THE EFFECTS OF EMOTION AND SLEEP ALTERATIONS ON HIPPOCAMPUS-DEPENDENT MEMORY CONSOLIDATION

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ABSTRACT

THE EFFECTS OF EMOTION AND SLEEP ALTERATIONS ON HIPPOCAMPUS-DEPENDENT MEMORY CONSOLIDATION

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The activity of hippocampal place cells is thought to generate the contextual representations in which episodic memories are embedded. However, new memories are susceptible to disruption until they undergo a process known as consolidation, becoming more permanent over time. This dissertation examines how changes in emotion and alterations in sleep patterns influence hippocampus-dependent memory consolidation. I first investigated the role of the dorsal and ventral hippocampus in predator odor fear learning by administering a histone deacetylase (HDAC) inhibitor into the dorsal or ventral hippocampus at different stages of contextual fear conditioning. Then, using long-term electrophysiological recordings of place cells in mice undergoing fear and extinction learning, I examined how changes in emotional valence affect spatial representations in the hippocampus. Last, I explored the effects of sleep deprivation on place cell activity in young and aged adult mice during the object-place recognition (OPR) task. I found that HDAC inhibition after context pre-exposure enhanced fear learning in the dorsal hippocampus but led to fear generalization in the ventral, suggesting that the dorsal hippocampus may encode the specific details of a context while the ventral encodes general aspects of the environment. Moreover, electrophysiological recordings in the dorsal hippocampus revealed that different place cells remapped preferentially at different stages of predator odor fear learning and extinction, suggesting that the extinction memory trace is a complex memory composed of old and new learning. Finally,

sleep deprivation (SD) impaired learning in young adult animals but enhanced it in aged animals, possibly due to enhanced consolidation of NREM during recovery. Old SD animals and young control mice exhibited stability throughout the experiment in one set of cells, while another set remapped in response to the introduction of objects and again to the moved object. This suggests that successful performance of the OPR task requires animals to maintain a stable representation of the static environment while updating a representation of object locations. Young SD animals exhibited long-term stability in both types of cells, reflecting a failure to update object-place representations, but control old animals exhibited complete global remapping, suggesting that their impairment stems from a failure to consolidate spatial representations formed during training. Together, these data demonstrate that emotion and sleep exert clear influences on hippocampus-dependent memory consolidation.

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CHAPTER 1: INTRODUCTION

Organisms are faced with a continual barrage of information from internal and external sources, making learning a constant and ongoing process. However, an animal cannot afford to deplete energy and cognitive resources by indiscriminately memorizing everything; in order to thrive, it must be able to selectively retain only adaptive information. Consequently, it is thought that newly formed memories remain susceptible to disruption until they undergo a process known as consolidation, after which the memory becomes more permanent and resistant to interference. Although this selective consolidation of specific memories offers considerable evolutionary advantages, the question remains of how and why certain memories become consolidated instead of others.

There is ample support in the literature that memory consolidation is a highly time-dependent process. Early experimental evidence came from rodent studies demonstrating that electroconvulsive shock treatments impaired memory if administered immediately after training (Duncan 1949, McGaugh & Alpern 1966). Subsequent studies in both humans and rodents using various pharmacological agents found a similar effect of timing, where immediate post-training administration had the strongest effect on long-term memory recall (For review, see McGaugh & Roozendaal 2009). Further investigation of the processes occurring in this time window has led to the current model of synaptic consolidation, which posits that a series of biochemical cascades after learning result in synaptic remodeling, thereby stabilizing the memory trace (For review, see Dudai 2004, Dudai 2012, Dudai et al 2015). Thus, factors or manipulations which disrupt or enhance these processes may in turn impair or enhance long-term memory formation.

Emotion appears to be one such factor, and perhaps is one means by which animals can identify relevant experiences for long-term retention. Many studies have reported that human subjects demonstrate better recall for emotionally arousing experiences or stimuli (For review, see LaBar & Cabeza 2006, McGaugh 2013), and adrenal stress hormones such as cortisol which are released during high emotional arousal have been demonstrated to have significant effects on

hippocampus-dependent learning (Cahill & McGaugh 1998). Similarly, there is evidence that physiological factors such as sleep or sleep loss can influence later performance on many memory tasks and in fact may be critical for consolidation (For reviews, see Rasch & Born 2013, Stickgold 2005, Stickgold 2013, Walker & Stickgold 2004).

This dissertation examines how neuromodulatory and physiological factors such as emotion and sleep can influence memory consolidation in the hippocampus. I will begin by briefly reviewing the literature on the role of the hippocampus in memory, with a description of place cells, the principal cells in this region. Then, I will introduce evidence that emotion affects memory consolidation, focusing on a discussion of research in fear and extinction learning. This will serve as background for the work described in Chapters 2 and 3, which focus on the role of the hippocampus in predator odor fear learning, and how the changes in emotional valence that occur during fear and extinction learning affect the stability of spatial representations in the hippocampus. Finally, I will discuss the evidence that sleep is important for memory consolidation which will introduce the research described in Chapter 4 on how alterations in normal sleep patterns can affect learning and place cell activity during the hippocampus-dependent object-place recognition task.

1. The Hippocampus

The hippocampus is an evolutionarily old structure present across a wide range of mammalian species, with homologous structures found in non-mammalian species. Originally classified as part of the limbic system, the hippocampus has since been implicated in a number of cognitive processes. There are many studies leading to the now widely accepted conclusion that the hippocampus is crucial to learning and memory, but perhaps none is more famous or pivotal than Scoville and Milner's report of H.M., am epileptic patient that underwent bilateral removal of his hippocampi and adjacent cortical areas. After the surgery, H.M. exhibited severe deficits in the ability to form new episodic memories, i.e. memories of events happening in specific spatial and temporal contexts; yet his recall of older episodic memories that happened several years prior to the surgery showed no significant impairments (Scoville & Milner 1957). Moreover, further

investigation revealed that he was also capable of forming new implicit memories that do not require conscious recollection, such as habits. Although a few prior studies had implicated the hippocampus in memory (Glees & Griffith 1952, Terzian & Dalle Ore 1955), it was H.M.'s remarkable symptoms that catapulted this brain region into the spotlight and sparked a wave of research into the role of the hippocampus in learning and memory.

1.1 Role of the hippocampus in learning and memory

The peculiar nature of H.M.'s memory deficits revealed that memory is not a single unitary system; rather it involves multiple dissociable processes that require distinct brain regions (For review, see Eichenbaum 2010, Squire 2004, Squire & Dede 2015). Many subsequent studies in humans, rodents, and a number of other species confirmed that the hippocampus was required for certain types of memory tasks but not others (Squire 1992, Squire et al 2001). In particular, the hippocampus seemed especially important in tasks that required learning spatial or contextual information (Bird & Burgess 2008, Martin & Clark 2007, O'Keefe & Nadel 1978). Hippocampal lesions in rats resulted in behavioral impairments in the Morris water maze, which required animals to use the configuration of distal cues to remember and locate a hidden platform. In contrast, performance was spared in the visible platform version, which could be solved using a beaconing strategy (Morris et al 1982). In agreement with these findings, learning deficits in hippocampal lesion studies were also reported in the radial arm maze (Olton et al 1979, Pothuizen et al 2004), T-maze (Deacon et al 2002, Gerlai 1998), and object-place learning tasks (Bussey et al 2000, Mumby et al 2002). Pharmacological inactivation of the hippocampus produced similar results. For example, after rats were trained to search for a food reward in a cross maze, bilateral hippocampal inactivation using lidocaine resulted in impaired place learning but not in simpler stimulus-response behaviors (Packard & McGaugh 1996). Other rodent studies found that bilateral hippocampal inactivation produced deficits in several other spatial tasks including an open field water maze (Riedel et al 1999) and a passive avoidance response paradigm (Lorenzini et al 1996).

However, several studies also linked hippocampal damage to deficits in non-spatial tasks, suggesting that the hippocampus was important for more than just simple spatial learning. Rats

with hippocampal lesions exhibited learning impairments in tasks that required animals to discriminate between cues of different odors (Eichenbaum et al 1988) or durations (Meck et al 1984). Additionally, animals with hippocampal damage were unable to learn configurations of cues, leading Rudy & Sutherland to propose that the memory impairments seen after hippocampal damage reflected an inability to integrate multiple features together into a cohesive mental representation to be used for future associations (Rudy & Sutherland 1989, Rudy & Sutherland 1995, Sutherland et al 1989, Sutherland & Rudy 1989). Moreover, unilateral hippocampus inactivation was sufficient to impair performance on a complex active avoidance task where distal and proximal cues were dissociated, suggesting that the hippocampus plays a particularly important role in organizing sets of stimuli into distinct frameworks (Cimadevilla et al 2001). Furthermore, recent studies suggest that the hippocampus also participates in complex processing involving imagery (Byrne et al 2007), relational memories (Eichenbaum 2004), and planning of routes (Ferbinteanu & Shapiro 2003, Frank et al 2000).

These complex cognitive functions are supported by the connectivity, neurochemistry, and structural organization of the hippocampus. At its dorsal pole, the hippocampus is heavily connected with regions involved in navigation and spatial processing, such as the subicular complex and retrosplenial cortex (Cenquizca & Swanson 2007, Harker & Whishaw 2004, Swanson & Cowan 1977, Van Groen & Wyss 2003, Vogt & Miller 1983). At its ventral pole, it exhibits strong bidirectional connectivity with regions involved in fear, anxiety, and stress such as the amygdala (Majak & Pitkanen 2003, Pitkanen et al 2000), and projects to the medial prefrontal cortex, a region critical for regulating the behavioral response expressed after fear learning (Orsini et al 2011, Roberts et al 2007, Sah et al 2013, Sierra-Mercado et al 2011). Additionally, the hippocampus contains a high concentration of glucocorticoid receptors in subfields CA1 and CA2 (Van Eekelen & De Kloet 1992, Van Eekelen et al 1988, Van Eekelen et al 1987), and axons from the ventral region project to the hypothalamus, supporting negative feedback inhibition of the hypothalamic-pituitary-adrenal (HPA) axis stress response (Jacobson & Sapolsky 1991). Moreover, axons from the ventral hippocampus terminate within regions that receive direct inputs from the

suprachiasmatic nucleus and dorsomedial hypothalamic nucleus, structures critical for generating the endogenous circadian rhythm (Cenquizca & Swanson 2007, Kishi et al 2000, Watts et al 1987). Since the dorsal and ventral regions are interconnected (For review, see Fanselow & Dong 2010), the hippocampus is well-positioned to be sensitive to factors that integrate contextual information with emotional and homeostatic states determined by the sleep/wake cycles.

1.2 Place cells

The role of the hippocampus in spatial processing is not only supported by lesion, pharmacological, and neuroanatomical studies but also physiological findings. The principal neurons of the hippocampus, known as place cells, fire in particular regions when an animal is moving in an environment (O'Keefe & Dostrovsky 1971). This distinctive characteristic led O'Keefe and Nadel to propose that the combined activity of place cells enable an animal to form a "cognitive map", an allocentric representation of space that facilitates navigation (O'Keefe & Nadel 1978). Interestingly, hippocampal place fields, e.g., the locations where each cell fires, are not hard-wired. These representations develop within minutes in each context, and each representation is unique to the specific environment; for example, place cells that fire robustly in one environment may cease firing altogether or fire in a completely different region when the animal is moved to a new environment. Moreover, it appears that specific spatial representations are modified through experience-dependent learning. When an animal enters a novel context, it forms a new representation that can be retrieved and modified upon reentry to the context to incorporate environmental changes and task-relevant information (Bostock et al 1991, Kentros et al 1998, Mehta et al 2000, Muzzio et al 2009a, Wilson & McNaughton 1994a).

Although visuospatial cues exert robust control over place fields, place cells continue firing in blind animals (Save et al 1998) and in the dark, even when most sensory cues are removed (Quirk et al 1990), suggesting that internal self-motion cues derived from vestibular and/or proprioceptive inputs are sufficient to trigger place cell activity. Conversely, disruption of vestibular information (Stackman et al 2002), completely disrupt place cell firing. These data suggest that internal self-motion cues, which normally allow animals to calculate displacements relative to a start

position, may be sufficient to support navigation. However, in the absence of external cues, the navigation signal accumulates errors (Gothard et al 1996a, McNaughton et al 1996). As a result, place cells continuously update information using external cues, a process known as path integration (McNaughton et al 2006).

Place cells respond to a variety of external cues including proximal and distal visuospatial cues, diffuse auditory or olfactory cues (Eichenbaum et al 1987, Wiener et al 1989), somatosensory information (Hill & Best 1981, O'Keefe & Conway 1978) and sounds (Moita et al 2003, Sakurai 1990). Importantly, learning contingencies strongly influence which cues modulate place cell firing (Kentros et al 2004, Markus et al 1995, Muzzio et al 2009c, Wiener et al 1989, Wood et al 2000). Moreover, there is evidence that the hippocampus encodes these different representations of the external world by using independent reference frames (Fenton et al 2010, Gothard et al 1996b, Kelemen & Fenton 2010, Zinyuk et al 2000). These characteristics suggest that the internal representations generated by hippocampal place cells may be used for more than just spatial navigation. Instead, place cells may serve to integrate spatial contexts with episodic events, respond to task-relevant information, and participate in future memory planning.

Place cells have been shown to respond to the aforementioned cues through changes in firing rates, cessation of firing, and shifts in the location of place fields. Collectively, these effects are termed remapping and can occur in two different ways: global and rate remapping. Global remapping is when place cells respond to environmental variation by firing in different spatial locations. This phenomenon occurs when animals are exposed to completely different spatial environments. On the other hand, rate remapping refers to changes in firing rate that are accompanied by little or no change in the location of the place fields. This phenomenon generally occurs in response to alterations within the same spatial environment, such as changes in wall color (Colgin et al 2008, Leutgeb et al 2005).

Many different studies have explored the circumstances under which remapping can be observed (Colgin et al 2008, Shapiro et al 1997). Early studies found that rotation of a single distal visual cue caused place fields to rotate accordingly (Muller & Kubie 1987, O'Keefe & Conway 1978);

likewise, rotation of a set of visual cues produced the same result as long as the topographical arrangement of cues was preserved (Hetherington & Shapiro 1997, O'Keefe & Conway 1978, O'Keefe & Nadel 1978). Moreover, removal of a single cue had no effect on place cell firing if the remaining cues were unaltered (O'Keefe & Conway 1978). However, when the cues were scrambled or removed entirely, place fields became degraded or exhibited complete remapping (Shapiro et al 1997). These results suggest that place cells encoded not only individual visual cues, but also the relationships among multiple cues, and even relationships between different sets of local and distal cues (Knierim 2002, Tanila et al 1997). Interestingly, when animals were trained in an environment with unreliable distal cues, place cells soon stopped responding to changes in distal cues (Jeffery & O'Keefe 1999, Muzzio et al 2009). However, when the configuration of distal cues needed to be memorized in order to perform a working memory task, even removal of all the cues failed to disrupt place cell firing, suggesting that learning affects the stability of hippocampal representations and that task-relevant information has more long lasting effects than task-irrelevant information (O'Keefe & Speakman 1987).

Finally, it has been shown that place field stability, the tendency of a cell to fire in the same location over successive exposures to the same environment, requires the same biochemical cascades that are necessary for memory consolidation and long-term forms of plasticity (Agnihotri et al 2004, Kentros et al 1998, Rotenberg et al 2000), suggesting that stability is a correlate of spatial memory. Moreover, as with episodic memory, place field stability requires selective attention for proper encoding and retrieval (Kentros et al 2004, Muzzio et al 2009a, Muzzio et al 2009b). Thus, it is possible to assess memory strength by tracking changes in place field stability, which provides a useful physiological model to assess memory changes in real time.

2. Emotion & memory

Although memories of mundane events fade easily, memories rife with emotion exhibit a striking tenacity within our recollections. Researchers and laypersons alike have long observed that strong emotional events have a powerful influence on memory. Physician reports from World War I noted that veterans suffering from "shell shock", a historical term used to describe a common

reaction to combat stress, frequently experienced changes in memory (Myers 1915). More recent studies have found that individuals suffering from post-traumatic stress disorder often experience greatly heightened recall of the traumatic event, i.e. flashbacks (Hellawell & Brewin 2004). In the lab, experimental evidence reveals a clear link between heightened emotional arousal and increased memory consolidation (Cahill & McGaugh 1998, McGaugh 2013, Phelps 2004, Roozendaal & McGaugh 2011). Human subjects consistently exhibit better recall for emotionally arousing stimuli compared to emotionally neutral ones (Hamann 2001, Hamann et al 1999). Furthermore, deliberate suppression of emotions while viewing emotional stimuli has been found to impair subsequent recall when tested at a later time (Richards & Gross 2000). Emotion thus appears to play a critical part in determining which experiences will be remembered later on and how strong those memories will be.

Critically, while both positive and negative emotions have been shown to enhance memory, negative emotions, such as fear, may have the greatest impact on recollection. Studies in humans have found better recall for negative stimuli compared to positive or neutral stimuli (Kensinger & Corkin 2003, Ochsner 2000). Moreover, several studies have revealed that negative emotion can enhance memory for the visual details of an event (Kensinger 2007, Kensinger 2009, Kensinger et al 2007). In rodents, fear learning often occurs rapidly and after a single trial, in sharp contrast to less emotionally salient tasks or tasks of positive valence. For example, rodents often require many trials to reach criterion when learning to navigate spatial mazes or dig for food rewards (Barnes et al 1980, Cheng 1986), but a single shock exposure is sufficient to induce fear learning (Blanchard & Blanchard 1969). Furthermore, these rapidly acquired fear memories can persist for months (Frankland et al 2004, Kim & Fanselow 1992), indicating that negative emotions can have lasting effects on memory.

In this section, I will discuss how emotion can interact with learning and memory. I will first provide a brief overview of fear conditioning, the most commonly used experimental paradigm for studying negative emotional memory. Then, I will review the role of the hippocampus in this form

of learning. Finally, I will discuss learning situations wherein the learned negative emotional valence of a cue becomes neutral again, as in extinction learning.

2.1 Fear conditioning

Emotion plays an important role in increasing the saliency of a given experience, and fear in particular seems to have an especially potent ability to enhance memory. The ability to remember dangerous situations confers an enormous survival advantage by helping organisms respond to and avoid threats. In classical Pavlovian fear conditioning (Pavlov 1927), a subject forms an association between the conditioned stimulus (CS), a neutral cue with no physiological relevance for the animal, and the unconditioned stimulus (US), an aversive stimulus that produces an innate fear response termed the unconditioned response (UR). After paired presentations of the CS and US, presentations of the CS alone elicit novel fearful behaviors termed conditioned responses (CR), which reflect associative learning. In contextual fear conditioning, the CS is the spatial context in which the aversive US is presented. Since the context involves the association of multiple environmental features into a single cognitive representation of the environment, contextual conditioning only occurs if animals first form a configural representation of the environment (Rudy et al 2004). Therefore, successful fear learning requires two sequential stages: the formation of the contextual representation (i.e. context pre-exposure phase) and the formation of the fear association (i.e. conditioning phase) (Fanselow 1990).

Many different studies have identified the hippocampus as a crucial brain region for contextual fear conditioning. Lesions of the hippocampus have been shown to impair contextual fear learning without affecting cued conditioning, a form of fear learning that involves the association of a discrete cue (CS) with an aversive stimulus (US) (Chen et al 1996, Kim & Fanselow 1992, Phillips & LeDoux 1992). Similarly, pharmacological studies have indicated that the hippocampus is required for contextual fear learning. Hippocampal acetylcholine levels have been shown to increase with contextual fear conditioning (Nail-Boucherie et al 2000), whereas intrahippocampal infusions of scopolamine, a muscarinic receptor antagonist, lead to impaired contextual conditioning (Gale et al 2001, Wallenstein & Vago 2001). Likewise, hippocampal

administration of muscimol, a gamma-aminobutyric acid (GABA) receptor agonist (Holt & Maren 1999) or anisomycin, a protein synthesis inhibitor (Barrientos et al 2002), have been shown to produce fear learning deficits. Interestingly, hippocampal inactivation produced learning deficits when performed after contextual pre-exposure, a day prior to conditioning (Matus-Amat et al 2004). These data suggest that the hippocampus is critical for forming the cognitive representation of the context to be associated with the US.

Although most of the research on the hippocampus has focused on the dorsal region, the ventral hippocampus has also been implicated in fear learning. Impairments of both contextual and cued conditioning have been found after lesions or inactivation of the ventral hippocampus (Esclassan et al 2009, Maren & Holt 2004, Richmond et al 1999, Trivedi & Coover 2004). Additionally, ventral hippocampal infusions of muscimol (Bast et al 2001, Rudy & Matus-Amat 2005), NMDA receptor antagonists (Zhang et al 2001), and anisomycin (Rudy & Matus-Amat 2005) have been shown to cause deficits in contextual fear conditioning. However, while both regions appear to be involved in fear learning, it has been suggested that the dorsal and ventral regions may have somewhat different functions (Fanselow & Dong 2010). In addition to the differing anatomical connections of each of these regions, dorsal and ventral place cells also display distinct firing characteristics. Ventral cells have large, overlapping receptive fields in contrast to the well-defined place fields of dorsal cells (Jung et al 1994, Keinath et al 2014, Kjelstrup et al 2008, O'Keefe & Dostrovsky 1971). These differences have led to the proposal that while the dorsal hippocampus codes specific details of a context, the ventral may be important for generalization among contexts (Keinath et al 2014, Komorowski et al 2013).

At present, only a few studies have examined the effects of fear conditioning on hippocampal place cell activity. These studies suggest that place cells do respond to emotional cues. Interestingly, even though the hippocampus is not necessary for cued conditioning, two studies found that rats trained to associate a tone with a shock showed increases in place cell activity while the animal was traversing particular place fields (Donzis et al 2013, Moita et al 2003). In a separate study, it was also found that contextual conditioning caused place cell remapping in

the short term (Moita et al 2004, Wang et al 2012). More recently, Wang et al. found that the new representations formed after conditioning stabilize in the long term (Wang et al 2012). These data suggest that emotions can have long-lasting effects on place cell activity, which correlates with the enduring quality of emotional memories.

2.2 Extinction

Although it can be advantageous for strong emotions to exert long-lasting effects on memory, organisms must also be able to update their responses accordingly when these emotions are no longer relevant. Persistent fearful responding in a context once the danger has passed can become detrimental due to high opportunity costs and wasted energy expenditure; therefore, organisms must be able to assimilate new information about the environment and change their behavior. The process by which an organism learns that a stimulus no longer predicts a consequence is known as extinction. Typically, extinction training consists of repeated or prolonged exposures to the conditioned stimulus alone, until the subject no longer exhibits a conditioned response (Delamater & Westbrook 2014, Myers & Davis 2002, Myers & Davis 2007). It is widely believed that extinction represents the formation of a novel association in which the CS predicts the absence of the US, rather than erasure of the original CS-US association. Furthermore, it is thought that the new memory trace formed during extinction competes with the original trace at the time of retrieval (Delamater 2004, Myers & Davis 2002, Myers & Davis 2007). Support for the idea that extinction does not involve memory erasure comes from behavioral phenomena such as spontaneous recovery, reinstatement, and renewal, which demonstrate that the conditioned fear response can be recovered after successful extinction training. Spontaneous recovery involves the return of an extinguished conditioned response once a period of time has elapsed after extinction training (Pavlov 1927, Rescorla 2004). Reinstatement involves exposing an animal to a mild US alone after extinction training, a procedure that reinstates the conditioned responses to the CS (Rescorla & Heth 1975, Westbrook et al 2002). Finally, contextual renewal shows that the conditioned response returns when the CS is presented outside the training context (Bouton & Ricker 1994). All these phenomena have been demonstrated across species in various fear

conditioning paradigms indicating that the original fear response is intact and can be retrieved under some circumstances.

Among the properties of extinction, its context specificity has been the subject of widespread attention in the field (Bouton 2002, Bouton et al 2006). The fact that conditioned fear responses can only be reduced in the context where extinction training occurs has limited the effects of therapies aimed at ameliorating anxiety disorders. Therefore, understanding the neural mechanisms of this process is of crucial importance. In this regard, the hippocampus has received substantial attention because current views suggest that this region provides a gating mechanism that modulates the expression of fear or extinction in a context-dependent manner. This is achieved through hippocampal projections to the prelimbic and infralimbic regions of the medial prefrontal cortex (mPFC), areas found to modulate the expression of fear and extinction, respectively (Ji & Maren 2007, Sierra-Mercado et al 2011, Sotres-Bayon et al 2012). This contextual gating hypothesis is supported by studies showing that inactivation of either the dorsal or ventral hippocampus disrupts the context specificity of extinction (Corcoran et al 2005, Corcoran & Maren 2001, Corcoran & Maren 2004) or impairs its acquisition and/or retrieval (Hobin et al 2006, Sierra-Mercado et al 2011). Lesion studies (Ji & Maren 2005) have found similar results, strongly suggesting that an intact hippocampus underlies context-specific extinction learning.

Given the evidence that the hippocampus is involved in both contextual fear conditioning and extinction, it is important to explore whether the same or different subpopulations of hippocampal neurons underlie these processes. Electrophysiological studies characterizing cell responses involved in cued conditioning have revealed distinct subpopulations in the basolateral (Herry et al 2008) and lateral (Knapska et al 2012) nucleus of the amygdala that are selectively active during fear conditioning or extinction. Moreover, these distinct subpopulations display differential connectivity to the hippocampus and mPFC. However, no studies have examined whether separate populations of hippocampal place cells are involved in forming contextual representations during fear conditioning and extinction.

3. Sleep & memory

Sleep can be defined behaviorally as a reversible state that occurs on a circadian cycle, during which the organism maintains a stereotypic posture and exhibits behavioral quiescence and reduced responsiveness to external stimuli. It is a homeostatic process wherein the pressure for sleep increases with time awake, and deprivation generally leads to a compensatory increase once the organism is able to sleep (Amlaner et al 2009). In most mammals and birds (Amlaner & Ball 1994, Tobler 2005), distinct physiological characteristics allow researchers to categorize sleep into two broad stages: rapid eye movement (REM) sleep and non-REM (NREM) sleep. REM sleep, also termed “paradoxical sleep” in older literature, is characterized by muscle atonia and random eye movements, and EEG activity recorded during REM displays relatively fast, low-amplitude waves that strongly resemble those seen in wake states. In contrast, NREM sleep is generally characterized by highly synchronized, low frequency brain waves. Human polysomnography allows for further division of NREM sleep into lighter sleep stages preceding a stage of deep, slow-wave sleep (SWS) (Amlaner et al 2009, Carskadon & Dement 2005, Rechtschaffen & Kales 1968, Zepelin & Rechtschaffen 1974).

Interestingly, sleep undergoes several notable changes over the course of the lifespan. Older subjects in a number of species have significantly more fragmented sleep, experiencing shorter sleep bouts and more awakenings (Dijk et al 2001, Espiritu 2008, Hasan et al 2012, Ingram et al 1983, Ohayon et al 2004, Van Gool & Mirmiran 1983). Moreover, both aged humans and rodents have been reported to exhibit more napping behavior, with a greater tendency to sleep during the typical active period for the species (Buysse et al 1992, Webb 1989, Welsh et al 1986). There is also evidence of diminished sleep efficiency and intensity, with an increased proportion of sleep spent in the lighter stages of NREM rather than slow wave sleep or REM (Ehlers & Kupfer 1989, Gillin et al 1981, Landolt & Borbély 2001). Changes in the sleep EEG have also been observed, most notably reductions in delta power (Landolt & Borbély 2001) and average peak amplitude of delta waves (Smith et al 1977). Some studies have also found decreases in the number, density, and duration of spindles, as well as a slight increase in intraspindle frequency

(Crowley et al 2002, Nicolas et al 2001). These changes in sleep may be the consequence of other biological changes that occur with age, such as alterations in neuroendocrine function (Kern et al 1996, Van Cauter et al 2000) and circadian modulation (Cajochen et al 2006, Czeisler et al 1992, Dijk et al 1999).

Although the overwhelming prevalence of sleep in the animal kingdom suggests that it performs some function vital to life, the actual purpose of sleep is still a heavily debated topic. Earlier hypotheses proposed that sleep might simply be a means of energy conservation or a behavioral adaption to keep organisms quiescent during times of vulnerability (Meddis 1975, Walker & Berger 1980). More recently, it has been posited that sleep may have restorative functions in clearing metabolites from the brain (Xie et al 2013). The most intriguing hypothesis however, may be the idea that sleep plays a key role in memory consolidation (Marshall & Born 2007, Stickgold 2005, Walker & Stickgold 2004). Evidence for a relationship between sleep and memory comes from a wide variety of studies across many species, including humans, rats, mice, birds, and cats (Peigneux et al 2001, Smith 1995).

In this section, I will discuss evidence that alteration in sleep patterns affect learning and memory. I will first describe research highlighting the way sleep characteristics have been reported to change after learning, and then review the evidence that learning is sensitive to sleep deprivation. This discussion will lead up to the research described in Chapter 4, where I examine how changes in sleep patterns affect learning and place cell activity in young and aged adult animals during the object-place recognition task.

3.1 Learning-related changes in sleep characteristics

Both human and rodent studies have found abundant evidence that sleep characteristics change after learning, lending credence to the idea that sleep may be involved in memory consolidation (Peigneux et al 2001, Smith 1995, Walker & Stickgold 2004). Unfortunately, perhaps due to the wide range of species and learning paradigms used, findings have frequently been conflicting or contradictory. In rodents, many early studies reported increases in REM sleep following training on a number of learning tasks, including spatial memory tasks (Smith & Rose

1996, Smith & Rose 1997), operant conditioning (Smith & Wong 1991), and active avoidance (Portell-Cortés et al 1989, Smith et al 1979). Additionally, while most researchers examined sleep occurring within a few hours of training, some studies that recorded sleep for longer periods have reported that increases in REM can be observed for days after learning (Smith et al 1979). However, not all studies found increases in REM sleep and in fact, several studies found reductions of REM sleep following fear conditioning (Jha et al 2005, Sanford et al 2003a, Sanford et al 2003b, Sanford et al 2003c). Sanford and colleagues found that this suppression of REM sleep could also be seen after exposure to aversive or anxiogenic stimuli (Tang et al 2004), highlighting the difficulty in pinpointing the exact nature of learning-related sleep changes.

Although much of the early research focused on REM sleep, several animal studies have since found evidence supporting a link between NREM sleep and memory as well. Notably, Hellman and Abel reported increases in NREM sleep, but not REM, following fear learning. Interestingly, these changes were only seen in animals undergoing cued fear conditioning, where a tone is followed by shock. Control groups that were exposed to the tone alone or to the tone and shock concurrently did not show this increase in NREM sleep, suggesting that the changes were specifically due to associative learning (Hellman & Abel 2007). Recently, Morawska et al. reported that enhancing NREM sleep after traumatic brain injury served to rescue memory deficits on the novel object recognition task in rats (Morawska et al 2016), providing additional support that NREM sleep may also be important for memory.

In human research, studies have similarly found strong evidence of a relationship between sleep and learning, yet contradictory results again make the specifics of that relationship unclear. Many studies demonstrated a positive correlation between post-training sleep and performance gains on a wide range of declarative and procedural memory tasks (Gais & Born 2004, Smith 2001). From these studies, two main hypotheses have emerged regarding memory consolidation during sleep. First, the dual process hypothesis posits that procedural memories are consolidated during REM sleep while declarative memories are consolidated during NREM sleep (Plihal & Born 1997). Second, the sequential hypothesis (Giuditta et al 1995) suggests that cycles of NREM and REM

sleep are necessary for memory consolidation (For review, see Rasch & Born 2013). Attempts to corroborate these different ideas by characterizing post-learning sleep have led to conflicting findings, sometimes even with the use of the same learning paradigm. Moreover, there is evidence that subject age may be an additional critical variable, as some studies have suggested that older subjects only benefit from post-training sleep on certain declarative memory tasks but not procedural memory tasks (Pace-Schott & Spencer 2011, Wilson et al 2012). Therefore, while it seems clear that post-training sleep interacts with memory, the precise mechanisms involved require further elucidation.

Some of the most convincing evidence for sleep-dependent memory consolidation comes from research documenting replay, a particularly interesting phenomenon which occurs during post-training sleep. Replay is the reactivation during sleep of specific patterns of neural activity seen during wake, and it has been suggested as a mechanism for memory consolidation (O'Neill et al 2010, Sutherland & McNaughton 2000). The active system consolidation hypothesis proposes that repeated reactivation of neurons during slow wave sleep may tag specific synapses that are then strengthened during REM sleep, thus enhancing learning and stabilizing the memory trace (Diekelmann & Born 2010, Marshall & Born 2007). Alternatively, it has been suggested that sleep may instead enhance memory by downscaling synapse strengths and restoring homeostasis, thus enhancing signal to noise ratio (Tononi & Cirelli 2003). In rodents, replay has been observed in several regions including the CA1 layer of the hippocampus (Pavlides & Winson 1989, Skaggs & McNaughton 1996, Wilson & McNaughton 1994b), the medial prefrontal cortex (Euston et al 2007, Peyrache et al 2009), and the visual cortex (Ji & Wilson 2007). Furthermore, replay has also been found to occur during sleep in the motor cortex analog of zebra finches, where it is thought to aid in song learning (Dave & Margoliash 2000), and in the non-human primate neocortex (Hoffman & McNaughton 2002). Additionally, although difficult to study with the same level of precision, there is evidence that in humans, brain regions active during learning become reactivated during sleep. Several neuroimaging studies using positron emission topography (PET) or functional magnetic resonance imaging (fMRI) techniques have found that, based on changes in cerebral blood flow,

some brain regions that displayed heightened activity during awake training also exhibited heightened activity during sleep (Maquet et al 2000, Peigneux et al 2004, Peigneux et al 2003, Yotsumoto et al 2009). Moreover, several studies have reported that these changes correlate with behavioral performance on a visuomotor task (Peigneux et al 2003) and on a hippocampus-dependent route-learning task where subjects navigated a virtual town (Peigneux et al 2004).

More recently, researchers have begun manipulating replay with external stimuli, thus establishing a more direct, causal link between replay and memory. In human studies, inducing reactivation of specific memories during sleep by means of auditory or olfactory cues have been found to correlate with better recall upon wake (Cousins et al 2014, Rasch et al 2007, Rudoy et al 2009, Schönauer et al 2014, Schreiner & Rasch 2014). Bendor & Wilson demonstrated that auditory cues could also be used to manipulate hippocampal replay in mice (Bendor & Wilson 2012), and de Lavilleon et al further extended this research by pairing intracranial rewarding stimulations with the spontaneous activity of a specific place cell during sleep, thus forming an artificial place-reward association reflected in the animal's waking preference for the place cell's firing field (de Lavilléon et al 2015). Taken together, there is a large body of evidence supporting the hypothesis that sleep plays an important role in memory consolidation.

3.2 Effects of sleep deprivation on memory

Sleep deprivation has a powerful physiological impact on the body, and in extreme cases, these effects can be striking. In 1983, Rechtschaffen and colleagues subjected rats to prolonged, total sleep deprivation and noted a number of major effects, including changes in body temperature, weight, energy expenditure, and overall health. By the end of the study, extended sleep deprivation had proven fatal for every animal in the study after approximately two to three weeks (Rechtschaffen et al 1983). Although these effects were quite dramatic, many other researchers have demonstrated that even an acute period of sleep deprivation can produce significant physiological changes, including hormonal and metabolic effects as well as effects on inflammation and the cardiovascular system (Mullington et al 2009).

In addition to the multitude of biological effects exerted by sleep deprivation, many researchers have documented the cognitive consequences of sleep loss as well (Durmer & Dinges 2009, Lim & Dinges 2010). In humans, sleep deprivation has been shown to have a marked negative effect on vigilant attention and reaction time (Lim & Dinges 2008). It has also been linked to impairments in tests of working memory (Chee et al 2006, Turner et al 2007), decision making (Killgore et al 2006), creativity (Horne 1988), and numerous other simple and complex tasks. Somewhat counter intuitively, many studies suggest that older subjects exhibit greater tolerance for sleep deprivation when assessed on several measures, compared to younger subjects. Specifically, although older subjects frequently had lower baseline performance than young subjects, they showed little change after sleep deprivation, whereas younger subjects displayed significantly impaired performance relative to baseline (Adam et al 2006, Duffy et al 2009, Philip et al 2004, Silva et al 2010, Stenuit & Kerkhofs 2005).

Consistent with the hypothesis that sleep may be particularly important for memory consolidation, sleep deprivation conducted immediately following training has also been linked to performance decrements in many forms of learning. In humans, sleep deprivation has been associated most strongly with impairments of procedural memory (Fischer et al 2005, Gais et al 2000, Stickgold et al 2000), although there is also evidence that sleep deprivation can impair declarative memory depending on the task used (Diekelmann et al 2009, Gais et al 2006). However, findings in the rodent literature strongly suggest that hippocampus-dependent memory is particularly sensitive to sleep deprivation. Smith & Rose reported that REM sleep deprivation after training impaired performance on the hidden platform version of the Morris water maze, but not on the visible platform version, which is hippocampus-independent (Smith & Rose 1996). Similarly, Graves et al found that hippocampus-mediated contextual fear conditioning in mice was impaired by post-training sleep deprivation while cued fear conditioning, which is largely amygdala-dependent, remained intact (Graves et al 2003). Many other studies have reported similar deficits following post-training sleep deprivation (Beaulieu & Godbout 2000, Smith & Kelly 1988, Smith & MacNeill 1994, Smith et al 1998). In sum, there is substantial consensus about the negative effects

of sleep deprivation on memory in young adult animals. However, much less is known about how sleep deprivation affects memory in aged animals.

Many attempts have been made to identify a critical time window following learning, during which sleep deprivation has the greatest impact. Periods of four to six hours of sleep deprivation have been demonstrated to impair learning on the Morris water maze (Smith & Rose 1996), contextual fear conditioning (Graves et al 2003), and novel object recognition (Palchykova et al 2006) when administered immediately after learning, but not if administered after a delay of four hours or more. More recently, a study by Prince et al. reported that a three-hour period of sleep deprivation, beginning one hour after training, impaired hippocampal long-term potentiation (LTP) and performance on the hippocampus-dependent object-place recognition task (Prince et al 2014). Despite a few early studies that have found evidence that sleep deprivation can impair learning even when administered days after training (Smith & Kelly 1988), the majority have found that sleep deprivation is most effective when administered closely after learning. Crucially, there has been little investigation of the critical time window for sleep in old animals. This is quite important, as it has been shown that consolidation processes in old animals may be much longer than those observed in younger populations (Ingvar et al 1985, Rattan 1996, Ryazanov & Nefsky 2002).

Although the overwhelming majority of studies has found sleep deprivation to have negative consequences, it has been demonstrated to have positive effects in some select cases. For example, 40-60% of patients who undergo acute total sleep deprivation show improvements in depressive symptoms the following day or during deprivation (Giedke & Schwärzler 2002). Moreover, because sleep is a homeostatic process that resists deprivation, acute total sleep deprivation generally leads to a period of recovery sleep characterized by enhanced REM and NREM sleep intensity, and improved sleep efficiency as compared to normal baseline sleep (Mistlberger et al 1983, Nakazawa et al 1978). Some studies have reported that sleep deprivation can have neuroprotective effects for stroke (Cam et al 2013) or traumatic brain injury (Martinez-Vargas et al 2012), possibly because of subsequently enhanced recovery sleep. Indeed, a recent study demonstrated that enhanced NREM sleep, whether induced pharmacologically or by acute

sleep restriction, rescued cognitive deficits seen after traumatic brain injury (Morawska et al 2016). Interestingly, there is evidence that recovery sleep may also differ by age. Some early studies in humans reported that while young subjects had increased amounts of slow wave sleep during recovery, older subjects showed much smaller increases in total sleep (Bonnet & Rosa 1987). Old rats similarly have been found to exhibit smaller increases in overall sleep time after sleep deprivation compared to younger animals (Mendelson & Bergmann 2000). In sum, numerous studies have found evidence suggesting that sleep may play a crucial role in memory consolidation, but these effects appear to be modulated by factors such as task demand and subject age. Furthermore, the neurophysiological consequences of sleep deprivation are not yet fully understood.

4. Conclusion

A growing body of evidence indicates that memory consolidation can be affected by many different factors, including emotion and sleep. However, the effects of these factors appear to be modulated by numerous other variables. Moreover, these variables and factors may also interact in ways that differentially impact memory consolidation. For example, some studies have reported that sleep enhances emotional memories more than neutral memories (Nishida et al 2009, Payne et al 2008, Wagner et al 2001), while others have found that emotional learning produces changes in subsequent sleep (Jha et al 2005, Madan et al 2008). Thus, it is plausible that alterations in sleep patterns play a role in the underlying mechanisms responsible for the persistent nature of emotional memory. Interestingly, both sleep architecture (Espiritu 2008) and emotion regulation (Carstensen et al 2003) have been found to change over the course of the lifespan, thus opening the possibility that some age-related changes in cognition may stem from interactions in this area. Elucidating how each of these factors can affect hippocampus-dependent memory consolidation paves the way for developing a more holistic understanding of learning and memory.

CHAPTER 2: HDAC I inhibition in the dorsal and ventral hippocampus differentially modulates predator-odor fear learning and generalization

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Abstract

Although predator odors are ethologically relevant stimuli for rodents, the molecular pathways and contribution of some brain regions involved remain elusive. Inhibition of histone deacetylases (HDACs) in the dorsal hippocampus has been shown to enhance shock-induced contextual fear learning, but it is unknown if HDACs have differential effects along the dorso-ventral hippocampal axis during predator odor fear learning. We injected MS-275, a class I HDAC inhibitor, bilaterally in the dorsal or ventral hippocampus of mice and found that it had no effects on innate anxiety in either region. We then assessed the effects of MS-275 at different stages of fear learning along the longitudinal hippocampal axis. Animals were injected with MS-275 or vehicle after context pre-exposure (pre-conditioning injections), when a representation of the context is first formed, or after exposure to coyote urine (post-conditioning injections), when the context becomes associated with predator odor. When MS-275 was administered after context pre-exposure, dorsally injected animals showed enhanced fear in the training context but were able to discriminate it from a neutral environment. Conversely, ventrally injected animals did not display enhanced learning in the training context but generalized the fear response to a neutral context. However, when MS-275 was administered after conditioning, there were no differences between MS-275 or vehicle control groups in either the dorsal or ventral hippocampus. Surprisingly, all groups displayed generalization to a neutral context, suggesting that predator odor exposure followed by the restraint necessary for the injections leads to fear generalization. These results may elucidate distinct functions of the dorsal and ventral hippocampus in predator odor-induced fear conditioning as well as some of the molecular mechanisms underlying fear generalization.

Introduction

Predator odors are ethologically relevant stimuli that have been shown to elicit a variety of defensive responses in rodents (Blanchard and Blanchard, 1990; Dielenberg and McGregor, 2001; Wallace and Rosen, 2000; Wang et al., 2013; Zangrossi and File, 1992), and, under some conditions, can also produce conditioning (Blanchard et al., 2001; Dielenberg et al., 2001; Takahashi et al., 2008). Contextual fear conditioning, including predator odor fear learning, involves the association of a context (the conditioned stimulus, CS) with a predator odor (unconditioned stimulus, US), which leads to the emergence of a conditioned fear response (CR) in response to the context CS (Anagnostaras et al., 2001; Fanselow, 2000; Maren and Holt, 2000; Rosen, 2004). We have recently developed and characterized a predator odor fear conditioning paradigm using coyote urine that is effective with mice. We showed that this paradigm produces moderate but consistent freezing, a stereotypic response to fear observed in rodents, during long-term retrieval tests. This response is not observed when animals are exposed to water (no odor) or an aversive non-fearful odor (2-methyl butyric acid), indicating that the freezing is a result of associative learning (Wang et al., 2013; Wang et al, 2015). Furthermore, the conditioned fear response is context specific since freezing is observed only in the training context, and it requires both the dorsal and ventral hippocampus (Wang et al., 2013).

Using this model, we recently found that spatial representations formed in the dorsal hippocampus after predator odor fear conditioning are stable in the long term (Wang et al., 2012) but become unstable again during extinction (Wang et al., 2015), suggesting that predator odor learning alters the stability of the dorsal hippocampal representation of context. These findings correlate with numerous studies indicating that the dorsal hippocampus receives preprocessed spatial information (Witter et al., 2013) and thus plays a critical role forming representations of context during conditioning (for review, see Maren and Holt, 2000); however, the role of the ventral region remains unclear. Clarifying the role of the ventral hippocampus for predator odor fear learning is particularly important because this region receives most of the olfactory inputs from the medial and posterior amygdala (Kemppainen et al., 2002; Pitkanen et al., 2000), areas that receive

projections from the main and accessory olfactory system involved in predator odor processing (Masini et al., 2010). Moreover, in addition to these neuroanatomical differences, ventral and dorsal cells display distinct firing characteristics, further suggesting that these regions may have different functions. Cells in the dorsal hippocampus fire in specific circumscribed locations, whereas ventral cells have large and overlapping receptive fields (Keinath et al., 2014; Kjelstrup et al., 2008; O'Keefe and Dostrovsky, 1971). Based on these differences, it has been suggested that the dorsal region may be important for minimizing memory interference by coding specific aspects of contexts, while the ventral hippocampus may play a role in contextual generalization (Keinath et al., 2014; Komorowski et al., 2013). However, no studies have directly tested if these differential functions play a role in contextual fear learning.

On the molecular level, the formation of new memories requires alterations in gene transcription, which lead to the translation of proteins necessary for the cellular changes implicated in long-term memory (for review, see Kandel, 2012). This occurs through modifications of chromatin, a DNA-protein complex. The basic unit of chromatin is the nucleosome, which consists of DNA wrapped around four histone proteins. Modifying these proteins through processes such as acetylation, phosphorylation, and methylation changes the state of the chromatin, influencing the rate of transcription by making the DNA more or less accessible (Levenson and Sweatt, 2005; Wood et al., 2006). The most studied and well understood of these modifications in relation to memory is histone acetylation, a process that facilitates gene transcription by relaxing chromatin structure. This, in turn, leads to synthesis of proteins necessary for long-term memory (Peixoto and Abel, 2013). Histone acetylation is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), enzymes that increase and decrease acetylation, respectively (for review, see Day and Sweatt, 2011; Levenson and Sweatt, 2005). Evidence from experiments investigating the correlation between histone modifications and long-term memory in mice suggests that changes in acetylation are essential for hippocampus-dependent fear learning using electrical shock (Bahari-Javan et al., 2012; Vecsey et al., 2007). However, it is unknown what role histone acetylation plays in predator odor conditioning and whether the dorsal and ventral hippocampus

differentially respond to chromatin alterations.

Here, we investigated the effects of the HDAC inhibitor MS-275, a class I-specific inhibitor (Beckers et al., 2007; Formisano et al., 2015; Khan et al., 2008; Simonini et al., 2006), in the dorsal and ventral hippocampus on innate anxiety and during predator odor fear conditioning. We found that MS-275 had no effect on traditional anxiety tests. However, injections of MS-275 after context pre-exposure (pre-conditioning injections) had different roles in the dorsal and ventral hippocampus, leading to enhanced fear and generalization, respectively. Interestingly, although injections after conditioning did not have effects in any of the groups, all conditions displayed fear generalization, suggesting that animals generalize fear to neutral contexts when a stressor, such as restraint, follows immediately after predator odor exposure. These results extend our understanding of hippocampal function during fear learning and provide insights about the learning contingencies that could lead to fear generalization.

Materials and Methods

Animals. Male C57BL/6 mice 2–5 months of age (Jackson Laboratories, Bar Harbor, ME) were housed individually, kept on a 12-h light/dark cycle, and allowed access to food and water *ad libitum* for at least 1 week prior to beginning behavioral experiments. All experiments were approved by the Institution of Animal Care and Use Committee of the University of Pennsylvania, and were carried out in accordance with NIH guidelines.

***Anxiety measures*.** Animals were run in the open-field test and black-white box to determine the effects of MS-275 on innate anxiety. MS-275 was infused bilaterally into either the dorsal or ventral hippocampus 1-2 hours before conducting anxiety tests. For the open field test, mice were placed in the center of a large cylindrical arena (70 cm in diameter) with a white base and walls. The arena was illuminated by a ceiling-mounted array of eight 60-watt lights arranged symmetrically around the perimeter, approximately 1.8m above the base. Explorative behavior was recorded for 20 minutes. Additionally, we evaluated freezing by calculating the percent time the animals remain immobile, except for respiratory movements. For the black/white two compartment box, we used a plastic test box divided into two equal compartments (22 x 24cm) connected by a small opening.

The black compartment was darkened with black contact paper and covered with a piece of cardboard, while the open-topped white compartment was lined with white contact paper and illuminated by three ceiling-mounted 60-watt lights approximately 1 m above the apparatus, aimed at the center of the compartment. Mice were placed in the center of the white compartment facing the black side, and explorative behavior was recorded for 3 minutes (see behavioral analysis for quantification details below).

Contextual fear conditioning and context discrimination. Prior to the start of behavioral experiments, animals were handled and restrained twice a day for two consecutive days. Animals were then conditioned using a predator odor contextual fear conditioning paradigm previously characterized in our lab (Wang et al., 2013). We have demonstrated in several studies that this paradigm produces moderate but consistent increases in freezing, which are not seen when animals undergo the same schedule of context exposures with no odor exposure or with exposure to a non-fearful odor (Wang et al., 2012, 2013, 2015). On day 1, mice were habituated to a cylindrical training context (baseline context A, blA) and an equivalently sized neutral context (baseline context B, blB) for 10 minutes each, one day before conditioning. Both contexts were 35 cm in diameter and had distinct configurations of black visual cues on the cylinder's white walls; additionally, the contexts were placed in separate rooms. The next day (day 2, 24 hr after baseline context exposures), a paper towel square (2cm x 2cm) saturated with 40 drops of coyote urine (Maine Outdoor Solutions, Harmon, MN) was placed in the center of context A, and mice were re-exposed to the context for 4 minutes in the presence of the odor (conditioning session, cond). A short-term retrieval test was conducted 1 hour later in context A without odor for 10 min. We have previously shown that this retrieval session is important for the consolidation of the fear representation in the long term (Wang et al., 2012). The following day (day 3, 24 hours after conditioning), a long-term retrieval test was conducted in both context A and context B for ten minutes without odor (24h A and 24h B). The order of context A and context B during baseline and the 24 hr retrieval test was counterbalanced across animals. At all time points other than the conditioning session, a paper towel square saturated with water was placed in the center of the

context. MS-275 or DMSO (4%) was administered bilaterally into either the dorsal or ventral hippocampus immediately following the baseline context exposures (pre-conditioning injections) or immediately after the conditioning session (cond; post-conditioning injections). We evaluated freezing as a measure of learning as described below.

Behavioral analysis. All behavioral measures were recorded and analyzed using the Limelight automated tracking system (Coulburn Instruments). For the open-field test, the context was divided into 3 equally spaced concentric circles and the percent time spent in these areas was measured. For the black-white box, the percent time spent in the white compartment of the white/black box was measured, along with the number of reentries to the white side. For conditioning and the open field, freezing was quantified as the percentage of time during which the velocity of the animal was lower than 0.6 cm/sec. Freezing was evaluated using both Lime Light and custom-written MATLAB code. For the MATLAB analysis, the position data were smoothed with a 1 sec boxcar to eliminate jitter in the tracking. Finally, we calculated percent freezing by calculating the percent ratio between freezing at 24 hr and freezing during baseline for each context

Surgery. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally and placed on a stereotaxic apparatus in a flat skull position (David Kopf Instruments, Tujunga, CA). 26 gauge guide cannulas (Plastics One, Roanoke, VA) were implanted bilaterally in either the dorsal or ventral hippocampus at the following coordinates, measured from Bregma in mm. Dorsal: AP, -1.7; ML, ± 1.5; DV: -1.0 (internal cannulas project an additional 0.7mm beyond guides for an injection depth of -1.7). Ventral: AP, -3.0; ML, ± 2.8; DV: -2.0 (internal cannulas project an additional 1.5 mm beyond guides for an injection depth of -3.5). An anchor screw was placed just anterior to lambda, and cannulas were affixed to the skull with cyanoacrylate and dental cement. After surgery, animals were allowed to recover for one week prior to behavioral experiments.

Bilateral hippocampal injections and drug concentration in the hippocampus. MS-275 (SelleckChem, Houston, TX) was diluted to 1 mM using 4% dimethyl sulfoxide (DMSO) in ACSF, then infused bilaterally into either the dorsal or ventral hippocampus through the implanted guide

cannulas using a standard infusion syringe pump (Harvard Apparatus, Holliston, MA). Total volume injected was 0.5 µl on each side at a rate of 0.5 µl/min. Controls were injected with an equivalent volume of vehicle (4% DMSO in artificial cerebrospinal fluid). We estimated the concentration of MS-275 in the hippocampus to be roughly 71µM, since the average volume of the hippocampus in C57bl6 mice is 28 mm^3, with each hemisphere being approximately 14 mm^3 and each dorsal and ventral sub-regions around 7 mm^3 (Peirce et al., 2003). This concentration is well above the dosages that are effective in inhibiting class I HDACs (Hu et al., 2003; Khan et al., 2008). The increase in acetylation produced by MS-275 (Formisano et al., 2015; Simonini, 2006) is evident 2 hr after drug treatment and persists for up to 8 hr (Simonini, 2006). Injections were performed in a room separate from all behavioral experiments and were given after contextual pre-exposure (pre-conditioning injections) or after predator odor exposure (post-conditioning injections).

***Histology*.** To verify cannula placements, animals were sacrificed after behavioral experiments. Brains were removed and fixed at 4°C with 10% formalin for at least 24 hours. They were then transferred to a 30% sucrose solution and kept for at least 48 hours at 4°C for cryoprotection. Brains were then cryosectioned (35 µm, coronal) and Nissl stained with cresyl violet using standard histological procedures (Powers and Clark, 1955).

Statistics. Independent t-tests were used to evaluate anxiety measures. Two-way ANOVAS with repeated measures were used to compare baseline and post-conditioning freezing in the training and neutral context. Student Newman Keuls post hoc tests were used to determine significant differences. Independent and paired t-tests were used to evaluate percent of freezing relative to baseline.

Results

MS-275 has no effect on innate anxiety

Since we wanted to establish the effects of HDAC inhibition on fear learning, we first investigated whether MS-275 had any effects on innate anxiety. We performed bilateral injections of MS-275 or vehicle into the dorsal or ventral hippocampus of animals 2-3 hours prior to behavioral testing in the open field and the black/white box (Figure 1A,B). The open field test, which consists

of free exploration in a large arena, evaluates the anxiety that rodents exhibit in open spaces. It is well established that rodents find the inner areas of the open field more anxiogenic than the outer areas (Hall, 1934; Prut and Belzung, 2003). Thus, differences in the amount of time spent in these areas normally reflect distinct levels of anxiety. Thirty-four animals were injected with MS-275 (dorsal: N=20; ventral: N=14), and 41 animals were injected with vehicle (dorsal: N=21; ventral: N=20). We divided the open field in three concentric areas and calculated the time spent in each of these zones. We did not find any differences between the groups in the percentage of time spent in the three concentric regions comprising the open field (Figure 1C,D; dorsal: center: $p = 0.80$, inner: $p = 0.36$, outer: $p = 0.62$; ventral: center: $p = 0.57$, inner: $p = 0.30$, outer: $p = 0.46$). Additionally, we did not find differences in the levels of freezing in the open field (Figure 1E,F; dorsal: $p = 0.45$; ventral: $p = 0.66$)

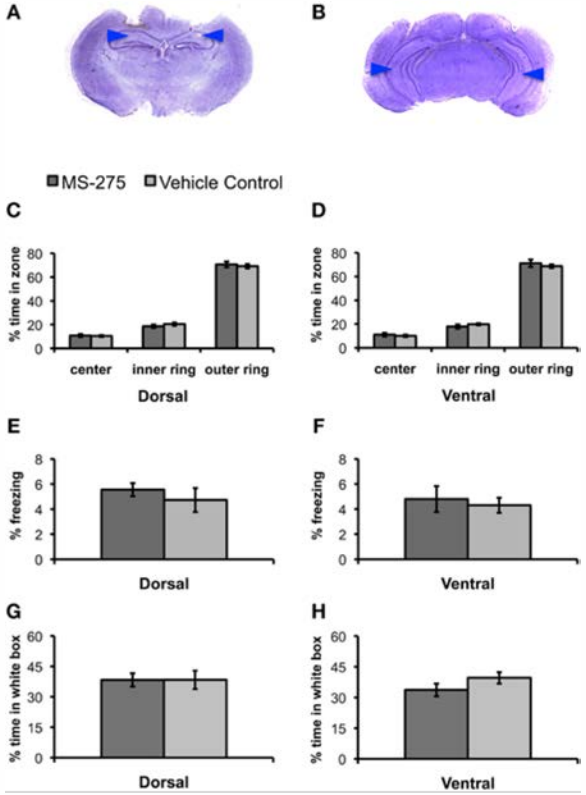

Figure 1 (A,B) Photomicrograph of Nissl-stained coronal section showing position of dorsal **(A)** and ventral **(B)** guide cannulas. Note that internal cannulas for dorsal injections project an additional 0.7 mm beyond guide cannulas, and internal cannulas for ventral injections project an additional 1.5 mm beyond guide cannulas. Blue arrows indicate estimated injection depth. **(C–H)** Animals injected with MS-275 or vehicle in either the dorsal or ventral hippocampus showed no significant differences in tests of anxiety. **(C–F)** Animals injected with MS-275 (dorsal: N = 20; ventral: N = 14) or vehicle (dorsal: N = 21; ventral: N = 20) showed no significant differences in behavior in the open field test. This was evident in the percentage of time spent in the center, inner ring, and outer ring of an open field in the dorsal **(C)** and ventral **(D)** groups, as well as the level of freezing observed in the open field in the dorsal **(E)** and ventral **(F)** groups. **(G,H)** Animals injected with MS-275 (dorsal: N = 12; ventral: N = 11) or vehicle (dorsal: N = 12; ventral: N = 11) in the dorsal **(G)** or ventral **(H)** hippocampus showed no significant difference in the percentage of time they spent in the white compartment of the black/white box. Means ± SEM are shown.

Next, we evaluated the effects of MS-275 on behavior in a black/white two-compartment box (Crawley and Goodwin, 1980; Sánchez, 1997). The animals are initially placed in the open-topped white

compartment, and the time they spend in this compartment versus the dark covered side of the chamber is measured while animal freely move across these areas. Rodents find bright, open environments more anxiogenic than closed, dark ones (Sánchez, 1995); therefore, increased anxiety is typically seen through a reduction in the amount of time spent exploring the white compartment. Twenty-three animals were injected with MS-275 (dorsal: N=12; ventral: N=11) and 23 animals were injected with vehicle (dorsal: N=12; ventral: N=11). We did not find significant differences between the groups in the percentage of time spent in the white compartment (Figure 1G,H, dorsal: $p = 0.99$; ventral: $p = 0.17$) or the number of reentries to the white compartment (dorsal: $p = 0.29$; ventral: $p = 0.24$; data not shown). These results suggest that MS-275 does not affect innate anxiety in the dorsal or ventral hippocampus.

Effects of MS-275 on fear learning

A. Inhibition of class I HDACs following contextual pre-exposure produces enhanced fear in the dorsal hippocampus and generalization in the ventral region

Several researchers have suggested that successful contextual conditioning consists of two stages. In the first stage, a representation of the context is formed, while in the second stage, the context is associated with the US (Fanselow and Rudy, 1998; Fanselow, 2000; O'Reilly and Rudy, 2001; Rudy et al., 2004; Rudy and O'Reilly, 1999; Rudy and O'Reilly, 2001; Young et al., 1994). This view is based on the observation that animals display learning deficits in the absence of contextual pre-exposure (Fanselow, 1990). Our predator odor paradigm is ideal for testing the contributions of the hippocampus to these two learning stages because animals are exposed to the context one day prior to conditioning, which provides an optimal time window to explore the effects of HDAC inhibition either during the context pre-exposure (pre-conditioning) or the associative phase (post-conditioning).

To determine the role of histone acetylation during the formation of a representation of context, MS-275 or vehicle (DMSO, 4%) was injected in either the dorsal or ventral hippocampus after exposure to the training context (baseline context A) and a neutral context (baseline context B) on Day 1 (pre-conditioning injections). Twenty-four hours after the injections (Day 2), these

groups were conditioned and tested 1 hr after conditioning in the training context A. On day 3, the four groups were retested in the training (A) and neutral (B) context 24 hr after conditioning (long-term retrieval test; see Figure 2A). The order of exposure to context A and B during baseline and the 24 hr retrieval tests was counterbalanced across animals.

Since the dorsal hippocampus has been implicated in coding specific information about contexts (Nadel et al., 2013), and cells in this region are sensitive to subtle contextual changes (Colgin et al., 2008), we hypothesized that dorsal injections of MS-275 after context pre-exposure (pre-conditioning injections) should lead to enhanced learning because animals would remember the training context in more detail. These injections, however, would not substantially decrease the ability of the animals to discriminate the training and neutral contexts because the specific information about each context would be remembered. Conversely, since spatial representations in the ventral hippocampus are large and overlapping, a characteristic that may facilitate generalization (Keinath et al., 2014; Komorowski et al., 2013), we hypothesized that ventral injections after context pre-exposure (pre-conditioning injections) would lead to fear generalization to a neutral context.

There were no differences in baseline freezing prior to conditioning in the dorsal groups (MS-275: N=13; vehicle control: N=16). In support of our hypothesis, we found that dorsal injections significantly increased freezing in the training context (context A) in the MS-275 group in comparison to the vehicle control group. (Figure 2B; effect of group: $F_{1,27}$=4.50, $p<0.05$, effect of session: $F_{3,81}$=17.56, $p<0.001$; interaction: $F_{3,81}$=2.95, $p<0.04$). Post hoc Student-Newman-Keuls tests (SNKTs) showed that freezing levels were comparable in the control and MS-275 groups during baseline (blA: p=0.40; blB: p=0.99), but were significantly different during the post-conditioning 24 hr test in context A (24hr: $p<0.003$). Furthermore, although MS-275 also produced a significant increase in freezing in the neutral context (B) (SNKTs: 24hrB: $p<0.03$), both the vehicle and MS-275 groups, were able to discriminate the training context A from the neutral context B, which was evident in significantly higher levels of freezing in context A (vehicle control: $p<0.05$; MS-275: $p<0.005$). The differences between the MS-275 and vehicle groups are also evident in a

significant percent increase in freezing observed 24 hr after conditioning relative to baseline in context A (Figure 2C; t_{27}=-2.07, p<0.05) and a trend in context B (t_{28}=-1.82, p=0.08). Again, when we compared the percent increase in freezing from baseline within each group, both the vehicle control and MS-275 groups displayed higher freezing in context A than B at 24 hr relative to the freezing baseline in each context (MS-275 (trend toward significance): t_{11}=2.13, p=0.056; vehicle control: t_{19}=2.63, p<0.02). These results suggest that MS-275 enhances the consolidation of training context memory without disrupting the ability of the animals to discriminate between the fearful context and a neutral one.

We did not observe differences in baseline freezing in the ventral groups (MS-275: N=17; vehicle control: N=15) receiving injections after contextual pre-exposure (pre-conditioning). Even though MS-275 did not significantly affect learning, the HDAC inhibitor produced fear generalization in response to the neutral context B. This was evident in higher freezing levels in the neutral context in animals injected with MS-275 than vehicle-injected controls (Figure 2D; effect of group: $F_{1,31}$=0.09, p=0.77, effect of session: $F_{3,89}$=25.19, p<0.001; interaction: $F_{3,89}$=2.75, p<0.05). SNKTs showed no differences prior to conditioning or after conditioning in the training context (P>0.05). However, the groups were significantly different in the neutral context B (24hr B: p<0.04). Furthermore, while the control animals discriminated between the training and neutral context (p<0.003), the MS-275 group did not (p=0.865). These effects are also evident when we examined the percent increase in freezing at 24hr after conditioning relative to baseline in each context, showing that there were no differences in the training context A (t_{35}=-0.97, p=0.34) but significantly higher freezing in the MS-275 group in context B (t_{35}=2.36, p<0.03; Figure 2E). Importantly, while vehicle control animals clearly discriminated between the contexts (t_{16}=2.36, p=0.03), MS-275 animals showed no difference in freezing in context A and B (t_{19}=0.08, p=0.94). These results suggest that HDAC I inhibition in the dorsal and ventral hippocampus plays different roles in predator odor fear memory. MS-275 leads to enhanced fear memory in the dorsal hippocampus and fear generalization in the ventral region.

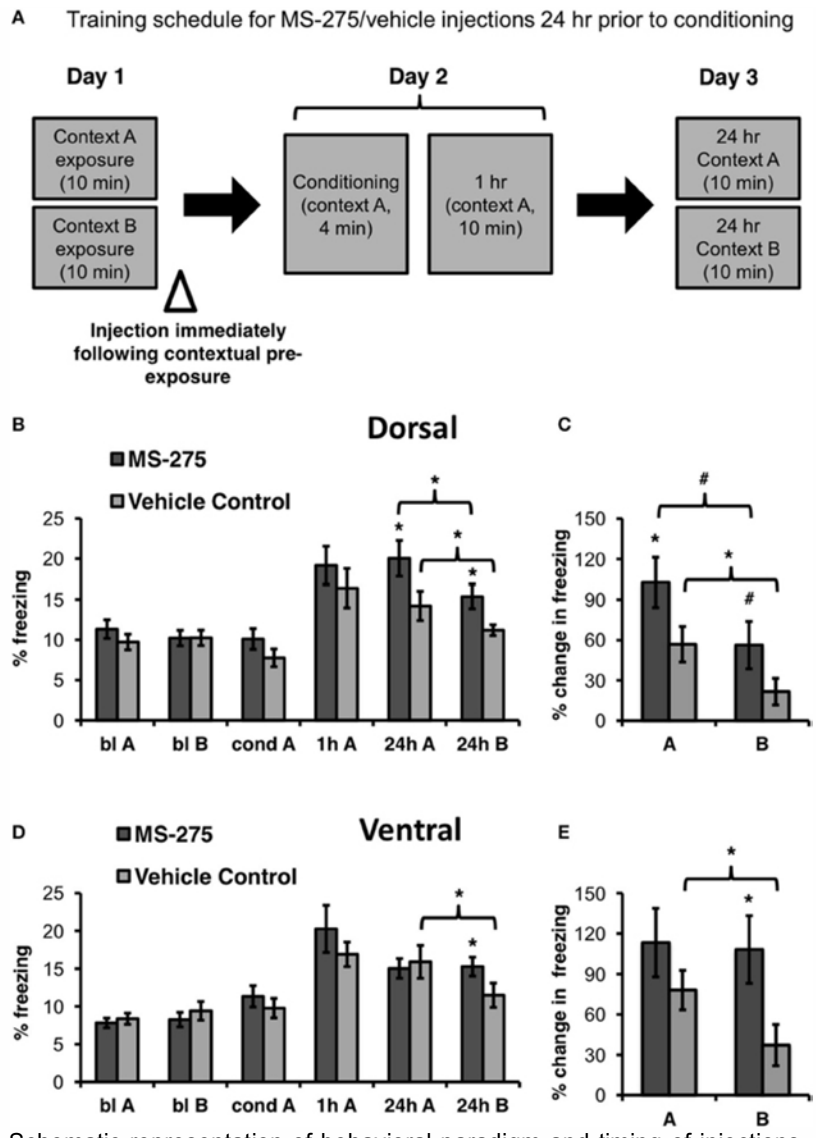

Figure 2 (A) Schematic representation of behavioral paradigm and timing of injections. Animals were injected with MS-275 or vehicle immediately following exposure to contexts A and B, 24 h prior to fear conditioning. **(B)** Animals injected with MS-275 (N = 13) in the dorsal hippocampus after contextual pre-exposure (bl A, bl B) exhibited enhanced fear learning in context A and elevated freezing in context B 24 h after conditioning in comparison to the vehicle-injected controls (N = 16). However, freezing was significantly higher in context A, the conditioning context, than in the neutral context B, indicating that these animals still discriminated between the environments. **(C)** Animals injected with MS-275 in the dorsal hippocampus exhibited a significantly greater percent change in freezing 24 h post-conditioning in context A and a trend toward enhanced freezing in context B relative to baseline freezing in each of these contexts in comparison to controls. **(D)** Animals injected with MS-275 (N = 17) in the ventral hippocampus after contextual pre-exposure exhibited fear generalization without showing enhanced fear learning 24 h after conditioning; This generalization effect was not observed in control animals injected with vehicle (N = 15). **(E)** Animals injected with MS-275 in the ventral hippocampus exhibit significantly greater percent change in freezing 24 h post-conditioning in context B relative to baseline in that context in comparison to vehicle-injected controls. No difference in percent change in freezing was observed in the training context A between the MS-275 and vehicle control animals. Means ± SEM are shown, *p < 0.05, #p < 0.09.

B. Inhibition of class I HDACs following conditioning has no effect on fear learning

To determine the role of histone acetylation along the longitudinal hippocampal axis after coyote exposure, we then examined groups of mice injected with MS-275 or vehicle (DMSO, 4%) after conditioning (see Figure 3A). The amygdala has been identified as a critical brain region for the associative phase of predator odor fear learning (for review, see Takahashi et al., 2008). In particular, it has been demonstrated that the medial amygdala plays a role during acquisition (Blanchard et al., 2005; Takahashi et al., 2007) and the basolateral amygdala during consolidation (Takahashi et al., 2007). Since the dorsal hippocampus receives very few projections from the basolateral amygdala and no projections from the medial amygdala, the area where olfactory information converges (Pikkarainen et al., 1999), we hypothesized that dorsal injections of MS-275 would not affect predator odor fear learning. Conversely, the ventral hippocampus receives strong projections from the posterior, medial, and basolateral amygdala (Kemppainen et al., 2002; Pitkanen et al., 2000) as well as structures associated with the hypothalamic-pituitary adrenal axis (Witter, 1986), which suggests that the ventral hippocampus may also be involved in processing anxiety and fear. However, since we did not observe any effects of MS-275 on anxiety measures in the ventral region, we hypothesized that HDAC I inhibition after conditioning would not have an effect on the conditioning phase in the ventral hippocampus.

In agreement with our predictions, we found no significant effect of MS-275 in the dorsal hippocampus (effect of group: $F_{1,33}$=0.25, p=0.62, interaction: $F_{3,91}$=0.06, p=0.98). The MS-275 (N=11) and vehicle control (N=11) groups only showed an effect of session reflecting that freezing was significantly higher after learning (Figure 3B; effect of session: $F_{3,91}$=17.04, p<0.001; post hoc comparisons showed that freezing was significantly higher after conditioning in comparison to baseline in both context A and B, p<0.05). In the ventral groups, we observed a similar pattern. There were also no significant differences between the MS-275 (N=13) and control (N=14) groups (effect of group: $F_{1,28}$=0.51, p=0.48, interaction: $F_{3,75}$=1.13, p=0.34), though the groups displayed a significant effect of session, which reflected that freezing changed during learning (Figure 3C; effect

of session: $F_{3,75}$=27.19, p<0.001, post hoc comparisons showed that freezing was significantly higher after conditioning in comparison to baseline in both context A and B, p<0.05). The fact that all groups exhibited significantly higher freezing in the neutral context B at 24 hours compared to baseline indicates that generalization is not produced by HDAC inhibition. Since all animals are restrained during the injections, we suggest that that the observed generalization occurs as a result of having an additional stressor following predator odor exposure. These findings indicate that predator odor fear learning provides a good model to study fear generalization.

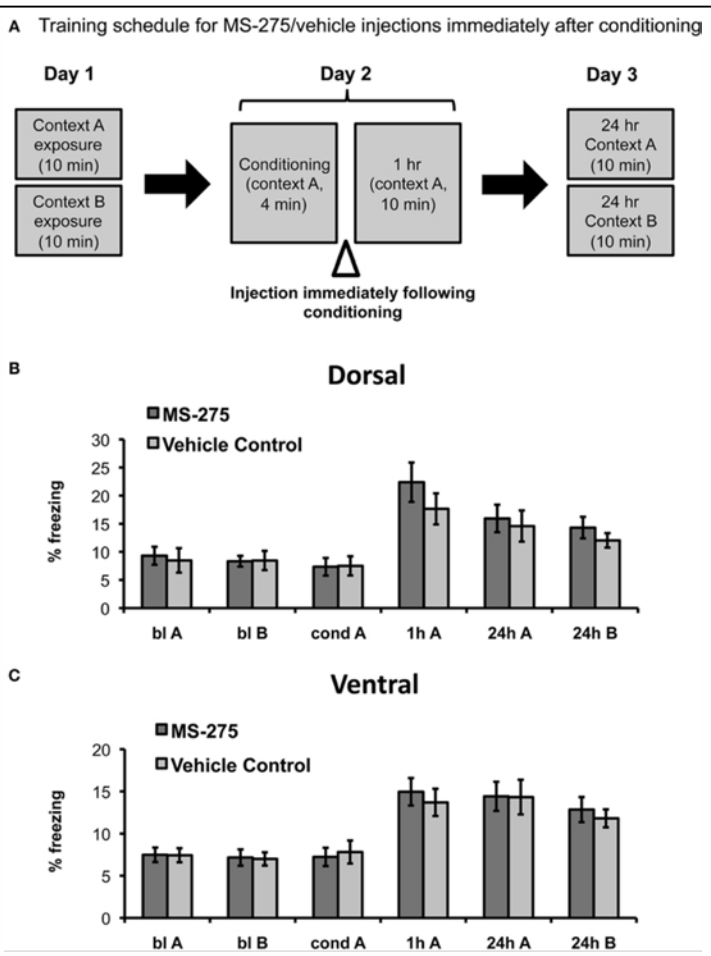

Figure 3 (A) Schematic representation of behavioral paradigm and timing of injections. Animals were injected with MS-275 or vehicle immediately following fear conditioning in context A. Animals injected in the dorsal (**B**; MS-275: N = 11, vehicle: N = 11) or ventral (**C**; MS-275: N = 13, vehicle: N = 14) hippocampus after the conditioning session exhibited fear generalization 24 h after conditioning. Means ± SEM are shown, *p < 0.05.

Discussion

Using predator odor conditioning, we tested the effects of the class I HDAC inhibitor MS-275 in the dorsal and ventral hippocampus on innate anxiety and at different stages of fear learning. We found that HDAC I inhibition after contextual pre-exposure (pre-conditioning) has different effects in the dorsal and ventral hippocampus. In the dorsal hippocampus, HDAC inhibition enhances fear learning, whereas in the ventral hippocampus, it leads to fear generalization.

However, this epigenetic mechanism does not affect learning when HDAC inhibition takes place after conditioning. Interestingly, the presentation of a predator odor followed by a stressor results in fear generalization to a neutral context.

There are several advantages associated with the use of predator odors in fear learning. First, odors are extremely relevant cues to rodents because identifying dangerous odors is critical for survival (Brennan and Keverne, 1997; Luo et al., 2003; Restrepo et al., 2004). Second, predator odors produce innate fear in many species, and thus, are ethologically relevant models (Apfelbach et al., 2005; Ferrero et al., 2011; Rosen et al., 2008). Moreover, predator stress produces long-term changes in behavior, and these changes correlate with persistent alterations in molecular fear and stress pathways (Adamec et al., 2001; Adamec et al., 1998; Adamec and Shallow, 1993; Blanchard and Blanchard, 1989; Wiedenmayer, 2004). Here, we demonstrate that predator urine is a good model to study the molecular mechanisms underlying fear generalization.

Field studies have shown that long-lasting smells, such as predator urine, produce long-term avoidance of spatial locations in many mammalian species (Swihart, 1991; Rossell, 2001), suggesting that animals can recall spatial locations where these odors have been encountered for long periods of time. In this study, as well as previous ones (Wang et al., 2012, 2013, and 2015), we found that coyote urine produces consistent but moderate levels of freezing, which, at first glance, appears surprising in the context of the effects on behavior observed in field studies. However, we have also previously demonstrated that exposure to predator urine has profound and long-lasting effects on hippocampal spatial representations. Specifically, we showed that the spatial map formed after predator odor exposure stabilizes in the long term (Wang et al., 2012), and these changes can only be reversed when animals learn to perceive the context as safe after extinction (Wang et al., 2015). These findings suggest that while conditioned freezing in response to predator urine is moderate, the neurological changes associated with fear learning induced with long lasting predator smells are persistent.

Prey animals are under significant evolutionary pressure to rapidly identify and avoid novel predators, since unguarded encounters may result in death. Consequently, it has been

demonstrated that after exposure to a specific predator, many species are capable of generalizing fear responses to completely novel predators that resemble the one initially encountered (Ferrari et al., 2007; Ferrari et al., 2008; Griffin et al., 2001). It follows that it would also be important for prey animals to generalize defensive fear responses from a particular dangerous context to novel but similar contexts that may also be unsafe. Fear generalization to environments that resemble one in which a threat is originally encountered may be evolutionarily advantageous, since particular types of predators are frequently found in similar habitats. Our data suggest that epigenetic mechanisms within the ventral hippocampus play a role in this process.

It is important to note that although animals injected with MS-275 in the dorsal hippocampus after contextual pre-exposure (pre-conditioning) display increased freezing in a neutral context, the level of generalization observed in these animals is minimal in comparison to ventrally injected mice. This suggests that these animals still differentiate between the training and neutral contexts. Conversely, animals injected with MS-275 in the ventral region cannot discriminate between neutral and fearful contexts. Several studies support the idea that the ventral hippocampus may play a role in fear generalization. In rats, hippocampal place cells have receptive fields of increasing size moving from the dorsal to ventral pole (Kjelstrup et al., 2008). We recently showed that even though ventral populations contain precise spatial information, the broadly tuned nature of the cell's receptive fields favors the involvement of this region in generalization processes (Keinath et al., 2014). Furthermore, the ability of ventral cells to generalize across situations is modulated by learning (Komorowski et al., 2013). These data suggest that in rodents, the ventral region may be critical for extracting commonalities across situations. Interestingly, studies investigating anatomical differences in humans found that in healthy adults, the anterior hippocampus (ventral in rodents) contains a smaller proportion of dentate gyrus than the posterior hippocampus (dorsal in rodents) (Malykhin et al., 2010). This distinction is remarkable because the dentate gyrus is implicated in pattern separation, the process of distinguishing between similar memories (Bakker et al., 2008; Marr, 1971; Rolls and Kesner, 2006), suggesting that the ability of the hippocampus to discriminate between similar memories may decrease along the dorso-ventral

longitudinal axis. In addition, fMRI studies in humans have shown that the anterior (ventral) and posterior (dorsal) hippocampi are activated in different kinds of recall tasks. Thinking of specific spatial details of an event activates the posterior (dorsal) region, while thinking about the general location of the same event activates the anterior (ventral) area (Poppenk et al., 2013). Therefore, data from both rodents and humans suggest that the dorsal and ventral hippocampus may serve different roles in encoding a representation of context. The dorsal hippocampus encodes particular features and allows animals to discriminate between similar situations, whereas the ventral hippocampus appears to facilitate generalization processes. Here we show that the consolidation of these memories involves epigenetic mechanisms.

Our data indicate that inhibition of class I HDACs does not have an effect on innate anxiety and/or the conditioning phase of predator fear learning. Since previous studies suggest that the ventral hippocampus plays a role in anxiety (Bannerman et al., 2004; Kheirbek et al., 2013), it is possible that other epigenetic mechanims modulate anxiety in this region. MS-275 preferentially inhibits HDAC1 over HDAC2/3, and has no effect on other HDACs (Formisano et al., 2015; Khan et al., 2008). Previous studies have found that HDAC1 regulates DNA repair in neurons (Wang, Pan et al., 2013) and modulates fear extinction (Bahari-Javan et al., 2012), HDAC2 plays an important role in several forms of spatial memory (Guan et al., 2009), and HDAC3 enhances long-term contextual fear memory (McQuown et al., 2011), all of which suggest that distinct epigenetic mechanisms modulate different aspects of memory consolidation. Interestingly, a recent study demonstrated that Class II HDAC inhibitors also regulate hippocampus-dependent learning and plasticity (Kim et al., 2012). It will be important to assess if class II HDACs play a role in the ventral hippocampus and whether this epigenetic pathway affects the conditioning phase or anxiety responses. Understanding the role of the hippocampus and the contribution of different epigenetic markers to fear learning may shed light on the mechanisms that lead to post-traumatic stress disorder (PTSD) and other anxiety disorders that result from deficits in contextual learning. Here, we demonstrate that predator odor fear conditioning provides a useful model to understand these processes.

Author Contributions: JH, AT, and EW performed experiments; RY performed experiments, analyzed data, and wrote the manuscript; IM designed experiments and supervised analysis and writing of the manuscript.

Conflicts of interest statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER 3: Extinction of learned fear induces hippocampal place cell remapping

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Note regarding author contributions

The work for this chapter was done in collaboration with my colleague, Dr. Melissa Wang, resulting in the finished co-first author publication printed in the *Journal of Neuroscience*. Wang had previously developed a contextual fear conditioning paradigm using coyote urine as the unconditioned stimulus (Wang et al 2012, Wang et al 2013), and I expanded upon her work to develop the paradigm for contextual fear extinction that is used in this paper. I ran all the behavioral experiments needed to develop this protocol, after which both Wang and I performed surgeries, recordings, and data analysis for the primary electrophysiological experiment. The data included for this section of the paper is evenly split between both of our efforts. In addition to the primary experiment, I also ran two control groups for the electrophysiological experiments: one group where I substituted an aversive, non-fearful odor in lieu of the predator odor to control for possible effects of arousal, and another group where extinction training was conducted outside of the conditioning context to control for the effects of prolonged exposure to a context. For these controls, I performed all the surgeries, recordings, and analysis. The first draft of the paper was written by Wang in 2013 and edited extensively by Dr. Muzzio and myself. After Wang left the lab in 2014, I completed data collection and rewrote the paper for submission to the Journal of Neuroscience with the finalized results and the inclusion of the additional experiments that I had run, which form a substantial portion of the paper. Because we both contributed equally to the work, we decided to be joint first authors, with her name listed first due to alphabetical ordering.

Abstract

The extinction of learned fear is a hippocampus-dependent process thought to embody new learning rather than erasure of the original fear memory, although it is unknown how these competing contextual memories are represented in the hippocampus. We previously demonstrated that contextual fear conditioning results in hippocampal place cell remapping and long-term stabilization of novel representations. Here we report that extinction learning also induces place cell remapping in C57BL/6 mice. Specifically, we observed cells that preferentially remapped during different stages of learning. While some cells remapped in both fear conditioning and extinction, others responded predominantly during extinction, which may serve to modify previous representations as well as encode new safe associations. Additionally, we found cells that remapped primarily during fear conditioning, which could facilitate reacquisition of the original fear association. Moreover, we also observed cells that were stable throughout learning, which may serve to encode the static aspects of the environment. The short-term remapping observed during extinction was not found in animals that did not undergo fear conditioning, or when extinction was conducted outside of the conditioning context. Finally, conditioning and extinction produced an increase in spike phase locking to the theta and gamma frequencies. However, the degree of remapping seen during conditioning and extinction only correlated with gamma synchronization. Our results suggest that the extinction learning is a complex process that involves both modification of pre-existing memories and formation of new ones, and these traces coexist within the same hippocampal representation.

Introduction

The extinction of learned fear is generally thought of as new learning rather than erasure of a fearful memory, due to behavioral processes wherein the conditioned fear response resurfaces after extinction. Notably, the conditioned response returns when a delay (spontaneous recovery) or a mild US (reinstatement) is introduced after extinction training, or when extinction is tested outside of the context where training occurred (contextual renewal) (Bouton, 2004; Ji and Maren, 2007; Myers and Davis, 2002). Thus, it is thought that fear extinction results in a novel memory trace that competes with the original fear memory. The contextual specificity of extinction suggests that the hippocampus is critical for this process (Corcoran et al., 2005; Corcoran and Maren, 2004; Ji and Maren, 2005). However, it is unknown how the hippocampus encodes these conflicting emotional representations.

The most striking evidence for the role of the hippocampus in spatial processing comes from the activity of its principal neurons, known as place cells, which fire in selective locations as an animal traverses a context (O'Keefe and Dostrovsky, 1971) and are thought to encode a neural representation of space (O'Keefe and Nadel, 1978). Recently, it has been shown that hippocampal place cells remap in response to fear learning (Moita et al., 2004), and this novel representation stabilizes in the long term (Wang et al., 2012). However, the physiological responses of these neurons to extinction learning are unknown. Since place cell stability is thought to be a neural correlate of spatial memory (Agnihotri et al., 2004; Kentros et al., 1998; Rotenberg et al., 2000; Rotenberg et al., 1996), our previous results showing place field stabilization after conditioning provide a unique basis for understanding hippocampal encoding of extinction because they allow us to evaluate how stable fear representations are affected by changes in the emotional valence of a context.

Place cells can represent multiple aspects of the same context in accordance with varying reference frames such as tasks and motivations (Ferbinteanu and Shapiro, 2003; Gothard et al., 1996; Zinyuk et al., 2000). Thus, it is possible that place cells may also encode different emotional representations of fear and safety. To assess this possibility, it is necessary to record from the

same neurons throughout fear learning. To this end, we developed an extinction paradigm using predator odor contextual fear conditioning. We found that extinction produces remapping in some cells, indicating that a new 'safe' contextual representation is formed. This remapping occurs in a subset of cells that previously remapped during fear conditioning as well as a subset that was primarily stable during encoding of the fear association, suggesting that extinction may both modify old memory traces and lead to the formation of new ones. Interestingly, some cells remain stable throughout fear learning and extinction, which suggests that some cells mainly respond to the static aspects of the spatial context rather than changes in valence. Finally, during extinction there is an increase in low (30-60 Hz) and high (60-80 Hz) gamma coherence, a synchronization mechanism involved in signal amplification that has been shown to correlate with attentional processes (Womelsdorf and Fries, 2006). These results indicate that the neural correlates of extinction include a complex spatial representation that incorporates distinct traces of varying emotional valence.

Materials and Methods

Subjects

Male mice 2-6 months of age (strain: C57Bl/6, Jackson Laboratory, Bar Harbor, ME) were housed individually on a 12-hour light/dark cycle and allowed access to food and water *ad libitum*. All experiments were carried out in accordance with NIH guidelines and approved by the Institution of Animal Care and Use Committee of the University of Pennsylvania.

Training of extinction and control animals during behavioral and electrophysiological testing

To evaluate the influence of extinction on hippocampal place cells, we devised an extinction paradigm based on our previously reported predator odor contextual fear conditioning paradigm (Wang et al., 2013). Mice were pre-exposed to the rectangular training context (30 cm x 40 cm) in two 10-minute sessions, both 24 hours and immediately prior to contextual fear conditioning (habituation and baseline sessions). Fear conditioning was then conducted by returning mice to the training context for 5 minutes while a paper towel square (2.5cm x 2.5cm) saturated with 40 drops of 100% coyote urine (Maine Outdoor Solutions, Hermon ME) was taped to the center of the context. Ten-minute retrieval tests were given at 1 hour and 24 hours after

conditioning. Immediately after the 24-hour memory retrieval test, mice undergoing extinction were exposed to the same context for 30 minutes to extinguish fear learning. A retrieval test 10 minutes in length was conducted 24 hours post-extinction (48 hours after coyote urine exposure). Since we were interested in testing extinction, we used a larger amount of coyote urine than we previously reported (Wang et al., 2012) during conditioning and introduced a learning criterion to ensure that all animals included in the analysis displayed consistent and robust learning. Specifically, only animals showing at least 20% increase in freezing at 24 hr were included in the extinction group. One animal did not reach the learning criteria and was excluded from the analysis.

To test the context specificity of extinction for predator odor fear conditioning, a third group of mice (ABA control) was conditioned in the training context (A), extinguished in a novel context (B), and tested 24 hours after extinction training in context A. To control for repeated contextual exposure and rule out non-associative effects, another group of mice (water control) was placed in the context following the same schedule as the extinction animals (including the 30 minute extinction session), but was not fear conditioned with the odor.

For electrophysiological experiments, the extinction, water control, and ABA groups were run as described above. Additionally, to evaluate the effect of an aversive, non-fearful odor on place cell representations, we added short-term and long-term odor controls. The short-term controls included three groups of mice that were exposed to either water, coyote urine, or 2-methy butyric acid (2-MB), a synthetic odor resembling the smell of spoiled food, in a context with no visual cues in order to assess electrophysiological responses in the presence and absence of the odors. The long-term odor control group consisted of a subset of animals following the same schedule as the extinction group but that were exposed to 2-MB instead of coyote urine during the conditioning session. We had previously shown that this odor does not produce long-term freezing to the context (Wang et al., 2013), and here we evaluated if it had some long-term effect during extinction. In all sessions, freezing was measured as an index of fear learning. Freezing was defined as period of total immobility except for respiratory movements, and computed as the percent of time during which the velocity of the animal was lower than 0.6 cm/s. Freezing was

measured using two independent measures including the automated Limelight system (Coulbourn Instruments, PA) and custom-written Matlab code. The position data were smoothed with a 1 sec boxcar prior to the assessment of freezing to eliminate jitter in the tracking.

Surgery

Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally and placed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA). They were then were implanted with drivable 6-tetrode microdrives in the dorsal hippocampus (from bregma (in mm): AP, -1.7; ML, -1.6; DV, -1.0). Tetrodes were implanted above the hippocampus and the tetrode bundle was slowly advanced by 15-20 μm steps per day into recording position. A ground wire was connected to a screw placed on the contralateral side of the skull. The headstages were affixed to the skulls with cyanoacrylate and dental cement.

Electrophysiology & Data Analysis

Electrophysiology and data analysis were done as previously described (Wang et al., 2012). Beginning one week after surgery, neural activity from each tetrode was screened daily. The search for cells was conducted in an environment different from the one used for actual experiments. The headstage was connected to a tethered unity gain amplifier with green and red LEDs for tracking the position of the animal. Units were amplified using a 32-channel amplifier (Neuralynx, Bozeman, MT) between 2,500 and 10,000 times and filtered between 400-9,000 Hz. The amplifier output was digitized at 30.3 kHz. The position of the animal and electrophysiological data were recorded by Cheetah Data Acquisition software (Neuralynx, Bozeman, MN). The electrode bundle was advanced by 15-20 μm steps per day, lowering the tetrodes in small steps to increase the stability of the recordings (Kentros et al., 2004; Muzzio et al., 2009). Pyramidal cells were identified by their characteristic firing patterns (Ranck, 1973), and experiments were begun only when recordings were stable for at least 24 hours. Long-term recordings were considered stable when cells had the same cluster boundaries over two sessions, and the waveforms obtained from all four wires of a tetrode were identical between sessions.

After completion of the experiments, units were cluster cut and analyzed using MClust software (developed by A. David Redish, University of Minnesota). Cells were only accepted for analysis if they formed isolated clusters with clear Gaussian ellipses and minimal overlap with surrounding cells and noise and displayed high waveform similarity throughout the experiment. To ensure that the quality of our clusters was similar in both groups over time, we computed isolation distance, a measure of how separated a cluster is from other spikes recorded on the same tetrode (Harris et al., 2001; Schmitzer-Torbert et al., 2005). The isolation distance parameter reflects the radius of the smallest ellipsoid from the center of the cluster under study to noise or other spikes, and we used this measure to compare cluster quality between groups in all sessions. Finally, all cells were inspected to rule out that there any events during the 2 msec refractory period.

The generation of place field maps was done in Matlab by first dividing the environment into 2 x 2 cm pixels, then calculating the number of spikes and the time spent in each pixel. Both the spike count and time maps were then smoothed with a 3 cm full-width at half maximum Gaussian kernel. The final place field map was obtained by dividing the smoothed spike map by the smoothed rate map. Only periods of movement were included in the place field analysis, during which the minimum walking speed was 2 cm/s. We excluded cells that fired less than 40 spikes during movement in a session or displayed peak firing frequencies lower than 1.3 Hz before smoothing after the speed threshold was applied. Sampling of the environment was calculated as the percentage of pixels sampled for more than 1 sec after smoothing. Place field stability was assessed by performing pixel-by-pixel Pearson R cross-correlations between maps.

To evaluate whether the remapping observed during extinction occurred gradually, we divided the extinction session in three consecutive blocks, extinction 1 to 3 (ext1, ext2, ext3) and performed pixel-by-pixel cross-correlations between these blocks. To visualize the remapping trends in the data and determine which cells were more stable than chance, we established a correlation threshold of 0.3 using the following randomization procedure. For each animal, place field maps were generated for the 24 hr session and the last block of extinction (ext3). Next, the map of each cell from the 24 hr session was correlated with a random map from the ext3 session,

and the mean correlation across comparisons was computed. These random correlations were computed 1000 times with replacement, e.g., for each animal, a particular cell participated in more than one random comparison. This procedure yielded a distribution of mean random correlation values for each animal. The highest 95% value of these distributions (0.3) was chosen to guarantee that stability values above this threshold were significant above a 0.05 probability level (data not shown). Cells with a pixel-by-pixel correlation below this threshold over the course of the short-term conditioning or extinction training sessions were considered to exhibit remapping in response to conditioning or extinction. This correlation threshold corresponds with previous studies in mice showing place field stability (Kentros et al., 2004; Muzzio et al., 2009), but does not have a direct physiological correlate. Therefore, it is used here only to capture remapping trends in the data.

A place field was defined as activity within 9 or more contiguous pixels (total area ≥ 36 cm^2). Place field size was calculated as the total area of the pixels comprising a field. If more than one place field was present, place field size was calculated as the average of all fields. Mean firing rate was calculated as the number of spikes during movement divided by the amount of time the animal was moving. Place field coherence, a measure of field organization, was calculated by correlating each pixel with its 8 neighboring pixels, before smoothing. The spatial information content for cell *i* was calculated as previously described (Skaggs et al., 1993) using the formula $IC=\Sigma p_i(R_i/R)\log(R_i/R)$, where p_i is the probability of occupying pixel *i*, R_i is the firing rate at location *i*, and *R* is the overall mean firing rate.

Spike Synchronization Analysis

The local field potential (LFP) signal was recorded from tetrodes used to obtain single unit activity. These data were recorded at 30 kHz, digitally filtered between 0.1-400 Hz. For further processing, data were imported to Matlab, then low-pass filtered and resampled to 10 kHz using the Matlab function *resample* to reduce the size of data files and make further analysis computationally tractable. We used these data to analyze spike-field coherence (SFC), a measure of phase synchronization between the LFP and spike times as a function of frequency (Fries et al., 2001). To this end, we first calculated the spike-triggered average (STA), by extracting the LFP

segment ±500 ms around each spike, and averaging across these spike-triggered epochs. In the absence of neuronal synchronization the STA would be flat, whereas when spikes are phase-locked to particular frequencies, a signal at these frequencies can be observed in the STA. We then estimated the power spectrum using the multitaper method (Matlab function *pmtm*), over the range of 4 to 80 Hz in log increments. To calculate SFC, the power spectrum of the STA was divided by the average of all power spectra of the spike-triggered epochs. We computed normalized coherence spectra graphs showing the change in SFC for all frequencies (mean±1SEM), without binning in frequency bands. Coherence spectra were normalized by the coherence during the baseline session for each cell. Additionally, we examined coherence in theta (4-12Hz), low gamma (40-60Hz), and high gamma (60-80Hz) frequency bands, by averaging the coherence spectra in these bands. Finally, we correlated the coherence in these frequency bands with place field stability, averaging across all cells within a session, to assess the relationship between remapping and synchrony in these bands. Place field stability was Fisher-transformed prior to these correlations to reduce any potential ceiling effects.

Statistics

A Two-way ANOVA with repeated measures was used to compare freezing between extinction and control animals across all sessions of conditioning or extinction. Statistical analyses of place cell correlations and gamma coherence were performed with a mixed-models approach (McCulloch and Searle, 2001) based on our previously reported statistical analyses (Wang et al., 2012). Post hoc multiple comparisons were conducted using Bonferroni corrections. Although no details are included, all statistical decisions in the overall analyses and post hoc multiple comparisons were confirmed by additional robust and conservative mixed-models and non-parametric tests. These additional statistical analyses have allowed for making conservative statistical decisions, in that they do not depend on the fulfillment of assumptions inherent to parametric tests or the possible presence of outliers.

In all mixed model analyses, we first tested group by session interactions. If there was a significant interaction, we tested for simple effects of group on each individual session (or session

on extinction group) using maxT multiple comparison tests for mixed model estimates (Maxwell and Delaney, 2004) to control for Type I error. If there was no significant interaction, we conducted post hoc tests for main effects of group. All mixed model analyses were performed using GNU R software, (R Foundation for Statistical Computing) with nlme, lme4, multcomp, contrast, lmerTest, nparLD, and nparcomp libraries. In all cases, a significant difference was determined with a probability <0.05, and error bars indicate ± SEM. Error bars were adjusted according to the number of cells included in each session.

Results

Prolonged exposure to the training context produces context specific extinction of predator odor fear

To examine the effects of fear extinction at the behavioral level, we tested three independent groups of animals. The extinction and ABA control groups were fear conditioned as previously described in our reported predator odor contextual fear conditioning study (Wang et al., 2013). These groups later underwent contextual extinction by being exposed to either the training context (extinction) or a novel context (ABA control) for 30 min. The extinction group tested if a prolonged exposure to the training environment decreased fear to the context and the ABA group tested if predator odor extinction learning was context-specific, as previously demonstrated in other extinction paradigms (Bouton, 2004; Ji and Maren, 2007). The third group (water control) was tested in a single context following the same schedule as the other groups, but was exposed to water rather than coyote urine during the conditioning session. This group controlled for possible non-associative effects such as habituation. All groups were then re-tested in the context 24 hours after extinction (post-ext session, Figure 4A). As an index of fear, percent of total time spent freezing was measured in all trials. It has previously been shown that a prolonged exposure to the conditioning context extinguishes the conditioned fear response for shock-induced fear (Mamiya et al., 2009; Suzuki et al., 2004); here we found that similar extinction training also produces predator odor fear extinction. Specifically, we observed that the prolonged exposure to the training context reduced freezing 24 hours after extinction (postext session) while the same length of exposure to

a novel context (ABA group) did not. Additionally, repeated exposures to the context (water control) do not significantly affects freezing at any time point (effect of group: $F_{2, 29} = 4.86$, $p<0.015$; effect of session: $F_{4, 112} = 10.07$, $p<0.001$; interaction: $F_{8, 112} = 2.52$, $p<0.015$; post-hoc tests indicated that freezing in both the extinction and ABA control groups was significantly higher than the water control during the 1 hr and 24 hr sessions, while in the post-extinction session only freezing in the ABA control was significantly higher than the water control, $p<0.036$, Figure 4B). Furthermore, the freezing observed in the extinction group 24 hours after extinction was not significantly different

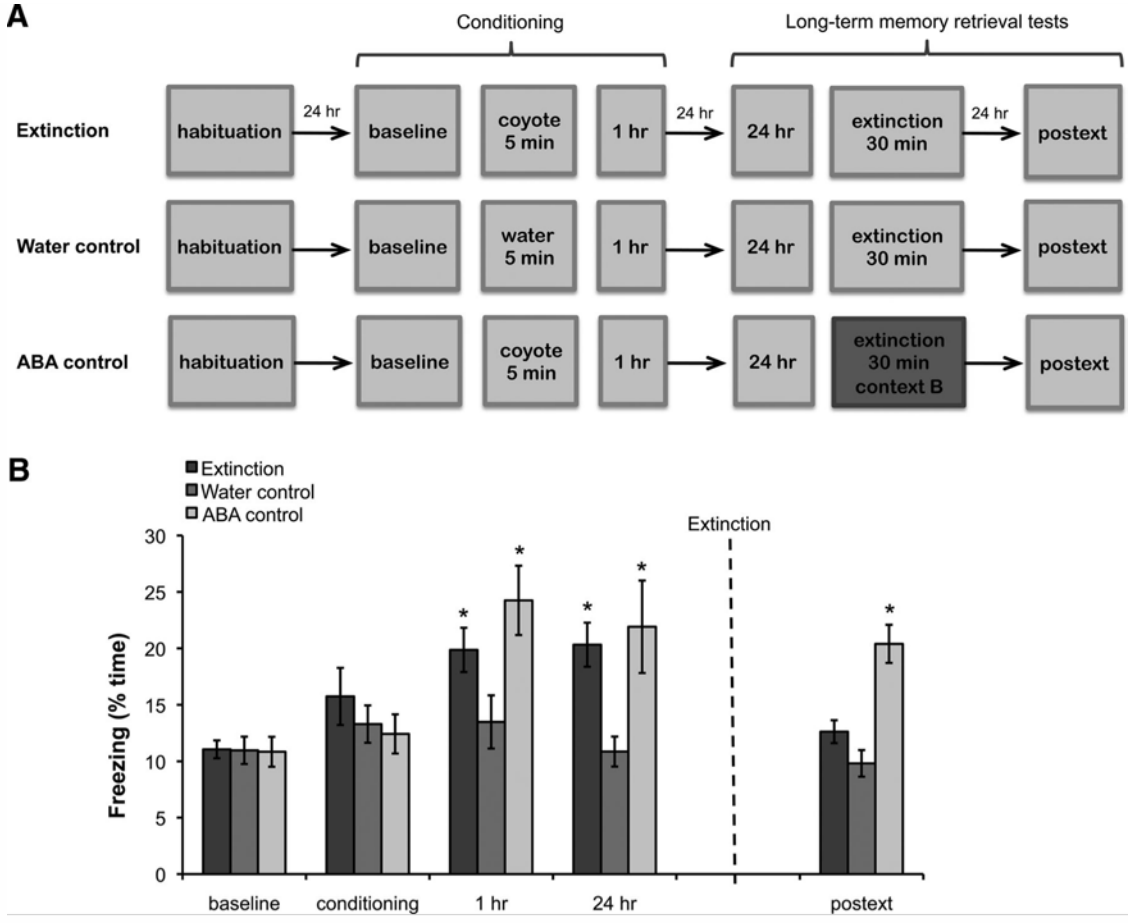

Figure 4 A, Schematic representation of behavioral paradigm. **B**, In extinction animals, freezing returns to baseline levels 24 h after extinction (postext). Animals exposed only to the context (water control) following the same schedule as the extinction animals do not display significant changes in freezing throughout training. Animals fear conditioned in the training context but exposed to a new context during extinction (ABA control) display fear in the training context when tested 24 h after extinction (extinction: n = 17, water control: n = 12, ABA control: n = 7). Bars represent means ± SEM, *p < 0.05.

from baseline freezing levels ($p>0.05$), indicating that the 30-minute extinction session was sufficient to extinguish behavioral fear responses.

Cellular and behavioral characteristics during periods of movement are equivalent between extinction and water control animals

To examine the neural correlates of extinction, we recorded 107 cells from the dorsal area CA1 of 5 animals, of which 74 cells were held throughout training. As a control, we recorded from 40 cells in 3 animals run in the water control condition described previously, of which 35 cells were held throughout training (Figure 5A). Cells accepted for analysis formed distinct clusters and exhibited stable waveforms and cluster boundaries between consecutive sessions (Figure 5B).

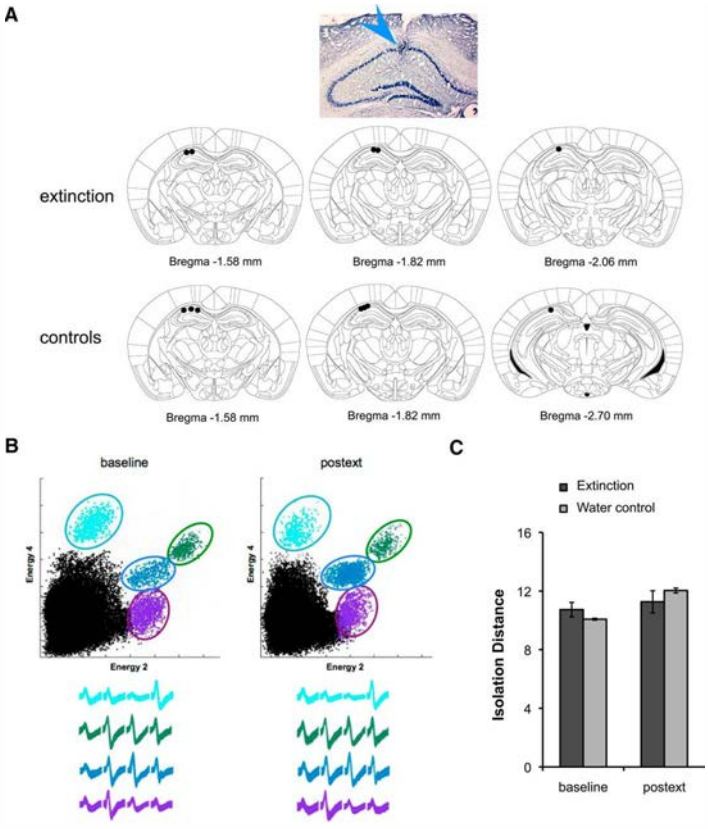

Figure 5 A, Photomicrograph of Nissl-stained coronal section showing position of electrode bundle and schematic coronal sections showing the position of all electrodes in the extinction (n = 5) and control (n = 7) groups. **B**, Cluster projections and waveforms recorded during baseline and 24 h postext showing recording stability. **C**, Analysis of cluster quality showing no significant differences between the extinction and control group throughout the recording period. Isolation distance, a measure of cluster quality that determines how separated each cluster is from noise and other cells, was computed during baseline and 24 h postext. For the clusters shown in B, isolation distance values are the following: light blue: 11.5, 9.2; green: 14.2, 17.1; blue: 22.3, 23.4; purple: 17.4, 10.4. Bars represent means ± SEM. *$p < 0.05$.

Additionally, there were no differences in mean isolation distance between clusters from each group at the beginning and end of training (t-tests between groups: baseline: t_{158}=0.16, p>0.05, 24 hr post-extinction: t_{148}=0.57, p>0.05, Figure 5C), indicating that cell quality was similar across conditions.

We found that the electrophysiology animals undergoing extinction displayed the same freezing patterns observed in the extinction group shown in Figure 4B (mean percent freezing at baseline [bl]: 13.94 ± 1.29; 24 hr: 26.02 ± 4.83; post-extinction [postext]: 13.09 ± 1.34). Importantly, the moderate differences in freezing between control and extinction animals did not affect sampling of the arena. All animals included sampled at least 75% of the context in all sessions, with no difference in mean sampling between groups (t_7=0.88, p>0.05, mean sampling across sessions: extinction: 97.5 ± 0.01%, control: 97.8 ± 0.02%; Figure 6A). Importantly, there were no differences in average speed (including periods of movement above 2 cm/sec, the speed threshold used for place cell analyses) between the two groups across all sessions (no effect of group: F<1; session: $F_{6,42}$=1.06, p>0.05; or interaction: $F_{2,42}$=1.16, p>0.05; mean across all sessions [cm/sec]: extinction: 5.72 ± 0.31; control: 5.98 ± 0.32; data not shown). Additionally, there were no differences between the groups in the percentage of data included in the analyses (e.g., mean percent of spikes firing during periods of movement across all sessions: extinction: 67 ± 4.4%; control: 65 ± 3.5%; t_7=0.17, p>0.05; data not shown).

Differential remapping of place cells was observed in response to emotional valence and static aspects of the context

At the cellular level, we found a high degree of variability in the stability of place fields during the short-term conditioning and extinction sessions. The majority of cells remapped in response to predator odor exposure during fear conditioning, 1 hr, or both sessions as previously shown (Wang et al., 2012). The cellular representations in the extinction group stabilized at the 24 hr session, whereas the water control group (Figure 6F) displayed the typical long-term remapping observed under no task contingencies (Kentros et al., 2004; Muzzio et al., 2009). Many place fields also appeared to shift during the extinction session (Figure 6, B and C), although some remained

stable throughout extinction (Figure 6, D and E). Among the cells that remained stable during extinction, some remapped more strongly during fear conditioning (Figure 6D), while others did not respond to either learning process (Figure 6E). To more closely examine place cell activity during extinction, we divided the 30-minute extinction session into three 10-minute intervals (ext1, ext2, and ext3). Over the course of extinction, we observed that place fields tended to shift gradually (Figure 7, A and B) and were less stable than those recorded in the water control animals (Figure 7, C and D), which displayed the high stability typically observed during a continuous short-term session (Kentros et al., 2004).

Since only a subset of cells displayed remapping during conditioning and extinction in the extinction animals, we used a remapping threshold to further characterize the trends in the data. This threshold (0.3) was generated by calculating random distributions of pixel-by-pixel cross-correlation values (see Methods) and coincides with previously reported values for stability in mice (Kentros et al., 2004; Muzzio et al., 2009). Cells with an average correlation below this threshold were considered remapping.

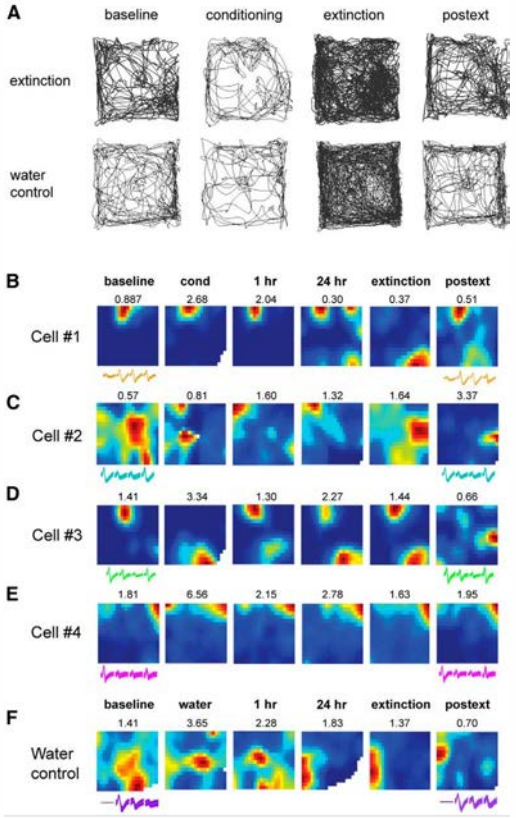

Figure 6 A, Example paths from a water control and an extinction animal showing complete sampling of the environment. **B–F**, Examples of place cell rate maps. Activity was normalized relative to the peak firing frequency shown on top of each rate map. Blue represents areas visited by the animal where a particular place cell does not fire, and more vivid colors represent areas of increasing firing activity for that cell. Remapping was observed both during conditioning and the 30 min extinction session, although different cells displayed different phenotypes. **B**, Example cell that exhibits remapping during extinction. **C**, Example cell that remaps during both fear conditioning and extinction. **D**, Example cell that remaps during fear conditioning but remains stable in extinction. **E**, Example cell that remains stable throughout fear and extinction learning (static cell). **F**, Example cell from a water control animal that did not undergo fear conditioning or extinction. Waveforms at the beginning and end of the recording period are shown below each map. The constancy in the waveforms indicates recording stability.

Using this threshold, we found that 45% of cells remapped during extinction in the experimental group. Among those, 27% remapped in response to both fear conditioning and extinction, while 18% remapped predominantly in response to extinction. In contrast, in the control group, 3% of cells remapped during both processes, and 6% remapped mainly during extinction. Additionally, we found that 25% of cells remapped primarily during fear conditioning in the extinction group, in comparison to 3% in the control group. Finally, 30% of cells in the extinction group remained stable during conditioning and extinction in comparison to 88% of cells in the control group that remained stable during all short-term sessions (Figure 8A). These data suggest that a subset of cells involved in fear conditioning is also involved in extinction learning (e.g., cells that remap during both learning processes), while other cells that were previously stable throughout conditioning may be recruited specifically for extinction learning (e.g., cells that remap preferentially during extinction). This indicates that extinction learning involves both the modification of previous contextual representations as well as the formation of novel representations.

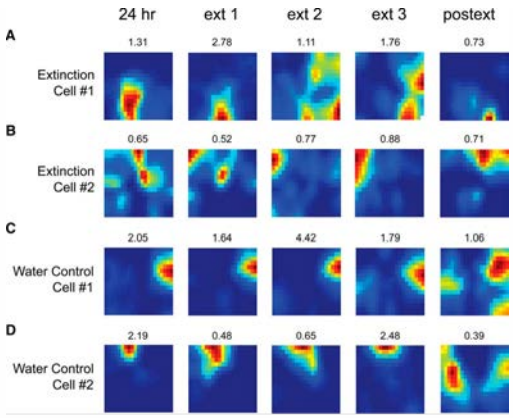

Figure 7 Place cell rate maps showing gradual remapping occurring over the course of the extinction session. Immediately following the 24 h retention test, the extinction session was divided into three 10 min intervals (ext1, ext2, and ext3). **A, B**, Cells exhibit gradual changes in their preferred firing location over the extinction intervals. Note that in most cases the fields remap further when the animal is tested 24 h after extinction (postext), suggesting that extinction training brings cells back to their typical long-term remapping state. **C, D**, Remapping was not observed in the water control group during a prolonged exposure to the context. Control cells also exhibited instability in the long term as has been previously shown in mice (Kentros et al., 2004; Muzzio et al., 2009). Peak firing frequency is shown on top of each map.

Fear conditioning and extinction decrease the stability of spatial representations

To quantify the overall remapping observed during conditioning and extinction, we calculated pixel-by-pixel cross-correlations between consecutive sessions and averaged across sessions. There was no significant difference between extinction and water control groups for the habituation/baseline comparison prior to conditioning, but correlations between consecutive

conditioning sessions were significantly lower in the extinction group than in the control group, except for the comparison 1hr/ 24 hr (effect of group: $F_{1, 118} = 10.563$, $p<0.002$; effect of session: $F_{7, 567} = 17.116$, $p<.001$; effect of interaction: $F_{7, 567} = 2.648$, $p<0.01$). Post-hoc multiple comparisons indicated that the correlation in the extinction group was significantly lower than the control during conditioning (baseline/coyote, coyote/1hr, $p<0.05$), corroborating our previous finding that conditioning produces short-term remapping (Wang et al., 2012). Additionally, average correlations between the consecutive extinction intervals were significantly lower in the extinction group than the controls ($p<0.05$). For the 1hr/24hr comparison, the trend was inverted and came close to statistical significance ($p=0.052$), showing that after conditioning, the place fields stabilize in animals that reach the learning criterion (Figure 8B).

We also calculated correlations between the third extinction interval and the first (extinction 3/extinction 1), as well as the third interval and the 24 hr session immediately preceding the extinction session (extinction 3/24hr). Again, place fields were significantly less stable in the extinction group than in the control group for both comparisons (effect of group: $F_{1,103} = 8.99$, $p<0.003$; session: $F_{1, 100} = 18.75$, $p<0.001$; interaction: $F < 1$; Figure 8C). It is important to note that on average, the remapping observed during extinction was less robust than the remapping observed during conditioning (mean place field similarity during conditioning: 0.37 ± 0.03, extinction: 0.50 ± 0.02), suggesting that the change in emotional valence of a context during extinction only partially changes the neuronal representation of that context.

Interestingly, the extinction 3 and 24hr post-extinction sessions were significantly less correlated in the extinction group compared to the control group ($p<0.05$, Figure 8B), indicating that the new map formed during extinction does not stabilize but remaps in the long term. To examine this further, we were able to record from 25 cells in 3 extinction animals in an additional 48-hour post-extinction session. Again, we found low stability compared with the 24hr post-extinction session, analogous to the correlation between extinction 3 and 24hr post-extinction (mean correlation between 24hr post-extinction/48hr post-extinction: 0.39 ± 0.04, mean correlation between extinction 3/24hr post-extinction: 0.38 ± 0.03; $p>0.05$; data not shown). The lack of stability

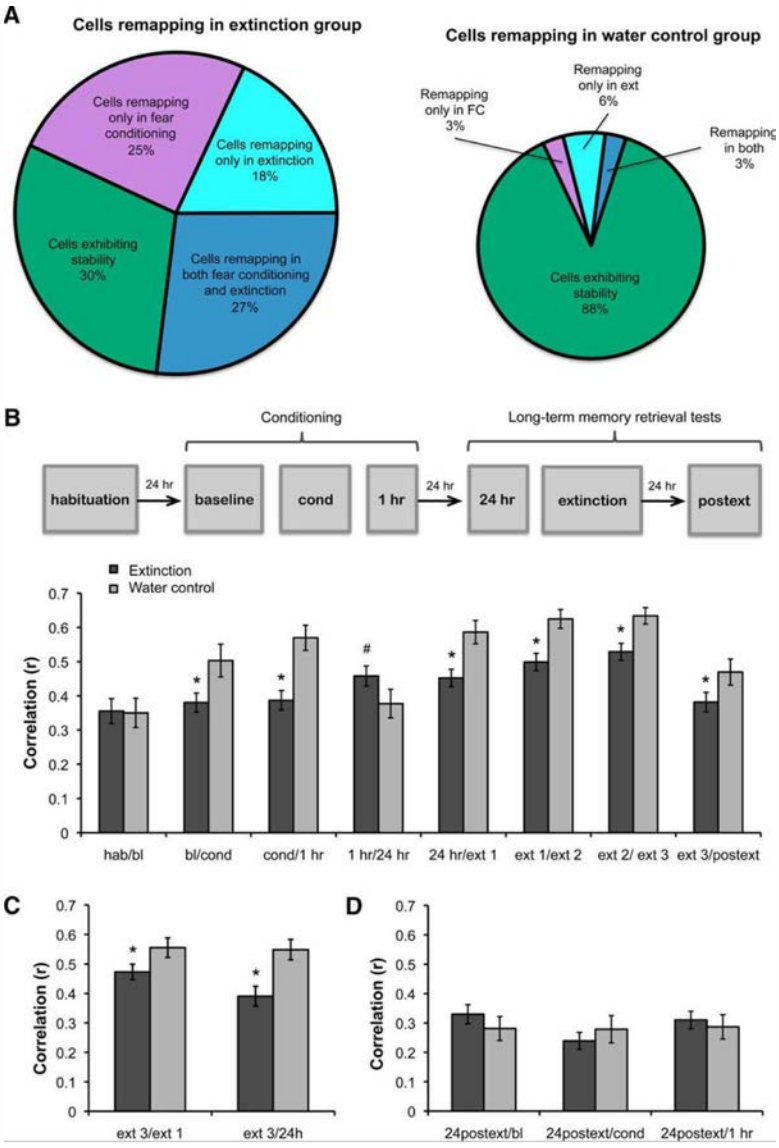

Figure 8 A, Pie charts showing percentages of cells remapping or remaining stable during conditioning and extinction in the extinction and water control groups. **B–D**, Pixel-by-pixel cross-correlations between sessions in the extinction and water control groups. **B**, In the extinction group (n = 5, 107 cells), place fields were significantly less stable during fear conditioning, extinction, and 24 h after extinction compared with the water control group (n = 3, 40 cells). **C**, This effect was also observed when comparing the third extinction interval with the first, and when comparing the third extinction interval with the 24 h session immediately before extinction. **D**, In the extinction and the water control groups, place field maps formed 24 h after extinction did not resemble previous maps from baseline, fear conditioning, and 1 h sessions. Bars represent means ± SEM, *p < 0.05.

observed after extinction contrasts with the long-term stabilization that we previously observed after fear conditioning (Wang et al., 2012). These data suggest that while fear learning results in long-

term stabilization of spatial representations, extinction learning returns cells to their inherent long-term instability (Kentros et al., 2004; Muzzio et al., 2009).

We then wanted to determine whether the map formed after extinction was a novel map or resembled a spatial representation from a previous session. Therefore, we calculated correlations between the 24 hr post-extinction session with the baseline, coyote, and 1 hr sessions and found that the similarities between maps were low and not significantly different from the control group (there was no effect of group, $F<1$,nor its interaction with session, $F_{2, 134} = 2.76$, $p>0.05$, Figure 8D). Similar low correlations with earlier sessions were also evident in the subset of cells that we recorded up to 48 hr post-extinction (mean correlations in extinction group, 48 hr post-extinction/baseline: 0.28 ± 0.04, 48 hr post-extinction /coyote: 0.27 ± 0.04, 48 hr post-extinction/1hr: 0.26 ± 0.04; data not shown). These results suggest that post-extinction representations from both extinction and control animals do not resemble maps from previous sessions. Importantly, since place cell activity was only analyzed during periods of movement and there were no differences in average speed or sampling of the environment between extinction and control groups, our findings indicate that the remapping observed in the extinction group reflects cellular responses to the altered emotional valence of the context.

Aversive non-fearful odors or general arousal do not produce significant place cell remapping

The observation that some cells remapped only in response to conditioning while others remapped only during extinction introduces the question of whether the cells that remap during coyote exposure are in fact responding in an emotion-dependent manner or merely to the presence of an odor. To address this, we performed a separate experiment in which mice were exposed to water (3 animals, 20 cells), coyote urine (7 animals, 46 cells), or 2-methylbutyric acid (2-MB, 3 animals, 51 cells), an odor shown to be aversive but not fearful to mice (Kobayakawa et al., 2007; Wang et al., 2013). This experiment was performed in a white environment, following the same context exposure schedule as the conditioning sessions (baseline context exposure, odor presentation, short-term test session one hour later). We found that introduction of 2-MB did not affect short-term place field stability since correlations between sessions were not significantly

different than those between presentations of water (Figure 9A). These results corroborate previous findings showing that the introduction of neutral odors only produces remapping in a very small number of dorsal cells (Anderson and Jeffery, 2003; Keinath et al., 2014). Conversely, presentation of coyote urine significantly decreased place cell stability compared to the water and 2-MB groups (effect of group: $F_{2, 114} = 10.914$, $p<0.00001$, session: $F<1$, interaction: $F<1$; post-hoc tests indicated coyote odor was significantly different from 2-MB, $p<0.0001$ and water, $p<0.0002$; but there were no significant differences between water and 2-MB, $p>0.05$). These results indicate that only fearful odors produce significant remapping in the dorsal hippocampus.

Finally, to rule out the possibility that a non-specific general state of arousal could produce short-term remapping during extinction, we also recorded in the long term from two additional groups of animals following the same schedule as the extinction and water control groups. One group was exposed to 2-MB during the conditioning session to determine if the arousal resulting from exposure to an aversive odor could produce short-term remapping during a prolonged exposure to the context 24 hr after the odor removal. The second group was trained using the ABA design to determine if a heightened arousal state produced by fear conditioning could produce remapping in a neutral context (B) during extinction. We found that only animals trained in the ABA condition displayed short-term remapping during the conditioning sessions, corroborating that 2-MB does not produce significant short-term remapping. Moreover, only the animals in the ABA group displayed place field stability during the 24 hr session, indicating that fear learning stabilized the representations formed after conditioning. Finally, the ABA animals displayed remapping between context A and context B, which was evident in the comparison between the 24 hr and extinction 1 (ext 1) sessions; however, neither the ABA animals nor the 2-MB mice displayed short-term remapping during extinction (Figure 9 B-F, effect of group: $F_{1,66} = 7.165$, $p<0.0094$; effect of session: $F_{7,364} = 24.248$, $p<0.0001$; effect of interaction: $F_{7,364} = 11.058$, $p<0.0001$. Post-hoc tests indicated differences between the two groups during bl/cond, 1h/cond, 1h/24h, and 24h/ext1, but not in habA/bl1, ext1/ext2, ext2/ext3, nor 24hpost/ext3). Interestingly, when we compared the representations formed during the 24 hr and the post-extinction sessions in the ABA group in the

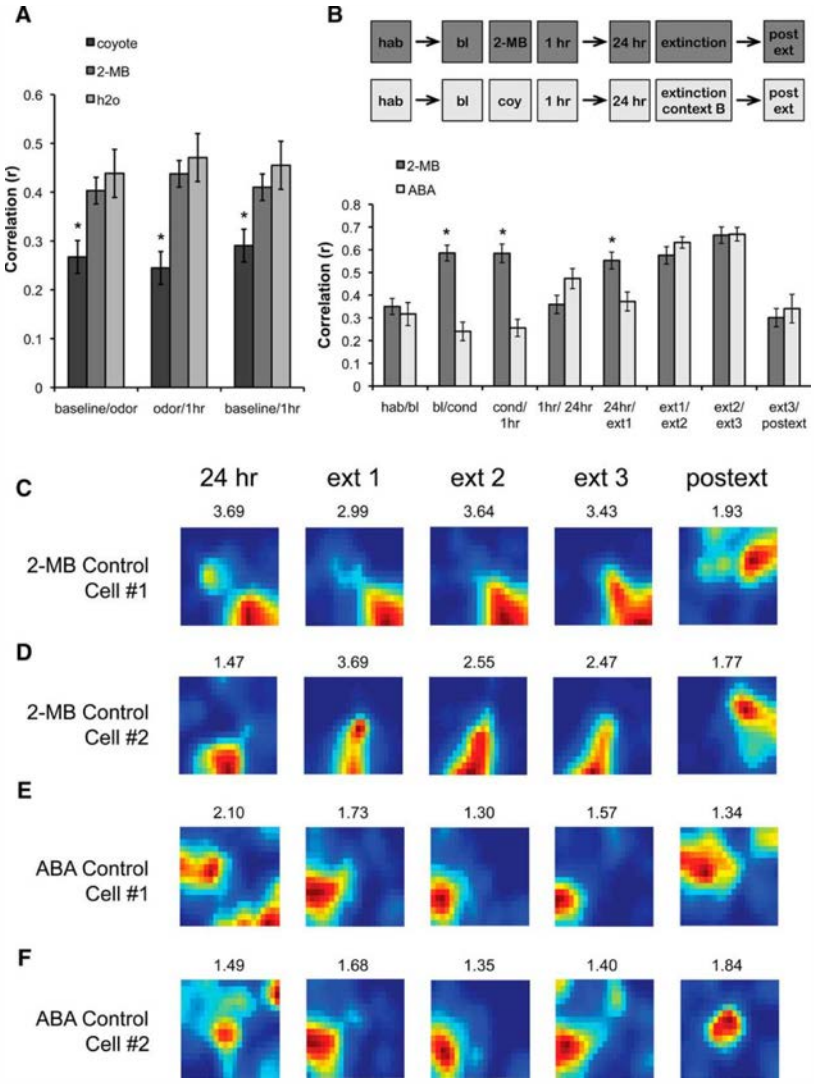

Figure 9 Nonfearful odors or general arousal do not produce remapping. **A**, The effects of coyote urine (fearful; 46 cells from 7 animals), 2-MB (aversive but nonfearful; 51 cells from 3 animals), and water (no odor; 20 cells from 3 animals) were compared by examining the short-term remapping produced when the odor was introduced after the baseline session (baseline/odor), removed 1 h later (odor/1 h), and before and after odor exposure (baseline/1 h). Only the fearful odor (coyote urine) produced significant short-term remapping. **B**, Pixel-by-pixel cross-correlations showing similarity scores across sessions in two groups of control animals. In the 2-MB group (2 animals, 31 cells), animals were trained as the extinction group but exposed to 2-MB during the conditioning session. In the ABA group (2 animals, 27 cells), animals were conditioned and tested for retrieval in context A but received extinction in context B. Bars represent means ± SEM, *$p < 0.05$. **C, D**, Rate maps showing example cells trained in the 2-MB group. Note the stability of these cells during all short-term sessions (24 h, ext1, ext2, and ext3) but not 24 h postext. **E, F**, Rate maps showing example cells trained in the ABA group. Note the remapping between 24 h and ext1 interval, indicating that the cells responded to the change from context A to context B. However, similarly to the 2-MB group, cells remained stable in context B (e.g., maps formed during ext1, ext2, and ext3). Additional remapping is observed when animals are reintroduced to context A during the postext session. The map retrieved during the postext session resembles the one formed at 24 h, indicating that in the absence of extinction in the training context, spatial representations stabilize in the long term. Peak firing frequency is shown on top of each map.

training context, we found high long-term stability (Correlation: 0.46±0.06) as we previously reported (Wang et al., 2012). Together, these results indicate that the cells remapping in response to predator odor conditioning or extinction primarily respond to the emotional aspects of the learning association rather than the presence of the odor itself, or even the aversive nature of the odor.

Changes in emotional valence are associated with increased excitability and reduced spatial information content in hippocampal place cells

Extinction animals displayed a significant increase in mean firing rate in comparison to control animals throughout learning (

Figure 10A, effect of group: $F_{1,191}$=6.03, p<0.02). The same pattern was observed for peak and infield firing rates ($F_{1,191}$=7.98, p<0.005 and $F_{1,191}$=4.96, p<0.03, respectively, data not shown). We also examined other place field parameters and did not observe significant group differences in mean field size, number of fields or place field coherence, a measure of place field organization (p>0.05, data not shown). However, we did find significant group differences in spatial information content, a parameter that evaluates how the firing rate of a cell predicts the location of an animal (Skaggs et al., 1993). Although there were no differences between the extinction and control groups during baseline, spatial information content increased in the water control group in comparison to the extinction group (

Figure 10B, effect of group: $F_{1,191}$ = 8.64, p<0.004). This may reflect the fact that the

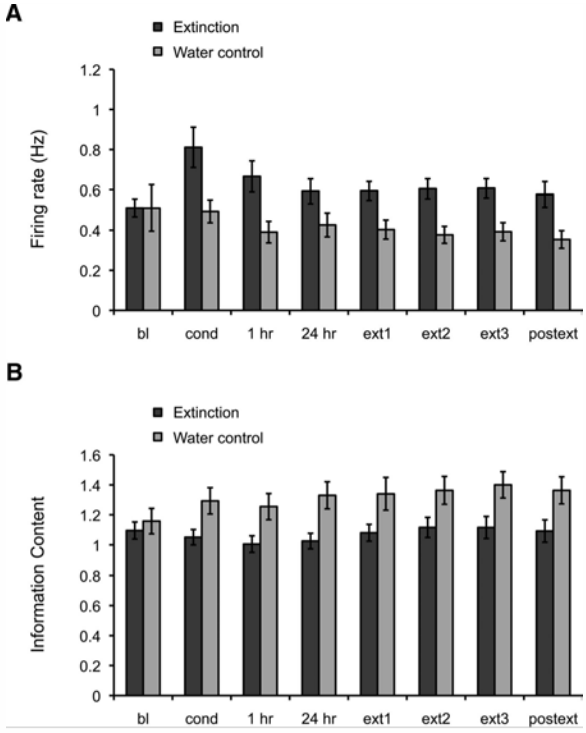

Figure 10 Changes in firing rate and spatial information content during conditioning and extinction. **A**, In extinction animals, mean firing rate was higher than in control animals throughout learning. **B**, Spatial information increases in water control animals after repeated exposures to the context, but remains relatively constant in the extinction group throughout learning (effect of groups < 0.05).

context is perceived as different during distinct stages of fear learning in the extinction group, which in turn may affect the spatial tuning of the place cells. Indeed, these results correlate with previous observations in CA1 showing that the spatial tuning of place fields decreases in novel environments and increases with repeated exposures in familiar ones (Karlsson and Frank, 2008). Together, these data indicate that fear learning induces several changes in place cells including increases in excitability and reductions in spatial information content.

Enhanced gamma coherence during extinction correlates with place cell remapping

Remapping and firing rate changes associated with it may reflect the manner in which spikes synchronize with local field oscillations. To investigate this possibility, we examined the effects of fear conditioning and extinction on neuronal coherence at the theta (4-12Hz) and the low and high gamma frequencies (30-60Hz, and 60-80Hz respectively). Normalized spectral graphs indicated that there was an enhancement in coherence across sessions in the extinction group in comparison to the water control group (Figure 11A). Analysis of band binned coherence showed a significant effect of group between the extinction and water control groups in theta coherence (Figure 11B, $F_{1,104} = 4.59$, $p<0.04$) but this effect was not modulated by the session (session: $F_{6,504} = 1.04$, p-0.39; interaction between group and session: $F_{6,504} = 1.74$, $p=0.11$). Additionally, we found that low and high gamma coherence were enhanced in the extinction group relative to water control animals and the effect was modulated by the session (Figure 11C and 8D, low gamma: effect of group: $F_{1,104} = 6.32$, $p<0.02$; session: $F_{6,508} = 3.46$, $p<0.002$; interaction: $F_{6,508} = 2.75$, $p<0.02$; high gamma: effect of group: $F_{1,104} = 3.33$, $p=0.07$; session: $F_{6,508} = 3.79$, $p<0.001$; interaction: $F_{6,508} = 3.66$, $p<0.001$). Specifically, there was a gradual decrease in gamma coherence in the water control animals during repeated exposures to the context, whereas both low and high gamma coherence were significantly enhanced during extinction and post-extinction sessions in the experimental group (low gamma: post hoc multiple comparisons indicated significant effects during extinction 1: $p<0.03$, extinction 2: $p<0.02$, extinction 3: $p<0.003$, and post-extinction: $p<0.003$; high gamma: post hoc multiple comparisons indicated significant effects during extinction 2: $p<0.005$, extinction 3: $p<0.02$, and post-extinction: $p<0.03$). These results suggest that sustained phase locking of spikes

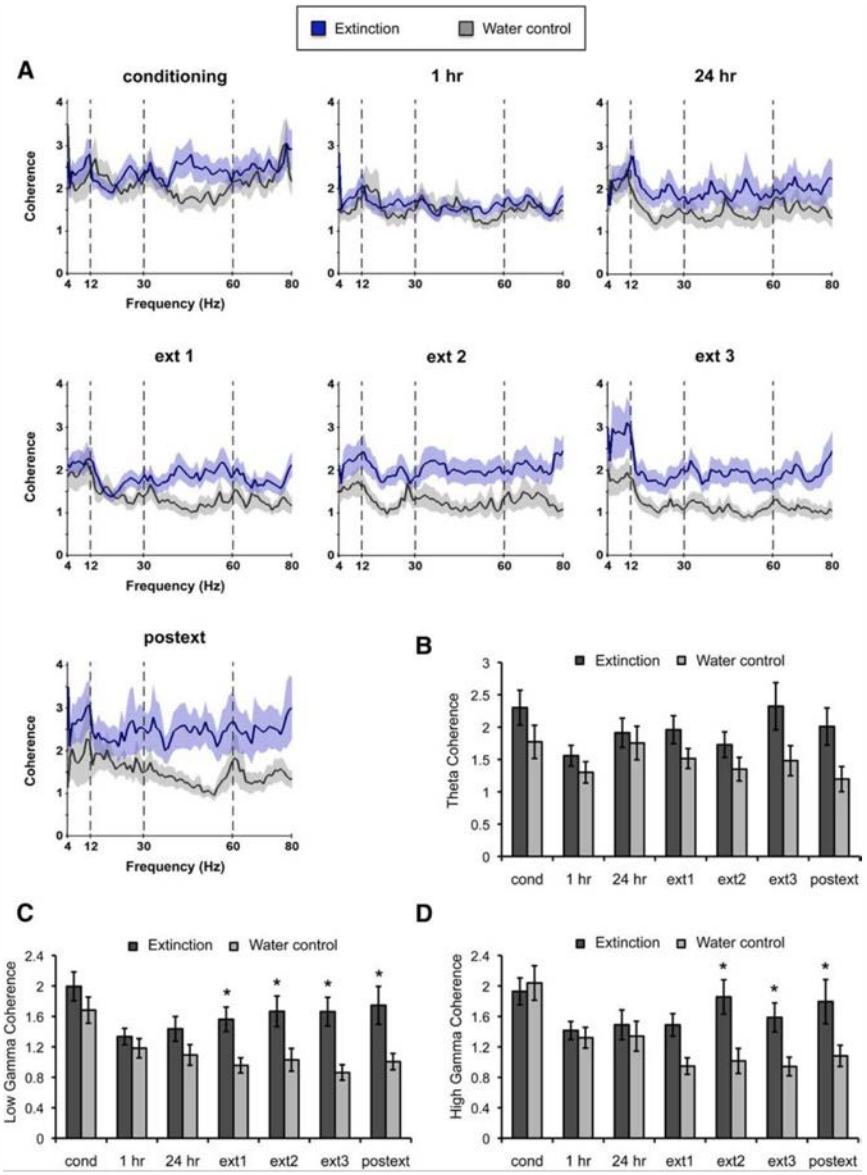

Figure 11 Extinction animals exhibit significantly higher gamma coherence during fear conditioning and extinction. **A**, Spectral graphs showing coherence normalized relative to baseline displaying levels of synchronization across frequencies in the extinction and control animals. Note the sustained level of low and high gamma coherence during extinction in experimental animals compared with water control animals. **B–D**, Histograms showing normalized binned coherence at theta and low and high gamma frequencies. **B**, There was a moderate increase in theta coherence throughout learning in the experimental group. **C, D**, Low (**C**) and high (**D**) gamma coherence increased during extinction and postext sessions in the experimental animals compared with controls. Bars represent means ± SEM, *p < 0.05.

to low and high gamma frequency may play a specific role in fear learning. Interestingly, the place cell remapping we observed 24 hours after extinction corresponded with an enhancement in

gamma coherence at this time point, suggesting that neuronal synchronization also increases with the formation of novel representations.

Due to the differential remapping we observed during conditioning and extinction, we next asked if the degree of remapping could predict the observed changes in theta and gamma coherence. To assess this, we performed correlations between the stability during learning and theta and gamma coherence. Although we observed no overall effect for theta coherence during fear learning (Figure 12A-B [left panel], Fisher transformed correlation, conditioning: r=-0.04p=0.70; extinction: r=-0.05, p=0.62), we found that during both conditioning and extinction, stability was inversely correlated with low gamma coherence (Figure 12A-B [middle panel], Fisher transformed correlation: conditioning: r= -0.27, p<0.02; extinction: r=-0.21, p<0.04). Additionally, we found that

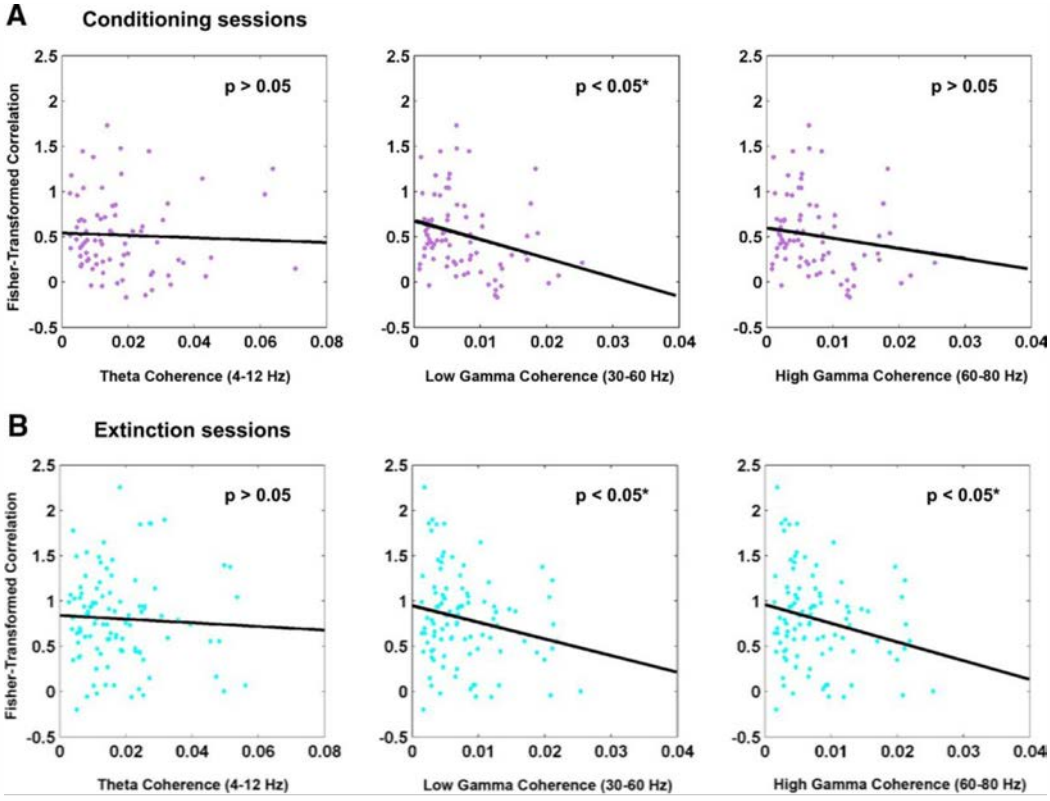

Figure 12 Correlations between stability and theta and gamma coherence. **A**, Correlations during conditioning sessions. The degree of remapping during conditioning did not show significant correlations with theta coherence (left) or high gamma coherence (right). However, it significantly correlated with low gamma coherence (middle). **B**, Correlations during extinction sessions. The degree of remapping during extinction did not show significant correlations with theta coherence (left). However, it significantly correlated with low (middle) and high (right) gamma coherence.

during extinction but not conditioning, place cell stability was inversely correlated with high gamma coherence (Figure 12A-B [right panel], Fisher transformed correlation: conditioning: r= -0.13, p=0.23; extinction: r=-0.23, p<0.03). Importantly, the results are similar whether the unit of analysis is single cells, sessions, or animals. These data indicate that place cell remapping in response to alterations in the valence of a context correlate with gamma synchronization, although low and high gamma frequencies may differentially affect distinct learning phases. The increase in gamma synchronization may facilitate cross talk with other brain regions.

Discussion

The idea that extinction is new learning rather than modification of a preexisting memory is largely based on behavioral findings indicating that the original memory trace re-emerges after extinction training under some conditions (Bouton, 2004; Myers and Davis, 2002). Since extinction is a context-dependent process that requires the hippocampus (Ji and Maren, 2007), this region is thought to regulate the context-specificity of extinction by providing contextual gating for the expression of fear after extinction, e.g., contextual renewal of fear memories (Goosens, 2011; Ji and Maren, 2007). Here we characterized for the first time the physiological changes in hippocampal neurons in response to fear extinction. Our findings indicate that extinction is a complex representation that encodes different characteristics of the context. There are hippocampal cells that remap primarily during extinction to form new representations of the context, suggesting that extinction indeed represents new learning. However, other cells remap both during conditioning and extinction, demonstrating that extinction may also modify pre-existing memories. Furthermore, there are cells that remap mainly during conditioning, which may serve to facilitate savings, the reacquisition of the original fear association at a faster rate than initially learned (Napier et al., 1992). Finally, there are cells that remain stable throughout fear learning, and may serve to encode the static aspects of the context. We also observed a moderate increase in theta coherence across sessions and a robust enhancement in low and high gamma coherence during extinction. Interestingly, the remapping associated with conditioning and extinction correlates with gamma coherence, suggesting that remapping may serve to amplify task-relevant information through

neuronal synchronization. Together, these findings indicate that memory traces of distinct emotional valence co-exist in the contextual representation of extinction.

Tronson et al. (2009) previously showed that different populations of principal cells in the hippocampus expressed cFos and pErk after fear conditioning and extinction, respectively, with less than 5% of cells exhibiting colocalization of both proteins. Their results suggest that on the molecular level, fear conditioning and extinction may be mediated by different populations of hippocampal neurons. However, variables such as the length of the extinction protocol may affect the specificity of immediate early gene markers (Radulovic and Tronson, 2010). Therefore, to understand how the hippocampus provides a gating mechanism for conditioning and extinction, it is necessary to record from the same neurons throughout fear learning. Using this approach, our results indicate that while some cells may be involved specifically in fear conditioning or extinction, many cells are involved in forming contextual representations of both learning processes.

It is interesting to note that representations formed after extinction do not stabilize in the long term, in contrast with our previous observation that cells display long-term stability after fear conditioning (Wang et al., 2012). In familiar environments, place fields in mice are generally unstable in the long term in the absence of task contingencies (Kentros et al., 2004; Muzzio et al., 2009). Therefore, it is possible that most cells revert to their original remapping state after fear extinction because the animal no longer needs to remember the context as fearful. Even though extinction eliminates the fear response, the animal likely still remembers the context to some degree, since processes such as spontaneous recovery and savings in the rate of relearning have been demonstrated previously (Macrae and Kehoe, 1999; Pavlov, 1927). It is possible that these processes bring back the original memory by re-establishing prior representations in specific subpopulations of cells. For example, cells that remapped primarily during conditioning may remap to locations previously associated with fear as the fear response returns.

Regarding the remapping we observed during predator odor fear conditioning, an alternative interpretation of our results is that the observed changes are merely due to the presence or absence of the odor rather than learning-dependent changes in the emotional valence of the

context. However, this possibility is unlikely because we also show that an aversive, non-fearful odor does not produce significant place field remapping in the short term either during odor exposure or during the extinction session. These results are in agreement with our previous study showing that dorsal place cells do not significantly remap in response to different odors (Keinath et al., 2014), as well as a previous finding showing that very few cells respond to changes in the odor of a context alone (Anderson and Jeffery, 2003). The absence of substantial remapping in response to non-fearful odors in the dorsal hippocampus may reflect the fact that olfactory inputs project more prominently to the ventral hippocampus (Kerr et al., 2007; Majak and Pitkänen, 2003). Conversely, the remapping observed in response to fearful odors may be mediated through the direct projections from the magnocellular portion of the basolateral amygdala to the dorsal hippocampus, which could serve to convey emotional valence (Pikkarainen et al., 1999).

One intriguing possibility is that the valence-specific cell populations that we identified in this study are differentially connected to other brain regions and circuits governing fear and extinction. For example, recent studies in the amygdala have found distinct circuits involved in switching fear memories on and off, and hippocampal afferents are directly connected to fear but not extinction cells in the amygdala (Herry et al., 2008). Thus, it is possible that the place cells responding primarily to fear conditioning are the same hippocampal cells that directly or indirectly project to fear cells in the amygdala. Likewise, place cells that remapped more strongly during extinction may be indirectly connected via the ventral hippocampus to other regions involved in extinction such as the prefrontal cortex (Hoover and Vertes, 2007). Future studies simultaneously recording units in the dorsal and ventral hippocampus in conjunction with activity in other brain regions will serve to clarify whether distinct subpopulations of place cells show differential synchronization across brain areas during fear and extinction learning.

In this study, the characterization of fear and extinction cells assumes that we recorded from the same cells during conditioning and extinction. Although the nature of extracellular *in vivo* recordings makes this difficult to prove, several observations support the contention that we indeed held the same cells throughout training. First, we observed waveform constancy for the full duration

of the experiment, which suggests minimal electrode drift. Second, we were able to over-impose cluster boundaries across successive sessions. Third, a substantial number of cells in the extinction group and the majority of the cells in the ABA control display long-term stability, suggesting that they were indeed the same cells throughout training. Therefore, we favor the interpretation that we were holding the same cells over time. However, it is important to note that even if there was electrode drift across days, the analysis of short-term remapping during the extinction session still reveals significant differences between the extinction and control groups. Critically, extinction-induced remapping is only seen in fear-conditioned animals undergoing extinction in the training context, and is not present in any of the control groups (i.e. water, 2-MB, or ABA control), indicating that extinction indeed produces instability in a subpopulation of cells. These results clearly demonstrate that some cells respond to the altered valence of the context, a phenomenon that is specific to extinction training.

Previous studies showed that gamma coherence between brain regions plays an important role in the routing of information (see Fries, 2005 and Colgin and Moser, 2010 for reviews), which may serve to coordinate the reactivation of memories (Carr et al., 2012). In the medial temporal lobe, synchronization at low and high gamma frequencies mediates cross talk between CA3/CA1 and medial entorhinal cortex/CA1 regions, respectively (Colgin et al., 2009). Low gamma coherence has been suggested to play a role in memory encoding, whereas high gamma coherence appears to participate in retrieval (for a review, see Colgin and Moser, 2010). In support of this hypothesis, we found that the degree of remapping correlates with low gamma coherence during conditioning, when the fear memory is encoded. Conversely, the degree of remapping correlates with both low and high gamma coherence during extinction, which involves encoding of new safe representations and retrieval of old fearful associations. Additionally, in the visual cortex, phase locking at the gamma frequency band has been suggested to underlie selective attention (Fries et al., 2001; Womelsdorf and Fries, 2007). Therefore, the enhancement in low and high gamma coherence observed during extinction may reflect an attention-like process that allows the animal to determine which emotional representation (i.e. ‘safe’ or ‘fearful’) is relevant during and

after extinction. We have previously found a similar enhancement in gamma coherence when animals had to determine whether visual or olfactory cues were relevant to find a hidden reward (Muzzio et al., 2009). It is possible that whenever there is competition between varying information sources, gamma coherence increases the salience of information relevant to the task at hand.

In conclusion, we have demonstrated that neurons in the dorsal hippocampus shift their preferred firing locations in response to fear and extinction learning, and that different subpopulations of cells appear to preferentially encode fearful, safe, and static aspects of a context. Furthermore, we have shown that extinction learning involves both modification of previous memory traces and the emergence of new representations. Finally, the degree of remapping observed during conditioning and extinction correlates with gamma synchronization. Together, these results indicate that extinction is a complex memory trace that integrates several aspects of different emotional valence.

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Conflicts of interest statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER 4: Effects of sleep deprivation on hippocampal neuronal activity in old and young adult mice during the object-place recognition task

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Abstract

Age-related changes have been documented in both sleep and cognition. Moreover, there is evidence that sleep may enhance memory consolidation. Here, we examined changes in sleep characteristics and place cell activity in young and aged adult male C57bl/6 mice performing a hippocampus-dependent object-place recognition task. Mice explored 3 objects in a novel context during training and underwent 5 hrs of sleep deprivation immediately afterward, followed by overnight recovery sleep before being tested the next day with 1 object displaced. We found that sleep deprivation impaired learning in young mice but enhanced it in old animals. Interestingly, sleep-deprived old mice also showed enhanced consolidation of NREM sleep during recovery. Additionally, we observed heterogeneous place cell responses to objects and spatial context in all groups. In young control animals and old sleep-deprived animals, a subpopulation of cells exhibited high place field stability throughout training and test sessions, suggesting that these cells may code the static aspects of the environment (context cells). However, another subpopulation of cells remapped when objects were introduced to the context and again when an object was displaced, suggesting that these cells may code object location (object cells). In sleep-deprived young mice, both context and object cells remained stable during the test session, suggesting that although they correctly remember the static environment, the observed behavioral deficit results from a failure to update the spatial representation of the objects. Meanwhile, old control animals exhibited global remapping in both types of cells, implying that their impairment stems from a failure to consolidate representations formed during training. Our results suggest that sleep deprivation may be able to rescue age-related memory deficits by enhancing consolidation of NREM during recovery sleep. Additionally, successful performance of the object-place recognition task may require animals to both maintain a stable representation of the context and simultaneously update a spatial representation of objects within the environment.

Introduction

Although the precise function of sleep remains under debate, one leading hypothesis is that sleep plays an important role in memory consolidation. Numerous studies in a wide variety of species have found evidence that post-training sleep improves performance on many different learning tasks (Gais & Born 2004, Gais et al 2006, Smith 2001). Additionally, sleep deprivation after training has been shown to impair learning, particularly on hippocampus-dependent memory tasks (Graves et al 2003, Prince et al 2014, Smith & Rose 1996). Hippocampal activity during sleep appears to be important for memory consolidation. Previous studies have found that networks of cells active during wake periods are reactivated during sleep. In this "replay" activity, cells fire following the same temporal sequences observed during wake states at a compressed time scale (Pavlides & Winson 1989, Skaggs & McNaughton 1996, Wilson & McNaughton 1994). However, it is unknown how hippocampal place cells respond to sleep deprivation. Moreover, density of hippocampal spindles during slow wave sleep correlates with the degree of sleep-dependent memory enhancement, further suggesting that this activity is critical for memory processes (Fuentemilla et al 2013).

Sleep and the response to sleep deprivation have been shown to undergo age-related changes across the lifespan (Huang et al 2002). Older subjects have generally been found to tolerate sleep deprivation better than young subjects (Adam et al 2006, Duffy et al 2009, Philip et al 2004, Silva et al 2010, Stenuit & Kerkhofs 2005), but they also exhibit more fragmented sleep and less slow wave sleep in comparison to young subjects (Espiritu 2008, Hasan et al 2012, Ohayon et al 2004). Not surprisingly, hippocampus-dependent cognitive tasks are particularly sensitive to age-related impairments (Barnes 1979, Miller & O'Callaghan 2005, Wimmer et al 2012), and have also been shown to be highly susceptible to sleep loss (Graves et al 2003, Prince et al 2014, Smith & Rose 1996); thus, it has been suggested that this age-related cognitive decline may be linked to changes in sleep patterns (Altena et al 2010). However, it is still unclear how changes in sleep patterns can affect neuronal activity and memory during wake periods. Furthermore, although it has been suggested that sleep deprivation in young animals may be useful as a model

of aging (Harrison et al 2000), this idea has not been tested by direct comparison of neuronal responses in young and old animals following sleep deprivation.

Here, we investigated how an acute period of sleep deprivation followed by recovery sleep affects place cell activity in old and young adult C57bl/6 mice performing an object-place recognition task, a hippocampus-dependent learning task that takes advantage of the rodent's innate preference for novelty (Oliveira et al 2010). We found that while sleep deprivation impaired performance in young mice, it surprisingly enhanced performance in old animals. We also found that dorsal hippocampal place cells remapped heterogeneously during acquisition and testing. In all groups, we identified cells that either responded to the context (context cells) or to the configuration of objects (object cells). However, the phenotype of these subpopulations differed depending on the experimental condition. In young control mice and old sleep-deprived mice, object cells remapped during training when the objects were introduced and again during the test session when an object was moved, whereas context cells remained stable both during training and the test session. These results suggest that the object-place task requires animals to both maintain a stable representation of the context and simultaneously update a representation of the configuration of objects in the environment. Interestingly, old sleep-deprived mice also showed a trend for enhanced consolidation of NREM sleep despite showing no increase in overall sleep compared to old control animals.

Conversely, both static and object cells recorded from young sleep-deprived mice exhibited long-term stability, suggesting that the learning deficit observed in this group may stem from a failure to update the memory of the object configuration rather than a failure to consolidate the memory of the context. Moreover, although young mice exhibited a trend for sleep rebound, this was primarily through an increase in the number of REM bouts. Finally, old control animals exhibited complete remapping of both context and object cells between the training and test sessions, indicating that their performance deficits resulted from impaired consolidation of the spatial representations formed during training. In sum, our results suggest that aging impairments can be rescued by enhancing the consolidation of NREM sleep through acute sleep deprivation.

Furthermore, impairments observed in young animals after sleep deprivation are not equivalent to those observed in aging.

Materials and Methods

Subjects

Young (8-24 weeks old) and aged (50-70 weeks old) adult male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were housed individually on a 12-hour light/dark cycle and allowed access to food and water *ad libitum* for at least two weeks prior to experiments. Animal living conditions were consistent with the standard required by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experiments were approved by the Institution of Animal Care and Use Committee (IACUC) of the University of Pennsylvania and of the University of Texas at San Antonio and were carried out in accordance with NIH guidelines.

Experimental procedures

The object-place recognition task was conducted in a square context (30cm x 30cm) with distinct visual features on each wall. Common everyday items (glass beer bottle, metal soda can, and plastic juice bottle) were used as objects after pilot testing determined mice showed roughly equal preference for all items on average. On day 1, animals were first habituated to the empty context for 6 minutes (hab). After the habituation session, 3 objects were arranged along one of the diagonal axes in the context and 3 object exploration trials were conducted (trial 1-3). Each object trial consisted of 6 minutes of free exploration and was conducted 2 minutes apart. In between object trials, the context was wiped down with 70% ethanol to eliminate any odor trails. Immediately following the third object trial, animals in the experimental group were sleep deprived for 5 hrs, whereas controls were housed in the same deprivation chamber but allowed to sleep. On day 2, one object in the context was moved from its original location to an adjacent corner. Mice were then allowed to freely explore the context with the moved object (test) for 6 minutes (Fig. 1A). All object positions were counterbalanced across trials, and all behavioral procedures were conducted during the first third of the light cycle (ZT4 or earlier).

Sleep Deprivation

An automated sleep deprivation apparatus (Pinnacle Technology, Lawrence, KS) consisting of a cylinder with a bar spanning the enclosure was used for all sleep deprivation procedures. It has been shown that automated sleep deprivation mechanisms that force movement are more consistent and effective than alternatives such as gentle handling (Fenzl et al 2007). Animals were individually housed in the apparatus starting at least 24 hours prior to beginning experiments with fresh bedding, food, and water, and were returned to the apparatus in between sessions. To induce sleep deprivation, a motor was turned on, causing the bar to rotate continuously at approximately 3 rpm, reversing direction at random to prevent animals from predicting the trajectory of the bar. Animals in the experimental group were sleep deprived for 5 hours at a time, while animals in the control group were housed in the apparatus on the same schedule while the bar remained stationary.

EEG analysis of a pilot group during a 5 hr period of sleep deprivation confirmed that sleep was minimal in both old and young adult animals (average percent time awake: 94.4±2.2% in old, 96.3±1.1% in young; data not shown). A plasma corticosterone assay using samples drawn after 5 hours of sleep deprivation in a pilot group revealed a small increase in corticosterone compared to controls in both age groups (young: t_8=1.94, p=0.09; old: t_8=4.68, p<0.05; data not shown). However, corticosterone levels were not significantly different from animals in a control group given 5 minutes of gentle handling (young: t_8=1.81, p>0.05; old: t_8=1.64, p>0.05; data not shown).

Surgery

All procedures were performed stereotaxically (David Kopf Instruments, Tujunga, CA). Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally, or with inhalatory isoflurane (1.5% by volume in O_2) and kept on a heating pad to maintain body temperature. Carprofen (5mg/kg) was administered after anesthetization and one day post-operatively for analgesic purposes. Animals were allowed at least one full week of recovery prior to beginning electrophysiological experiments.

For place cell recordings, drivable 6-tetrode microdrives were implanted in the hippocampus at the following coordinates from bregma (in mm): AP, -1.7; ML, -1.6; DV, -1.0. A

ground wire was attached to a screw placed on the contralateral side of the skull, and headstages were secured to the skulls with cyanoacrylate and dental cement. For EEG recordings, prefabricated 2 channel EEG/1 channel EMG headmounts (Pinnacle Technology) were implanted. 4 holes were drilled in the skull and screw-type electrodes were inserted to rest on the surface of the brain at the approximate coordinates from bregma (in mm): frontal leads: AP: +3.2mm, ML: +/- 1.2mm, and parietal leads: AP: -1.8mm, ML: +/- 1.2mm. Two EMG leads were placed under the nuchal musculature and affixed with VetBond. Cyanoacrylate and dental cement were used to anchor the headmount to the skull and cover all exposed electrodes.

Histology

Electrode placement was verified after completion of recordings. Animals were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), and final electrode positions were marked by passing a current (0.1 mA for 5 seconds) through the tetrodes that yielded unit data (53500 Lesion Making Device, Ugo Basile, Comerio VA, Italy). The brains were removed and fixed at 4°C for at least 48 hours in 10% formalin containing 3% potassium ferrocyanide (J.T. Baker, Deventer, Netherlands). They were then transferred to a 30% sucrose solution for at least 48 hours at 4°C for cryoprotection. Brains were then cryosectioned (30 μm, coronal) and Nissl stained with cresyl violet using standard histological procedures (Powers & Clark 1955).

Behavioral Analysis

All object exploration trials were video recorded using Limelight (Actimetrics, Wilmette, IL) and manually scored offline by three blind independent scorers. Performance in the object-place recognition task was assessed by tracking explorative behavior exhibited by the animals in reference to the objects. All instances when the animal was both oriented toward and touching the object with nose, vibrissae, and/or forelegs were recorded as “object exploration”; contacting the object while passing or oriented away were not counted. Animals with an average object exploration time less than 10 seconds on day 1 or during the test session on day 2 were excluded from analysis. Behavioral data from animals used for EEG analysis was combined with data from animals used for place cell recordings.

To quantify object preference, we calculated the percentage of total object exploration time spent exploring the moved object. Object preference on Day 1 was calculated using average exploration times in trials 2 and 3 (trial 1 was excluded due to a significant novelty effect on exploration time; young: main effect of session, $F_{(3,108)}$=19.46, p<0.001; old: main effect of session, $F_{(3,102)}$=4.63, p<0.05; *post hoc* multiple comparisons for both age groups showed significant differences between trial 1 and other sessions, p<0.05). We then calculated the difference in percent preference for the moved object (Δ % preference) between Day 2 and Day 1 as follows:

$$\Delta\,\%\,preference = 100 * \left(\frac{moved\ object\ exploration_{test}}{total\ object\ exploration_{test}} - \frac{moved\ object\ exploration_{trial\ 2-3}}{total\ object\ exploration_{trial\ 2-3}}\right)$$

Thus, larger values indicate greater increases in preference for the moved object during the test session, while lower values indicate little change in preference from day 1 to day 2, after the object is displaced. Importantly, this equation eliminates potential bias introduced by individual pre-training preferences for a particular object.

Place Cell Analysis

Place cell recordings and data analysis were done as previously described (Wang et al 2012, Wang et al 2015). Beginning one week after surgery, neural activity from each tetrode was screened daily while animals explored a standard context different from contexts used for experiments. The headstage was connected to a tethered unity gain amplifier with green and red LEDs for tracking the position of the animal. Units were amplified using a 32-channel amplifier (Neuralynx, Bozeman, MT) between 2,500 and 10,000 times and filtered between 400-9,000 Hz. The amplifier output was digitized at 30.3 kHz. The position of the animal and electrophysiological data were recorded by Cheetah Data Acquisition software (Neuralynx, Bozeman, MN). The electrode bundle was advanced by 15-20 μm steps per day, lowering the tetrodes in small steps to increase the stability of the recordings (Kentros et al 2004, Muzzio et al 2009). Pyramidal cells were identified by their characteristic firing patterns (Ranck 1973), and experiments were begun only when recordings were stable for at least 24 hours. Long-term recordings were considered stable when cells had the same cluster boundaries over two sessions, and the waveforms obtained from all four wires of a tetrode were identical between sessions.

After completion of the experiments, units were cluster cut and analyzed using MClust software (developed by A. David Redish, University of Minnesota). Cells were only accepted for analysis if they formed isolated clusters with clear Gaussian ellipses and minimal overlap with surrounding cells and noise, and displayed high waveform similarity throughout the experiment. Finally, all cells were inspected to rule out that any events during the 2 msec refractory period. The generation of place field maps was done in Matlab by first dividing the environment into 2 x 2 cm pixels, then calculating the number of spikes and the time spent in each pixel. Both the spike count and time maps were then smoothed with a 3 cm full-width at half maximum Gaussian kernel. The final place field map was obtained by dividing the smoothed spike map by the smoothed rate map. Only periods of movement were included in the place field analysis, during which the minimum walking speed was 2 cm/s. We excluded cells that fired less than 30 spikes during movement in a session or displayed peak firing frequencies lower than 1.3 Hz before smoothing after the speed threshold was applied.

Place field stability was assessed by calculating pixel-by-pixel Pearson R cross-correlations between maps. Object cells were defined as cells with a correlation below 0.3 between the habituation session and first object trial, while cells with a correlation of 0.3 or higher between the first two sessions were defined as context cells. This value falls 2 standard errors above the mean correlation between habituation and trial 1 for all cells. Cells with this correlation value are generally reported in the literature as being stable (Kentros et al 2004, Muzzio et al 2009), and we have used this value previously to differentiate remapping and stable cells (Wang et al 2015).

Sleep State Analysis

EEG/EMG signals were recorded for five hours following sleep deprivation on day 1 using hardware from Pinnacle Technology (Lawrence, KS). Animals were implanted with prefabricated headmounts that contained two EEG channels and one EMG channel for muscle activity. For recordings, the headmounts were attached to a preamplifier unit that provided 1^{st}-stage amplification (100x) and an initial high-pass filter (1^{st}-order 0.5 Hz for EEG and 10 Hz for EMG). All signals were then passed to the acquisition system via a tether and low-torque commutator and

sampled at 300 Hz, digitized and routed to a PC-based acquisition and analysis software package (Sirenia Acquisition, Pinnacle Technology). Animals with excessive noise in any channels (>10% of epochs classified as artifact) were discarded from EEG analysis, although behavioral analysis was retained.

Recordings were divided into 4 second epochs, and each epoch was then classified as wake, rapid eye movement (REM) sleep, or non-REM (NREM) sleep using a semi-automated cluster scoring program in the SleepPro software package (Pinnacle Technology, Lawrence, KS). Wake periods were identified as epochs with high EMG activity. Sleep epochs were identified by low EMG activity, with REM epochs exhibiting higher theta power and NREM epochs containing more delta power. All ambiguous epochs were hand scored. Bouts were defined as periods of 3 or more continuous epochs of the same state. To analyze sleep fragmentation, Sirenia SleepPro was used to determine the number and average length of bouts occurring in each state.

Statistics

Statistical analysis was performed with SigmaStat (Aspire Software International, Ashburn, VA) and Excel (Microsoft, Redmond, WA). Control and sleep deprivation groups were compared within each age group. A 2-way ANOVA with repeated measures was used to compare place cell correlations across sessions and percent sleep, number of bouts, and bout length between control and sleep deprived animals. Independent t-tests were used to compare rate change from trial 3 to the test session between controls and sleep deprived animals, as well as object preference during the test session, and place cell correlations between object and context cells for each group. For all statistics, a significance level of 0.05 was used. In all figures, an asterisk denotes a significant difference with a probability <0.05, and error bars indicate ± SEM.

Results

Sleep deprivation impairs object-place memory in young adult mice but enhances performance in old mice

To examine the effects of sleep deprivation on memory at the behavioral level, we tested mice in the object-place recognition task (Figure 13A), a learning task that has previously been

shown to be hippocampus dependent (Oliveira et al 2010). We found no differences between control and sleep deprived young adult animals (Figure 13B) or old animals (Figure 13D) in total object exploration times in any sessions either before or after sleep deprivation (for both ages, no effect of group: $F<1$, nor its interaction with session: $F<1$, $p>0.05$). Consistent with previous literature, we found that young sleep deprived mice performed worse than their age-matched controls, showing significantly less increase in preference for the moved object ($t_{37}=2.23$, p=0.03, Figure 13C). Similarly, old control mice showed minimal increase in preference for the moved object, as reported by other studies (Wimmer et al 2012). Surprisingly however, old sleep-deprived mice showed enhanced performance characterized by an increase in preference for the moved object compared to old control animals ($t_{36}=2.48$, p=0.02, Figure 13E).

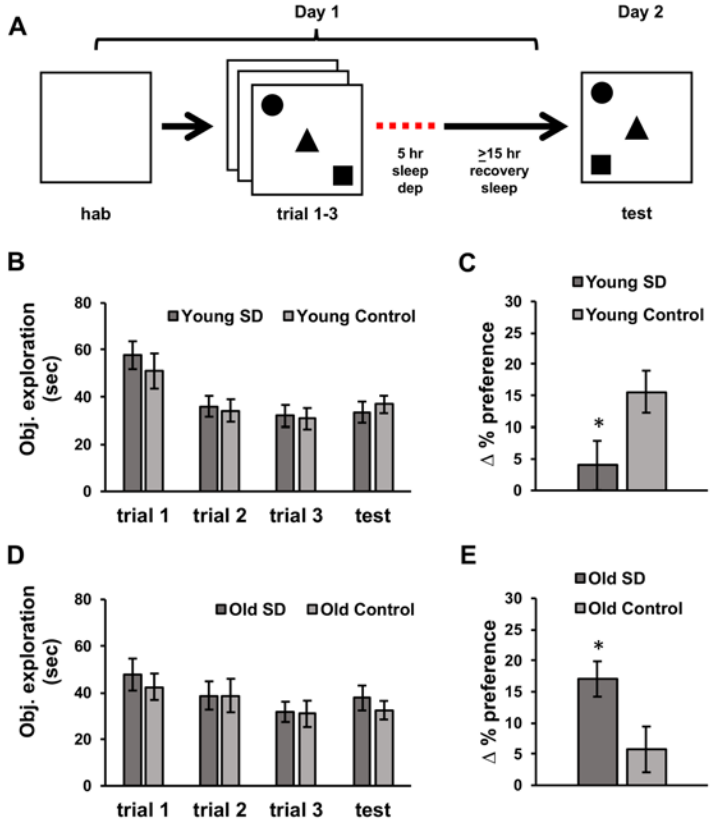

Figure 13 A. Schematic representation of behavioral paradigm. **B.** There were no significant differences between young control and SD mice in average object exploration time across sessions. **C,E.** Preference for the moved object during the test session, plotted as a change in percent preference from day 1 trials. **C**. Young SD animals show reduced preference for the moved object compared to controls. **D.** There were no significant differences between old control and SD mice in average object exploration time across sessions. **E.** Old SD mice showed greater preference for the moved object compared to controls.

Place cells remapped heterogeneously in response to objects and static aspects of the context

To examine how place cell activity responded to sleep deprivation and recovery sleep in the context of the object-place recognition task, we recorded 41 cells from dorsal area CA1 of four

young adult mice, of which 38 cells were held throughout training. As a control, we recorded 54 cells in seven young animals that were not sleep deprived, of which 50 cells were held throughout training. In old animals, we recorded 43 cells from five mice, 30 of which were held throughout training. We recorded an additional 34 cells from five old control mice, of which 33 were held throughout training. Cells accepted for analysis formed distinct clusters and exhibited stable waveforms and cluster boundaries between consecutive sessions.

Previous studies have shown that dorsal hippocampal cells respond to objects (Cohen et al 2013). Therefore, we expected strong remapping between the habituation session (no objects) and trial 1 (same context with objects). Interestingly, we observed a high degree of variability in place field remapping, with some cells displaying strong remapping and others remaining stable. To quantify these observations, we calculated pixel-by-pixel cross-correlations between sessions to examine place field stability, i.e. the degree of similarity between the fields, throughout the experiment. We then further classified the cells using a remapping threshold of 0.3, which corresponds with previous thresholds we have used to identify stable and unstable place fields (Wang et al 2015). This classification allowed us to divide cells into two categories: "object cells", which remapped between habituation and trial 1 when the objects were first introduced, and "context cells", which displayed high stability between both the habituation and trial 1 sessions. In young animals, each of these categories made up roughly half of the cells recorded, with 47% remapping in response to the objects and 53% remaining stable (Figure 14A). However, in old animals, the majority of cells recorded were object cells (63%), while only 37% were context cells (Figure 14B).

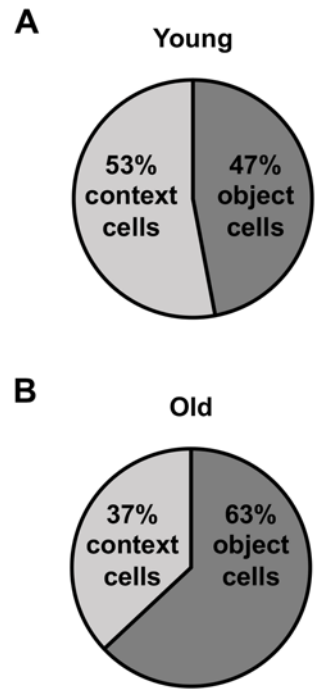

Figure 14 A. Of all cells recorded from control and SD young adult mice, 53% were context cells and 47% were object cells. **B.** Of all cells recorded from control and SD old mice, 37% were context cells and 63% were object cells.

Sleep deprivation reduces global place field remapping in young adult mice

Although we found no significant differences between sleep deprived and control young adult animals in average pixel-to-pixel cross correlations of consecutive sessions occurring prior to sleep deprivation (hab/trial 1, trial 1/trial2, trial 2/trial 3), there was a trend in the main effect of group ($F_{(1,287)}$=3.74, p=0.06), which reflected a difference in the comparison of the last training trial to the test session. Additionally, we found an effect of session ($F_{(3,287)}$=23.18, p<0.001) showing that place field stability was higher during short-term training sessions with the objects than habituation or testing, but no significant interaction (F<1, p>0.05; Figure 15A).

We then separated the cells into context and object categories and examined remapping within each. We found that in control young adult mice, context cells remained stable throughout the entire experiment, including trial 3 to the test session (Figure 15B). However, object cells per definition remapped between habituation and trial 1, and then displayed high, short-term place field stability in between object trials. Critically, object cells remapped again during the test session when an object was moved, suggesting that these cells responded to the displacement of the object (Figure 15C). Meanwhile, context cells in sleep-deprived young adult mice also displayed high place field stability throughout the experiment (Figure 15D). However, object cells in this group failed to remap when the object configuration was changed on day 2 and remained stable between trial 3 and the test session instead, indicating that they did not recognize the object displacement (Figure 15E).

Next, we examined the average correlation between trial 3 and the test session for context and object cells. There was no significant difference between young control and sleep-deprived mice in the stability of context cells (t_{43}=0.50, p>0.05), suggesting that sleep-deprived young mice successfully retrieved context representations formed during training. However, object cells in young controls showed a trend indicating more remapping between trial 3 and the test session compared to object cells in sleep-deprived young mice (t_{37}=1.79, p=0.08). In young control animals,

object cells also had a significantly lower average correlation than context cells (t_{44}=3.55, p<.05), and the average correlation of object cells fell below the remapping threshold while the average correlation of context cells fell above the threshold. In contrast, there was no significant difference in average correlation between context and object cells in young sleep-deprived animals (t_{36}=0.66, p>0.05), and the average correlation of both was above the remapping threshold (Figure 16A). In sum, these data indicate that object cells remap after the object is displaced in young control animals, but that this does not occur in young sleep deprived young animals.

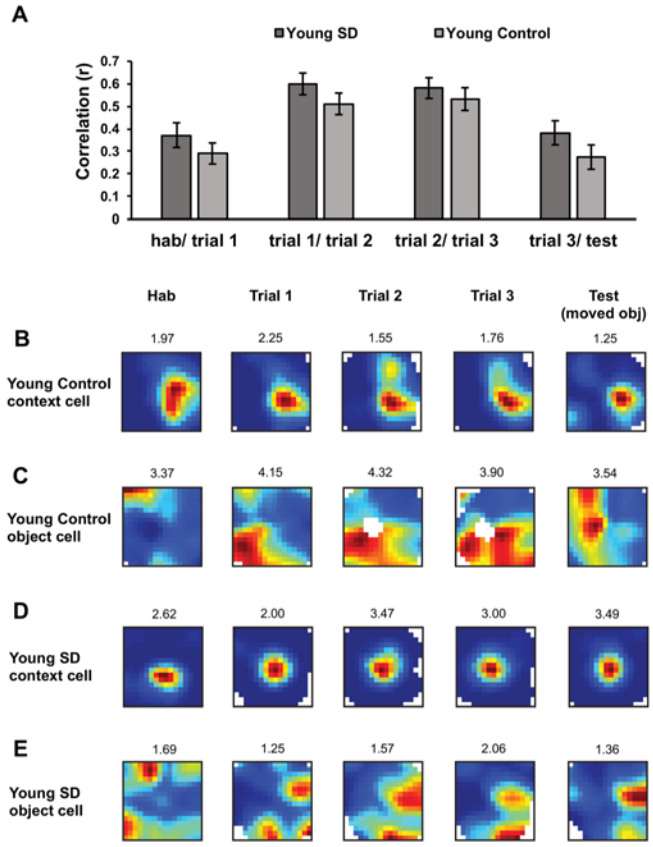

Figure 15 A. There were no significant differences between young control and SD mice in average pixel-to-pixel cross-correlations of consecutive sessions. **B-E,** Examples of place cell rate maps. Activity was normalized relative to the peak firing frequency shown on top of each rate map. Blue represents areas visited by the animal where a cell does not fire, and more vivid colors represent areas of increasing firing activity for that cell. Prior to sleep deprivation, two types of cells were identified: context cells, which remained stable from habituation to trial 1 when objects were introduced to the environment; and object cells, which remapped between habituation and trial 1 when objects were introduced. In young control animals, (**B**) context cells remained stable throughout but **(C**) object cells remapped between hab and trial 1, remained stable in the short-term between trials 1-3, and then remapped again from trial 3 to the test session. In young SD animals, (**D**) context cells remained stable throughout while (**E**) object cells remapped between hab and trial 1 but remained stable between trials 1-3 and from trial 3 to the test session.

Sleep deprivation reduces firing rate changes in response to a displaced object in young adult mice

We examined average mean firing rates across all sessions and found no significant differences between control and sleep-deprived young animals (no effect of group or interaction, *F*<1, p>0.05; Figure 16B). However, firing rate changes (rate remapping) in individual cells have previously been shown to code subtle

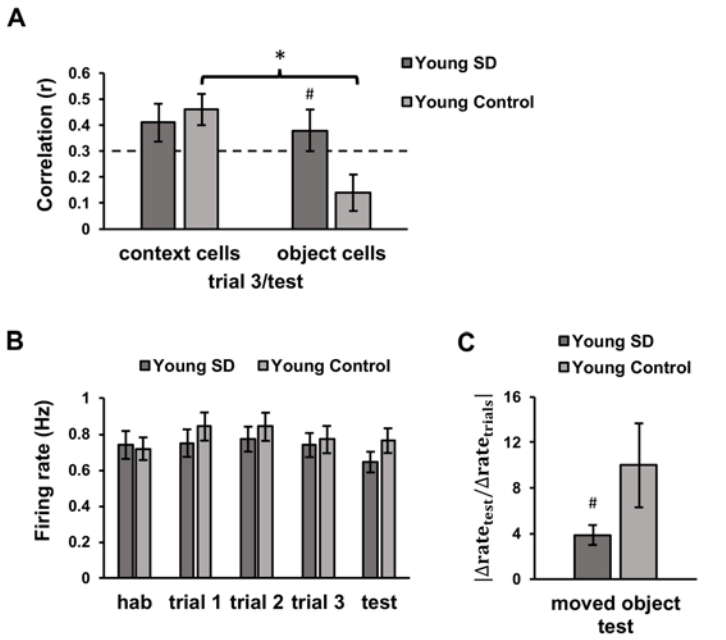

Figure 16 A. Average pixel-to-pixel cross-correlations of context and object cells between trial 3 and the test session with the moved object. Context cells in young control mice are significantly more stable than object cells. In contrast, context and object cells in young SD mice show no significant difference in stability. Moreover, object cells in young SD mice are more stable than those in control animals. Dotted line indicates remapping threshold of 0.3. **B.** Average mean firing rates across all sessions show no differences between young control and SD animals. **C.** Compared to controls, young SD mice show a trend for reduced absolute rate change between object trials on day 1 and the test session with the moved object.

changes in the environment. Since individual cells may exhibit firing rate changes in opposite directions, it is possible that these rate alterations are obscured by calculating the overall average firing rate. Therefore, we calculated a ratio of the absolute rate change between trial 3 and the test session to the absolute rate change seen within object trials 1-3. This allows us to capture the magnitude of rate change occurring in response to the moved object. We found that young control animals exhibited a trend toward greater rate change in response to the moved object in comparison to sleep-deprived animals (t_{109}=1.58, p=0.06; Figure 16C).

Young adult animals exhibit a trend toward NREM rebound and increased number of REM bouts during recovery sleep

We recorded the first five hours of *ad lib* recovery sleep following sleep deprivation in a separate group of five young adult animals, and five hours of a time-matched period of *ad lib* sleep in six control young adult animals. Consistent with accounts in the literature (Mistlberger et al 1983), we found that following five hours of sleep deprivation, young adult mice exhibited a slight rebound in NREM sleep, showing a trend toward spending a greater percentage of time in NREM sleep

compared to control young animals and a smaller percentage of time awake (group x stage interaction: $F_{(2,16)}$=2.35, p=0.12; *post hoc* multiple comparisons show a slight trend toward greater time in NREM sleep, p=0.11 and less time spent in wake, p=0.06; Figure 17A). We also observed a trend toward increased number of REM bouts following sleep deprivation (group x stage interaction: $F_{(2,16)}$=3.05, p=0.07; *post hoc* multiple comparisons indicated a trend for greater number of REM bouts in the sleep deprived group than the control group, p=0.07; Figure 17B), but with no significant differences in bout length (no effect of group, *F*<1, nor its interaction with stage, *F*<1, p>0.05; Figure 17C). These results indicate that following sleep deprivation in young adult mice, overall NREM tends to increase but remain unchanged in quality. Conversely, overall REM does not change, but the number of REM bouts increases in recovery sleep.

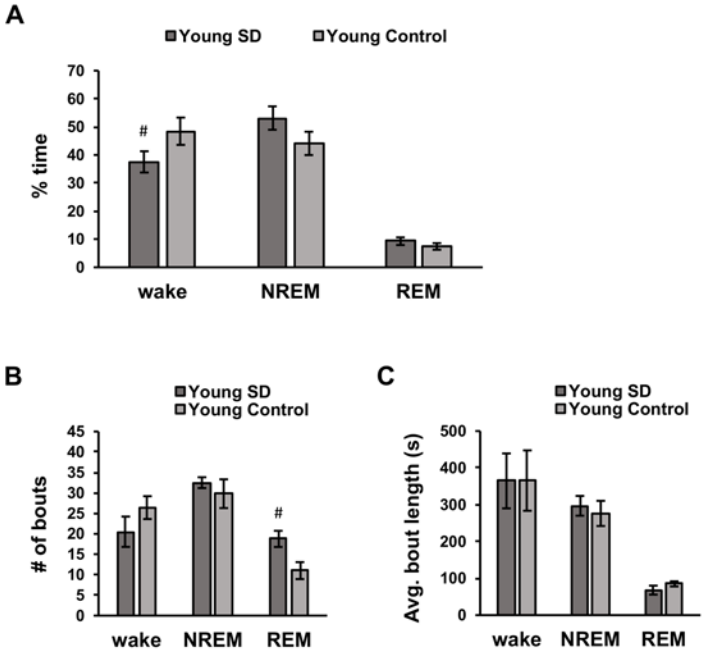

Figure 17 A-C. Analysis of first 5 hours of recovery sleep after sleep deprivation. **A.** Young SD animals show a trend for reduced time awake compared to controls. **B.** Young SD animals show a trend for increased number of REM bouts compared to young controls. **C.** No significant differences were seen between young SD and control animals in average wake, NREM, or REM bout lengths.

Sleep deprivation enhances place field stability in old mice

We found no significant differences between sleep-deprived and control groups in pixel-to-pixel cross-correlations when comparing sessions that occurred prior to sleep deprivation (hab/trial 1, trial 1/trial 2, trial 2/trial 3). However, old sleep deprived animals exhibited a significantly higher average correlation between trial 3 and the test session compared to controls (significant main effect of session, $F_{(3,204)}$=39.28, p<0.001, interaction between group and session, $F_{(1,204)}$=4.06,

p<0.05; *post hoc* multiple comparisons revealed a significant difference between sleep-deprived animals and controls only in the comparison between trial 3 and the test session, p<0.05; Figure 18A). On Day 1 of training, place cells in old control animals displayed the same pattern of stability observed in the young, with context cells remaining stable while object cells remapped from habituation to trial 1. However, both context and object cells in old control mice remapped again when the last training trial was compared to the test session with the moved object (Figure 18B-C). In contrast, place cells in sleep-deprived old animals exhibited a very similar pattern of remapping to that seen in young adult control animals. While context cells remained stable throughout, object cells remapped from trial 3 to the test session with the moved object (Figure 18D-E).

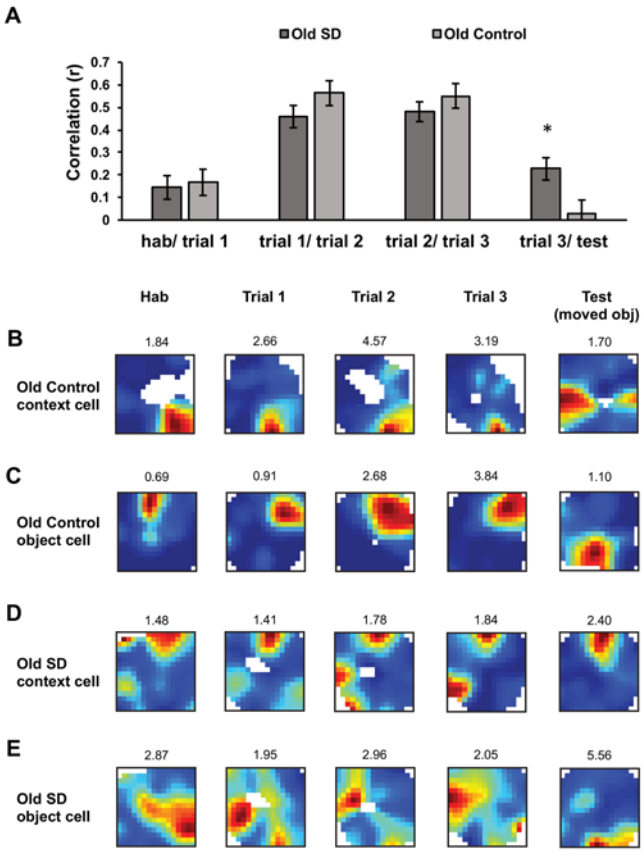

Figure 18 A. Average pixel-to-pixel cross-correlations of consecutive sessions in old control and SD mice. Old SD animals show higher place field stability between trial 3 and the test session compared to controls. **B-E,** Examples of place cell rate maps. Activity was normalized relative to the peak firing frequency shown on top of each rate map. Blue represents areas visited by the animal where a cell does not fire, and more vivid colors represent areas of increasing firing activity for that cell. Prior to SD, two types of cells were identified: context cells, which remained stable from hab to trial 1, and object cells, which remapped between hab and trial 1 when objects were introduced. In old control animals, (**B**) context cells remained stable until trial 3 but remapped between trial 3 and the test session. (**C**) Control object cells remapped between hab and trial 1, remained stable in the short-term between trials 1-3, but then remapped again between trial 3 and the test session. In old SD mice, (**D**) context cells remained stable throughout, while (**E**) object cells remapped between hab and trial 1, remained stable in the short-term between trials 1-3, and then remapped again from trial 3 to the test session.

We then examined the average correlation between trial 3 and the test session for context and object cells. Both context and object cells in old sleep-deprived animals had higher average correlations compared to controls (context cells: t_{19}=1.54, p<0.05; object cells: t_{37}=2.04, p<0.05). Additionally, context cells in old sleep-deprived mice were significantly more stable than object cells (t_{26}=2.23, p<0.05), and the average correlation of context cells fell above the remapping threshold while the average correlation for object cells fell below the remapping threshold. In old control mice however, there was no significant difference in stability between context and object cells (t_{30}=0.65, p>0.05), and the average correlation between trial 3 and the test session for both types of cells were well below the remapping threshold (Figure 19A). These findings suggest that an acute period of sleep deprivation was able to rescue an age-related deficit in place-field stability.

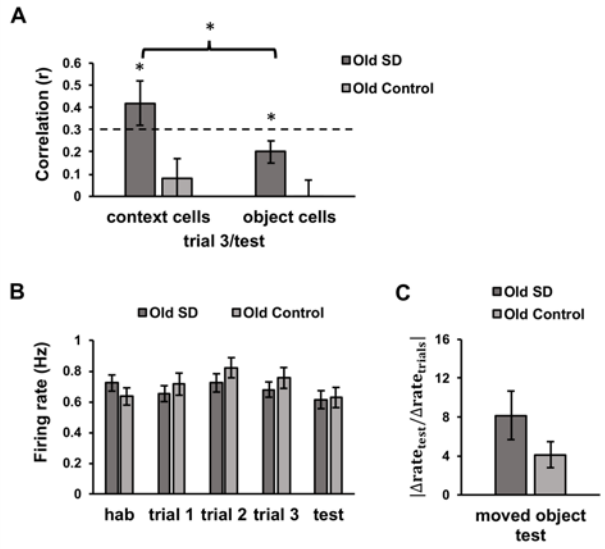

Figure 19 A. Average pixel-to-pixel cross-correlations of context and object cells between trial 3 and the test session with the moved object. Context and object cells in old SD mice are significantly more stable than those in old controls. Additionally, context cells in old SD mice are significantly more stable than object cells. Dotted line indicates remapping threshold of 0.3. **B.** Average mean firing rates across all sessions show no differences between old control and SD animals. **C.** Compared to controls, old SD mice show a trend for increased absolute rate change between object trials on day 1 and the test session with the moved object.

Sleep deprivation increases firing rate changes in response to a displaced object in aged adult mice

We examined average mean firing rate across all sessions and found no significant differences between sleep-deprived and control old animals (no effect of group, *F*<1, p>0.05; Figure 19B). We then examined the absolute rate change between trial 3 and the test session, using the same ratio calculation described earlier. Interestingly, we observed the reverse trend to that seen in young adult animals. Although the differences were not significant, the sleep-deprived group

showed a trend toward increased absolute rate change observed in response to the moved object, compared to the control group (t_{79}=1.26, p=0.10; Figure 19C).

Aged adult mice do not exhibit a sleep rebound after deprivation, but show a trend toward increased NREM consolidation

We recorded the first five hours of *ad lib* recovery sleep after deprivation in a separate group of five old animals, and a time-matched period of *ad lib* sleep in eight control aged mice. We observed no differences between sleep-deprived and control aged animals in the percentage of time spent in wake, NREM, or REM stages (no effect of group, F<1, nor its interaction with stage, F<1, p>0.05; Figure 20A), a finding that suggests older subjects exhibit reduced compensatory increases in sleep following sleep deprivation as previously shown (Bonnet & Rosa 1987, Mendelson & Bergmann 2000, Shiromani et al 2000, Webb 1981). However, in comparison to control old animals, sleep-deprived old mice exhibited a trend toward fewer number of wake and NREM bouts (significant group x stage interaction, $F_{(2,22)}$=6.46, p<0.05; *post hoc* multiple comparisons revealed trends showing differences between control and sleep-deprived animals in wake, p=0.06, and NREM, p=0.07; Figure 20B), although increases in bout length did not reach significance. These findings suggest that in old mice, sleep restriction may enhance subsequent NREM sleep quality during recovery sleep by decreasing fragmentation.

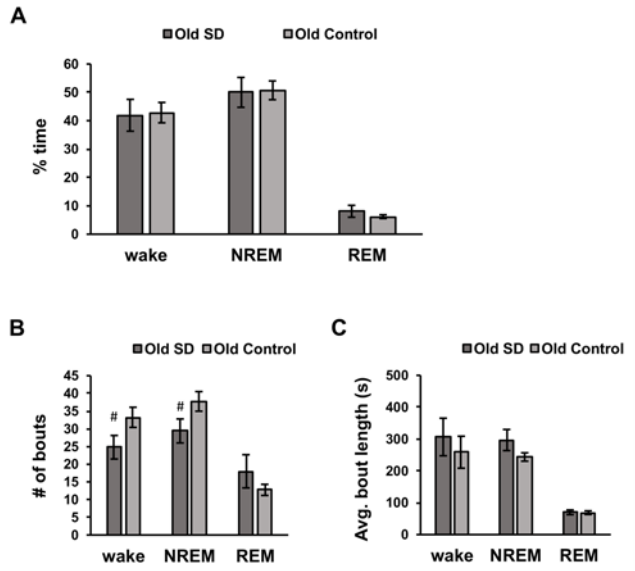

Figure 20 A-C. Analysis of first 5 hours of recovery sleep after sleep deprivation. **A.** Old SD animals show no significant differences with controls in percent time spent in wake, NREM, or REM. **B.** Old SD animals show a trend for fewer number of wake and NREM bouts compared to controls. **C.** No significant differences were seen between old SD and control animals in average wake, NREM, or REM bout lengths.

Discussion

Many findings in the literature indicate that sleep is critically important for memory consolidation, with ample evidence that sleep deprivation can cause memory deficits in a wide variety of behavioral tasks. Interestingly, there is also some evidence that tolerance of sleep deprivation may vary depending on age. Here, we characterized for the first time place cell activity in old and young adult mice performing the object-place recognition task after an acute period of sleep deprivation and recovery sleep. We found that sleep deprivation resulted in impaired object-place memory in young adult mice, but surprisingly enhanced performance in old mice. Place cells in all groups remapped heterogeneously during training, with some cells remapping when the objects were introduced and others remaining stable. Interestingly, both young control mice and old sleep deprived mice exhibited similar patterns of place field stability during the test session with the moved object. In these groups, cells that previously remapped in response to the objects remapped further during the test session when one object was moved. In contrast, cells which remained stable between habituation and object exploration continued exhibiting stability in the long term. Moreover, while both old control animals and sleep deprived young adult mice performed poorly on the task, they displayed different patterns of stability during the test session. Old control animals showed strong remapping in all cells, while place cells in sleep deprived young mice exhibited long-term stability. These results suggest that in order to perform the object-place recognition task successfully, animals must maintain a stable representation of the context in which the task is performed while also updating a concurrent representation of relevant spatial landmarks in the environment. Furthermore, although old control animals and young sleep-deprived animals both show impairment on the object-place task, these impairments may stem from different deficits in the spatial representations underlying this learning paradigm.

Several studies have previously investigated how post-training sleep or sleep deprivation affects performance on the object place recognition task, although these studies have been done exclusively with young adult animals. Despite a number of variations in experimental protocol, there is general consensus that immediate post-training sleep seems to be particularly critical for

memory. Using a two-hour retention interval between training and testing, Binder et al. (2012) found that memory performance was best in rats when this retention interval occurred during the inactive phase and the animal was permitted to sleep (Binder et al 2012). Similarly, periods of three and five hours of immediate post-training sleep deprivation have been shown to impair object-location memory in mice (Havekes et al 2014, Prince et al 2014). Our behavioral findings that five hours of total sleep deprivation immediately after training leads to memory deficits in young adult mice are thus consistent with the prevailing literature.

Interestingly, although the behavioral deficits seen in the object-place recognition task have typically been attributed to disrupted memory consolidation, our results suggest otherwise. Instead, the pattern of place field stability we observed in young adult mice after sleep deprivation indicate that memory consolidation may remain intact, but that there is a failure to encode novel changes to the context. Like young control animals, sleep-deprived young mice had a subset of cells that remained stable throughout the entire experiment including the test session. However, unlike controls, cells in sleep-deprived young animals that previously remapped in response to the objects failed to remap further when one object was moved, instead exhibiting long-term stability between the training and test sessions. These data demonstrate that sleep-deprived young animals are correctly retrieving the spatial representations formed during training, implying that memory consolidation has occurred successfully. However, they are unable to update the retrieved representation with new information. Several studies have demonstrated that under normal circumstances, memories become labile again in a short window after retrieval and can be modified before being reconsolidated (For review, see Nader 2003). Our results suggest that in young adult animals, sleep deprivation may interfere with this process, at least with regards to spatial representations in the hippocampus. In the future, it will be important to test if old animals also have deficits in reconsolidation, a procedure that updates memories and is dependent on protein synthesis, and if so, whether acute sleep deprivation can ameliorate these deficits as well.

Previous studies examining place cell activity during the object-place recognition task have been conducted in young adult rats under normal sleep conditions. Larkin et al. found that CA1

place fields exhibited changes in firing rate but not stability during the moved object test (Larkin et al 2014). Although we also observed high stability in the short term during object exploration trials, we observed only moderate overall stability between the training and test sessions and no changes in the average mean firing rate in young adult animals. However, we did observe some evidence of rate remapping between the object exploration trials and the test session in young control mice and old sleep-deprived mice. Furthermore, it is important to note that the Larkin et al. study was done in rats rather than mice, and used a five-minute retention interval so that all training and test sessions were conducted in close temporal proximity. We used a retention interval of over 15 hours in order to allow time for sleep deprivation and recovery. Place fields in mice have been shown to be less stable in the long-term in the absence of task contingencies or particularly salient conditions (Muzzio et al 2009), so the moderate stability we report is typical for the species. One other study examining place cell activity in the object-place task found evidence that changes in hippocampal gamma rhythms accompanied encoding of novel object-place associations (Zheng et al 2016). Unfortunately, technical limitations produced by electrical noise originating from the animal/object interactions prevented us from investigating if similar changes in gamma synchronicity can be found in mice, but this may be a fruitful avenue for future research.

Unexpectedly, we found that sleep-deprived old mice showed enhanced performance on the object-place recognition task relative to old controls. Many studies have found that older populations frequently have higher tolerance for sleep deprivation compared to younger populations, showing less change from baseline in measures such as vigilant attention, subjective sleepiness, and reaction time (Adam et al 2006, Duffy et al 2009, Stenuit & Kerkhofs 2005). Here, an acute period of sleep deprivation followed by a longer period of recovery sleep appears to have had an enhancing effect on both memory consolidation and reconsolidation. Cells in the old sleep-deprived mice that were stable during training when objects were introduced continued exhibiting high stability in the long-term between the training and test trial with the moved object. Moreover, similar to cells in young control animals, place cells in old sleep-deprived mice that originally remapped in response to the objects remapped further in response to object displacement during

the test session. Since sleep fragmentation has been associated with impaired memory consolidation and learning (Sportiche et al 2010, Tartar et al 2006, Ward et al 2009), it is possible that the subsequent enhancement of NREM sleep during recovery sleep was able to rescue normal age-related cognitive deficits. Indeed, several studies have found that enhancing NREM sleep via pharmacological interventions or sleep restriction can provide protective effects on cognitive impairments following stroke or traumatic brain injury (Cam et al 2013, Martinez-Vargas et al 2012, Morawska et al 2016).

We observed behavioral deficits in the control old mice, in agreement with existing literature demonstrating age-related impairment on the object-place recognition task (Wimmer et al 2012). Additionally, they displayed a different pattern of place field stability than that seen in similarly impaired young sleep-deprived mice. Cells in control old mice showed low stability in the long-term, remapping strongly between the training and test session regardless of their stability during training. In particular, cells that were stable throughout training and most likely encoded static aspects of the context remapped again during the test session, suggesting that the old mice failed to retrieve the correct spatial representation for the environment. Reduced place field stability has been previously reported in old animals (Barnes et al 1997), along with correlated behavioral impairments in several spatial tasks (Rosenzweig et al 2003). It is likely that the impaired long-term stability of context cells in control aged animals underlies the observed performance deficits on the object-place task. Critically, these deficits were different than those observed in young sleep-deprived animals, where the representation of context was stable but the object-place representation failed to update. Our data suggest that models that attempt to use young sleep-deprived animals as models of aging may be flawed (Harrison et al 2000), since the long-term memory impairments observed in these groups stem from different deficiencies.

In conclusion, we have demonstrated that sleep deprivation impairs performance on the object-place recognition task in young adult mice, but enhances performance in old mice, possibly through increased consolidation of NREM during recovery sleep. In both age groups, place cells in the dorsal hippocampus remap heterogeneously during the object-place recognition task, and

different subpopulations may form distinct representations of the static aspects of the context and the spatial configuration of objects within the context. The phenotype observed in both old sleep-deprived mice and young control mice suggests that successful performance on the object-place task requires both the maintenance of a stable representation of the context as well as an updated representation of object locations within the environment. Sleep deprivation appears to impair performance in young adult mice by disrupting their ability to update object-place representations. However, impairments in old control animals appear to stem from a failure to consolidate the spatial representation of context formed during training. Our findings contribute to a better understanding of how sleep deprivation disrupts hippocampus-dependent spatial memory, and have potential clinical implications for rescuing age-related cognitive deficits.

Author Contributions: AST performed behavioral experiments and analyzed data; RKY performed experiments, analyzed data, and wrote the manuscript; IM designed experiments and supervised analysis and writing of the manuscript.

Conflicts of interest statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER 5: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

In summary, it is clear that both neuromodulatory factors such as emotion and physiological factors such as sleep can influence the consolidation of hippocampus-dependent memory. Here, we have clarified the role of the hippocampus in emotional learning and further elucidated the effects of emotion on hippocampal activity. Consistent with literature suggesting that the dorsal and ventral regions of the hippocampus display different patterns of gene expression and connectivity (Fanselow & Dong 2010, Leonardo et al 2006, Thompson et al 2008), we found evidence that certain chromatin modifications may have differential effects along the longitudinal hippocampal axis during predator odor contextual fear conditioning. Specifically, we demonstrated that fear learning was enhanced when a class I histone deacetylase (HDAC) inhibitor was administered to the dorsal hippocampus after context pre-exposure, but that administration of the same HDAC inhibitor to the ventral hippocampus resulted in fear generalization (Yuan et al 2015). These data join the increasing body of evidence that the dorsal hippocampus codes specific details to allow for better context discrimination, while the ventral region facilitates generalization across similar contexts. Moreover, we showed that in the dorsal hippocampus, stable spatial representations that are formed during contextual fear conditioning can be further modified when the emotional valence of the context is changed during extinction training. We found that different subpopulations of cells respond to distinct aspects of learning during extinction: while some cells coded static aspects of the context, others responded either to fearful or safe associations. These data suggest that the extinction memory trace contains various elements that represent the past and current emotional valences of a context (Wang et al 2015). Together, these findings indicate that emotion exerts a strong effect on hippocampus-dependent learning, and can directly impact spatial representations in the hippocampus.

Although there is a large body of evidence supporting a general role for HDACs as negative regulators of long-term memory, it is still unclear whether specific HDACs play different roles in mediating particular types of learning. Class I HDACS, consisting of HDACs 1, 2, 3, and 8, have received the most attention in the field of learning and memory, with many studies demonstrating

that manipulations of these HDACs can influence cognition (For reviews, see Levenson & Sweatt 2005, Peixoto & Abel 2013, Roth & Sweatt 2009). We examined epigenetic mechanisms in the dorsal and ventral hippocampus using MS-275, a class I HDAC inhibitor with preferential inhibition of HDAC1 (Formisano et al 2015, Khan et al 2008). Interestingly, we observed effects on fear learning only when administration occurred following context pre-exposure, suggesting a role for HDAC1 in mediating the encoding of contextual information rather than fear learning specifically. Consistent with this finding, inhibition of HDAC1 has also been found to mediate fear extinction, a highly context-specific form of learning (Bahari-Javan et al 2012), as well as enhance long-term memory on the object-place task (Hawk et al 2011). However, Guan et al (2009) found evidence that overexpression of HDAC2 rather than HDAC1 in the hippocampus caused memory deficits in contextual fear conditioning and the Morris water maze, while HDAC2 knockout mice showed enhanced learning. Moreover, they demonstrated that overexpression of HDAC2 suppressed dendritic spine formation and impaired hippocampal LTP (Guan et al 2009). Additionally, increased levels of HDAC2 but not HDAC1 have been reported in two different mouse models of Alzheimer's disease (Gräff et al 2012). Critically, other researchers have also identified HDAC3 as a regulator of long-term memory, with focal deletions of HDAC3 or HDAC3 inhibition leading to enhanced contextual fear learning (McQuown et al 2011). These widely varied findings highlight the importance of continued research in this area, particularly as mounting evidence suggests that epigenetic mechanisms are at play in mediating cognitive and circadian changes in aging as well as neurodegenerative and neuropsychiatric diseases (Calvanese et al 2009, Fischer et al 2010, Orozco-Solis & Sassone-Corsi 2014).

Our finding that different subpopulations of cells in the hippocampus appear to respond preferentially to fear or extinction is especially interesting in light of studies demonstrating similar segregation of function at a cellular level in other brain regions. In the basolateral amygdala, separate populations of neurons have been identified which are selectively active only during fear or extinction (Herry et al 2008). These subpopulations exhibit differential connectivity with the hippocampus and prelimbic and infralimbic regions of the medial prefrontal cortex, regions that

modulate the expression of fear and extinction, respectively (Herry et al 2008, Senn et al 2014). Recent findings suggest that these neurons are also differentially inhibited by local interneurons (Vogel et al 2016), allowing for rapid shifts in activity that drive behavioral transitions between fear and extinction (Herry et al 2008, Vogel et al 2016). Similar subpopulations have been found in the lateral nucleus of the amygdala, where neurons active during fear received inputs primarily from the ventral hippocampus and prelimbic cortex while those active during extinction received inputs mainly from the infralimbic region (Knapska et al 2012). Since the hippocampus has been identified as being crucial for mediating the context specific expression of extinction (Corcoran et al 2005, Corcoran & Maren 2001, Corcoran & Maren 2004), it may indirectly provide contextual modulation of this amygdalar activity through these projections.

Interestingly, even though it is the ventral hippocampus that projects to the medial prefrontal cortex and is heavily connected with the amygdala (Sierra-Mercado et al 2011, Sotres-Bayon et al 2012), nobody has recorded from place cells in this region during fear conditioning and extinction learning. Our lab has previously shown that cells in this region respond to olfactory stimuli of high emotional valence and exhibit spatial tuning both on a single cell and population level (Keinath et al 2014). These findings, taken in conjunction with the anatomical connectivity of this region and the evidence that it is critically involved in the circuitry of fear extinction (Ji & Maren 2007, Sierra-Mercado et al 2011, Sotres-Bayon et al 2012), make it highly likely that ventral hippocampal place cells would respond strongly to the changes of emotional valence that occur during contextual fear and extinction learning.

However, we have also found that the ventral hippocampus may mediate generalization while the dorsal plays a more dominant role in context discrimination. Thus, another avenue of research could be to investigate whether subpopulations of cells in both the dorsal and ventral hippocampal areas exert contextual modulation through differential synchronization with other brain regions. Coordination of neuronal population activity produces synchronized oscillations of local field potentials at specific frequencies, which may influence crosstalk between other regions. This oscillatory activity has recently garnered a great deal of interest, with evidence that coherence in

the theta and gamma frequencies may be especially important for learning and attention (Fell et al 2003, Jones & Wilson 2005, Nyhus & Curran 2010). Indeed, changes in theta-gamma coupling in the basolateral amygdala and medial prefrontal cortex (mPFC) have been found to occur during periods of fear and safety (Stujenske et al 2014), and there is evidence of significant changes in theta coherence between the dorsal hippocampus and medial prefrontal cortex during fear conditioning and extinction (Lesting et al 2011). Neuronal firing in the mPFC has also been shown to entrain to hippocampal theta rhythms depending on the behavior being expressed (Hyman et al 2005). It is possible that different populations of cells in the hippocampus exhibit differential synchronization with these regions during fear and extinction learning.

Finally, some of the strongest evidence that extinction constitutes new learning comes from behavioral phenomena such as spontaneous recovery and fear reinstatement, cases in which the conditioned fear response returns after extinction training (Bouton 2004). Considering our results, it would be interesting to investigate patterns of place field stability in situations where the conditioned fear response returns. Our findings suggest that the spatial representations underlying extinction incorporate previous representations formed during fear learning as well as new representations, with different cells remapping preferentially during fear and extinction. Thus, one possibility is that during spontaneous recovery or reinstatement, cells would continue to remap heterogeneously, with fear-responsive cells remapping while extinction-responsive cells remained stable. However, since we did not find long-term stability in cells that remapped during extinction (Wang et al 2015), it is also possible that a new spatial representation could be formed, or that the return of fear behaviorally could lead to the reemergence of spatial representations formed during fear learning.

In addition to emotion, we also investigated how changes in sleep patterns could influence learning and cellular activity in the hippocampus during the object-place recognition task, a spatial memory task. Curiously, we found that while sleep deprivation impaired learning in young adult mice, it enhanced performance in aged mice. Place cells in both young control animals and sleep-deprived old animals displayed similar patterns of stability in two major subpopulations of cells:

context and object cells. Context cells most likely code static aspects of the environment and remained stable throughout, while object cells remapped both when the objects were introduced and again when an object was moved. However, sleep-deprived young adult animals exhibited high stability in both context and object cells during the test session, while control old animals displayed low stability in both context and object cells. Moreover, although the learning deficits seen in old control animals seem to stem from impaired memory consolidation, the deficits observed after sleep deprivation in young adult animals do not appear to come from the same impairment, since they successfully retrieve spatial representations formed during training. Instead, it is possible that sleep deprivation in young animals renders memories more rigid and less labile upon retrieval, thus leading to difficulties with incorporating new information during memory reconsolidation. These findings suggest that sleep deprivation immediately after training can influence the stability of long-term spatial representations, although the nature of this effect may depend on age.

The object-place recognition task that we used is a variant of the novel object recognition task, where a familiar object is substituted with a new, unfamiliar object in the same location (Antunes & Biala 2012). Although place cells have been found to respond to rotations or movements of three-dimensional objects within the environment (Cressant et al 1997, Cressant et al 1999, Lenck-Santini et al 2005, Renaudineau et al 2007), object substitutions without changes in location have largely failed to provoke a response in the form of either rate or global remapping, even though animals exhibit increased exploratory behavior of the novel object (Cohen et al 2013, Lenck-Santini et al 2005). This and other findings from early lesion studies have been interpreted as evidence that the hippocampus mediates object location memory and not object recognition memory (Ennaceur et al 1997, Winters et al 2004), but this conclusion remains a contentious topic (Cohen & Stackman Jr 2015). Indeed, one study that recorded from hippocampal neurons in rats encountering a mix of novel and familiar objects in different positions on a circular track found that a small percentage of cells showed firing rate changes in response to object identity, even though the majority responded only to object location (Manns & Eichenbaum 2009). It has been previously

suggested that place cell firing is controlled by external cues in a hierarchical manner, such that cells anchor to certain types of cues preferentially (Renaudineau et al 2007, Shapiro et al 1997). It is possible that, while place cell activity primarily underlies object location memory, these cells may also respond to object identity secondarily (Manns & Eichenbaum 2009).

Old mice have previously been shown to have severe impairments on the object-place recognition task (Wimmer et al 2012), a finding we replicated in our old control animals. Unexpectedly, we found that old animals showed improved performance after undergoing an acute period of sleep deprivation followed by a longer session of recovery sleep. Age-related impairments have been found in many other forms of learning, including spatial memory tasks such as the Barnes circular platform task, the Morris water maze and radial arm maze (Barnes 1979, Barnes et al 1980, Oler & Markus 1998, Shukitt-Hale et al 2004, Wyss et al 2000), as well as trace and contextual fear conditioning (Kishimoto et al 2001, Moyer Jr & Brown 2006). Thus, it would be very interesting to see if a similar regimen of sleep deprivation and recovery can also rescue age-related deficits in other forms of learning. However, there is also evidence that post-training alterations in sleep characteristics may be highly dependent on the nature of the learning task (Diekelmann et al 2009), which may limit possible therapeutic effects of sleep deprivation.

In view of the potential clinical relevance of our finding, it will be particularly important to investigate the boundaries of the sleep deprivation and recovery window in order to optimize the memory enhancing effect we observed. Many researchers have attempted to identify a critical window for sleep after learning, but no clear consensus has been reached. Early studies found evidence of altered sleep patterns up to seven days after training (Smith & Lapp 1986), with sleep deprivation producing memory deficits even when induced as late as two days after training (Smith & Kelly 1988). More recently, Prince et al. demonstrated that a delayed, 3-hour period of sleep deprivation beginning one hour after training effectively impaired memory and hippocampal long-term potentiation, but that three hours of immediate sleep deprivation after training had no effect (Prince et al 2014). However, these studies have been overwhelmingly conducted on young adult animals. There is some evidence that the time course of some molecular processes involved in

learning and memory may be altered in aged animals (Ingvar et al 1985, Rattan 1996, Ryazanov & Nefsky 2002), which could potentially influence the timing of a critical period for sleep and sleep deprivation after learning. Additionally, it has been suggested that some age-related changes in sleep occur in a triphasic fashion rather than showing a steady linear change over the entire lifespan; for example, Floyd et al reported that in humans, the ability to initiate sleep declined steadily until age 30 but then plateaued for many years, only continuing to decline again after age 50 (Floyd et al 2000). Thus, it may also be important to make a finer distinction among different age groups to appropriately capture this effect.

Because we believe that the enhanced learning seen in old animals may be attributable to the more consolidated recovery sleep they exhibit following sleep deprivation, it will be critical to analyze this period more closely. We have currently done only a broad analysis of the average amount of wake, NREM and REM sleep recorded in each group, along with average bout length and number of bouts, but it is possible that a more detailed analysis could reveal additional insights. Bout lengths have been reported to be highly variable across the day (Hasan et al 2012), so an examination of when the most consolidated recovery sleep is occurring could serve to identify when recovery sleep has the most impact. Moreover, many studies have found learning-related increases in the density of sleep spindles, bursts of 10-15hz waves seen in the EEG during NREM sleep (Fogel & Smith 2006, Mednick et al 2013, Schabus et al 2004). Meanwhile, sleep deprivation has been reported to suppress spindle activity during subsequent recovery sleep in young subjects (Dijk et al 1993, Knoblauch et al 2003), and there is some evidence that spindle activity may also be reduced in aging (Crowley et al 2002, Nicolas et al 2001). It is possible that we could find increased spindle activity in the recovery sleep of old animals as additional evidence of sleep-related memory consolidation mechanisms.

Sleep fragmentation has also been associated with impaired neurogenesis (Guzman-Marin et al 2007, Sportiche et al 2010), a topic of particular interest considering that the dentate gyrus is the major site of adult neurogenesis in most mammals (Eriksson et al 1998, Gage 2002). Moreover, there is a large body of correlational evidence demonstrating that increased neurogenesis is

positively associated with learning and memory (Aimone et al 2006, Deng et al 2010, Leuner et al 2006). Conditions that suppress neurogenesis have been shown to impair certain types of learning (Jessberger et al 2009, Shors et al 2001, Snyder et al 2005) while conditions that increase neurogenesis appear to enhance memory performance (Nilsson et al 1999, Sahay et al 2011, Van Praag et al 2005). Additionally, the rate of neurogenesis has been shown to decline with age, with evidence that both the proliferation rate and the rate at which new cells survive to maturity are severely reduced in aged animals compared to young animals (Galvan & Jin 2007, Klempin & Kempermann 2007, Lazarov et al 2010). Thus, it is plausible that the more consolidated period of sleep seen in recovery boosts neurogenesis in sleep-deprived old animals, enabling better memory performance.

In conclusion, our studies indicate that neuromodulatory and physiological factors such as emotion and sleep exert clear influences on hippocampus-mediated memory consolidation. However, further research is needed to fully delineate the extent of these effects. It is increasingly evident that a better understanding of the functional differentiation along the dorsoventral axis of the hippocampus and its interaction with other brain regions may be critical to developing a more complete view of the neural circuitry underlying fear and extinction learning. Moreover, our findings reveal a potentially valuable effect of acute sleep deprivation as a means of rescuing age-related cognitive deficits. Both areas of research may have important clinical relevance to populations suffering from age-related neurodegenerative or anxiety-related psychiatric disorders.

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Chapter 1

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