasticity describes the repeated potentiation and depression of the same synapses induced by the optical LTP and LTD protocols in this study. The profound capacity of neural connectivity to retain memory under synaptic structural construction (LTP) and deconstruction (LTD) has important implications for the dynamical model of memory storage over time.

1.5 The Engram as a Physical Substrate of Memory

The engram, a physical substrate of memory, was first postulated by the German biologist Richard Semon in the early 1920's. Semon's engram theory is composed of the "Law of Engraphy" for memory encoding and the "Law of Ecphory" for memory retrieval. The Law of Engraphy describes how experience-induced "simultaneous excitations" form a connected, physical trace that persists after the experience The Law of Ecphory describes the cue driven "partial return of an energetic situation," or reactivation of the engram trace in memory recall (Semon 1921, 1923). Essentially, engram theory hypothesizes that experiences create an enduring physical trace which can be reactivated to *relive* the experience in memory. Semon's proposal of the engram was novel for the 20th century and upholds remarkably well with current theories of memory. Modern engram theory is best captured in the "Identification and Manipulation of Memory Engram Cells":

> When a subject undergoes an experience, or episode, a set of selected stimuli from the experience, or episode, activate populations of neurons to induce enduring biochemical and electrophysiological changes in these cells and their connections, each contributing to the storage of the memory. Subsequently, when a part of the original stimuli returns, these cells are

reactivated to evoke the recall of the specific memory (Liu, Ramirez, Redondo and Tonegawa 2015).

Today, the engram describes the lasting biophysical modifications between cells active during the encoding of an experience. Hebbian plasticity as a function of LTP and LTD provides a plausible mechanism for these changes in cellular connectivity. The engram stores information present during encoding and represents a long-term trace that is reactivated in the recall of past episodic events. The engram can exist in active or dormant states and is distributed brain wide.

To properly identify a neural ensemble as an engram: synaptic and cellular *changes* must be observed and *sufficiency* in inducing recall as well as *necessity* for memory expression must be demonstrated (Tonegawa et al 2015; Ramirez thesis, 2015). The advanced understanding of neurons, synaptic connections and neural circuits combined with novel neurotechnologies has been applied to validate the role of engrams in memory.

1.6 Activity-Dependent Genetic Tagging of Neurons

Genetic strategies capitalize on the activity-dependent nature of immediate early genes (IEGs) to selectively label cells that are sufficiently recruited during an experience. The cells are selected based on activity: high activity induces downstream cellular cascades that impact the expression of IEGs such as c-Fos, Arc and Zif268 (Guzowski et al 1999;Guzowski, 2002; Guzowski et al 2006; Jones et al 2001; Okuno et al 2016). The IEG c-Fos is a regulatory transcription factor that is transcribed within minutes and has peak protein levels between 60 to 90 minutes post activation (Guzowski, 2002). Thus, c-Fos can be referenced as a correlate of recent neural activity. The Tet-Tag system utilizes activity-driven c-Fos expression to express tetracycline-transactivator (tTA) by coupling the c-Fos promoter to the tTA gene (Reijmers et al 2007). Then tTA activates the tetracycline response element (TRE) promoter, driving expression of the fluorescent protein coupled to the TRE promoter. For the purposes of this project, the TRE promoter is coupled to mCherry, a red fluorescent reporter (figure B).

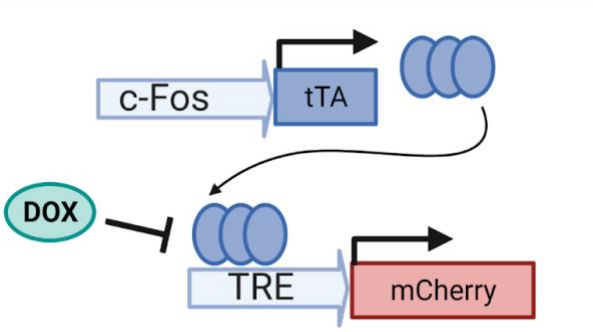

Figure B. Viral Constructs for Tet-Tag System.

Critically, doxycycline (DOX) diet is administered to restrict expression of the fluorescent reporter to the designated "tagging window." DOX binds to tTA, inhibiting the activation of the TRE promoter and preventing expression of the TRE-coupled protein (Reijmers et al 2007). In the absence of DOX, the Tet-Tag system labels highly active cells with the desired fluorescent reporter. The Tet-Tag system has been applied to label or "tag" neurons active during the acquisition of a contextual fear memory in a temporally and spatially specific manner (Liu et al., 2012; Tayler et al 2013). Additionally, c-Fos staining allows visualization of neural activity during fear memory recall, by collecting the brain 90 minutes after this behavioral experience. In Tayler et al. 2013, the researchers examined the co-labelling of neurons in hippocampal regions during the memory acquisition and recall events, to study how reactivation of the postulated engram cells changes from recent to remote recall. The "Reactivation of Neural Ensembles During the Retrieval of Recent and Remote Memory" is a primary influence for my project, and a strong example of the application of the activity-dependent Tet-Tag system and IEG c-Fos staining in studying engrams and the changing cellular representation of memory over time (Tayler et al, 2013).

1.7 Light on the Brain: Optogenetics Characterization of the Engram

Optogenetics revolutionized neuroscience by placing light-driven temporal control of specific neural populations in the hands of researchers (Boyden et al 2005). Just short of magic, optogenetics "genetically tricks brain cells to respond to light, and thus confers millisecond control over defined sets of

cells in the brain" (Ramirez thesis, 2015). The technology applies transgenic strategies to express a light activatable ion-channel in neurons in an activity dependent manner similar to the Tet-Tag system. The famous light-activated ion channel channelrhodopsin (ChR2) is permeable to cations and induces a depolarizing, excitatory effect on the neurons. Other rhodopsins are permeable to chlorine anions and induce hyperpolarizing, inhibitory effects in the neurons. With wild optogenetic engineering, the algae-derived ChR2 allowed the first manipulation of neurons which are highly active during memory acquisition events (Liu and Ramirez, 2012).

Optogenetics, the light-activation of neurons, has enabled the identification and manipulation of mouse hippocampal cell populations involved in contextual fear memory. For example, optogenetic stimulation of dentate gyrus (DG) cells active during the initial fear conditioning event induced freezing in a non-associated context, demonstrating their *sufficiency* for memory retrieval (Liu, 2012). Optogenetic inhibition of similarly tagged DG cells disrupted fear memory recall, demonstrating the tagged cells' *necessity* in memory retrieval (Denny et al 2014). Together, these optogenetic experiments support localization of a fear memory in DG cell ensembles. A breadth of optogenetic studies have explored engram and hippocampal region dynamics in memory beyond the DG.

1.8 Manipulation of CA1 in Memory Models

Optogenetic and pharmacological inhibition studies have explored the role of region CA1 in associative fear memory and have demonstrated the necessity of dorsal CA1 (dCA1) activation for memory acquisition and recall. The optogenetic inhibition to dCA1 in mice during fear conditioning leads to significant impairments in memory acquisition (Deisseroth et al 2011). The inhibition of neural activity in dCA1 prevents the formation of a potentiated neural substrate for the associative fear memory. In a separate experiment, temporal (short-term) optogenetic inhibition of dCA1 during recall tests at recent (1 day) and remote (28 days) time points impaired the mice's ability to recall the associative fear memory (Deisseroth et al 2011). The temporal optogenetic inhibition of CA1 was further tested and the impairment on remote recall was replicated at 9 weeks and 12 weeks. The recall impairment implicates

CA1 in both recent and remote memory retrieval, contrary to previously reported findings. Pharmacological inhibition and lesion studies on CA1 and the hippocampus report an impairment in recall at recent, but not remote time points (a million citations). The role of CA1 in retrieval of associative contextual fear memory at remote time points is not clear cut.

To reconcile the discord with older research that states that CA1 is not necessary for remote memory recall and new research which clearly demonstrates that CA1 is implicated in remote memory recall - the researchers extended the optogenetic inhibition time to match the time course of pharmacological inhibition (Deisseroth et al 2011). Interestingly, the prolonged optogenetic inhibition experiments replicated the results of pharmacological inhibition experiments. Specifically, the 30 minute optogenetic inhibition of dCA1 led to impairments in recent but not remote recall. The replicability of this finding, that CA1 is not necessary for remote recall, provides grounds and inspiration for the theory of systems consolidation: the uploading of memory from the hippocampus to the cortex and distributed brain regions.

1.9 Memory Uploading: Synaptic and Systems Consolidation

In 1950, Karl Lashley's Mass Action Principle brought forth the idea that the memory engram is not localized to any single brain region but rather distributed across many. Lashley pursued lesion studies in attempt to localize a specific region contributing to memory impairments by damage to the engram. Instead, Lashley discovered that the degree of memory impairment correlated with the extent of lesion damage but not with the location of the lesion. The results provided an early indication that memory is stored not only in the hippocampal circuitry, but across many brain systems.

1.9.1 Defining Synaptic and Systems Consolidation

Consolidation, the translation of memory from a temporary to long-lasting representation, can be considered at a synaptic and a systems neuroscience level. Synaptic consolidation refers to a stimulus

induced change in local synaptic strength within a broader neural circuit that encodes the memory (Dudai et al 2012). Synaptic consolidation creates the initial memory representation to be stored long-term by systems consolidation: a "process in which an experience-dependent internal representation is converted to a long-term form and reorganized over a distributed brain circuit" (Dudai et al 2012). Together, synaptic and systems consolidation provide a comprehensive framework for understanding memory consolidation.

The standard systems consolidation model describes the transfer of memory from the hippocampus to a distributed memory trace across brain regions. A human metabolic neuroimaging study on memory observed increased hippocampal activity during recent retrieval (5 days) compared to remote retrieval (25 days) (Bontempi et al 1999). Memory is hippocampal dependent in the encoding and early consolidation phases. Alternatively, the study observed low activity in the frontal cortex, anterior cingulate cortex, and the temporal cortex during the recent retrieval and observed significantly increased activity in these regions at the remote retrieval (Bontempi et al 1999). Over time, the memory moves to cortical regions and becomes hippocampal independent. The reduction in hippocampal activity and increase in cortical activity from recent to remote retrieval provides strong evidence for the standard systems consolidation model.

1.9.2 Systems Consolidation: The Role of CA1

Systems consolidation is further supported by studies which demonstrate that hippocampal regions are necessary for memory retrieval at recent but not remote time points. For example, the prolonged inactivation of CA1 during recall tests did impair recent but not remote memory retrieval (discussed in 1.8; Deisseroth et al 2011). During the recent recall test, consolidation mechanisms are actively processing the memory and the hippocampus remains critical for memory retrieval. During remote recall, however, the memory trace has been consolidated across many brain regions which can compensate for the hippocampal impairment in memory retrieval. A candidate region that may provide compensation for dCA1 inhibition in fear memory retrieval is the anterior cingulate cortex (ACC).

Following prolonged inhibition of dCA1 during recall, an increase in c-Fos activity was observed in the ACC demonstrating that the ACC is involved in remote memory retrieval. Additional experiments show that both precise and prolonged optogenetic inhibition of the ACC induce memory impairments at the *remote,* but not recent recall tests. The specific impairment by ACC inhibition at remote recall validates the transfer of memory dependence to cortical regions over time. Furthermore, an increase in c-Fos activity was observed in the prefrontal cortex (PFC) during remote memory retrieval. The increased activity of the ACC and PFC, as well as the role of ACC in remote fear memory recall, support the translation of memory to cortical regions in the systems consolidation model.

The role of hippocampal region CA1 in recent and remote memory recall is more complicated as the region does not follow the standard systems consolidation model. CA1 is proposed to recruit the brainwide physical trace of the fear memory in natural memory retrieval at recent and remote recall. In opposition to the lack of memory impairment at remote recall by prolonged dCA1 inhibition, the precise optogenetic inhibition of dCA1 during recall tests *did* impair remote memory retrieval (Deisseroth et al 2011). The dCA1 induced memory impairment, and other dCA1 inhibition studies, motivate the idea that dCA1 acts to recruit relevant cortical regions in remote memory retrieval. Inactivation of dCA1 disrupts memory-specific activity in the subiculum, retrosplenial cortex (RSC), and lateral/perirhinal entorhinal cortex (LEC/PER) during fear-memory recall (Tanaka et al 2014). In fact, dCA1 inhibition during recall led to a significant reduction in the reactivation of tagged "fear engram" cells in the subiculum, RSC, and LEC/PER (Tanaka et al 2014). The reactivation was assessed by comparing the tagged cells to c-Fos activity during recall tests. The reduced reactivation of "fear engram" cells in cortical regions following dCA1 inactivation during retrieval provides strong support for the role of CA1 in the recruitment of the distributed memory trace and systems consolidation.

The role of CA1 in recruiting a distributed memory trace is connected to how the engram captures brain-wide activity during an experience. Prioritization of the hippocampal dependence of memory during early phases of consolidation does not make the brain-wide nature of the engram clear. The hippocampus *is* significantly recruited during the memory acquisition and encoding phase to assemble information from

the sensory cortices, motivational nuclei, and other relevant regions in a coordinated manner. However, the cellular trace of activity, the engram, is not restricted to the hippocampus. The brainwide pattern of activity exists immediately following an episodic event, but the physical trace is initially weak. Thus, the hippocampus plays a critical role in driving the repeated reactivation, or replay, of activity patterns across cortical regions. The replay occurs in a highly temporally compressed manner and often in reverse order. Hippocampal directed replay provides a plausible mechanism for systems consolidation that fits within the broadly accepted framework of synaptic consolidation: the repeated reactivation of a synaptic trace by hippocampal replay drives activity dependent synaptic modifications which continually update and consolidate the memory to a longer-term cortical representation.

Let's briefly return to the paradigm of CA1's role in remote memory retrieval with the context of synaptic and systems consolidation. It is proposed that the remaining trace of a memory in region CA1 at remote time points reflects an efficient, compressed representation of the memory that is used in memory retrieval and memory updating. In memory retrieval, the compressed representation in CA1 may act as a guide to find and activate the cortical representations specific to that memory. Remember, however, that CA1 is not necessary for memory retrieval at remote timepoints and the brain may employ alternative mechanisms for the recruitment of the cortical memory trace. In memory updating, the compressed representation may be used to passively reactive the memory trace to protect it against synaptic dynamics to be discussed in the following section.

1.10 From Memory Trace to Representational Drift

Memories "do not simply conserve veridical representations of the past but must continually integrate new information to ensure survival in dynamic environments" (Mau et al 2020). This statement describes the foundational principle of representational drift: memory is not a static entity. Synaptic dynamics are constantly modifying neural connectivity and the brain must have mechanisms to protect memory against change. Thus far, much emphasis has been placed on the engram as an immediate synaptic trace of activity - with systems consolidation being a general mechanism for the long-term

storage of memory. In this section, we delve deeper into the synaptic mechanisms which contribute to the encoding, consolidation, and representational drift as the long-term evolution of memory.

1.10.1 Transient and Persistent Synaptic Dynamics

Synaptic structure is incredibly dynamic, yet the stability of synaptic connectivity is critical to the storage of information in a network state. How can a synaptic trace, the proposed physical representation of memory, reliably store information if synaptic structure is constantly changing? The stability-plasticity dilemma, which describes the discord between synaptic dynamics and synaptic storage models, is one of the biggest unanswered questions in memory research today.

Evidence for synaptic dynamics comes from longitudinal *in vivo* neuroimaging of dendritic spines, small protrusions from the dendrite which typically receive excitatory stimulus. Spines, a correlate of synaptic structure, may be classified as *transient* with a mean lifetime of 2 days or *persistent* with a lifetime ranging from months to years (Berry and Nedivi, 2017). Thin spines are classified as transient and constitute approximately ⅔ of synapses within the adult mouse hippocampus and cortex. Bulbous, excitatory mushroom spines are classified as persistent and constitute approximately ¼ of the synapses. To simplify the synaptic storage model, consider transient spines as reflecting "how much space is available" and persistent spines as the "space where stuff is stored." The anatomical ratio of transient and persistent spines is significant because transient spines reflect a capacity for new memory storage while persistent spines reflect the volume of occupied "storage space." The model of transient and persistent spines provides a framework to better approach the synaptic plasticity-stability dilemma.

Dynamic structural changes of synaptic connectivity are proposed to facilitate the transformation of the initial connectivity network, the engram, to a more efficient long-term representation. This transformation can be accomplished by synaptic sampling of a broader "sample space" for new, improved connectivity (Mau et al 2020). Transient spines have been observed to form, disappear, and reform over the course of days, providing a plausible mechanism for synaptic sampling (Attardo et al 2015, Berry and Nedivi 2017). In this model, transient spines test new synaptic connections for reliability and efficiency in

synaptic transmission. The successful transient spine would then be strengthened to a persistent spine. Transient and persistent spine dynamics evolve at a neural ensemble level to orchestrate the long-term consolidation of memory.

The sampling model describes the construction of an optimal memory-storage network; yet the model does not explain how the cells, synapses, and neurophysiological elements of learning and memory retain information while performing the "sampling." A neurobiological mechanism is required to retain information while in this labile, sampling state. The transient spines appear to retain "knowledge" of their location, allowing them to re-appear. Critically, the transient spine reflects a component of information storage in memory. To narrow the question, consider what mechanisms drive the formation, elimination and return of transient spines. I propose that coordinated activity-dependent genetic changes across the relevant ensemble of cells could temporarily store information while a transient synapse is in its hidden state. The genetic changes may include the up-or-downregulation of proteins for receptors, receptor scaffolds, and general synaptic growth. The genetic activity and synaptic structural modifications may act in an oscillatory pattern such that one retains information while the other is absent.

1.10.2 Synaptic Dynamics in CA1

To be completed!

Methods

ANIMALS

Male C57BL/6J (Charles River Laboratories) mice aged 1-3 months were used for the overlap experiments. All mice were maintained on a 12:12 hour light cycle with light beginning at 0700. Mice were placed on 40 mg/kg doxycycline diet (DOX) for a minimum of 24 hours prior to viral injection surgery and allowed access to food and water ad libitum. A minimum 10-day postoperative recovery period was given prior to experiments. 48 hours prior to contextual fear conditioning, the DOX diet was replaced with normal food to allow activity-dependent viral expression. The off-DOX period is termed the tagging window. At the end of the 48 hour period mice underwent contextual fear conditioning (500 second session with 3 shocks, 1.0 mA, 2 sec long). The mice were placed back on the DOX diet for the remainder of the overlap experiments. All animals were treated in accordance with protocol 201800579 approved by the Institutional Animal Care and Use Committee at Boston University.

VIRUSES

A Tet-Tag viral construct was used to label cells which are highly active within a period of time restricted by DOX. The cFos-tTA viral construct couples the c-Fos promoter to the tetracycline transactivator (tTA) transcription factor which binds to the tetracycline response element (TRE) in the absence of DOX. Activation of the TRE-mCherry construct drives the expression of mCherry, a red fluorescent protein, in cells with high activity during the tagging window. Doxycycline binds to tTA, preventing the expression of mCherry during the on-DOX period.

Vendor: UMass Viral Vector Core

Viral Construct: AAV-cFos-tTA + TRE-mCherry

STEREOTAXIC SURGERY

The mouse's weight was recorded prior to the procedure. Isoflurane was administered at 3.0 % for induction and maintained at 1.5 %. The mouse was placed in the stereotaxic frame with the nose cone and ear bars properly adjusted (Kopf instruments). 5 mg/kg of meloxicam and 0.2 mL of saline were injected subcutaneously. A rectal thermometer with an associated heat pad was administered to maintain the animal temperature between X degrees. Ophthalmic ointment (Puralube) was applied and maintained to protect the eyes. A chemical depilatory (Veet) was applied to the scalp to remove hair. The scalp was disinfected by applying 3 successive betadine and ethanol washes. 0.1 mL of lidocaine was administered under the scalp prior to making an incision along the midsagittal plane. Bregma and lambda were identified and used to level the skull along the anterior-posterior (AP) axis within 0.01 mm. The injection coordinates were used to level the skull along the medial-lateral (ML) axis within 0.01 mm.

A craniotomy was performed bilaterally at the injection site coordinates for dorsal CA1 (dCA1). The dCA1 coordinates are AP: -2.0, ML: +/- 1.5, DV: -1.5. The Hamilton needle was lowered to the injection site and held for three minutes before injecting 300 nL of the Tet-Tag viral cocktail at a rate of 100 nL/min (delivery system?). The needle was held for five minutes post injection. The scalp was sutured together and tissue adhesive (3M Vetbond) was applied. Following the procedure, 0.03 mg/kg of buprenorphine was injected intraperitoneally. The mice were monitored until they regained normal motor function.

Within 12-24 hours postoperatively, 0.03 mg/kg of buprenorphine was injected intraperitoneally and 5 mg/kg of meloxicam was injected subcutaneously. The mouse's weight was recorded and compared to the preoperative weight.

ANIMAL BEHAVIOR

Contextual Fear Conditioning

Prior to behavioral experiments, each mouse was handled for five to ten minutes on two separate days. Mice were placed in the fear conditioning chamber for 500 seconds receiving a 1.0 mA shock of two second duration at 198, 278, and 358 seconds (Colburn; 7 in L x 7 in W x 12 in H). Mice were kept in separate cages during the CFC protocol to prevent communication between cagemates. CFC was administered at the end of a 48 hour off DOX period.

Recall

2, 4, 7, 14, or 28 days after the CFC event mice were placed in the same chamber used for CFC for 10 minutes. No shock was administered and all animals were on DOX. All mice were transcardially perfused 90 minutes post placement in the chamber with 4 % PFA.

Behavioral Scoring

All freezing data was scored using FreezeFrame by manually setting a threshold for the motion index. FreezeFrame collects motion data by measuring the total change in pixel values between successive frames. Freezing is defined by a minimum bout of 1.25 seconds below the motion index threshold. The CFC behavioral data was segmented by time period between shocks. The first five minutes of the 10 minute recall event were scored.

HISTOLOGY

Following the 90-minute timed perfusion, brains were collected and post-fixed in 4% paraformaldehyde (PFA) for 48 hours. Brains were sliced coronally using the vibratome (Leica VT1000 S) at 40 um thickness and collected in PBS or stored in 0.01% sodium azide. If not sliced after the 48 post-fixation period, the brains were transferred to 0.01% sodium azide.

IMMUNOHISTOCHEMISTRY

Six slices were selected per animal. The slices are washed three times for 10 minutes in protein buffer solution (PBS). The slices were then blocked for 2 hours in a PBS solution containing 0.2% Triton X, 5% normal goat serum (NGS), and 5% bovine serum albumin (BSA). The slices were transferred to the primary antibody solution containing 0.2% Triton X in PBS (PBST), 5% NGS, and 1% BSA. The primary antibody solution containing rat anti-RFP (Chromotek) and rabbit anti-cFos (Abcam) was prepared at a 1:1000 dilution. Following 48 hours in the primary antibody solution at 4C, the slices were washed three times for 10 minutes in PBST. The slices were then transferred to the secondary antibody solution containing PBST, 5% NGS, and 1% BSA. The secondary antibody solution with goat anti-rat 555 (ThermoFisher) and goat anti-rabbit 488 (ThermoFisher) was prepared at a 1:200 dilution. The slices were incubated in the secondary antibody solution for 2 hours at room temperature. Next, the slices were washed with PBS three times for 10 minutes. Finally, the slices were mounted on slides with Vectashield DAPI mounting medium (Vector Laboratories). The slides were dried overnight and nail-polished the following day to seal from air exposure. All washes and staining periods were performed on a shaker.

CONFOCAL IMAGING

All slices were imaged using the Zeiss LSM 800 confocal microscope with a 20x/0.8NA air objective and Zen 2.3 software. Dorsal CA1 was imaged by creating nxn micron tiles with a z-stack defined by the signal range. Each slice within the z-stack was separated by 2 um. The following parameters applied across all images: pinhole 25 um; digital gain 1.0; averaging: 4 scans. For **overlap experiments** three channels were imaged: DAPI/blue 405 nm; mCherry/red 561 nm; c-Fos/green 488 nm. 6 to 8 images were acquired per animal. For **mCherry quality**

experiments two channels were imaged: DAPI/blue 405 nm; mCherry/red 561 nm. 2 to 4 images were acquired per animal. All mCherry quality images were acquired with the same parameters (561 nm: laser power 0.15%, master gain 630V; 405 nm: laser power 0.24%, master gain 700V).

CELL COUNTING

To draw the region of interest (ROI) a composite image of all three channels was max z-projected and the user manually drew an ROI around stratum pyramidale based on fluorescence signal in ImageJ. The user manually selected one z-slice from each image with even mCherry and c-Fos expression to quantify with the automated counting Jupyter Notebook adapted from Joe Zaki and updated by Amy Monasterio. The automated output includes the number of c-Fos + cells, the number of mCherry cells, and the number of co-labelled mCherry and c-Fos+ cells.

mCHERRY QUALITY ANALYSIS

3 to 6 brain slices were selected per animal and mounted without staining. To draw the region of interest (ROI) the DAPI channel was max z-projected and the user manually drew an ROI around stratum pyramidale based on DAPI in ImageJ. The ROI was applied to the max z-projected mCherry channel to measure mean intensity, standard deviation, and area. The variance was calculated as the square of the standard deviation.

Results & Discussion

To research how the cellular representation of memory changes over time we applied the Tet-Tag system to label cells active during contextual fear conditioning and c-Fos staining to identify cells active during recall sessions at 2, 4, 7, 14 or 28 days (figure 1,2). The change in overlap, or reactivation of tagged cells, provides insight into how similar or different the cellular representation of memory is between encoding and recall. The recall events are performed at multiple time points up to one month with the intention of capturing various stages of memory acquisition and consolidation. The overlap experiments were performed in dorsal CA1 (dCA1) which is critically engaged in the processing, storage, and retrieval of contextual information.

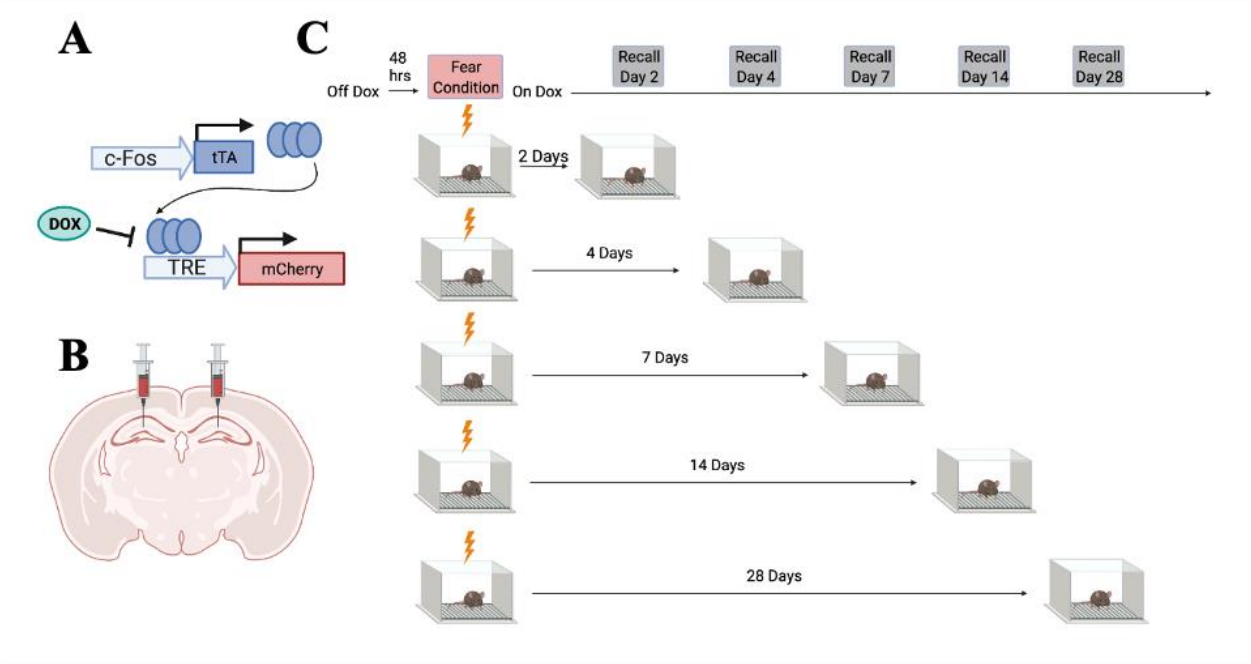

Figure 1 Tet-Tag Schematic and Experimental Timeline. (A) The Tet-Tag system was applied to label highly active cells during the off-DOX tagging period. (B) The Tet-Tag viral cocktail was injected bilaterally into dCA1. (C) Mice were fear conditioned at the end of a 48-hour off-Dox period. Mice were immediately placed back on Dox and tested for recall at 2, 4, 7, 14 or 28 days. Each time point is a separate group of mice.

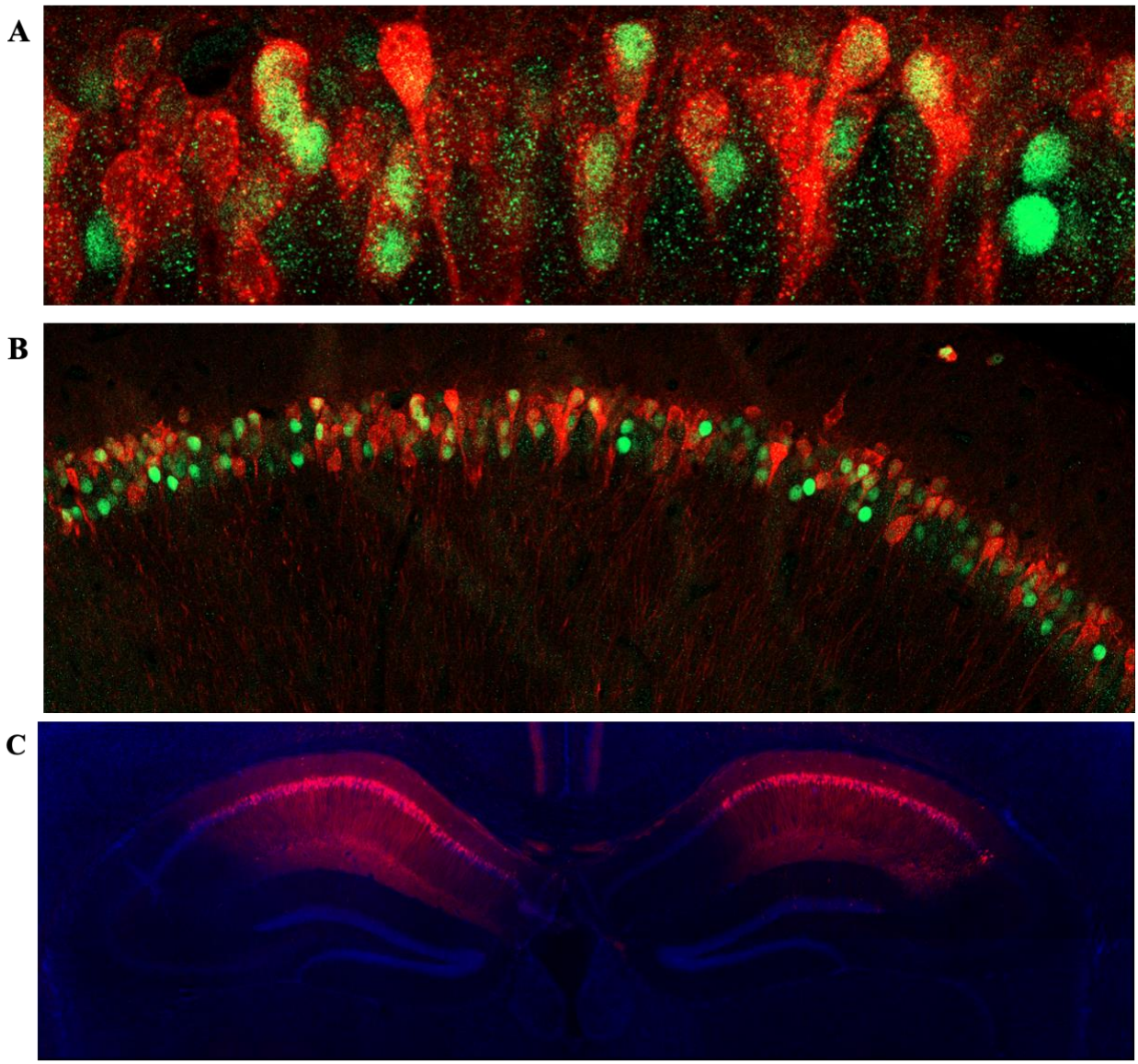

Figure 2 Representative Histology. (A) Red cells are Tet-Tag labelled with mCherry. Green cells are c-Fos+. Yellow cells are co-labelled with mCherry and c-Fos. (B) Typical image acquired of dCA1. (C) Full hippocampal image showing bilateral mCherry expression (pink) and cell nuclei (DAPI).

During contextual fear conditioning all groups of mice demonstrated fear learning with an increase in freezing from pre-shock to post-shock 3 (figure 3). Variability was observed between groups in percent freezing post-shock 3; this result may be attributed to differences in age between groups or differences in behavioral expression of fear between mice (figure 3). The percent freezing during recall was comparable across sessions performed at 2, 4, 7, 14 or 28 days showing sufficient memory retention and retrievability up to one month.

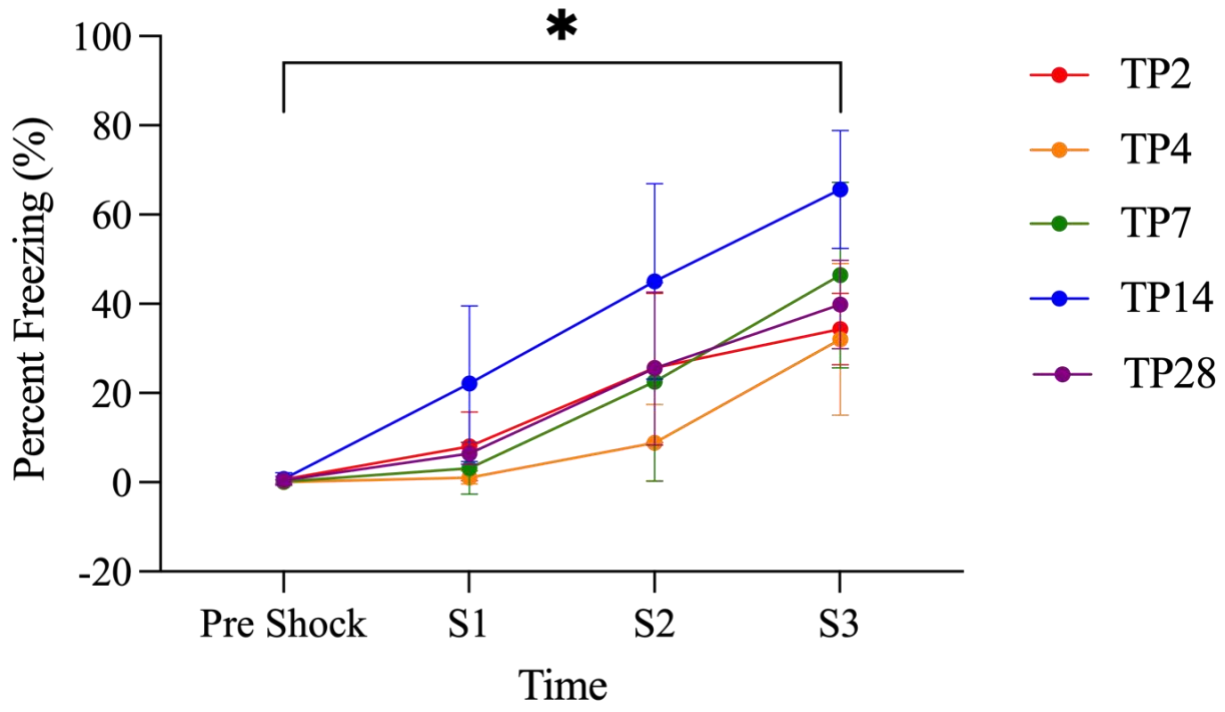

Figure 3 Percent Freezing During Contextual Fear Conditioning over Time Segmented by Shock. Shock 1 (S1) delivered at 198 s, Shock 2 (S2) delivered at 278 s, and Shock 3 (S3) delivered at 358 s. Each group demonstrated an increase in freezing with the presentation of each successive shock (two-way RM ANOVA: $F=89.47$, $p < 0.0001$, Time). Each group demonstrated a significant increase in percent freezing between pre-shock and post S3 time bins (Tukey's multiple comparison's test: TP2 ***, $p=0.0010$; TP4 *, $p = 0.0137$; TP7 *, $p=0.0176$; TP14 **, $p = 0.0012$; TP28: **, $p =0.0017$). A significant difference in percent freezing following S3 is observed between TP2 and TP14 (Tukey's MCT: *, $p=0.0179$) as well as between TP4 and TP14 (Tukey's MCT: *, $p =0.0309$).

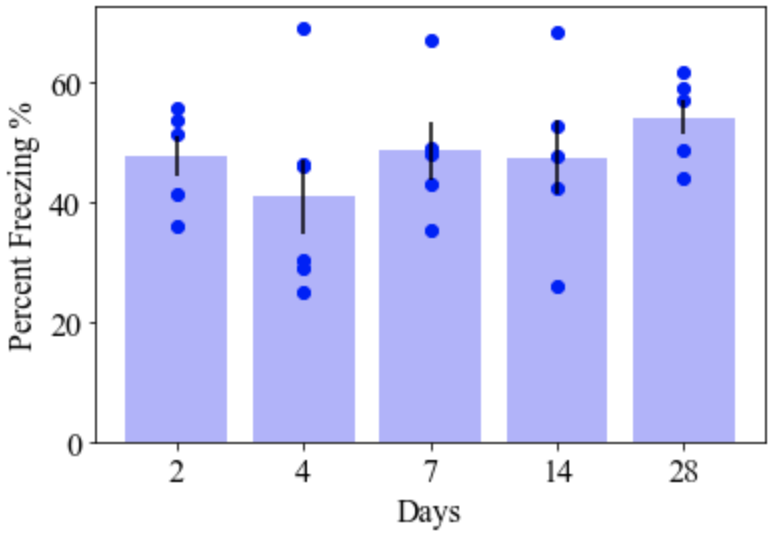

Figure 4 Percent Freezing during Recall. No significant difference was observed in percent freezing during recall between groups. All groups pass the Shapiro test for normality and the Levene test of variance for homogeneity. No statistical difference was observed between percent freezing across groups (one-way ANOVA: F=0.748, p=0.570).

The percent overlap, or number of co-labeled c-Fos+ and mCherry cells divided by total number of mCherry cells, remained stable across all time points (figure 5A). Thus, reactivation of the initial cellular representation of memory remains stable between memory retrieval at 2, 4, 7, 14 and 28 days. Additionally, the stable percent overlap corresponds to the stable expression of fear recall across all time points (figure 4). The mean percent overlap ranges from 25 to 40 % indicating that less than half of tagged cells are activity-specific to the cellular representation of fear memory. The non-specificity of the tagged cells may be a combination of the high basal activity of CA1 and the longer tagging window of 48 hours (Deisseroth et al 2011). The 90-minute post recall fixation of brain tissue provides a more temporal snapshot of activity in CA1. 2-photon *in vivo* imaging of tagged cells and calcium transient in dCA1 provides the cellular resolution and temporal clarity to properly address the changing cellular representation of memory and reactivation over time.

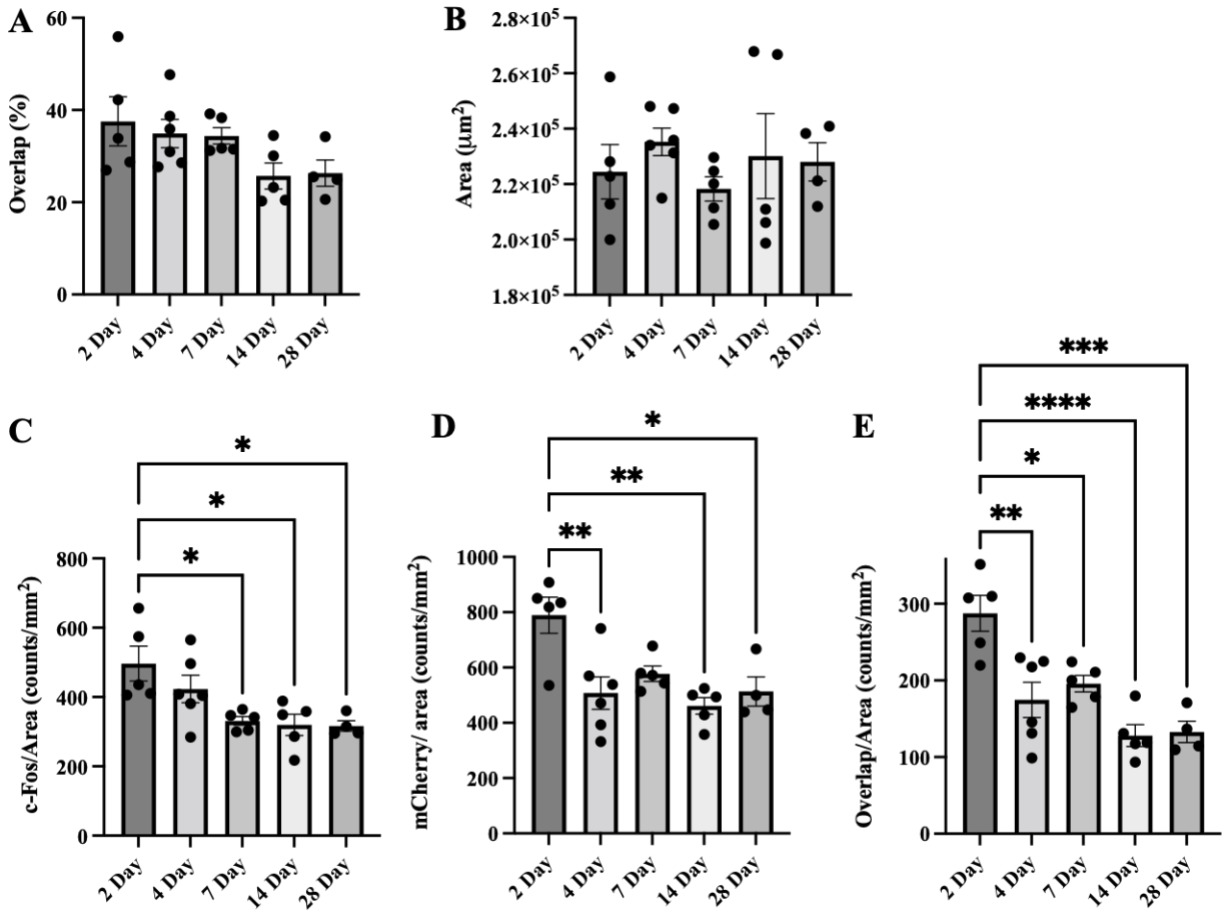

Figure 5 Reactivation of CA1 Tagged Cells during Recall at Various Time Points. (A) The percent overlap, the number of co-labeled c-Fos+ and mCherry cells divided by total number of mCherry cells, is consistent across time points (one-way ANOVA: F=2.398, p=0.0844). (B) The ROI area is consistent across time points (one-way ANOVA: F=0.5219, p=0.7207). (C) The number of c-Fos+ cells per area in mm^2 varies across time points (one-way RM ANOVA: F=5.106, p=0.0053). A significant decrease in c-Fos+ cells per area was observed from day 2 to day 7, day 14, and day 28 (Tukey's MCT: day 7 *, p= 0.0242; day 14 *, p= 0.0146; day 28 *, p= 0.0200). (D) The number of mCherry cells per area in mm^2 varies across time points (one-way RM ANOVA: F=6.546, p=0.0015). A significant decrease was observed in the number of mCherry cells per area from day 2 to day 4, day 14 and day 28 (Tukey's MCT: day 4 **, p=0.0044; day 14 **, p=0.0015; day 28 *, p=0.0128). (E) The number of overlapping, co-labelled c-Fos + and mCherry cells per area in mm^2 varies across time points (one-way RM ANOVA: F=11.36, p<0.0001). A significant decrease in overlap per area was observed from day 2 to day 4, day 7, day 14, and day 28 (Tukey's MCT: day 4 **, p=0.0022; day 7 *, p=0.0194; day 14 ****, p<0.0001; day 28 ***, p=0.0002).

In the following analysis we normalized c-Fos and mCherry counts to the area of the region of interest (ROI), CA1 stratum pyramidale. A significant decrease in c-Fos counts per area was observed from the 2 day to 7 day, 14 day, and 28 day time points (figure 5C). No significant difference in c-Fos per

area was observed between day 4 and other time points, but a stepwise decrease was observed from the 2 day to 4 day to 7 day recall (figure 5C). The decrease in c-Fos over time supports the model of systems consolidation and the efficient remodeling of hippocampal representations of memory over time. A significant decrease in mCherry counts per area was observed from the 2 day to 4 day, 14 day, and 28 day time points. The decrease in mCherry counts is not consistent with our hypothesis that the initial cellular representation of the contextual fear memory is comparable across groups. The quality of the mCherry reporter over time and performance of the automated cell counter are likely responsible for this effect. The mean intensity and variance of pixel values obtained from the unstained fluorescent reporter mCherry provide conflicting insight into the quality of mCherry expression over time: the mean intensity increased significantly at day 28 compared to day 2 and day 14 yet the variance increased significantly at day 28 compared to day 2, 4 and 14 (fig 6). Based on the increased mean intensity we would expect the number of identified mCherry cells to increase at the 28 day time point, but the opposite is observed (fig 5D). The increase in variance, the 'spread' of pixel values about the mean, provides more valuable insight into the variability of mCherry counts across groups. Visually, hyperlocal "dots" of higher intensity and regions of lower intensity are observed across the cell body at late time points, possibly a result of protein clustering and protein trafficking. Increased variance in pixel values may lead to decreased performance of the automated cell counter, as the image analysis functions perform optimally on clearly segmentable cells with even fluorescent signal across the soma. Further validation of the automated cell counter is necessary to conclusively identify mCherry reporter quality as the reason for decreased mCherry counts per area.

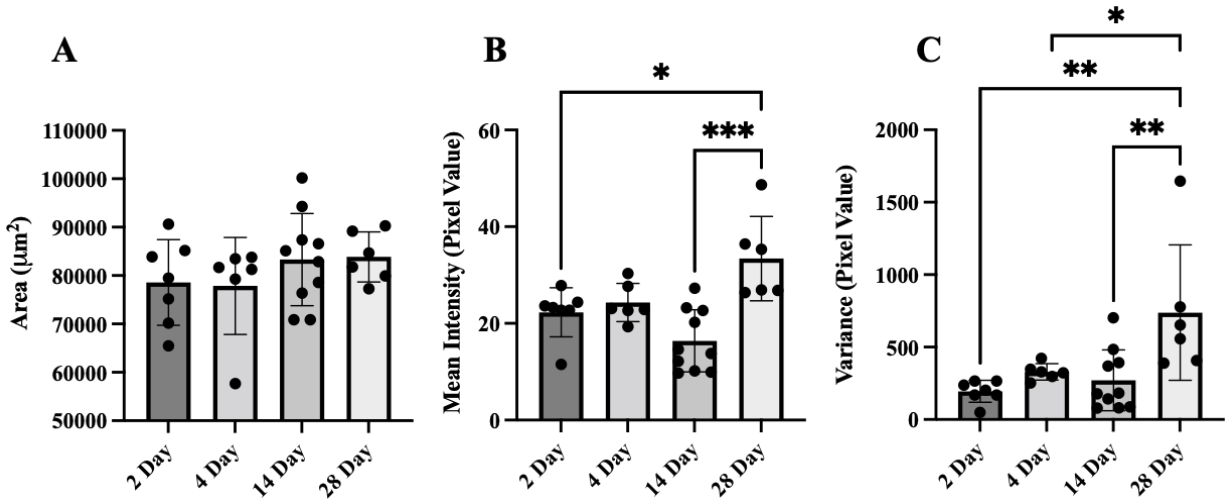

Figure 6 Quality of mCherry Reporter at Various Time Points. (A) The ROI area is consistent across time points (one-way ANOVA: F = 0.8766, p=0.4665). (B) The mean intensity of mCherry reporter varied across time points (one-way RM ANOVA: F= 9.343, p = 0.0003). A significant increase in mean intensity was observed at day 28 compared to day 2 and day 14 (Tukey's MCT: day 2 *, p=0.0188; day 14: ***, p = 0.0001). (C) The variance in pixel intensity varied across time points (one-way RM ANOVA: F=6.146, p=0.0028). A significant increase in variance was observed at day 28 compared to day 2, day 4 and day 14 (Tukey's MCT: day 2 **, p = 0.0031; day 4 *, p = 0.0397; day 14 **, p = 0.0062).

Finally, we normalize the overlap counts to the ROI area and observe a significant decrease over time. Specifically, we observe a significant decrease in overlap over area from day 2 to day 4, day 2 to day 7, day 2 to day 14, and day 2 to day 28. The overlap normalized to area tells a very different story from the overlap normalized to total mCherry counts. Considering the stable reactivation of tagged cells across all time points (5A) and the significant reduction in reactivated cells per area (5E) - I propose that there is an increased efficiency in the cellular representation of the contextual fear memory in CA1 over time. However, it is important to recognize that the decrease in identified mCherry cells at later time points may lead to undercounting of overlap cells. Further validation of the mCherry quality at remote time points, or application of a CRE-dependent labeling system to maintain expression levels over time, is required to eliminate this bias in raw overlap counts.

The stable reactivation of tagged cells in CA1 across 2, 4, 7, 14 and 28 day recall is consistent with the published report of stable reactivation between 2 and 14 day recall (Figure 5A; Tayler et al 2013). Our results expand on the work of Tayler et al 2013 by performing recall tests and assessing

overlap at many time points: 2, 4, 7, 14 and 28 days. Importantly, the stable reactivation of fear-tagged cells in CA1 indicates that CA1 is consistently implicated in natural recall from recent to remote time points up to 28 days. The decrease in c-Fos counts per area provides key insight into the degree of hippocampal, specifically CA1, activation in memory retrieval over time. The c-Fos activity significantly decreased from the early retrieval (day 2) to late retrieval (day 7, day 14, day 28). This finding is consistent with systems consolidation - how the initially hippocampal dependent and localized memory trace redistributes to cortical regions - decreasing, but not eliminating hippocampal engagement in remote memory retrieval. Together, the decrease in c-Fos activity and stable reactivation of tagged cells suggest that the cellular representation of memory becomes more specific and efficient over time. The efficient model of memory is further supported by the decrease in total number of reactivated cells per area - because this requires a smaller subset of cells to reliably retain the memory. In conclusion, the cellular representation of memory is consistent, and possibly more efficient, over time. Future experiments to visualize synaptic and cellular dynamics *in vivo* during learning and memory can expand the understanding of how stable and dynamic mechanisms contribute to the time evolution of a memory trace.

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