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Investigating the Role of HDAC3 in Memory Reconsolidation

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ABSTRACT

Memory reconsolidation is a process by which existing memories re-enter a labile state and become susceptible to enhancement or impairment. During reconsolidation, epigenetic mechanisms such as histone acetylation and deacetylation are thought to control expression of memory relevant genes to modify synaptic connections. One enzyme of interest, Histone Deacetylase 3 (HDAC3), is highly expressed in the brain and acts as a critical negative regulator of long-term memory formation (Kwapis et al., 2018). The role of HDAC3 in regulating memory reconsolidation molecularly and behaviorally remains unclear, and my work aims to investigate how inhibiting its activity can affect the strength of a memory. I utilized the Objects in Updated Locations (OUL) behavioral paradigm alongside contextual fear conditioning to investigate behavioral outputs of memory reconsolidation after HDAC3 inhibition. Our data show that inhibiting HDAC3 in the brain after the update session leads to enhancement of the updated memory, but impairment of the original memory, suggesting that there may be competition between the original and updated memory. When we conducted a separate experiment with a subthreshold update session where mice had less time to learn the update in the OUL paradigm, we found that the original memory was restored without affecting the updated memory, consistent with our competition hypothesis. These findings prompted us to investigate whether our results could transcend paradigms, and we began to consider memory reconsolidation in contextual fear conditioning. In this experiment, we tested whether we could use memory updating to persistently reduce fear memory. Specifically, we compared whether updating fear memory could produce a more persistent reduction in fear than standard extinction procedures, which are notoriously susceptible to fear relapse. We observed that memory updating produced a more persistent reduction in fear memory (in both spontaneous recovery and reinstatement)

compared to mice that received standard extinction training. Overall, our work suggests that memory updating can manipulate existing memories of both incidental spatial information and more salient aversive information and may be a potential strategy to improve memory function and prevent relapse.

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personal mentor. I always felt comfortable coming to him for advice on life topics outside of science. He has provided me with valuable advice on life as a graduate student and made me super excited about the next steps. I always looked forward to working with him and chatting about the progress of our projects and what implications they have. He is a very special scientist with a lot of passion for research, and I can't wait to see all the cool things he will do in the future!

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Chapter 1

Introduction: The Flexible Brain and Memory Updating

1.1 Defining Memory Reconsolidation

Memory reconsolidation is a process by which existing memories enter are modified when new information is presented and susceptible to enhancement or impairment. One real-life example to illustrate memory reconsolidation is learning something in a class and then revisiting the material during studying. When studying we may create a mnemonic to remember a concept and we incorporate it into our memory, in this case enhancing it and making the information easier to recall when we take the exam. Memory is a dynamic process, and reconsolidation allows humans to adapt to their ever-changing environment. Researchers have established that short-term memories transition into long-term memories via a process termed memory consolidation (Nader & Hardt, 2009). Memories can exist in two different states, labile and stable. Memories in a labile state are susceptible to modification, while those in a stable state are effectively consolidated (Haubrich & Nader, 2018). During memory consolidation, a memory stabilizes as neurons produce new RNA and proteins (Kandel 2001). These changes are thought to modify synaptic plasticity, producing stronger synaptic connections between neurons involved in learning and storing that memory (Nader & Hardt, 2009). Long term memory formation requires both transcription and de novo protein synthesis; when either protein synthesis or transcription is blocked around the time of learning, long-term memory is blocked but short-term memory persists (Schafe & LeDoux, 2000). Once memory is fully consolidated, however, it is relatively resistant to disruption and inhibitors of either protein or mRNA synthesis are unable to disrupt the stored information.

Reactivation of a consolidated memory, however, can return a stable memory to a labile, unstable state, in which it must reconsolidate or restabilize (Nader et al., 2000). Recent work has demonstrated that new, relevant information must be presented during retrieval to initiate the reconsolidation process, suggesting that one purpose of reconsolidation is to allow existing memories to be updated with new information. On the systems level, memories that are similar

are likely to activate overlapping neurons. Kwapis et al, 2018 has demonstrated this by showing that there is significant overlap in the neurons activated during the training and update sessions of the OUL paradigm. While memory reactivation can lead to reconsolidation it is important to note that upon retrieval not all consolidated memories will destabilize. Two characteristics of a memory that determine if it will destabilize are memory strength and memory age. Memories that are very strong are unlikely to destabilize when they are retrieved. Further, when the time gap between memory consolidation and memory reactivation is large, memories are less likely to destabilize (Haubrich & Nader, 2018). The retrieval session also determines whether a memory will be destabilized. Retrieval sessions that are short prevent memory reconsolidation from occurring (Bustos et al., 2009), while those that are too long lead to memory extinction (Eisenberg et al., 2003).

1.2 Relevance of Studying Memory Reconsolidation

Studying memory updating will provide a new depth of knowledge about the brain that can be applied to understanding the molecular underpinnings of diseases that impact memory. Memory serves as a critical component of cognitive, addiction, mood, anxiety, and developmental disorders. Much memory reconsolidation research focuses on aversive memories. Some studies provide evidence that interfering with memory reconsolidation during memory retrieval of a trauma event could help ameliorate trauma related pathologies (Haubrich & Nader, 2018). Targeting reconsolidation (memory updating) as opposed to extinction (forgetting) may offer better treatments for patients. Memory extinction occurs when a patient experiences a threatening stimulus repetitively without the aversive consequence, leading the patient to forget the negative valence of the memory. While psychologists often use extinction as a treatment for anxiety, it proves to be ineffective in the long term as fear memories can return with the passage of time. Memory extinction is believed to form a new memory rather than modifying the initial fear memory, which is why fear rebound is common (Rescorla & Heth, 1975). All of this evidence provides reason for focusing memory research on reconsolidation, especially now with mental health issues on the rise worldwide.

1.3 Behavioral Paradigms for Memory Reconsolidation

Objects in Updated Locations (OUL)

There are a few behavioral paradigms that can be used to investigate memory reconsolidation. This thesis, which focuses on hippocampal dependent memory, utilized two different paradigms. The first task, Objects in Updated Locations (OUL) was developed to measure spatial memory by testing animals' ability to remember the location of objects. This behavioral paradigm can determine whether an animal remembers original and updated information in a single test session (Kwapis et al., 2020). The OUL task relies on animals' innate preference for novelty and contains three phases: training, updating, and testing. During the training session mice learn the location of two objects, A1 and A2 (Fig 1, phase 1). This information is then updated by moving one of the objects, A3 (Fig 1, phase 2). Finally, during the test session both the initial and updated object location information are presented along with a novel object, A4 (Fig 1, phase 3). Work from our lab has validated the OUL paradigm in young mice, showing that the task causes retrieval of the original information followed by reconsolidation-based memory updating, rather than formation of a new memory. To confirm that OUL relies on memory updating, our past work infused the protein synthesis inhibitor anisomycin into the dorsal hippocampus right after the update session. The results found that post-update anisomycin not only prevented updating, but also impaired the original memory, suggesting that the original memory was indeed destabilized by the memory update. This study also identified that aged animals have impaired memory updating ability, making this paradigm useful for studying age-related memory impairment (Kwapis et al., 2020). Another recent study that utilized the OUL behavioral paradigm investigated the effects of radiation on hippocampal dependent memory (Alaghband et al., 2023). This research found that female mice that were acutely and chronically irradiated, along with chronically irradiated males had memory updating impairments (Alaghband et al., 2023). Beyond this work, there is little known about the molecular mechanisms that support memory updating in the OUL task.

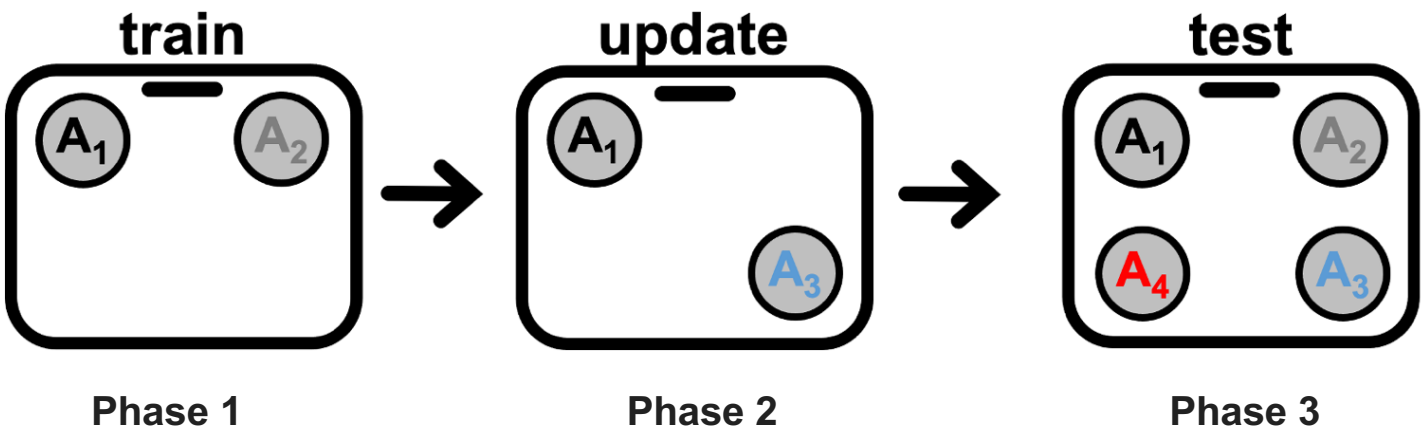


Figure 1: Objects in Updated Locations (OUL) Paradigm

The OUL task contains three phases: training, updating, and testing. In the first phase animals learn the locations of two identical objects A1 and A2. During the next phase one object is moved (A3), serving as the memory update. Finally, in the test session animals are exposed to the original information (A1 and A2), update information (A3), and a novel information (A4).

Contextual Fear Conditioning

The other behavioral paradigm we used was contextual fear conditioning. In this paradigm mice learn to associate a context with an aversive foot shock. In research the contextual fear conditioning paradigm is frequently used to model Post Traumatic Stress Disorder (Chaaya et al. 2018). This paradigm acts as an effective model for PTSD, as patients often experience relapse when exposed to contexts similar to one where they experienced trauma (Boschen et al. 2009). Contextual fear conditioning is dependent on the hippocampus and basolateral amygdala (Anagnostaras et al. 2001). It is believed that the hippocampus combines all the cues that represent a context while the amygdala is responsible for the sensory aspect of the fear (Fanselow 2010). Traditionally, memory extinction has served as the main treatment for attenuating fear memories. During extinction the conditioned stimulus- the context, is presented without the unconditioned stimulus- the foot shock (Bouton et al. 2012). However, extinction is often ineffective as a long-term solution for treating fear memories because it leads to formation

of a new memory, rather than disrupting the initial context and shock association (Rescorla & Heth, 1975). On the other hand, memory reconsolidation may have therapeutic potential for reducing fear memories more persistently. Since memory reconsolidation allows for existing memories to be updated, it could be used to modify traumatic memories to a less aversive form (Haubrich & Nader, 2018). In our contextual fear conditioning paradigm, we trigger memory updating by presenting mice with a significantly weaker shock than the training session when they are re-exposed to the context. Mice then reconsolidate their memory for the context with the weak shock information. Fear memory is measured through analysis of freezing to determine the resilience of the memory. Much research has made use of fear conditioning as a means of studying memory reconsolidation. One study found that memory updating was more effective at reducing fear than memory extinction (Popik et al., 2020). In this experiment, the researchers conducted auditory fear conditioning in rats. The animals were trained with a 0.5 mA foot shock and underwent a memory reactivation session for three days where they either received a weaker 0.1 mA shock or no shock. The researchers found that the mice that received a weaker shock had lower freezing levels than the group that received no shock. We decided to use these two different paradigms to explore memory updating comprehensively. Although contextual fear conditioning and the OUL task engage different types of memory updating, we aimed to investigate whether they may share similar mechanisms.

1.4 Molecular Mechanisms of Memory Updating

For a memory to reconsolidate (update) it must destabilize first. Memory destabilization occurs via ubiquitin/proteasome dependent protein degradation (Lee et al., 2012), thought to ‘break down’ synapses storing a memory to enable modification. Further, changes in AMPA receptor expression are associated with memory retrieval and destabilization (Haubrich & Nader, 2018). Once the memory is destabilized, other molecular mechanisms modulate the re-stabilization process. As previously mentioned, de novo protein synthesis is critical for memory reconsolidation (Haubrich & Nader, 2018). Further, transcription factors act as important regulators of reconsolidation. Specifically, disruption of cyclic AMP response element binding

protein (CREB) in the hippocampus, amygdala, and prefrontal cortex has been shown to impair reconsolidation in auditory and contextual fear conditioning (Kida et al., 2001). The transcription factor nuclear factor- κB (NF-κB) has also been shown to regulate synaptic plasticity during retrieval and reconsolidation (Merlo et al., 2005).

One outstanding question is whether memory consolidation and reconsolidation are molecularly distinct, however some differences have been described. The transcription factor ZIF268 has been identified as unique to reconsolidation, while BDNF is unique to consolidation (Lee et al., 2004). This may only hold true for certain memory types, as this study was conducted in a contextual fear conditioning paradigm, and another study using an object recognition task found that ZIF268 was required for memory consolidation in addition to reconsolidation (Bozon et al., 2003). As transcription factors are important for reconsolidation, immediate early genes are as well. Specifically, ZIF268 is expressed in the hippocampus and prefrontal cortex after retrieval of a contextual fear memory (Hall et al. 2001) and cFos and JunB are expressed in the CA1 region of the hippocampus after retrieval of a contextual fear memory (Strekalova et al. 2003).

Memory reconsolidation is also modulated by epigenetic mechanisms, which are thought to modify synaptic plasticity by regulating transcription (Jarome & Lubin, 2014). One epigenetic mechanism that has been identified as an important regulator or memory reconsolidation is histone acetylation, which is associated with increased gene expression (Jarome & Lubin, 2014). Histone acetylation is regulated by two groups of enzymes: Histone deacetylases (HDACs) and Histone Acetyltransferases (HATs). Histone deacetylation is generally associated with a closed chromatin state that represses gene expression, while histone acetylation is associated with an open chromatin state which enhances gene expression (FIG 2). HDACs have been shown to be powerful negative regulators of long-term memory (McQuown & Wood, 2011). Research has found that HDAC inhibition with trichostatin A enhanced hippocampus dependent memory and plasticity (Vecsey et al., 2007). Many studies have investigated how class 1 HDACs (HDACs 1, 2, 3, and 8) impact memory through pharmacological inhibition (McQuown & Wood, 2011). These studies have determined that class 1 HDACs are important regulators of long-term memory. Overexpression of HDAC2 for example, impairs memory and synapse formation,

whereas loss of HDAC2 enhances memory and synapse formation (Guan et al., 2009). Within the class 1 HDACs, HDAC3 serves as an enzyme of interest in our lab and will be the focus of this thesis. HDAC3 is the most highly expressed class 1 HDAC in the brain, with the highest levels being in the hippocampus, cortex, and cerebellum (Broide et al., 2007). HDAC3 activity decreases gene expression in a couple of ways. Firstly, the removal of acetyl groups increases the attraction between DNA and histone tails, making it more tightly bound and less accessible for transcription (McQuown & Wood, 2011). Additionally, histone deacetylation reduces recruitment of transcription activators, such as HATs (Zeng & Zhou, 2002). Both deletion and inhibition of HDAC3 in mice leads to long-term memory formation after a subthreshold object recognition learning task (McQuown et al., 2011). Since HDAC3 impacts long-term memory, but not short-term memory, it likely mediates memory through increases in gene expression (McQuown & Wood, 2011). To follow up on this hypothesis, researchers investigated gene expression changes in HDAC3 deletion mice. They determined that the mice had increased expression of Nr4a2 and cFos in the dorsal hippocampus after a subthreshold learning event. These are both genes that have been implicated in long-term memory (McQuown et al., 2011). One question that remains unanswered is how HDACs regulate whether a long-term memory will form. One leading hypothesis is that HDAC3 functions as a "molecular brake pad," which assumes that HDACs and corepressors form complexes which keep genes silenced until a strong learning event occurs, leading to the brake pad being removed so gene expression occurs, and memories consolidate (McQuown & Wood, 2011).

My thesis aims to investigate how HDAC3 contributes to memory reconsolidation/updating. To gain a better understanding of this enzymes role in memory updating we conducted a series of experiments using the OUL paradigm and contextual fear conditioning. Our goal is to determine how inhibition of HDAC3 during updating of different memory types alters behavioral outcomes. The goal at large is to determine common mechanisms for updating of different types of memory that can be applied to improving our understanding of both age-related cognitive decline and post-traumatic stress disorder.

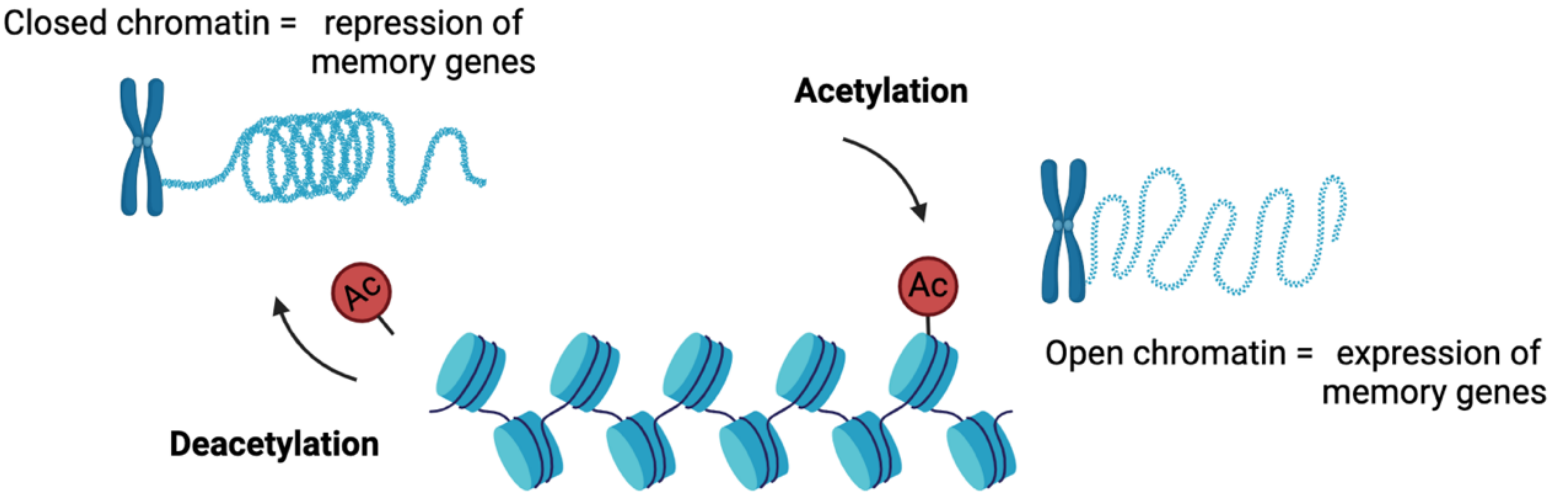


Figure 2: Histone Acetylation and Memory

Acetylation changes chromatin structure. Adding acetyl groups to histones relaxes chromatin, allowing for expression of relevant memory genes. Removing acetyl groups condenses chromatin, preventing expression of memory relevant genes.

Chapter 2
Methods

Subjects

The subjects were young adult (2-6-months) male C57BL/6J mice. Mice had free access to food and water. Lights followed a strict 12-hour light/dark cycle; all behavioral experiments occurred during the light cycle. Animals were housed with four mice per cage for OUL experiments and to avoid stress-induced fighting, mice were single-housed for all fear conditioning experiments. Experiments were conducted in accordance with National Institute of Health guidelines for animal care and use and were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University.

RGFP966 Administration

Vehicle solution: The vehicle was made by adding 20-30 mL of sterile water, 15g Hydroxypropyl-beta-cyclodextrin (HPBCD), and 0.41g Sodium acetate (NaOAc) to a 100mL beaker. Once all salts were dissolved the pH of the solution was brought down to 5.4 using 1:4 diluted hydrochloric acid (HCl). The solution was brought to 50mL with sterile water. The HPBCD stock solution was then collected through sterile vacuum filtration and stored at room temperature. On injection day, 500uL of dimethyl sulfoxide (DMSO) was added to 4.5mL of the HPBCD stock solution and loaded into syringes.

RGFP966 solution: On injection day, 500uL of DMSO was added to 5mg of lyophilized RGFP966, vortexed, and moved to a scintillation vial. Next, 4.5mL of the stock HPBCD solution was added to the stock RGFP966 and DMSO solution, vortexed, and loaded into syringes.

Injections: Animals were weighed with a scale in a fume hood immediately before injection. Mice were injected with 10mg/kg subcutaneously of the RGFP966 solution or an equivalent volume of the vehicle solution (30g mouse = 0.3mL injection).

Objects in Updated Locations (OUL) Paradigm

The OUL paradigm encapsulates 5 components: handling, habituation, training, updating, and testing. The general experimental timeline is as follows, with each experimental phase described below:

Day 1-2	Day 3-6	Day 7-8	Day 9	Day 10	Day 11
handling	handling + habituation	habituation + scruff	training (A1 and A2)	updating (A1 and A3)	testing (A1, A2, A3, A4)

Handling: Mice underwent handling for six consecutive days. Mice were carted into the behavior room, and two mice were handled at a time for two minutes. During handling, proper protective equipment (PPE) was worn, including a gown and gloves.

Habituation: After the first two days of handling mice were handled and then placed into the empty OUL arena to freely explore for five minutes. Mice underwent habituation for six days. On the final two days of habituation mice were scruffed to minimize injection stress.

Training Session: Mice were placed into the OUL arena for 10 minutes and allowed to freely explore. They were exposed to objects in locations A1 and A2, which represent the original training information.

Update Session: Mice were placed into the OUL arena for 5 minutes and allowed to freely explore. They were exposed to objects A1 and A3. Introduction to object A3 represents the updated information.

Test Session: Mice were placed into the OUL arena for 5 minutes and allowed to freely explore. They were exposed to objects A1 and A2 (original memory), object A3 (memory update), and object A4 (novel).

Measuring learning: Learning was measured by analyzing differences in exploration time for each object. Mice are attracted to novelty, so if mice learn and successfully form a memory for an object location, they will spend less time exploring the location compared to an identical object in a novel location during subsequent trials. The difference in exploration time was calculated using a discrimination index. The discrimination index was calculated using the equation below. If mice have intact memory formation and successful updating, they should explore object A4 the most and objects A1, A2, and A3 equally during the test session.

Discrimination index (DI)= $\left(\frac{\text{time exploring novel location} - \text{time exploring familiar location}}{\text{time exploring novel location} + \text{time exploring familiar location}}\right) \times 100$

Statistics: Behavioral videos were manually scored to determine object exploration times for training, updating, and testing sessions. Experimenters were blind to experimental conditions when scoring. Behavior was scored following a specific criterion; the animal's head must be oriented toward the object within approximately one centimeter, or with its nose touching the object (Kwapis et al., 2018). Šidák-corrected t-tests were conducted to compare the experimental groups. All analyses were two-tailed and required a p-value of 0.05 for significance. Additionally, all statistics were completed using GraphPad Prism 10 software. Data are all shown with as mean ±SEM.

Contextual Fear Conditioning Paradigm

Equipment:

Context A: Gooseneck lamps and a white noise machine were turned on in the behavior room for Context A. The context consisted of even grid shock bars, clear chamber walls, and isopropyl alcohol scent. A small square of paper towel was sprayed with isopropyl alcohol and placed in the bottom tray of each chamber as a distinguishing odor.

Context B: blackout boxes, walked to behavior room, Windex, curved insert, flat white floor, checkered walls, red light only.

Handling: Prior to fear conditioning, mice underwent handling for five consecutive days. Mice were carted into the fear conditioning behavior room and handled for two minutes each. During handling PPE was worn including a gown and gloves. The overhead light and white noise machine were turned on.

Training sessions: Four mice were carted into the fear conditioning room at a time and placed in individual chambers. The training session lasted one day and was 10 minutes long. The training session started with a 3-minute baseline followed by 5 strong 2-second 0.8mA shocks and ended with a 3-minute post-session baseline. The shocks were spaced one minute apart.

Updating/extinction sessions: Mice were carted into the fear conditioning room four at a time and placed in individual chambers. Animals underwent 30-minute update sessions for five days. During the update session, mice received 10 weak 2-second 0.2 mA shocks. The mice in the extinction control group were placed in the context for 30 minutes without receiving shocks.

Context test sessions: Mice were carted or carried into the fear conditioning room four at a time and placed in individual chambers. Animals were placed in either context A or B for 5 minutes without receiving shocks.

Spontaneous recovery and reinstatement test sessions: Following training, mice were left in their homecage undisturbed for 15 days. After this time, mice were placed back into context A for five minutes, and freezing levels were measured to assess spontaneous recovery. At the end of the five minutes, mice received one 0.8 mA reinstatement shock and were left in the context for one minute. The next day, mice were placed back into context A, and freezing levels were measured to assess reinstatement.

Statistics: Fear responses were recorded as freezing behavior and was evaluated using EthoVision software. Freezing fear behavior is presented as mean of percent time freezing ± standard error of the mean (SEM). Two-way ANOVAs followed by Šidák's multiple comparison post hoc analyses was used to test for significance, with $p < 0.05$ being considered significant. All calculations were performed using GraphPad Prism 10 software.

Chapter 3

Results

Three major experiments were conducted:

1. Role of HDAC3 in the Young Brain
2. Subthreshold Update and HDAC3 Inhibition in the Young Brain
3. Memory Updating in Contextual Fear Conditioning

Experiment 1: Role of HDAC3 in the Young Brain

Previous studies in our lab have shown that aged animals have impairments with updating memory in the OUL paradigm (Kwapis et al., 2020). However, when we inhibit HDAC3 with the drug RGFP966 after the update session, we see that memory updating ability is rescued (FIG 3). While this data shows that HDAC3 inhibition may have therapeutic potential for age related memory decline, it remains unclear how HDAC3 regulates memory reconsolidation in the young brain. Since it was previously determined that young mice can successfully update memory in the OUL paradigm (Kwapis et al., 2020), we hypothesized that we would observe no differences between vehicle and RGFP966 mice due to a ceiling effect. To test this, this experiment consisted of two groups: vehicle injected control mice, and RGFP966 injected HDAC3 inhibited mice. Both groups were trained in the OUL paradigm to learn the locations of objects A1 and A2 which acted as the original information. The next day they underwent the update session where they were exposed to one familiar object (A1) and an updated object (A3). Immediately after the update session, mice received a subcutaneous injection of either vehicle or RGFP966. The following day, mice were given the test session, in which they were exposed to both the original object locations (A1 and A2), the updated location (A3), and a completely novel location (A4). The exploration time for objects A1, A2, and A3 were compared against A4 to determine how well animals learned original and updated information. Our results indicated that both vehicle and RGFP mice successfully learned the update as there was no significant difference in the discrimination index between the groups for

object A1 and A3 ($p=0.3943$) (FIG 4). During the test session both groups also recalled A1 and had no significant difference in the discrimination index between A1 and A4 ($p=0.5063$) (FIG 5). Both mice also successfully recalled the update with no significant difference in the discrimination index between A3 and A4 ($p=0.9769$) (FIG 5). Vehicle mice successfully recalled the location of A2 during the test session, however, RGFP mice exhibited a deficit in memory for the location of A2. RGFP mice had a significantly lower discrimination index between this object and the novel object (A4) compared to vehicle mice ($t(24)=2.339$, $p=0.028$) (FIG 5). A low discrimination index indicates that mice did not preferentially explore one object over the other. Since mice have an innate preference for novelty, they should explore the novel object (A4) more than the original object (A2) if they remember it. These results indicate that inhibition of HDAC3 in young mice improves the update memory at the cost of the original memory, leading us to hypothesize that there may be memory competition between the original and updated information.

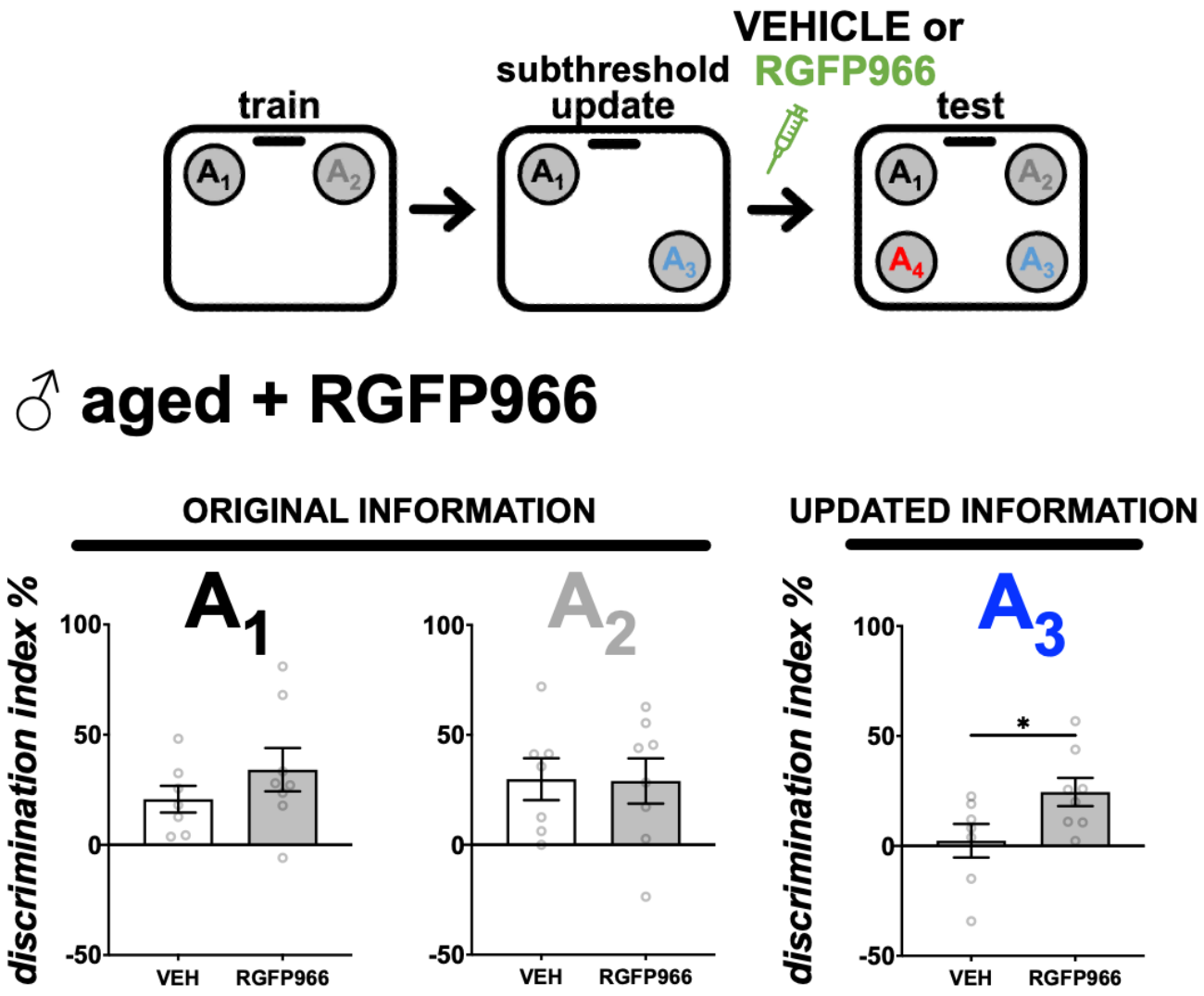


Figure 3: Inhibiting HDAC3 in Aged Animals Rescues Memory Updating

Aged animals successfully learn the location of the original objects (A1 and A2) but cannot learn the location of the updated object (A3). When animals are injected with HDAC3 inhibitor RGFP966 after the update session, memory for the updated object location is rescued. RGFP966 mice have a significantly higher discrimination index between the update object (A3) and novel object (A4) compared to the vehicle group ($t_{(13)}$=2.238, p=0.0434).

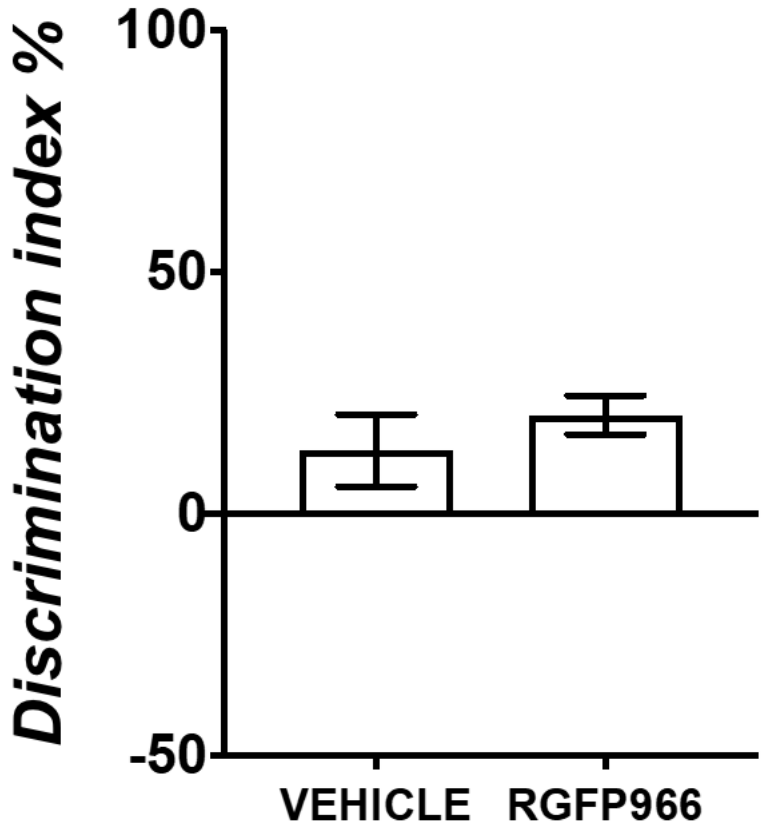


Figure 4: Young Vehicle and RGFP Mice Successfully Update Object Location Memory During the update session both vehicle and RGFP mice successfully recall the original object (A1) and preferentially explore the update object (A3). The data indicates no significant difference in the vehicle and RGFP groups for discrimination index between A1 and A3 ($p=0.3943$).

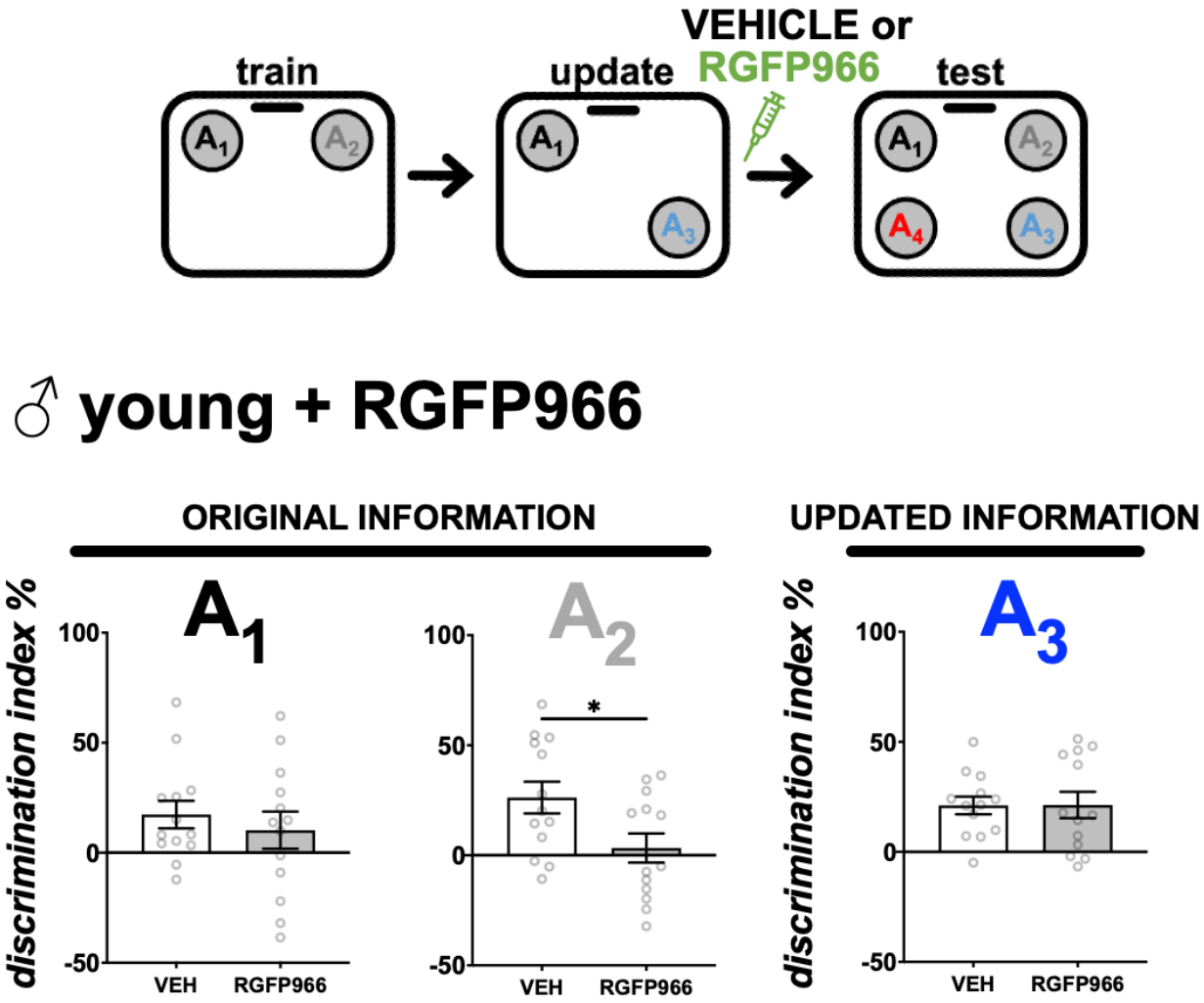


Figure 5: Inhibition of HDAC3 in Young Animals Impairs Original Object Location Memory

During the test session young vehicle and RGFP mice successfully recall the A1 object with no significant difference in discrimination index for A1 and A4 between the two groups (p=0.5063). Both groups also recall the update object A3 with no significant differences in discrimination index between A3 and A4 (p=0.9769). Vehicle mice successfully recall object A2 from the training session, but RGFP mice exhibit a memory deficit for this object (t(24)=2.339, p=0.028) The deficit observed for the original (A2) location memory in the RGFP mice indicates that updated information may compete with the original information.

Experiment 2: Subthreshold Update and HDAC3 Inhibition in Young Mice

To further explore the hypothesis that memory competition occurs between the original memory and the update in young mice, we conducted a follow up experiment where we manipulated the update session in the OUL paradigm. This experiment was identical to experiment 1 except mice received a subthreshold update that was not sufficient to induce learning of the updated information (A3). The subthreshold update session lasted only one minute instead of the standard 5 minutes which is sufficient for learning. The purpose of the subthreshold session was to put the updated information in a deficit in hopes that it could be enhanced by RGFP966. We hypothesized that giving the mice a subthreshold update would equalize the strength of the original memory and update memory, and they would no longer compete.

The results indicate that both vehicle and RGFP mice successfully recall the locations of both original objects during the test session. There was no significant difference between groups in the discrimination index between object A1 and A4 ($p=0.5187$) (FIG 6). Similarly, there was no significant difference between groups in the discrimination index between object A2 and A4 ($p=0.4314$) (FIG 6). This means that when we enhance a subthreshold update with RGFP966 we no longer see a deficit in the original memory during the test session. Unlike mice given RGFP966 after a full update session (experiment 1), here, mice given RGFP966 after the subthreshold update showed intact memory for the original information (A2). The discrimination index between this object and the novel object (A4) was high, indicating that the mice preferentially explored the novel object since they remembered the original information. RGFP966 mice also successfully recalled the update object A3, but vehicle mice failed to remember this object ($p=0.0313$) (FIG 6). This was expected as they received a one-minute subthreshold update which was not enhanced by HDAC3 inhibition. Together, this suggests that a weaker, subthreshold update can be strengthened by HDAC3 inhibition without impairing memory for the original object location.

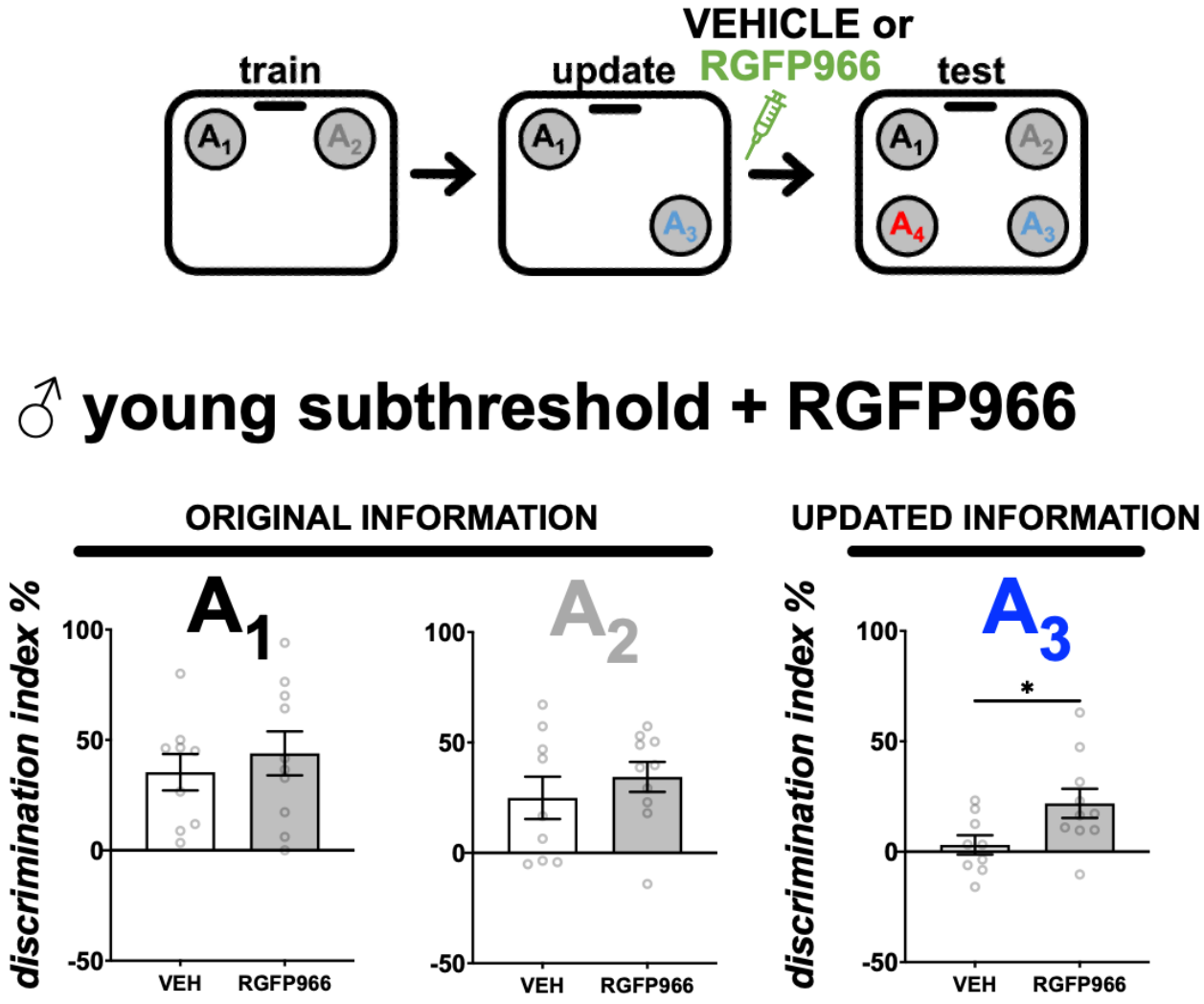


Figure 6: Inhibition of HDAC3 After a Subthreshold Update Restores Original and Updated Memories to Equal Strength

Young vehicle and RGFP mice successfully learned the original information (A1 and A2) with no significant difference between groups in discrimination index for A1-A4 ($p=0.5187$) and A2-A4 ($p=0.4314$). When mice receive a subthreshold update which is enhanced by RGFP966 they no longer have a deficit in memory for the original object A2, as the discrimination index is high for A2-A4. RGFP mice also successfully recall the update object A3, but vehicle mice do not, due to receiving a subthreshold update which is not enhanced ($p=0.0313$).

Experiment 3: Memory Updating in Contextual Fear Conditioning

The main goal of our work is to determine mechanisms of memory updating, and if there are similarities in reconsolidation of different types of memories. Because of this, it is critical that we utilize different memory reconsolidation paradigms, so we know that our understanding of memory updating isn't limited to one type of memory, such as spatial memory with the OUL paradigm. Therefore, the following experiments utilized contextual fear conditioning to explore memory updating.

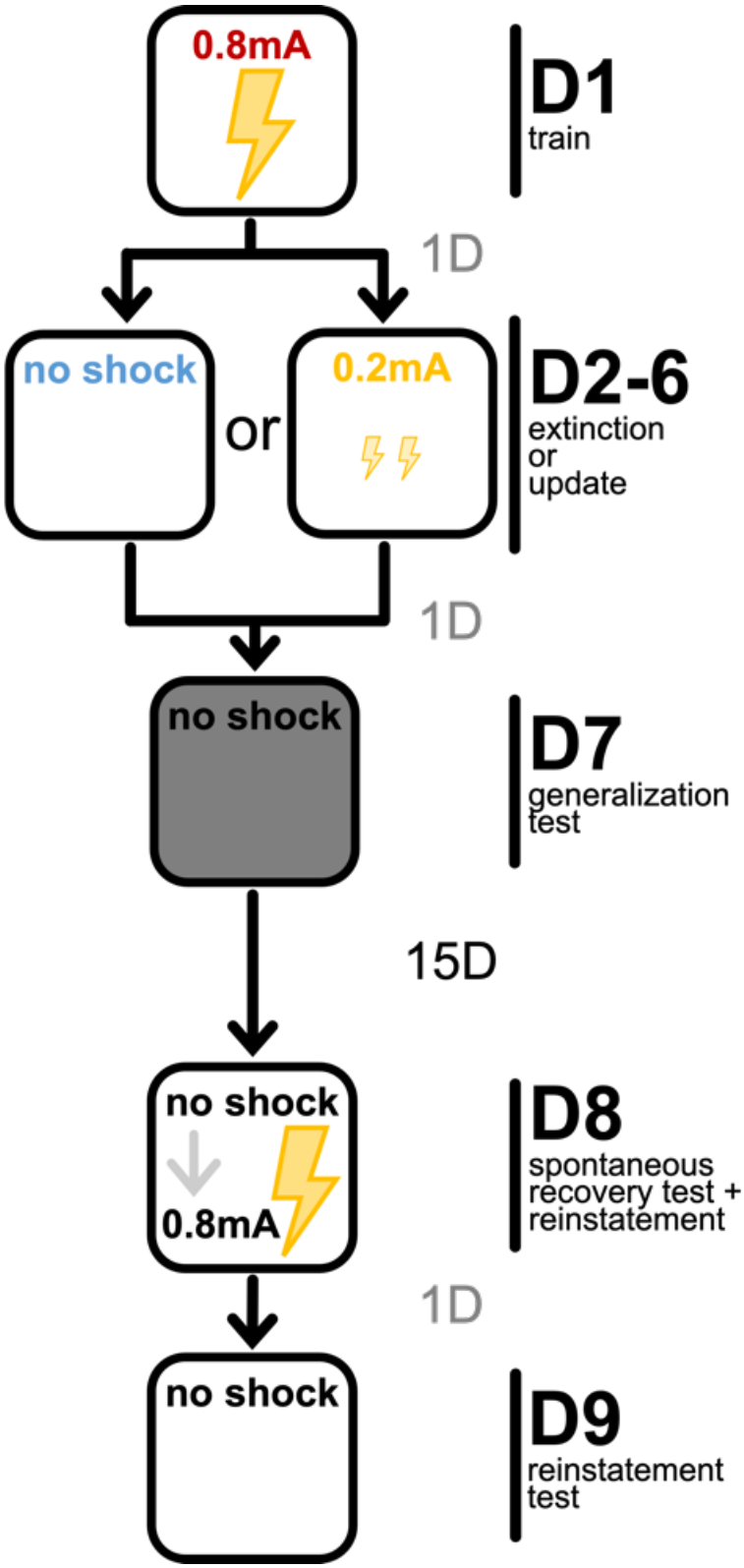
In the following experiment we investigated memory reconsolidation as an alternative to memory extinction. We were curious if updating and extinction lead to differences in the persistence of fear memory. To get at this question we analyzed spontaneous recovery and reinstatement of fear. These tests are often used to determine whether memory extinction is permanent. During spontaneous recovery animals are re-exposed to the context where they underwent extinction after a period without exposure to the context. Freezing levels are measured to determine if there is a rebound in fear. At the end of this session, mice receive a foot shock as reminder of the initial fear memory. The next day they undergo the reinstatement test where they are placed back into the context and their freezing levels are measured again.

To induce a memory update in our contextual fear conditioning paradigm, we decreased the shock intensity from the training intensity of 0.8 mA to a weaker 0.2 mA. We believe that this would lead to a memory update rather than formation of a new memory because it is exposing the animal to the same stimulus as the initial training (the shock) rather than presenting the context in the absence of this shock as extinction does. For this experiment, mice were trained in context A and received five strong 0.8 mA shocks. The following five days, mice underwent either updating or extinction sessions. The update group received 10 weaker 0.2 mA shocks, while the extinction group received no shocks. After 15 days, the mice were placed in the context with no shocks to measure spontaneous recovery of fear. At the end of this session, they received one strong 0.8 mA shock to reinstate the fear memory. The next day their freezing levels were measured, and we compared the persistence of fear memory between the groups.

We first compared the changes in freezing across different test sessions within each group. The results indicated that the extinction group experienced a significant rebound in fear during the

spontaneous recovery ($t(9)=7.972$, $p=<0.0001$) and reinstatement test ($t(9)=8.674$, $p=<0.0001$) compared to the last day of extinction (FIG 7). In contrast to these results, the update group experienced a much lower increase in freezing during the spontaneous recovery ($t(9)=2.630$, $p=0.0273$) and reinstatement test ($t(9)=2.685$, $p=0.025$) compared to the last day of updating (FIG 7). Furthermore, fear rebound is present in the extinction group when we compare the spontaneous recovery test to the reinstatement test ($t_{(9)}=6.994$, $p=<0.001$), but not in the update group (FIG 7). These results indicate that memory updating may stabilize freezing levels more than extinction does. We also compared freezing levels between the extinction and update groups during the different test sessions. The update group has increased freezing during the last day of extinction ($t_{(17.84)}=4.285$, $p=0.0005$), likely due to the last update containing shocks (FIG 8). The update group also freezes more than the extinction group during the spontaneous recovery test ($t_{(16.72)}=2.238$, $p=0.0222$) (FIG 8). However, during the reinstatement test the extinction group freezes more than the update group ($t_{(15.72)}=2.563$, $p=0.0211$) (FIG 8). These results provide evidence that memory updating may be more effective in reducing fear response than extinction.

Figure 7: Contextual Fear Conditioning Timeline



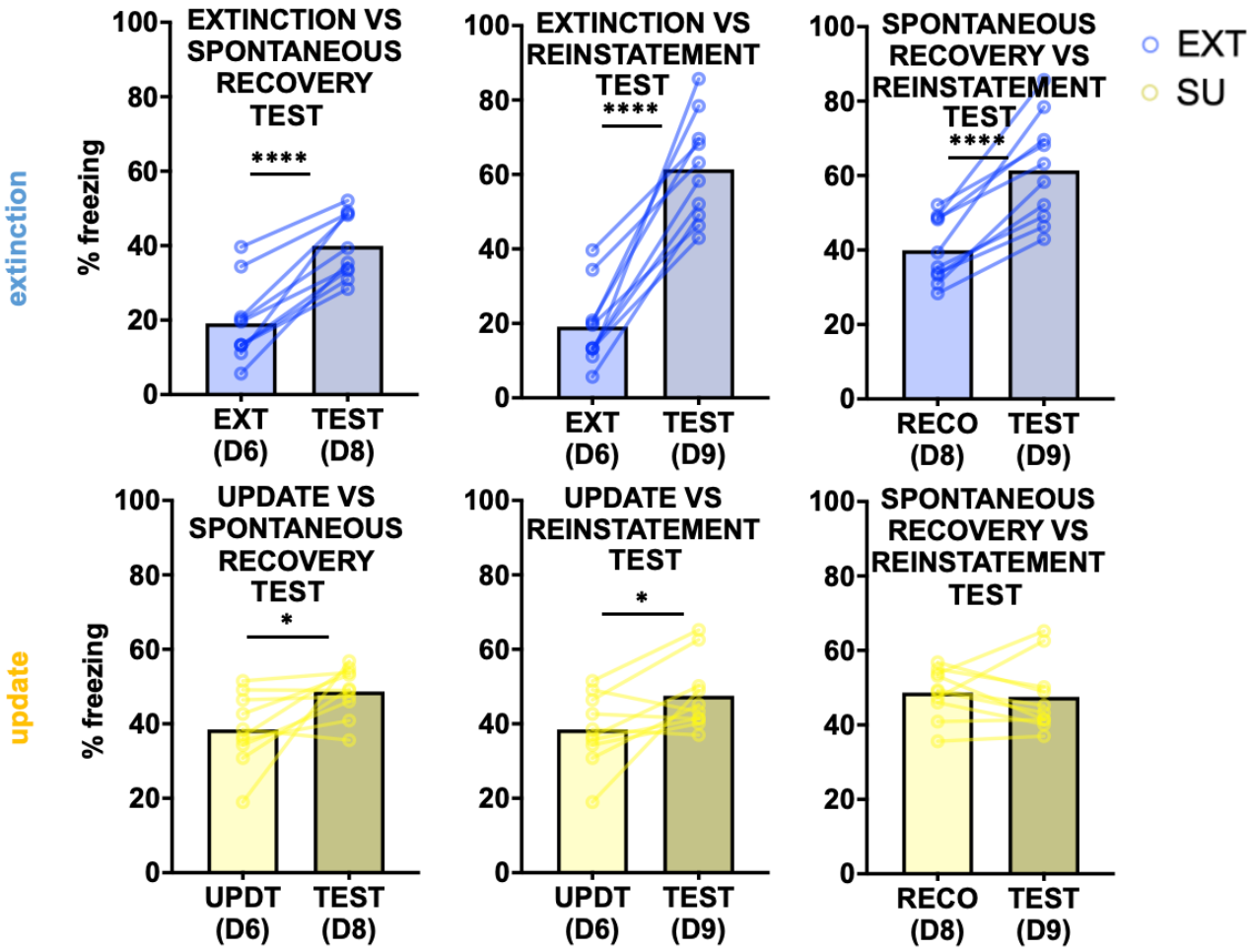


Figure 8: Memory Updating in Contextual Fear Conditioning Stabilizes Freezing during Spontaneous Recovery and Reinstatement Tests

Mice in the extinction group had significantly increased levels of freezing during the spontaneous recovery test (t(9)=7.972, p=<0.0001) and reinstatement test (t(9)=8.674, p=<0.0001) compared to the last day of extinction. When comparing the spontaneous recovery test to the reinstatement test in the extinction group, even more significant increases in freezing were observed ($t_{(9)}$=6.994, p=<0.001). Mice in the update group had much lower increases in freezing during the spontaneous recovery test (t(9)=2.630, p=0.0273) and reinstatement test (t(9)=2.685, p=0.025) compared to the last update day. These mice did not have significant differences in freezing when we compared the spontaneous recovery test to the reinstatement test.

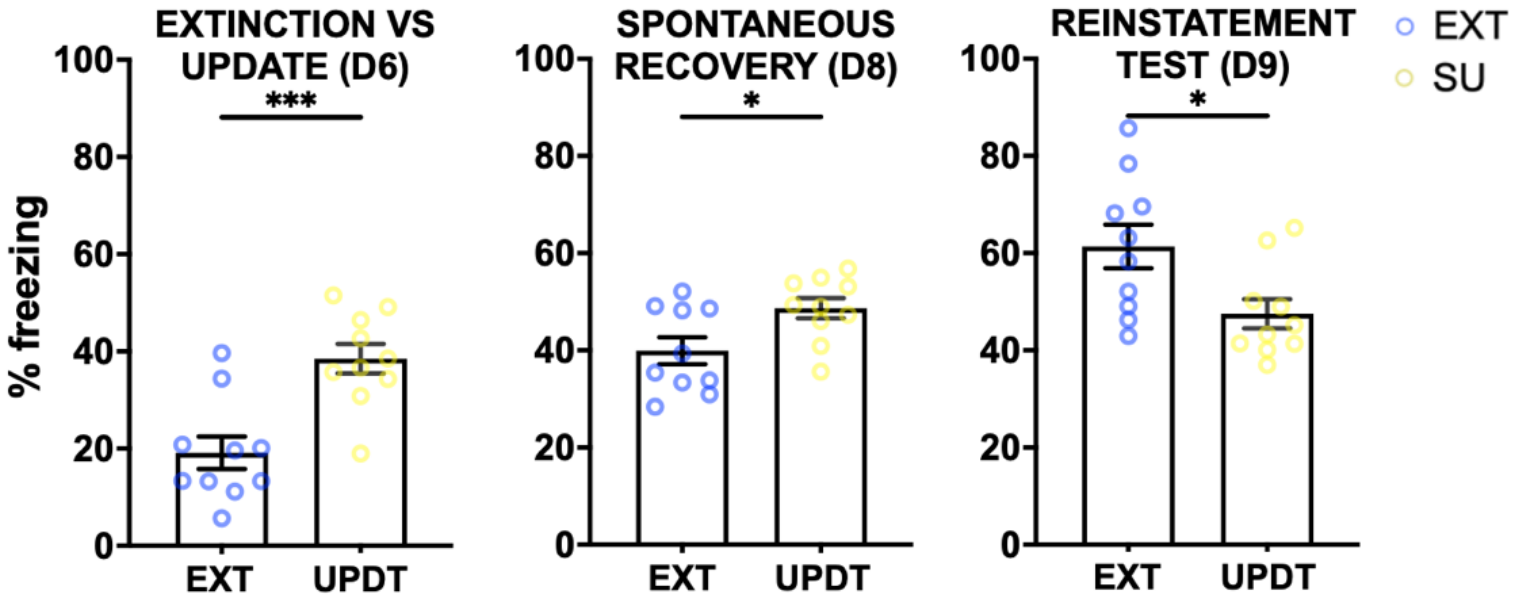


Figure 9: Fear Extinction Increases Freezing during Fear Reinstatement Compared to Memory Updating

During the last day (D6) of updating or extinction mice in the update group had higher levels of freezing ($t_{(17.84)}$=4.285, p=0.0005), likely due to the last update containing shocks. The update group also had higher levels of freezing than the extinction group during the spontaneous recovery test ($t_{(16.72)}$=2.238, p=0.0222). This effect reversed during the reinstatement test as the extinction group had significantly higher freezing ($t_{(15.72)}$=2.563, p=0.0211).

Discussion

Major Findings

In this thesis we utilized two behavioral paradigms to investigate the role of HDAC3 in memory updating. The first experiment made use of the OUL paradigm to answer how inhibition of HDAC3 affects memory updating in the young brain. Our results determined that control vehicle mice successfully learn both the original and updated information, while RGFP966 mice learn the updated information but have deficits in the original information. This result was surprising to us as we expected that HDAC3 inhibition would have no effect on memory in young mice since they normally learn the original and updated information without issue. In other words, we believed that RGFP966 would not improve memory of the updated information due to a ceiling effect. Since we observed a deficit in the original memory when the update was enhanced, we hypothesize that memory competition is occurring between the original information and updated information. It is possible that strengthening the update memory too much causes it to outcompete the original memory. The mechanisms by which this occurs remain unclear, and future studies must be completed to uncover why memory competition happens. One potential hypothesis is that the brain inactivates or even erases the original memory once the update becomes too strong. To further test the hypothesis that memory competition exists between the original and updated information, we tested whether a subthreshold update strengthened with HDAC3 inhibition would still be sufficient to impair the original memory. Our results from this experiment showed that RGFP966 could turn a subthreshold learning event into a strong memory that no longer outcompeted the original information. This is consistent with our hypothesis that the original and updated memories compete for expression and HDAC3 inhibition can shift this balance.

Next, we decided to investigate how memory updating and memory extinction in a fear conditioning paradigm differentially impact fear memory persistence. Our results indicated that the extinction group experienced significant fear rebound during both spontaneous recovery and reinstatement. In contrast, animals given a memory update, with weak shocks presented during the ‘extinction’ session, showed a much more persistent extinction that resisted reinstatement. These results sparked the hypothesis that fear updating may stabilize a fear memory and prevent

cases of extreme rebound. One potential theory we have for why this may occur goes back to the idea of memory competition. We believe that the update sessions work to modify the original fear neural engram in a way that stabilizes it, while the extinction sessions form a new memory which competes with the original memory during the reinstatement test. It seems that the initial fear memory outcompetes the extinction memory during the spontaneous recovery and reinstatement sessions even though the extinction sessions were more recent than the initial training. It is unclear why this happens, but it could be because the initial fear memory is more salient than the extinction memory, causing it to be stronger. Other studies have also proposed that reconsolidation changes the expression of the original memory, but extinction forms a new memory that is independent from the initial fear memory (Suzuki et al., 2004). One study found that fear retrieval and extinction activate distinct neural ensembles, and that silencing the fear acquisition tagged neurons prevented spontaneous recovery (Lacagnina et al., 2019). If memory reconsolidation modifies the initial fear memory and extinction forms a new separate memory, reconsolidation may prove to be a more effective therapeutic intervention than extinction. In humans, it could be possible induce retrieval of a maladaptive memory and deliver a drug that impacts reconsolidation and modifies the memory to a less detrimental form (Haubrich & Nader, 2018). One study found that administering the beta blocker propranolol after reactivation selectively disrupted the emotional component of spider phobia (Soeter & Kindt, 2015).

Limitations and Future Directions

While these studies provide many insights that will help us better understand memory updating there are some limitations that must be addressed through future work. Firstly, all these experiments were conducted in male mice. We cannot generalize these results to a broader population and would need to repeat these studies with female mice to identify any sex differences in memory updating. Second, while we know that HDAC3 acts as a critical regulator of memory reconsolidation, it is still unclear which genes HDAC3 acts on, and if it acts on different genes during memory consolidation and reconsolidation. Previous research has identified Per1 as gene that is regulated by HDAC3 during memory consolidation (Kwapis et al., 2018), however our studies did not assess gene expression changes during memory reconsolidation after HDAC3 manipulation. Future work could repeat these experiments and

collect brain tissue after behavior to perform RNA sequencing analysis and compare between groups. Another limitation is that it is still unclear how RGFP966 inhibits HDAC3. We know that HDAC3 forms a complex with corepressors NCoR and SMRT before manipulating chromatin (McQuown & Wood, 2011), but it is unknown how RGFP966 acts on this complex to inhibit deacetylation activity. We also lack a method to monitor when HDAC3 is active in the brain, so we don't know the best timepoint to inhibit the enzyme. Lastly, these experiments all rely on behavior as a secondary measure of memory reconsolidation. We have yet to investigate memory updating at the cellular level. One valuable future direction is to identify neural ensembles that are active during different phases of memory. We could use a tagging system during contextual fear conditioning to identify the group of neurons active during memory formation, reconsolidation, and extinction. After identifying each of these groups we could determine if there is overlap between any of the populations. For example, it would be useful to understand whether neurons active during fear acquisition overlap with neurons activated during the memory update. If there is significant overlap this could provide additional evidence for memory reconsolidation as an alternative treatment for trauma since extinction activates a separate population of neurons than fear acquisition (Lacagnina et al., 2019). More cellular overlap between the fear acquisition engram and update engram would suggest that memory updating does indeed modify the original memory and can transform a fear memory to be less aversive.

Conclusion

Memory updating is a critical process that allows us to adapt and survive in our ever-changing environment. Studying memory updating will be extremely useful in understanding general memory impairment and how memory contributes to trauma-related disorders such as PTSD. Our work demonstrates that the OUL task and contextual fear conditioning are two useful behavioral paradigms for investigating memory updating. Using the OUL paradigm we were able to manipulate HDAC3 activity and identify that inhibition of HDAC3 in aged mice restores memory updating, while inhibition of HDAC3 in young mice

leads to competition between original and updated information. Through contextual fear conditioning we found that memory updating stabilizes freezing levels and leads to decreased fear relapse compared to extinction, making reconsolidation useful as a potential therapy for aversive fear memories. Overall, our work suggests that memory updating can be used to manipulate different types of memory including spatial information and salient aversive information. Future work will determine mechanisms of memory updating we can use to improve memory function and prevent relapse of aversive memories.

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