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The Role of HDAC3 in Memory Updating via the Objects in Updated Locations (OUL) Task

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Reviewed and approved* by the following:

Janine Kwapis
Assistant Professor of Biology
Thesis Supervisor

Stephen W. Schaeffer
Professor of Biology
Honors Adviser

* Electronic approvals are on file.

ABSTRACT

Recent work provides evidence that epigenetic mechanisms play a vital role in memory formation. One epigenetic mechanism in particular, the repressive histone deacetylase HDAC3, operates in the hippocampus as a key negative regulator of memory; HDAC3 disruption or deletion transforms subthreshold learning into stable long-term memory. In older brains, HDAC3 contributes to age-related memory decline; HDAC3 deletion ameliorates age-related hippocampal memory impairments. Presently, it remains unclear whether HDAC3 induces age-related impairments in memory updating—the process through which an existing memory is updated with new information. The goal of this experiment was to determine the impact of HDAC3 on memory updating in young and old mice. To test this, we injected a pharmacological HDAC3 inhibitor, RGFP966, immediately after a hippocampal memory update in the Objects in Updated Location (OUL) paradigm in young (3-m.o.) and old (18-m.o.) mice. In old mice, HDAC3 blockage ameliorated age-related memory updating impairments, so that these mice now expressed intact memory for the update and memory for the original information at test time. In young mice, however, which already show robust memory updating, blocking HDAC3 after the update session led to disruption of the original memory, so that only memory for the update was robustly expressed at test. These results demonstrate that HDAC3 contributes to age-related impairments in hippocampus memory updating. Further, our work indicates that the original memory and the updated information appear to compete for expression, with HDAC3 helping to regulate which memory dominates at test.

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Chapter 1

Introduction

Memories are dynamic, flexible entities capable of changing in response to new information rather than fixed, static records of past experiences. The brain retains a vast capacity to incorporate current information into an existing memory; an ability broadly termed "memory updating," or more specifically, "reconsolidation." As humans and other organisms navigate new or changing environments, reconsolidation-dependent memory updating is critical to keep memories relevant and anticipate future outcomes—a necessity to augment survival and adaptation. Most memories (including those involved in human diseases) are not newly formed associations; existing memories undergo alterations or additions (updates), especially in the aged, experienced brain. Despite the integral importance of memory updating, the underlying mechanisms supporting this process at the molecular, cellular, and circuit levels remain enigmatic. Furthermore, dysregulation of these unclear mechanisms might contribute to the cognitive decline experienced in aging. More knowledge about the underlying mechanisms that modify memories in response to new information is required to improve memory across the lifespan.

Prior research has established the existence of the reconsolidation-based updating process. Memory retrieval (reactivation of an existing memory) induces a period of lability, termed reconsolidation, during which memories are modified or strengthened. The reconsolidation process consists of two phases: (1) an initial destabilization phase characterized by protein degradation, then (2) a restabilization phase marked by protein synthesis (Jarome et

al., 2011; Jarome et al., 2015; Lee, 2008; Nader & Hardt, 2009; Lee et al., 2017; Nader et al., 2000; Parsons et al., 2006). Previous studies demonstrate that reconsolidation only occurs when new information is presented during retrieval; when retrieval only contains familiar information, the original memory retains its stability and resists amnesic agents such as protein synthesis inhibitors (Jarome et al., 2015; Kwapis et al., 2017; Morris et al., 2006; De Oliveira et al., 2013). This suggests that new information prompts reconsolidation, initiating memory alterations. Despite previous research indicating that reconsolidation alters the original memory content (Jarome et al., 2015; Lee, 2008; Kwapis et al., 2017; Lee, 2010) and reorganizes the memory at the circuit level (Kwapis et al., 2017), the neural mechanisms facilitating this process are largely uncharacterized.

Both long-term memory formation and memory updating require transcription (Alberini, 2009), a process that is regulated in part through various epigenetic mechanisms. Specific chromatin modifications promote transcription via modulating histone-DNA interactions (Kouzarides, 2007). Enzyme modifiers, histone acetyltransferases (HATs) and histone deacetylases (HDACs), regulate the state of acetylation on histone tails. In general, during consolidation, HAT activity works to relax the chromatin at memory-relevant genes, promoting their transcription to enable memory consolidation. In contrast, histone deacetylase (HDAC) activity tightens up the chromatin and represses transcription, impairing memory consolidation (Peixoto & Abel et al., 2013). Previous research has shown that broad-spectrum HDAC deletion or inhibition facilitates transcription and enhances both synaptic plasticity and long-term memory (LTM) (Bredy and Barad, 2008; Guan et al., 2009; Maddox and Glenn, 2011; Vecsey et al., 2007). For example, a learning event that fails to produce lasting memory can be transformed into a learning event that drives robust and persistent long-term memory via HDAC inhibition

(Stefanko, et al., 2009). In genetic models of Alzheimer's disease, HDAC inhibitors ameliorate cognitive deficits, suggesting that memory modulation via HDAC inhibition provides considerable therapeutic potential for numerous cognitive disorders (Fischer et al., 2007; Kilgore et al., 2010).

Currently, little is known about how individual HDACs impact learning and memory. Recently, one specific HDAC has become increasingly more relevant in studies concerning memory formation: HDAC3 (histone deacetylase 3). HDAC3 is a Class 1 HDAC that is highly expressed in brain tissue—including the hippocampus—and a key negative epigenetic regulator of LTM (Broide et al., 2007). Although HDAC3 is known to play a vital role in age-related cognitive decline (including difficulties in memory formation and storage (McQuown et al., 2011), whether HDAC3 plays a role in memory updating is unknown.

To date, most of the work on reconsolidation-dependent memory updating focuses on fear memories, which are robust, long-lasting, and rapidly acquired. However, fear memories have a few disadvantages when investigating memory updating. First, fear memories often resist modification as they are extremely robust and persistent (Eisenberg et al., 2003; Suzuki et al., 2004). Additionally, in fear conditioning, the original and updated information is indistinguishable. Since rodents freeze for the duration of a conditional stimulus (CS), it becomes difficult to behaviorally differentiate between freezing related to the original information and freezing related to the updated information; the freezing behavior can reflect either the original or the updated information (Kwapis et al., 2017). As a final note, fear conditioning entails aversive, stressful stimuli and may not reflect the type of memories that occur in everyday life and are often affected during the normal aging process.

For these reasons, this study used a new behavioral paradigm, Objects in Updated Locations (OUL), that our lab developed to overcome the limitations of fear conditioning (Wright et al., 2020). OUL is a novel, non-stressful, hippocampus-dependent task that assesses the original and updated information in a single test session. The paradigm avoids unnecessary stress by relying on incidental learning, making it appropriate for studying age-related deficits in memory updating. Further, unlike fear conditioning, OUL allows the researcher to assess the strength of both the original memory and the updated information in a single test session.

The OUL protocol includes five phases: handling, habituating, training, updating, and testing. Handling involves physical interaction between the experimenter and the mice, which allows the mice to become well-habituated to the experimenter and the handling procedure. In all experiments, mice were handled for two minutes per day for four days. Then, habituation began for six consecutive days during which mice were placed in the training context (objects absent) and allowed to explore for five minutes. Following habituation, mice underwent training, which involved two identical objects (A_1 and A_2) in specific locations (Fig. 1A). Depending on age, mice received either a single 10-minute training session or three consecutive days of 10-minute training sessions. One day after training, mice were given a five-minute update session: young mice were assigned to either the No Update condition (mice re-exposed to training objects in same locations) or the Update condition (one object moved to a new location known as A_3) in a counterbalanced fashion (Fig. 1A); contrarily, older mice were assigned to only the Update condition (as aging mice are difficult to obtain, for old mice we focused exclusively on the Update condition). Mice possess an innate preference for novelty, allowing us to assess memory for the original training session during the update by comparing the exploration times between the moved object (A_3) and the unmoved object (A_1). Memory for the training session is

demonstrated by increased object exploration in a new location. Thus, the update session enables us to verify that animals successfully learned the training information and updated the original memory. Finally, after the update session, all mice were given a five-minute test session, which assesses their memory for both the original training information and the updated information. In the test session, mice were exposed to four identical objects: three objects in previous locations (A_1, A_2, and A_3) and a fourth object in a novel location, A_4. By comparing exploration of each of the three familiar locations with exploration of the novel location, we can assess whether each animal remembers the original (A_1 and A_2) or updated (A_3) locations. After completion, we hand-scored object exploration and performed statistical analysis to assess memory for the original and updated information.

Previous research by our group (Kwapis et al., 2020) has validated the OUL task and showed that memory updating is impaired in aging mice. When older mice (approximately 18 months old) performed the task, they acquired the original information successfully but showed age-related impairments in their ability to update this memory with the novel object location A_3 (Kwapis et al., 2020).

Therefore, in this present study, we investigated the role HDAC3 plays in memory updating using OUL. We aimed to test whether HDAC3 inhibition might ameliorate cognitive decline in the aging brain and improve memory updating. To accomplish this, we conducted two experiments: (1) testing the role of HDAC3 in updating in young mice and (2) testing the role of HDAC3 in updating in old mice. Based on the previous studies presented thus far and the known role of HDAC3 in the brain, we hypothesized that HDAC3 inhibition would improve memory updating in old mice but have no effect in young mice that already successfully learn memory

updating. The overarching goal of this project is to find an effective way of modulating memory via HDAC inhibition for considerable therapeutic potential in various cognitive disorders.

Chapter 2

Methodology

Subjects

The subjects were young adult (2-6-months) and old (18-20-months) male C57BL/6J mice. Mice were given free access to food and water. Lights followed a strict 12h light/dark cycle; all behavioral experiments occurred during the light cycle. 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 (IACUC number: PROTO201800406).

Drug Administration

Drug preparation involved two steps: (1) dissolved RGFP966 in DMSO, and (2) diluted in a vehicle of 30% (wt/vol) hydroxypropyl-β-cyclodextrin and 100 mM sodium acetate (pH 5.4). For both drug and vehicle, the final DMSO concentration was 10% (vol/vol). Mice in the drug condition group received a subcutaneous injection of the pharmacological HDAC3 inhibitor, RGFP966 (Repligen), immediately after a hippocampal memory update; the control group was given a vehicle. Research in rodents confirmed that RGFP966 effectively penetrates the blood-brain-barrier within 15 minutes, which establishes the rationale for a single-dose, single-inhibitor approach (Malvaez et al., 2013; Bieszczad et al., 2015). During handling sessions, mice were scruffed in preparation for the injection to decrease anxiety and allow for

proper injection. Weight of mice are recorded to prepare correct dosage and observations are recorded concerning amount injected or any leakage seen.

Experimental Design: OUL Paradigm

The task entails five key sessions: handling, habituation, training, updating, and testing. As mentioned in the introduction, subsequent of handling and context habituation, the mice underwent a training session. Then, following 24h after the training session, the mice were given an update session in which one object was moved to a new location (termed A_3; Update). A control group (No Update) was presented with the objects in the same locations as during training (A_1 and A_2). Immediately following the update session, mice received either a subcutaneous injection of RGFP966 or saline; treatment groups were randomly assigned. 24h after the update session, mice were tested to assess their original and updated memory; mice were exposed to four identical objects: two objects in the original training locations (A_1 and A_2), one in the updated location (A_3), and one in a novel location (A_4) (Fig. 1B)

Rodents possess an innate preference for novelty; mice that remember the original and updated locations will exhibit increased exploration time for the novel location. (Vogel-Ciernia & Wood, 2014; Kwapis et al., 2018). Thus, in the test session, if a mouse remembers objects A_1, A_2, and A_3, it should preferentially explore the novel location, A_4. Therefore, we can assess memory for each of the familiar locations by comparing its exploration to that of A_4 and calculating a discrimination index (DI) for each object (A_1-A_3). The formula for DI is:

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

Example DI calculation: $(A_4\text{-}A_1)/(A_4+A_1) \times 100$.

In the No Update group where object locations A_3 and A_4 are equally novel, experimenters randomly assigned either A_3 or A_4 to serve as the novel location, taking care to counterbalance this choice across all animals and conditions.

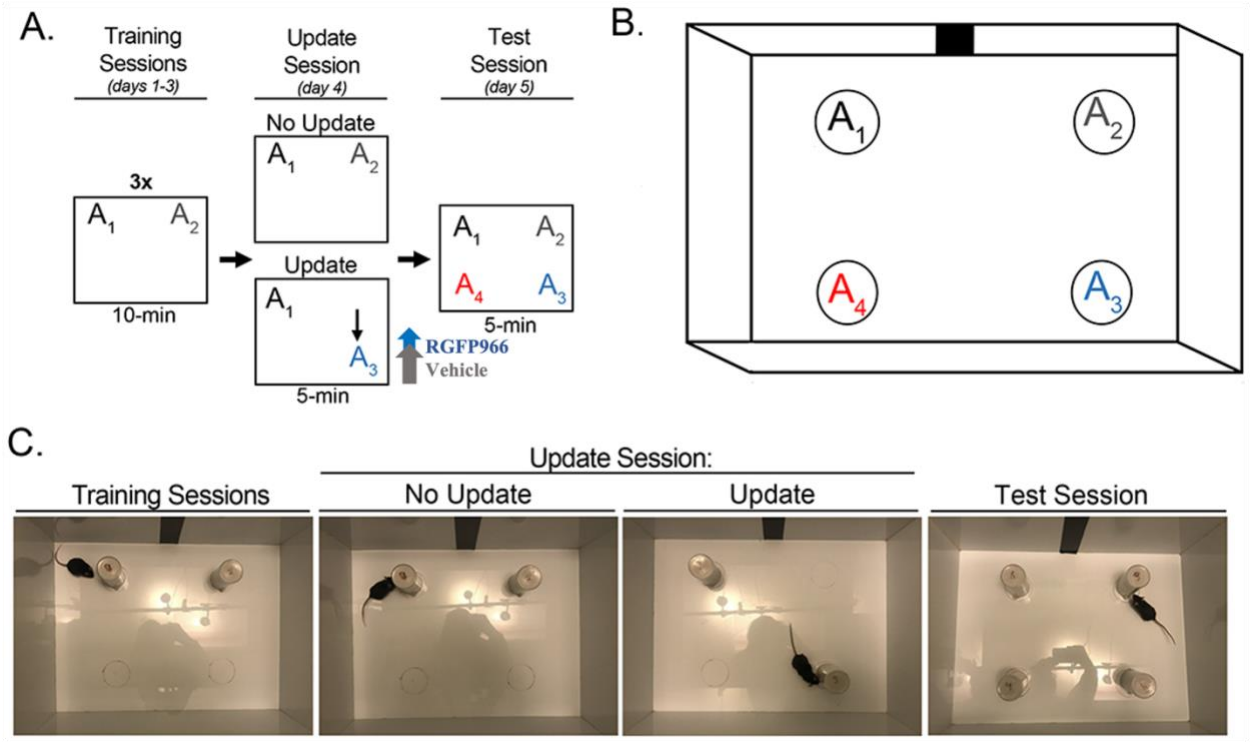

Figure 1. OUL Schematic and Experimental Setup

(A) General experimental timeline for OUL. Young mice underwent one 10-minute training session while older mice underwent three 10-minute subsequent training sessions to ensure the original memory was successfully acquired. The update session is shown with the right object moved; in the actual experiment, the displaced object was counterbalanced across groups. Immediately following the update session, mice were injected with either RGFP966 (HDAC3 inhibitor) or saline (Vehicle). (B) Diagram of object location and context proportions. (C) Images of experimental setup for OUL in sessions: training, update, and test. The mice were 3-6-

month-old C57Bl/6J. Objects were beakers filled with gray cement. *Figure adapted from Wright et al., 2020.*

Statistical Analysis

Behavioral videos were manually scored offline 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 (Vogel-Ciernia & Wood, 2014; Kwapis et al., 2018).

Statistical analysis was performed using a two-way ANOVA followed by Sidak-corrected *t*-tests and Dunnett's multiple comparison test to compare the experimental groups. All analyses were two-tailed and required a p-value of 0.05 for significance. Additionally, all statistics were performed via Graphpad Prism 9 software. Data are all shown with as mean ±SEM.

Exclusion Criteria

Exclusion from further analysis was determined by several factors: if mice indicate a location/object bias (DI > ±20) during all days of training, young mice with an exploration time of less than three seconds total, old mice with an exploration time of less than two seconds during training, updating, or testing, and values ±2SD outside the group (considered outliers). If mice show a DI > ±20 during training, when both objects and locations are unfamiliar, they have a location or object bias. Object bias leads to unsuccessful learning of both

the training locations, which results in failure to learn in subsequent sessions; therefore, these mice must be excluded to avoid confounding conclusions due to decreased preference of novel locations paired with familiar locations. For young and old mice, exclusions were made if exploration times were lower than three or two seconds respectively. As aging mice generally show less exploration than young mice, the movement criteria are lower for these animals. Low exploration times indicate that mice did not adequately explore the objects enough during training to properly learn the object locations or did not show enough total exploration at test to calculate an accurate DI.

Chapter 3

Results

Two major experiments were conducted: (1) the role of HDAC3 in memory updating in young mice and (2) the role of HDAC3 in memory updating in old mice. In our first experiment, we aimed to determine whether HDAC3 disruption immediately after the OUL update session would affect memory in young mice. In our second experiment, we tested whether HDAC3 disruption might ameliorate the age-related impairments in memory updating in old mice. After applying the exclusion criteria, we had a sample size of 47 young mice and 23 old mice. None of our sample sizes were predetermined with statistical analysis; the sample sizes are similar to those generally used throughout the field (Jarome et al., 2011; Nader & Hardt, 2009) and previously in our lab (Urban et al., 2021; Kwapis et al., 2017). Results from the update and test session shown. Data presented as ±SEM, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, NU, no update; U, update.

Experiment 1: Role of HDAC3 in Young Mice

To determine whether HDAC3 plays a role in memory updating in young mice, we first looked at behavior during the update session to confirm that the mice successfully learned the original memory, or the training information. The update session for young mice confirms that the original object locations (A_1 and A_2) were successfully acquired. Mice in the No Update group demonstrated DI values relatively close to zero, indicating equal preference for the familiar locations A_1 and A_2 (Fig. 2). Contrarily, mice in the Update group preferentially explored the moved object, A_3 compared to the unmoved object, A_1, showing a significantly

higher DI compared to the No Update group (Fig 2; two-tailed Student's *t*-test: $t_{(29)} = 0.2415$, *p <0.05). Together, these results verify that the original object locations were learned in young mice exposed to a 10-minute training session, consistent with previous studies (Vogel-Ciernia & Wood, 2014; Kwapis et al., 2018). Immediately following the update session, the HDAC3 inhibitor, RGFP966, was subcutaneously injected.

Next, to determine whether the original memory was successfully modified to integrate the updated object location (A_3), mice underwent a test session in which each familiar location (A_1, A_2, and A_3) was tested against the novel object location, A_4. Raw percent exploration time for each of the four objects was recorded (Fig. 3E) alongside total overall exploration time during the test session (Fig. 3D; two-way ANOVA (significant main effect of update by drug interaction ($F_{(1, 45)} = 7.677$, $df = 1$, **p < 0.01); Sidak's post hoc comparing vehicle vs RGFP966: No Update; $t_{(22)} = 1.690$, p > 0.05 and Update; $t_{(27)} = 2.265$, p > 0.05). As expected, the No Update groups showed preferential (and equal) exploration of the two novel object locations (A_3 and A_4) at test compared to the familiar locations A_1 and A_2 (Fig. 3E; two-way ANOVA, significant group by object interaction ($F_{(9, 132)} = 3.118$, $df = 9$, **p < 0.01)). On the other hand, the Update group should have learned both the original and the updated object locations (A_1-A_3) and were expected to preferentially explore the novel location, A_4, during the test session. This expectation was met in the Update group treated with the vehicle, in which mice showed significantly more exploration of the novel location compared to the familiar objects (Fig. 3E; two-way ANOVA, significant group by object interaction ($F_{(9, 132)} = 3.118$, $df = 9$, **p < 0.01); Dunnett's multiple comparison test: A_1 (*p < 0.05), A_2 (*p < 0.05), and A_3 (***p < 0.001)). Individual object analysis also depict the Update group treated with the vehicle exploring A_4 more than the familiar object locations (A_1, A_2, and A_3): A_1 (Fig. 3A; two-way ANOVA (no

significant main effects or update by drug interaction); Sidak's post hoc comparing Update vehicle vs RGFP966 *t*-test: $t_{(26)} = 0.7117$, $p > 0.05$), A_2 (Fig. 3B; two-way ANOVA (no significant main effect or update x drug interaction); Sidak's post hoc comparing Update vehicle vs RGFP966 *t*-test: $t_{(26)} = 2.539$, $^*p < 0.05$), and A_3 (Fig. 3C; two-way ANOVA (significant main effect of update ($F_{(1, 43)} = 9.870$, $df = 1$, $^{**}p < 0.01$), no significant effect of update by drug interaction); Sidak's post hoc comparing Update vehicle vs RGFP966 *t*-test: $t_{(26)} = 0.02888$, $p > 0.05$). Surprisingly, in the Update group treated with RGFP966, we found that although mice successfully learned the updated information, RGFP966 *impaired* memory for the original information. Both Update groups showed successful memory for the update and showed significantly more exploration of the novel location A_4 compared to location A_3 (Fig. 3C; Sidak's post hoc comparing Update vehicle vs RGFP966 *t*-test: $^*p < 0.05$). For the original information, however, RGFP966 mice showed impaired memory. Most notably, the amount of time RGFP966 Update mice showed equal exploration of the novel location, A_4, and the "old" original object, A_2, suggesting that the mice failed to remember the "old" original training object location, A_2 (Fig 3B; two-way ANOVA (no significant main effect or update by drug interaction); Sidak's post hoc comparing Update vehicle vs RGFP966 *t*-test: $t_{(26)} = 2.539$, $^*p < 0.05$). Additionally, despite being re-exposed to A_1 during the update, the mice explored A_1 preferentially more than A_3 (Fig. 3E). Update mice given vehicle, on the other hand, showed explored the familiar locations (A_1, A_2, and A_3) with equal preference in comparison to the novel location, A_4. These results in the Update group treated with RGFP966 indicate a competition between the original information and the update information; although the mice showed intact memory for the updated location A_3, post-update RGFP966 impaired memory for the original information, suggesting that these memories are in competition. These results suggest that

HDAC3 disruption or inhibition might lead to strengthening of update information (A_3) at the cost of the original information (A_1 and A_2).

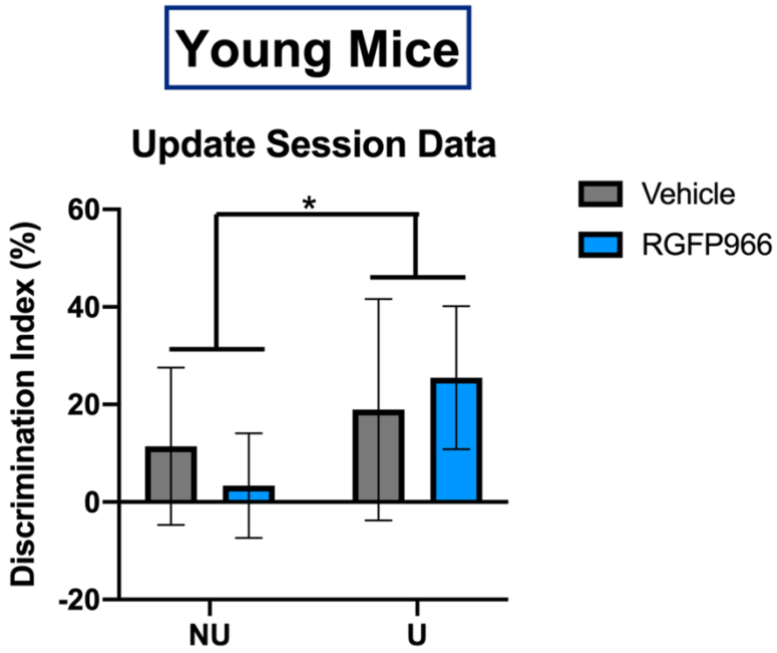

Figure 2. Young Mice Successfully Learned in Update Session

In the young mice, the update session for the No Update group was re-exposed to the original training information (A_1 and A_2). The No Update group has low DI values, indicating that they explored the objects without bias. In the Update group where the mice were presented with the displaced object, A_3, the mice preferentially explored A_3, indicated by higher DI values. A significant difference ($*p \leq 0.05$) between exploration times in the update session indicates that the young mice learned the original information.

RGFP966 Causes Competition Between Original and Updated Memory

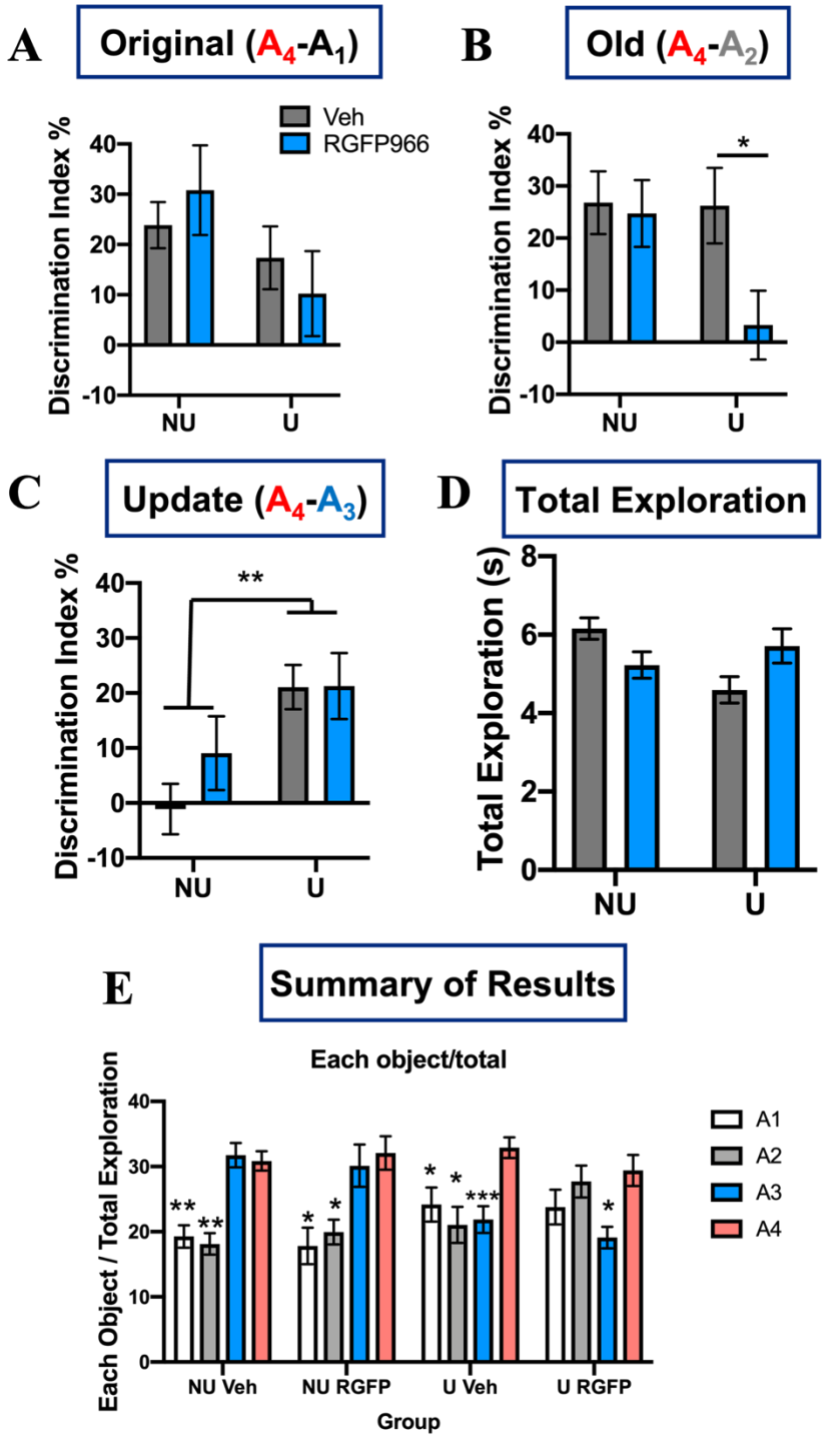

Figure 3. Young Mice HDAC3 Disruption in Test Session

A. **Original object, A1, compared to novel object, A4.** (i) No significant difference within the No Update group in the comparison between A_1 (present throughout the entire extent of all sessions) and A_4. High DI values indicate the mice learned the training information and explored the novel object more regardless of drug treatment. (ii) There was a moderate difference in the Update group between A_1 and A_4. The vehicle group has a larger DI than the RGFP966 group, suggesting that the vehicle group explored the novel object more while the RGFP966 group showed an equal preference with a DI closer to zero; this implies that the drug treatment impaired memory of the original training information.

B. **Original object (A_2)—for the update group, this was the "old" original since it was introduced during the training session then taken away until introduced again in the test session (while A_1 is present throughout all sessions)—compared to A_4.** (i) The No Update group had high DI values meaning they explored A_4 more and remembered the original information (A_2) regardless of drug treatment. (ii) There is a significant difference in the Update group between the vehicle and RGFP966 groups. The vehicle group remembered the original information and preferentially explored A_4 while the RGFP966 group significantly explored the old original information, A_2, greater than A_4; this indicates that the drug treatment impaired memory of the original training information.

C. **Update information, A_3, compared to novel information, A_4.** (i) The No Update group depicted a DI value near zero meaning there was an equal preference between the two objects (update and novel) regardless of drug treatment. (ii) The Update

group had high DI values meaning they remembered the update information and preferentially explored the novel object, A_4. (iii) There was a significant difference between the two groups regardless of drug treatment.

D. Total exploration of each object across all animals and conditions. (i) The No Update vehicle group equally explored A_1 and A_2 while preferring the novel objects A_3 and A_4. The No Update RGFP966 group explored A_1 less than A_2 while equally exploring A_3 and A_4. (ii) The Update vehicle group equally explored A_1-A_3 and preferred the novel object A_4. The Update RGFP966 group explored A_3 less than A_1 while exploring A_2 and A_4 the most. (iii) No significant difference was observed in total exploration time in the test session across all animals and conditions.

Experiment 2: Role of HDAC3 in Old Mice

To determine whether HDAC3 plays a role in memory updating in old mice, we first looked at behavior during the update session to confirm that the mice successfully learned the original memory, or the training information. For this experiment, due to a limited number of old mice and previous research conducted by Kwapis et al., 2018, there was only an Update group. In the update session for old mice, the results confirmed that the original object location (A_1) was successfully acquired, as mice preferentially explored the moved object, A_3 compared to the unmoved object, A_1, depicted by high DI values (Fig 4; two-tailed Student *t*-test: $t_{(14)} = 1.105$, $p > 0.05$). These results verify that the original object locations were learned in old mice exposed to three consecutive 10-minute training sessions, consistent with previous studies (Vogel-Ciernia & Wood, 2014; Kwapis et al., 2018). Immediately following the update session, the HDAC3 inhibitor, RGFP966, was subcutaneously injected.

24h after the update session and the subcutaneous injection of RGFP966, the old mice underwent a test session, which served to determine whether the original memory was successfully modified to integrate the updated object location (A_3). In the test session, mice were re-exposed to each familiar location (A_1-A_3) and a novel object location, A_4. Raw percent exploration time for each of the four objects was recorded (Fig. 5E) alongside total overall exploration time during the test session (Fig. 5D; two-tailed Student *t*-test: $t_{(14)} = 0.7688$, $p > 0.05$). Based on the results from the Kwapis et al., 2018 study, older mice in the vehicle group were expected to show poor memory for the update object location, A_3, while remembering the original training information (A_1 and A_2). Therefore, we expected our old vehicle mice to equally explore the update object location, A_3, and the novel location, A_4, during the test session. Indeed, the vehicle group explored the update location, A_3, slightly more (nonsignificant) than the novel

location, A_4, but explored the original training locations (A_1 and A_2) the least (Fig. 5E; two-way ANOVA (significant effects of drug ($F_{(1, 7)} = 9.374$, $df = 1$, *$p < 0.05$) and object ($F_{(1.874, 13.12)} = 7.926$, $df = 3$, **$p < 0.01$), but no significant main effect of object by drug interaction); Dunnett's multiple comparison test: A_1 ($p > 0.05$), A_2 ($p > 0.05$), and A_3 ($p > 0.001$)). This confirms our past research demonstrating that aging mice show difficulty updating existing memory.

Here, to test whether blocking HDAC3 would ameliorate this age-related impairment in memory updating, we injected RGFP966 immediately after updating. Consistent with our hypothesis, we found that mice treated with RGFP966 showed intact memory for both the original locations and the updated location. The RGFP966 group significantly explored the novel location, A_4, more than the updated location, A_3, in comparison to the vehicle group (Fig. 5C; two-tailed Student *t*-test: $t_{(14)} = 2.321$, *$p < 0.05$). Specifically, we found that both vehicle and RGFP966 mice showed intact memory for location A_1, presented during both the training and update sessions (Fig. 5A; two-tailed Student *t*-test: $t_{(14)} = 0.7719$, $p > 0.05$). For object location A_2, which was absent during the update session and thus considered to be the "old" original object location (with a longer retention interval of 48h between training and testing), RGFP966 slightly (though nonsignificant) improved memory. Although vehicle mice showed very weak memory for location A_2, suggesting that the original memory is not persistent in old mice, mice given RGFP966 showed slightly better retention of location A_2 (Fig. 5B; two-tailed Student *t*-test: $t_{(14)} = 1.435$, $p > 0.05$). Thus, RGFP966 given after updating seemed to strengthen the original memory itself.

RGFP966 also significantly improved memory for the updated information in old mice. Old mice showed more exploration of the novel location, A_4 than the updated location, A_3; importantly, old mice given post-update RGFP966 showed a significantly higher DI for location

A_3 than mice given vehicle (Fig. 5C; two-tailed Student *t*-test: $t_{(14)} = 2.321$, *$p < 0.05$), suggesting that blocking HDAC3 improved memory updating in these old mice. Thus, post-update HDAC3 disruption ameliorates age-related impairments in memory updating.

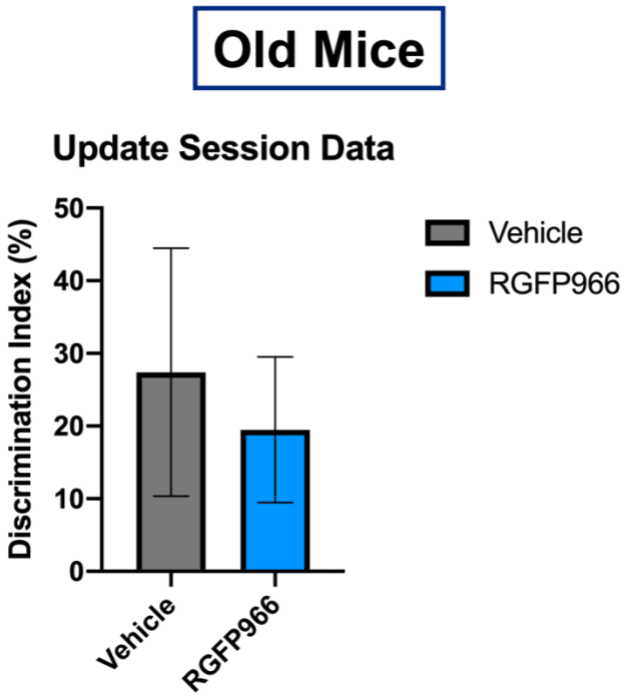

Figure 4. Old Mice Successfully Learned in Update Session

During the update session (before drug injection) both groups have high DI values, showing that they learned the original training information and preferred to explore A_3, which was the novel, updated information. Note that RGFP966 is not injected into the mice until immediately after the update session.

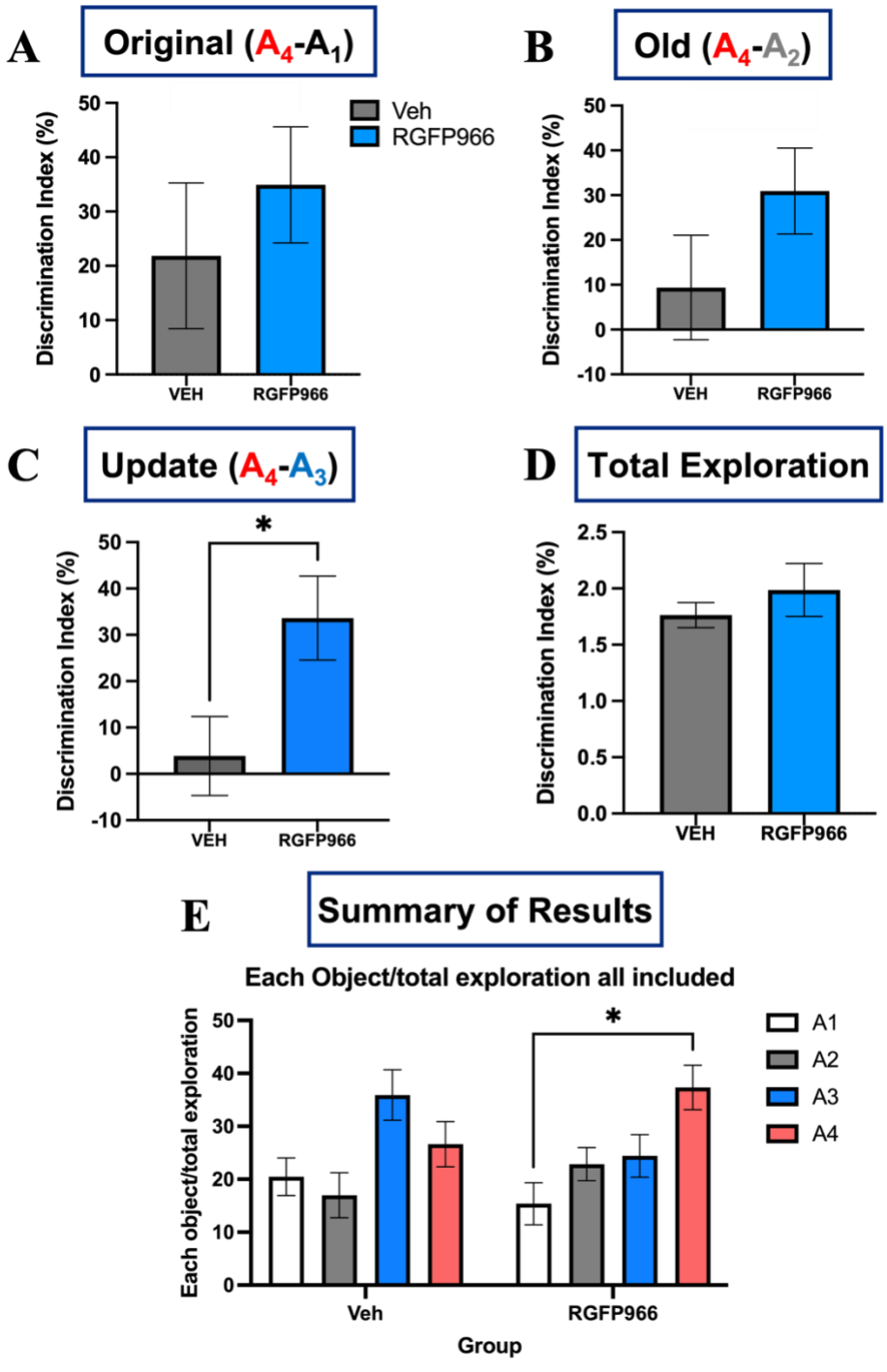

Figure 5. RGFP966 Impact on Old Mice Original and Updated Memory in Test Session

A. **Original object, A_1, compared to novel object, A_4.** High DI values indicate the mice learned the original training information and explored the novel object more regardless of drug treatment

B. **Original object (A_2) compared to A_4.** Both the vehicle and RGFP966 group remembered the old original information, A_2, and explored A_4 more; this indicates that regardless of drug treatment, the mice had intact memories of the old original training information. Despite the lack of significance, note that the vehicle group explored both A_2 and A_4 nearly equally via the depicted low DI value.

C. **Update information, A_3, compared to novel information, A_4.** The vehicle group depicts a DI value near zero meaning there was an equal preference between the two objects. The RGFP966 had higher DI values meaning they remembered the updated information and preferentially explored the novel object. There was a significant difference between the two treatment groups ($*p \leq 0.05$).

D. **Total exploration of each object across all animals and conditions.** The vehicle group equally explored the original training information, A_1 and A_2, while preferentially exploring the update information, A_3, more than the novel information, A_4; this depicts the age-related memory updating impairment in older mice. The RGFP966 group equally explored the familiar objects, A_1-A_3, while preferentially exploring the novel object location, A_4; this suggests that the HDAC3 inhibitor ameliorates impaired reconsolidation in old mice. There was a significant difference between the original training object, A_1, and the novel location, A_4, ($*p \leq 0.05$) while no significant difference was observed in total exploration time in the test session.

Chapter 4

Discussion

Major Findings

In this study, we found that HDAC3 contributes to age-related impairments in memory updating. We showed that by systemically blocking HDAC3, age-related impairments in memory updating are ameliorated; this is consistent with our hypothesis that HDAC3 restricts memory updating in addition to its known role in memory formation and storage. Furthermore, we found evidence that the original memory and the updated information compete in the OUL paradigm. In young mice that successfully learned memory updating, blocking HDAC3 immediately after updating led to no effect on memory updating but impaired retention of the original information. This indicates that the original and updated information compete for expression in the epigenetic mechanisms driven by HDAC3 (and potentially other individual HDACs/HATs); enhancing the update information with RGFP966 came at the expense of the original memory. Thus, RGFP966 may be able to strengthen a weak update in old mice, but further enhancing a robust memory in young mice may erode the initial memory itself.

In the Kwapis, et. al (2018) study, a comparison of old mice in both the No Update and Update groups were made; this investigation found that older mice successfully acquired the original information, however, they failed to learn the updated information. In the Update conducted in this study with old mice, the vehicle group expressed a similar result by exploring the updated location, A_3, more than the novel location, A_4, in the test session (Fig. 5E) despite

exposure to A_3 24h prior in the update session. In contrast, the old mice that received the drug treatment remembered the locations: original; A_1, old original; A_2, and the update; A_3. The RGFP966 old mice explored the novel location more in comparison to the other three (Fig. 5E). This result suggests that HDAC3 inhibition ameliorates age-related impairments in memory updating.

Age-related memory deficits are documented across numerous studies and species along with impaired behavioral flexibility. Research shows that HDAC3, a class I epigenetic regulator, plays a key role in memory consolidation via various behavioral tasks such as fear conditioning and Morris water mazes (Nader et al., 2000; Parsons et al., 2006; Morris et al., 2006). This study contributes to this knowledge by depicting how HDAC3 also plays a critical role in reconsolidation-dependent updating through the non-aversive OUL paradigm. Epigenetic mechanisms, which modulate gene expression, control transcription by modifying the structure of chromatin rather than altering the DNA sequence itself (Hemstedt et al., 2017). Although few studies have manipulated epigenetic mechanisms to investigate its role in reconsolidation, the research conducted by Kwapis and Wood 2014 found evidence suggesting epigenetic changes that facilitate transcription enhance restabilization while epigenetic changes that repress transcription inhibits restabilization. For example, by blocking HDAC activity, which typically opens chromatin and allows transcription, the restabilization phase is enhanced (Maddox & Schafe, 2011) whereas by blocking HAT (histone acetyltransferase] activity, which represses chromatin, impairs transcription (Maddox et al., 2013). As epigenetic mechanisms play a critical role in reconsolidation-dependent memory, it is necessary to understand how the individual chromatin modifiers contribute and potentially modulate an organism's response to memory

updates. In the case of this study, RGFP966 blocks HDAC3 activity, meaning it enhances restabilization phase of reconsolidation to improve memory updating across a lifespan.

In the old mice, RGFP966 appears as a potential candidate to ameliorate age-related memory updating impairments. Blocking HDAC3 seems to enhance the restabilization phase, improving reconsolidation. However, the most interesting finding is what occurs in the young, unexperienced brain when RGFP966 is introduced and blocks HDAC3 activity. Regardless of drug treatment, we expected the young mice to learn normally; they would remember all the locations and preferentially explore the novel location. Instead, the young mice with RGFP966 significantly failed to remember the old original location introduced during training; these mice explored the old original, A_2, and novel location, A_4, without preference. Thus, our work suggests that systemically blocking HDAC3 after a hippocampal update further strengthens an already robust memory, creating an overly strong memory that outcompetes the original information. Contrarily, in older mice that cannot learn the updated information on their own, HDAC3 blockage rescues the weak update memory without impairing the original memory. The underlying mechanism behind such a finding remains unclear.

Limitations and Opportunities in Future Studies

This study introduced the RGFP966 via a subcutaneous injection immediately following the update session to enhance reconsolidation. However, given the young mice findings, there are three future directions for further investigations. As previously mentioned, HDAC3 blockage in the young hippocampus, which typically depicts robust memory updating, results in the original memory weakening because it is creating an overly strong memory for the update that

outcompetes. Thus, to test this, one future direction is to manipulate the strength of the original memory and the update memory in young mice. Specifically, through three different avenues: (1) weakening the update session at which mice undergo a subthreshold update that is too short to drive successful updating prior to RGFP966 injection (mimics what normally occurs in older mice who depict memory updating impairment, meaning it should enhance the update in young mice while having no effect on retention of the original memory), (2) strengthen the training information by having young mice undergo three consecutive days of training rather than one so that the original memory can compete with the robust update memory created by RGFP966, and lastly (3) strengthen the original training with RGFP966 (injecting RGFP966 immediately following the training session) to test whether it creates an overly robust training memory that prevents updating. Furthermore, all the mice in this experiment were male, meaning there is potentially a different outcome for female mice. Thus, this study should aim to work with female samples and observe if these findings are consistent across sex. Lastly, there is also further research on other potential key candidates in reconsolidation that can utilize the OUL task to investigate the mechanisms such as AMPA receptor subunit exchange (Rao-Ruiz et al., 2011), protein degradation (Jarome & Helmstetter, 2013), and synthesis of key proteins such as zif268 and BDNF (Lee, 2010).

Conclusion

Understanding the mechanism behind how existing memories undergo modifications in the face of new and relevant information is a critical area of research. Most studies conducted in this area utilize fear-conditioning, a powerful system that has contributed key information

concerning mechanisms in reconsolidation-dependent memory updating. Here, we use the Objects in Updated Locations (OUL) task to assess the relationship more specifically between the original memory and the updated information. Through this paradigm, we found that blocking HDAC3 ameliorated age-related memory updating impairments in old mice whereas blocking HDAC3 in young mice impaired the original memory, possibly enabling the update memory to dominate. Identifying the mechanisms that support a successful reconsolidation-dependent memory updating is a vital step toward improving memory updating when it fails and improving it in age-related cognitive disorders.

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ACADEMIC VITA

DESTINY WRIGHT

Linkedln: https://www.linkedin.com/in/destiny-wright-1a33541a5/
dsw5269@psu.edu

EDUCATION

Pennsylvania State University, University Park — June 2018-Present
Schreyer Honors College
Graduation: May 2022
Major: Biology (Neuroscience option)

HONORS AND AWARDS

Presidential Leadership Academy — Aug 2019-Present
Millennium Scholars Program — June 2018-Present
Dean's List — 5 Semesters

RESEARCH EXPERIENCE

Undergraduate Researcher, Columbia University, New York — June 2021-May 2022
Principal Investigator: Dr. Yunglin Gazes

Undergraduate Researcher, Pennsylvania State University — Feb 2019-May 2022
Principal Investigator: Dr. Janine Kwapis

PUBLICATIONS

Wright, D. S., Bodinayake, K. K., & Kwapis, J. L. (2020). Investigating Memory Updating in Mice Using the Objects in Updated Locations Task. Current protocols in neuroscience, 91(1), e87

PRESENTATIONS

Poster Presentation, "Investigating Tau Pathology in Gray Matter Regions via Diffusion MRI," International Neuropsychological Society, Virtual 2021.

Oral Presentation, "Investigating Tau Pathology in Gray Matter Regions via Diffusion MRI," STAR U Symposium, 2021.

Poster Presentation, "Investigating the Role of HDAC3 in Memory Updating," Annual Biomedical Research Conference for Minority Students, Virtual 2020.