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Long-term stability of the hippocampal neural code as a substrate for episodic memory

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LONG-TERM STABILITY OF THE HIPPOCAMPAL NEURAL CODE AS A SUBSTRATE FOR EPISODIC MEMORY

by

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No man ever steps in the same river twice, for it’s not the same river and he’s not the same man - Heraclitus

DEDICATION

I would like to dedicate this work first and foremost to my wife, Susan. Without your love and encouragement I would never have started exploring the wonders of the brain.

I would also like to dedicate this work to my mother: you taught me the word “dendrites” and pushed me into this endeavor in your own way.

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ABSTRACT

The hippocampus supports the initial formation and recall of episodic memories, as well as the consolidation of short-term into long-term memories. The ability of hippocampal neurons to rapidly change their connection strengths during learning and maintain these changes over long time-scales may provide a mechanism supporting memory. However, little evidence currently exists concerning the long-term stability of information contained in hippocampal neuronal activity, likely due to limitations in recording extracellular activity *in vivo* from the same neurons across days. In this thesis I employ calcium imaging in freely moving mice to longitudinally track the activity of large ensembles of hippocampal neurons. Using this technology, I explore the proposal that long-term stability of hippocampal information provides a substrate for episodic memory in three different ways.

First, I tested the hypothesis that hippocampal activity should remain stable across days in the absence of learning. I found that place cells – hippocampal neurons containing information about a mouse's position – maintain a coherent map relative to each other across long time-scales but exhibit instability in how they anchor to the

external world. Furthermore, I found that coherent maps were frequently used to represent a different environment and incorporated learning via changes in a subset of neurons. Next, I examined how learning a spatial alternation task impacts neuron stability. I found that splitter neurons whose activity patterns reflected an animal's future or past trajectory emerged relatively slowly when compared to place cells. However, splitter neurons remained more consistently active and relayed more consistent spatial information across days than did place cells, suggesting that the utility of information provided by a neuron influences its long term stability. Last, I investigated how protein synthesis, known to be necessary for long-term maintenance of changes in hippocampal neuron connection strengths and for proper memory consolidation, influences their activity patterns across days. I found that along with blocking memory consolidation, inhibiting protein synthesis induced a profound, long-lasting decrease in neuronal activity up to two days later. These results combined demonstrate the importance of rapid, lasting changes in the hippocampal neuronal code to supporting long-term memory.

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LIST OF ABBREVIATIONS

AAV……Adeno-associated virus

AD……Alzheimer's disease

BTSP…….Behavioral Time Scale Plasticity

CA1……..Cornu Ammonis 1

CA2……..Cornu Ammonis 2

CA3……..Cornu Ammonis 3

CNMF-E……..Constrained Non-Negative Matrix Factorization for Microendoscope data

DBB……Diagonal Band of Broca

DG……...Dentate Gyrus

EC……..Entorhinal Cortex

ECII…….Entorhinal Cortex Layer II

ECIII……..Entorhinal Cortex Layer III

ER……Endoplasmic Reticulum

FOV…….Field-of View

GECI……...Genetically Encoded Calcium Indicator

GRIN……..Gradient Index

LC……Locus Coereleus

LEC……..Later Entorhinal Cortex

LTP……..Long Term Potentiation

L-LTP……...Long-Lasting Long Term Potentiation

MCI……Mild Cognitive Impairment

MEC……..Medial Entorhinal Cortex

mPFC…….Medial Prefrontal Cortex

PCA-ICA……Principal Components Analysis-Independent Components Analysis

MTL……..Medial Temporal Lobe

MTT……...Multiple Trace Theory

PFC…….Prefrontal Cortex

REM …… Rapid Eye Movement Sleep

ROI……Region of Interest

SLM……Stratum Lacunosum Moleculare

SL……...Stratum Lucidum

SM……..Stratum Moleculare

SNR……..Signal-to-Noise ratio

SO……Slow Oscillation

STDP……..……Spike Timing Dependent Plasticity

STSC…….Standard Theory of Systems Consolidation

SWR……Sharp Wave Ripple

SWS……...Slow Wave Sleep

S-LTP……...Short-Lasting Long Term Potentiation

TENASPIS……...A Technique for Extracting Neuronal Activity

..……...from Single Photon Image Sequences

VTA……Ventral Tegmental Area

1 CHAPTER ONE

On the Stability of the Hippocampal Neural Code as a Substrate for Episodic Memory

Introduction

One hypothesized function of memory is that it allows us to make predictions about future events based on current conditions. What mechanisms support proper memory formation and recall? Throughout this thesis, I will approach this idea from a simple perspective: that learning induces changes in the brain which are reflected in the neural code, and that proper memory maintenance requires preserving these changes across long time scales. This statement raises many questions. First, which brain regions must undergo plasticity to support memory? Historical evidence supports the hippocampus as vital for encoding episodic memories and consolidated them into long-term memories. However, depending on the content of the memory, different cortical regions may also become necessary for long-term recall. Thus, I will begin with a general overview of different types of memory and the brain regions involved in their expression. How do these brain regions change throughout learning/memory? To provide a context for how these changes occur, I will next discuss mechanisms supporting plasticity in hippocampal neurons. Since proper memory consolidation requires coordination between the hippocampus and other brain regions, I will next review theories concerning the role of cortical and subcortical structures in supporting long-term memory. However, in order to provide context I will first provide a synopsis of the anatomy and functional properties

of these structures, their connectivity with the hippocampus, and mechanisms supporting the strengthening of functional connections within the entire hippocampal-cortical circuit.

Finally, across what time scale do these changes persist? There is ample evidence that the consolidation window – the period over which a memory transitions from short-term to long-term memory – lasts from hours to days after initial memory encoding. Tracking activity of the same neurons across this time scale was not possible until recently due to the challenges of tracking long-term neuronal activity with traditional recording technologies. Thus, I will conclude with a discussion of the advantages and disadvantages of using *in vivo* calcium imaging to track the long-term activity of large ensembles of neurons in the rodent hippocampus. This background on plasticity mechanisms and calcium imaging sets the stage for three experiments I performed to examine the stability of the hippocampal neural code prior to learning, through acquisition and mastery of a spatial memory task, and finally during pharmacological disruption of memory consolidation.

1.1 A Brief History of Episodic Memory and its Relationship with the Hippocampus

In 1953, the 27 year old Henry Molaison, better known as patient H.M., underwent a radical surgery to address intractable epilepsy: the bilateral removal of a large portion of his medial temporal lobe (MTL), including most of the hippocampus, prepyriform gyrus, uncus, amygdala, and parahippocampal gyrus (Corkin, 1984). The surgery was a success – his epilepsy, resistant to all other treatments, was put in check, and remained so

until he died in 2008. However, the removal of his hippocampus resulted in profound anterograde amnesia: he was unable to form new memories lasting longer than a few minutes. Additionally, he lost a significant portion of his more recently formed long-term memories (retrograde amnesia), ranging from 2 to 11 years prior (Corkin, 1984; Milner, Corkin, & Teuber, 1968).

H.M.'s memory deficit was very specific. Despite his amnesia for recent experiences, he could learn new motor skills, such as drawing or tracing objects in a mirror (Milner et al., 1968). Thus, his surgery challenged the notion of equipotentiality which held that memory function (along with other cognitive and sensorimotor functions) was distributed throughout the cortex (Bruce, 2001; Lashley, 1931). This early theory was supported by Lashley's findings that memory deficits in rats correlated with the size but not with the location of brain lesions, suggesting that no single brain region supported memory on its own (Lashley & Franz, 1917). H.M.s specific memory deficit thus highlighted that the hippocampus is crucial for the formation of new episodic memories and their successful consolidation: the transformation of short-term to long-term memory. However, since much of his earliest memories were spared, it also indicated that other brain regions were capable of supporting long-term memory recall in the absence of reliable communication with the hippocampus.

1.1.1 Important Caveats from the Legacy of Patient H.M.

H.M.s impact and legacy are not completely cut-and-dry, however. Anecdotal evidence indicates that, while H.M. could not form new episodic memories, he was

capable of forming more general declarative memories, e.g. the name of the current president in 1973, Richard Nixon (Corkin, 1984). This hinted that the hippocampus might not be necessary for producing long-term memories for facts and general knowledge, only for specific events in one's life. Alternatively, it could indicate that part of his hippocampus was spared; indeed, a recent postmortem examination of his brain confirmed the presence of "residual hippocampal tissue" (Annese et al., 2014). Furthermore, H.M. also demonstrated significant retrograde amnesia that seemed to worsen later in life (Corkin, 1984; Milner et al., 1968), indicating that the hippocampus might be important for recall of all but much older, well-consolidated memories. However, this directly contradicts subsequent work suggesting that cortical regions, and not the hippocampus, support long-term memory (Frankland, 2006; Frankland, Bontempi, Talton, Kaczmarek, & Silva, 2004; Squire, Genzel, Wisted, & Morris, 2015; Winocur, Frankland, Sekeres, Fogel, & Moscovitch, 2009; Winocur, Moscovitch, & Bontempi, 2010). Thus, if cortical regions truly support long-term memory H.M.'s retrograde amnesia could indicate that he sustained an unidentified injury to his cortex around the time of surgery or shortly afterward; indeed, a postmortem study revealed focal damage to the left orbitofrontal cortex and widely distributed pathology in white matter tracts (Annese et al., 2014). Thus, while H.M's memory deficits suggest that the hippocampus is vital to the initial encoding and consolidation of long-term episodic memories and cortex is devoted to the long-term retention of memories, uncertainties about when his cortical damage occurred, as well as the sparing of parts of his hippocampus leave open the possibility of a more nuanced interpretation: that long-term

memory consolidation and retention depend on a continual interplay between hippocampus and cortex.

1.1.2 Links between Patient H.M.'s Pattern of Amnesia and Memory Deficits in Alzheimer's Disease

The memory deficits observed in H.M. and others with significant MTL damage mirrors what occurs in Alzheimer's disease. Alzheimer's disease (AD) is characterized by a variety of cognitive and physical deficits, most notably a temporally graded retrograde amnesia that begins with lapses in newly acquired short-term memories and slowly progresses to prevent the recall of older and older memories. This progression from short-term to long-term memory loss occurs concurrently with the movement of Alzheimer's pathology from the MTL to other cortical regions (Hasselmo, 1994; Smith, 2002). Specifically, early stages of AD are characterized by the presence of neurofibrillary tangles of tau protein (normally important for stabilizing microtubules, see Cleveland, Hwo, & Kirschner, 1977a, 1977b) and extracellular aggregations of Aβ proteins in the subiculum/regions CA1 of the hippocampus and the entorhinal cortex (Arnold, Hyman, Flory, Damasio, & Van Hoesen, 1991), a MTL region that provides highly processed inputs to the hippocampus (see 1.3.2-1.3.3 below). Aggregations of tau tangles begin in the entorhinal cortex years before AD symptoms emerge and progresses next to parts of the hippocampus; by the time an individual exhibits symptoms of Mild Cognitive Impairment (MCI), such as short-term memory loss or navigation deficits in familiar environments, these tangles have invaded the bulk of the MTL and migrated to

prefrontal cortical regions. When an individual is finally diagnosed with AD, tau tangles have infiltrated virtually all association cortices, i.e. most of the neocortex excluding primary sensory area, (Braak & Braak, 1991; Ohm, Müller, Braak, & Bohl, 1995; Smith, 2002). In contrast to tau tangles, the locations of Aβ plaques does not correlate as well with the progression of AD (Braak & Braak, 1991; Murphy & LeVine, 2010). Thus, short-term memory loss during MCI occurs when AD pathology is restricted to the MTL and long-term memory loss during late-stage AD only occurs when AD has invaded neocortical areas. Notably, the progression of tau pathology in different cortical regions correlates with their proximity of connection to the hippocampus and entorhinal cortex, with immediate input/output areas being affected first (Pearson, Esiri, Hiorns, Wilcock, & Powell, 1985). This simultaneous progression of memory deficits and AD pathology from MTL to cortical regions supports the notion that the hippocampus and its adjacent areas in the MTL are important for initial memory encoding and consolidation, whereas cortical output regions are also involved in long-term memory recall.

1.2 Different Memory Classifications

The specific memory deficits of H.M. not only highlighted the importance of the hippocampus to episodic memory but also sparked research into how other brain regions contributed to different types of memory. For discussion purposes, I will divide up memory in to three broad classes that I review in this section: procedural memory, emotional memory, and declarative memory. Learning and memory can occur in purely sensory domain too – commonly referred to as perceptual learning/memory – but will not be discussed in this dissertation.

1.2.1 Procedural Memory

Procedural learning and memory refers to the acquisition and retention of a repeated motor action, typically supported by the striatum. In humans, the dorsal striatum consists of a curved structure (the caudate nucleus) which generally resides directly above to the hippocampal formation but incrementally moves more medial as it curves upward and terminates in the putamen (Hawrylycz et al., 2012). The ventral striatum consists of the olfactory tubercle, which receives input directly from the olfactory bulb and lies medial and rostral to the hippocampus, and the nucleus accumbens, which resides medial and slightly rostral/superior to the hippocampus (Hawrylycz et al., 2012) and is highly innervated with dopaminergic projections originating in the ventral tegmental area (VTA, see Ikemoto, 2010). Despite its proximity to the hippocampus, H.M.'s striatum was generally spared post-surgery (Corkin, 1984). As a result, H.M. was capable of learning new skills, most notably the ability to trace a line that he could only observe in a mirror (Corkin, 1968; Milner et al., 1968). This result was later replicated in other patients exhibiting episodic memory loss due to a variety of causes (Cohen & Squire, 1980). These results hinted that the learning and retention of motor skills might not require the hippocampus. However, H.M also had difficulty learning to repeatedly navigate the same complex route through a maze (Corkin, 1984), hinting that for more complex habitual actions, like the making the series of turns required to get from work to home in an urban environment, might require some sort of hippocampal input. However, not all procedural learning occurs independently of the hippocampus, since amnesic

patients with hippocampal damage are incapable of learning a procedural learning task guided by the recognition of different visual contexts (Chun & Phelps, 1999).

What dictates when a repeated behaviors ceases to require input from the hippocampus? Early work in rodent navigation elucidated the timeframe over which map-based navigation of a new environment eventually transitioned to habit/response based navigation. In their pioneering work, Packard & McGaugh (1996) trained rats to go west from the south arm of a plus maze to retrieve a food reward over the course of 16 days. On day 8, they performed probe tests on animals, starting them in the previously un-encountered north arm, and found that rats overwhelmingly continued to go west, using a place-based or allocentric navigation strategy ("go west") that utilized external cues for reference. Lidocaine infusions into the caudate nucleus, which silence a particular region by initiating long-term inactivation of voltage-gated sodium channels, had no effect on this behavior, whereas infusions into hippocampus resulted in chance level behavior. This supported the importance of the hippocampus in performing map-based or allocentric learning. At 16 days, however, rodents placed in the north arm overwhelmingly used a response-based or egocentric strategy ("go left"), and caudate inactivation caused rats to revert to the place-based strategy while hippocampal inactivation had no effect. Thus, at later time points rats relied upon a habit based navigation strategy supported by the striatum, but could still utilize the map-based, hippocampal strategy if needed. This experiment highlighted the interplay between two different brain regions and memory systems through the course of learning, with the

hippocampus being instrumental in early learning of the task, while the striatum slowly took over as the task became well learned.

1.2.2 Emotional/Visceral Memory

Emotional or visceral memory refers to the body's learned, automatic response to a given stimuli. Its roots lay in the work of Ivan Pavlov, who successfully associated the body's automatic, reflexive reaction (unconditioned response) to one stimulus (unconditioned stimulus) and with a different (conditioned) stimulus. In his seminal experiment establishing classical conditioning, Pavlov trained dogs to salivate (unconditioned response) after hearing a bell (conditioned stimulus) by playing the bell around the same time as the dog was presented with a piece of meat (unconditioned stimulus). Thus, the dogs were capable of learning that one type of stimulus led to a particular outcome, and the expression of that memory occurs in visceral changes in the dog's body (Gould, 2004). This work set the stage for years of work studying the boundary conditions under which classical conditioning works. In general, classical conditioning is amygdala-dependent (Fendt & Fanselow, 1999), highlighting the necessity of the amygdala for proper expression of autonomic responses to fearful stimuli (Iwata, LeDoux, Meeley, Arneric, & Reis, 1986). For example, when normal rodents are presented with a tone and then given a foot shock at the end of that tone, they exhibit high levels of freezing in subsequent tests when the tone is played without the shock (Fendt & Fanselow, 1999; Gale et al., 2004). Mice with amygdala lesions however, exhibit no change in behavior (Gale et al., 2004), indicating they are unable to associate a

CS with the visceral output of freezing. Importantly, the hippocampus is also required for learning a similar task where there is a delay between the end of the tone and the applied shock, or for learning to associate a shock with a particular arena (Kim & Fanselow, 1992). Thus, while the amygdala is required the learned expression of a visceral response to a fearful stimuli, the hippocampus is also necessary for associating a fearful response with more complex stimuli and for bridging temporal delays between the conditioned stimuli and conditioned response.

1.2.3 Declarative Memory

The last type of memory I will discuss is declarative memory. In contrast to procedural memory or emotional/visceral memory, declarative memory refers to the conscious recall of a previously learned facts or experiences. Declarative memory can be roughly divided into two categories: semantic memory, which refers to memory for general facts/knowledge but may or may not include memory of exactly when/where these facts were learned, and episodic memory, which refers to memory for events occurring at a specific place and time (Tulving, 1972). Word definitions, the rules of trigonometry, the types of neurotransmitters in the brain, etc. are all considered semantic knowledge, whereas what a person had for breakfast yesterday, or where a person met their spouse for the first time, are dubbed episodic memories. These two terms are not mutually exclusive, however, since for example, most individuals born in or before the mid-1990s can tell you not only many of the facts related to the 9/11 bombings but also their exact location when they found out it had occurred (Hirst et al., 2015). Whether

these two types of knowledge are truly independent types of memory is a subject of much debate, since many argue that semantic memory is the natural outgrowth of repetition of episodic memories (for example see section 1.4.2.2.2 below). While the case of patient H.M and ensuing work leaves little doubt that the hippocampus is involved in the acquisition and consolidation of episodic memories, its role in the acquisition and retention of semantic memories is not clear. This is further supported by evidence that H.M. was capable of some new semantic learning post-operation (Corkin, 1984). The distinction between episodic and semantic memory, as well as the brain regions involved in supporting each, is intimately related with the concept of systems consolidation and will be discussed in-depth below (see section 1.4.2).

1.3 Anatomy and Functional Properties Supporting Memory of the Hippocampus and Its Input/Output Structures

Section 1.4 provides a detailed discussion of brain regions involved in long-term memory consolidation. Thus, in order to provide a context for this discussion, I will first review anatomy/functional properties of the MTL and several important input/output structures involved in long-term memory expression. Each section below will focus on anatomy and connectivity in the rodent brain, though human anatomy will occasionally be referenced.

1.3.1 Hippocampus

The hippocampus (Greek for seahorse, which it resembles) is a curved structure in the MTL. Its elongated, slightly curved shape spans from posterior to anterior in humans.

It has distinct functional properties and connectivity that varies along its long-axis. This variance is homologous in the rodent hippocampus, with the dorsal (or septal) pole of the rodent hippocampus corresponding to the posterior pole in humans and the ventral (or temporal) pole corresponding to the anterior pole in humans. In general, the anterior/ventral pole of the hippocampus connects to frontal cortices, medial parts of the amygdala/striatum, and ventromedial entorhinal cortex (EC), while the posterior/dorsal pole of the hippocampus connects with more posterior cortices (e.g. parietal and retrosplenial cortex), more medial parts of the amygdala/ventral striatum, and dorsolateral parts of the entorhinal cortex (Skelin, Kilianski, & McNaughton, 2018). The functional properties of place cells – ubiquitous hippocampal neurons which fire consistently in a particular area of an arena (their place fields, see O'Keefe, 1976) – also vary along this axis, with their fields being much smaller in dorsal hippocampus and much wider in ventral hippocampus (Kjelstrup et al., 2008). Additionally, hippocampal inputs/outputs vary along the hippocampal axis with ventral hippocampus exhibiting stronger connectivity with non-spatial processing regions (Risold & Swanson, 1996; Witter & Amaral, 2004). These observations support the idea that the dorsal hippocampus is primarily employed for encoding fine details and establishing a map whereas ventral hippocampus is important for more general encoding of the context as a whole (Lyttle, Gereke, Lin, & Fellous, 2013).

Additionally, the hippocampus is further subdivided into three main areas with distinct cytoarchitectonic features: 1) dentate gyrus (DG), 2) the hippocampus proper which consists of three subfields, cornu ammonis 3 (CA3), cornu ammonis 2 (CA2), and

cornu ammonis 1 (CA1), and 3) the subiculum. The most-well characterized hippocampal circuit is dubbed the trisynaptic loop due to its almost exclusive feedforward connectivity occurring in three stages: 1) perforant path projections from the EC to DG (thus named since its fibers perforate the subiculum on their way to the DG), 2) mossy fiber inputs from DG to CA3, and 3) Schaeffer Collateral inputs from CA3 to CA1. CA2 lies between CA1 and CA3 and has been relatively neglected compared to DG/CA3/CA1 until recently, and its unique properties will be discussed below. DG/CA3/CA1 each have unique patterns of connectivity that might support different neural computations supporting memory which will be discussed in detail below (Treves & Rolls, 1994).

1.3.1.1 Dentate Gyrus

The DG consists of the densely packed granule cell layer (GCL) sandwiched between the essentially cell-free molecular layer and the polymorphic layer whose number/density of cells is still low but well above that of the molecular layer (Amaral, Scharfman, & Lavenex, 2007). Unsurprisingly, the GCL contains primarily granule cells (GCs) whose dendritic arbors extend to the molecular layer, stratum moleculare (SM), where they receive perforant path input from EC layer II (ECII) cells. GCs provide feed-forward excitatory projections via unmyelinated axons dubbed mossy fibers to CA3 as well as to DG mossy cells (MCs) residing in the polymorphic or hilar area; the GC efferents to CA3 constitute the lone extrinsic projections of the DG (Witter & Amaral, 2004). MCs in turn provide feedback inhibition to GCs (Amaral et al., 2007; GoodSmith et al., 2017).

Treves & Rolls (1994) proposed that the feedback inhibition from MCs to GCs combined with sparse connectivity from mossy fibers to CA3 principal neurons uniquely positioned the DG to provide strong, highly distinct inputs to CA3 in response to similar but slightly different stimuli, a process termed pattern separation. This idea is supported by an elegant study demonstrating that rats with DG lesions have trouble remembering which of two identical objects cover a previously encountered food well when the two objects are close together but not when they are far apart (Gilbert, Kesner, & Lee, 2001). Evidence for pattern separation also occurs in the neural code of the DG, since DG neurons are generally only active in one of multiple environments or tend to fire in different locations in different arenas (Alme et al., 2014; Danielson et al., 2017; GoodSmith et al., 2017). However, recent studies using calcium imaging or careful positioning of electrodes to track layer information found that this sparse neuronal code occurred only in GCs; in contrast, MCs were much more likely to be active in different rooms and have similar firing fields in these rooms (Danielson et al., 2017; GoodSmith et al., 2017), which might support the postulate of Treves & Rolls (1994) that MC feedback inhibition helps shape the sparse firing patterns of GCs. Interestingly, homologs of the DG are not found in most non-mammals, suggesting that its development might provide a unique evolutionary advantage to mammals by allowing them to remember more locations (Striedter, 2016).

The discovery of continual neurogenesis through adulthood in the rodent DG provides a more nuanced view of the contribution of the DG to pattern separation. Adult born DG GCs are much more excitable and tend to have much poorer spatial tuning than

mature GCs (Danielson et al., 2016). Furthermore, a study found that inhibiting mature GC activity *in vivo* enhanced a mouse's ability to discriminate between a neutral and fearful context but diminished their ability to perform a spatial memory task requiring the use of a subset of cues for orientation (Nakashiba et al., 2012). This study hints that adult born GCs are integral for pattern separation but shift to support pattern completion as they mature. Furthermore, a recent comparative study found that neurogenesis drops to undetectable levels in adult humans (Sorrells et al., 2018). However, this report directly conflicts with another study that found neurogenesis in the human DG persisting into adulthood through the use of an alternate method to identify adult-born neurons (Boldrini et al., 2018). These studies thus warrant a careful consideration of the role that adult neurogenesis in the human DG might play in supporting cognition (Kempermann et al., 2018; Snyder, 2018), especially since even studies finding human adult neurogenesis acknowledge that it diminishes with time (Boldrini et al., 2018). That DG neurons are vital to proper memory recall is not in doubt, however, as numerous studies have demonstrated that optogenetic reactivation of sparse populations of DG neurons involved in initial memory encoding can robustly induce artificial recall of that memory (Liu et al., 2012; Ramirez et al., 2013; Redondo et al., 2014; Roy et al., 2016; Ryan, Roy, Pignatelli, Arons, & Tonegawa, 2015).

1.3.1.2 Cornu Ammonis 3

Cornu Ammonis 3 (CA3) lies adjacent to the DG and forms the second stage of the trisynaptic loop. In humans, but not rodents, the hippocampal fissure separates DG from CA3; however, in both rodents and humans the densely packed cell layer of CA3 is

separate from the GCL in the DG (Witter & Amaral, 2004). Unlike DG, CA3 consists of four main layers, listed from superficial to deep: 1) stratum lacunosum moleculare (SLM) which is generally devoid of excitatory cells but contains direct entorhinal afferent input, 2) stratum radiatum (SR) which receives mossy fiber input from DG as well as recurrent collateral axons – also called associational fibers – from other ipsilateral CA3 neurons as well as commissural collaterals from contralateral CA3, 3) stratum pyramidale (SP) which contains densely packed excitatory pyramidal cell bodies as well as parvalbumin (PV), cholecystokinin (CCK), and vasoactive intestinal polypeptide (VIP) interneurons, and 4) stratum oriens (SO) which also receives recurrent collateral input from other ipsilateral CA3 neurons and also contains somatostatin (SOM) and oriens-lacunosum moleculare (O-LM) interneurons (Witter & Amaral, 2004). Nestled between SP and SR in CA3 only is a cell-free layer named stratum lucidum (SL) containing Mossy Fiber axons from DG (Witter & Amaral, 2004).

The recurrent projections of CA3 neurons to other CA3 neurons stands in contrast to connectivity in other hippocampal subregions, leading Treves & Rolls (1994) to postulate that CA3 might provide a unique role in performing memory related computations. Specifically, they argued that during initial encoding of a memory sparse inputs from the DG to CA3 would activate a population of CA3 neurons whose extensive recurrent connectivity would work together as an "autoassociative" network to further amplify firing in those neurons. This autoassociative nature of the CA3 network would then allow the same pattern of activity in CA3 to be recapitulated even when presented with degraded input from the DG. Thus, CA3 could facilitate recall of a vivid memory

such as the birth of one's child based on only a subset of cues present at initial encoding (e.g. a baby's cry). Additionally, the highly recurrent connectivity of CA3, along with its divergent projections to all other hippocampal subregions (Witter & Amaral, 2004), might play a role in coordinating the transmission of short periods of synchronous spiking activity to other hippocampal subregions and cortex (Sullivan et al., 2011) in support of memory consolidation (see 1.4.2.2.1.1 below). However, the role of subcortical projections from CA3 to the lateral septum (LS), a key arbiter of aggressive behavior, must also be considered (Leroy et al., 2018) as a potential route for memory consolidation.

Compared to the role of DG in pattern separation, there is relatively less behavioral and physiological data supporting the postulate of Treves & Rolls (1994) that CA3 supports pattern completion. Specifically, if CA3 supports pattern completion, then CA3 neurons should exhibit similar firing patterns in similar arenas. However, in a notable experiment, CA3 neurons tended to be active in only one of two similar arenas, and those that were active in both had much lower spatial correlations between different arenas than within the same arena (Leutgeb, Leutgeb, Treves, Moser, & Moser, 2004). The opposite result occurred in CA1 neurons, which were much more likely to be active in multiple rooms and displayed highly similar patterns between the two. These results support a role of CA3 in pattern separation rather than pattern completion since CA3 neurons produced a map of place field locations for similar arenas. The lack of identifiable CA3 outputs supporting pattern completion is further bolstered by a study finding that CA3 only maintains its firing patterns in different arenas in aged rats

(Wilson, Gallagher, Eichenbaum, & Tanila, 2006; Wilson, Ikonen, Gallagher, Eichenbaum, & Tanila, 2005) who have also been shown to have spatial memory impairments (Barnes, Suster, Shen, & McNaughton, 1997), suggesting that a shift toward pattern completion in CA3 could actually detract from proper memory function. However, a follow up study to Leutgeb et al. (2004) found that, depending on experimental methodology, CA3 could sometimes induce similar firing patterns in two different arenas that closely mirrored the patterns observed in CA1 (Colgin et al., 2010). Furthermore, another study using an identical task to Gilbert et al. (2001; see 1.3.1.1 above) found that CA3 lesions produce a general impairment in working memory in rats that was not specific to the separation between object locations (Gilbert & Kesner, 2006). Thus, the unique architecture of CA3 provides a means for coordinating neural firing within a region, though its theoretical role in supporting memory remains murky.

1.3.1.3 Cornu Ammonis 2

CA2 occupies a narrow region between CA3 and CA1, and has similar anatomical features and connectivity patterns to CA3. It receives inputs from DG and sends outputs to all hippocampal regions including associational connections to other CA2 neurons and CA3 neurons as well as feedforward connections to CA1 (Witter & Amaral, 2004). CA2 has at least two notable differences from CA3: it receives extensive projections from the supramammilary area of the posterior hypothalamus (Witter & Amaral, 2004) and sends projections to a distinct region of the lateral septum (Leroy et al., 2018). Despite these relatively subtle anatomical differences from CA3, CA2 exhibits several distinct functional differences from other hippocampal regions. First, its projection to the lateral

septum regulates social aggression by ultimately disinhibiting the ventromedial hypothalamus (Leroy et al., 2018). Second, projections from dorsal CA2 to ventral CA1 are necessary for acquisition, consolidation, and recall of social memories (Meira et al., 2018). Third, CA2 neurons adjust their firing activity in response to social stimuli or novelty to a much great extent than CA1 neurons (Alexander et al., 2016). Last, CA2 neuron spatial patterns change to a much greater extent over time than they do between different arenas (Mankin, Diehl, Sparks, Leutgeb, & Leutgeb, 2015). Thus, the differential connectivity patterns and functional properties of CA2 neurons implicate the region less in spatial navigation/memory and more in supporting social memory, novelty and the passage of time than other regions of the hippocampus.

1.3.1.4 Cornu Ammonis 1

CA1 is the most widely studied hippocampal region for two main reasons: 1) its location at the final stage of the trisynaptic loop makes it the main staging area for sending hippocampal process information out to other brain structures, and 2) its physical location in the rat, close to the superficial brain surface, makes the dorsal portion of CA1 easily accessible for *in vivo* recordings with electrodes or using calcium imaging. CA1 is positioned between CA2 and the subiculum and is cytoarchitectonically almost identical to CA3, containing all the same layers with the exception of SL (Witter & Amaral, 2004). CA1 pyramidal neurons located in SP receive two main types of afferent signals: 1) internally processed information from CA3 Schaeffer Collaterals projecting to dendrites located closer to the cell body in SR, and 2) direct inputs of highly processed, multimodal information relayed via the temporoammonic (TA) branch of the PP from layer III

neurons in the entorhinal cortex (ECIII) to their distal dendrites located in SLM (Witter & Amaral, 2004). Furthermore, recent evidence indicates that neurons located more superficially in SP layer receive stronger input from CA3 than ECIII, while deep SP neurons receive stronger input from ECIII than CA3 (Fernández-Ruiz et al., 2017). Anatomical studies suggest that CA3 provides the main drive to CA1, with ~30,000 synapses onto each CA1 neuron versus ~1800 from ECIII (Ahmed & Mehta, 2009).

There are several theories ascribed to the convergence of EC and CA3 inputs onto the same neurons. Treves & Rolls (1994) proposed that, by the nature of DG and CA3 connections and their rapid plasticity, information provided by SC inputs contains less detail than that provided by the original ECII inputs to the DG. Thus, direct TA inputs from ECIII could provide information richer in details which would help integrate more plastic, newly formed memory information in CA3 with less processed information coming from EC. Hasselmo, Bodelón, & Wyble (2002) fleshed out this idea with a computational model based on findings that ECIII and CA3 input to CA1 was modulated by the phase of theta, a prominent 3-10Hz rhythm present in LFP recording in the rat hippocampus during attention/locomotion and rapid eye-movement sleep. Specifically, they proposed that since EC input is highest at the trough of theta (Fernández-Ruiz et al., 2017) this enabled stronger input from EC to the hippocampus that could induce plasticity and encode memories. Conversely, since CA3 input is highest at the peak of theta (Dragoi & Buzsáki, 2006) and EC input is the weakest, this resulted in heightened transmission from CA3 to CA1 that could facilitate the recall of previously encoded memories. Both theories thus acknowledge the convergence of information that has been

internally processed by the hippocampus with more raw external information in the same neurons. Another study indicated that EC inputs could influence hippocampal processing by regulating theta phase precession – the tendency of a place cell to fire at progressively earlier phases of theta as the rat passed through that cells' place field (Zugaro, Monconduit, & Buzsáki, 2005). A different study found hinted how CA3 inputs might impact CA1 activity: they found that experience-dependent backward shifts in CA3 neuron place fields (see the following paragraph and Mehta, Quirk, & Wilson, 2000) occurred one day prior to the same backward shifts in CA1 place fields (Lee, Rao, & Knierim, 2004). They concluded that CA3 is necessary for rapid, one-trial learning, and supports the idea that DG and CA3 interact to create predictions about what events might occur in the immediate future based on past experience, while CA1 then evaluates how these predictions compare to reality. Thus, congruent information from ECIII and CA3 could reinforce activity patterns in CA1 whereas conflicting information from ECIII and CA1 could adjust CA1 activity and potentially even CA3 activity via back-projections from EC to DG (Levy, 1989). This could refine CA1 outputs for that could later be relayed to neocortical areas during systems consolidation (see 1.4.2 below).

The functional properties of CA1 neurons during behavior are well documented. The discovery of place cells, individual CA1 neurons which fire in specific locations (their place fields) in a given environment (Muller & Kubie, 1987; Muller, Kubie, & Ranck, 1987; O'Keefe, 1976), is perhaps the best known behavioral correlate of these cells. This discovery led to the proposal that CA1 might support memory by providing a mechanism for binding memories to a specific location (O'Keefe & Nadel, 1978). More

recent research has highlighted that CA1 neurons can also track the passage of time by reliably firing at the same time point during a fixed delay (Gill, Mizumori, & Smith, 2011; Kraus, Robinson II, White, Eichenbaum, & Hasselmo, 2013; MacDonald, Lepage, Eden, & Eichenbaum, 2011; Pastalkova, Itskov, Amarasingham, & Buzsáki, 2008; Robinson et al., 2017). These same neurons can also provide information about the passage of time across longer time scales by gradually adjusting their firing rates (Mankin et al., 2012; Manns, Howard, & Eichenbaum, 2007; Mau et al., 2018; Rubin, Geva, Sheintuch, & Ziv, 2015; Ziv et al., 2013). Other research spanning the past two decades has found neurons that reliably respond to different variables such as odors and or tones if these features are relevant for performing a task (Aronov, Nevers, & Tank, 2017; Herzog et al., 2019; Muzzio et al., 2009; Wood, Dudchenko, & Eichenbaum, 1999). Finally, in more complex tasks CA1 neurons exhibit a high degree of heterogeneity and can even respond to different combinations of task features, e.g. the presence of an object in a particular corner of a particular arena (McKenzie et al., 2014). Thus, CA1 neurons appear capable of encoding whatever features are most relevant to proper performance of a task, highlighting their flexibility to support spatial and non-spatial memory.

CA1 sends projections to/receives projections from a wide variety of cortical regions in addition to its well documented afferent inputs from EC and CA3 (see above) that vary substantially from the temporal (ventral) to septal (dorsal) pole. CA1 has been shown to coordinate with retrosplenial cortex (Mao et al., 2018), parietal cortex (Khodagholy, Gelinas, & Buzsáki, 2017), and even auditory cortex (Rothschild, Eban, &

Frank, 2016) during tasks. Below I will discuss several prominent extrinsic connections from region CA1 that are integral to memory consolidation: those to the medial prefrontal cortex (mPFC, see 1.3.4), the amygdala (see 1.3.5), and the lateral septum (LS, see 1.3.8).

1.3.2 Medial Entorhinal Cortex

The medial entorhinal cortex (MEC) is situated immediately posterior to the perirhinal cortex and slightly posterior but more inferior to the postrhinal cortex (Witter & Amaral, 2004). While MEC receives afferents from both perihinal and postrhinal cortex, the postrhinal cortex (known as the parahippocampal cortex in humans) provides the primary source of input in the form of highly processed spatial information (Eichenbaum, Yonelinas, & Ranganath, 2007). As noted above (see 1.3.1), MEC layer II neurons provide direct excitatory inputs to DG neurons in SM whereas MEC layer III neurons provide direct input to layer SLM of CA1. This direct input is concentrated in the proximal (closer to CA2) portion of CA1 (Witter & Amaral, 2004). However, recent studies have identified clusters of MEC layer II neurons (dubbed "island" cells) that also project directly to CA1 (Kitamura et al., 2014). MEC layer V neurons also receive strong projections from CA1 (Witter & Amaral, 2004). Thus, the MEC provides strong inputs to DG and CA1 and receives back projections from CA1.

Functionally, superficial MEC is best known for its high proportion of grid cells: neurons which fire in a regular hexagonal pattern as rodent's explore an open field (Fyhn, Molden, Witter, Moser, & Moser, 2004). This regular firing pattern is hypothesized to provide a mechanism for tracking an animal's progress through space in the absence of

landmarks for navigation (known as path integration) that could support place cell firing in the hippocampus (McNaughton, Battaglia, Jensen, Moser, & Moser, 2006). This idea is bolstered by studies demonstrating that lesioning the pathway from MEC layer III to CA1 produces poorer quality place fields in CA1 but not CA3, which receives intact MEC layer II inputs via DG (Brun et al., 2008) and that complete MEC lesions reduce CA1 place field stability in repeated exposures to the same arena (Schlesiger et al., 2018). There is also evidence that MEC is important for maintaining precise spike-timing relationships in CA1 neurons via regulation of the theta rhythm (Schlesiger et al., 2015), which are critical for preserving structure in reactivations of CA1 neurons during post-learning sleep periods (Drieu, Todorova, & Zugaro, 2018, see also 1.4.2.2.1.1). Despite this, superficial MEC inputs to CA1 do not appear to be activated in a coherent manner (or at best weakly activated) with hippocampal neurons during post-behavior rest periods (O'Neill, Boccara, Stella, Schoenenberger, & Csicsvari, 2017; Ólafsdóttir, Carpenter, & Barry, 2016; Trimper, Trettel, Hwaun, & Colgin, 2017), while deep layer MEC neurons do appear to reactivate in a coordinated manner with neurons from CA1 (Olafsdottir, Carpenter, & Barry, 2017). These results suggest that MEC layer II/III inputs are important for establishing structured hippocampal activity during learning, but not during later reinstatements that could support long-term memory consolidation.

Nonetheless, a recent study showed that silencing of MEC inputs to DG during acquisition of a contextual fear memory impaired memory formation but did not block it since artificial memory recall via optogenetic stimulation of DG neurons tagged as active during initial memory encoding was still successful (Roy, Muralidhar, Smith, &

Tonegawa, 2017). This study thus indicates that MEC-DG plasticity is not required even for initial memory formation. In support of this, Remondes & Schuman (2004) found that lesioning MEC layer III to CA1 direct projections did not impair acquisition and short-term recall of a spatial memory. However, these same rats exhibited significant impairments in long-term memory recall, even when lesions occurred after initial memory acquisition. Furthermore, another recent study demonstrated that deep layer MEC neurons projecting to the basolateral amygdala (BLA) and medial prefrontal cortex (mPFC) were crucially involved in memory consolidation (Kitamura et al., 2017). These results combined support the idea – which directly contradicts the conclusion of the preceding paragraph – that superficial MEC inputs help shape proper hippocampal function during and after learning via different pathways, are necessary for long-term memory recall, and that deep layer MEC neurons are also crucial for long-term memory stabilization. Despite these apparent contradicitons, there remains no doubt that MEC input is crucial for shaping functional properties of hippocampal neurons and that the MEC is critical for long-term memory consolidation.

1.3.3 Lateral Entorhinal Cortex

The lateral entorhinal cortex (LEC) sits lateral to the MEC in rodents; it is also immediately posterior/inferior to the perirhinal cortex, from which it receives the majority of its afferent connections (Lein et al., 2007; Witter & Amaral, 2004). Similar to MEC, LEC layer II neurons provide performant path input to DG neurons in SM whereas LEC layer III neurons provide direct to CA1 neurons in SLM. In contrast to MEC, LEC

layer III input is concentrated in the distal (closer to subiculum) sub-region of CA1 (Witter & Amaral, 2004). Despite its close proximity and similarity to the MEC and similarity in afferent projections, the LEC occupies a very different functional role in the hippocampal system. In support of this, a recent study found that during development MEC layer II stellate cells matured first, followed in order by CA3/MEC layer II pyramidal cells, then CA1/DG neurons. Subicular and deep layer MEC/LEC neurons developed next, followed finally by LEC layer II/III neurons (Donato, Jacobsen, Moser, & Moser, 2017). Thus, LEC inputs to CA1 come online only after the rest of the circuit develops, suggesting that different information might be provided to the hippocampus by MEC versus LEC.

This idea is bolstered by numerous studies highlighting the differences in functional outputs of LEC neurons as well as their influence on hippocampal activity. Unlike the regular spatial firing patterns of MEC grid cells, LEC neurons exhibit strong responses to the introduction of objections into an arena (Deshmukh & Knierim, 2011; Wilson et al., 2013) with each cell sensitive to an object's bearing relative to the rat (Wang et al., 2018). During orientation, LEC neurons preferentially also respond to local (close to the rat) arena features whereas MEC neurons preferentially respond to distal (further from the rat) features (Neunuebel, Yoganarasimha, Rao, & Knierim, 2013). Furthermore, rats with LEC lesions showed impairments associating objects with distinct locations (Wilson et al., 2013), supporting the importance of the LEC to encoding sensory attributes of a memory while the MEC supports encoding of the spatial context of a memory. Interestingly, in another study LEC neuron firing patterns also evolved more

reliably/robustly over ~20 seconds time (but not shorter time spans) than did firing patterns in MEC/CA3/CA1 neurons, potentially supporting a role for the LEC in encoding the passage of time over this time scale (Tsao et al., 2018). Thus, LEC provides distinct inputs to the hippocampus that support the encoding of different aspects of a memory. If phylogeny recapitulates ontogeny, MEC development preceding LEC development (Donato et al., 2017) combined with the above listed functional properties of LEC neurons supports the idea that early hippocampal adaptations supported navigation and spatial memory which later evolved to include the ability to code non-spatial attributes of a memory.

1.3.4 Medial Prefrontal Cortex

The medial prefrontal cortex (mPFC) resides at the rostral end of the rodent brain and consists of three main regions, listed here from most dorsal to most ventral: 1) anterior cingulate cortex (ACC), located medial/inferior to secondary motor cortex, 2) prelimbic cortex (PL), situated directly medial to the white matter tracts of the cingulum and forceps minor corpus callosum, and 3) infralimbic cortex (IL), situated directly superior to the dorsal peduncle (Paxinos & Watson, 2009). mPFC neurons are critical for proper performance of a hippocampal-dependent (see section 1.4.2.1) learning task and demonstrated lasting changes in their neural code reflecting task stimuli that develop slowly over the course of two weeks (Takehara-Nishiuchi & McNaughton, 2008), suggesting that dialog between the HPC and mPFC results in a critical role of the mPFC in the recall of long-term memories. This is supported by studies demonstrating

substantial coupling between CA1 and mPFC during active behavior (Place, Farovik, Brockmann, & Eichenbaum, 2016), rest (Tang & Jadhav, 2018; Tang, Shin, Frank, & Jadhav, 2017) and sleep (Wierzynski, Lubenov, Gu, & Siapas, 2009). Unlike its connectivity with the EC, CA1 does not also receive direct input from mPFC (Vertes, 2006).

While ventral hippocampus projects directly to mPFC (Preston & Eichenbaum, 2013), dorsal CA1 connects to the mPFC indirectly through its extensive reciprocal connections with nucleus reuniens (NR), a thalamic structure with anatomical and functional connections with the mPFC (Vertes, 2006). NR sends projections directly to distal dendrites in CA1, but has little-to-no connectivity with DG, CA2, or CA3 (Vertes, 2006). Proper NR function is necessary for discrimination between two memories at the behavioral level (Xu & Südhof, 2013), and silencing its activity likewise significantly reduced the separation between two different trajectories that is normally present in the CA1 neural code (Ito, Zhang, Witter, Moser, & Moser, 2015). Thus, connectivity between CA1 and the mPFC via the NR is important for memory expression at both the neural and behavioral level.

The importance of hippocampal-prefrontal communication to proper memory function is further supported by an experiment that utilized simultaneous recordings in CA1 and mPFC during a context-guided memory task requiring rats to remember which of two objects was rewarded in a given arena (the opposite object was rewarded in a second arena). In this study, Place et al. (2016) found that information flowed

preferentially from the hippocampus to mPFC when the rat initially entered an arena, but reversed course as the rat made his decision about which object to approach. This study supports the idea that bidirectional communication between the hippocampus and mPFC is vital to proper memory function, with the hippocampus providing information about the memory context and the mPFC helping guide retrieval of the appropriate memory in the hippocampus.

The induction of long-term plasticity and changes in mPFC firing patterns supporting long-term memory recall could result from functional coupling between the mPFC and hippocampus post-learning. For example, one study found that PL area neurons fired consistently after hippocampal neurons during deep sleep epochs (Wierzynski et al., 2009). This coupling could provide a mechanism for inducing the slow changes in PL neuron activity patterns that occurred over the course of weeks after rats were trained on a hippocampal-dependent trace conditioning task (Takehara-Nishiuchi & McNaughton, 2008). Consistent with this idea, impairing spontaneous hippocampal activity post-learning of contextual fear conditioning task prevented long-term, learning-related changes in mPFC spine densities from occurring and also impaired long-term memory recall (Kitamura et al., 2017). Thus, hippocampal-prefrontal interactions during learning, sleep, and recall are important to proper episodic memory function.

1.3.5 Amygdala

The amygdala is a subcortical structure positioned lateral to the temporal (ventral) third of the hippocampus in the rodent brain (Lein et al., 2007). It is further divided in the more lateral amygdala (LA) and basolateral amygdala (BLA), with the LA residing slightly more anterior and superior to the BLA (Paxinos & Watson, 2009). Functionally, the LA is necessary for memory acquisition in classical conditioning tasks like auditory fear conditioning that do not require the hippocampus and directly associate a conditioned sensory stimulus with an unconditioned stimulus like a foot shock (Rashid et al., 2016).

In contrast, the BLA becomes necessary, as does the hippocampus, when the task requires associating a tone with a foot shock across a delay (trace fear conditioning) or associating a foot shock with a given arena (contextual fear conditioning). The BLA has direct reciprocal projections with ventral CA1 (Felix-Ortiz & Tye, 2014), while it receives indirect inputs from dorsal CA1 via its connections with the MEC (Kitamura et al., 2017; Witter, Groenewegen, Lopes da Silva, & Lohman, 1989). In an elegant experiment, Kitamura et al. (2017) demonstrated that neurons in the hippocampus, MEC, PFC, and BLA were all activated during contextual fear conditioning. The hippocampus and MEC were vital to memory acquisition, short-term memory recall, and its consolidation; in contrast the PFC was capable of supporting long-term but not short-term recall, suggesting a substantial reorganization of the circuitry supporting the fear memory (see 1.4.2 below). Despite this reorganization, however, the BLA was necessary for fear

memory recall at all time-points, further supporting its role in expression of the visceral output (in this case, freezing) of emotional memories.

1.3.6 Ventral Tegmental Area/Locus Coereleus

Dopaminergic inputs to the hippocampus providing a signal of novelty or reward originate from two main sources: the Ventral Tegmental Area (VTA) and the Locus Coereleus (LC). Early studies supported the idea that novel experiences activated dopaminergic VTA inputs to hippocampus that enhanced LTP (Frey & Morris, 1998) and facilitated learning as well as long-term memory consolidation (Lisman & Grace, 2005). This is supported by a recent study demonstrating that simultaneously recorded VTA and CA1 neurons exhibit coordinated, synchronous activity during immobile periods within a novel experience but not during post-learning sleep (Gomperts, Kloosterman, & Wilson, 2015). Furthermore, another study demonstrated that optogenetic stimulation of CA1 projecting VTA neurons strengthened long-term memory recall (McNamara, Tejero-Cantero, Trouche, Campo-Urriza, & Dupret, 2014), supporting the role of VTA dopaminergic inputs to memory stabilization *in vivo.* However, another recent study demonstrated that LC inputs to CA1 far outweighed VTA inputs. Furthermore, this study showed that LC neurons changed their firing rates in response to novelty to a much greater extent than did VTA neurons, suggesting that LC might provide the dominant novelty signal to CA1 (Takeuchi et al., 2016). Regardless, this study also showed that optogenetic stimulation of CA1 projecting LC neurons also produced stronger long-term

memories, supporting the role of dopaminergic inputs from either VTA or LC in memory consolidation.

1.3.7 Diagonal Band of Broca

The Diagonal Band of Broca (DBB) is a basal forebrain region that, along with the medial septum, provides significant cholinergic input to all regions of the hippocampus and also received back-projections from dorsal CA1 (Witter & Amaral, 2004). Functionally, these cholinergic inputs are hypothesized to be important for regulating transmission of information within the hippocampus. Acetylcholine levels are high during periods of wakefulness and rapid eye movement sleep (REM) but not during periods of slow wave sleep (SWS) known to be important for memory consolidation (see Hasselmo, 1999 and 1.4.2.2.1.1 below). Furthermore, studies in brain slices have demonstrated that cholinergic agonists dampen transmission of neural activity through CA3 recurrent collaterals (Hasselmo, Schnell, & Barkai, 2018) and from CA3 to CA1 (Hounsgaard, 1978; Valentino & Dingledine, 2018) but do not suppress EC layer II to DG and DG to CA3 communication (Hasselmo, 1999). This supports a model where high acetylcholine levels facilitate memory formation/encoding during wakefulness by allowing plasticity to occur in the early stages of the trisynaptic loop while limiting plasticity elsewhere in the circuit (Hasselmo, 1999). In contrast, low acetylcholine levels during SWS could do the reverse and limit changes to EC-DG-CA3 circuitry while enabling plasticity in CA3 recurrent connections, CA3 to CA1 connections, and CA1 outputs to cortex that could support retrieval of previously encoded memories (Hasselmo,

1999). This idea is reinforced by the high incidence of synchronous, high frequency discharges of hippocampal neurons during SWS that originate in CA3 and spread to CA1: these discharges result in coordinated activity between CA1 and cortex and are important for memory consolidation in cortical regions (see 1.4.2.2.1.1 below). Thus, both DBB cholinergic inputs and LC/VTA dopaminergic inputs play an important role in strengthening memories via different mechanisms.

1.3.8 Septal Nuclei

The septal nuclei are located directly inferior to the corpus callosum and straddle the midline of the rodent brain (Paxinos & Watson, 2009). They consist of the centrally located medial septum (MS) and the lateral septum (LS) which is located (unsurprisingly) lateral to the MS. The LS also contains a dorsal portion superior to the MS that also straddle the midline. The MS provides cholinergic and GABAergic input to the hippocampus and EC which can modulate transmission of neural activity in hippocampal sub-circuits (see 1.3.7 directly above) and is also vital for generating theta: a prominent 3-8 Hz rhythm observed in local field potential recordings that occurs during REM and periods of wakefulness (Green & Arduini, 1954; Hasselmo et al., 2002; Stewart & Fox, 1990). Theta is thought to be important for inducing intra-hippocampal plasticity during wakefulness by organizing neuron activity into rapid, reliable sequences (Dragoi & Buzsáki, 2006; McNaughton et al., 1996; O'Keefe & Recce, 1993) that could later be reinforced via replay during sleep (see 1.4.2.2.1.1 below). It could also help prevent interference between memory encoding and retrieval by segregating when each stage

occurs into different parts of the theta cycle (Hasselmo et al., 2002); this is supported by a number of recent studies demonstrating that CA3 inputs to CA1 arrive preferentially during the rising phase of theta while EC activity transmits preferentially during the trough of theta (Dragoi & Buzsáki, 2006; Fernández-Ruiz et al., 2017; Zugaro et al., 2005). Further supporting this idea, disruptions to theta via lesions to the septal nuclei can cause memory impairments that mirror those observed during hippocampal lesions (Winson, 1978).

In contrast to the MS, the LS provides no documented afferents to the hippocampus but does receive prominent inputs from all hippocampal subregions except DG (Witter & Amaral, 2004). CA2 to LS projections are also implicated in mediating social aggression (Leroy et al., 2018), while CA3 to LS projections are important for establishing the specificity of a contextual fear memory (Besnard et al., 2019). Destroying hippocampal-septal connectivity through fornix lesions also prevented the flexible expression of memory in a transitive inference task in rats, suggesting that the septal nuclei could play a role in proper performance of complex cognitive tasks (Dusek & Eichenbaum, 1997). Thus, MS inputs to the hippocampus could support memory acquisition and consolidation via several different mechanisms, whereas hippocampal outputs to LS could provide an underexplored sub-cortical route for long-term systems consolidation (see 1.4.2).

1.4 Memory Consolidation

Memory consolidation refers to the process whereby semantic or episodic knowledge transitions from short-term to long-term memory. Consolidation can further

be broken up into two broad categories: synaptic consolidation and systems consolidation, which I review here.

1.4.1 Synaptic Consolidation

Synaptic consolidation refers to the persistent strengthening of synapses as a result of learning. Though strengthening can occur both presynaptically and postsynaptically, in the following sections I will focus on post-synaptic changes.

1.4.1.1 Long Term Potentiation

Donald Hebb famously postulated that neurons which fired closely together in time should increase their connection strength (Hebb, 1949). At the time, no such physiological mechanism for strengthening synapse had been uncovered; the first such evidence did not emerge for over twenty years with the discovery of long term potentiation (LTP). In their seminal study, Bliss & Lomo (1973) stimulated perforant path axons leading from layer II neurons in the EC through the subiculum to the DG in anesthetized rabbits while simultaneously recording population responses from DG neurons. They found that the response of DG neurons to a single stimulating pulse of electricity substantially increased immediately after providing a tetanic (15Hz) stimulus to the performant path, and that this potentiation of the synapses persisted on the range of 1-10 hours. This study was the one of the first to illustrate a mechanism, LTP, through which neurons could strengthen their connections and induce Hebbian plasticity. The immediate rise in post-synaptic strength typically occurs via insertion of new channels, typically α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors,

into the synapse. This insertion of AMPA receptors into results in the growth in size of the dendritic spine (Hayashi & Majewska, 2005; Rudy, 2008); critically, although NMDA receptors are generally not thought to be inserted into the synapse, their function as a calcium permeable channel is vital for initiating the signaling cascades necessary for LTP induction (Frey & Morris, 1997, 1998; Malenka & Bear, 2004; Rudy, 2008). I discuss specific mechanisms for LTP induction and maintenance further in the following section.

1.4.1.2 Different Time Scales for Persistence of Long Term Potentiation

Later work elucidated the molecular mechanisms through which LTP transpires and led to a distinction in types of LTP based on differences in time scale and mechanism. Short term LTP (S-LTP) occurs immediately through the insertion of locally stored AMPA receptors from inside the dendritic spine to available space on the membrane of the synapse (Bredt & Nicoll, 2003; Malenka & Bear, 2004; Rudy, 2008). S-LTP does not require large stimulus trains for induction and occurs very quickly since neither protein translation to form new channels nor structural changes to the spine are necessary (Rudy, 2008). Conversely, because it does not result in large structural changes, S-LTP is relatively short lived, lasting hours at most (Rudy, 2008). Long lasting LTP (L-LTP), on the other hand, can last as long as a slice is viable *in vitro* and for months *in vivo* (Abraham, Logan, Greenwood, & Dragunow, 2002). L-LTP occurs when a large stimulus, sufficient to open NMDA receptors, allows calcium influx into the dendrite. This calcium influx initiates a signaling cascade resulting in transcription and translation of new plasticity proteins at the soma after which these proteins are trafficked

down the dendrite and inserted into the spine (Frey & Morris, 1997, 1998, also see 1.4.1.3 below). Of course, there are many forms of LTP, and it must be acknowledged that non-NMDA receptor dependent LTP requiring neurotrophins for induction is supported by local transcription/translation in the dendritic spine/compartment (Kang & Schuman, 1996). However, due to the large body of animal literature establishing the importance of NMDA receptor activity to learning and memory, the following discussion will focus on NMDA receptor dependent LTP (Agnihotri, Hawkins, Kandel, & Kentros, 2004; Dupret, O'Neill, Pleydell-Bouverie, & Csicsvari, 2010; Luscher & Malenka, 2012; Rossato et al., 2018).

In contrast to S-LTP, L-LTP induction occurs much more slowly due to protein synthesis and the need for trafficking proteins from the nucleus to the synapse, as well as the frequent need to increase spine size in order accommodate the new receptors (Hayashi & Majewska, 2005). In fact, Raymond & Redman (2006, 2012) further broke down the timescale of plasticity. They elegantly demonstrated a) that the quickest/most transient LTP (~1 hour) occurs via local, NMDA receptor dependent calcium signaling in endoplasmic reticulum (ER) ryanodine receptors located in the dendritic spine, b) that intermediate lasting LTP (~2 hours) required activation of ER IP3 receptors located in the dendritic compartment via NMDA receptor mediated calcium signaling, and c) that long lasting LTP (> 4 hours) required activation of voltage gated calcium channels located at the soma but not NMDA receptor activation. Thus, longer lasting forms of LTP appear to require calcium influx very far from the post-synaptic densities whereas shorter lasting

LTP requires calcium signaling much closer to the synapse/spine where strengthening occurs.

1.4.1.3 Synaptic Tag-and-Capture Hypothesis

If L-LTP depends on protein translation in the soma and is generally input specific (Frey & Morris, 1997), how does the neuron "know" where to insert new plasticity proteins? One possibility is that potentiated synapses retain some sort of "tag" indicating where new proteins are to be inserted (Frey & Morris, 1998). Indeed, Frey & Morris (1997) demonstrated that a weak stimulus to one set of inputs in a hippocampal slice was sufficient to produce L-LTP in those inputs but only if a larger stimulus had previously been applied to a different set of inputs. This suggested that local calcium-dependent signaling in the synapse created a "tag," lasting several hours and indicating where plasticity related proteins should be sent. This tag can enable a weakly potentiated synapse to "hijack" plasticity proteins whose manufacture in the soma was initiated by a strong stimulus applied to other synapses (Frey & Morris, 1998). However, this strong stimulus and subsequent protein synthesis/trafficking must occur within the approximately three hour lifetime of the tag. Interestingly, this tag can spread to adjacent spots on the dendrite, resulting in potentiation of neighboring synapses formed with different presynaptic neurons and local clustering of dendritic spines (Rogerson et al., 2014). Importantly, both the long-scale duration of a tag and its local diffusion could facilitate associative learning since different/weak inputs are potentiated only if they closely follow a strong stimulus, allowing long-term encoding of incidental information that might become relevant later.

1.4.1.4 Mechanisms Supporting the Induction of Long Term Potentiation

The stimulation protocol invoked by Bliss & Lomo (1973) likely does not accurately mimic the types of neuronal inputs occurring physiologically. Thus, the question remains: what sort of processes occur *in vivo* to support the induction of long term LTP? I will review two mechanisms supporting LTP induction *in vivo* in the following sections.

1.4.1.4.1 Spike-Timing Dependent Plasticity

One mechanism for inducing plasticity, known as spike timing dependent plasticity (STDP), involves temporally correlated spiking between a pre-synaptic and post-synaptic neuron. In order for plasticity to occur at a synapse, a presynaptic spike (action potential) must occur shortly (~20 ms) before a spike occurs in the post-synaptic neuron (Dan & Poo, 2004; Levy & Steward, 1983; Markram, 1997). One likely mechanism for STDP induction is that glutamate release at the pre-synaptic terminal opens NMDA receptors, following which the magnesium ion blocking calcium entry into NMDA receptors is expelled via a back-propagating action potential from the soma (Kampa, Clements, Jonas, & Stuart, 2004). Thus, the nonselective cation channel that forms the NMDA receptor allows calcium influx that initiates a signaling cascade resulting in LTP. Indeed, the original finding that staggered spikes occurring in the pre/postsynaptic neurons lead to a potentiated response (Markram, 1997) has been corroborated many times over (Dan & Poo, 2004; Magee & Johnston, 1997; Magee, Hoffman, Colbert, & Johnston, 1998). Importantly, if the post-synaptic spike arrives *before* the pre-synaptic spike, researchers observed the opposite: a de-potentiation of the

response (Markram, 1997). Thus, STDP facilitates either strengthening or weakening of connections between neurons based on precise coordination of spiking; for this reason, it is frequently referred to as Hebbian plasticity in deference to Hebb's famous postulate. Therefore, if learning is the association of two different stimuli with one another STDP provides a physiological mechanism for learning: the strengthening of connections between neurons.

1.4.1.4.2 Behavioral Time Scale Plasticity

Of course, associations frequently occur between events that do not occur within milliseconds of one another. One possible mechanism for linking together a series of events occurring much further apart in time is by shortening the window in which spikes happen, a phenomena that occurs during periods of immobility and sleep following a learning episode (see 1.4.2.2.1.1 below). Alternatively, there might exist a different mechanism for inducing plasticity between neurons that are active within a larger time window. Indeed, recent work has highlighted that plasticity can occur even when inputs to a given neuron arrive seconds apart, in a phenomenon dubbed behavioral time scale plasticity (BTSP). In a series of elegant studies, researchers in the Magee lab recorded intracellularly in awake behaving mice and found that CA1 spiking activity rode upon a subthreshold "ramp" potential, a long timescale membrane depolarization event (Bittner et al., 2015) that occurs ubiquitously in the hippocampus (Kolb et al., 2017). This ramp potential depended critically upon coordinated input from EC layer III neurons projecting to the distal CA1 dendrites located in SLM and CA3 neurons projecting to apical CA1 dendrites located in SR (Bittner et al., 2015; Takahashi & Magee, 2009). Importantly, the

presence of this intracellular ramp, either naturally or through electrical stimulation, can drive the formation of stable CA1 place fields (Bittner, Milstein, Grienberger, Romani, & Magee, 2017). Since the duration of this ramp potential lasts on the order of seconds, rather than the millisecond timescale required for STDP, it suggests that there might exist a mechanism for potentiating a synapse even if inputs to it arrive relatively far apart in time. Indeed, *in vitro* follow up work by Bittner et al. (2017) found that CA3 inputs arriving 1-3 seconds prior to or after an intracellular membrane ramp potential induced a potentiation of subsequent CA3 inputs. The result is a model whereby local inputs produce a relatively long lasting subthreshold response in dendrites that could later interact with a more "global" depolarization resulting from coordinated input from other neurons to induce plasticity for the local inputs. Thus, this recent work provides another possible mechanism for inducing LTP *in vivo*, though much follow up work is required to characterize the underlying cellular and molecular processes supporting BTSP.

1.4.2 Systems Consolidation

H.M.s loss of recent memories with spared early/long-term memories gave the first hint that episodic memories could be supported by different brain structures. Indeed, studies have repeatedly found that rodents with hippocampal damage/inactivation can reliably recall memories formed at remote (more than one day prior) but not recent time points (Cohen et al., 2013; Frankland & Bontempi, 2005; Winocur et al., 2009; Zola-Morgan & Squire, 1990). Interestingly, the reverse pattern occurs for cortex, supported by studies identifying an upregulation in a variety of neocortical structures at remote, but

not recent, time points (Bontempi, Laurent-Demir, Destrade, & Jaffard, 1999; Frankland, Bontempi, et al., 2004). Importantly, disruption of protein synthesis in the hippocampus during memory encoding preserves short-term memory while disrupting its consolidation into long-term memory (Frankland, Josselyn, et al., 2004); protein synthesis inhibition also preserves early stage learning while disrupting late-stage learning (Squire & Barondes, 1973). Similarly, hippocampal 24 hours after memory acquisition, but not 28 days later, impaired long-term recall of a contextual fear memory (Winocur et al., 2009). As a result, long-term memories that can be recalled without hippocampal input are frequently labeled as "hippocampal independent" memories; see 1.4.2.1 below for an important discussion regarding this terminology. These results support the central premise of systems consolidation: that during the consolidation period immediately following initial episodic memory encoding by the hippocampus, coordinated activity between the hippocampus and cortex induces plasticity in cortical neurons that eventually allows successful recall of the memory through cortical regions alone.

A recent study (Kitamura et al., 2017) elegantly supported this theory by tagging neurons that were highly active in the hippocampus, amygdala, and mPFC during a fear memory acquisition, and demonstrating that optogenetic stimulation of tagged mPFC neurons was sufficient to artificially induce memory recall at long, but not short time points. Furthermore, tagged mPFC neurons showed little functional change in their activity patterns until days later, consistent with previous work demonstrating a delayed onset of mPFC changes during a trace-eyeblink task (Takehara-Nishiuchi & McNaughton, 2008). These studies support the idea that initial connections between the

HPC and mPFC formed during memory acquisition require a period of time to mature such that the mPFC on its own is sufficient to support memory recall at remote time points.

The reasons why this temporally-graded memory signal might be adaptive and the roles that the hippocampus and cortex play in memory recall at different time points remain controversial. To address the putative role of the hippocampus in long-term memory recall, I will first briefly discuss a few caveats concerning experimental design related to hippocampal independent memories. Then, I will review a number of competing theories underlying systems consolidation in the following paragraphs.

1.4.2.1 Hippocampal (In)dependence of Long-Term Memories

The ability of animals and humans to recall more remote memories in the absence of a properly functioning hippocampus is frequently taken as evidence that a) the hippocampus is not necessary for long-term memory recall and that b) long-term memory recall can be supported entirely by neocortical/subcortical structures (Frankland & Bontempi, 2005). However, assuming that the hippocampus is not involved in the recall of remote memories is misleading for a number of reasons. First, the literature regarding which memory tasks become hippocampal independent with time is complex and conflicts on whether a given task becomes hippocampal independent and when this independence occurs (Sutherland, Sparks, & Lehmann, 2010). This could result from competition between different brain regions/systems (Packard & McGaugh, 1996) or from methodological differences in task design (Finnie et al., 2018).

Second, many of the studies cited as evidence of hippocampal independence utilize lesions of the brain (Kim & Fanselow, 1992; Winocur et al., 2009; Zola-Morgan & Squire, 1990). Just as significant plasticity occurs in the human brain post-traumatic brain injury or stroke (Nudo, 2013), lesions could cause compensatory plasticity in input/output structures to the hippocampus that would drastically affect the circuitry involved in memory formation/recall. On the other hand, imaging of metabolic activity demands at distinct time points after learning found that hippocampal energy demands were highest immediately after memory formation and declined steadily with time (Bontempi et al., 1999). Furthermore, much recent work has demonstrated that different regions of the hippocampus are involved in distinct types of memory, both within the trisynaptic loop and more grossly along the dorsal-ventral axis (Jimenez et al., 2018; Kheirbek et al., 2013; Leroy et al., 2018; Meira et al., 2018). Thus, incomplete lesions of the hippocampus could easily impair one type of memory while preserving others, with more complex tasks being impaired by even small lesions. Indeed, recent evidence demonstrated that while the ventral hippocampus is not required for spatial memory, it IS required for performance of a task that requires switching dynamically between two navigation strategies for proper performance (Torres-Berrío, Vargas-López, & López-Canul, 2018). Therefore, the location and extent of lesion are confounding factors in determining the hippocampal dependence of a task, as is the propensity for plasticity following any lesion.

Last, necessity does not imply sufficiency, and chronic damage/inactivation of the hippocampus may provide a signal to the rest of the brain that hippocampal input is

unreliable. This could result in the recruitment of secondary circuits that are capable of (mostly) accurate memory recall but that are not normally prioritized during non-pathological recall. For example, rats frequently use different strategies and brain regions for performing memory tasks, potentially masking the contribution of the hippocampus to proper memory function (Farovik, Place, Miller, & Eichenbaum, 2011; Fortin, Wright, & Eichenbaum, 2004). Several studies support this idea. In 2011, Goshen et al. demonstrated that mice were capable of recalling a long-term fear memory under chronic hippocampal inactivation using halorhodopsin, a light-gated chloride pump. However, when inactivation was limited to a focal time point eclipsing only the memory recall session, mice demonstrated no recollection of the fear memory, suggesting that without an extended period of inactivation, the brain could not activate compensatory/secondary circuitry to support memory recall. In another recent study, Meira et al. (2018) demonstrated a similar effect via chemogenetic inactivation of CA2 during remote recall of a social memory. These studies and others (Sparks, Lehmann, Hernandez, & Sutherland, 2011; Sutherland, O'Brien, & Lehmann, 2008; Wang, Teixeira, Wheeler, & Frankland, 2009; Wiltgen et al., 2010) indicate that under non-pathological conditions the hippocampus may be necessary for proper memory recall, and that other brain regions supporting episodic memory will only fill-in for the hippocampus under chronic hippocampal pathology or damage. The fact that similar deficits in memory recall occur with inactivation of cortical structures at remote time points suggests that the interplay between the hippocampus and cortical regions, rather than the proper function of one region alone, plays an important role in long-term memory recall.

1.4.2.2 Theories of Systems Consolidation

1.4.2.2.1 Standard Theory

It is well established that the hippocampus is capable of rapidly inducing plasticity in neurons (Bliss & Lomo, 1973; see also discussion of LTP above in section 1.4.1.1). Combined with the heightened excitability of new-born adult neurons in the DG (Danielson et al., 2016, 2017; Nakashiba et al., 2012), the hippocampus is thus well-situated for rapid learning of new information via induction of plasticity within subsets of neurons. However, there are tradeoffs associated with heightened plasticity: while experimental interventions to increase adult neurogenesis in the DG result in quicker task acquisition, they also result in heightened forgetting of previously learned information (Frankland, Köhler, & Josselyn, 2013). Furthermore, neural network simulations of learning often fail catastrophically when plasticity occurs during the retrieval of previous memories (Hasselmo, 1994; Newman, Shay, & Hasselmo, 2012), indicating that there must exist some mechanism to separate the encoding and retrieval phases of memory. Indeed, recent work in Alzheimer's transgenic mice hints that heightened excitability in hippocampal neurons, potentially due to excessive plasticity, might lie at the root of neuronal dysfunction in dementia (Busche et al., 2008; Busche & Konnerth, 2015). Thus, there must exist some mechanism in the brain to prevent rapid learning from overwriting previously encoded information.

Rooted in theories championed by David Marr (Marr, 1970, 1971), the Standard Theory of Systems Consolidation (STSC) addresses this conundrum by positing that

memory consolidation occurs through interactions between the fast learning hippocampus and the slower learning cortex (McClelland, McNaughton, & O'Reilly, 1995; Squire & Alvarez, 1995; Zola-Morgan & Squire, 1991). Specifically, this theory argues that each new experience is robustly encoded through changes in synaptic weights within the hippocampus (McClelland et al., 1995). Following each new experience, there is a dialog between the hippocampus and cortical regions where the new learning is compared with existing memories in the neocortex and any differences are reconciled via changes in cortical synapses (McClelland et al., 1995; Squire & Alvarez, 1995). Thus, the hippocampus is responsible for learning all the salient details of a new experience, while hippocampal-cortical interactions are responsible for "interleaving" consistent details of this new experience into more "structured" knowledge systems in the neocortex (McClelland et al., 1995). In this manner, new learning that conflicts with existing knowledge (e.g. that a penguin is a bird and not a fish despite swimming gracefully and not being able to fly) does not quickly overwrite a body of existing evidence that all birds have wings and can fly.

Several predictions arise from the details of the STSC. First, the hippocampus and cortex must obey different rules regarding the plasticity and stability of individual neurons. Despite a relative dearth of research into cortical plasticity mechanisms, particularly in those regions closely connected to the hippocampus, there is ample evidence that both LTP and LTD exist in cortical neurons and share many of the same mechanisms for their induction (Malenka & Bear, 2004). This indicates that the ability to undergo rapid structural changes is not unique to the hippocampus. However, recent work

combined high-resolution microscopy and computational modeling to project that 100% turnover of dendritic spines – sites of excitatory synaptic connections – would occur in the mouse hippocampus over the course of 30 days, while only 70% of spines in the mouse somatosensory cortex would do so (Attardo, Fitzgerald, & Schnitzer, 2015). This work supports the idea that there exists different levels of plasticity and stability in the hippocampus versus the neocortex, though follow-up work replicating this result in areas with more direct projections to/from the hippocampus is necessary to validate its application to the STSC. Second, recent revisions to this theory predicted that new knowledge consistent with existing knowledge structures should be more rapidly learned than knowledge conflicting with an existing schema since it requires less modifications to the slow-learning neocortical system (McClelland, 2013). These revisions were spurred by an experiment demonstrating that information can be rapidly incorporated into the cortex in a hippocampal-task if the rules of the task are well-learned (Tse et al., 2007). Last, the STSC predicts that hippocampal activation during memory recall should decrease with time whereas neocortical activation should increase with time, a finding that has been confirmed many times over (Frankland, Bontempi, et al., 2004; Kitamura et al., 2017; Maviel, Durkin, Menzaghi, & Bontempi, 2004).

The STSC posits that successfully consolidated long-term memories are effectively transferred to the cortex from the hippocampus. However, recent studies highlighting the continued role of the hippocampus in the recall of long-term memories provide a challenge to this premise (Goshen et al., 2011; Meira et al., 2018; see also 1.4.2.1 above for a detailed discussion). Additionally, hippocampal lesions produce large

deficits in certain tasks even at remote time points and have no effect in others, hinting that different tasks might differentially involve the hippocampus (Sutherland et al., 2010). In a related manner, perturbations of information relayed to the hippocampus from the mPFC via the NR can bi-directionally modulate an animal's behavior during contextual fear recall, hinting that dialog between the hippocampus and cortex is important for memory recall even AFTER that memory is consolidated (Xu & Südhof, 2013). Finally, the premise of reconsolidation challenges the STSC by demonstrating that memories can be weakened with hippocampal perturbations following memory reactivation, even at remote time points after the memory recall can putatively be supported solely by neocortical structures (see 1.4.2.2.3 below). Thus, while many of the predictions of the STSCC hold true today, there still exist a number of challenges to its basic premise which have resulted in alternative formulations of systems consolidation. However, prior to discussing these alternative theories, I will address one likely mechanism supporting dialog between the hippocampus and neocortex following initial memory encoding.

1.4.2.2.1.1 Neuron Ensemble Reactivation During Sharp-Wave Ripples

The STSC postulated that hippocampal-neocortical interactions post-learning could support the transfer of memory from hippocampus to cortex. However, at the time of the STSC's conception (Zola-Morgan & Squire, 1991) no mechanism had yet been discovered to support these interactions. However, in 1992, Buzsáki, Horvath, Urioste, Hetke, & Wise discovered the presence of synchronous, high frequency discharges of pyramidal CA1 neurons during periods of immobility and slow-wave sleep and speculated that this discharges might speculated might help induce plasticity between

CA1 and neocortical neurons. To pay homage to O'Keefe, (1976), Buzsáki, (2015) later dubbed these discharges sharp-wave ripples (SWRs) due to the characteristic v-shaped deflection (the sharp wave) in the local field potential (LFP) followed by a short, high-frequency oscillation (the ripple, ~100ms @ ~200Hz). SWRs are believed to originate in CA2/CA3 (Oliva, Fernández-Ruiz, Buzsáki, & Berenyi, 2016; Sullivan et al., 2011), whose synchronous discharge is tightly coupled with acetylcholine levels (Hasselmo, 1999). Furthermore, the likelihood of SWR occurrence in the cortex is influenced by cortical state during sleep (or vice versa), further supporting the premise that SWRs facilitate hippocampal-cortical communication and plasticity (Battaglia, Sutherland, & McNaughton, 2004; Peyrache, Khamassi, Benchenane, Wiener, & Battaglia, 2009; Skelin et al., 2018).g

Importantly, single-unit recordings during SWR discharges contain information relevant to memory formation. Hippocampal neurons known as place cells fire reliably in the same spatial location, designated their place fields (O'Keefe, 1976). If a rat traverses back-and-forth on a linear track, these place cells thus fire in a reliable sequence that reflects the rat's trajectory. A. K. Lee & Wilson (2002) found that these sequences of place cell activations, which fired over the course of several seconds during active running, reactivated in the same order but in much quicker succession during SWR events in sleep sessions immediately following linear track traversals. This result was later extended to include replay of trajectories in reverse order (Diba & Buzsáki, 2007; Foster & Wilson, 2006) and to open-field exploration (Pfeiffer & Foster, 2013, 2015) during awake immobility, suggesting that SWR related replay could provide a

mechanism for remembering from where one came or for more complex navigational planning, respectively. Another study found that the number of place cells reactivated during SWRs and the fidelity of the replay sequence correlated with performance on a spatial memory task (Dupret et al., 2010); conversely, the density of SWRs depends on the amount of plasticity induced in neurons during learning (Girardeau, Cei, & Zugaro, 2014). Additionally, SWR reactivation of trajectories during sleep, which normally last 1 hour maximum following exposure to a familiar environment, extends for significantly longer following exposure to a novel environment (Giri, Miyawaki, Mizuseki, Cheng, & Diba, 2019). These results support the idea that the stability of learning related plasticity in hippocampal neurons depends upon SWR reactivations. This maintenance of plasticity could occur by temporal compressing spikes that would normally occur seconds apart to much short timescales, facilitating the induction of STDP (see 1.4.1.4.1 above and Buzsáki, 2015). Crucially, a recent study demonstrated that SWR replay events required the presence of temporally compressed sequential replay of place cells occurring within a given theta cycle (theta sequences) during behavior in order to reach their full strength during post-behavior sleep sessions (Drieu et al., 2018).

SWRs are also strongly associated with different cortical states, bolstering their potential role as mediators of hippocampal-cortical plasticity. SWRs occurring during the presence of delta waves (1-4Hz LFP oscillations, see Amzica & Steriade, 1997), which indicate down-states in cortical slow oscillations (SOs, 0.5-2Hz, see Skelin, Kilianski, & McNaughton, 2018) and are frequently followed by sleep spindles, 10-20Hz oscillations depending upon thalamic input (Maingret, Girardeau, Todorova, Goutierre, & Zugaro,

2016). Importantly, increasing the coupling between SWRs and delta waves/spindles through artificial electrical stimulation induced changes in mPFC neuron firing patterns and also increased long term memory performance in hippocampal-dependent object location task (Maingret et al., 2016). Furthermore, cortical neuron activity can influence hippocampal content during SWRs (Rothschild et al., 2016), suggesting that memory related information does not flow exclusively from hippocampus to cortex during consolidation. Conversely, silencing of SWRs during sleep impairs long-term memory performance (Ego-Stengel & Wilson, 2009; Girardeau, Benchenane, Wiener, Buzsáki, & Zugaro, 2009; Nakashiba, Buhl, McHugh, & Tonegawa, 2009). Interestingly, a recent study implicated SWR replay even in consolidation of a memory whose acquisition does no rely on the hippocampus (Sawangjit et al., 2018). Thus, SWRs provide a plausible mechanism for post-learning strengthening of hippocampal-cortical circuits that could enable bidirectional communication supporting long-term memory recall.

1.4.2.2.2 Multiple Trace Theory

As mentioned above, there are a number of shortcomings within the STSC, most notably the frequent finding of complete (non-graded) retrograde amnesia after hippocampal damage in many tasks (Sutherland et al., 2010) and the relative sparing of more semantic knowledge versus more complete eradication of autobiographical damage (Rosenbaum, Winocur, & Moscovitch, 2001). This hints that many of the assumptions of the STSC – in particular that memories are transferred in their intact form from the hippocampus to cortex and that all declarative memories are treated equally by the hippocampus – need revision. To address these concerns, Nadel & Moscovitch (1997)

created the Multiple Trace Theory (MTT) of systems consolidation. The MTT posits that each new experience results in the encoding of that memory in both the hippocampus and cortical regions. Upon each reactivation of the memory, either by free recall or triggered by an external reminder, a new trace is created in both the hippocampus and cortex. Partially consistent with earlier theories of memory (Marr, 1970, 1971), the MTT argues that the hippocampus encodes all relevant information from an experience (with a special emphasis on encoding spatial information) whereas the cortex encodes different classes of information pertaining to each region. Thus, frequently reactivated memories have a widely distributed ensemble of neurons encoding the experience. Moreover, this facilitates the formation of semantic knowledge via the extraction of statistical regularities in similar/related experiences, since these regularly occurring experiences have a high likelihood of reactivating similar sets of neurons in the cortex. In contrast, hippocampal traces are much less likely to overlap since they encode all the details of an experience, which almost always occur at a different place and time. Indeed, this idea is supported by recent work demonstrating a drift in hippocampal neuron activity patterns over time (Mankin et al., 2015, 2012; Manns & Eichenbaum, 2009; Mau et al., 2018) and decreased overlap in the ensembles encoding different experiences as the time between them increases (Cai et al., 2016; Rubin et al., 2015).

The MTT helps explain many physiologically observed memory phenomena. First, it helps explain the fact the hippocampal lesions produce profound retrograde amnesia in autobiographical memory but not semantic memory (Sutherland et al., 2010), since all the details of an experience are encoded in the hippocampus but only the

common elements of similar experiences remain encoded in cortical neurons. Second, a more recent updates to the MTT (Winocur et al., 2010) predicts that the strongest memories are those that have been reactivated the most and thus have the most overlap in their cortical traces. Consequently, however, MTT also predicts that the strongest cortical traces represent only the shared details of experiences, making them more "schematic" and less detail-rich. Thus, memories should demonstrate a loss of specificity with the passage of time. Indeed, rodent students have demonstrated that the memory for a specific environment becomes less specific with time, as indicated by contextually fear conditioned mice exhibiting freezing behavior in both fearful and neutral contexts (Wiltgen & Silva, 2007; Winocur et al., 2009).

The MTT also has several shortcomings. First, like the STSC, it does not adequately account for studies demonstrating impairment of remote memories via focal inactivation of the hippocampus (Goshen et al., 2011; Meira et al., 2018), or via disruption of hippocampal plasticity after memory reactivation (see 1.4.2.2.3 below). Second, it predicts that for each experience there should be equivalent levels of activity in cortical and hippocampal regions at each time point. This is contradicted by several studies demonstrating more lasting activity in hippocampus following novel experiences (Giri et al., 2019), and more plasticity induced in the cortex following reactivation of familiar/remote memories (Frankland, Bontempi, et al., 2004; Maviel et al., 2004; Miyawaki & Diba, 2016; Tse et al., 2011). Second, its view that the hippocampus is involved only in forming more detail-rich but less schematic memories is inconsistent with the role of the hippocampus in the flexible application of memory. Thus, it cannot

adequately account for experiments demonstrating that rats with hippocampal damage cannot perform a transitive inference task (Dusek & Eichenbaum, 1997) which requires making an association between two previously encountered stimuli. Last, it does address the growing literature on the dynamic interplay between the hippocampus and cortex that occurs during and after memory tasks (Gomperts et al., 2015; McNamara et al., 2014; Place et al., 2016; Tang & Jadhav, 2018; Yu, Liu, Loback, Grossrubatscher, & Frank, 2017).

1.4.2.2.3 Reconsolidation

In 1968, two studies challenged all of the above theories by demonstrating that even old, putatively well-consolidate memories could be disrupted if an electroconvulsive shock was given to rats shortly after being presented with a reminder to reactivate a previous memory (Misanin, Miller, & Lewis, 1968; Schneider & Sherman, 1968). Many years later (Nader, Schafe, & Le Doux, 2000) extended this result, demonstrating that administration of anisomycin – a protein synthesis inhibitor – into the amygdala following reactivation of a remote fear memory induced amnesia for that memory. Importantly, amnesia was only induced when rats were first allowed to explore the arena in which they were shocked. This seminal experiment demonstrated that memories became labile for a brief period of time upon reactivation and thus required further consolidation, or reconsolidation, to again be established. This result was later recapitulated in the hippocampus in a variety of paradigms (Inda, Muravieva, & Alberini, 2011; Lee, 2008; Lux, Masseck, Herlitze, & Sauvage, 2015; Winocur et al., 2009), as well as in other species/brain regions (Eisenberg, Kobilo, Berman, & Dudai, 2003).

Reconsolidation occurs even in the peripheral nervous system (Bonin & De Koninck, 2014), hinting that it might provide a more general mechanism for updating the strength of synaptic connections (Bonin & De Koninck, 2015). Follow-up work fleshed out the boundary conditions of reconsolidation and demonstrated that memories only became labile if significant new information was introduced during the reactivation session (Finnie et al., 2018; Rossato et al., 2007). These findings challenged both the STSC and MTT theories: if consolidated memories ultimately reside in widely distributed neocortical areas, why should focal interruption of hippocampal activity disrupt their later expression?

1.4.2.2.4 Schema Theory of Systems Consolidation

Several recent theories incorporate the findings of reconsolidation to form a comprehensive model of systems consolidation, suggesting that successful recall of remote memories requires a delicate interplay between all the brain regions involved with that memory. In support of this, Fanselow (2010) suggested that all memories are encoded in a variety of brain regions; however, consistent with the early work of Packard & McGaugh (1996), certain brain regions might encode a memory more efficiently than others and thus take precedence as the primary system mediating control of that memory. Competition between memories can even occur between different memory representations within the same brain region (Eisenberg et al., 2003; Park et al., 2016; Rashid et al., 2016). Thus, proper expression of a consolidated memory frequently requires mediating between different competing memories, a role that could be served by the medial prefrontal cortex (Preston & Eichenbaum, 2013; Xu & Südhof, 2013).

McKenzie & Eichenbaum (2011) took this idea a step further, arguing that all memories are encoded upon a sea of existing information. Thus, consolidation (the strengthening of new memories) and reconsolidation (the updating of existing memories to incorporate new information) are indistinguishable, since all memories require making adjustments to previous knowledge. Consistent with the learning theories espoused by Piaget and Bartlett (Bartlett, 1932; Piaget, 1926, 1929), schema theory argues that learning should occur quicker when new information is consistent with prior knowledge since fewer brain circuits require updating. This is supported by a recent study demonstrating that rats learn the location of hidden food wells much quick after they have sufficient experience in a similar task (Tse et al., 2007). This idea is also consistent with a revision to one conception of the STSC (McClelland, 2013), and supported by the recent finding that reactivation of hippocampal cell ensembles representing previous experiences (one putative metric of the consolidation period) extends over a much longer time frame when novelty is involved (Giri et al., 2019). Furthermore, schema theory predicts that since all memories share content with other memories, any new learning likewise requires updating all the circuits involved with those memories. This prediction was supported by a follow-up study finding upregulation of plasticity related genes in the hippocampus and cortex when rats learned new information (the location of hidden food reward) in a well understood task (Tse et al., 2011). A more recent study confirmed and extended this result by showing that plasticity continued to occur in hippocampal neurons even after mice experienced a familiar environment, but that the number of neurons undergoing plasticity decreased asymptotically with time (Attardo et al., 2018).

Importantly, these findings indicate that the hippocampus, as well as other cortical regions, are almost always involved during recall of remote memories, and in many cases might be the dominant/primary mediator of a particular memory even at remote time points (see section 1.4.2.1, Goshen et al., 2011 and Meira et al., 2018).

Two recent studies provide insights into potential hippocampal mechanisms supporting the incorporation of new knowledge into existing schemas. In the first, Grosmark & Buzsáki (2016) found that replay of hippocampal cell sequences following post-learning sleep consisted of reactivations of two cell groups. The first group, which they dubbed "rigid" cells, fired in a reliable pattern that did not change from before to after learning. The second group, "plastic" cells, significantly increased/decreased their firing following learning. Another recent study (van de Ven, Trouche, McNamara, Allen, & Dupret, 2016) took this a step further. They likewise encountered ensembles that did not change their firing patterns during reactivation and other ensembles whose strength of reactivation gradually increased during exposure to a novel environment. Furthermore, they found that silencing of SWR events during post-learning sleep affected reinstatement of only the gradually strengthened ensembles in subsequent sessions. The rigid cells encountered by both groups could provide a backbone representing the prior knowledge in a schema upon which new learning in the plastic cells could occur. Furthermore, they suggest that tracking the long-term stability and plasticity of hippocampal neurons is important for understanding how long-term memories are either properly consolidated and later remembered or improperly consolidation and later forgotten.

1.5 Methodological Considerations Concerning the use of Calcium Imaging to Track Long Term Neuronal Activity

1.5.1 Background on Electrophysiology

Electrophysiology has long been considered the gold-standard for recording neuronal activity *in vivo* due to the ability to record electrical activity from neurons with a high degree of temporal resolution (Buzsáki, 2004). Post-recording computational techniques further allow researchers to disambiguate neighboring neurons recorded in closely packed brain regions (e.g. the CA1 pyramidal cell layer in the hippocampus) with a high degree of accuracy as long as multiple electrodes are used (Buzsáki, 2004). However, drift in electrical signal due to factors like slippage between the brain and electrodes or impedance changes due to gliosis makes it difficult to reliably record activity from large numbers of the same neurons across long time scales (Harris, Quiroga, Freeman, & Smith, 2016). Additionally, there is an inherent limit to maximizing cell yield: lowering the impedance of a given electrode increases the number of cells that can be detected by that electrode but makes it harder to disambiguate neighboring cells due to the introduction of noise (Harris et al., 2016). Adding more electrodes will also increase cell yield; however, eventually the size of the electrode bundle/shank becomes excessively large and results in brain damage that can significantly lower the expected neuron yield and could adversely affect the stability of recordings (Buzsáki, 2004). Thus, electrophysiology provides a tried-and-true method for recording from moderate numbers of neurons with high temporal resolution, but has several important limitations. Calcium

imaging addresses these limitations to enable reliable recordings from a large number of neurons across long time scales. However, this does not come without a cost: thus, I will review the methodology underlying calcium imaging as well, its advantages, and its disadvantages, in the following sections.

1.5.2 Calcium Indicators and Their Interaction with Physiology

Unlike electrophysiology, calcium imaging does not directly measure changes in local electric potentials (or currents) via the movement of ions. Instead, calcium imaging uses the influx of calcium into a cell, detected by a calcium indicator, as a proxy for neuronal activity. Early experiments employed chemical indicators – molecules that directly chelated calcium – such as fura-2 or Oregon-Green (Grienberger & Konnerth, 2012) which allowed for a relatively fast and direct measure of calcium influx. The downside to chemical indicators is that they must be bath applied to the tissue for each recording session, thus limiting the range of experiments to those that can be performed acutely under head fixation; however, for many researchers this is a relatively easy/robust method for introducing the calcium indicator into cells (Grienberger & Konnerth, 2012; Russell, 2011). Additionally, most chemical indicators do not easily allow introduction into specific cells/cell types. Chemical indicators continued to be employed due to their ease of use and the relative speed of their signal; additionally, many chemical indicators shift their excitation/emission wavelength (see 1.5.3) upon binding calcium, allowing accurate assessment of calcium levels despite uneven indicator levels or photobleaching (Russell, 2011). The predominance of chemical indicators has been surpassed by recent

advances with another class of calcium indicators whose speed and signal-to-noise ratio now surpass that of the more common chemical indicators (Chen et al., 2013; Helassa, Podor, Fine, & Török, 2016) and whose fluorescence is more photostable, allowing longer-term imaging (Tian et al., 2009).

The other method for determining neuronal calcium influx is through the use of a Genetically Encoded Calcium Indicators (GECI), first developed with Cameleon in 1997 (Miyawaki et al., 1997) and re-engineered in 2001 to improve its signal-to-noise ratio as GCaMP (Nakai, Ohkura, & Imoto, 2001). GCaMP has been vastly improved since 2001 to further increase its signal-to-noise ratio and dynamics resulting in an explosion of research employing GCaMP to measure calcium activity (Hamel, Grewe, Parker, & Schnitzer, 2015). GCaMP works by fusing a green fluorescent protein (GFP) molecule, connected to the N-terminus of the M13 fragment of myosin light chain kinase with calmodulin, which is connected to the C-terminus of M13 (Nakai et al., 2001). Subsequent calcium binding with GCaMP introduces a conformational change of the entire molecule which causes a corresponding increase in fluorescence intensity (Nakai et al., 2001). Rapid binding requires that GCaMP have a high affinity for calcium; the trade-off is that while GCaMP increases its fluorescence relatively fast in the presence of calcium, its subsequent decrease after calcium influx stops is slow (Hendel et al., 2008). The entire rise/fall in fluorescence is collectively referred to as a “calcium transient”, though only the rising phase is associated with spiking activity since that is when calcium influx occurs (Chen et al., 2013). The slow decay time of calcium transients is related to both the high affinity of GCaMP for calcium and the relative concentrations of

GCaMP/calcium, and can pose issues for disambiguating fluorescence changes occurring in neighboring neurons (see section 1.5.5 below). This is further complicated by the fact that neurons in which GCaMP has invaded the nucleus exhibit much longer decay times than those without GCaMP in the (Tian et al., 2009). Even the fastest of the most commonly-used variant (GCaMP6f), however, has a rise-time on the order of 0.1s (Chen et al., 2013), making it difficult to resolve individual action potentials in most preparations. Careful consideration/evaluation of these factors is vital to producing high-quality data that can be post-processed to obtain interpretable calcium traces (see 1.5.5 below).

How GCaMP is introduced to neurons is a key factor influencing its concentration within the neuron and if/when it will begin to invade the nucleus. There are two main methods for inserting GCaMP into a neuron: via the use of stable mutations in the genome (i.e. transgenic animals) and via viral introduction of the gene for GCaMP. The use of transgenic animals requires careful editing of the genome, generally in mice over rats, to ensure stable expression of GCaMP proteins within a given class of neurons specified by the promoter used. One key advantage of this approach is that, since any genome level changes are introduced at conception, they must result in reliable and stable expression of GCaMP within cells (Dana et al., 2014), which generally precludes any of the deleterious effects related to overexpression, e.g. slower calcium transients and traveling waves (see discussion above/below). Another advantage to this approach is that it can be used to selectively target neurons depending on the promoter used, and/or by cross-breeding GCaMP reporter mice with mice expressing Cre in the neurons of interest

(Zariwala et al., 2012). However, there are a couple disadvantages concerning the use of transgenic mice. First, extensive work must be done to breeding and genotyping these mice. Second, depending on the mouse line and promotor used there can be a variety of expression and brightness levels within neurons, with certain lines exhibiting high levels of expression in one brain region at the expense of others or producing highly fluorescent neurons but in only a sparse subset of cells (Dana et al., 2014). Thus, careful attention to the expression attributes of each mouse line is important to obtain high quality imaging, though many mouse lines are capable of producing adequate data for a wide range of regions (e.g. lines 5.11 or 5.17 in Dana et al. (2014)).

The other ubiquitous method for introducing GCaMP into neurons is through viral transduction. In this method, the gene for GGaMP is packaged into a viral vector which is then introduced into the brain area of interest, usually through stereotactic injection. After introduction, the virus proceeds to insert itself into the cell, resulting in the translation and transcription of the protein of interest (Kaspar et al., 2002) which in this case is GCaMP. Construction of the appropriate viral vector involves combining the GCaMP gene with 1) a virus and 2) a promoter to infect the appropriate cells. Both the virus type and promoter interact to influence the eventual expression profile of GCaMP. For example, the commonly used adeno-associated virus (AAV) has 11 serotypes (Mori, Wang, Takeuchi, & Kanda, 2004), each with a drastically different infection patterns/expression profile: in conjunction with a synapsin promoter (specific to neurons but excluding glial cells), AAV5 injection results in sparse labeling of cells while AAV9 results in abundant but promiscuous infection of neurons in the mouse hippocampus that

could also include non-specific infection of non-neuronal cells (Aschauer, Kreuz, & Rumpel, 2013), Overexpression of GCaMP can in turn lead to abnormal cell responses or even cell death (Resendez et al., 2016; Tian et al., 2009). Thus, careful attention to viral titer is required for any experiment using virally introduced GCaMP, usually via performing a study to assess expression levels and cell health at a time point matching the intended time of performing the experiment after injecting virus at various dilutions (Resendez et al., 2016). Despite these potential pitfalls, viral introduction of GCaMP is a robust and relatively easy means of inserting the calcium indicator into neurons.

1.5.3 In Vivo Fluorescence Imaging Techniques

Unlike traditional bright field microscopy, fluorescence microscopy relies on the emission of light from a fluorophore for visualization, which occurs when a fluorophore transitions from a higher quantum energy state to a lower energy state (Svoboda & Yasuda, 2006). This is most commonly achieved by first exciting a given fluorophore with a photon of light at a shorter (higher-energy) wavelength than the wavelength at which it emits light. Thus, each fluorophore has a unique range of wavelengths of light that will excite it and at which it emits light: its absorption and emission spectra, respectively. For GCaMP, the absorption spectra is centered on ~480nm while the emission spectra is centered on ~510nm (Akerboom et al., 2012). There are a variety of microscopy methods employed for visualizing the fluorescence changes resulting from calcium-GCaMP binding *in vivo*. Thus, in the following section I will review the three

most common techniques current employed: two-photon microscopy, single-photon microscopy, and fiber photometry.

1.5.3.1 Two-photon microscopy

In contrast to the typical excitation pattern outlined above, in two-photon microscopy excitation of the fluorophore occurs using lower energy, longer wavelength photons than the expected emission wavelength of the fluorophore. On their own, each photon is insufficient to produce fluorescence and due to its much lower energy is less damaging to surrounding tissue. However, if both photons arrive near simultaneously, they can combine to impart sufficient energy to excite the fluorophore. This requires delivering the two-photons to a fluorophore in quick succession, usually via a femtosecond laser (Svoboda & Yasuda, 2006). The probability of photons being excited is extremely high near the point where the excitation laser is focused and low elsewhere. This pattern of emission is dubbed the point-spread function of the microscope, and is much wider in the x-y plane than the z-plane, allowing for excellent z-plane resolution (Helmchen & Denk, 2005). Thus, spatial resolution is achieved by sending excitation light to a specific point and collecting all emitted photons as the signal from that point, then scanning through all the remaining points in the field of view to achieve a full picture of the area of interest.

In this manner, two-photon microscopy achieves excellent spatial resolution and signal-to-noise ratio by eliminating almost all background fluorescence from the areas above and below the desired focal plane. Additionally, high quality images can be

obtained much deeper using two-photon than single-photon microscopy, since lower wavelength light is attenuated less by brain tissue, particularly highly anisotropic white matter which produces much higher levels of light scattering than gray matter (Yaroslavsky et al., 2002). It also facilitates visualizing if GCaMP is restricted to the cell membrane/cytosol or if it has invaded the nucleus (see section 1.5.2), since its optical resolution in the z-plane is much smaller than the typical thickness of a neuron. The cell yield also often meets or surpasses that achieved with high-density electrophysiological recordings, and the ability to visualize cells expedites tracking the same cells across multiple recording sessions (Grienberger & Konnerth, 2012; Hamel et al., 2015). Despite the high quality images produced by two-photon microscopy, there are a number of drawbacks to using this approach. First, independently scanning each point requires careful calibration of expensive optics and is slow, with typical acquisition rates for a 200μm x 200μm field-of-view (FOV) on the order of 10-20 Hz (Dombeck, Harvey, Tian, Looger, & Tank, 2010; Runyan, Piasini, Panzeri, & Harvey, 2017). Second, the excellent z-plane resolution can actually diminish cell yield in tightly packed areas like the pyramidal cell layer in the hippocampus. Third, even small amounts of motion in the z-plane can drastically change the image, resulting in a loss of data. Fourth, photobleaching – the gradual diminishing of fluorescence due to depletion of active fluorophores – can occur relatively quickly under high levels of excitation, though this problem is shared by all imaging techniques discussed in this dissertation (Combs, 2010). Finally, due to the large number of expensive optics required, two-photon *in vivo* imaging generally requires head-fixation of an animal. This severely limits the range of behavioral tasks that can be

performed in conjunction with imaging. Innovative engineering techniques, such as allowing a head-fixed mouse to navigate through a virtual-reality maze by running on a spherical treadmill (Dombeck et al., 2010; Runyan et al., 2017), can partially address this limitation, though even this fix cannot provide proper vestibular inputs which can lead to abnormal spatial responses in hippocampal place cells (Acharya et al., 2016). However, the advent of miniaturized two-photon microscopes that would enable imaging in freely moving animals is currently on the horizon (Obenhaus et al., 2018). Despite these limitations, two-photon imaging provides an excellent method for visualizing calcium activity of neurons *in vivo*.

1.5.3.2 Single-photon microscopy

Single-photon microscopy uses the more traditional method of exciting a tissue sample with photons of a higher-energy, shorter wavelength than emission photons. To obtain high spatial resolution, confocal microscopy utilizes a pinhole aperture to filter out any light emitted lateral to the pinhole and from above/below the plane of interest (Combs, 2010). Like two-photon microscopy, this generally requires scanning each point independently, but heightens concerns of photobleaching and toxicity to cells due to the higher-energy photons employed. However, recent improvements in GCaMP variants have allowed for high-fidelity detection of fluorescence even without the optical sectioning provided by two-photon or confocal microscopy. Thus, one can perform single-photon microscopy by simultaneously exciting and collecting light from an entire FOV, focusing at a specific level in the tissue to obtain z-plane resolution. Neither a laser nor machinery for scanning the FOV is required to capture an image, thus increasing

acquisition rate. Moreover, the simplified optics can be miniaturized, allowing the construction of lightweight microscopes (miniscopes) that can be easily carried by a mouse (Ghosh et al., 2011; Ziv et al., 2013). Combining a miniscope with a gradient index (GRIN) lens extends the FOV from deep within the cortex to above the skull, thus facilitating *in vivo* imaging in freely moving animals from superficial and deep brain structures (Resendez et al., 2016). While large FOVs and faster acquisition rates (Mohammed et al., 2016) can be achieved with single-photon imaging in a head-fixed preparation, I will focus below on the advantages and disadvantages of *in vivo* single-photon using a miniscope.

Miniscope calcium imaging affords many advantages. First and foremost, its lightweight construction allows collection of neural data in freely moving animals, limited only by the length of the cord required to tether the animal to the data acquisition system. Second, single-photon microscopes can frequently track calcium activity in many more cells than their two-photon counterparts because light is collected from areas immediately above/below the focal plane; disambiguation from highly overlapping neurons above/below is frequently still possible despite their lower resolution. This poorer spatial resolution also, counterintuitively, makes it easier to correct for motion artifacts in the z-plane since the change in the image due to small up-down motions is not as dramatic as with two-photon imaging. Third, the visualization of neurons and landmarks like vasculature facilitates tracking activity in the same neurons across days to weeks (Mau et al., 2018; Rubin et al., 2015; Ziv et al., 2013). Fourth, photobleaching is less of a concern than with confocal or two-photon imaging (Combs, 2010). Last, due to

the simplified optics, building or purchasing a miniscope is much easier/cheaper than building/purchasing a two-photon microscope, and there are currently a wide variety of options available (Cai et al., 2016; Ghosh et al., 2011; Liberti et al., 2016).

Of course, there are numerous disadvantages related to the single-photon technique and miniaturization of optics. First, the signal-to-noise ratio is much lower than with two-photon imaging owing to collection of fluorescence from above/below the cell layer (Combs, 2010; Hamel et al., 2015). The net effect is that it becomes difficult to resolve individual action potentials with this technique; each calcium transient most likely represents a high-frequency burst of activity in the typical excitatory neuron (Hamel et al., 2015). Thus, miniscope imaging further biases toward the detection of high firing rate over low firing rate neurons. Second, motion artifacts can be exaggerated due to the additional momentum induced during animal locomotion, particularly in stronger/larger species like rats, though most current image registration algorithms are capable of correcting this easily. Third, the optics employed induce a pronounced optical aberration such that neuron regions-of-interest (ROIs) quickly become distorted toward the edge of the GRIN lens (Kitano, Toyama, & Nishi, 1983). This problem is not unique to deep brain region imaging since most of the commonly used miniscopes also utilize a GRIN lens as the objective. Additionally, GRIN lenses induce chromatic aberration in images; the result is that different wavelengths of light are collected from different viewing planes (Leiner & Prescott, 1983), making it difficult to track different neuron types through tagging with a different colored fluorophore. Last, miniscopes can be easily damaged by stronger animals like rats, though this problem is not unique to imaging and

is readily solvable. Thus, *in vivo* calcium with miniscopes is a powerful technique to answer questions about the long-term activity of large neuronal ensembles whose use must take into account the disadvantages associated with it.

1.5.3.3 Fiber photometry

Fiber photometry is one further method commonly used for obtaining calcium imaging data *in vivo*. In fiber photometry, a single fiber is used to both excite and collect fluorescence in a given brain region. Unlike the techniques used above, which achieve spatial resolution by dividing the FOV into thousands of individual points or pixels, fiber photometry utilizes only 1 channel for collection (Jimenez et al., 2018). Thus, the main disadvantage of fiber photometry is that it collects bulk fluorescence from a large number of neurons simultaneously and cannot disambiguate one neuron's activity from another. Despite this limitation, fiber photometry has several advantages. First, due to the smaller size of fibers used, it is generally much less invasive than using a GRIN lens (and potentially even high density electrodes) to image from deep brain structures. However, the largest fiber widths can approach the width of smaller GRIN lenses frequently used. Second, it is relatively easy/robust to obtain data using this technique, particularly in areas like the hippocampus where careful alignment of the GRIN lens with the pyramidal cell layer is required to obtain good imaging (see 1.5.4 below). Third, since GCaMP transport in an anterograde fashion through neurons, it enables easy imaging of the bulk signal coming from one brain region to another. Overall, fiber photometry offers an easy method for tracking the calcium activity of a brain region as a whole.

1.5.4 Invasiveness and Other Considerations for Hippocampal Imaging

Both excitation light and emission light scatters as it passes through tissue. All things being equal, the amount that light scatters increases with distance and decreases as the wavelengths of light becomes longer. Thus, the amount of scattering produced by single-photon imaging is much greater than that with two-photon imaging since the former utilizes much shorter, higher energy wavelengths to excite the fluorophore. Still, the light emitted from excited fluorophores undergoes the same amount of scattering in both single and two-photon imaging, since emitted light must pass through the same tissue with each technique. Thus, the practical depth limit of imaging with two-photon is ~900μm (Kondo, Kobayashi, Ohkura, Nakai, & Matsuzaki, 2017), significantly deeper than with single-photon imaging (Combs, 2010). This does not pose a problem for imaging of superficial structures, such as layer II/II neurons in the dysgranular retrosplenial cortex, as these neurons reside very close to the dorsal surface of brain and can be directly imaged with a miniscope or two-photon microscope after skull removal. However, removal of the cortex overlying the area of interest is necessary to access deeper brain regions (Combs, 2010; Resendez et al., 2016; Ziv et al., 2013), even with two-photon imaging (Dombeck et al., 2010).

Imaging in the dorsal CA1 pyramidal layer of the mouse hippocampus thus entails several steps. First, cortex overlying the region of interest in a 1-2mm diameter is aspirated out until the start white medial-later striations of the corpus callosum are in view. In the case of two-photon imaging, a steel cannula with a glass coverslip is then implanted since the distance from the corpus callosum to the cell layer (~125-150μm) is

well within its imaging depth limits (Dombeck et al., 2010). In the case of single-photon miniscope imaging, however, the top layer of the corpus callosum is carefully removed until only anterior-posterior white striations, signifying the intra-hippocampal connections and hippocampal efferents of the alveus, remain (Cai et al., 2016; Kinsky, Sullivan, Mau, Hasselmo, & Eichenbaum, 2018; Mau et al., 2018; Rubin et al., 2015; Ziv et al., 2013 and section 2). This additional step is crucial: without it, the amount of tissue through which one must image often exceeds the limits of single-photon microscopes. After this aspiration is finished, a GRIN lens is inserted to extend the FOV from below the brain surface to above the skull and then is cemented in place. Generally, the hippocampus must be gently depressed during either GRIN lens or cannula insertion (see section 2) to compensate for the brain swelling that occurs during surgery and to lower the lens to appropriate point relative to the mouse's skull (Resendez et al., 2016). In the case of imaging from deeper brain structures such as the amygdala, a guidehole is first created via aspiration of the upper ~1mm of cortex or via slow insertion of a small diameter needle followed by slow lowering of a GRIN lens directly into the brain until the desired imaging depth is attained (Resendez et al., 2016). Imaging obtained from deeper brain structures therefore must take into account the possible delirious effects of the invasiveness of the imaging surgery.

Deeper imaging with a miniscope could induce pathological responses through surgery induced distortions of the brain and/or through removing critical inputs to the brain region being imaged. First, the brain swells upward during the course of the aspiration, and if the GRIN lens/cannula is not depressed the proper amount during

insertion the result is a significant distortion of the brain region(s) below the aspiration. Whether or not this stretching/depression of tissue is related to presence of slow-traveling waves of calcium activity observed by us and others (Chiang et al., 2018) in some mice remains to be determined. Conversely, there is the potential to over compress tissue below the GRIN lens, particularly when targeting deeper brain regions, which could also affect normal cell function by disrupting connections to/from the neurons of interest. This could also result in ambiguity concerning the exact area/layer from which imaging occurs unless these areas are confirmed histologically. Second, accessing many brain regions involves removing significant input/output connections to/from the regions of interest. In the case of dorsal CA1 imaging, care must be taken during surgery to ensure limited damage to the entorhinal inputs traveling through the alveus, and this must be later confirmed with histology. However, frequently removal of efferent/afferent connections is unavoidable. For example, imaging from mouse dorsal dentate gyrus (DG) requires significant damage to CA1 (Danielson et al., 2016) which, given the highly recurrent nature of hippocampal connections, could induce non-physiological cell firing in the cells being imaged.

The lack of studies using single-photon imaging in the rat hippocampus is perhaps the most telling example of the tradeoffs between brain access and invasiveness. This is likely due to the increased size of the rat brain relative to the mouse brain: the distance from the top of the alveus to the pyramidal cell later in dorsal CA1 is ~200μm in rats, just beyond the viable depth limit for single-photon imaging with blue excitation and green emission light. As a result, the only viable imaging I obtained from rats (1 of 15 attempts)

revealed an extremely sparse neuron population, likely corresponding to interneurons in SO immediately overlying the pyramidal cell layer. One possible solution is to gain depth by removing the alveus; however, this would also remove a number of direct inputs from the entorhinal cortex that would impact normal neuron physiology. Another potential solution that avoids deafferentiation of the neurons being imaged is to use a red-shifted calcium indicator, such as jRCaMP (Dana et al., 2016), which allows for deeper imaging due the reduce scattering of the longer wavelength light employed. Overall, the invasive requirements for brain access can impact both the ability to image from certain brain regions as well as the quality of imaging/physiology of cells being imaged and must be taken into consideration for any imaging experiment.

1.5.5 Neuron Extraction

The extraction of neuron ROIs and their corresponding calcium traces from raw imaging data is not trivial. Prior to any data processing, the raw imaging movies must first be corrected for any motion artifacts to ensure that putative calcium transients are not the result of shifts in the imaging plane. Motion corrected movies can then be analyzed by a number of different cell extraction algorithms to identify putative neuron ROIs and their corresponding calcium traces. While a plethora of method exist for two-photon imaging data, here I will limit my discussion to three different algorithms used for cell detection in single-photon miniscope data and their advantages/disadvantages: Principal Components Analysis-Independent Components Analysis (PCA-ICA), Constrained Non-Negative Matrix Factorization for microendoscope data (CNMF-E),

and A Technique for Extracting Neuronal Activity from Single Photon Neuronal Image Sequences (TENASPIS).

PCA-ICA was the initial method utilized by Ziv et al. (2013) in their seminal work demonstrating the feasibility of using miniscopes for long-term recordings of neuronal activity. PCA-ICA uses advanced mathematical techniques to identify clusters of imaging pixels that reliably explain the variance observed in pixel intensities (putative neurons). Its main advantage is that it is a principled algorithm which has been reliably used to detect and confirm a number of hippocampal phenomena first discovered with electrophysiology, e.g. place fields (Rubin et al., 2015; Ziv et al., 2013). However, PCA-ICA was originally employed using GCaMP3 with a relatively sparse neuronal population relative to more current GCaMP variants. As such, it is not ideally suited for disambiguating highly overlapping neurons that occur in hippocampal imaging (Hamel et al., 2015). In fact, one report using simulated data found that as the correlation between neuron ROIs increased, reflecting more overlap between ROIs and more similar ROI shapes, PCA-ICA eventually became incapable of separating neuron ROIs (Zhou et al., 2018). Additionally, it also ended up producing much poorer reconstructions of the actual calcium trace (Zhou et al., 2018). The end result is that PCA-ICA may end up detecting many fewer cells and including more crosstalk than other algorithms (like CNMF-E and TENASPIS) that use image segmentation techniques to identify neurons (Hamel et al., 2015). Additionally, the details of the cell selection process are effectively hidden from the experimenter, making troubleshooting of imaging artifacts and poor quality traces difficult to impossible (Pnevmatikakis et al., 2016). Thus, while a good tool for analyzing

early miniscope imaging data, the shortcomings of PCA-ICA highlighted the need for more sophisticated analysis tools.

CNMF-E was adapted from a similar algorithm designed for two-photon day analysis partially to address this issue. Briefly, CNMF-E assumes that the fluorescence changes observed in each imaging movie are composed of four basic components: 1) spatial footprints for each neuron ROI each with a 2) corresponding time series of calcium activity restrained to that ROI, 3) background composed of a homogeneous global fluorescence changes encompassing the entire FOV and heterogeneous local fluorescence changes related to the activity of neighboring neurons and those above/below the viewing plane, and 4) temporally and spatially uncorrelated noise (Zhou et al., 2018). These four components are then assembled into an equation whose output represents the observed intensity in each pixel across the entire movie. Placing reasonable constraints on each of these components (e.g. ROIs approximating the size/shape of neurons and a sparsely active, non-negative time series of activity within each ROI, etc.) makes this equation solvable. CNMF-E has a significant number of advantages over PCA-ICA. First, it is open-source and thus accessible for troubleshooting and quality control. Second, it produces higher SNR traces due to accurate subtraction of background fluorescence and noise. Third, it produces more accurate results when compared to PCA-ICA on simulated data, missing less neurons and providing much more accurate ROI and calcium trace estimates, particularly as the SNR of the imaging movie decreases. Most importantly, it significantly outperformed PCA-ICA in accurately disambiguating highly overlapping neurons and faithfully reproducing their actual spatial footprints and calcium

traces. Most of the disadvantages are related to the implementation of the algorithm. For example, there are a number of parameters that must be carefully calibrated for proper implementation of CNMF-E, and these can vary drastically for different brain regions, acquisition rates, pixel sizes, etc. Additionally, utilizing CNMF-E on large/long movies can overwork the resources of all but the most top-end commercially available computers. However, these disadvantages are tractable problems to fix. Thus, CNMF-E improved significantly upon PCA-ICA for the extraction of neuronal signal from miniscope imaging data.

Like CNMF-E, TENASPIS originated to address the inability of PCA-ICA to disambiguate signal from highly overlapping neurons. The full implementation of TENASPIS is discussed in depth below (see 2.4.3.1). Briefly, TENASPIS first employs a spatial filtering technique to assist in disambiguating neighboring neurons. It then uses image segmentation techniques to identify highly correlated activity patterns in each imaging frame, corresponding to activity of putative neurons, and then to connect these patterns across frames to create neuron ROIs and their corresponding traces. TENASPIS is capable of accurately extracting neuronal data from imaging movies, as demonstrated by replications of reliable place field activity (section 2) and coding of elapsed time (Mau et al., 2018) in hippocampal neurons. Additionally, TENASPIS can accurately disambiguate calcium events from neurons with overlapping neuron ROIs (see Figure 2.1 and Figure 3.1). Like CNMF-E, it is open source and employs a heuristic that facilitates quality control. However, unlike CNMF-E it has not undergone rigorous benchmarking against simulated data and other cell extraction methods. Additionally, since it was

developed explicitly for extracting data from imaging the mouse hippocampus, TENASPIS requires adjustment of many parameters to accommodate data extraction from other brain regions. Overall, TENASPIS is one viable method for extracting neuronal data from miniscope imaging movies, and is used to analyze data throughout this thesis, but it requires cross-validation with other methods to remain a viable/competitive in the future.

Regardless of the method used to extract putative ROIs and their events, matching calcium activity to spiking remains difficult. In some two-photon recordings, it is possible deduce single action potentials from calcium activity (Chen et al., 2013). However, the accuracy of spike detection via the use of deconvolution algorithms declines with the signal-to-noise ratio (Pnevmatikakis et al., 2016), making it difficult to infer individual spikes from imaging data obtained via single-photon imaging.

1.5.6 Neuron Registration Across Recording Sessions

Likewise, tracking the same neurons across multiple sessions – commonly referred to as neuron registration – is not a trivial exercise. Neuron registration generally entails two steps. First, the FOV for each movie and its corresponding ROIs must be aligned to one another. This is necessary to correct small shifts/rotation in the FOV due to camera reattachment prior to imaging and independent correction of motion artifacts. Second, the ROIs in the aligned FOVs must be matched between sessions. In cortical regions or preparations with sparse labeling of cells with GCaMP this step can sometime be reliably done manually. However, densely labeled regions like the hippocampus

necessitate the use automated cell registration algorithms, both for efficiency and accuracy of neuron registration. This problem compounds as the number of sessions being registered increases. Here I will review two different algorithms developed for neuron registration between sessions.

The first method was developed by Sheintuch et al. (2017) to directly quantify registration quality. This method aligns FOVs by searching for the rotation/translation of the second session that maximizes the two-dimensional cross-correlation between neuron ROI footprints between sessions. It then models how similar neighboring cells from different sessions appear, probabilistically estimates their likelihood to be the same cell, and then uses a clustering algorithm (Bansal, Blum, & Chawla, 2004) to match neurons across multiple sessions. The main advantage of this method is that it assigns a registration statistic to each neuron registration indicating the likelihood the two neurons are the same. Combining these individual registration statistics also yields an estimate of the registration quality between each session as a whole. Furthermore, it is versatile and can be used with a variety of neuron extraction techniques (e.g. PCA-ICA and CNMF-E) as well as with two-photon data. One disadvantage of this registration process is its circularity. Neuron registration requires matching neuron ROIs between sessions with aligned FOVs, and these same ROIs are used to initially align FOVs. In most cases this procedure works well. However, in many cases, particularly for sessions occurring further apart in time, neuron overlap may be maximized when FOVs are clearly not aligned. Despite this limitation, which is mitigated if all registrations are subsequently verified by eye, this technique is a reliable method for performing neuron registration,

particularly since it can accommodate data that has been pre-aligned using any number of other image alignment methods that do not rely upon neuron ROI footprints.

One such method for neuron registration (Mau et al., 2018), which is used extensively in this dissertation (section 2 and section 3), utilizes anatomical features such like vasculature to perform the initial FOV alignment. This method is discussed in depth below (section 2). Briefly, FOV alignment is first performed using the minimum projection from each movie, which results in an image that maximizes contrast between areas with high levels of GCaMP and areas with little to no GCaMP like vasculature. Next, neurons are matched based on the distance between their centroids, and in the rare case that there are multiple potential matching neurons the pair with the highest correlation between ROIs is paired. Last, for multiple sessions, the principle of transitivity is applied to ensure accurate registration. For transitivity to hold, neurons pairings calculated by registering session A to session B and then registering session B to session C should match those calculated by registering session A to session C directly. Any neuron-pairs not passing the transitivity test are discarded from future analysis. Finally, since neuron ROIs are generally elliptical and have a heterogeneous distribution of orientations, registration quality is evaluated by calculating the distribution of changes in orientation between sessions for all neuron pairs. This distribution is then compared to chance by shuffling neuron identity between sessions and then calculating orientation difference. However, this method could provide a non-conservative chance distribution since, in some cases, neurons of the same orientation cluster together in the imaging window. To address this issue, chance distributions are also calculated after intentionally,

incrementally shifting the FOV from one session and then registering it to itself. This analysis provides a more conservative estimate of chance level orientation differences and additionally helps indicate how sensitive neuron registration is to small errors in FOV alignment. Finally, any registrations not significantly different from chance are discarded. Additionally, ALL registrations were evaluated by eye, paying particular attention to those whose orientation difference curves approached chance.

There are several advantages to this method over Sheintuch et al. (2017). First, FOV alignment uses anatomical features rather than neuron ROIs to avoid circularity. Nevertheless, errors can still occur with this (and any) FOV alignment procedure, highlighting the importance of evaluating each registration by eye for both methods. Second, neuron pairs that fail the transitivity test are excluded. Sheintuch et al. (2017) find a similar amount of neurons failing the transitivity test – approximately 10% in Figure 5B in Sheintuch et al. (2017) – but unlike my method, they retain these cells. Still, it is relatively easy to identify these neurons and subsequently exclude them. Thus, both method reviewed provide a good method for neuron registration.

Careful consideration of registration errors is important when interpreting calcium imaging data. Aside from caveats in the algorithms discussed above, there are several other sources of registration error. First, for both single-photon and two-photon imagining, changes in alignment between the objective and GRIN lens/glass window will induce corresponding changes in the FOV. These changes are accentuated by the thin optical sectioning properties of two-photon microscopes, making it more difficult to

obtain proper alignment between sessions but simultaneously easier to evaluate by eye if alignment is off. However, since single-photon imaging collects fluorescence from a much thicker plane, evaluating small differences in alignment between sessions can be difficult to do by eye while the miniscope if reattached. Second, even if this concern is mitigated through chronic implantation of the miniscope, subsequent creep of the dental cement used to attach the camera results in a systematic lowering of the objective that can result in large changes in FOV depth over the course of several weeks.

Finally, even small changes can be particularly problematic when interpreting single-photon data, particularly when considering "new" cells or "silent" cells that become active/have no observable calcium activity on the subsequent session. A recent report (Katlowitz, Picardo, & Long, 2018) employing two-photon imaging in the premotor region of the zebra finch during a highly stereotyped behavior found high levels of persistent activity in neuron activity patterns between days. This contrasted with a previous reporting finding that a relatively larger subset of cells became active or turned off across days (Liberti et al., 2016). Katlowitz et al. (2018) argue that the root cause of this discrepancy is due to methodological issues with single-photon miniscope imaging: neurons toward the top/bottom of the viewing window might appear to be silent/new cells when in fact the changes in their activity are due to slight shifts in the z-plane that either bring the cell into focus or drop it out of focus. Thus, careful consideration must be taken when interpreting the existence of new/silent cells found in single-photon imaging data. As far as addressing the first two sources of error raised above, however, both methods discussed here calculate (different) metrics to assess the quality of registration between

each neuron-pair and each session-pair. Importantly, these metrics can later be used to evaluate if systematic changes in the neural representation (Eichenbaum, 2017a; Kraus et al., 2013; Mau et al., 2018; Robinson et al., 2017; Tsao et al., 2018) are real or if they are actually the result of registration errors between sessions.

1.5.7 What Does Calcium Imaging Actually Measure?

Calcium is one of most important signaling molecules utilized by neurons. This fact is often overlooked due to the power of using calcium influx as a proxy for neuronal activity. Furthermore, while calcium influx is closely correlated with neuronal spiking, calcium indicators operate on a much slower time scale than do action potentials, increases in intracellular calcium can frequently occur independent of action potentials, and action potentials can even occur in the absence of significant calcium influx. These points warrant careful consideration. Thus, I will discuss two important points regarding the interpretation of calcium imaging data in this section. First, what are the caveats/pitfalls of using calcium imaging as a proxy for neuronal activity/how should a calcium trace be interpreted? Second, what does measuring calcium influx (as opposed to electrical activity) mean for subsequent changes occurring within the cell? Though most of the following discussion pertains to all of the modern GECIs, the following discussion will focus on the GCaMP family of indicators.

The typical calcium transient corresponding to a single action potential has a fast rise time and slow decay (~0.1 second and 1 second respectively, see Chen et al., 2013); this is due to the dynamics of calcium binding with GCaMP (Nakai et al., 2001).

However, even the relatively fast rise time of a calcium transient, which occurs during spiking activity of a neuron, is much slower than the time-scale of the ion influx/efflux causing a typical action potential (Hodgkin & Huxley, 1952). Individual action potentials can be inferred from calcium activity in some cases (Chen et al., 2013). However, even with high signal-to-noise preparations, e.g. sparsely labeled neurons recorded using a two-photon microscope, deducing action potentials underlying the calcium signal can be difficult (Pnevmatikakis et al., 2016). This is further compounded by the fact that the amplitude and rise time of a calcium trace increases with the number of spikes occurring (Chen et al., 2013). This increased signal is likely due to the interaction of calcium ions entering the cell through voltage-gated calcium channels (VGCCs) at the soma and through calcium-permeable NMDA receptors in the dendrites (Magee et al., 1998; Markram, Helm, & Sakmann, 1995). While calcium entry into the soma occurs primarily through VGCCs (Mao, Hamzei-Sichani, Aronov, Froemke, & Yuste, 2001), large amounts of calcium can induce additional calcium release from internal stores in the endoplasmic reticulum (Grienberger & Konnerth, 2012; Kano, Garaschuk, Verkhratsky, & Konnerth, 1995; Tsien & Tsien, 1990), further magnifying the detected calcium signal. The detection of single action potentials thus becomes difficult to impossible in a lower signal-to-noise preparation, e.g. single-photon imaging with a miniscope, since the amplitude of these events is unlikely to exceed the noise floor. The non-linear amplification of transient amplitude with increasing number of action potentials also makes interpreting the height of a calcium transient difficult. On the other hand, single action potentials can sometimes even occur in the absence of significant calcium entry

into the cell (Magee et al., 1998). Calcium imaging is therefore biased toward recording from high firing rate neurons and indicates significant amounts of calcium entry into the cell.

Bursting events require calcium entry through both the soma and dendrites, and the main source of dendritic calcium influx is through NMDA receptors (Bloodgood & Sabatini, 2009). Calcium entry through NMDA receptors is also vital for induction of long-term plasticity in synapses (Malenka & Bear, 2004; Shepherd & Bear, 2011). Thus neurons detected with calcium imaging are likely those which will undergo significant remodeling/plasticity at their afferents. Is this the primary reason why a number of recent calcium imaging studies have demonstrated significant turnover in the active cell population over time (Cai et al., 2016; Kinsky et al., 2018; Mau et al., 2018; Rubin et al., 2015; Ziv et al., 2013)? Would we see no turnover/slower turnover using different methods that detected activity from less bursty cells which were subsequently less likely to undergo plasticity? Evidence suggests that the answer is no, since previous studies have found significant turnover in hippocampal activity patterns using electrophysiology over short to intermediate time-scales (Mankin et al., 2015, 2012; Manns et al., 2007). Furthermore, recent studies using different, non-calcium related, imaging techniques have found that plasticity is ubiquitous in hippocampal neurons even in highly familiar circumstances (Attardo et al., 2018), presumably because afferent inputs to CA1 exhibit 100% turnover over the course of a month (Attardo et al., 2015). Still, this bias toward higher firing rate, more plastic neurons must be considered, especially since the rules regarding how downstream structures interpret hippocampal outputs are poorly

understood (Lisman et al., 2017). On the other hand, vesicle release due to a single action potential is unreliable (Korn, Triller, Mallet, & Faber, 1981); bursting of neurons may provide a method for reliable transmission of signal at downstream synapses (Lisman, 1997). Furthermore, calcium entry through low-threshold VGCCs is linked with BDNF mediated long-term cell survival in cultured neurons (Ghosh, Carnahan, & Greenberg, 1994; Lyons & West, 2011). Thus, calcium imaging's bias toward the recording of more plastic neurons could also provide a readout of the more reliable signals transmitted to downstream regions.

Much of the early work utilizing calcium imaging focused on the interaction between calcium signal in different compartments of the neuron during spontaneous and evoked activity and how that contributed to firing activity/plasticity (Davie, Clark, & Hausser, 2008; Mao et al., 2001; Markram et al., 1995; Schiller, Schiller, Stuart, & Sakmann, 1997; Yuste & Denk, 1995). Recent work has continued this trend, using head-fixed two-photon imaging in conjunction with active behavior to elucidate how/why place cells emerge (Sheffield, Adoff, & Dombeck, 2017; Sheffield & Dombeck, 2014). Calcium entry through dendrites is intimately tied to the presence of plateau potentials – sustained, sub-threshold rises in membrane potential result from precisely timed input between ECIII and CA3 inputs to distal/proximal regions of CA1 dendrites (Bittner et al., 2015; Sheffield & Dombeck, 2019). Current injections during plateau potentials, observed with whole-cell patch recordings, were sufficient to induce place field formation (Bittner et al., 2015). Likewise, the prevalence of dendritic calcium transients ramped up immediately prior immediately prior to, and in the same location as, the

formation of a place field for a neuron (Sheffield et al., 2017). These two studies suggest that the detection of somatic calcium implies the existence of high calcium influx through dendrites. Furthermore, another study found that neurons with reliable activity in most of their dendritic branches also had the most stable place fields (Sheffield & Dombeck, 2014), suggesting that careful monitoring of the reliability of calcium activity could provide valuable information about the inputs to a given neuron. Though these studies all considered the formation of place fields, the same mechanisms could also apply to the emergence of hippocampal neurons responding to non-spatial features of a task (Aronov et al., 2017; Muzzio et al., 2009; Pastalkova et al., 2008; Robinson et al., 2017; Wood et al., 1999). Thus, though there are several caveats underlying the use of calcium imaging that warrant careful consideration, calcium imaging can also provide valuable information about both the gross spiking activity of neurons as well as their inputs.

1.6 Hippocampal Remapping as a Mechanism for Learning

The induction of LTP in hippocampal neurons is thought to provide a neural mechanism for learning new information (Malenka & Bear, 2004). Is there a corresponding signature of learning observable in the activity patterns of hippocampal neurons? One idea is that the reorganization of place field firing locations, a phenomena dubbed remapping (Muller & Kubie, 1987; Muller et al., 1987), might provide a discernible readout of learning in the neural code (Leutgeb & Leutgeb, 2014). Here, I will briefly review definitions of remapping to clarify the many ways in which place fields can change and then discuss how these changes could relate to learning.

Rodents frequently, but not always, utilize different configurations of place fields to represent different arenas. In highly distinct arenas, all the place fields randomly reorganize their firing locations in the phenomena of global remapping (Leutgeb et al., 2005). However, place fields frequently retain the same location but modulate their peak firing rate when rodents are placed in a different, but highly similar arena in the phenomena of rate remapping (Leutgeb et al., 2005). Partial remapping can also occur when a subset of cells change their firing field locations in response to a new object or barrier in an arena (Bostock, Muller, & Kubie, 1991; Muller & Kubie, 1987). Place fields can also all coherently rotate together in response to the rotation of an orienting cue in a given arena (Muller & Kubie, 1987) and sometimes even when all cues remain fixed (see Chapter Two and Kinsky et al., 2018). Thus, remapping occurs to different extents depending on the changes made to an arena and could provide a neural substrate for learning. In support of this idea, one study found that rats which underwent contextual fear conditioning exhibited a marked remapping of place fields in the shock arena but not in a neutral arena (Moita, 2004). Another study confirmed that fear conditioning in mice also produced remapping, but also increased the stability of place field firing locations thereafter (Wang et al., 2012). The robust changes to spatial maps in the same arena due to fear conditioning therefore strongly support the idea of hippocampal remapping as a neural readout of/substrate for learning.

1.7 Stability of the Hippocampal Code as a Substrate for Long-Term memory

Place cells might serve as a substrate for spatial memory by providing a signal to downstream regions of an animal's location. However, to do so they must reliably fire in the same location across long time scales, otherwise downstream neurons requiring coincident input from neurons with the same place field will not become sufficiently depolarized to fire. Evidence in older rats who exhibit deficits in the induction and maintenance of LTP (Barnes & McNaughton, 1985) supports this idea, since aged rats likewise exhibit instability in the spatial firing locations of hippocampal CA1 neurons and impaired performance on spatial memory tasks (Barnes & McNaughton, 1985; Barnes et al., 1997). These results support the idea that the inability to recall the proper map of a given environment could underlie failures in spatial memory recall. Recent and older studies demonstrating reliable hippocampal neuron responses to a plethora of non-spatial features of a task (see 1.3.1.4) could extend this idea to more general, non-spatial episodic memories.

Unfortunately, the ability to track firing properties of neurons across long time scales using traditional electrophysiology is difficult (see 1.5.1). However, recent advances in imaging technology have facilitated tracking the activity of large ensembles of hippocampal neurons across long time scales, though one must be cognizant of the potential pitfalls of using single-photon imaging to track neuron activity across days (see 1.5.6). Given the complete remodeling of afferent input to hippocampal neurons over the course of a month (Attardo et al., 2015) and the continual drift exhibited in the hippocampal neural code (Mankin et al., 2012; Manns et al., 2007; Mau et al., 2018),

understanding the rules governing which neurons maintain stable information across days could provide valuable insights into how the hippocampus supports long term memory.

I will address this idea in the following three chapters of this dissertation. In Chapter 2, I will examine the hypothesis that, in the absence of learning, hippocampal place cells should maintain stable locations for their place fields. In Chapter 3, I will scrutinize how learning a spatial alternation task impacts the stability of hippocampal neurons with the hypothesis that, due to their greater utility in obtaining reward, neurons whose activity patterns reflect information more relevant to task performance will likewise exhibit more stability than neurons carrying less relevant information. Finally, in Chapter 4, I will examine how the disruption of protein synthesis, known to block memory consolidation, impacts the stability of hippocampal activity patterns over short and long time-scales.

2 CHAPTER TWO

Hippocampal Place Fields Maintain a Coherent and Flexible Map Across Long Time Scales[1]

SUMMARY

To provide a substrate for remembering where in space events have occurred, place cells must reliably encode the same positions across long time scales. However, in many cases place cells exhibit instability by randomly reorganizing their place fields between experiences, challenging this premise. Recent evidence suggests that, in some cases, instability could also arise from coherent rotations of place fields, as well as from random reorganization. To investigate this possibility, we performed *in vivo* calcium imaging in dorsal hippocampal region CA1 of freely moving mice while they explored two arenas with different geometry and visual cues across eight days. The two arenas were rotated randomly between sessions, and then connected, allowing us to probe how cue rotations, the integration of new information about the environment, and the passage of time concurrently influenced the spatial coherence of place fields. We found that spatially coherent rotations of place field maps in the same arena predominated, persisting up to six days later, and that they frequently rotated in a manner that did not match that of the

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[1] Chapter 2, in full, is a reprint of the following published article as it appears in press: Kinsky, N.R., Sullivan, D.W., Mau, W., Hasselmo, M.E., and Eichenbaum, H.B. (2018). Hippocampal Place Fields Maintain a Coherent and Flexible Map across Long Timescales. Current Biology. *28*.

arena rotation. Furthermore, place field maps were flexible, as mice frequently employed a similar, coherent configuration of place fields to represent each arena despite their differing geometry and eventual connection. These results highlight the ability of the hippocampus to retain consistent relationships between cells across long time scales and suggest that, in many cases, apparent instability might result from a coherent rotation of place fields.

2.1 INTRODUCTION

The well-established place coding properties of hippocampal neurons (O'Keefe, 1976; O'Keefe & Dostrovsky, 1971) are generally thought to provide a neural mechanism for remembering where events occurred in an environment (O'Keefe & Nadel, 1978). A key assumption underlying this mechanism, supported by early studies in single neurons in the rat, is that the spatial map composed of place cells remains stable over long periods (Muller et al., 1987; Thompson & Best, 1990). While these and other studies have demonstrated that place cells in mice and rats can remain remarkably stable across long time-scales (Rubin et al., 2015; Thompson & Best, 1990; Ziv et al., 2013), other recent studies have demonstrated that place cells in mice are unstable across days in the absence of strong attention trained to specific landmarks. This manifests as global remapping: a complete reorganization of place fields when the animal is re-exposed to the familiar environment (Jeantet & Cho, 2012; Kentros, Agnihotri, Streater, Hawkins, & Kandel, 2004; Muzzio et al., 2009). Still, other recent findings suggest that this instability might instead reflect the use of different cues across days, in the form of a reorientation (or rotation) of the same overall map during the re-exposure. These studies have shown

that, when disoriented, rodents utilize the geometry of an environment rather than single visual cues to reorient (Cheng, 1986; Cheng, Huttenlocher, & Newcombe, 2013; Cheng & Newcombe, 2005). Recent studies extended this result by demonstrating that spatially tuned cells in the hippocampus and medial entorhinal cortex concurrently reorient their firing locations in accordance with the animal's behavior during reorientation to environmental geometry (Keinath, Julian, Epstein, & Muzzio, 2017; Weiss et al., 2017) (see (Julian, Keinath, Marchette, & Epstein, 2018) for review). Thus, while in many cases global remapping underlies place field instability observed upon repeated exposures to an environment, in other cases this instability might result from the coherent re-alignment of the spatial map to a cue undetected by the experimenter. To address how this re-alignment behaves longitudinally, we employed *in vivo* calcium imaging in dorsal CA1 while mice explored two distinct environments that were rotated daily for eight consecutive days. This allowed us to investigate the stability of spatial maps in each environment, the differences in maps between environments, responses of maps to cue rotations, and their evolution over time. Additionally, we connected the environments on two days to investigate whether this would disrupt the configuration of established maps. By leveraging the strength of calcium imaging to identify large numbers of the same neurons across multiple recording sessions, we were able to compare ensemble spatial firing patterns during exposures to the same and different environments across days. We predicted that comparisons between sessions in the same environment would yield largely the same map, and that comparisons between sessions in different environments would reveal global remapping. We hypothesized that place field maps within the same

arena would either rotate with the arena in accordance with the mouse's use of arena cues for orientation, or globally remap consistent with the observation of long-term instability during open-field recordings (Kentros et al., 2004). While some comparisons produced results consistent with these predictions, in most cases we found that mice utilized a coherent map: a configuration of place fields that maintain the same angle and distance from one another. Consistent with recent studies (Keinath et al., 2017; Weiss et al., 2017), however, we found that coherent maps frequently rotated in a manner that does not utilize specific arena cues or larger room cues for orientation. Maps were flexible as mice frequently employed a coherent map between the two different arenas while simultaneously modulating activity in a subset of cells to discriminate between them. Arena connection caused the neuronal population to temporarily sharpen discrimination between the arenas without permanently disrupting coherent maps in each arena afterward. Finally, coherent maps persisted in a significant proportion of the neuronal population across all eight days of the experiment despite constant turnover of cells actively participating in the ensemble each day. These findings highlight that in many cases place fields can maintain structure across days, and suggest that while instability frequently indicates global remapping it may sometimes also reflect the re-orientation of a coherent map to different cues.

2.2 RESULTS

2.2.1 Experimental Outline

Mice (n = 4) explored two arenas – a square and an octagon of equal area, painted the same color and with distinct visual cues (horizontal/vertical black stripes) – over the course of eight days (Figure 2.1A). The arenas were surrounded by curtains designed to obscure any extra-maze (room) cues and heighten the salience of arena cues that the mice might use for orientation. Initially, each mouse underwent two 10 minute sessions per day in the same arena with the arena pseudorandomly rotated 90 degrees clockwise or counterclockwise between sessions. This repeated until the mouse experienced two days in each arena (SQUARE1-2 and OCTAGON1-2). On days 5/6 (CONN1/CONN2) the arenas were connected via a previously hidden hallway and the mouse explored the combined arena in one continuous session broken up into alternate 5 minute blocks in each arena. On the last two days (SQUARE3 and OCTAGON3) the arenas were again separated.

We employed in-vivo calcium imaging with a microendoscope to track neural activity in the dorsal CA1 region of the hippocampus via virally expressed GCaMP6f (Chen et al., 2013; Ziv et al., 2013). Using this technique we recorded large numbers of neurons (n = 194 to 548 per 10 minute session, (Figure 2.1B, Figure 2.7) from which we extracted calcium traces, identified putative spiking epochs (Figure 2.1C), and created calcium event rate maps exhibiting the well-established spatial tuning of hippocampal neurons (O'Keefe, 1976; O'Keefe & Dostrovsky, 1971) (Figure 2.1D). The number of

neurons remained steady throughout the experiment ($p = 0.73$, Kruskal-Wallis ANOVA for SQUARE1-3 and OCTAGON1-3). Additionally, we were able to reliably identify the same neurons across all 8 days of the experiment (Figure 2.1E, Methods). These results establish the feasibility of tracking calcium events in large numbers of spatially tuned neurons across all eight days of the experiment.

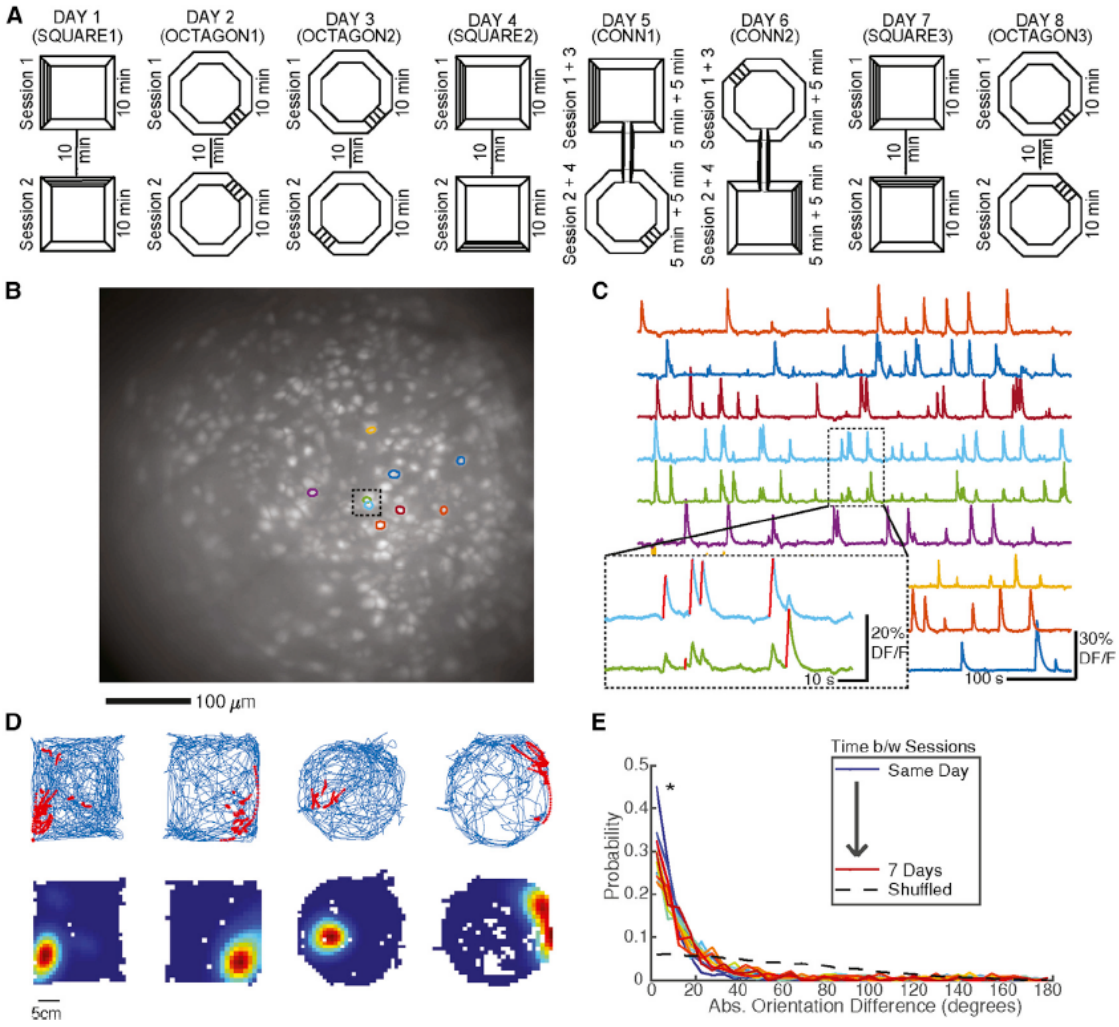

Figure 2.1: Experimental Setup

A) Mice explored two different arenas across 8 days. SQUARE1-3 and OCTAGON1-3: 2 - 10 minute sessions with arena pseudorandomly rotated between sessions. CONN1 and CONN2: Arenas were connected with a hallway and mice were given two 5 min blocks in each in alternating fashion.
B) Maximum projection from a recording session with nine neuron ROIs overlaid. Dashed box indicates two closely spaced ROIs. See also Figure 2.7.
C) Example calcium traces for ROIs highlighted in A. Dashed box demonstrates the ability of the cell/transient detection method to disambiguate crosstalk between neighboring neurons by assigning putative spiking epochs (red lines) to the appropriate neuron.
D) Example place fields. *Top:* Blue = mouse's trajectory, red = calcium event activity. *Bottom*: Occupancy normalized calcium event rate maps. Red = peak calcium event rate, Blue = no calcium activity.
E) Distribution of ROI orientation (major axis angle) differences between sessions for one mouse. Since the majority of ROIs are elliptical, the small changes in ROI orientation shown here indicate that neurons are properly registered between sessions. *p < 1e-28 all session-pairs, one-sided Kolmogorov-Smirnov test vs shuffled.

2.2.2 Coherent Maps Predominate in the Hippocampus

Does instability of hippocampal spatial representations in mice always result from a random reorganization of place fields, or might place fields retain structure between sessions? As noted in the introduction, instability observed in previous studies during random foraging (Agnihotri et al., 2004; Rotenberg, Abel, Hawkins, Kandel, & Muller, 2000) could result from global remapping of place fields (Figure 2.2A, top). Alternatively, we hypothesized that the trial-by-trial rotations of place fields observed in a recent study (Keinath et al., 2017) might persist over longer time scales (Figure 2.2A, bottom). In agreement with this study, we likewise found that place fields frequently rotated together coherently between sessions by maintaining the same distance and angle from each other (Figure 2.2C-D). To quantify the level of coherent rotation we identified the angle θ that each neuron's place field rotated between sessions (Figure 2.2B) and

plotted the distribution of θ for each session-pair (Figure 2.2E-G). In line with a previous study (Paz-Villagrán, Save, & Poucet, 2004), we reasoned that if a significant proportion of place-fields maintained a coherent structure and rotated the same amount, then the distribution of θ should exhibit clustering around the mean angle of the distribution, θ_{mean} (Figure 2.2E-F). On the other hand, if all the place fields independently reorganized between sessions (global remapping), we would observe a uniform distribution of θ (Figure 2.2G). We applied this approach to every pair of sessions in the same arena, regardless of arena rotation and day lag between sessions, and found that mice predominantly utilized a coherent spatial map to represent each arena (Figure 2.2J); nonetheless, some mice still exhibited global remapping between sessions in a minority of cases (percentages for each mouse: 36%, 14%, 0%, and 0% in square, 21%, 7%, 14%, and 0% in the octagon). We obtained similar results using an alternative analysis method to identify place field rotations (Figure 2.8).

Are coherent rotations an all-or-nothing phenomenon, or does some of the population deviate and randomly remap between sessions? To address this, we defined a place cell as coherent if its field's rotation matched that of population mean within a 30 degree range ($|\theta - \theta_{mean}| < 30$); we designated the remaining cells as randomly remapping. Using these classifications, we found that approximately half the population typically stayed coherent between sessions (Figure 2.2H-I). These results combined confirm previous studies finding a mixture of stability and dynamics in hippocampal neurons (Rubin et al., 2015; Ziv et al., 2013) and extend their work to a two-dimensional, non-goal directed task.

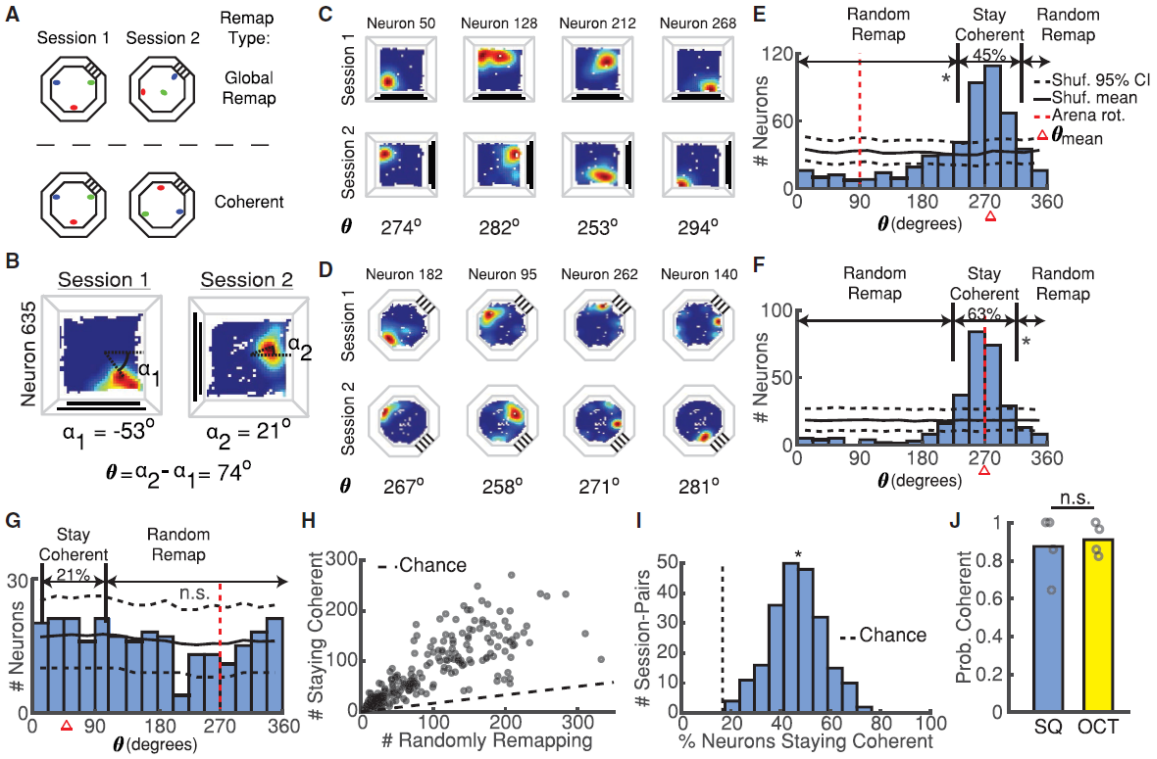

Figure 2.2: Coherent Maps Predominate in the Hippocampus

A) Schematic of null hypothesis of global remapping between sessions (top) and alternate hypothesis of coherent mapping between sessions (bottom) using three example place fields (red, green, blue). In global remapping, all place fields randomly reorganize. In coherent mapping, place fields retain the same configuration but may or may not rotate.

B) The place field rotation between sessions (θ) was calculated as the difference between α_1 and α_2, the angle from the arena center to the occupancy bin with the peak calcium event rate in session 1 and session 2, respectively. See also Figure 2.8.

C) Calcium event rate maps from 4 simultaneously recorded neurons between two square arena sessions demonstrating coherent mapping. The rotation of each neuron's place field is indicated at the bottom. Note that all rotations are close to 270 degrees.

D) Same as C, but for two octagon sessions from a different mouse.

E) Distribution of place field rotations for the coherent session-pair shown in C demonstrates clear clustering of rotations. Percentages of neurons staying coherent ($|\theta - \theta_{mean}| < 30$) or randomly remapping ($|\theta - \theta_{mean}| >= 30$) are indicated above the distribution. Red triangle = θ_{mean}. Black solid/dashed lines = shuffled mean and 95% CI. Red dashed line = arena rotation. *p < 0.001, shuffle test.

F) Same as E, but for the coherent session-pair shown in D. *p < 0.001.

G) Same as E-F but for an infrequent session-pair exhibiting global remapping. p = 0.15.

H) Number of neurons staying coherent versus randomly remapping for all session-pairs. Dashed line indicates numbers expected by chance.
I) Percentage of neurons whose place fields stay coherent for all mice/session pairs. *p = 1.8e-108 (t-test vs chance).
J) Probability of using a coherent map in each arena. Open circles indicate proportions for each mouse. p = 1, Wilcoxon rank-sum test.

2.2.3 Coherent Maps Do Not Consistently Utilize Arena Cues For Orientation

Previous studies have found that CA1 place fields will move together to follow the rotation of visual cues within an arena (Muller & Kubie, 1987), indicative of the mice using these cues to orient themselves. Alternatively, a recent study (Keinath et al., 2017) demonstrated that disoriented mice frequently ignore purely visual cues and instead utilize geometry to concurrently reorient themselves and their place field maps. In support of this work, in many cases where two sessions shared a coherent spatial map, place fields rotated incongruously with arena cues (Figure 2.3A). We thus examined the relationship between arena and place field rotations, reasoning that if mice utilized arena cues to orient their spatial map, the rotation of the map (θ_{mean}) would match the arena rotation (θ_{arena}) between sessions. Applying this criteria, we found that coherent maps did frequently rotate with the arena (Arena designation: $\theta_{mean} \approx \theta_{arena}$, Figure 2.3B), but just as often rotated in a different direction (Mismatch designation: $\theta_{mean} \neq \theta_{arena} \neq 0$, Figure 2.3A). One possible explanation of this effect could be that despite our best efforts to minimize extra-maze cues, mice oriented themselves in the larger room (Room designation: $\theta_{mean} \approx 0$, Figure 2.3C). However, these session-pairs only occurred at chance levels (Figure 2.3D), suggesting mice were unable to reliably extract extra-maze cues for

orientation. The high prevalence of mismatch session-pairs was consistent within arenas (Figure 2.3D), and coherent rotations occurred even when the arena was not rotated between sessions (32% ± 24% of session-pairs). Thus, while spatial maps typically remained coherent between sessions, they frequently did not use arena cues for map alignment.

If mice utilize the geometry of the room to orient their spatial maps (Keinath et al., 2017; Weiss et al., 2017), is the stability of these maps worse in more rotationally uniform arenas? To investigate this, we divided the arena into 45 degree bins and examined the distribution of mean place field rotations between sessions (Figure 2.3E). We found that almost all mismatch sessions rotated in 90 degree increments in the square whereas few did so in the octagon (Figure 2.3F). Thus, place field instability resulting from coherent rotations might be exaggerated in more rotationally uniform arenas like the octagon. We found no relationship between the mouse's initial location/orientation upon entering the maze and the rotation of maps in mismatch sessions (Figure 2.9). Taken together, our findings extend the work of Keinath et al. (Keinath et al., 2017) by demonstrating that coherent rotations controlled by arena geometry occur even when mice are not intentionally disoriented and are more common in circular arenas. Thus, instability in place fields could result from a rotation as well as from global remapping.

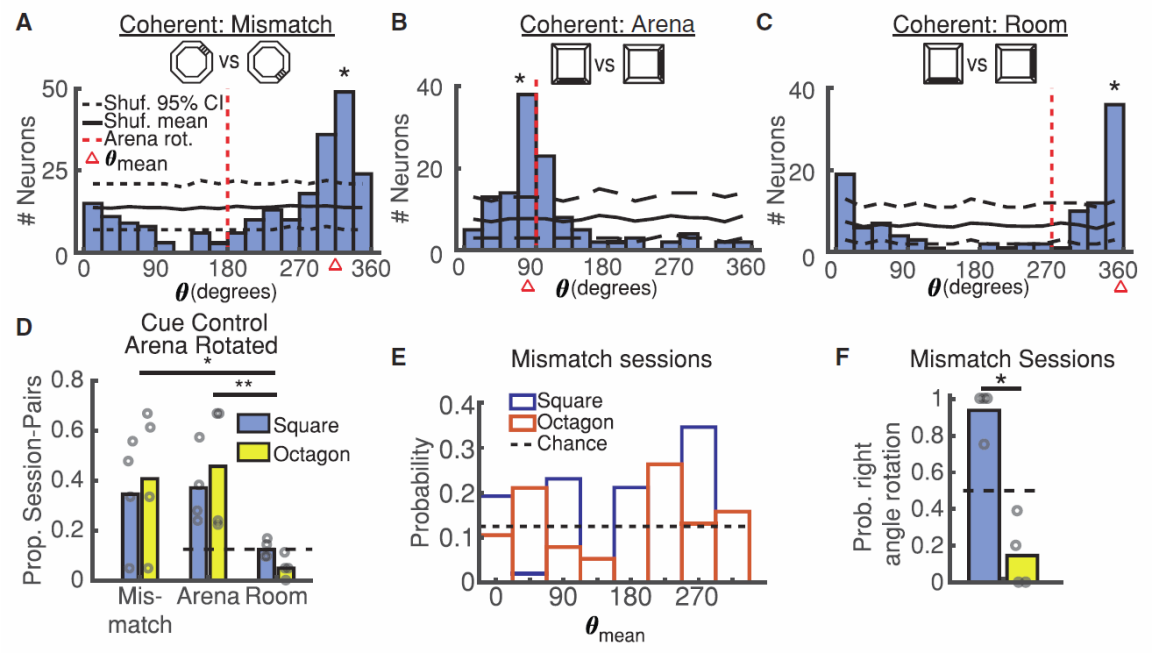

Figure 2.3: Coherent Maps Do Not Consistently Utilize Arena Cues For Orientation

A) Session-pair in the octagon arena demonstrating a **mismatch** between arena and place field rotations ($|\theta_{mean} - \theta_{arena}| > 30$). Black solid/dashed lines = shuffled distribution mean and 95% CI. Red dashed line = arena rotation. Red triangle = θ_{mean}. *p < 1/1000, shuffle test. See also Figure 2.2C,E.

B) Session-pair in the square arena demonstrating control of place field rotations by **arena** rotations ($\theta_{mean} \approx \theta_{arena}$). Same conventions as A. *p < 1/1000. See also Figure 2.2D,F.

C) Session-pair in the square arena demonstrating a lack of place field rotations ($\theta_{mean} \approx 0$), consistent with orientation in the larger **room**. Same conventions as A. *p < 1/1000.

D) Probability mice orient their place field maps per A-C indicates a high prevalence of mismatch session-pairs. Open circles indicate individual mouse probabilities. Dashed line indicates chance. p = 0.0042, Kruskal-Wallis ANOVA, *p = 0.028, **p = 0.0057 post-hoc Tukey test. All comparisons between square and octagon are not-significant (p > 0.05, Wilcoxon rank-sum test).

E) Distribution of mean place field rotation angles for all square (blue) and octagon (orange) mismatch session-pairs. See also Figure 2.9.

F) Proportion of mismatch session-pairs with place field rotations at right angles. Same conventions as D. Dashed line indicates chance. *p = 0.014, Wilcoxon rank-sum.

2.2.4 Coherent Maps Generalize Across Different Environments

Mice could employ a unique place field map in each arena to reflect differences between them (Leutgeb et al., 2004; Wilson & McNaughton, 1993). Alternatively, they could utilize a similar map to link common experiences in both arenas (Eichenbaum, 2004). We thus tested if, and to what extent, mice utilized the same map between arenas. Applying the method described above for within-arena comparisons, we found that mice often employed a coherent map to represent both arenas despite their distinct visual and geometric cues (Figure 2.4A-C). Of course, the use of coherent maps between arenas might also indicate that mice failed to perceive the arenas as different. To address this question, we utilized a population vector (PV) analysis, which is sensitive to changes in both neuron firing location and calcium event rate. The PV analysis allowed us to compare the relative similarity of the neuronal population between visits to different arenas, after taking into account the mean place field rotation of the population. We found that PV similarity between sessions in the same arena was significantly higher than between sessions in different arenas (Figure 2.4D-E, Figure 2.10A), indicating that the mice were capable of discriminating arenas at the neuronal level. We obtained similar results using PVs constructed without taking into account place field rotations and using only the maximum event rate for each neuron ($p=1.4e-8$, Wilcoxon rank-sum test of PV correlations in same vs. different arena), indicating that event rate changes alone were sufficient for the population to distinguish between arenas. Thus, the use of a similar map

of place fields between arenas might support the role of the hippocampus in providing a contextual memory that links memories occurring in each arena (Eichenbaum, 2004) across space and time (Redish, 2001).

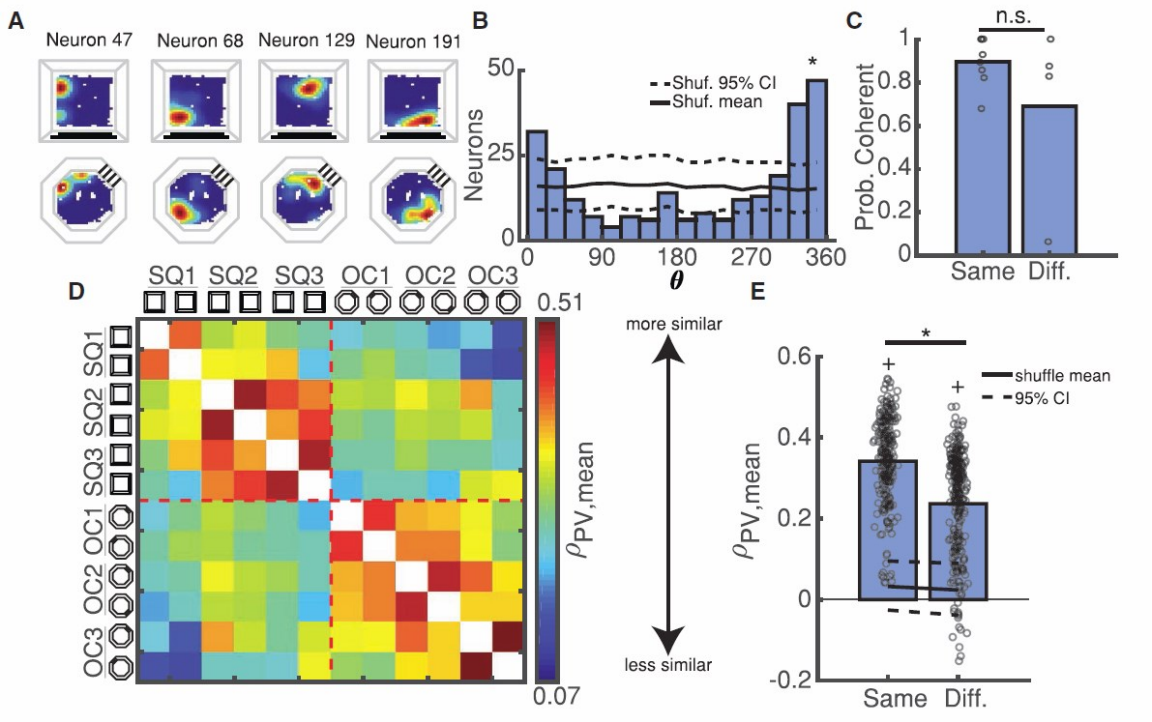

Figure 2.4: Coherent Maps Generalize Across Different Environments

A) Calcium event maps from 4 simultaneously recorded neurons indicate place fields stay in the same location between arenas.

B) Distribution of place field rotations for coherent session-pair shown in A. Black solid/dashed lines = shuffled mean and 95% CI. *p < 0.001, shuffle test.

C) Probability of using a coherent map remains high within and between arenas. Open circles = mean for each mouse/comparison-type. *p = 0.48, Wilcoxon rank-sum test.

D) Mean population vector (PV) similarity between all non-connected sessions in each arena, grouped by arena and averaged across mice. Warmer/cooler colors indicate higher/lower PV similarity between sessions. See also Figure 2.10A.

E) PVs are more similar within arenas than between arenas. Open circles indicate mean PV correlations for all mice/session-pairs. Black solid/dashed lines = shuffled distribution mean and 95% CI. *p = 1.3e-28 Wilcoxon rank-sum test. +p < 1e-37, sign-rank test vs upper 95% CI.

2.2.5 Connecting Arenas Temporarily Sharpens Discrimination

A notable previous study demonstrated that rats initially exposed to two connected arenas discriminated between them to a much greater extent than counterparts who only experienced each arena separately (Colgin et al., 2010), suggesting that rodent's prior experience in an arena has a strong effect on its subsequent representations therein. We thus wondered if we could also induce arena discrimination via connection, but after mice had already established representations of each arena. In support of this, we observed relatively low PV similarity for repeated trips to different arenas versus trips to the same arena during connection (Figure 2.5A-B, Figure 2.10B). Furthermore, PVs on CONN1 and CONN2 exhibited lower similarity than PVs on un-connected days while still staying above chance (Figure 2.5C, Figure 2.10A-B). Despite this sharpened discrimination, we found that the probability of utilizing a coherent map was no different than before/after connection ($p = 0.34$, balanced Kruskal-Wallis ANOVA). In fact, the presence of the hallway seemed to align place fields between arenas (Figure 2.5D, Coherent neuron), potentially by providing a unique geometric feature that could be used for orientation (Keinath et al., 2017). In support of this, the mean place field rotation angle between arenas was near zero (0.93 ± 1.38 degrees) during CONN1 and CONN2. Thus, introducing the hallway increased discrimination between the octagon and square arenas by the population without inducing a wholesale shift to global remapping.

Several mechanisms could support simultaneous discrimination and coherent mapping of the two environments. First, the subpopulation of neurons staying coherent (Figure 2.5D, Coherent neuron) could decrease in size. In support of this, we observed a reduction in the proportion of neurons staying coherent between different arenas versus in the same arena (Figure 2.5E); as a result, after accounting for rotation, the neuronal population also tended to shift its place fields more between arenas than within arenas (Figure 2.10F). Second, in line with previous electrophysiological studies (Leutgeb et al., 2005; Lever, Wills, Cacucci, Burgess, & O'Keefe, 2002; Wills, Lever, Cacucci, Burgess, & O'Keefe, 2005), neurons could modulate their calcium event rate both up and down between arenas (Figure 2.10C,G) with some neurons highly active in one arena or the other (On/Off Cells in Figure 2.5D and selective cells in Figure 2.10G). Indeed, we observed a substantial increase in the proportion of on/off cells occurring between different arenas (Figure 2.5F). These two mechanisms (reducing the coherent population size and modulating event rate between arenas) were sufficient to support arena discrimination, since PVs formed with only non-selective, coherent neurons failed to distinguish between arenas (Figure 2.10H). Despite inducing substantial changes on CONN1 and CONN2, however, we found little lasting effect of connection as PV similarity and the proportion of neurons staying coherent remained unchanged in the sessions following arena connection (Figure 2.10D-E). However, we did observe a small but significant increase in the number of on/off cells from before to after arena connection (Figure 2.5G), indicating that rate modulation effects (Leutgeb et al., 2005; Lever et al., 2002; Wills et al., 2005) might provide a mechanism for persistent arena

discrimination. These results indicate that arena connection temporarily sharpened discrimination between arenas without inducing substantial long-term changes in the hippocampal representation of each.

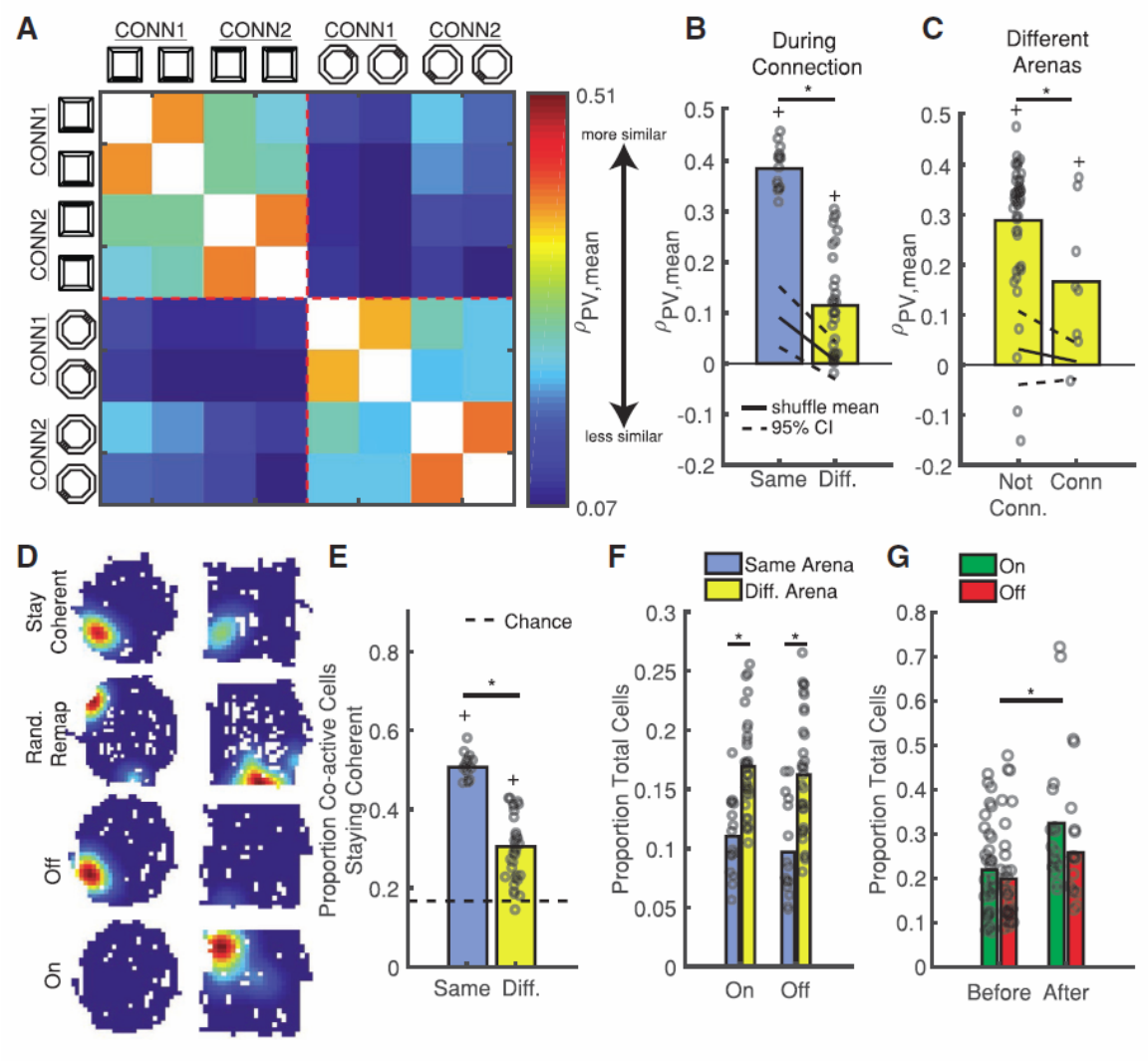

Figure 2.5: Connecting Arenas Temporarily Sharpens Discrimination

A) Mean PV similarity on connected days, grouped by arena and averaged across mice. Same color scale as Figure 2.4D. See also Figure 2.10B.

B) PVs are more similar within arenas (blue) than between arenas (yellow) during connection. Open circles are for all mice/session-pairs. Black solid/dashed lines =

shuffled distribution mean and 95% CI. *p = 2.3e-8, Wilcoxon rank-sum test. +p < 0.001, sign-rank test vs upper 95% CI.

C) PV similarity between arenas on un-connected days are higher than on connected days. Same conventions as B. All session-pairs considered were 1 day apart. *p = 0.041, Wilcoxon rank-sum test. +p < 0.04, sign-rank test vs upper 95% CI.

D) Example event rate maps for neurons that either stay coherent, randomly remap, turn "off" (active in 1st arena, inactive in 2nd), or turn "on" (inactive in 1st arena, active in 2nd) between arenas.

E) The size of the population staying coherent decreases between arenas. Open circles indicate proportions for all mice/session-pairs during connection. Black Dashed black line = chance. *p=2.3e-8, Wilcoxon rank-sum test. +p = 2.3e-8 sign-rank test vs chance.

F) More neurons turn on/off between different arenas than within the same arena. Same conventions as E. *p < 2e-4, Wilcoxon rank-sum test.

G) Arena connection induces a lasting increase in the number of on/off neurons. Same as F but for session-pairs before and after connection. *p = 0.026, Wilcoxon rank-sum test.

2.2.6 Properties of Activity Across Long Time Scales

How stable are coherent maps across long time-scales? Based on previous work establishing that population spatial representations evolve over hours to days (Mankin et al., 2012; Rubin et al., 2015; Ziv et al., 2013) we hypothesized that the probability of two sessions utilizing a coherent map would decrease with time. In support of this, and in agreement with previous studies (Cai et al., 2016; Rubin et al., 2015; Ziv et al., 2013), we found that the percentage of cells reactivated in a later session decreased with time between sessions (Figure 2.6A). These findings indicate that time influenced which neurons made up the active ensemble. However, θ histograms exhibited significant clustering around one angle for sessions at short and long time lags (Figure 2.6B-C), indicating that maps stayed coherent even at long time scales. Furthermore, the probability of maintaining a coherent subpopulation of neurons did not change with increasing time lag, remaining high for sessions up to six days apart (Figure 2.6D). This

finding is further supported by PV analyses demonstrating that ensemble similarity remains high up to six days later (Figure 2.6E), even when putative silent neurons are included in the population (Figure 2.11A). These results combined indicate that, while neurons continuously dropped in/out of the active population, those that remained active between sessions tended to retain the same, coherent map of place fields at long time scales.

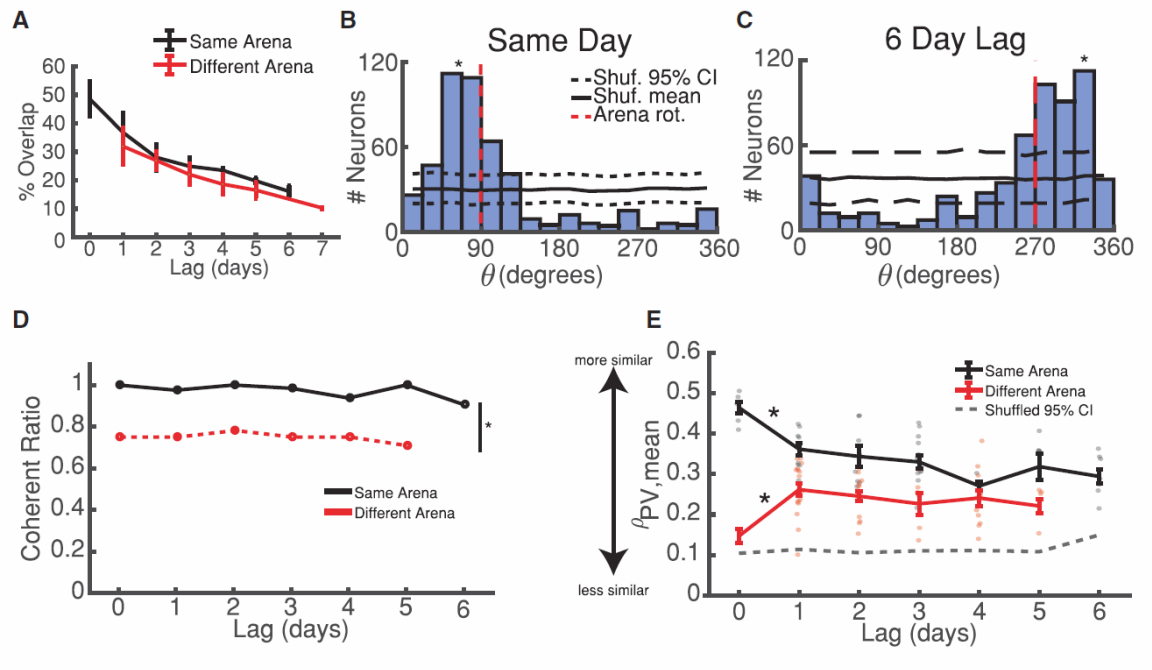

Figure 2.6: Properties of Activity Across Long Time Scales

A) % Cell overlap vs. time lag between sessions demonstrates that fewer neurons are reactivated with time. Data are shown as mean ± s.e.m. Black = same arena, red = different arena.

B) θ distribution for session-pair occurring the same day. Black solid/dashed lines = shuffled mean and 95% CI. Red dashed line = arena rotation. *p < 0.001, shuffle test.

C) θ distribution for session-pair occurring 6 days apart. Same conventions as B. *p < 0.001.

D) Time does not influence the probability of maintaining a coherent map between sessions. p > 0.5 Kruskal-Wallis ANOVA for same (black) and different (red) arena session-pairs across time. *p = 6.5e-5 Wilcoxon rank-sum test.

E) High PV correlations at ~θ_{mean} supports the use of coherent maps at all time lags between sessions. Grey dashed = upper 95% CI from shuffled distribution. Colored dots indicate mean for each session-pair across mice. Error bars = s.e.m. *p < 0.001, Wilcoxon rank-sum test vs upper 95% CI at all time lags. See also Figure 2.11.

2.3 DISCUSSION

While place cells can remain remarkably stable (Thompson & Best, 1990; Ziv et al., 2013), the finding of long-term instability in mice place fields by others (Jeantet & Cho, 2012; Kentros et al., 2004) challenges the proposed role of the hippocampus in supporting memory of where events occur in space (Agnihotri et al., 2004; Rotenberg et al., 2000), raising the question: how can a randomly changing spatial representation reliably retrieve the appropriate memory of previously encountered arena? We argue that, in some cases, the hippocampus can maintain an intact configuration of place fields, and that the aforementioned instability might sometimes result from a change in orienting this coherent map between arena exposures (Keinath et al., 2017). Here, we performed *in vivo* calcium imaging in the mouse hippocampus to record from large numbers of cells across eight days and comprehensively address this hypothesis. We found that within the same arena, place fields largely stayed coherent between sessions (Figure 2.2), even at long time scales (Figure 2.6). However, we also occasionally observed global remapping between sessions (Figure 2.2J). Consistent with previous studies (Keinath et al., 2017; Weiss et al., 2017), coherent maps frequently ignored visual cues for alignment between sessions (Figure 2.3). Analyses done assuming adherence of spatial maps to arena cues revealed relatively low correlations (Figure 2.11B). Thus, our data support the view that instability in place fields could stem from either global remapping or from a map

orientation change relative to arena cues. These results also build upon previous findings (Keinath et al., 2017) by extending this phenomenon to longer time scales (Figure 2.6), by demonstrating that coherent rotations exist even when mice are not intentionally disoriented, and by indicating that coherent rotations are not an all-or-nothing phenomenon (Figure 2.2H-I). Additionally, our results suggest that more rotationally uniform arenas contribute to rotational instability of place fields (Figure 2.3E-F). Taken together, our work suggests that future studies could perform rotation analyses (e.g. see (Fuhs et al., 2005; Law, Bulkin, & Smith, 2016; Paz-Villagrán et al., 2004)) to determine whether global remapping or coherent place field rotations underlie instability, especially in arenas with high degrees of symmetry.

Our results appear to conflict with recent studies (Rubin et al., 2015; Ziv et al., 2013) that found stable place field maps across long time-scales without random, coherent rotations. However, this discrepancy is likely due to task differences: the linear track paradigm employed in the previous studies results in stereotypic, goal-directed behavior that causes highly directional firing of hippocampal neurons (Markus et al., 1995). This could also sharpen attention to external cues, thus enhancing place field stability relative to visual cues between sessions (Kentros et al., 2004; Muzzio et al., 2009). Notably, after taking into account coherent rotations of place fields between arenas, our results appear entirely consistent with these studies (Rubin et al., 2015; Ziv et al., 2013), which demonstrate a stable yet dynamic hippocampal spatial representation

across long time-scales. Our results thus extend previous work (Rubin et al., 2015; Ziv et al., 2013) to a non-goal directed task in a two-dimensional arena.

Hippocampal place field maps might support contextual learning and memory by providing a neural substrate for triggering the appropriate behavioral response in a given context (Smith & Bulkin, 2014). However, task demands can also play an important role in dictating hippocampal representations of context (McKenzie et al., 2014; Smith & Bulkin, 2014). Supporting this idea, non-spatial cues can modulate activity of spatially tuned hippocampal neurons when task demands differ in the same physical location (Ferbinteanu & Shapiro, 2003; Smith & Mizumori, 2006; Wood, Dudchenko, Robitsek, & Eichenbaum, 2000). Here, we find that the complement also holds: while performing a similar task, mice use the same map to represent different arenas (Figure 2.4). Of course, this could occur due to poor processing of spatial information by mice (see below). Alternatively, coherent place field rotations might go hand-in-hand with map generalization, since mapping the relationships between spatial locations without regard to specific visual cues could provide flexibility to utilize the same hippocampal map across multiple arenas (Eichenbaum, 2017b) and group them into similar learning contexts (Redish, 2001). This idea is supported by recent work demonstrating the ability to artificially reactivate memories by optogenetic stimulation (Kitamura et al., 2017; Liu et al., 2012; Ramirez et al., 2013; Ryan et al., 2015). In these studies, the simultaneous stimulation of hippocampal neurons tagged during contextual fear conditioning was sufficient to trigger expression of the fear memory even in a different, neutral arena. Thus, one possibility is that the natural reactivation of the same set of neurons, as would

occur when mice utilized the same place field configuration in different arenas, could also elicit a similar behavioral response in each arena. This idea warrants future studies testing whether the degree to which fear conditioned mice utilize a coherent map between shock and neutral arenas predicts how much they generalize freezing to the neutral arena.

Our work appears to directly contradict previous studies demonstrating remapping between arenas (Law et al., 2016; Lever et al., 2002; Wills et al., 2005) and even within arenas (Jeantet & Cho, 2012; Kentros et al., 2004). Much of these discrepancies likely result from differences in methodology. The arenas in our study differed only in shape/visual cues, whereas rats in the other studies demonstrating remapping between environments explored arenas that also varied in combinations of color, texture, odor, etc. Consistent with our results, in the one study (Lever et al., 2002) using arenas with the same color/texture but different shape, rats initially utilized similar place field configurations between arenas. The gradual divergence of place fields between arenas observed in this study over time could result from interference between jointly acquired memories (Law et al., 2016) since rats experienced arenas in alternating fashion each day (Lever et al., 2002), whereas mice in our study explored only one arena per day. The level of attention rodents pay to an environment, as well as what cues rodents attend to, has been shown to influence the stability of place fields (Kentros et al., 2004; Muzzio et al., 2009). Thus, the differences in methodology employed by these studies, as well as ours, could strongly influence how stable place fields remain between sessions by adjusting the level/locus of the rodent's attention. The heterogeneity in results highlights that future studies are warranted to uncover what dictates when mice utilize a coherent

but rotationally unstable spatial map and when their place fields exhibit global remapping. Yet another seminal study demonstrated that rats can in fact maintain the same place field configuration between arenas while modulating neuron firing rates to distinguish between arenas in the phenomenon of "rate remapping" (Leutgeb et al., 2005). Furthermore, they showed that rate remapping varies depending on the magnitude of differences between arenas. Our results are entirely consistent with this study, as we observe similar drops in PV correlations between arenas while still remaining above chance (Figure 2.4D-E, Figure 2.5B), and demonstrate that changes in neuron event rate contribute to PV discrimination between arenas (Figure 2.10H). Furthermore, our results demonstrate that calcium imaging can identify coarser aspects of rate remapping in the form of neurons turning on/off between arenas (Figure 2.5F).

Another explanation for the discrepancies between previously mentioned studies (Lever et al., 2002; Wills et al., 2005) and ours could be the use of different species. Perhaps mice simply have a greater tendency to perceive two different arenas as similar? In support of this, a previous study (Cho, Giese, Tanila, Silva, & Eichenbaum, 1998) demonstrated that mouse place cells orient to local cues to a greater extent than rats, indicating that mice attend less to distant cues. This could result in a greater tendency of mice to view the arenas as similar and to exhibit coherent place field rotations, since more immediate features like arena material are less useful than other visual cues for disambiguating similar arenas or re-orienting in the same arena. Thus, increased attention to local cues by mice could increase the likelihood that they utilize the same map between arenas. While this effect might predominate in mice, it still likely exists in rats.

First, as mentioned above, rats do frequently use the same map of place fields between two arenas ((Leutgeb et al., 2005) and early sessions in (Lever et al., 2002)), even when they are permanently connected (Skaggs & McNaughton, 1998; Spiers, Hayman, Jovalekic, Marozzi, & Jeffery, 2013). Second, disoriented rats frequently utilize geometry to reorient (Cheng, 1986; Cheng et al., 2013; Cheng & Newcombe, 2005) and sometimes exhibit coherent rotations in the firing fields of upstream grid and head-direction cells in medial entorhinal cortex (Weiss et al., 2017) and place cells in the hippocampus (Knierim, Kudrimoti, & McNaughton, 1995). Thus, rats can also form spatial maps not tied to specific arena features. However, since studies that explicitly test for coherent rotations of maps between arenas are the exception (Fuhs et al., 2005; Law et al., 2016; Paz-Villagrán et al., 2004) rather than the rule, future studies will be needed to comprehensively address this.

Perhaps the best explanation for discrepancies between our results and others is that an animal's prior experiences can have a strong effect on its subsequent hippocampal representation of an arena. This is demonstrated by an elegant study (Colgin et al., 2010) which found that hippocampal neurons in rats that first experienced arenas as connected discriminated between them to a much greater extent than in rats that only experienced them as separate. Consistent with this study, we found that connecting arenas mid-way through the experiment sharpened discrimination between them (Figure 2.5C). Despite this, however, the connection induced no substantial effect on later representations of each arena (Figure 2.10D-E). Thus, our results support the importance of prior knowledge in accommodating future learning (McClelland, 2013; McClelland et al.,

1995; Tse et al., 2007) since the initial formation of coherent maps allowed for temporary modification but rendered them resistant to permanent disruption.

Overall, our results highlight the capability of the hippocampus to retain stable relationships between place fields across long time scales while simultaneously encoding the differences between experiences. These findings warrant future studies that investigate if non-spatially tuned hippocampal neurons (Aronov et al., 2017; Kraus et al., 2013; Muzzio et al., 2009; Pastalkova et al., 2008) also maintain a consistent structure over days to weeks.

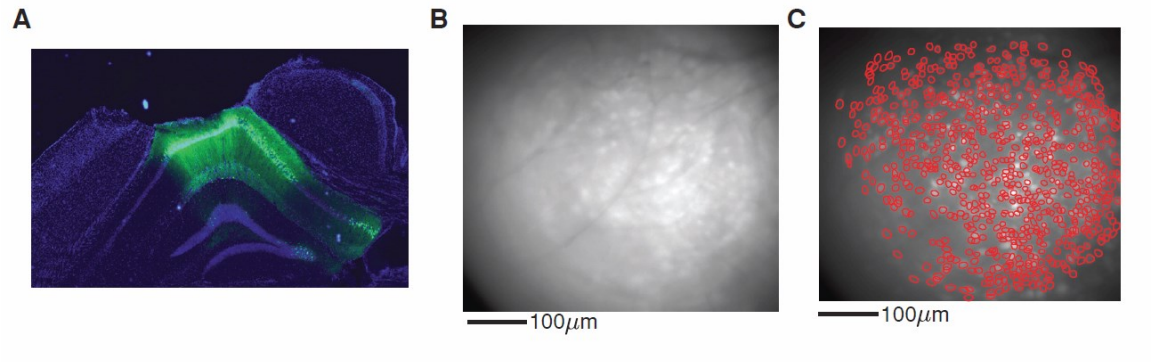

Figure 2.7: Details of Calcium Imaging (Related to Figure 2.1)

A) Histological confirmation of recording location, showing the presence of GCaMP6f primarily localized to dorsal CA1. Note the intact cell layer below where superficial cortex was removed to enable insertion of the GRIN lens. Blue = DAPI, Green = GCaMP6f
B) Example imaging window. Light areas indicate regions of background fluorescence. Dark lines indicate blood vessels. Scale bar = 100 μm.
C) Maximum projection of imaging window for mouse in b with all neuron ROIs overlaid in red. Scale bar = 100 μm.

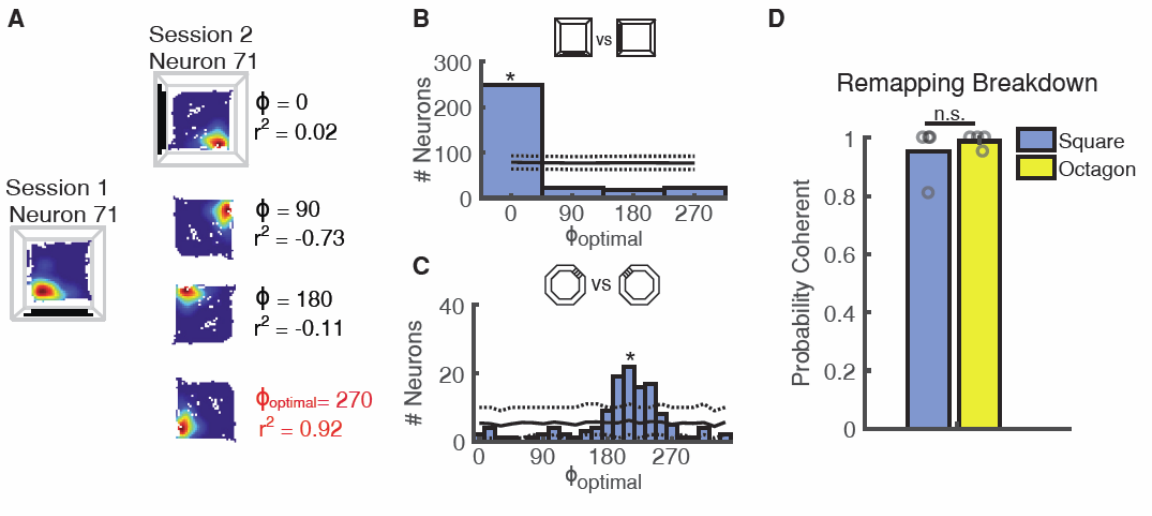

Figure 2.8: Correlation Based Rotation Method for Identifying Coherent Maps (Related to Figure 2.2)

A) Methodology for identifying the angle of rotation of a neuron's spatial firing between sessions. The correlation between calcium event maps for session A and session B was calculated after rotating the mouse's trajectory in session B by the angle ϕ in 90 degree increments in the square and 15 degree increments in the octagon. $\phi_{optimal}$ was defined as the rotation that maximizes the correlation between calcium event maps. This method is less sensitive than the center-out method (Figure 2.2B, Methods) because the minimum angle change it can resolve is equal to the increments of ϕ noted above (90 degrees in the square and 15 degrees in the octagon). However, it does not require making any arbitrary assumptions required to identify place fields.

B) $\phi_{optimal}$ distribution between two sessions recorded the same day in the square arena. Clustering of $\phi_{optimal}$ values at 0 degrees indicates coherent mapping between sessions. Black solid line = shuffled distribution mean, black dashed line = 95% CI of shuffled distribution. *p = 0, $\chi 2$ = 8.4e4, df = 3.

C) $\phi_{optimal}$ distribution between two sessions recorded the same day in the octagon arena. Clustering of $\phi_{optimal}$ values at ~210 degrees indicates coherent mapping between sessions. Same conventions as B. *p = 0, $\chi 2$ = 6.1e4, df = 23.

D) Probability of session-pairs utilizing a coherent map in each arena. Open circles indicate proportions for each mouse. p = 0.46, Wilcoxon rank-sum test.

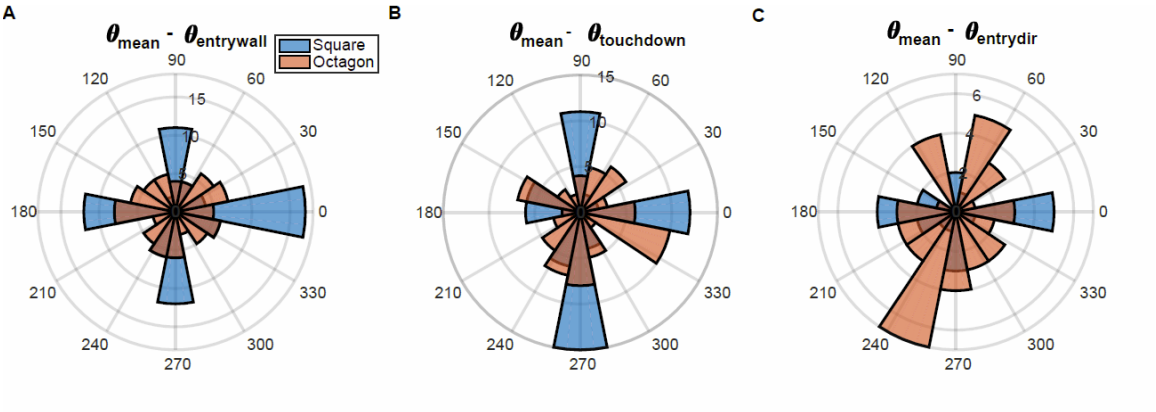

Figure 2.9: The Direction of Mouse Entry to the Arena Does Not Predict Coherent Place Field Rotations (Related to Figure 2.3)

A) Circular histogram of mean place field rotation (θ_{mean}) between sessions minus the angle difference between the mouse's entry walls ($\theta_{entrywall}$). If mice utilized the wall over which they entered the arena to anchor their place field maps between sessions, then the distribution should cluster around 0. Since values do not preferentially cluster at 0 over other orientations, we conclude that mice do not utilize the wall over which they entered the arena to orient their place field maps between sessions. p = 0.12 (square), p = 0.25 (octagon) shuffle-test.

B) Mouse orientation upon touching the arena floor does not dictate coherent place field rotations. Same as A, but for mouse orientation when his paws first touch the floor ($\theta_{touchdown}$). p = 0.06 (square), p = 0.31(octagon) shuffle-test.

C) Mouse orientation while crossing over the entry wall does not dictate coherent place field rotations. Same as A, but for mouse orientation (nose direction) upon first crossing into the arena while being carried ($\theta_{entrydir}$). p = 0.57 (square), p = 0.15 (octagon) shuffle-test.

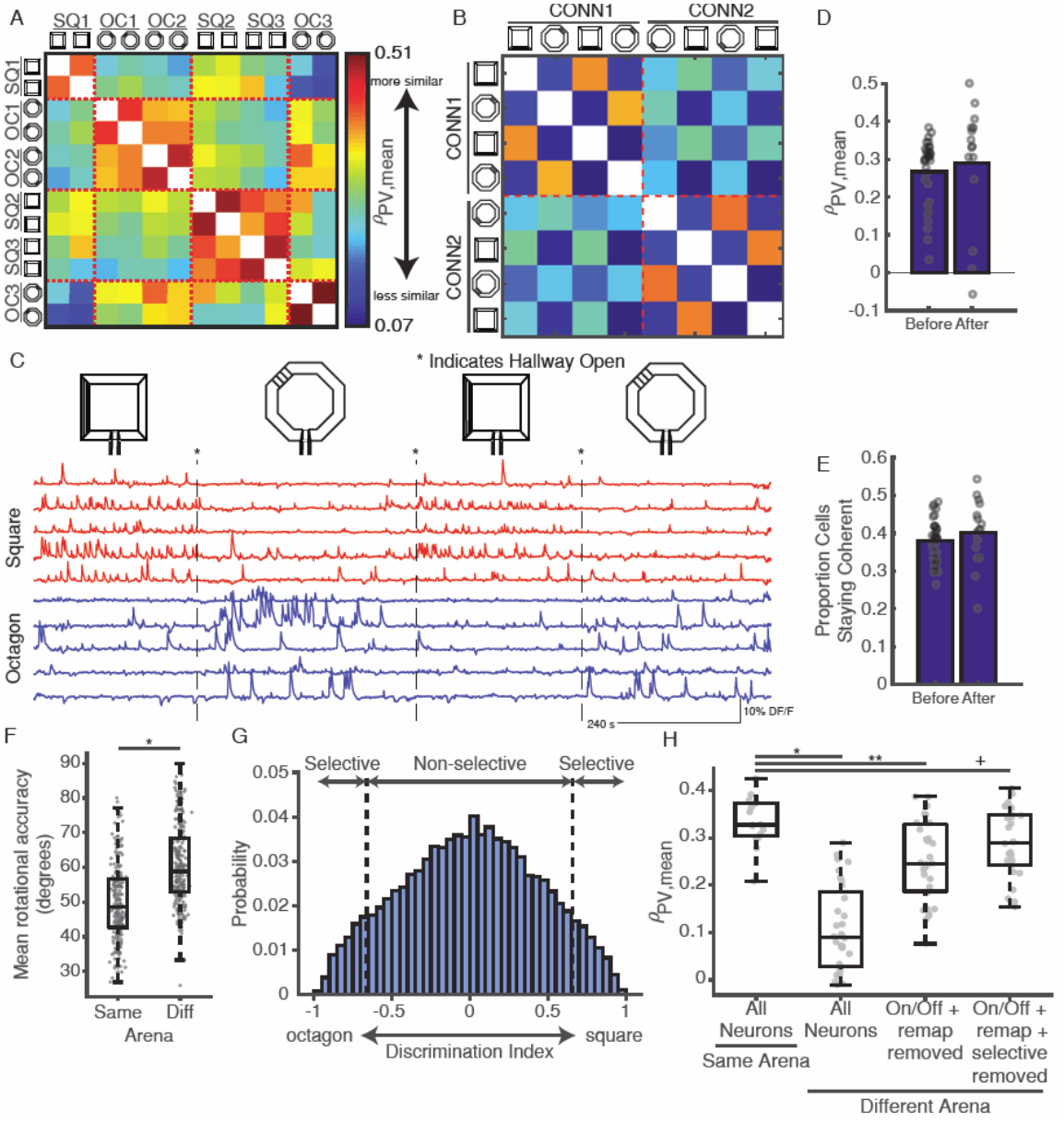

Figure 2.10: Neuronal Population Segregation (Related to Figure 2.4 and Figure 2.5)

A) Mean PV similarity between all non-connected sessions in each arena, sorted chronologically. Same color scheme as Figure 2.10B (and Figure 2.4D).

B) Mean PV similarity between all sessions in each arena on connected days, sorted chronologically. Same color scheme as Figure 2.10A (and Figure 2.5A).

C) Neurons modulate calcium event rate between arenas. Example neuron traces during arena connection (CONN1). Arena occupied by the mouse indicated at top. Traces are color coded as follows: red = highly selective for square, blue = highly selective for

octagon. Vertical dashed lines denote time points when the mouse crossed between arenas via the hallway.

D) Mean PV similarity between arenas does not change from before to after arena connection. Open circles = mean PV for all mice/session-pairs exactly one day apart. p = 0.23, Wilcoxon rank-sum test.

E) Proportion of cells staying coherent between sessions from before to after arena connection. Open circles = proportion all mice/session-pairs exactly one day apart. p = 0.22, Wilcoxon rank-sum test.

F) Place fields rotate less accurately between different arenas than in the same arena. Close circles = mean rotational accuracy of all place fields between sessions <= 6 days apart. *p = 8.7e-21, Wilcoxon rank-sum test.

G) Neurons active in both arenas modulate calcium event rates in both directions between different arenas, as indicated by the spread of Discrimination Index values from -1 (active only in the octagon) to 1 (active only in the square). All mice/session-pairs (On/Off cells excluded for clarity, see Figure 5F). Dashed lines denote extent of neurons that are active in each arena but "selective" for one arena versus the other (i.e. those neurons that produce at least 66% more calcium events in one arena than the other).

H) On/Off neurons, random remapping neurons, and "selective" neurons (see Figure 2.10G) all contribute to PV discrimination between arenas during connection, since the neuronal population fails to distinguish between arenas only when all three subpopulations are removed from the PV. *p = 6.2e-8, **p = 0.0016, +p = 0.072 (n.s.) Wilcoxon rank-sum test.

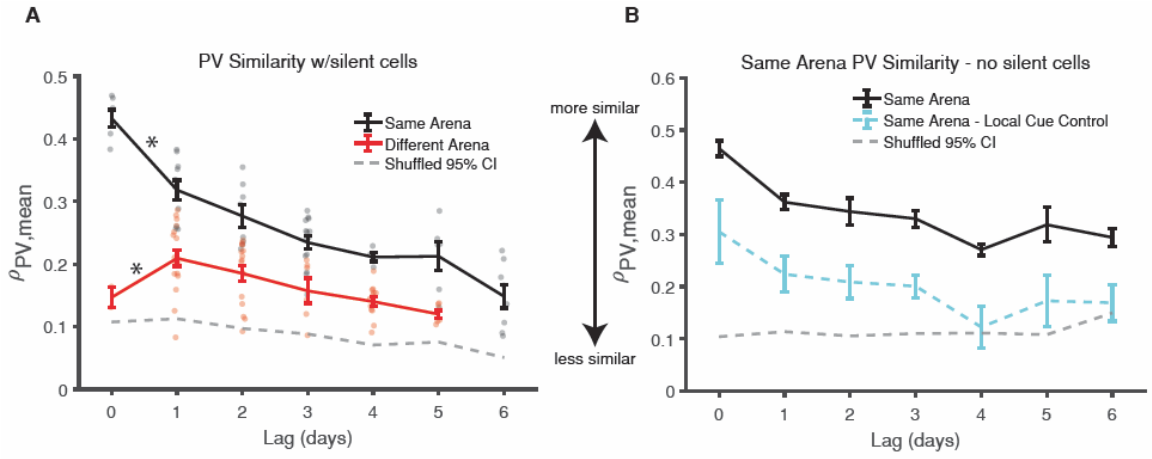

Figure 2.11: Population Similarity Versus Time (Related to Figure 2.6)

A) PV correlations at $\phi_{optimal,p}$ vs. time between sessions including silent cells. The distribution of PV correlations remains above chance at each time point, indicating the population remains coherent at short and long time lags even with neurons becoming silent/active between sessions. Black = same arena, red = different arena, gray dashed =

upper 95% CI from shuffled distribution. Colored dots indicate mean PV correlation of each session-pair across all mice. Error bars indicate s.e.m. *p < 0.001, Student's t-test of mean PV vs. chance at all time lags.

B) Assuming mice use local clues exclusively for orientation produces low PV correlations. Black = same arena PV correlations at $\phi_{optimal,p}$. Blue dashed = same arena PV correlations with place fields calculated after rotating mouse trajectories such that arena cues are aligned between sessions (assumes mice utilize local cues for place field alignment), gray dashed = upper 95% CI from shuffled distribution. Error bars indicate s.e.m.

2.4 METHODS

2.4.1 EXPERIMENTAL MODEL AND SUBJECT DETAILS

2.4.1.1 Animal Subjects

Subjects were 5 male C57/BL6 mice (Jackson Laboratories) weighing 25-30g, age 3-8 months. One mouse was excluded from the study after performing the experiment due to the inability to correct motion artifacts in his imaging videos. Mice were socially housed with 1-3 cage mates in a vivarium on a 12hr light/12hr dark cycle with lights on at 7am and given *ad libitum* access to food and water. Mice were singly housed after surgery. All procedures were performed in compliance with the guidelines of the Boston University Animal Care and Use Committee.

2.4.1.2 Viral Constructs

We obtained an AAV9.*Syn*.GCaMP6f.WPRE.SV40 virus from the University of Pennsylvania Vector Core at a titer of ~$4x10^{13}$GC/mL and diluted it to ~ 5-$6x10^{12}$GC/mL with 0.05M phosphate buffered saline prior to infusion into CA1.

2.4.2 METHOD DETAILS

2.4.2.1 Stereotactic Surgeries

Naïve mice, age 3-8 months, underwent two stereotaxic surgeries and one base plate implant for calcium imaging (Ziv et al., 2013). All surgeries were performed under 1% isoflurane mixed with oxygen, and were given 0.05mL/kg analgesic buprenorphine, 5.0mL/kg anti-inflammatory Rimadyl (Pfizer), and 400mL/kg antibiotic Cefazolin (Pfizer) subcutaneously immediately after induction. Mice received the same dosage of buprenorphine, Rimadyl, and Cefazolin twice daily for three days following surgery. In the first surgery, a small craniotomy was performed at AP -2.0, ML = +1.5 and 250nL of GCaMP6f virus was injected 1.5mm below the brain surface at 40nL/min. 10 minutes after the infusion was finished, the needle was slowly removed, the mouse's scalp was sutured, and then the mouse was removed from anesthesia and allowed to recover.

3-4 weeks after viral infusion, mice received a second surgery to attach a gradient index (GRIN) lens (GRINtech, 1mm x 4mm). After inducing anesthesia and providing pre-operative analgesia/antibiotics, a 2mm craniotomy centered at AP = 2.25, ML = 1.5 was performed and the cortex overlying region CA1 of the dorsal hippocampus was aspirated under constant irrigation with cold sterile saline. Aspiration stopped after removing the medial-lateral striations of the corpus callosum, which revealed anterior-posterior striations. Successive rounds of Gelfoam and cold saline were applied for 5-10 minutes to control bleeding, after which any saline left on the brain was suctioned out.

The GRIN lens was then stereotactically lowered to the brain surface and depressed an additional 50 µm to compensate for brain swelling during the surgery. Kwik-Sil (World Precision Instruments) was applied to seal any gaps between the skull edge and the GRIN lens, and the lens was cemented in place using Metabond (Parkell). After building up a well of Metabond, the GRIN lens was subsequently covered with Kwik-Cast (World Precision Instruments) for protection. For two of the mice used in this study, the GRIN lens was not implanted directly. Rather, a 2mm cannula with a glass cover plate was implanted, filled in Kwik-Sil for protection, and the GRIN lens was later cemented in the cannula with Metabond during the camera attachment. For these mice, the cannula was not depressed 50 µm but allowed to rest upon the surface of the brain while Kwik-Sil and Metabond were applied. Mice received the same post-operative care and injections as occurred after they first surgery.

After one week of convalescence, mice underwent a final procedure to attach a miniature epifluorescence microscope (Inscopix). No tissue was cut during this procedure – the mouse was put under anesthesia solely to make him immobile and facilitate camera attachment. After induction, a baseplate was attached to the microscope, which was set at the middle of its focal range, and the camera was lowered toward the GRIN lens until a clear picture of the brain was achieved (~50-100µm below any visible vasculature, and/or when any calcium events from putative neurons were observed). When the ideal distance between GRIN lens and microscope objective was achieved, the camera was then raised up 50µm to compensate for the subsequent downward pull of Metabond during curing. The bottom of the base plate was first attached to the well of dried Metabond below using

Flow-IT ALC Flowable Composite (Pentron), followed by a layer of opaque Metabond, which adhered to the sides of the base plate for reinforcement and blocked out ambient light.

After recovery from the second surgery, mice were food deprived and maintained at no less than 85% of their pre-surgery weight. 2-3 weeks prior to the experiment, they were allowed to forage randomly for chocolate sprinkles in a variety of arenas in order to identify the focal depth that maximized the number of in focus neurons. This also allowed us to habituate each mouse to the general experimental procedure, and to establish a baseline level of background fluorescence and calcium activity.

2.4.2.2 Experimental Outline

The experimental set-up consisted of a square and octagon of approximately equal area constructed from 3/8" plywood. All direction references (e.g. north, southwest, etc.) are given in reference to the standard configuration (see below). The square arena was 25cm x 25cm x 15cm. The octagon area had 8 – 11cm x 15cm sides (approximate diameter of 28cm). One wall of each arena was marked with a polarizing visual cue for orientation: vertical black stripes for the square and horizontal black stripes for the octagon. The arenas were oriented in the same manner (hereafter referred to as the standard configuration) for the first session of each day (with the exception of day 6). The standard configuration occurred when the arenas were rotated such that the visual cues were located on the south wall of the square and the northeast wall of the octagon. In this

configuration the east wall of the square and the west wall of the octagon each had a 5cm gap that was hidden by a removable wall. Each arena was wiped down thoroughly with 70% ethanol prior to each recording session to eliminate any olfactory cues. Room cues were minimized as follows: by placing opaque plastic sheeting around the arenas, by playing white noise, by carrying the mouse from his homecage to the recording arena in a random, circuitous manner for each session, and by having the experimenter move every 15-30 seconds.

Prior to the first recording session of each day, the imaging camera was attached and the focal depth was verified by eye. Two mice were lightly anesthetized (30-60s) in order to attach the microscope and given 30 minutes to recover prior to recording; the other two were gently handled and kept awake during camera attachment 5-10 minutes prior to recording. Mice began each session in their homecage, which was placed just outside the plastic sheeting. On days 1, 4, and 7 (SQUARE1, SQUARE2, and SQUARE3, respectively), mice underwent two 10 minute sessions in the square arena; on days 2, 3, and 8 (OCTAGON1, OCTAGON2, and OCTAGON3, respectively) the mice underwent two 10 minute sessions in the octagon arena. The 1st session on each of these days occurred in the standard configuration, after which the mouse was removed to his homecage while the arena was cleaned and then rotated pseudorandomly 90 degrees clockwise (CW) or counterclockwise (CCW) for the 2nd recording session. The arena was also randomly moved between one of three different, adjacent positions between sessions

SQUARE1-3 and OCTAGON1-3. For one mouse, the arena was not rotated between sessions on day 1 and day 8.

On day 5 (CONN1) the mice received one continuous 20 minute (minimum) recording session. The session began the same as day 4 with the mouse placed in the square arena in the standard configuration. After 5 minutes of exploration, the hidden east wall was lifted to reveal a hallway connected to the west wall of the octagon for the first time. After the mouse entered the octagon arena, the west wall was lowered to contain the mouse in the octagon, and he was allowed to explore this arena for 5 minutes. The same procedure was repeated twice more until the mouse had explored each arena twice. Day 6 (CONN2) was similar to day 5 except the mouse started in the octagon arena, which was rotated 180 degrees from the standard configuration, and ended in the square. See Figure 2.1A for a pictorial outline summarizing the above procedure.

2.4.2.3 Image Acquisition and Processing

All brain imaging data was acquired using nVista HD (Inscopix) v2 and v3. All movies were obtained at 1440 x 1280 pixels and 20 frames/second. Raw imaging data was first pre-processed in Mosaic software (Inscopix) by spatially downsampling by a factor of 2 (final pixel size = 1.18 microns/pixel) performing motion correction, and cropping to eliminate any dead pixels due to motion correction or areas with no clear calcium activity. A minimum projection (Figure 2.71b) of the final motion corrected, cropped movie was produced for later neuron registration across sessions/days (in the

instances where 2 sessions were recorded on the same day they were both motion corrected to the same reference frame to ensure trivial session registration, see Neuron Registration section below). Isolated dropped frames (maximum 2 consecutive frames) were replaced with the previous good frame. In the rare case where extended chunks of dropped frames occurred, these frames were excluded from all analyses.

2.4.2.4 Behavioral Tracking

Position tracking of mice was performed using Cineplex v2/v3 (Plexon) software at 30 frames/sec. Brain imaging data and behavioral data were synchronized by sending a TTL pulse sent from the Cineplex computer, which signaled the beginning of behavioral tracking to the nVistaHD data-acquisition and triggered image acquisition. Each behavioral video was visually inspected, and any errors in tracking were corrected using custom-written software in MATLAB (available at https://github.com/SharpWave/PlacefieldAnalysis) which also interpolated behavioral imaging data to match imaging data.

2.4.2.5 Histology

Mice were perfused transcardially with 10% phosphate buffered saline (KPBS) followed by formalin. Brains were then extracted and post-fixed in formalin for 2-4 additional days, and were transferred to a 30% sucrose solution in KPBS for 1-2 additional days. Brains were then frozen and sliced in 40 μm sections on a cryostat (Leica CM 3050S), mounted, and cover slipped with Vectashield Hardset mounting medium

with DAPI (Vector Laboratories). Slides were then imaged using a Nikon Eclipse Ni-E epifluorescence microscope at 4x, 10x, and 20x to verify viral expression levels, location, and GRIN lens placement above the CA1 cell layer.

2.4.3 QUANTIFICATION AND STATISTICAL ANALYSIS

All significance values are reported in figure legends or directly in the text. Unless otherwise noted, all statistics are done using either a Kruskal-Wallis ANOVA, a Wilcoxon rank-sum test, or using a bootstrap shuffling procedure (details provided in the appropriate section below). If the Kruskal-Wallis ANOVA is significant ($p < 0.05$), post-hoc Tukey test p-values are reported in the text/figure legend. All data points shown in figures are for all mice/session-pairs unless otherwise noted. We utilized custom MATLAB by Berens (Berens, 2009) to perform all circular statistics.

2.4.3.1 Neuron and Calcium Event Identification

Neuron regions-of-interest (ROIs) and calcium events were identified using a custom written, open source algorithm employed in MATLAB 2016b called A Technique for Extracting Neuronal Activity from Single Photon Neuronal Image Sequences (Tenaspsis) (Mau et al., 2018). Tenaspis is open-source and available at: https://github.com/SharpWave/TENASPIS. First, Tenaspis filters each calcium imaging movie with a band-pass filter per (Kitamura et al., 2015) to accentuate the separation between overlapping calcium events. Specifically, Tenaspis smooths the movie with a 4.5 μm disk filter and divides it by another movie smoothed with a 23.6 μm disk filter.

Second, it adaptively thresholds each imaging frame to identify separable pockets of calcium activity, designated as blobs, on each frame. Blobs of activity are accepted at this stage of processing only if they approximate the size and shape of a mouse hippocampal neuron, as measured by their radius (min = ~6μm, max = ~11μm), the ratio of long to short axes (max = 2), and solidity (min = 0.95), a metric used by the regionprops function of MATLAB we employ to exclude jagged/strange shaped blobs. Third, Tenaspis strings together blobs on successive frames to identify potential calcium transients and their spatial activity patterns. Fourth, Tenaspis searches for any transients that could result from staggered activity of two neighboring neurons. It rejects any transients whose centroid travels more than 2.5μm between frames and whose duration is less than 0.20 seconds. Fifth, Tenaspis identifies the probable spatial origin of each transient by constructing putative regions-of-interest (ROIs), defined as all connected pixels that are active on at least 50% of the frames in the transient. Sixth, Tenaspis creates initial neuron ROIs by merging putative transient ROIs that are discontinuous in time but occur in the same location. Specifically, it first attempts to merge all ROIs whose centroids are less than a distance threshold of ~0.6μm from each other. In order to merge two transient ROIs, the two-dimensional Spearman correlation between the ROIs must yield $r^2 > 0.2$ and $p < 0.01$. Tenaspis then successively increases the distance threshold and again attempts to merge ROIs until no more valid merges occur (at a distance threshold of ~3μm, typically). Seventh, Tenaspis integrates the fluorescence value of each neuron ROI identified in the previous step across all frames to get that neuron's calcium trace, and then identifies putative spiking epochs for each neuron. Specifically, it first identifies the

rising epochs of any transients identified in earlier steps. Then, it attempts to identify any missed transients as regions of the calcium trace that have a) a minimum peak amplitude > 1/3 of the transients identified in step 3, b) a high correlations ($p < 0.00001$) between active pixels and the pixels of the average neuron ROI identified in step 6, and b) a positive slope lasting at least 0.2 seconds. Last, Tenaspis searches for any neuron ROIs that overlap more than 50% and whose calcium traces are similar and merges their traces and ROIs.

2.4.3.2 Place cells

Calcium transients were spatially binned (4cm x 4cm) and normalized by occupancy. Spatial mutual information (SI) was computed from the following equations, adapted from (Olypher, Lánský, Muller, & Fenton, 2003):

$$I_{pos}(x_i) = \sum_{k=0}^{1} P_{k|x_i} \log\left(\frac{P_{k|x_i}}{P_k}\right)$$

$$SI = \sum_{i=1} P_{x_i} I_{pos}(x_i)$$

where:

- P_{xi} is the probability the mouse is in pixel x_i
- P_k is the probability of observing k calcium events (0 or 1)
- $P_{k|xi}$ is the conditional probability of observing k calcium events in pixel x_i.

The SI was then calculated 1000 times using shuffled calcium event timestamps, and a neuron was classified as a place cell if it 1) had at least 5 calcium transients during the session, and 2) the neuron's SI exceeded 95% of the shuffled SIs. We obtained similar results using smoothed occupancy rate maps, which were constructed using 1cm x 1cm bins and applying a Gaussian filter (σ = 2.5cm). We defined the extent of a place field as all connected occupancy bins whose smoothed event rate exceeded 50% of the peak event rate occupancy bin.

2.4.3.3 Neuron Registration

Neuron registration occurred in two steps: session registration and neuron registration.

2.4.3.3.1 Session registration

Prior to mapping neurons between sessions, we determined how much the imaging window shifted between sessions. In order to isolate consistent features of the imaging plane for each mouse (such as vasculature or coagulated blood), we created a minimum projection of all of the frames of the motion-corrected and cropped brain imaging movie for each recording session. One session ("registered session") was then registered to a base session using the "imregtform" function from the MATLAB Image Processing Toolbox, assuming a rigid geometric transform between images, and the calculated transformation object was saved for future use.

2.4.3.3.2 Neuron Registration

Next, each ROI in the registered session was transformed to its corresponding location in the base session. Each neuron in the base session was then mapped to the neuron with the closest center-of-mass in the registered session, unless the closest neuron exceeded our maximum distance threshold of 3 pixels (3.3 μm). In this case the base session neuron was designated to map to no other neurons in the registered session. If, due to high density of neurons in a given area, we found that multiple neurons from the base session mapped to the same neuron in the registered session, we then calculated the spatial correlation (Spearman) between each pair of ROIs and designated the base session ROI with the highest correlation as mapping to the registered session ROI.

For multiple session registrations, the same procedure as above was performed for each session in two different ways. First, we registered each session directly to the first session in the experiment and updated ROI locations/added new ROIs to the set of existing ROIs with each registration. This helped account for slight day-to-day drift in neurons ROIs due to shifts in vasculature, build-up of fluid underneath the viewing window, creep/shrinkage of dental cement, etc. Second, to ensure that neuron ROIs did not drift excessively across sessions we also performed all the above steps but did NOT update ROI locations allowing us to register each set of ROIs to those furthest away chronologically. The resulting mappings were then compared across all sessions, and any neuron mappings that differed between the two methods (e.g. ROIs that moved excessively across the duration of the experiment) were excluded from analysis. Those that remained in the same location, and were included.

2.4.3.4 Place Field Rotation Analysis

We employed two methods to identify how much the spatial calcium activity of each neuron rotated between sessions.

2.4.3.4.1 Center-out Method

First, occupancy normalized calcium event maps were generated for each session by summing up calcium activity (defined as any frames with a rising calcium trace) for each neuron when the mouse was moving faster than 1cm/s in 1 cm bins and smoothing with a Gaussian kernel (σ = 2.5 cm). Next, we identified the location of each neuron's place field(s) (see Place Fields section above), and calculated the angle from the center of the arena to the place field. We designate this angle as α. For neurons with multiple place fields, we defined this angle as the circular mean of the angles for all its place fields. We then calculated the place field rotation, θ, as the difference between α values in each session. We also calculated a metric of how well the population rotated together between sessions as follows:

$$rotational\ accuracy = \sum_{i=1}^{n} |\theta_i - \theta_{mean}|/n$$

Where θ_i is the rotation of the *ith* neuron and θ_{mean} is the circular mean rotation of all neurons.

2.4.3.4.2 Correlation Method

First, occupancy normalized calcium event maps were generated for each session by summing up calcium activity (defined as any frames with a rising calcium trace) for each neuron when the mouse was moving faster than 1cm/s in 1 cm bins and smoothing with a Gaussian kernel (σ = 2.5 cm). Second, the Spearman correlation between smoothed calcium event maps was then calculated for each neuron active in both sessions (the Spearman correlation is undefined for neurons that have no calcium events when the mouse is running above the speed threshold). Third, the mouse's trajectory in the second session was rotated by the angle ϕ (in 90 degree increments for the square and 15 degree increments for the octagon, following the right hand rule) and the above process was repeated for each rotation between 0 and 360 degrees. Finally, the optimal rotation (ϕ_{opt}) of each neuron was taken as the rotation of the mouse's trajectory in the second session that produced the maximum correlation. Chance-level ϕ_{opt} values were obtained by randomly shuffling each neuron's identity in the second session and performing the above procedure 1000 times. For octagon-to-square comparisons, we first transformed rotated octagon arena trajectories to square trajectories using the method utilized by Lever, et al. (Lever et al., 2002). We obtained similar results utilizing calcium event maps created using 4cm occupancy bins and without smoothing. To quantify the use of coherent maps between the two arenas, we first transformed the mouse's trajectory in the octagon arena to square coordinates (Lever et al., 2002). This method is not as sensitive as the center-out method, since the resolution of ϕ_{opt} values is equal to the increments in

which the data is rotated. However, unlike the center-out method, it does not require making any assumptions about the location of each place field.

2.4.3.5 Coherent and Remapping Designations

2.4.3.5.1 Center-out Method

A significantly large number of place-fields had to rotate together in order for a session-pair to be designated as sharing a coherent map. To quantify this, we first identified the circular mean of all place field rotations, designated as θ_{mean}. We then calculated n_{close}, the number of neurons that were < 30 degrees from $\theta_{mean.}$ We then compared this number to chance n_{chance}, calculated in the same manner but after randomly shuffling neuron identity between sessions. We the repeated this step 1000 times, and calculated a p-value for each session-pair, defined as 1 – (# times $n_{close} > n_{chance}$)/1000. In order to be designated as coherent, a session-pair had to have a p-value < 0.05 after Bonferroni correction ($p < 0.05/m$, where m = the number of session-pairs, i.e. 28 for square-to-square and octagon-to-octagon comparisons, and 64 for octagon-to-square comparisons).

2.4.3.5.2 Correlation Method

In order for session-pairs to be identified as coherent, they had to satisfy a stringent, two-pronged criteria. First, the distribution of ϕ_{opt} had to significantly differ from a uniform distribution ($p < 0.05$ for χ^2 test). Second, a permutation test was performed in order to rule out the possibility that the population breaks into multiple

coherent subpopulations (Lee, Yoganarasimha, Rao, & Knierim, 2004). The permutation test was performed as follows. Tuning curves for the population were constructed by calculating the mean correlation between the calcium event maps of all neurons at each rotation. Then, the p-value was calculated as the number of times that the peak value of the shuffled tuning curve exceeded that of the actual data, divided by the number of shuffles. In order to be designated as coherent, a session-pair had to have a p-value < 0.05 after Bonferroni correction ($p < 0.05/m$, where m = the number of session-pairs, i.e. 28 for square-to-square and octagon-to-octagon comparisons, and 64 for octagon-to-square comparisons). Sessions that did not meet both criteria were designated as global remapping sessions. The χ^2 test p-value is reported throughout the text unless it is smaller than the permutation test p-value, in which case both are reported. Note that neurons with poor spatial firing properties will have lower correlations than neurons with punctate firing fields and/or high spatial information. Thus, to bias our results against the hypothesis of predominantly coherent spatial representations, we included all neurons in the coherency analysis, regardless of their spatial information content.

2.4.3.6 Coherent Rotation Designations

Coherent session-pairs within arenas (square-to-square and octagon-to-octagon) were further sub-divided into groups based on which cues the mice appeared to use to orient their place field maps. Coherent session-pairs where the arena was rotated between sessions were designated as "Coherent: Arena" if $|\theta_{mean} - \theta_{arena}| < 30$ degrees, "Coherent: Room" if $\theta_{mean} < 30$, and "Coherent: Mismatch" otherwise. When there was no arena

rotation between sessions (and thus there was no mismatch between arena and room cues), coherent session-pairs were designated as "Coherent: Arena/Room" if $\theta_{mean} < 30$ and "Coherent: Mismatch" otherwise. θ_{mean} was calculated using the center-out method above.

2.4.3.7 Entry Angle vs. Rotation Analysis

We manually identified the wall over which the mouse was carried into the arena ($\theta_{entrywall}$), the angle he was facing when he crossed over this wall ($\theta_{entrydir}$), and the angle he was facing when he first touched down ($\theta_{touchdown}$) in the arena. Since place fields tended to rotate in 90 degree increments in the wall, we likewise defined the angle of entry for the mouse in 90 degree increments (e.g. the east wall = 0 degrees, the north wall = 90 degrees, etc.). We then calculated the change in entry angle between sessions, subtracted it from the change in θ_{mean} between sessions, and designated it as $\Delta\theta_{mean,entrywall}$. We reasoned that, if the mouse utilized the wall over which he entered to anchor this place field map, then his entry angle rotation should match the place field rotation between sessions for all mismatch sessions and $\Delta\theta_{mean,entrywall}$ should equal zero. We thus counted up all the $\Delta\theta_{mean,entrywall}$ values <= 15 degrees from zero and compared this number (n_{data}) to chance ($n_{shuffle}$), determined by randomly shuffling entry angle between session and repeating the procedure above 1000 times. We then calculated a p-value as 1 – the number of times n_{data} exceeded $n_{shuffle}$. We then repeated the above procedure for the angle the mouse was facing when he first entered the maze ($\Delta\theta_{mean,entrydir}$) and the angle he was facing upon touchdown in the arena ($\Delta\theta_{mean,touchdown}$).

2.4.3.8 Population Vector Calculations

Population vectors (PV) were constructed for each occupancy bin (4cm x 4cm) by taking the calcium event rate of each neuron from the corresponding occupancy bin in its unsmoothed calcium event map. In order to account for coherent rotations of the map between sessions, the mouse's trajectory in the second session was rotated by $\sim\theta_{mean}$ (in 90 degree increments for the square and 15 degree increments for the octagon) before calculating the PV in the second session. Spearman correlations were calculated for each occupancy bin, and the mean correlation across all bins was taken as the mean spatial PV correlation between sessions. Chance level for all PV analyses was calculated by shuffling occupancy bins and calculating mean correlations 1000 times for each session-pair. We also performed rate-only PV similarity analyses by forming PVs without rotating the data between sessions and using each neuron's maximum event rate to form the PV.

For the connected day analyses and time lag analysis without silent cells, PVs included neurons only if they met the following criteria instituted to exclude any low event rate neurons whose changes could artificially skew our results: 1) the neuron was active (at least one calcium transient) each day, and 2) the neuron produced more than 5 calcium transients and had a p-value for spatial MI (see Placefields section) of < 0.05 on at least one day. For connected day analyses, inactive cells were those that produced no calcium transients when the mouse was above the 1cm/s speed threshold, but had at least one calcium transient during the session. For all comparisons within CONN1 and

CONN2, we considered each continuous 5 minute visit to an arena as its own session. However, we combined visits in each arena into one 10 minute session in each arena for any PV analyses between CONN1/CONN2 and un-connected days in order to ensure similar length sessions for between day comparisons.

For the time lag analysis, included neurons had to pass the transient number and p-value thresholds on at least one session but only needed to be active in one session. A silent neuron (one that became active in the second session or were active in only the first session) was only considered if its ROI in one session did not overlap with any other neighboring ROIs in the other session. This precluded neurons that might be misidentified as silent due to overly conservative neuron registration between sessions (e.g. those that overlapped with another ROI but were just outside the stringent distance threshold required to be mapped as the same cell). Additionally, we have not shown comparisons exactly 7 days apart due to a scarcity of data at that time lag between sessions; the removal of this data does not alter the results shown in Figure 2.6e.

2.4.3.9 Single Neuron Classifications

On/off cells were identified by first registering all neurons, without filtering for spatial selectivity or transient number, across sessions and then identifying any neurons that were active in the second/first session but not the first/second session. Additionally, since neuron registration does not take into account the speed threshold of 1cm/sec we applied for all analyses above, we identified additional on/off neurons as those that were

detected by Tenaspis in each session, but did not produce any calcium events while the mouse was above the speed threshold in the first/second session. Additionally, we calculated a Discrimination Index for each neuron to determine its preference for being active in one arena versus the other, defined as $(ER_{square} - ER_{octagon})/ (ER_{square} + ER_{octagon})$, where ER = calcium event rate. We defined neurons as "selective" if $|DI| > 0.66$, which indicated that they had 66% or more calcium events in the square than the octagon, or vice versa. Neurons with $|\theta - \theta_{mean}| < 30$ were designated as staying coherent. Neurons were designated as random remapping otherwise. We obtained similar results to Figure 2.2i for cutoffs of 15 degrees, 30 degrees, and 45 degrees.

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3 CHAPTER THREE

Hippocampal Neuron Phenotype Influences the Stability of its Neural Code

3.1 INTRODUCTION

Place cells in the hippocampus encode the current position of rodents (Muller & Kubie, 1987; Muller et al., 1987; O'Keefe, 1976; O'Keefe & Dostrovsky, 1971), bats (Geva-Sagiv, Romani, Las, & Ulanovsky, 2016), and even humans (Ekstrom et al., 2003; Miller et al., 2013; Niediek & Bain, 2014), supporting the known role of the hippocampus in spatial memory (Morris, Garrud, Rawlins, & O'Keefe, 1982; Vorhees & Williams, 2014). However, the hippocampus is also widely known for its role in supporting the encoding, retrieval, and consolidation of non-spatial long-term memories (Corkin, 1984; Eichenbaum, 2004; Milner et al., 1968), suggesting that it must encode variables beyond an animal's current location. Indeed, a plethora of recent studies have demonstrated that the hippocampus encodes the dimensions of a given task, from odors (Muzzio et al., 2009; Wood et al., 1999) to time (Kraus et al., 2013; MacDonald et al., 2011; Manns et al., 2007; Pastalkova et al., 2008; Robinson et al., 2017; Salz et al., 2016) to tones (Aronov et al., 2017). One early demonstration that the hippocampus encodes dimensions beyond an animal's current location was the discovery of trajectory-dependent neurons or splitter cells (Frank, Brown, & Wilson, 2000; Wood et al., 2000). These cells modulated their activity levels while a rat was in the exact same spatial location based on its past or future trajectory in a spatial alternation task. These cells provided a glimpse at how the hippocampal spatial code could also reflect the animals future planning or past experience.

Several studies have demonstrated that place fields move, or remap, their locations toward goal locations in a spatial learning task (Dupret et al., 2010; McKenzie, Robinson, Herrera, Churchill, & Eichenbaum, 2013). These studies highlight that the flexibility of place fields to adjust their firing locations is important to learning new information. Conversely, the ability of hippocampal neurons to maintain the same firing location in the absence of learning might support long-term memory retrieval. Indeed, a recent study illustrated that neurons with place fields located near a hidden goal maintained more stable firing fields than place cells with fields in other locations, suggesting that the utility of a neuron's information to task performance influences its long-term stability (Zaremba et al., 2017).

Thus, since context-dependent splitter cells provide immediately relevant information for performing a spatial alternation task, we hypothesized that these neurons might be important for proper task performance. Furthermore, we hypothesized that downstream structures might provide feedback to splitter cells that would result in different long-term dynamics for these cells when compared to place cells. Specifically, we addressed three lines of inquiry. First, does better-trajectory dependent information conveyed to downstream structures correlate with better performance? Second, given the steady evolution of hippocampal cells across days (Cai et al., 2016; Mau et al., 2018; Rubin et al., 2015; Ziv et al., 2013), do splitter cells turn over at a slower rate than other cells, thus providing a longer-lasting influence on downstream activity? Last, since trajectory-dependent activity is established in some neurons, are these neurons less prone to remapping than other neurons?

Many of these questions require the ability to accurately identify and track the same neurons across long-timescales, a task that was extremely difficult at the time of the discovery of splitter cells (Frank et al., 2000; Wood et al., 2000). To address this question, we revisited one of the original paradigms used to discover these neurons and paired it with *in vivo* miniscope recordings of GCaMP6f activity in dorsal CA1 of freely-moving mice. This technology allowed us to not only track the long-term activity of neurons, but also to adequately characterize the heterogeneity of trajectory-dependent activity in the hippocampus, since we can simultaneously record from a large number of neurons in each session. Using this task/recording method, we first found that trajectory-dependent coding correlates with task performance, indicating its importance for supporting memory-guided behavior. Second, we found that trajectory-dependent neurons display more consistent information about an animal's location. Third, we established that neuron phenotype is important for predicting the long-term activity levels of individual neurons: splitter cells were more likely to be persistently active in the days following their onset than were return arm place cells, indicating that phenotypes providing more adaptive information might provide longer lasting input to downstream structures. Fourth, we found that the population as a whole displayed a rapid onset of trajectory-dependent activity followed by stable coding of trajectory thereafter. Last, we discovered that recruitment of context-dependent splitter cells peaked several days into training, whereas place cell recruitment peaked on the first day. These results combined suggest that neuron phenotype influences the short and long-term impact that a neuron provides to downstream structures, and paves the way for future studies investigating

how heterogeneity in the neural code might support acquisition and retention of more complex behavioral tasks.

3.2 RESULTS

3.2.1 Behavior and Imaging

Food deprived mice (n=4) were trained to perform a continuous spatial alternation task on a figure-8 maze while we simultaneously recorded calcium activity using a miniaturized microscope in GCaMP6f expressing neurons in region CA1 of the dorsal hippocampus (Figure 3.1A). In order to habituate mice to the maze, all mice received one 30 minute session where they freely explored the maze prior to performing the task. Three of the four mice were also forced to loop on each side of the maze in 5-10 minute blocks in order to establish the location of the wells where food reward was delivered. Mice learned the task quickly (Figure 3.1B) and performed the task at > 70% on average (Figure 3.1C) throughout the course of the experiment (27, 16, 29, and 36 days for the four mice involved).

In conjunction with the alternation task, we performed *in vivo* imaging using a miniaturized epifluorescence microscope to record calcium activity from large numbers of GCaMP6f expressing neurons in dorsal region CA1 the hippocampus. We utilized custom-written software (Kinsky et al., 2018; Mau et al., 2018) to extract neuron ROIs (Figure 3.1D), construct their corresponding calcium traces, and identify each ROI's putative spiking activity (Figure 3.1E), even in cases where the putative neuron ROI overlapped significantly with another ROI (Figure 3.1F). Using this technique, we

recorded from large numbers of neurons (243-1205 neurons per ~30 minute session) and successfully tracked them across days by comparing the distance between neuron ROI centroids (Figure 3.1G) and verifying that ROIs did not change orientation between sessions (Figure 3.1H).

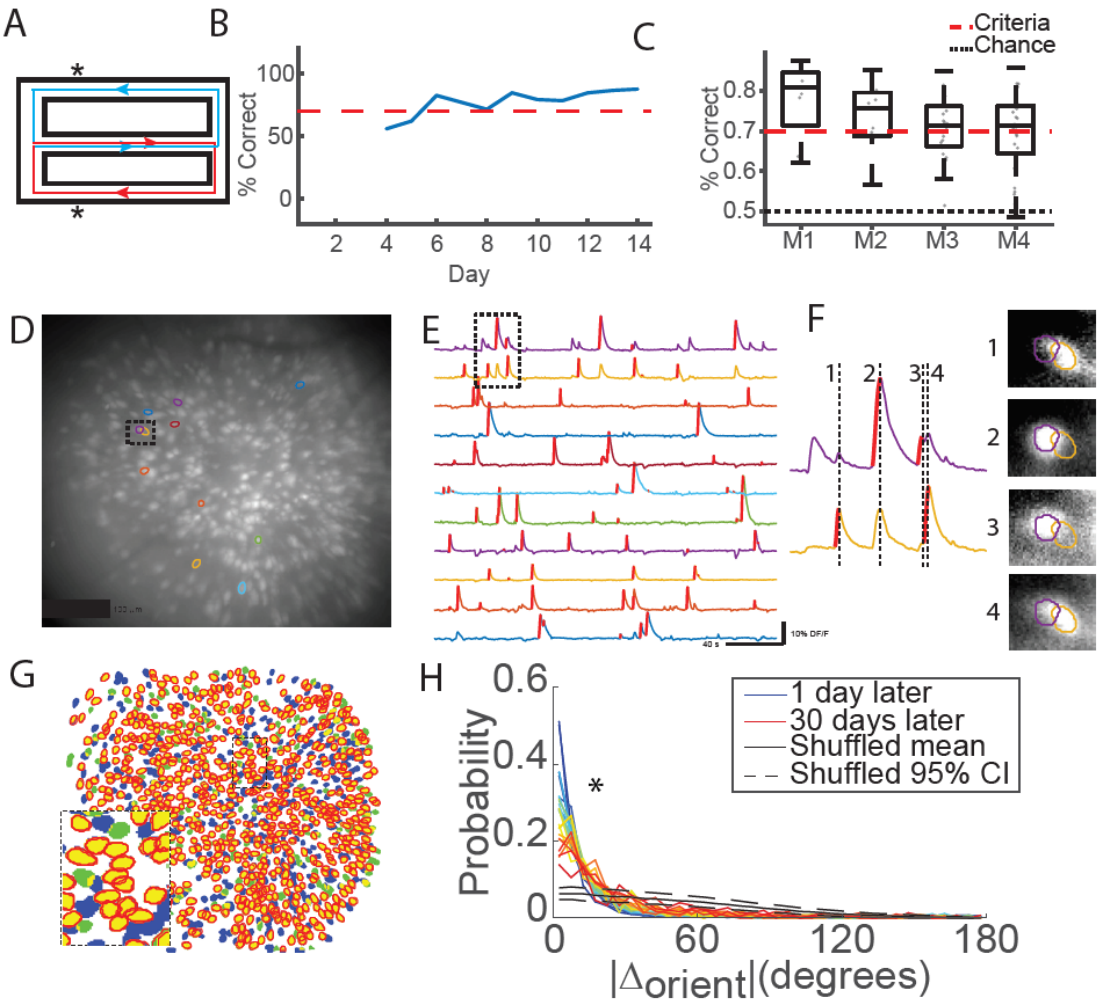

Figure 3.1: Experimental Setup and Imaging

A) Alternation Maze. Blue = Left turn trajectories, Red = Right turn trajectories, *= location of food reward.
B) Example learning curve for one mouse.

C) Performance summary for all four mice, all sessions included. Red dashed = criteria (70%), black dashed = chance.
D) Example maximum projection from one imaging session with 10 putative neuron ROIs overlaid.
E) Example calcium traces for ROIs depicted in D. Red lines on the ascending phase of each calcium event indicate putative spiking activity.
F) *Left:* Expansion of traces for overlapping ROIs from dashed boxes in D and E highlights the ability of cell detection algorithm to disambiguate overlapping traces. *Right*: Localization of pixel intensities at each time point indicated to the left.
G) Example neuron registration between two sessions. Blue/Green = pixels corresponding to putative ROIs extracted in the $1^{st}/2^{nd}$ session only. Yellow = pixels corresponding to portions of ROIs active in both sessions. Red = outline of ROIs matched as the same neuron between sessions.
H) The small size of changes in ROI orientation between sessions indicate proper neuron registration between sessions.

3.2.2 Trajectory-Dependent Activity is Maintained Across Days

The initial studies establishing the existence of trajectory-dependent splitter cells in the hippocampus were performed using electrophysiology in rats (Frank et al., 2000; Wood et al., 2000). Thus, we first wondered if we could detect trajectory-dependent activity in a different species while using a technique with much lower temporal resolution. To do so, we constructed tuning curves representing the probability a given neurons had calcium activity at each spatial bin (1cm) along the stem in correct trials only, and classified neurons as trajectory-dependent if at least 3 bins displayed a significant difference between their tuning curves (permutation test). We found that we were capable of not only identifying trajectory-dependent cells on a given day (60 ± 23, mean ± s.e.m. across all four mice), but that in many cases these neurons maintained the same phenotype across multiple days (Figure 3.2A-B). Trajectory-dependent neurons occurred as an average of 10%/5% of neurons active on the stem/all neurons across all sessions (12%/6%, 5%/3%, 12%/6%, and 9%/4% for individual mice). Of course,

trajectory-dependent activity could result from factors other than the mouse's current and past position, such as its lateral position along the stem. We addressed this by limiting the portion of the maze we considered the stem to exclude any areas where the mouse exhibited stereotypical turning behavior by eye (Figure 3.2A-B, bottom). Together, these results indicate that trajectory-dependent coding exists in mice and in many cases maintains the same activity profile across both short and long timescales.

We also noticed that positional location of place cell firing along the stem progressed backward during the task, such that calcium activity occurred at earlier and earlier portions of the stem with time (Figure 3.2). This is consistent with a study reporting the backwards-migration of spatial firing with experience (Mehta et al., 2000). We did not find any evidence of consistent place field migration between sessions (Figure 3.2E). To the best of our knowledge, this is the first demonstration of hippocampal trajectory-dependent activity in mice and using calcium imaging.

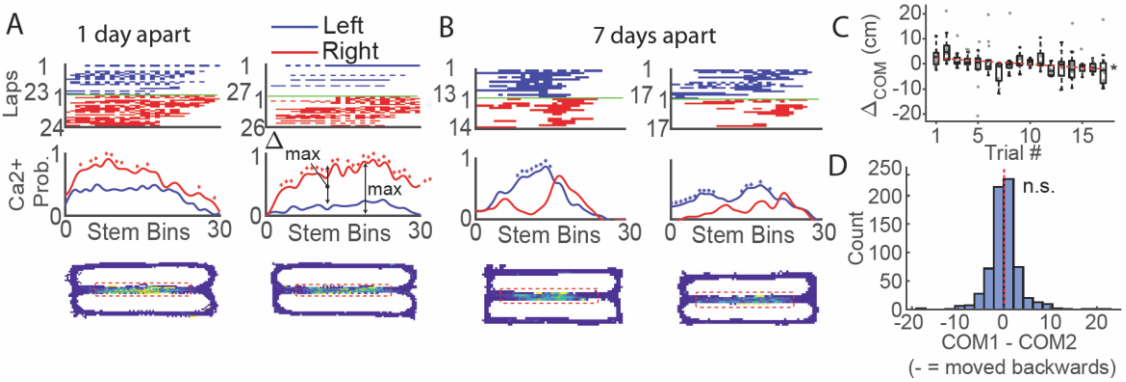

Figure 3.2: Trajectory-Dependent Activity Persists Across Days

A) *Top:* Calcium event rasters along the stem for correct trials for two sessions recorded one day apart, sorted by turn direction at the end of the stem. Blue = left, Red = right. *Middle:* Calcium event probability curves for each turn direction.*p<0.05, shuffle-test. *Bottom:*

occupancy normalized calcium event map. Red dashed = extent of stem considered in above plots.

B) Same as A, but for a different mouse and for sessions 7 days apart.

C) The centroid of spatial firing on the stem relative to its mean location across the entire session drifts backwards throughout the session. Circles = centroid shifts for each neuron active on the stem. Example session from one mouse for right turns only. *r = -0.28, p=5.1e-5, t = -4.1 for null hypothesis that slope = 0.

D) Average change in centroid location between adjacent sessions for all mice between two sessions indicates that place field location does not drift between sessions. p = 0.67 t-test.

3.2.3 The Magnitude of Trajectory-Dependent Activity Correlates with Performance

Trajectory-dependent neurons provide information vital to task performance that might be utilized by downstream structures to inform proper motor actions (Albouy et al., 2008; Kahn et al., 2017; Wise & Murray, 1999). Thus, we wondered if the presence of task performance might be associated with trajectory-dependent information in the neural code of all neurons active on the stem. We measured the magnitude of trajectory-dependent activity for each neuron as the maximum difference between its tuning curves Δ_{max}, normalized by the maximum of the curves overall ($\Delta_{max,norm}$). We found a significant positive correlation between Δ_{max}/max and performance across all sessions from all mice (Figure 3.3A). We also obtained a significant correlation when we considered the average Δ_{max}/max and performance values for each mouse (Figure 3.3B), suggesting that this effect was not driven by different numbers of sessions performed by each mouse. To bolster this argument, we trained a decoder to classify future turn direction using a linear discriminant analysis (LDA) at each spatial bin along the stem based on the neural activity of the population. We found that the accuracy of the LDA decoder positively correlated with the animal's performance on a given day, indicating that better separation between upcoming left and right trajectories by the neural code was

related to increased memory (Figure 3.3C-D). We also obtained results approaching significance for individual mice but not across all mice/sessions when we calculated the correlation between left and right tuning curves as another metric for trajectory-dependent information (Figure 3.3E-F, p = 0.054 for individual mice). This metric is very conservative, however, since it produces low values (indicating high-trajectory dependent information) for splitters that shift their location along the stem between trial type (Figure 3.2B) but not for splitters that modulate event rates in the same location (Figure 3.2A). These results indicate that trajectory-dependent activity broadcast to information to downstream structure might prove valuable to proper task performance.

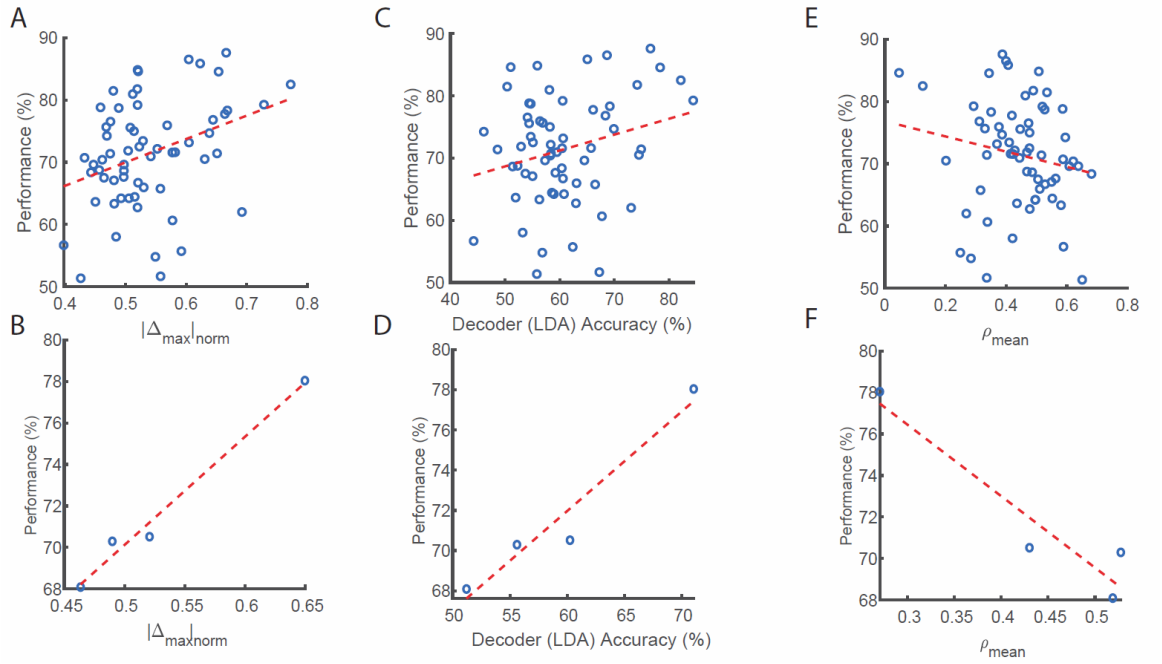

Figure 3.3: The Quality of Trajectory-Dependent Activity Predicts Performance

A) Performance for each session versus the average Δmax_{norm} value for all cells from that session. Circles = all sessions, all mice.*ρ=0.35, p=0.0058 Spearman (Pearson?) correlation.
B) Same as A, but for each mouse. **ρ=0.98, p=0.0088.

C) Performance for each session versus the average linear-discriminant decoder accuracy across all stem bins for correct trials. Circles = all sessions, all mice.*ρ=0.25, p=0.047.
D) Same as C but for each mouse. *ρ=0.94, p=0.03.
E) Performance for each session versus the mean correlation between left and right calcium event probability curves for each neuron. ρ=-0.18 p=0.17.
F) Same as E but for each mouse. +ρ=0.90, p=0.054.

3.2.4 Trajectory-Dependent Neurons are More Persistently Active over Long Timescale than Place Cells

Multiple studies (Cai et al., 2016; Kinsky et al., 2018; Mau et al., 2018; Rubin et al., 2015; Ziv et al., 2013) have shown that hippocampal neurons exhibit significant turnover across days (Figure 3.1G) with fewer and fewer staying active as time progress. We thus wondered if neuron phenotype might influence how quickly (or not) a neuron became inactive. We predicted that there might exist a drive to maintain activity in neurons that provided information immediately relevant to performing the correct turn (splitter cells) over return arm place cells (PCs) active elsewhere on the maze (Figure 3.4A). We found that, for individual mice, splitter cells were more likely to be consistently present/active at both short (Figure 3.4B) and long (Figure 3.4C) timescales than place cells without activity on the stem (PCs). This effect persisted up to 15 days later when considering all mice together (Figure 3.4D-F). One explanation for this result could relate to the higher event rate of splitter neurons (Figure 3.8A) since bursts of action potentials are more effective at transmitting information to downstream structures (Lisman, 1997), enabling these structures to provide feedback (via an unknown mechanism) regarding the utility of information they received. However, we obtained

similar results when we performed the same analysis after down-sampling the population of splitter cells such that their mean event rate matched that of PCs (Figure 3.8), suggesting that increased stability we observed in splitter cells was related to their information content. This result suggests that neuron phenotypes providing valuable information exert a longer-lasting influence on downstream structures than do other neuron phenotypes.

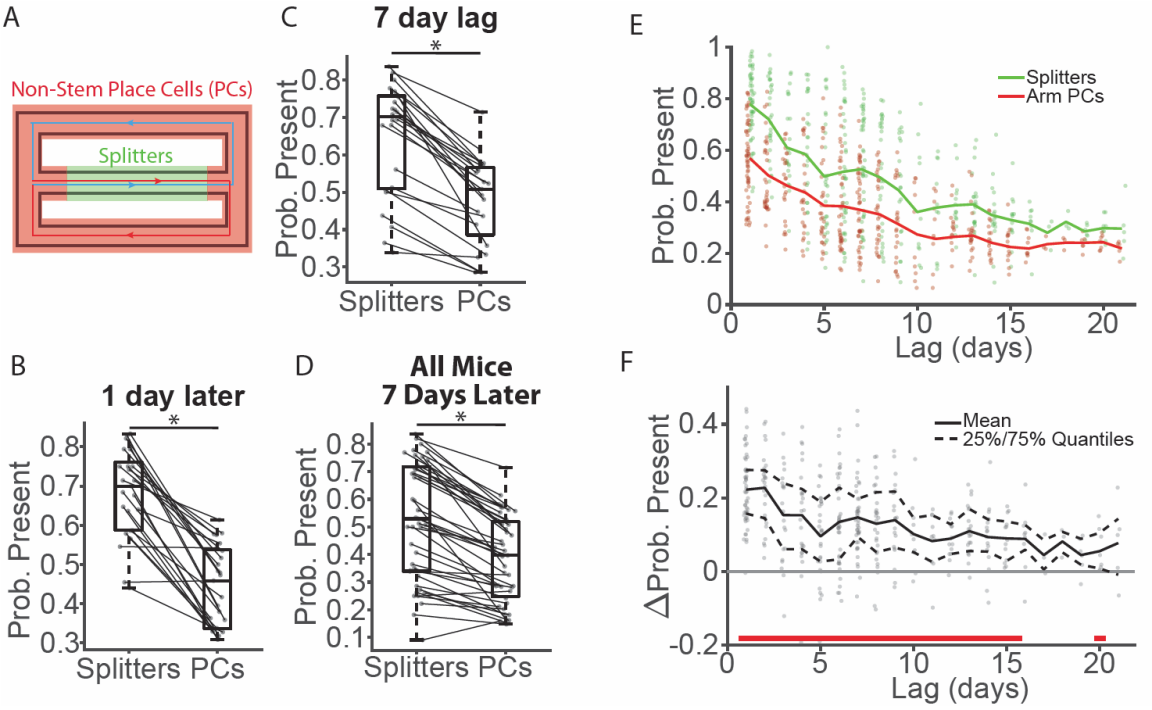

Figure 3.4: Splitters Display Decreased Long-term Turnover Rates

A) Schematic for locations of two neuron phenotypes considered: Green = splitters, Red = Non-Stem Place Cells (PCs)
B) Probability splitters and PCs are present one day later for one mouse. *p=8.3 x10^{-6}, signed-rank test
C) Probability splitters and PCs are present seven days later for a different mouse. *p=9.7 x10^{-6}, signed-rank test.
D) Same as C but for all mice. *p = 1.3x10^{-8}, signed-rank test.

E) Probability splitters and arm place cells are present versus lag between sessions. Seven day lag data shown in D. Dots: probabilities from individual session-pairs, lines: mean probability at each time lag. Green = Splitters, Red = Arm PCs.
F) Difference between splitter and arm PC probability present. Dots: probability present differences for individual session-pairs. Black solid/dashed lines: Mean and 25%/75% quantiles of data at each time point. Red bars = significant differences after Holm-Bonferroni correction of one-sided sign-test. See
G) Table 1 for signed-rank test p-values at all lags.

Table 1: One-sided Signed-Rank Significance Values for Probability Splitter Vs. Non-Stem Place Cells are Present

Lag (days)	1	2	3	4	5	6	7
All Cells (Figure 3.4)	1.3e-10	9.1e-7	9.4e-5	4.3e-5	2.2e-4	4.5e-7	2.7e-8
Matched Rate Cells (Figure 3.8)	2.6e-6	1.5e-4	0.016	2.1e-3	3.7e-3	4.0e-4	7.2e-6
Lag (days)	**8**	**9**	**10**	**11**	**12**	**13**	**14**
All Cells (Figure 3.4)	1.2e-5	1.0e-4	4.9e-3	0.014	1.2e-4	2.5e-5	8.9e-4
Matched Rate Cells (Figure 3.8)	1.1e-4	5.1e-3	0.11	0.14	0.042	0.011	0.16
Lag (days)	**15**	**16**	**17**	**18**	**19**	**20**	**21**
All Cells (Figure 3.4)	1.2e-4	9.8e-3	0.25	0.13	0.047	3.9e-3	0.094
Matched Rate Cells (Figure 3.8)	9.7e-4	0.047	0.25	0.13	0.81	1	0.56

3.2.5 Trajectory-Dependent Neurons Provide More Consistent Spatial Information than Return Arm Place Cells

We next wondered how the information provided by splitter cells differs from that of other neuron phenotypes. To do so, we decided to compare the long-term spatial coding properties of trajectory-dependent splitter neurons on the stem (Figure 3.5A) to

other neurons active on the stem that did not meet the statistical criteria to be considered a splitter. When comparing spatial calcium activity over the entire map across sessions, we found that splitter neurons had a significantly higher 2D event map correlation values than did PCs up to at least seven days later (Figure 3.5B). This indicates that trajectory-dependent splitter neurons might preferentially influence downstream structures by providing a more consistent representation of space than other neurons.

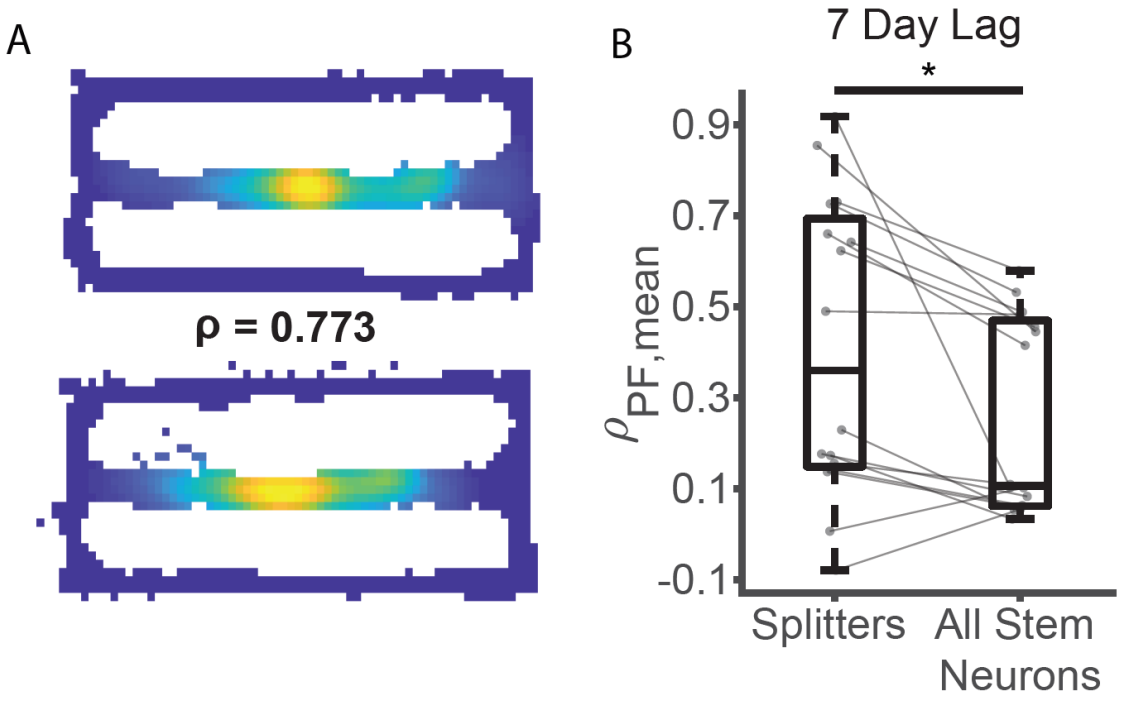

Figure 3.5: Splitters Maintain More Consistent Spatial Information

A) Example 2D occupancy normalized calcium event maps the same neuron with a place field on the stem for two sessions recorded seven days apart. The high Spearman correlation between maps indicates consistent spatial firing between sessions.
B) Mean spatial correlations for splitter neurons and all neurons active on the stem for all sessions seven days apart for one mouse. *p=0.002, signed-rank test.

3.2.6 Trajectory-Dependent Neurons Display a Rapid Onset Followed by Stable Activity

Next, we examined the ontogeny of trajectory-dependent behavior. We hypothesized that three different scenarios could support the emergence of splitters. In line with a study showing that unstable neurons can support well-learned behavior (Liberti et al., 2016), splitters could slowly ramp up/down their splitting behavior (Figure 3.6A, top), or they could come online suddenly and turn off just as suddenly (Figure 3.6A, middle). Finally, and consistent with the idea that there might be a drive to maintain stability in neurons that convey valuable information to downstream regions, neurons could quickly begin to exhibit trajectory-dependent activity and then maintain that activity thereafter (Figure 3.6A, bottom). Previous work (Monaco, Rao, Roth, & Knierim, 2014) presented the idea that neurons pre-disposed to a phenotype can come online suddenly after a head-scanning/attention event. To address this question, we identified the day when each neuron we recorded first exhibited significant trajectory-dependent activity, and then tracked how much that activity changed in the days before and after becoming a splitter, taking $\Delta_{max,norm}$ as a metric of how well the neurons differentiate between trajectories. We found evidence for heterogeneity in the ontogeny of splitting, with some neurons exhibiting a rapid onset of trajectory-dependent activity (Figure 3.6C) and others ramping up their trajectory-dependent activity in the days prior to becoming a splitter (Figure 3.6B); both cell phenotypes appeared to maintain stable trajectory-dependent activity afterward. This trend became apparent when we combine results across all cells. At shorter time scales (+/- 2 days from splitting onset), individual

mice exhibited a mixture of ramping up activity in the days prior to splitting onset, followed by stable splitting (Figure 3.6D). However, the rapid onset followed by stable activity phenotype was readily apparent when examining group data over longer time scales (+/- 9 days from splitting onset, Figure 3.6E). We observed a similar trend for return arm place cells using mutual information as a metric of place coding strength (Figure 3.6F), suggesting that similar rules govern the onset and fate of trajectory-dependent and spatial coding in hippocampal neurons.

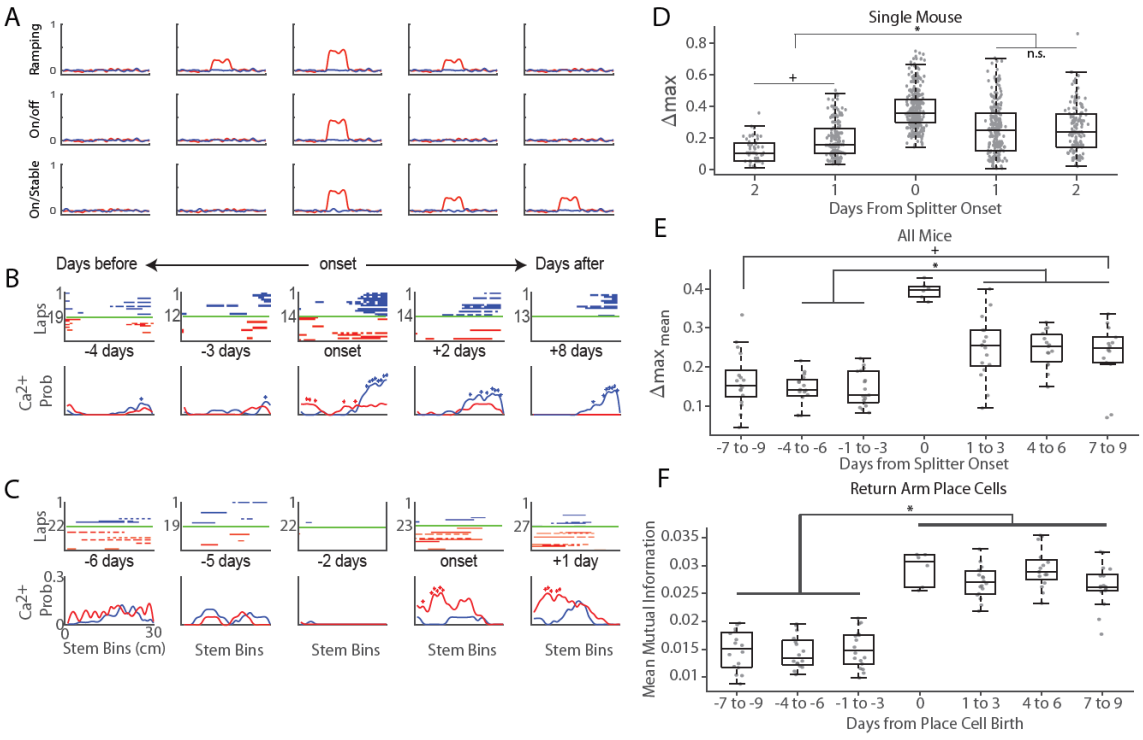

Figure 3.6: Splitters Come Online Abruptly and Maintain Stable Fields

A) Schematic illustrating theoretical splitter ontogeny scenarios. *Top:* slow ramp up/down of trajectory-dependent behavior in days preceding/following splitter onset. *Middle:* transient, short lasting trajectory-dependent activity persisting only during splitter onset day. *Bottom:* sudden onset of trajectory-dependent activity followed by stable splitting thereafter.

B) Example splitter across days from one mouse roughly matching On/Stable cell in schematic in A. Red box, splitting onset day.
C) Same as B but for a different mouse.
D) Δmax for all neurons +/- 2 days from splitter onset for one mouse. p = 2.3x10-9 Kruskal-Wallis ANOVA, *p < 0.001, +p = 0.026 post-hoc Tukey test.
E) Δmax_{mean} +/- 9 days from splitter onset for all mice. p = 2.3x10-9 Kruskal-Wallis ANOVA, *p < 0.02, +p = 0.18 post-hoc Tukey test.
F) Mean Mutual Information +/- 9 days from place cell birth for all mice. p = 3.2x10-15 Kruskal-Wallis ANOVA, *p < 0.001 post-hoc Tukey test.

3.2.7 Place Cell Onset Coincides With or Precedes Splitter Onset

We next wondered if hippocampal neurons displayed significant spatial tuning before, during, or after they exhibited trajectory-dependent firing. As shown above, splitter cells are a special type of place cell (Figure 3.5) and have a similar onset/offset trajectory to place cells (Figure 3.6); thus, we hypothesized that the onset of trajectory-dependent firing in hippocampal neurons would either coincide with or follow their birth as place cells. To test this idea, we first tallied the onset day of each cell phenotype. We found that both cell phenotypes were present from day 1 and continued to come online throughout the experiment (Figure 3.7A, C). The bulk of place cells were immediately recruited on day 1. In contrast, and in agreement with a previous study (Bower, Euston, & McNaughton, 2005), the recruitment of splitter cells did not peak until several days later (Figure 3.7A,C), suggesting that trajectory-dependent activity tended to emerge slower than did spatial activity. This could occur independently in two different groups of neurons, or it could occur serially with each neuron first becoming a splitter cell only after becoming a place cell. Thus, to test if this delay in splitter cell ontogeny occurred in the same cells, we directly compared the day a cell became a splitter to the day it became a place cell, including only neurons with activity on the stem of the maze. We found that

in the bulk of neurons, splitting occurred simultaneously with place field onset, while a different population of neurons exhibited trajectory-dependent activity only after first becoming place cells (Figure 3.7B,D). Thus, place cells and splitter cells occupy an overlapping population of neurons with spatial responsivity coinciding with or preceding trajectory-dependent coding.

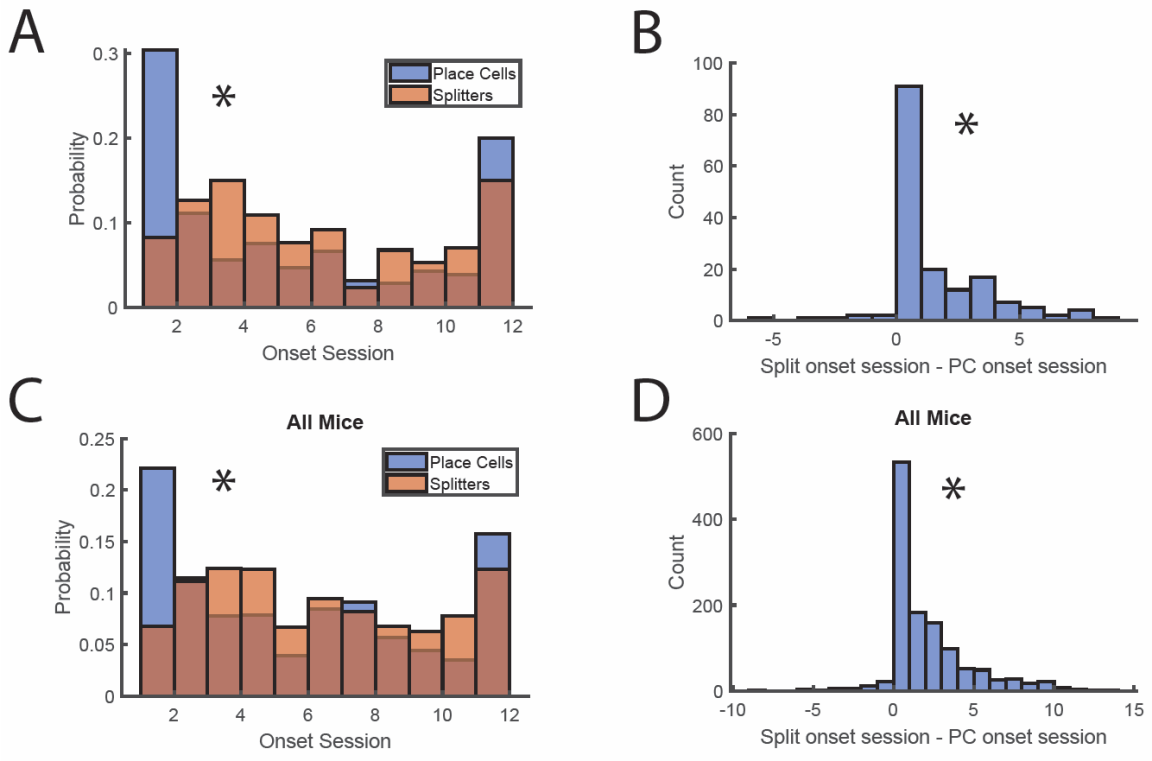

Figure 3.7: Place Cell Onset Coincides With or Precedes Splitter Onset

A) Histogram of the first (onset) day a neuron exhibits a splitter cell or place cell phenotype for one mouse. *p = 2.7x10-^{13}, one-sided Kolmogorov-Smirnov test.
B) Difference between splitter cell onset day and place cell onset day for one mouse. *p=1.9x10^{-38} χ^2 goodness-of-fit test, mean = 2.3, median = 1.
C) Same as A) but for all mice. *p = 6.1x10-^{16}, one-sided Kolmogorov-Smirnov test.
D) Same as B) but for all mice. *p=4.9x10^{-118} χ^2 goodness-of-fit test, mean = 1.6, median = 1.

3.3 DISCUSSION

From an evolutionary perspective, one adaptive function of memory is the ability to provide information vital to survival. Thus, maintaining activity and consistency in neurons encoding information pertinent to survival might provide a mechanism for preferentially strengthening connections with downstream structures via consistent replay of the same sequences (Buzsáki, 2015a; Diba & Buzsáki, 2007; Louie & Wilson, 2001; Maboudi et al., 2018; Pfeiffer & Foster, 2013). Conversely, if the pool of neurons available to encode a given memory remains fixed, then forgetting of incidental information through the turnover/silencing of neurons not required for survival is actually adaptive (Hardt, Nader, & Nadel, 2013) because it could increase the numbers of neurons available to encode other relevant information (Richards & Frankland, 2017). Here, we utilize *in vivo* calcium imaging with miniaturized microscopes to explore this idea (Figure 3.1). Since trajectory-dependent splitter neurons (Figure 3.2) contain information relevant to proper task performance (Figure 3.3), we hypothesized that they would exhibit relatively high stability when compared to other neuron phenotypes. To the best of our knowledge, this is the first demonstration that trajectory-dependent hippocampal activity exists in mice and that it can be detected with calcium imaging.

We find support for this idea in a number of ways. First, splitter neurons are more persistently active across long time scales than neurons that only provide information about the animal's current location on the return arm (Figure 3.4). Second, splitters come online abruptly and then maintain a stable readout of trajectory up to 9 days after becoming a splitter (Figure 3.6). Splitters also provide a more consistent signal of the

animal's current location than do other neurons (Figure 3.5), further supporting their long-term stability. Last, we found that splitter cells are a dynamic subpopulation of place cells; while many begin acting as place and splitter cells simultaneously, others act as place cells prior to becoming a splitter cell (Figure 7). This finding concurs with the slow increase of trajectory-dependent activity with experience found in a previous study (Bower et al., 2005). These data combined support the idea that neuron phenotype influences its subsequent stability (Zaremba et al., 2017) and the consistency of the information it provides to downstream structures.

Our study utilizes single-photon imaging to perform longitudinal tracking of hippocampal neuron activity and confirm existing studies showing increasing turnover of coactive neurons with time (Cai et al., 2016; Rubin et al., 2015; Ziv et al., 2013). However, a recent study performed in songbirds demonstrated that imaging artifacts, specifically small shifts in the z-plane of single-photon imaging, could entirely account for putative cell turnover (Katlowitz et al., 2018). Thus, the turnover we and others observe in hippocampal neurons could likewise be artefactual. While relevant, this concern is mitigated in our study for a number of reasons. First, the Katlowitz et al. (2018) study was performed in the basal ganglia of songbirds while they performed a stereotyped behavior supported by highly stable firing responses of neurons over short and long timescales (Guitchounts, Markowitz, Liberti, & Gardner, 2013; Margoliash & Yu, 2009; Richard H. R. Hahnloser, Alexay A. Kozhevnikov, & Michale S. Fee, 2002). In contrast, our study was performed in CA1 of the mouse hippocampus, a highly plastic brain region exhibiting complete, monthly turnover of afferent connections (Attardo et

al., 2015) that also exhibits a high degree of drift in neuron firing responses over relatively short time-scales (Mankin et al., 2012; Manns et al., 2007). Second, studies utilizing activity-dependent tagging of neurons also find that the overlap between active cells in the mouse hippocampus declines with time between sessions (Cai et al., 2016; Kitamura et al., 2017), supporting long-term hippocampal cell turnover as a real phenomenon. Most importantly, our study compares the *relative* turnover rates of two different cell phenotypes: splitter cells and place cells. Thus, even if day-to-day misalignments in the z-plane forced neurons out of focus, this would occur equally for both splitters and place cells. Therefore, concerns about imaging artifacts cannot explain our finding that splitter cells are more persistently active across long time scales than place cells.

One notable study found that optogenetic silencing of nucleus reuniens, an important communication hub between the medial prefrontal cortex and dorsal CA1 of the hippocampus, significantly reduced trajectory-dependent activity in rat CA1 neurons while having no impact on the rat's performance of a spatial alternation task (Ito et al., 2015). Those results directly challenge our finding that the quality of trajectory-dependent information contained in CA1 activity patterns correlates with a mouse's performance (Figure 3.3). One potential reason for this discrepancy is that their intervention only partially reduced trajectory-dependent information without eliminating it, potentially allowing the splitter cells remaining to provide adequate information for proper task performance. Second, relatively easy tasks might be less resistant to a partial disruption and rats performed at close to ceiling levels in the Ito et al. (2015) study. Our

mice performed at lower levels, though still well above chance, indicating that the spatial alternation task might place higher attentional and cognitive demands on mice than on rats. Third, Ito et al. (2015) utilized the difference in peak firing rate on left versus right trials as a metric for trajectory-dependent activity. However, this calculation does not account for trajectory-dependent information provided by neurons that maintain similar firing rates, but shift their firing location along the stem between left and right trials (see Figure 3.2B, left). Last, silencing of nucleus reuniens eliminated trajectory-dependent activity predicting future trajectories only; information related to past trajectories, which could be utilized by downstream structures to help make the correct upcoming turn, was maintained. Thus, trajectory-dependent neural activity could still be important for proper task performance.

Rodents with hippocampal lesions are capable of performing a continuous alternation task (Ainge, van der Meer, Langston, & Wood, 2007). This raises the question: how important is trajectory-dependent activity if mice can perform the task without the hippocampus at all? We have two responses to this question. First, long-term lesions test necessity, not sufficiency, since these lesions can induce compensatory plasticity that could allow non-hippocampal regions to support the task (Packard & McGaugh, 1996). Second, under normal conditions the hippocampus might still be the default brain region for task performance. This is emphasized by Goshen et al. (2011), who demonstrated that mice cannot perform long-term recall of a putatively hippocampal-independent contextual fear memory (Bontempi et al., 1999; Debiec, LeDoux, & Nader, 2002; Frankland, Bontempi, et al., 2004; Kim & Fanselow, 1992;

Kitamura et al., 2017, 2009; Winocur et al., 2009) when hippocampal inactivation is limited to a short time period before the task; however, mice became capable of successful long-term memory recall when this inactivation was extended over a long time period prior to performing the task. This study and others (Meira et al., 2018; Sparks et al., 2011; Sutherland et al., 2008; Wang et al., 2009; Wiltgen et al., 2010) support the idea that the hippocampus is vital for long-term recall under normal conditions and that redundant pathways are recruited for episodic memory retrieval only if chronic aberrant activity is detected in the hippocampus.

Through what mechanism do trajectory-dependent neurons maintain a greater stability across long time-scales? After the initial onset of trajectory-dependent behavior, these neurons could receive feedback from dopaminergic neurons originating in the ventral tegmental area (VTA) during learning (Gomperts et al., 2015) or from locus coeruleus (LC) neurons during post-learning sleep (Takeuchi et al., 2016) that could strengthen afferent connections to splitter neurons. This could also occur during sharp-wave ripple related replay of prior trajectories (Diba & Buzsáki, 2007; Pfeiffer & Foster, 2013) in conjunction with simultaneous dopaminergic inputs from VTA (Gomperts et al., 2015). However, this mechanism would also strengthen all cells active en route to the goal location, whether they carried information about trajectory or not. One possibility, however, is that since trajectory-dependent neurons appear to be more active than other cells (Figure 3.8A) they might be preferentially reactivated during sharp-wave ripple events, an idea that warrants future testing.

Taken together, our results highlight the influence of cell phenotype on its subsequent stability, and suggest that the emergence of task-related trajectory-dependent coding coincides with or follows the emergence of spatial coding in neurons. Future work could investigate mechanisms supporting the stability and emergence of this neuron phenotype.

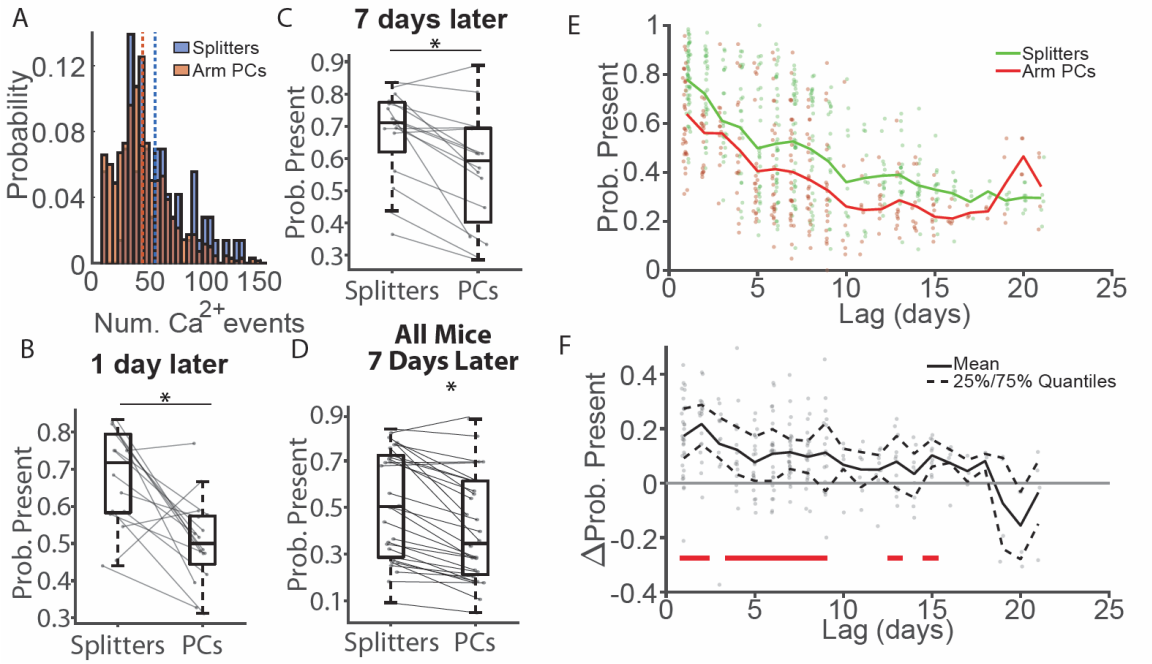

Figure 3.8: Decreased Long-term Turnover Rates for Event-Rate Matched Splitters (Related to Figure 3.4)

A) Histogram from one session showing that Ca^{2+} event rates are higher for splitters for than for arm PCs. Blue/red dashed: mean number Ca^{2+} events for splitter/arm PCs.
B) Probability splitters and PCs are present one day later for one mouse. *p=0.002, signed-rank test. Note that in B-F the population of splitters used was downsampled such that its mean Ca^{2+} event rate matched that of the population of non-stem PCs.
C) Probability splitters and PCs are present seven days later for a different mouse. *$p=8.8x10^{-4}$, signed-rank test.

D) Same as C but for all mice. *p = 3.7×10^{-6}, signed-rank test.
E) Probability splitters and arm place cells are present versus lag between sessions. Dots: probabilities from individual session-pairs, lines: mean probability at each time lag. Green/Red: Splitters/Arm PCs.
F) Difference between splitter and arm PC probability present. Dots: probability present differences for individual session-pairs. Black solid/dashed lines: Mean and 25%/75% quantiles of data at each time point. Red bars = significant differences after Holm-Bonferroni correction of one-sided sign test. See Table 1 for one-sided signed-rank test p-values at all lags.

3.4 METHODS

3.4.1 Animals

Five male C57/BL6 mice (Jackson Laboratories), age 3-14 months and weighing 25-30g were used. One mouse was excluded from analysis after performing the experiment due to the inability to correct motion artifacts in his imaging videos. Mice were housed socially with 1-3 other mice in a vivarium on a 12hr light/dark cycle with lights on at 7am and given free access to food and water. All mice were singly house after surgery. All procedures were performed in compliance with the guidelines of the Boston University Animal Care and Use Committee.

3.4.2 Viral Constructs

We used an AAV9.*Syn*.GCaMP6f.WPRE.SV40 virus from the University of Pennsylvania Vector Core at an initial titer of ~4×10^{13} GC/mL and diluted it to ~5-6×10^{12} GC/mL with sterilized 0.05 phosphate buffered saline (KPBS) prior to infusion into CA1.

3.4.3 Stereotactic Surgeries

All surgeries were performed in accordance with previously published procedures (Kinsky et al., 2018; Resendez et al., 2016) in accordance with the Boston University Animal Care and Use Committee. Briefly, we performed two stereotactic surgeries and one base-plate implant on naïve mice, aged 3-8 months. Surgeries were performed under 1-2% isoflurane mixed with oxygen. Mice were given 0.05mL/kg buprenorphine (Buprenex) for analgesia, 5.0mL/kg of the anti-inflammatory drug Rimadyl (Pfizer), and 400mL/kg of the antibiotic Cefazolin (Pfizer) immediately after induction. They received the same dosage of Buprenex, Cefazolin, and Rimadyl twice daily for three days following surgery and were carefully monitored to ensure they never dropped below 80% of their pre-operative weight during convalescence. In the first surgery, a small craniotomy was performed at AP -2.0, ML +1.5 (right) and 250nL of GCaMP6f virus was injected 1.5mm below the brain surface at 40nL/min. The needle remained in place a minimum of 10 minutes after the infusion finished at which point it was slowly removed, the mouse's scalp was sutured, and the mouse was removed from anesthesia and allowed to recover.

3-4 weeks after viral infusion, mice received a second surgery to attach a gradient index (GRIN) lens (GRINtech, 1mm x 4mm). After performing an ~2mm craniotomy around the implant area, we carefully aspirated cortex using a blunted 25ga and 27ga needle under constant irrigation with cold, sterile saline until we visually identified the medial-lateral striations of the corpus callosum. We carefully removed these striations using a blunted 31ga needle while leaving the underlying anterior-posterior striations

intact, after which we applied gelfoam to stop any bleeding. We then lowered the GRIN lens until it touched the brain surface and then proceeded to lower it another 50µm to counteract brain swelling during surgery (note that in two mice we first implanted a sleeve cannula with a round glass window on the bottom without depressing an additional 50µm and then cemented in the GRIN lens during base plate attachment). We then applied Kwik-Sil (World Precision Instruments) to provide a seal between skull and GRIN lens and then cemented the GRIN lens in place with Metabond (Parkell), covered it in a layer of Kwik-Cast (World Precision Instruments), and then removed the animal from anesthesia and allowed him to recover after removing any sharp edges remaining from dried Metabond and providing any necessary sutures.

Finally, after ~2 weeks we performed a procedure in which the mouse was put under anesthesia but no tissue was cut in order to attach a base plate for easy future attachment of the microscope. To do so, we attached the base plate to the camera via a set screw, carefully lowered the camera objective and aligned it to the GRIN lens by eye, and visualized fluorescence via nVistaHD v2.0/v3.0 until we observed clear vasculature and putative cell bodies expressing GCaMP6f (Resendez et al., 2016), then raised the camera up ~50µm before applying Flow-It ALC Flowable Composite (Pentron) between the underside of the baseplate and the cured Metabond on the mouse's skull. After light curing we applied opaque Metabond over the Flow-It ALC epoxy to the sides of the baseplate to provide additional strength and to block ambient light infiltration.

3.4.4 Imagine Acquisition and Processing

Brain imaging data was obtained using nVista HD (Inscopix) v2/v3 at 1440 x 1280 pixels and a 20 Hz sample rate. Two mice were lightly anesthetized (~60 seconds) to facilitate camera attachment and then given ~15 minutes to recover prior to any recordings; the camera was attached to the other two mice while they were awake. Prior to neuron/calcium event identification we first pre-processed each movie using Mosaic (Inscopix) software which entailed a) spatially downsampling by a factor of 2 (1.18 μm/pixel), b) performing motion corrections, and c) cropping the motion-corrected movie to eliminate any dead pixels or areas with no calcium activity. We then extracted a minimum projection of the pre-processed movie for later neuron registration. We replaced isolated dropped frames (maximum 2 consecutive frames) with the previous good frame, and in the rare case where more than 2 frames dropped in a row these frames were excluded from all analyses.

3.4.5 Neuron and Calcium Event Identification

We utilized custom-written, open-source MATLAB software (available at https://github.com/SharpWave/Tenaspis) to identify putative neuron ROIs and their calcium events in accordance with previously published results (Kinsky et al., 2018; Mau et al., 2018).

3.4.6 Across-Session Neuron Registration

We utilized custom-written, open-source MATLAB software (available at https://github.com/nkinsky/ImageCamp) to perform neuron registration across sessions in

accordance with previously published results (see 2.4.3.3). We checked the quality of neuron registration between each session-pair in two way: 1) by plotting the distribution of changes in ROI orientations between session and comparing it to chance, calculated by shuffling neuron identity between session 1000 times, and 2) plotting ROIs of all neurons between two sessions and looking for systematic shifts I neuron ROIs that could lead to false negatives/positives in the registration. During the course of these checks, we noticed the quality of registration between sessions dropped significantly approximately halfway through the experiment. Thus, we excluded any registrations occurring between the first and second halves of the experiment for these two mice.

3.4.7 Behavioral Tracking and Parsing

Behavioral data was recorded via an overhead camera with Cineplex v2/v3 software (Plexon) at a 30Hz sample rate. Cineplex produced automated tracking of the animal's position by comparing each frame to a baseline image without the animal in the arena. Imaging and behavioral data were synchronized by TTL pulse at the beginning of the recording. Each video was inspected by eye for errors in automated tracking and fixed manually via custom-written MATLAB software. After fixing all erroneous data points, the animal's position was interpolated to determine his location at each imaging movie time point.

3.4.8 Histology

Mice were killed and transcardially perfused with 10% KPBS followed by formalin. Brains of perfused mice were then extracted and post-fixed in formalin for 2-4

more days after which they were placed in a 30% sucrose solution in KPBS for 1-2 additional days. The brains were then frozen and sliced on a cryostat (Leica CM 3050S) in 40 μm sections after which they were mounted and coverslipped with Vectashield Hardset mounting medium with DAPI (Vector Laboratories). We then imaged slides at 4x, 10x, and 20x on a Nikon Eclipse Ni-E epifluorescence microscope to verify proper placement of the GRIN lens above the CA1 pyramidal cell layer.

3.4.9 Experimental Outline

After recovery from surgery, mice were food deprived to maintain no less than 85% of their pre-surgery weight. Mice were subsequently exposed to a variety of arenas in order to habituate them to navigating with the camera attached. Prior to training on the alternation task, all mice were given 1-4 habituation sessions on the alternation maze. The maze floor (inner dimension = 64 x 29 cm) and walls (height = 18cm) were constructed from 3/8 inch (0.95cm) thick plywood and the barriers between arms were constructed from two 53cm long 1.5 x 5.5 inch (3.8 x 14 cm) pine framing studs. The finished maze consisted of a central stem and two return arms, each 7.5cm wide with 5.7cm wide openings at each of the central stem through which mice could exit or enter the return arms. Two food wells ~ 0.25 cm deep were created toward the end of each return arm to hold chocolate sprinkles: they were centered 12.5 cm from the end of the maze where mice exited the return arm/entered the center stem. Food was placed in these wells through a small opening in the side of the maze. Prior to exploring the arena it was sealed with urethane.

Three of the mice were first trained to loop on each side of the maze independently in 3-10 minutes blocks by blocking off access to the other side with Plexiglas dividers in order to familiarize mice with the general task demands, arena, and location of food reward (chocolate sprinkles); the other mouse received one habituation session where he was allowed to freely traverse the maze. Following habituation, three of the four mice were placed in the center stem and rewarded regardless of the first turn direction. On subsequent trials, mice were only rewarded if they turned the opposite direction of the previous trial. These mice were allowed to run freely and were only blocked when they a) attempted to reverse course on the central stem, b) attempted to exit the return arm after they had committed to it, or c) attempted to run straight across to the other arm without turning down the central stem after obtaining reward. We considered a mouse committed to an arm after his tail entirely crossed from the edge of the central arm into the stem. These mice generally ran ballistically down the center stem and were allowed to pause once they entered the return arm and after they obtained reward. Food reward was only delivered once the mouse had committed to a return arm in order to avoid providing an auditory cue of reward location. One mouse encountered a lapse in performance mid-way through the experiment and began perseverating on one turn direction in blocks: he was subsequently given a number of trials at the beginning of each session where he was forced to turn each direction by blocking off one turn direction with a Plexiglas divider, after which he was then allowed to freely choose turn directions. The fourth mouse was initially forced to alternate at the end of his habituation looping

sessions. All forced trials were not considered during later data analysis. Two of the mice received one session per day; the other two received 1-2 sessions per day.

3.4.10 Place Cell Identification

Place Cell Identification was performed as described in Kinsky et al.(2018), see 2.4.3.2.

3.4.11 Trajectory-Dependent/Splitter Cell Identification

Prior to performing any analysis, each mouse's trajectory data was aligned to that from the first habituation session. This was done by 1) manually rotated the data to correct for any day-to-day changes in maze angle relative to the recording camera, 2) calculating the edges of the mouse's trajectory as the data points located at the 2.5% and 97.5% points in the cumulative density function of his x/y position data, and 3) adjusting the data by applying the necessary translation and scaling (minimal) to overlay each session's trajectory on the first session. After aligning data across sessions, the mouse's trajectory on each trajectory was parsed into his progression through the different sections of the maze, starting at the a) **base**, then moving down the b) **center stem** into the c) **choice** point, then turning into the d) left/right **entry** to the e) **return arm**, and finally entered the f) **approach** to the center stem just after the reward port. The center stem portion was manually identified for each mouse as the point where the mouse's trajectory into/out of each return arm stopped diverging. This was done in order to mitigate the possibility that trajectory-depending activity was controlled entirely by

stereotyped sensory inputs, e.g. the mouse hugging/whisking the left side of the center stem after right turn trial.

After parsing the animal's behavior into these sections, the center stem was broken up into ~1cm bins and the event rate for each neuron was calculated for each trial. Tuning curves for each trial type (left or right turn) were then constructed, which consisted of each neuron's mean event rate for all correct trials at each spatial bin. The difference between these curves was then calculated. To assess significance, we again constructed tuning curves for left/right trials and calculated their difference, but after randomly shuffling trial turn identity 1000 times to establish the likelihood the observed difference between tuning curves could emerge by chance. We then defined splitters/trajectory-depending cells as neurons which had at least three bins whose real tuning curve difference exceeded 950 of shuffled values. In order to exclude spurious identification of splitters we excluded any neurons that did not have at least 5 transients on the center stem.

We calculated three different metrics to quantify the level of trajectory-dependent activity in each neuron. First, we calculated $|\Delta_{max,norm|}$ as absolute value of the maximum difference between the two turning curves found at any bin along the stem, divided by the overall max of either tuning curve. Second, we calculated $|\Sigma\Delta_{max}|_{norm}$ as the sum of the absolute value of the difference between tuning curves divided by their sum at each spatial bin. Third, we calculated the correlation between left and right tuning curves. Note that this metric is very conservative since it produces low correlations for splitters who shift the location of their peak activity between left and right trials along the length of the

stem (Figure 3.2B) but not for splitters who modulate their event rate in the same place along the stem (Figure 3.2A).

3.4.12 Linear Discriminant Decoding Analysis

A linear discriminant decoder was trained on data from 50% of trials on a given session using the fitdiscr function in MATLAB. Calcium event activity for each neuron at each time point when the mouse was on the center stem were used as the input variables and the mouse's upcoming turn direction was used as the response variable. Only correct trials were considered for training. The decoder was then used to predict the turn direction of the other 50% of trials, after which the process was repeated 999 times using a different random 50% of trials for training/decoding. The decoding accuracy was then calculated in ~3.3cm bins along the stem, and the mean accuracy across all bins was taken as the decoding accuracy for that session.

3.4.13 Persistent Activity/Probability of Recurrence Analysis

We first performed neuron registration between sessions and classified persistently active neurons as those that were identified by our cell extraction algorithm on both sessions and produced at least five calcium events through the course of the first recording session being considered in the registration. We then categorized cells into two different phenotypes, context-dependent splitter cells or place cells. Splitter cells were designated based on the criteria listed above. Neurons that produced no calcium activity on the stem of the maze and met our place cell criteria were defined as non-stem place cells. A session-pair was excluded from analysis if there were fewer than ten cells in

either category in the first session being registered. This analysis was performed in two ways: 1) including all cells found for each phenotype, and 2) matching mean event rate between neuron phenotype by excluding the lowest firing rate place cells. In the event that place cells had a higher mean firing rate than splitter cells, no place cells were excluded.

3.4.14 Phenotype Ontogeny Analysis

We tracked the ontogeny splitter cells in three steps. First, we registered all the neurons we recorded across the entire experiment. Second, we identified the first day/session that a neuron passed our statistical criteria to be considered a splitter and defined that session as its onset. Finally, we calculated multiple metrics for the quantity of trajectory-dependent activity produced by each of these neurons (see 3.4.11 above) in all the sessions preceding and following onset, excluding any sessions that occurred on the same day. The methodology for tracking place cell onset was identical, except mutual information (see 2.4.3.2) was used a metric of spatial information provided by each cell.

4 CHAPTER 4

Temporarily Arresting Protein Synthesis to Block Memory Consolidation Induces a Long-Term Disruption of Hippocampal Activity

4.1 Introduction

The hippocampus is vital for the encoding and retrieval of short-term episodic memories (see 1.1). Additionally, hippocampal injury/lesions result in the permanent loss of recently encoded memories (Kim & Fanselow, 1992; Winocur et al., 2009) suggesting the hippocampus also supports consolidation of short-term memories into lasting, long-term memories. The consolidation process is thought to require structural changes in hippocampal neurons since inhibiting protein synthesis blocks long-term contextual fear memory consolidation while leaving short-term memory intact (Flood, Rosenzweig, Bennett, & Orme, 1973; Winocur et al., 2009). Inhibiting protein synthesis also prevents the stabilization of newly formed place-fields without affecting firing locations of existing place cells (Agnihotri et al., 2004), suggesting that changes in neural activity patterns might underlie the observed consolidation deficits. Despite the importance of protein synthesis in stabilizing both memories and hippocampal activity patterns, there is no current evidence directly linking memory consolidation deficits to instability in hippocampal activity. To address this, we paired a contextual fear conditioning task in mice with *in vivo* calcium imaging using a miniaturized epifluorescence microscope (miniscope) to capture activity of the same, large ensemble of hippocampal neurons across long-time scales. We induced amnesia in half our mice by injecting the protein

synthesis inhibitor anisomycin immediately after applying a foot shock in one arena. Importantly, the foot shock was applied only after mice had two days to explore the arena and establish reliable activity patterns. We hypothesized that preventing the synthesis of new proteins, anisomycin would prevent the long-term stabilization of hippocampal activity patterns, thus resulting in unreliable long-term inputs to downstream regions that could produce amnesia.

4.2 RESULTS

4.2.1 Imaging and Behavior

In a typical contextual fear conditioning (CFC) paradigm, the level of foot shock applied produces a robust increase in freezing behavior as a readout of the strength of the fear memory (Frankland, Josselyn, et al., 2004). However, the number of active hippocampal neurons scales with the size of the arena explored by rodents (Rich, Liaw, & Lee, 2014). Thus, a strong fear-conditioning paradigm sharply limits the number of active neurons available for recording since mice with strongly fear conditioned mice tend to only partially explore an arena in the days following foot shock. To combat this, we devised a CFC task designed to produce significantly elevated levels of freezing but without severely limiting the extent of the arena the mice explored. In this task, mice were first allowed to explore a shock arena (Coulbourn Instruments, 7”x7”x12”) for 10 minutes followed by a neutral arena (identical to the square arena utilized in Chapter 2) on two separate days (Days -2 and -1) to habituate to each arena and to allow us to

observe multiple days of hippocampal activity prior to learning (note that day number indicates time relative to foot shock). On day 0, mice (n=4) were first placed in the arena for 60 seconds after which they were given a pair of mild foot shocks and then removed from the arena 60 seconds later. They then had ten minutes of exposure to the neutral arena after which they were given an intra-peritoneal injection of anisomycin (150mg/kg) or vehicle (phosphate buffered saline). Mice were then allowed to explore the same arena four hours later in a short term memory (STM) test, as well as three separate long term memory (LTM) tests performed one, two, and seven days later (Figure 4.1A). We carefully titrated the strength of the contextual fear memory (measured as the amount the mouse froze) by adjusting the amplitude (0.25 to 0.5mA) and number of shocks (1-3) and found that a pair of 0.25 mA foot shocks produced significant freezing from day -1 to day 1 without reducing the extent of arena explored in behavioral mice (Figure 4.1B). Anisomcyin administration successfully induced amnesia since we observed no increase in freezing from day -1 to day 1 (Figure 4.1C). Freezing at 4 hours in these mice could result from either short-term memory recall or from side-effects of the injection, since a separate cohort of mice given anisomycin but no foot shock exhibited significantly increased freezing levels 1 and 4 hours after injection but not 6 hours after injection (data not shown). Thus, we were able to successfully induce a contextual fear memory and impair its consolidation.

Following a previously established paradigm (Kinsky et al., 2018), we utilized a miniaturized epifluorescence microscope to visualize the activity of hippocampal neurons virally expressing GCaMP6f across long-time scales. Using this technique, we recorded

from large numbers of neurons and tracked them across all days of the experiment (Kinsky et al., 2018). We verified that undergoing two prior surgeries as well as the presence of the camera did not affect the magnitude of freezing for our imaging mice at all time points (Figure 4.1D-E). We observed a significant difference in absolute freezing in the shock arena from day -1 and day 1 for our control but not our anisomycin imaging mice (Figure 4.1D-E), though it must be noted that this difference approached significance in our anisomycin mice ($p = 0.07$, Figure 4.1E). However, control imaging mice exhibited significantly higher freezing in the shock arena relative to the neutral arena when compared to the anisocmycin group on days 1 and 2 ($p < 0.04$, unpaired one-sided t-test). Thus, administration of anisomycin successfully blocks long-term memory consolidation in our cohort of imaging mice.

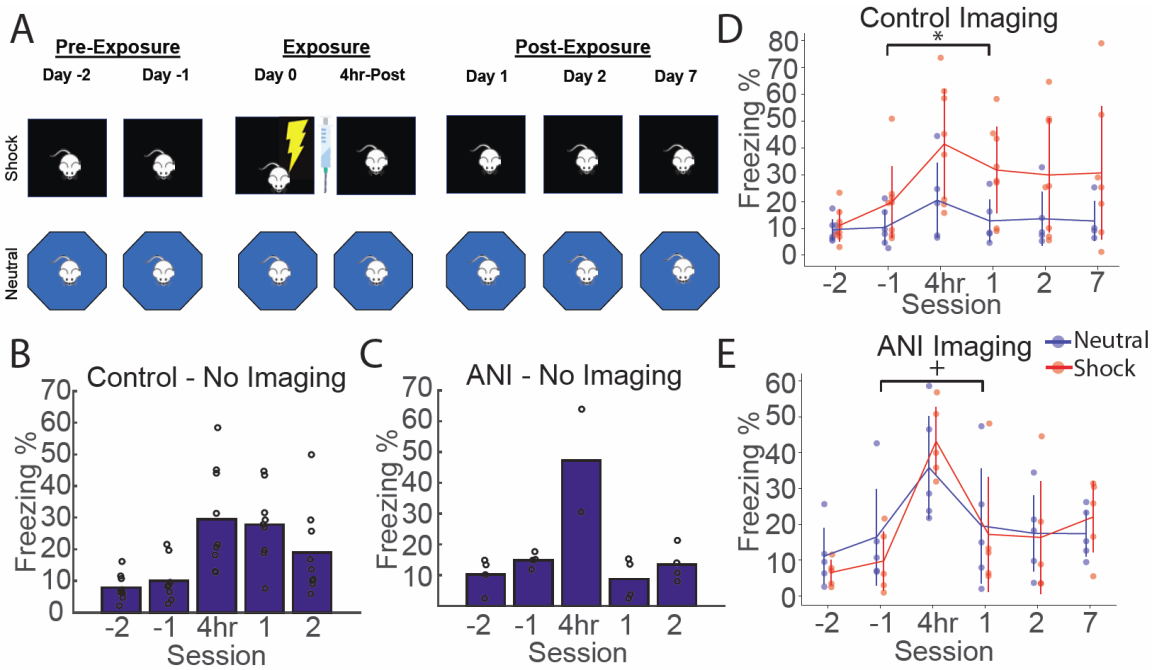

Figure 4.1: Anisomycin Administration Successfully Blocks Consolidation of a Context-Specific Fear Memory

A) Behavioral schedule. 2 – 10 minutes sessions in each arena. Session noted at top is time relative to foot shock. Anisomycin/vehicle control was injected immediately following the neutral arena Day 0 session into mice.
B) Foot shock results in lasting freezing in control behavioral mice (n=4). *p<0.024, +p=0.059, one-sided paired t-test.
C) Anisomycin induces amnesia in behavioral mice for foot shock at day 1 and 2 sessions (n=4). All comparisons with day -1 are not significant. Day -1 to 4 hour session was not evaluated due to missing data (video capture error) for two mice at the 4 hour session.
D) Foot shock results in lasting freezing in control behavioral mice (n=4 mice). Red = shock arena, blue = neutral arena. *p = 0.004, one-sided paired t-test. Note that one mouse was excluded from this comparison due to excessive freezing in the shock arena prior to shock on day -1.
E) Same as D but for imaging mice that received anisomycin infusion after shock session. +n.s., p = 0.07, one-sided paired t-test.

4.2.2 Anisomcyin Disrupts Basal Turnover Rate of Hippocampal Neurons

Multiple studies have demonstrated that hippocampal activity patterns change continually over time (Mankin et al., 2012; Manns et al., 2007; Mau et al., 2018), perhaps due to a complete remodeling of its afferent connections over the course of a month (Attardo et al., 2015). One way in which this change is reflected in the number of previously active cells that are reactivated at later time points: as time between sessions progresses, fewer and fewer of the cells active in the first session become active again (Cai et al., 2016; Kinsky et al., 2018; Rubin et al., 2015; Ziv et al., 2013). We confirmed this effect in our control mice: the overlap ratio (the number of cells active in both sessions divided by the total number of cells active in either session) decreased as time between sessions increased (Figure 4.2A). Furthermore, the number of cells active on any

given day in our control mice remained constant throughout the experiment (though there was a small increase from 4 hours and 1 day post shock to day 7) suggesting that this turnover was not due to a concomitant decrease in cell activity but through a continual drift in which cells participated in the active cell population (Figure 4.2B).

Since protein synthesis is required for potentiation of connections between neurons (Frey & Morris, 1997, 1998; Nguyen, Abel, & Kandel, 1994), we hypothesized that the administration of anisomycin would reduce basal rate of neuron turnover, resulting in a higher proportion of neurons that were persistently active at long time points. Surprisingly, we observed that the rate of cell turnover increased for the session following anisomcyin administration ($p = 0.0028$ two-sided t-test for decrease in overlap ratio from day -1 to 4 hour session between Anisomycin and Control groups, Figure 4.2C). This increased turnover returned to normal one day after anisomycin administration as there was no difference in the rate of cell turnover from the 4 hour session to day 1 ($p = 0.91$ two-sided t-test for decrease in overlap ratio from day -1 to 4 hour session between Anisomycin and Control groups). This increased turnover could result from a change in the total number of active cells in sessions performed four hours to two days post-injection (Figure 4.2D). This decrease was accompanied by a concomitant drop in event rate post-injection (Figure 4.2E), suggesting that anisomcyin produced a long-term drop in neuron firing rate (Sharma, Nargang, & Dickson, 2012). This firing rate drop could explain the drop in the number of detected cells since lower firing rates produce smaller changes in fluorescence (Chen et al., 2013) and increase the probability neuron activity does not exceed the noise floor in our recordings. Thus,

anisomycin induces lasting changes in hippocampal activity patterns, perhaps due to disruption of constitutive AMPA receptor trafficking (Anggono & Huganir, 2012; Kessels & Malinow, 2009), along with inducing amnesia.

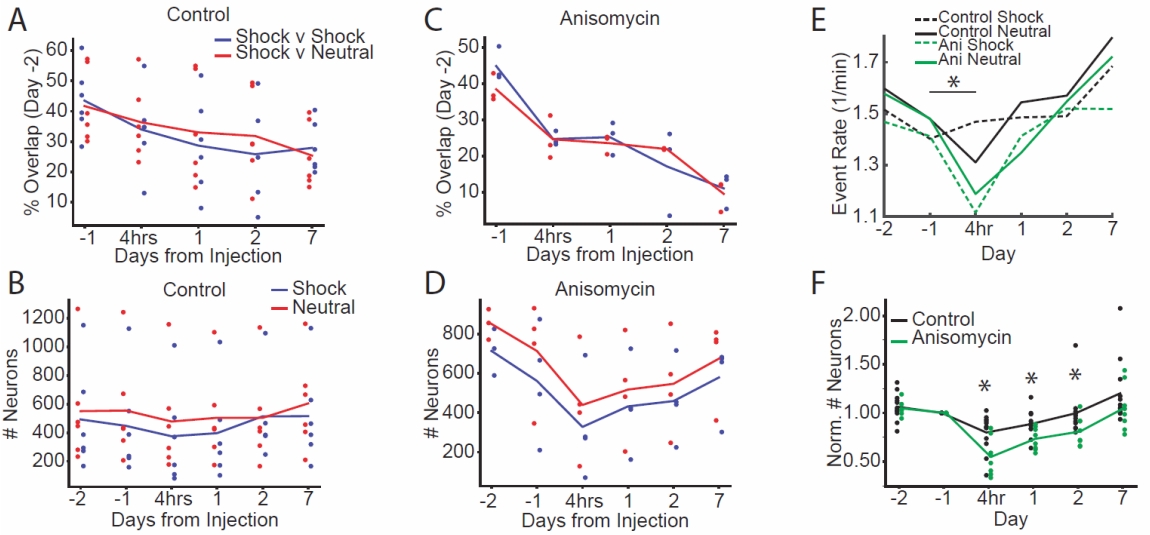

Figure 4.2: Inhibiting Protein Synthesis Induces a Lasting Decrease in the Number of Active Cells

A) The overlap between cells active cells from day -2 consistently decreases as time between sessions increases in control mice. Blue = overlap within shock arena, Red = overlap between shock arena and neutral arena. n = 6 mice.

B) Number active neurons detected across all sessions remains constant across days in each arena for control mice. p=0.0004 ANOVA, however post-hoc Tukey tests reveal this is due to an increase from the 4 hour and 1 days sessions to day 7, not a decrease post-injection.

C) Anisomycin administration (between lag of 1 and 2 days) accelerates the decrease in cell overlap immediately after injection. Same conventions as A. n = 3 mice.

D) Anisomcyin induces a decrease in the number of active cells 4 hours to 2 days later. Same conventions as B. $p = 4.7 \times 10^{-5}$ ANOVA performed on data normalized to day -1 neuron counts. *p<0.05 post-hoc Tukey test. n = 4 mice.

E) Anisomycin produces a decrease in Ca^{2+} event rate of detected neurons. *p<0.03 in each arena, Wilcoxon ranksum test for change in mean event rate from day -1 to 4 hour session. All other changes in event rate from day -1 are not significant.

F) The number of neurons detected is different between anisomycin and control groups after anisomycin administration. Neuron number was normalized to day -1. *$p<0.05$, Wilcoxon ranksum test.

4.3 DISCUSSION

Protein synthesis supports the long-term maintenance of synaptic potentiation in the hippocampus (Frey & Morris, 1997, 1998) and thus could provide a mechanism for stabilizing learning-related changes in the brain. Accordingly, there is much research into the importance of protein synthesis to memory consolidation (Debiec et al., 2002; Flood et al., 1973; Frankland, 2006; Milekic & Alberini, 2002; Squire & Barondes, 1974) and the disruption of normal hippocampal activity across short (Sharma et al., 2012) and long timescales (Agnihotri et al., 2004). However, to the best of our knowledge, there is no evidence directly linking disruptions of hippocampal activity with the memory deficits induced by arresting protein synthesis. This is likely due to a) difficulty in obtaining a reliable behavioral readout of memory strength that is both susceptible to disruption with a protein synthesis inhibitor AND does not completely stop animal locomotion, and b) the inability, until recently, to reliably track neuronal activity of the same neurons across both short and long timescales. Here, we connect these two disparate fields of research by combining long-term imaging of hippocampal neuronal activity in mice with a contextual fear conditioning task (Figure 4.1A) which produced a reliable increase in freezing behavior at both short (four hours) and long (1-7 days) post-shock intervals (Figure 4.1B,D). Importantly, our protocol did not result in excessive freezing, which could limit the number of active neurons since multiple studies have shown that the number of neurons recruited directly correlates with how much an animal explores a

given environment (Monaco et al., 2014; Rich et al., 2014). Finally, arresting protein synthesis via systemic administration of anisomycin immediately following foot shock prevented memory consolidation by reducing freezing levels to pre-shock levels 1-7 days later (Figure 4.1C,E). We also observed a significant decrease in freezing relative to control group in the shock arena versus neutral arena, suggesting that anisomycin blocked memory consolidation of a context-specific fear memory. However, it must be acknowledged that we observed only a trend toward significantly more absolute freezing in our control versus our anisomycin group.

A recent report found that intra-hippocampal administration of anisomycin produced a pronounced decrease in spontaneous and evoked neuronal activity that lasted for several hours (Sharma et al., 2012). Here, we extend this finding to show that arresting protein synthesis resulted in a suppression of neuronal activity that persists for at least two days following administration of anisomycin (Figure 4.2B,D). There are at several possible explanations for this prolonged reduction in hippocampal activity following anisomycin administration. First, protein synthesis is necessary for both plasticity as well as regularly occurring constitutive processes to maintain normal neuronal function (see 1.4.1). Thus, disrupting protein synthesis would prevent any learning related changes as well as normal maintenance operations from occurring in neurons. This is supported by the fact that we observe a concomitant dip in the number of active neurons active in both the shock and neutral arenas. Second, short-term suppression of spontaneous hippocampal activity could reduce intra-hippocampal and hippocampal-cortical plasticity by disrupting the quantity of sharp-wave ripples:

synchronous, high-frequency discharge events observed in the hippocampal LFP (Buzsáki et al., 1992) that are vital for long-term memory consolidation (Girardeau et al., 2009; Maingret et al., 2016). Sharp-wave ripple related replay is hypothesized to support induction and maintenance of plasticity (Buzsáki, 2015a) by effectively mimicking the tetanic stimuli utilized by Bliss & Lømo (1973) to induce hippocampal long-term potentiation (LTP). Thus, short-term disruption of spontaneous hippocampal activity (Sharma et al., 2012) could effectively prevent intra-hippocampal plasticity induction via this mechanism. It could also prevent hippocampal-cortical plasticity important for the induction of supporting long-term expression of episodic memories without the involvement of the hippocampus in the phenomenon of systems consolidation (see 1.4.2). Third, short-term disruption of spontaneous hippocampal activity could even induce long-term depression in synapses by effectively reducing their firings rates to very low frequencies (Malenka & Bear, 2004). None of these mechanisms are mutually exclusive. Last, two recent studies found that neurons undergoing plasticity during learning integrated themselves with more rigid neurons in post-learning replay events (Grosmark & Buzsáki, 2016; van de Ven et al., 2016). Thus, if anisomycin does indeed disrupt constitutive neuron upkeep along with plasticity, this would further weaken the backbone of neurons supporting the schema into which the new fear memory would normally be assimilated (McKenzie & Eichenbaum, 2011). Therefore, there are several potential mechanisms through which anisomycin could produce long term decreases in hippocampal activity.

Many studies utilizing a CFC protocol similar to ours perform freezing tests shortly post-anisomcyin injection as a metric of short-term memory retention (Frankland, Josselyn, et al., 2004; Suzuki et al., 2004). However, we found that systemic administration of anisomycin induced substantial freezing in naïve mice who did not receive a foot shock (data not shown) 1-4 hours later, but not six hours later. Therefore, it must be acknowledged that using freezing metric of short-term memory while anisomycin is still actively inhibiting protein synthesis (~4 hours after systemic injection, see Flood et al., 1973) is problematic since it is impossible to attribute freezing to a context-shock associational memory or to non-specific effects of anisomycin. Despite this, there is still ample evidence that anisomycin blocks long-term memory consolidation by specifically inhibiting plasticity related learning that a shock occurs in a specific context. First we found that freezing significantly increases in control but not anisomycin mice from day -1 to day 1. Second, we found a significant increase in freezing relative to the neutral arena ($p < 0.01$, $t = 2.88$ independent t-test between groups). This is consistent with the finding that inhibiting protein synthesis prevents long-term stabilization of learning induced changes induced in the hippocampus at both the synaptic (Frey & Morris, 1997, 1998) and neural coding levels (Agnihotri et al., 2004).

Pioneering work by Frey & Morris (1997, 1998) highlighted the importance of protein synthesis to the maintenance of long-term but not short-term synaptic potentiation. In line with this work, Agnihotri et al. (2004) demonstrated that anisomycin administration immediately following exposure to a new environment disrupted the long-term stability of hippocampal place field locations in that environment but not in a

familiar environment. This suggests that the maintenance of late-phase synaptic potentiation observed by Frey & Morris (1997, 1998) could also support the maintenance of stability in the hippocampal spatial code. Furthermore, previous studies have demonstrated that shock induces a selective remapping of place fields in the fear arena only (Wang et al., 2012) and that anisomycin only impacts stability in newly formed place cells (Agnihotri et al., 2004). Therefore, we would expect any induced stability to occur for place fields in the shock arena only, since learning is specific to the shock environment (Figure 4.1D). Thus, follow up work examining place field stability in both cohorts of mice is warranted to determine the relationship, if any, between disrupted place field stability and long-term memory retention.

Overall, our study begins to elucidate how arresting protein synthesis dependent synaptic strengthening might prevent memory consolidation by through long-term disruption of hippocampal activity. Follow-up analysis will further examine the effects of this disruption on the information content of hippocampal neurons that could be conveyed to downstream structures.

4.4 METHODS

Behavioral data was recorded at 30Hz in Cineplex v3 software (Plexon) in the neutral arena and synchronized with imaging data via TTL pulse as described previously (Kinsky et al., 2018); a similar procedure was performed in shock arena, but behavioral data was acquired using Freeze Frame tracking software (Coulbourn Instruments) at 3.75Hz. Freezing epochs were defined as any set of 10 or more frames in a row (2.67 seconds) where the mouse's velocity fell below 1.5 cm/sec; data from the neutral arena

was downsampled to match the acquisition rate in the shock arena prior to calculating freezing. All other experimental and analytical details are described in sections 2.4 and 3.4.

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5 CHAPTER 5

CONCLUSION

Decades of research support two distinct roles for the hippocampus: 1) its necessity for the encoding and consolidation of episodic memories and their recall at recent (and perhaps remote) time points, and 2) its ability to automatically and reliably create maps of space. One proposed way to unite these seemingly disparate functions of the hippocampus is the proposal that its spatial coding might provide a mechanism for associating memories with a particular place (O'Keefe & Nadel, 1978). However, for this theory to hold true, place fields of hippocampal neurons must remain relatively stable across the time-scales of episodic memory formation and consolidation; rampant remapping of place fields (see 1.6) would render any learned associations between place cells and other neurons void since a completely different set of neurons would be active when an animal later returned to the same place. This could be particularly problematic for episodic memories containing information vital to survival. For example, if place cells remapped in the days following a discovery of a predator by a particular tree, this could impair their ability to activate a flight-or-fight response via associations formed with hypothalamic neurons during the initial encounter with the predator. While seminal work by Barnes et al. (1997) provided a link between the stability of place fields and animals' ability to perform a spatial memory task, inherent limitations in performing longitudinal neural recordings with electrophysiology (1.5.1) have prevented fully testing this theory across time scales spanning the consolidation of episodic memories. However, recent

advances in neural recording technology have enabled reliable long-term tracking of neuronal activity in large ensembles of neurons (see 1.5), allowing scientists to re-examine the work of Barnes et al. (1997) over longer time scales. This idea warrants further consideration for two opposing reasons: 1) proper hippocampal activity immediately following memory acquisition is necessary for its consolidation (see 1.4.2) and, despite the ability of cortical regions to support long-term memory after hippocampal injury, the hippocampus normally support long-term memory in non-pathological conditions (see 1.4.2.1), and 2) despite its role in supporting episodic memory, hippocampal afferents completely remodel over the course of a month (Attardo et al., 2015) and hippocampal neurons exhibit significant drift in their information content over the course of minutes to days (Mankin et al., 2012; Manns et al., 2007; Mau et al., 2018; Rubin et al., 2015; Ziv et al., 2013). Thus, much work is required to reconcile the continually occurring plasticity in the hippocampus necessary for learning with the stability required to support short and long term episodic memory.

In this thesis, I examined three ideas that begin to address how plasticity and stability of hippocampal neurons interact over long time scales. First, I explored the idea that there should be little change in the long-term stability of place cell firing locations while animals randomly forage in the same arenas since there are no salient learning events to induce plasticity. Second, I addressed the dynamics of the birth and fate of different neuron phenotypes that emerge during learning and mastery of a spatial learning task, with the hypothesis that the utility of information carried by a given neuron influences its subsequent stability. Last, I investigated how disrupting protein synthesis –

necessary for the induction and maintenance of long-term changes in synaptic transmission due to learning – impacts the long-term activity of hippocampal neurons in conjunction with blocking memory consolidation.

Changes in synaptic transmission are hypothesized to provide a mechanism for learning (Malenka & Bear, 2004). This idea is supported by studies demonstrating that a dopamine-mediated novelty signal increases memory strength, synaptic strength, and post-learning hippocampal activity (McNamara et al., 2014; Takeuchi et al., 2016), and that heightened hippocampal activity occurs in sleep sessions following exposure to a novel, but not a familiar, environment (Giri et al., 2019). Thus, I first explored the hypothesis that, since there is little change in synaptic weights or overall hippocampal activity in the absence of learning, there should likewise be little change in the spatial firing patterns of place cells. In Chapter 2, I demonstrate that place cells generally maintain a consistent pattern of spatial firing in relation to one another (a coherent map) across short and long time scales (Figure 2.2, Figure 2.6). However, in line with previous studies (Keinath et al., 2017; Weiss et al., 2017) they exhibit instability in how they anchor their place field map to the external environment (Figure 2.3). Additionally, perhaps this lack of stable anchoring to the external environment is advantageous since it provided a mechanism for mice to efficiently use the same neural firing patterns to represent a similar but differently shaped arena (Figure 2.4, see also Buzsáki, 2015b) and potentially even to link memories between the two environments (Eichenbaum, 2004). Notably, we found that new learning was accommodated through remapping of a subset of neurons while simultaneously maintaining a coherent map in another subset of neurons

(Figure 2.5), providing one potential mechanism for integrating learning-required plasticity and memory-mandated stability in the same brain region. These findings could also help explain why mice performing a random foraging task appeared to have much lower levels of instability in their hippocampal firing patterns than mice performing a spatial memory task in the same arena (Kentros et al., 2004). Overall, this chapter provides support for the idea that place fields exhibit sufficient stability in their firing locations relative to one another to provide a cohesive substrate for supporting long-term episodic memories.

However, several findings from Chapter 2 warrant future consideration. First, we found that only a small, but significant, number of cells maintains a coherent map at long time scales since a large number of cells turn on/off as time between sessions increases (see Figure 2.6, Rubin et al., 2015, and Ziv et al., 2013). Thus, even though the information in these recurrently active neurons remains consistent, the information being conveyed to downstream structures is still extremely different since these coherent mapping neurons constitute only a small portion of the active neurons. Perhaps the constant remodeling of hippocampal connections (Attardo et al., 2015) serves to incrementally refine hippocampal activity patterns even when no learning occurs such that the new patterns of activity can elicit the same behavioral response as the original activity pattern. Or, perhaps the consistent activity of these few coherent mapping neurons is sufficient to drive downstream regions appropriately on their own. Second, consistent with a previous study (Keinath et al., 2017), we found that mice utilized arena geometry to anchor maps (Figure 2.3), but discovered no rules governing how they

selected the appropriate geometrical feature for map orientation (Figure 2.9). Furthermore, Kentros et al. (2004) demonstrated that dopamine agonists/antagonists selectively increase/decrease place field stability. These findings highlight that much work remains to uncover the mechanisms supporting stable anchoring of maps to the external environment.

What rules govern how the activity patterns of neurons change/stabilize, given the constant turnover of active hippocampal neurons with time (Attardo et al., 2015; Cai et al., 2016; Mankin et al., 2012; Mau et al., 2018; Rubin et al., 2015; Ziv et al., 2013)? In Chapter 3 I explore how different hippocampal neuron phenotypes emerge over the course of learning and mastery of a spatial alternation task and how phenotype influences long-term hippocampal activity. I first confirmed that neurons which modulate their calcium event rate according to a mouse's previous/future trajectory exist in mice and are detectable with calcium imaging (Figure 3.2). The relative magnitude of trajectory-dependent activity occurring in these so-called trajectory-dependent/splitter neurons correlated with task performance (Figure 3.3), suggesting the information they provide to downstream regions could influence task performance. Thus, I wondered if, just as environmental pressures select for adaptive traits in animals, perhaps there exists an adaptive pressure in the brain to maintain stability in patterns of activity in these neurons since they provide information to downstream regions that is vital to task performance. Indeed, I found that splitter cells exhibited a lower rate of turnover than did place cells on the return arms of the maze, suggesting that they provided a longer-lasting influence on downstream structures (Figure 3.4). This hints at a mechanism whereby neurons that

exhibit less useful phenotypes for task performance (e.g. for survival in the real world) are preferentially recycled into the pool of silent neurons that can later be recruited to act as a more useful phenotype. Those splitter cells that remained active furthermore provided more consistent spatial information across days than did their non-splitter cells counterparts, bolstering the hypothesis that neuron phenotype influences the long-term stability of its information content (Figure 3.5). Last, I found that splitters as well as place cells emerged rapidly and then sustained their respective phenotypes for at least nine days (Figure 3.6), providing a cellular substrate for long-term memory of both the arena and task. In line with a study finding a gradual increase in trajectory-dependent activity with experience, I found that recruitment of splitter cells peaked 3-4 days into performing the task; in contrast, place cell recruitment was highest on the first day of training (Figure 3.7). Thus, these results support the hypothesis that cell phenotype influences its subsequent stability: though both place and splitter cells displayed rapid onsets followed by stable neural coding, splitter cells provided more consistent and longer-lasting information to downstream structures.

How do intracellular processes supporting plasticity maintenance impact the hippocampal neural code? One recent study found that temporarily arresting protein synthesis, which is necessary for normal cell upkeep and the manufacture of new channels/proteins supporting induction of long-lasting LTP (see 1.4.1.1), produces a pronounced short-term drop in neuronal activity (Sharma et al., 2012). Thus, in Chapter 4 of this thesis I investigate how blocking memory consolidation via administration of a protein synthesis inhibitor immediately following encoding of a contextual fear memory

influences hippocampal activity over longer time scales. I found that anisomycin administration successfully blocked consolidation of a context-specific fear memory (Figure 4.1). We hypothesized that temporarily arresting protein synthesis following learning would prevent any learning-related, long-term plasticity from occurring, effectively stopping changes in neural activity that reflected either learning or normal cellular turnover observed by us and others (Cai et al., 2016; Kinsky et al., 2018; Mau et al., 2018; Rubin et al., 2015; Ziv et al., 2013). Surprisingly, we found that anisomycin administration accelerated the rate of cell turnover by substantially reducing the number of active neurons up to two days later (Figure 4.2). This likely occurs since protein synthesis is vital not only for the manufacture of plasticity-inducing proteins but also for operations of normal cell upkeep (see 1.4.1.1). Thus, temporarily disrupting protein synthesis not only prevents any learning-related changes from stabilizing but also disrupts normal cellular communication for an extended period of time. The implication of this finding is that anisomcyin does not simply prevent encoding of a new memory, but likely impacts how the hippocampal neural code reflects previous experience through disruption of intra-hippocampal connection strengths.

The overarching theme of this dissertation is that the stability of information contained in hippocampal activity patterns supports memory. However, as noted throughout, all hippocampal activity persists upon a continual background of change. This occurs at the level of synaptic inputs (Attardo et al., 2015), gradual drift in spiking/calcium activity (Mankin et al., 2015, 2012; Manns et al., 2007; Mau et al., 2018), increases in the number of cells turning on/off with time (Cai et al., 2016; Kinsky

et al., 2018; Mau et al., 2018; Ziv et al., 2013), and levels of expression of plasticity-related genes (Attardo et al., 2018). How can the hippocampus support memory acquisition, consolidation, and retrieval if its underlying circuitry is constantly rewired? One idea is that gradual drift in hippocampal representations could provide a mechanism for remembering the temporal order of events (Manns et al., 2007; Rubin et al., 2015; Schiller et al., 2015), which in turn is important for determining causality. Thus, this drift could actually be essential for inferring the temporal context of a given memory and linking discrete events across time (Eichenbaum, 2013; Wallenstein, Hasselmo, & Eichenbaum, 1998). Another idea is that the adaptive function of memory is not to reminisce or reliably reproduce previous experiences, but rather to make useful predictions about upcoming events based on their similarity to past experiences. From this vantage point, too much rigidity in representing past experiences is not only unnecessary but could prove harmful since preserving incidental information would occupy valuable brain resources that could be devoted to encoding new information that is more relevant to survival. In this sense, forgetting is actually beneficial since it frees up neurons representing memories less relevant to survival and allows them to be recycled into the pool of neurons that could be recruited to represent more valuable information (Hardt et al., 2013; Richards & Frankland, 2017). Thus, continual change could reflect a refinement of the hippocampal code based on continual experience to more reliably inform future actions than a rigid adherence to past events might allow. This change in hippocampal output patterns could help shape the responses of cortical neurons to support long-term memory in the process of systems consolidation (Kitamura et al., 2017;

McClelland et al., 1995). Alternatively, input from cortical regions could also help shape hippocampal output patterns in a context-dependent manner to obtain the best behavioral outcome in well-learned tasks (Place et al., 2016). Perhaps this cortical input could even help dictate which neurons undergo plasticity and which remain stable (Grosmark & Buzsáki, 2016; van de Ven et al., 2016) when new information is integrated into an existing schema (McClelland, 2013; McClelland et al., 1995; McKenzie & Eichenbaum, 2011). Thus, continual drift could provide a mechanism for improving the adaptive function of memory, but truly understanding its influence on memory will require much work to better elucidate how downstream regions interpret incoming hippocampal signals.

Overall, this thesis utilizes *in vivo* calcium to investigate how hippocampal neuron activity changes (or ceases to change) across long time-scales. My dissection of how the hippocampal neurons maintain structure in their spatial maps in the absence of learning supports the idea that place fields might provide a substrate for anchoring episodic memories to spatial locations. My investigation of how cell phenotypes emerge supports LTP as a mechanism for learning and memory maintenance. Furthermore, my finding that the information content of a neuron influences its long-term stability supports the idea that there are rules governing the turnover and plasticity of hippocampal neuron activity patterns (Zaremba et al., 2017). Finally, my exploration of the long-term effects of anisomcyin on neuronal activity provide insight to how arresting protein synthesis might disrupt memory consolidation.

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