THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

The Role of HDAC3 in Stress-Enhanced Fear Learning

MARC LUSSIER
SPRING 2023

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Premedicine
with honors in Biology

Reviewed and approved* by the following:

Janine Kwapis
Assistant Professor of Biology
Thesis Supervisor

Bernhard Luscher
Professor of Biochemistry and Molecular Biology
Thesis Honors Adviser

* Electronic approvals are on file.

ABSTRACT

Certain disorders like post-traumatic stress disorder (PTSD) occur when long-term memory becomes pathogenic. Previous studies have identified epigenetic mechanisms that contribute to memory formation, including histone acetylation. One class of histone deacetylases (HDAC), HDAC3, has been shown to act as a negative regulator of fear memory formation in the amygdala, a part of the brain that is essential for encoding emotional memories. Here, we investigated the role of HDAC3 in trauma learning and subsequent context fear memory. We hypothesized that HDAC3 inhibition would transform a weak stressful event into a stronger, more traumatic event. We injected the amygdala of mice with an attenuated viral vector that blocks HDAC3 activity or an empty control viral vector and subjected the mice to a modified stress-enhanced fear learning (SEFL) procedure. The SEFL protocol, a rodent model of PTSD, involves initially exposing mice to a strong traumatic event via 10, 2-second, 0.7 mA electric shocks in a specific context. These shocks are delivered at randomized time points during a 56-minute period. Later, in a different context, freezing behaviors are assessed to measure fear memory formation after fear conditioning. In a modified procedure, a weaker initial traumatic event is used that is insufficient to elicit maximal fear learning during subsequent fear conditioning. In female mice, inhibiting HDAC3 failed to amplify the effect of trauma on subsequent fear conditioning; all female mice showed the same high level of fear memory when tested by subsequent context fear conditioning, irrespective of the strength of the initial traumatic event However, in male mice injected with an empty vector a weak traumatic event resulted in a submaximal fear response to fear conditioning, which was enhanced if the mice were injected with the HDAC3 inhibitor. This enhanced fear response of the males was similar in strength to

the invariant fear response of female mice. The results indicate that there is a basal sex difference in sensitivity of mice to trauma, and HDAC3 inhibition transforms a weak traumatic event into one that causes a persistent exaggeration of subsequent fear learning and memory in male mice. In female mice, this weak traumatic event induced strong learning during subsequent fear conditioning, independent of HDAC3 manipulation. Analyses of transcribed genes and “fear proteins” present during fear conditioning following trauma should provide a potential avenue for future research in the field of PTSD.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

This project would not have been possible without the help of some amazing people. Without all the people listed below, I would not be the person that I am today.

I would first like to thank my research supervisor, Dr. Janine Kwapis, for accepting me into her lab four years ago. Her wisdom, advice, and guidance have been irreplaceable, and I am indebted to Dr. Kwapis for all that she has done for me. I also owe my thanks to my research mentor, Dr. Mark Urban, who helped teach me the ins and outs of research and was essential in helping to run both the surgeries and fear conditioning trials involved in this project. I will never forget the countless hours we spent together and am truly thankful for his mentorship.

I would also like to thank multiple Kwapis Lab members for their help in various aspects of this project, such as graduate students Chad Brunswick for teaching me some of the statistical methods and Chad Smies for his help running fear conditioning trials. I would not have been able to complete this project without their support and encouragement. I also want to thank my honors adviser Dr. Bernhard Luscher for taking the time to read this thesis and offer his feedback.

Finally, I would like to thank my family and friends for providing me with unconditional love and support throughout my life. They have pushed and encouraged me at every turn along my journey, for which I am eternally grateful.

This material is based upon work supported by the NIA under Award No. K99/R00 AG056596, the Whitehall Foundation under grant # 2020-05-06, and the AFAR under Junior faculty grant # A21105, all to Janine L. Kwapis. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the NIA, Whitehall Foundation, or AFAR.

Chapter 1

Introduction

Memory is the process by which information is gathered from an organism's internal and external environments and is stored, modified, or discarded. Memory consolidation is the process by which information is converted into long-term storage (Nadel & Moscovitch, 1997), while memory recall is the retrieval of these encoded experiences. Traditional theories of memory implicate the hippocampus in both the consolidation and retrieval of episodic memory, a type of explicit memory during which conscious recall of an event occurs (Nadel & Moscovitch, 1997; Squire et al., 2015). Implicit memory differs from explicit memory in that it does not require active recall on the part of an organism. Implicit memory has been shown to be strongly linked to emotion, where an unconscious recall of an event can trigger an emotional response (Tobias et al., 1992). This is especially significant if long-term memory acquisition, which is critical for the daily function of complex organisms, becomes pathological. Exposure to a traumatic event triggers an emotional response that is processed by the amygdala, which has been shown to influence hippocampal plasticity (Izquierdo et al., 2016). Long-term memory of the event, consisting of the context and the emotions associated with it, are jointly encoded by the hippocampus and amygdala. However, rodent studies have shown that these brain regions have different roles. The hippocampus is critical for context and spatial memories (Broadbent et al., 2004; O'Keefe & Nadel, 1979), while the amygdala is necessary for emotional memories (Phelps & Anderson, 1997). Both implicit and explicit memories are important in order to navigate a changing world. However, memories also have the potential to be problematic. For example, a

memory of a traumatic event can cause excessive fear and avoidance behaviors that interfere with daily life and can contribute to the development of anxiety disorders and post-traumatic stress disorder (PTSD). Understanding how these maladaptive memories form is critical for developing treatments for these disorders.

PTSD develops when individuals are exposed to a traumatic event such as a war combat experience or sexual assault. These events leave them predisposed to exhibiting an exaggerated fear response when exposed to a mild stressor that is reminiscent of the original traumatic event. PTSD is characterized by a combination of distress, impairment, and avoidant and self-destructive behaviors in response to a traumatic event (Kilpatrick et al., 2013), the memory of which can persist indefinitely; the lifetime prevalence of PTSD is between 5% and 10% (Kilpatrick et al., 2013; Jakovljević et al., 2012). Though PTSD is one of the most recognized mental disorders, its pathophysiology is not well understood (Jakovljević et al., 2012). Women are twice as likely to develop PTSD, with a lifetime prevalence of 5.7% for men and 12.8% for women (Kilpatrick et al., 2013). The reasons for this are disputed. Hypotheses include differences in societal expectations and emotional processing of fearful events (Street & Dardis, 2018), differences in sex hormones (Ramikie & Ressler, 2018), and the increased risk of sexual abuse or trauma which increases the risk of developing PTSD (Kessler et al., 1995). Even when controlling for the type of traumatic event, this sex prevalence disparity was still present (Christiansen & Elklit, 2012). It is likely that a combination of factors leads to the development of PTSD, including male-female differences in the molecular mechanisms induced by traumatic learning in the hippocampus and amygdala, a brain region known to play a role in fear memory formation (Davis, 1992).

One potential mechanism is epigenetic modification, which is important for memory formation. During memory formation, epigenetic modifications dynamically alter access to the transcriptome to enable the transcriptional changes that support long-term memories including fear memories. While the specifics are not well understood, epigenetic changes have been implicated in the stress response (Stankiewicz et al., 2013) in a way that may create an exaggerated fear memory upon exposure to subsequent traumas. Epigenetic modifications are changes in the structure of DNA in which different "markers" are attached to the genome to modify gene expression and are critical regulators of long-term memory formation (Lubin et al., 2011). One epigenetic mechanism that is important for memory is the acetylation of histones. Histones are proteins around which DNA is wound to form chromatin, the collection of which forms chromosomes. There are two distinct forms of chromatin: euchromatin and heterochromatin. Euchromatin is a looser, unwound form of DNA that is associated with higher levels of transcription, while heterochromatin is a more tightly wound form that is associated with gene repression. Adding an acetyl group to a histone loosens the DNA coils, allowing access for transcriptional machinery to bind to the DNA to transcribe genes necessary for long-term memory (Agranoff et al., 1967). Because transcription is required for long-term memory formation (Agranoff et al., 1967), the acetylation state of the histones during a memorable event can influence whether this event will be consolidated into long-term memory. Generally, as histones become more acetylated, the chromatin becomes more unwound, leading to transcriptional activation and greater memory formation. Conversely, deacetylating histones leads to a more closed chromatin state, transcriptional repression, and a decrease in memory formation. Two enzymes that facilitate the addition and removal of an acetyl group to a histone are histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. HDAC3,

a type of Class I HDAC, is found in the greater HDAC complex that facilitates the deacetylation of histones (Hayakawa & Nakayama, 2011). HDAC3 activity has been shown to negatively affect long-term memory acquisition (McQuown et al., 2011), and in the amygdala and hippocampus of male mice, HDAC3 has been shown to be a negative regulator for context-based fear learning (Kwapis et al., 2017).

However, how HDAC3 contributes to trauma-based learning of males and females is currently unknown. Trauma-based learning and PTSD can be modeled in rodents with the Stress Enhanced Fear Learning (SEFL) paradigm (Rau et al., 2005). In this paradigm, rodents are given an acute trauma event, usually 10 unsignaled, randomized shocks of constant intensity and time for mice (Rajbhandari et al., 2018) in a specific context (Context A), to mimic the trauma event that initiates PTSD. Then, the rodents are exposed to a very weak shock in a new context (Context B). As with humans, mice with a history of trauma show an exaggerated response to this subsequent mild fear conditioning that results in a robust and persistent fear memory for Context B (Fig. 1). This exaggerated subsequent fear memory mimics the symptoms commonly affiliated with PTSD, such as a heightened reaction to aversive stimuli that remind the individual of a long past trauma event. Despite its usefulness as a model for PTSD, there is very little known about the molecular mechanisms in the amygdala and hippocampus that enable SEFL learning. Specifically, the mechanisms that enable the trauma event to persistently sensitize the animal's response to subsequent fear conditioning are largely unknown. However, it is possible that HDAC3 and other similar mechanisms create an enduring change in the genome in response to this trauma, similar to PTSD (Ponomarev et al., 2010), thereby enabling this exaggerated fear memory formation (Fig. 2).

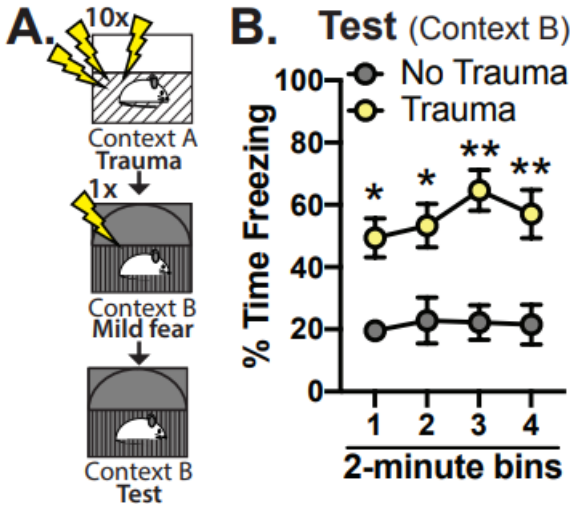

Figure 1. SEFL set up and representative data

(A) Schematic of the SEFL paradigm. Mice are first given 10 shocks (the trauma event) or no shocks (control) in Context A. Then, in Context B, a mild shock is administered. Finally, freezing behavior is measured the following day in Context B. (B) Representative data showing that mice exposed to the traumatic event in Context A freeze more in Context B following a single mild foot shock. This exaggerated fear response is significantly greater when compared to mice that were not exposed to that initial trauma. *Data from our pilot SEFL runs.*

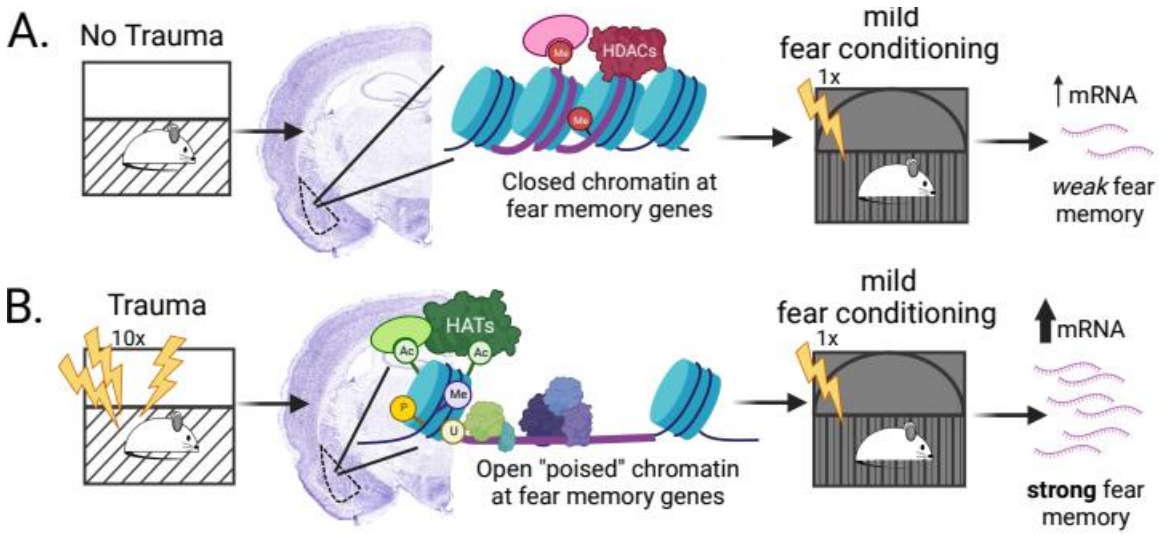

Figure 2. SEFL hypothesis: Trauma drives an epigenetic molecular memory at key fear memory genes

(A) In the absence of trauma, the presence of HDACs prevent transcription of fear memory genes, leading to weak memory for mild fear conditioning. (B) Trauma activates histone acetyltransferases (HATs), opening the chromatin at key fear memory genes. In response to subsequent mild fear conditioning, these genes are strongly and persistently transcribed, leading to a strong fear memory for subsequent mild fear conditioning. *Figure made from Biorender.*

This project will specifically look at HDAC3 and its role in SEFL in male and female mice. We used a dominant-negative viral vector, HSV-HDAC3(Y298H) that expresses HDAC3 with a single amino acid substitution that disrupts the enzymatic activity of HDAC3 without affecting its protein-protein interactions (Lahm et al., 2007) allowing us to specifically test whether its catalytic activity is required for fear memory. Here, we injected HSV-HDAC3(Y298H) into the amygdala of male and female mice during SEFL to determine whether HDAC3 contributes to the persistent sensitization observed following trauma. We found that female mice show fear sensitization following a weak, 2-shock “trauma” event, even without HDAC3 inhibition. Male mice, on the other hand, showed no lasting sensitization following this weak trauma event unless HDAC3 was inhibited; blocking HDAC3 in the amygdala of male mice transformed a weak trauma into an event that sensitized subsequent fear learning and memory. This indicates that HDAC3 modulates the lasting effects of trauma and may contribute to male-female differences in trauma susceptibility in PTSD and other anxiety disorders.

Chapter 2

Methods

Overview of Experiments

In Experiment 1, mice were handled for five days for at least 1 minute each. Afterwards, they were subjected to the SEFL paradigm. In Experiment 2, mice were handled for five days for at least 1 minute each. Afterwards, surgery took place to inject either the HDAC3 inhibitor or the empty viral vector. Three days after surgery, when viral expression was at its maximum, mice were subjected to the modified SEFL paradigm, where a weak trauma event was delivered in Context A that was below the expected limit that can encode a traumatic event (Hassien et al., 2020).

Subjects

Subjects were 8-week-old C57BL/6J mice obtained from Jackson Laboratories. All mice were individually housed during the experiment, were given free access to food and water, and were kept on a 12-hour light/dark cycle. All trials occurred during the light phase. The experiments were performed in accordance with the United States National Institutes of Health guidelines for animal care and were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University.

Virus

2 different viruses were used in experiment 2, both neuron-specific herpes simplex virus (HSV). An empty viral vector (HSV-EV) was used as a control, while an HDAC3-inhibiting virus was used for experimental mice. The single amino acid substitution in this dominant-negative viral vector, HSV-HDAC3(Y298H), selectively disrupts HDAC3 function without inhibiting protein-protein interactions (Lahm et al., 2007).

Surgical Procedure

Before surgery was performed, mice were handled for five days prior for at least one minute each. On surgery day, mice were first anesthetized using 2% isoflurane dissolved in oxygen. Their heads were shaved and cleaned with betadine and an eye gel was applied to prevent drying out. They were then injected with Ketoprofen, an analgesic. Their heads were then secured to a stereotaxic frame. Before any surgical cut was made, the toe-pinch reflex was performed to assess consciousness. If no reflex was present, an approximately-half inch cut was made to the skin on the head using a sterile surgical blade, exposing the occipital and parietal bones. The skin was then pulled apart, dried using 70% ethanol, and the bregma was located. For each hemisphere, using a 0.7 mm burr, the skull was drilled at 1.8 mm caudal and 3.45 mm lateral to the bregma and a micro pump injector filled with 1.5 µL of either viral or empty vector was placed at the surface of the skull. The needle was then lowered 0.2 mm to place the injector at the surface of the brain. The injector was lowered 4.40 mm at a rate of 0.2 mm/15 seconds until it reached the amygdala, upon when it was allowed to sit for two minutes. Injection then began at a rate of 6 µL/hour. After injection was complete, it was allowed to sit for five minutes.

It was then pulled up 0.1 mm and allowed to sit for an additional five minutes. After, the injector was removed from the brain at a rate of 0.1 mm/15 seconds. The skin was moistened using sterile saline and sealed using vetbond. For recovery, mice were injected with saline, single-housed inside a warm and clean enclosure until the anesthetic wore off, and finally returned to the vivarium for continued recovery and monitoring.

Verification of Injection

To ensure the needle was injecting into the amygdala, fluorescent secondary antibodies were injected using coordinates of 1.8 mm caudal, 3.45 mm lateral, and 4.6 mm ventral to the bregma. Afterwards, the mice were immediately killed, and their brains were cut to see where the fluorophore was in the brain tissue. The coordinates were verified as having hit the amygdala.

Fear Conditioning, SEFL Experiment

Mice were handled for five days prior for at least 1 minute each prior to fear conditioning. Fear conditioning was performed in four identical plastic chambers housed within sound-dampening boxes. First was the Trauma Event in “Context A.” Context A conditions were as follows: 0% tone, 0% white noise, 35% white light, 100% IR light, and an even grid bar floor capable of administering electric shocks. Between each subject, the floor, chamber, and waste catcher were cleaned with 70% isopropyl alcohol. Trauma mice were placed in the chamber for 3 minutes, after which 10 randomized, 2-second, 0.7 mA shocks were delivered over the course of 56 minutes. Following this, mice were allowed to remain in the chamber for 1-minute post-shock. No-trauma control mice were given no shocks for 60 minutes. After the hour, all mice

were immediately removed from the chamber, placed back into their cage, and returned to the vivarium. 24 hours later, the Context Training in “Context B” took place. Conditions for Context B were as follows: 0% tone, 5% white noise, 2% white light, 100% IR light, an uneven grid bar floor capable of administering electric shocks, and a U-shaped plastic insert with a grid-like pattern. On Context B days, mice were transported from the vivarium in concealed boxes, and the fear conditioning room was illuminated in red light only. Between each subject, the floor, chamber, concealed boxes, and waste catcher were cleaned with Windex. Once in the chamber, all mice were allowed a 3-minute baseline period with no shocks, after which a single 2-second, 0.35 mA shock was delivered. Mice remained in the chamber for 2 minutes post-shocks with no shocks administered before being immediately removed from the chamber, placed back into their cage, and returned to the vivarium. 24 hours later, the Context Test took place in Context B. All mice were placed into the chamber for 8 minutes, after which they were immediately removed, placed back into their cage, and returned to the vivarium.

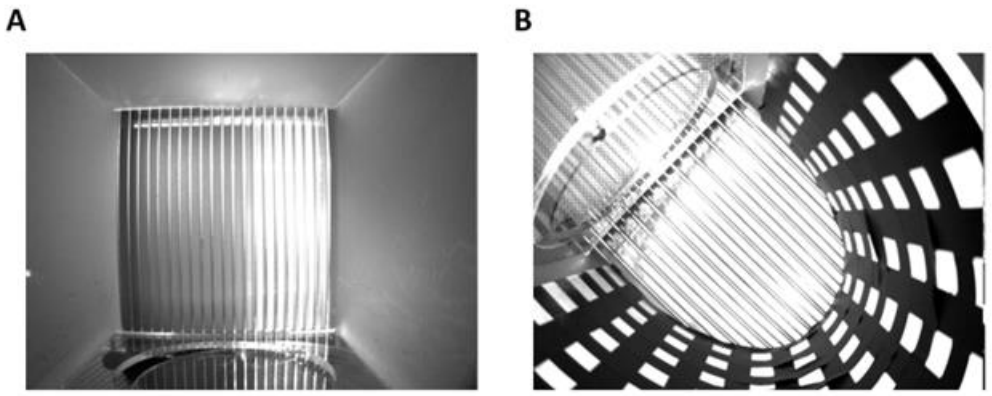

Figure 3. Context A and Context B for SEFL paradigm.

(A) Context A. (B) Context B. The plastic chamber is turned relative to the camera because the plastic insert partially blocks the light which interferes with motion capture.

Fear Conditioning, Modified SEFL Experiment

For the modified SEFL paradigm, the only difference is the strength of the Trauma Event in Context A. Trauma experimental mice were placed into Context A for 3 minutes in which there were no shocks, after which 2 randomized, 2-second 0.7 mA shocks were administered over the course of 10 minutes. Following this, mice were allowed to remain in the chamber for 1 minute with no shocks before being immediately removed and returned to their cages and the vivarium. No-trauma control mice were placed in the chamber for 14 minutes with no shocks before being removed. 7 days later, the Context Conditioning and Context Test administration were followed as described above. The 2 shocks were thought to be below the necessary threshold to prime the genome to be able to strongly encode the contextual fear memory in Context B.

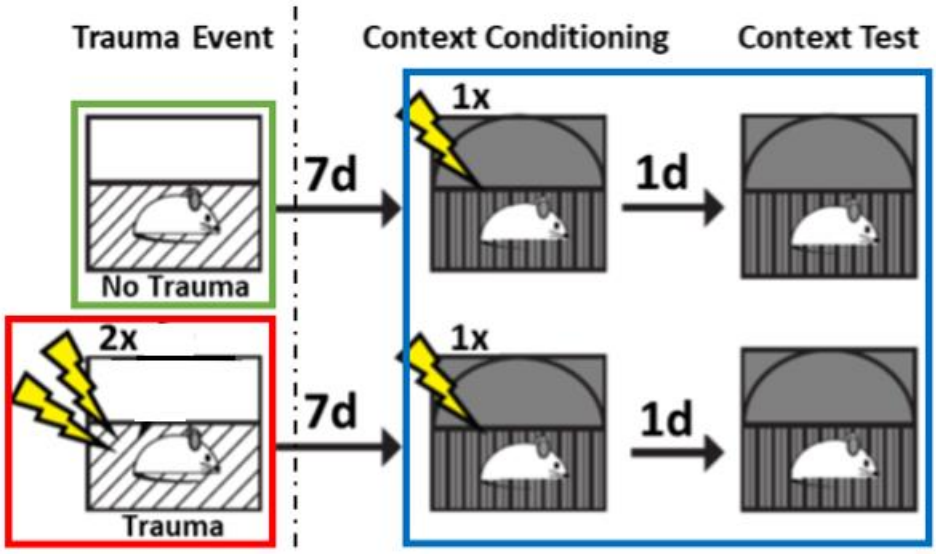

Figure 4. SEFL Set Up with Subthreshold Trauma Condition

The experimental setup for SEFL involves two treatments; one where mice receive no trauma, and another where mice receive a trauma condition. In “Context A,” mice that receive the weak trauma condition will receive two 0.7 mA shocks at separate randomized time points,

both within the same 10-minute period, while control mice will receive no trauma. One week later, mice will be placed into a separate context (Context B) with different surroundings and scent, and all will be given a single 2 second 0.35 mA shock after 3 minutes. The next day, their fear response will be measured within Context B. *Figure adapted from Urban, 2022.*

Statistical Analysis

In all contexts, 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 and three-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 9 software.

Chapter 3

Results

Data are presented as ± standard error of the mean (SEM), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Experiment 1: Trauma Persistently Sensitizes Mice to Future Fear Conditioning

In the first experiment, we verified that the stress-enhanced fear learning (SEFL) paradigm served to create an exaggerated fear response upon subsequent exposure to mild context fear conditioning in a new environment. Mice were either exposed to a trauma (male: n = 6; female; n = 6) or no trauma (male: n = 6; female: n = 6) condition in Context A, where freezing was used to verify that the electrical shocks acted as a fearful event. During the Trauma Event, 10 random, unsigned shocks were given to the Trauma group. Mice in this group showed increased freezing over time due to the increasing number of shocks. Meanwhile, because the No Trauma group did not get shocked, mice in that group showed none to minimal freezing (Fig. 5; two-way repeated measures (RM) ANOVA, main effect of Time: $F_{(7.446,163.8)} = 8.800$, $p < 0.0001$; two-way RM ANOVA, main effect of Trauma: $F_{(1,22)} = 354.5$, $p < 0.0001$; two-way RM ANOVA, main effect of Interaction: $F_{(59,1298)} = 8.866$, $p < 0.0001$). 24 hours later, mice were subjected to the fear conditioning (FC) portion of SEFL. In the first part of FC, the Context Conditioning, all mice were given a single mild shock in Context B. The second part of FC, the Context Test, involved placing mice back in Context B and measuring their freezing response. During the Context Conditioning on Day 2, freezing percentages revealed that mice in the Trauma group froze significantly more than mice in the No Trauma group. Additionally, freezing

during the pre-shock (baseline) and post-shock periods revealed that Context A and Context B were sufficiently different from each other. In addition, mice in the Trauma group froze more overall. Finally, post hoc analysis showed that mice in the Trauma group froze significantly more post-shock when compared to mice in the No Trauma group, indicating that the traumatic event led to a more extreme fearful reaction upon exposure to a mildly fearful stimulus (Fig. 6; two-way RM ANOVA, main effect of Trauma: $F_{(1,21)} = 33.47$, $p < 0.0001$; two-way RM ANOVA, main effect of Shock: $F_{(1,21)} = 38.68$, $p < 0.0001$; two-way RM ANOVA, main effect of Interaction: $F_{(1,21)} = 20.40$, $p = 0.0002$; Šidák's multiple comparisons post hoc analysis between No Trauma and Trauma mice showed no significant difference in freezing percentage during baseline time ($p = 0.2049$) and a significant difference during post-shock time ($p < 0.0001$)). Finally, during the Context Test on Day 3, mice in the Trauma group froze significantly more across the eight minutes when compared to mice in the No Trauma group (Fig. 7A; two-way RM ANOVA, main effect of Trauma: $F_{(1,22)} = 23.07$, $p < 0.0001$; two-way RM ANOVA, main effect of Time: $F_{(3.946,86.82)} = 4.755$, $p = 0.0017$; two-way RM ANOVA, main effect of Interaction: $F_{(7,154)} = 2.620$, $p = 0.0139$), indicating that trauma predisposes mice to show an exaggerated fear response during fear conditioning.

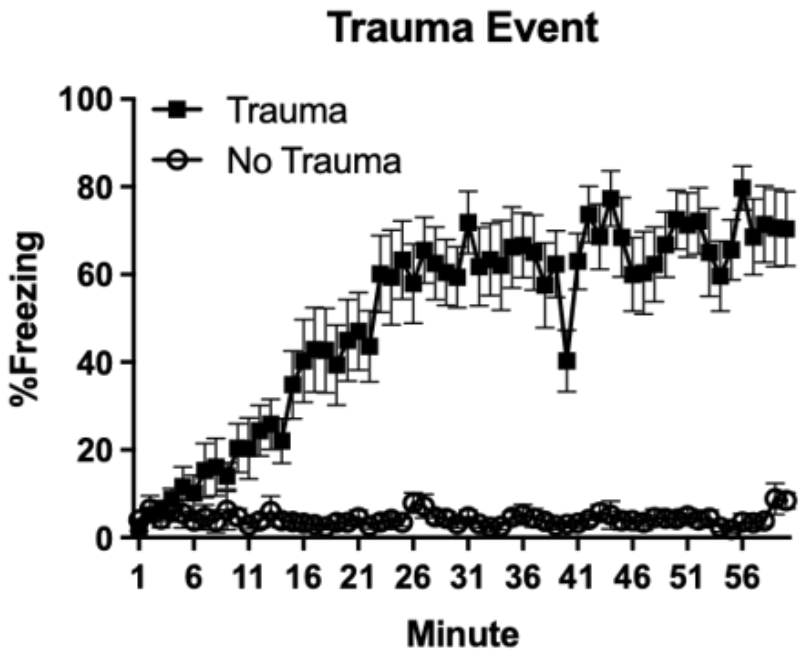

Figure 5. Shocks increase freezing in mice across the duration of the Trauma Event.

Mice administered shocks after the baseline period show an increased percent freezing compared to No Trauma control mice, indicating that the shocks were successful in creating a fearful event.

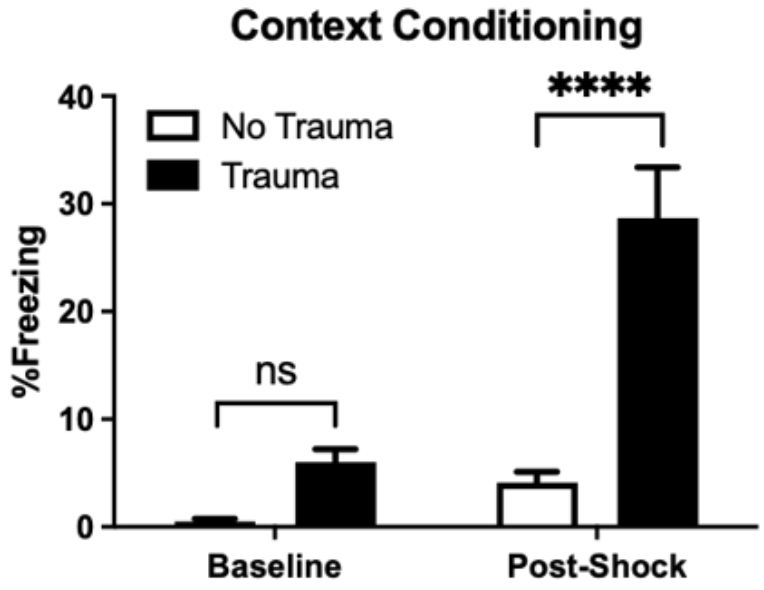

Figure 6. Trauma mice showed increased freezing post-shock in Context B.

Mice in both the No Trauma and Trauma groups showed low percent freezing during the baseline period in Context B, indicating that Context A and B were sufficiently different from each other. In other words, because they were not significantly different from each other, Trauma mice were not generalizing the fear from the trauma event in Context A (Šidák's multiple comparisons post hoc analysis between No Trauma and Trauma mice post-shock: $p = 0.205$). Additionally, Trauma mice had a more severe reaction to the shock in Context B when compared to No Trauma mice, meaning that the trauma event served to sensitize mice to future mild traumatic stimuli (Šidák's multiple comparisons post hoc analysis between No Trauma and Trauma mice post-shock: $p < 0.0001$).

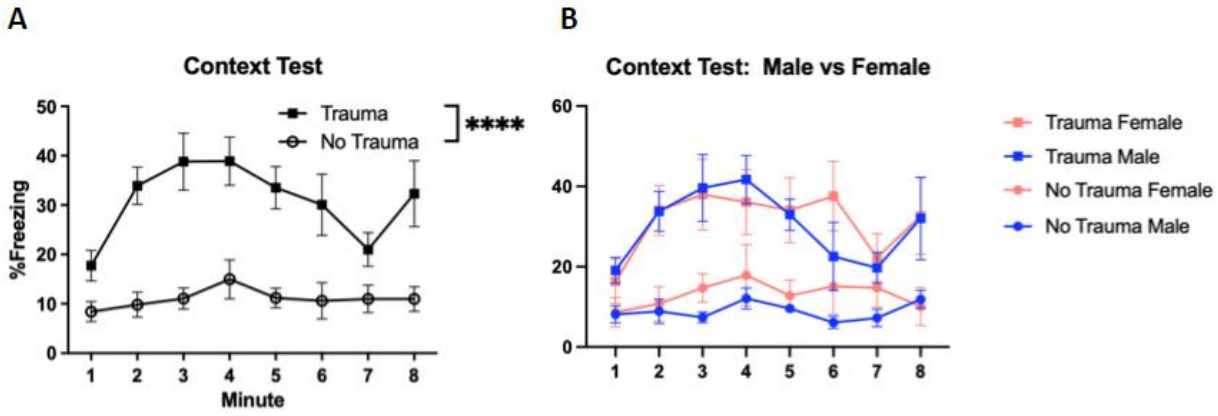

Figure 7. Trauma mice compared to No Trauma mice show an exaggerated fear response during the Context Test

(A) Mice in the Trauma group compared to No Trauma controls show significantly greater percent freezing across the eight minutes of the Context Test, indicating that trauma facilitates the formation of a strong conditioned fear memory. The low percent freezing in the initial minute of the Context Test in Trauma mice could mean that these mice need some time to recognize Context B as an environment in which they were previously shocked. Because they have been primed by the Trauma Event, this recognition leads to a rapid exaggerated fear

reaction. The significant main effect of the interaction of trauma and time can be seen in this graph (two-way RM ANOVA, main effect of Interaction: $F_{(7,154)} = 2.620$, $p = 0.0139$). The freezing percentages of the No Trauma and Trauma mice throughout the Context Test were different from each other. This further shows that Trauma mice have been substantially affected by the trauma event and have learned to show a stronger fearful reaction when placed back into the context where a mild trauma had occurred. (B) The data shown here are representative of the same mice in Figure 7A, just separated by sex. By inspection, there is no significant difference between male and female Trauma mice. These results indicate that trauma successfully predisposes both male and female mice to show an exaggerated fear response during future fear conditioning.

Experiment 2: Inhibiting Amygdalar HDAC3 Sensitizes Male Mice to Future Trauma

In the second experiment, an HDAC3 inhibitor or an empty vector was injected into male and female mice, which were then subjected to the modified SEFL protocol. The modification occurred during the Trauma Event in Context A; Trauma mice received 2 randomized shocks over the course of 10 minutes instead of 10 randomized shocks over the course of 56 minutes. In Experiment 2, because No Trauma controls were not administered any shocks in Context A, their freezing during the Context Test resembled what was seen in Experiment 1 (Fig. 7); both No Trauma male and female groups exhibited around 20% freezing during the Context Test. As such, the data presented here are representative of the HSV-HDAC(Y298H) (male: n = 17, female: n = 22) and HSV-EV (male: n = 16, female: n = 20) Trauma condition mice.

During the Trauma Event, both HSV-EV and HSV-HDAC(Y298H) mice compared to empty vector controls showed increased freezing behavior after the 3-minute baseline period. This is similar to what was seen in the Experiment 1 Trauma Event (Fig. 5; two-way repeated measures (RM) ANOVA, main effect of Time: $F_{(7.446,163.8)} = 8.800$, $p < 0.0001$), indicating that the weak 2-shock trauma successfully acted as a fearful event for the mice. In addition, these findings indicate that this weak trauma may amplify fear learning during subsequent fear conditioning. This increase in freezing behavior was significant with time (Fig. 8A; two-way RM ANOVA, main effect of Time: $F_{(13,806)} = 34.18$, $p < 0.0001$) throughout the duration of the Trauma Event, meaning that mice froze more as time went on. In addition, there was no significant difference of freezing behaviors between Y298H and EV mice (Fig. 8A; two-way RM ANOVA, main effect of Virus: $F_{(1,62)} = 0.01357$, $p = 0.91$), indicating that all mice reacted to the shocks equally. 7 days later, during the Context Conditioning, freezing percentages revealed that female-EV mice and male-Y298H mice froze significantly more over the three minutes following the single mild shock when compared to the two minute baseline period. Female-Y298H mice trended towards a significant difference in freezing percentages, while male-EV mice showed no significant difference in their freezing percentages pre- and post-baseline. The results from the Context Conditioning indicate that a weak trauma sensitizes female mice and male mice that have had amygdalar HDAC3 inhibited to future mild stimuli during fear conditioning (Fig. 9B). Based on these results, we expected to see a similar trend in the Context Test. Our prediction turned out to be true; 24 hours after the Context Conditioning, mice were placed back into Context B for the Context Test. Freezing during the Context Test revealed that male-Y298H mice froze significantly more than male-EV mice across the eight minutes (Fig. 10A; two-way RM ANOVA, main effect of Virus: $F_{(1,31)} = 8.711$, $p = 0.006$), while freezing

between female-Y298H and female-EV mice was not significantly different (Fig. 10B; two-way RM ANOVA, main effect of Virus: $F_{(1,40)} = 0.098$, $p = 0.76$), indicating that there is a sex-dependent effect of trauma that can influence future fear conditioning. When exposed to a weak trauma, all females show an enhanced fear memory formation during subsequent fear conditioning. Males do not show this effect until amygdalar HDAC3 is inhibited. Additionally, female mice as a group froze significantly more than male mice, while mice injected with the Y298H virus froze significantly more than mice injected with the empty virus (Fig. 10C; two-way ANOVA, main effect of Sex: $F_{(1,71)} = 23.01$, $p < 0.0001$; two-way ANOVA, main effect of Virus: $F_{(1,71)} = 4.120$, $p = 0.046$; two-way ANOVA, main effect of Interaction: $F_{(1,71)} = 5.96$, $p = 0.017$). The results indicate that a weak trauma can enhance fear memory formation during subsequent fear conditioning in female mice and HDAC3-inhibited male mice that will lead to an exaggerated fear response.

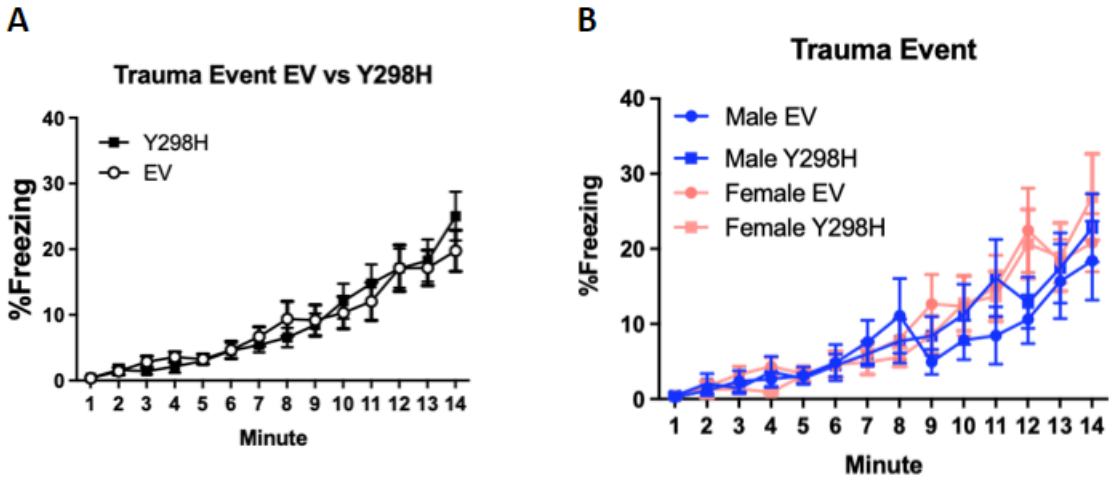

Figure 8. Experiment 2: Weak Trauma Event Y298H vs EV

(A) Both HSV-HDAC3(Y298H) and HSV-EV mice showed increased freezing post-baseline during the traumatic event itself. This indicates that a weak 2-shock trauma can act as a

fearful event that may enhance future fear learning. The significant effect of time showed that mice froze significantly more over time throughout the Trauma Event, while the lack of significance for virus showed that both Y298H and EV groups froze an equal amount (two-way RM ANOVA, main effect of Virus: $F_{(1,62)} = 0.014$, $p = 0.91$; two-way RM ANOVA, main effect of Time: $F_{(13,806)} = 34.18$, $p < 0.0001$; two-way RM ANOVA, main effect of Interaction: $F_{(13,806)} = 0.7388$, $p = 0.73$). (B) There is no indication that the Trauma Event affected males and females in either virus condition differently, meaning that the weak trauma was successful in acting as a fearful event independent of sex.

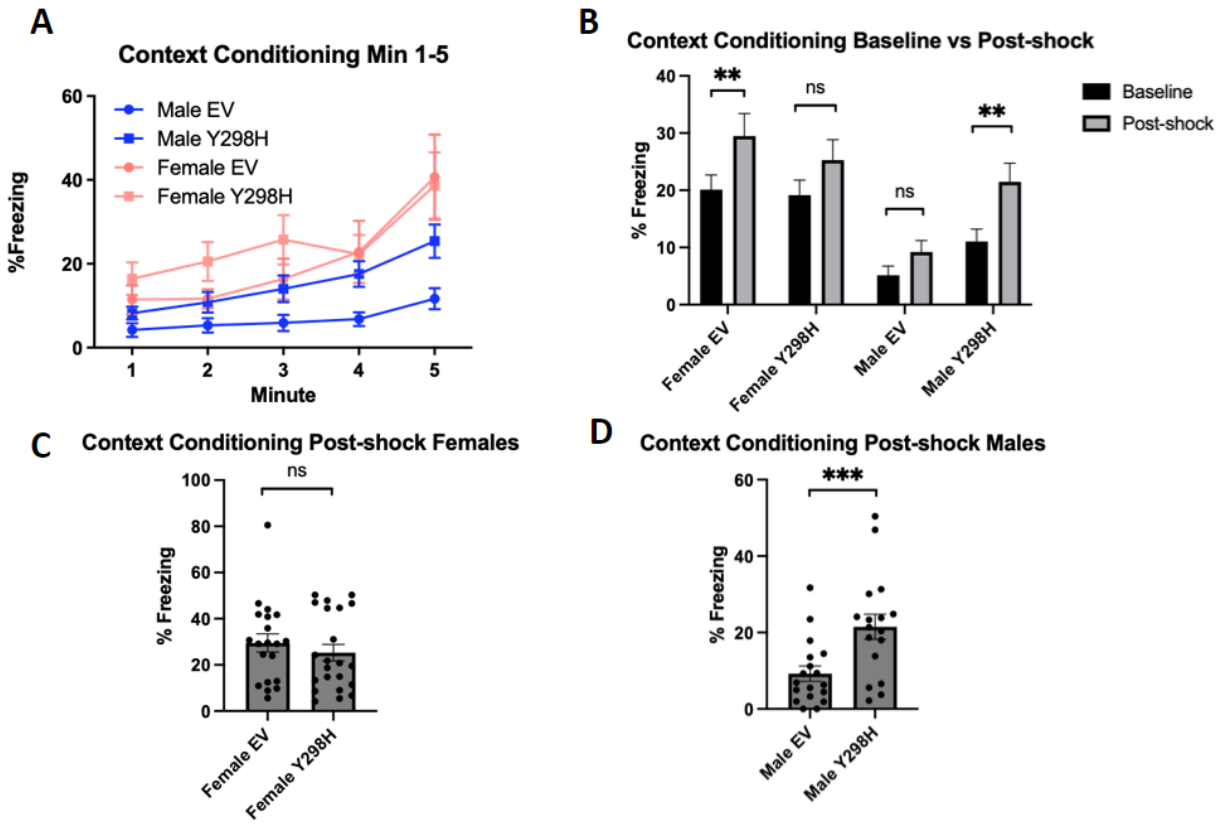

Figure 9. Female and Male Y298H mice show increased freezing post-shock in Experiment 2 Context Conditioning

(A) Tracking the percent freezing across the 5-minute Context Conditioning revealed a slight increase in freezing post-shock starting at the 2-minute mark for all four groups. (B)

Comparison of baseline and post-shock freezing percentages for all four groups (three-way RM ANOVA and Šidák's multiple comparisons post hoc). Female-EV ($p = 0.0069$) and male-Y298H mice ($p = 0.0050$) show a significant difference in freezing percentages pre- and post-shock, while female-Y298H mice approach significance pre- and post-shock ($p = 0.11$). Male-EV mice showed no significant difference in freezing percentage pre- and post-shock ($p = 0.55$), implying that for male mice with functional amygdalar HDAC3, a weak trauma event is not sufficient to form a strong fear memory. (C) Post-shock comparison of female-EV and female-Y298H mice show no significant difference in freezing percentages (two-way RM ANOVA and Šidák's multiple comparisons post hoc; $p = 0.59$), meaning that all female mice may freeze similarly during the Context Test regardless of if HDAC3 is inhibited or not. (D) Post-shock comparison of male-EV and male-Y298H mice reveals that male-Y298H mice freeze significantly more following the mild shock during fear conditioning when compared to male-EV mice (two-way RM ANOVA and Šidák's multiple comparisons post hoc; $p = 0.0008$). These data indicate that Y298H, and thus inhibiting amygdalar HDAC3, may predispose male mice to show an exaggerated fear reaction during the Context Test following fear conditioning after a weak trauma. This is confirmed in Fig. 10.

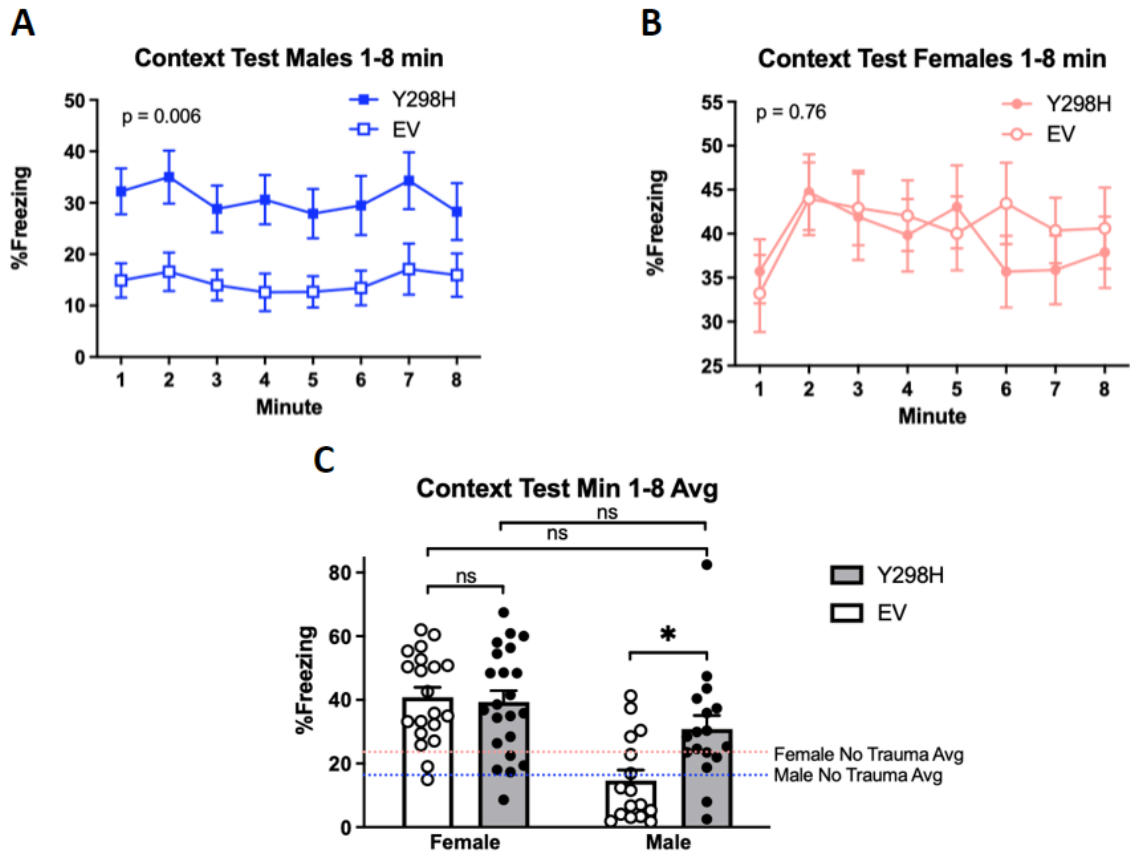

Figure 10. HDAC3 inhibition in male mice causes significantly greater freezing during fear conditioning following a weak traumatic event

(A) Comparison of male-Y298H and male-EV mice show a significant difference in freezing percentages across the eight minutes of the Context Test, indicating that HDAC3 inhibition in the amygdala is at least partially responsible for the lasting sensitization to fear-inducing stimuli. Male mice injected with the HSV-HDAC3(Y298H) virus are reacting more strongly to Context B after the mild shock in Context Conditioning, indicating that the weak trauma event from Context A has primed these mice to having an exaggerated fear response during fear conditioning. (B) Comparison of female-Y298H and female-EV mice show no significant difference in freezing percentages across the Context Test. (C) Average percent freezing across the Context Test for all four groups showed a significant difference between

male-Y298H mice and male-EV mice (two-way ANOVA, main effect of Sex: $F_{(1,71)} = 23.01$, $p < 0.0001$; two-way ANOVA, main effect of Virus: $F_{(1,71)} = 4.120$, $p = 0.0461$; two-way ANOVA, main effect of Interaction: $F_{(1,71)} = 5.963$, $p = 0.0171$. Šidák's multiple comparisons post hoc analysis revealed a significant difference between male-Y298H and male-EV mice; $p = 0.0229$. There are no significant differences between the female-Y298H and EV groups ($p = 0.9998$), the female-Y298H and male-Y298H groups ($p = 0.4469$), and the female-EV and male-Y298H groups ($p = 0.2871$)). These results indicate that the Y298H virus, and thus inhibiting HDAC3, was directly responsible for the increase in freezing behaviors seen in male mice. This is evident because there is no significant difference between either of female groups and the male-Y298H group, implying that the Y298H virus restored the males' ability to form a strong fear memory to the level of the females in response to a weak trauma. However, the similarity in freezing percentage of the male-EV and male No Trauma mice (which was about 20% for Experiment 2) indicate that the EV mice view the mild stimuli as a novel trauma event, similar to the No Trauma mice. In other words, the weak trauma event in Context A did not sufficiently prime the EV mice to react more strongly to Context B during the Context Test following the mild shock during Context Conditioning.

In summary, in the second experiment, HDAC3 inhibition was shown to predispose male mice exposed to a weak trauma event to show an exaggerated fear response in future fear conditioning. Male mice without HDAC3 inhibition did not show this enhanced freezing behavior during fear conditioning trials. Female mice exposed to this weak trauma event showed an exaggerated fear response during fear conditioning regardless of whether or not HDAC3 was inhibited.

Chapter 4

Discussion

The results indicate that females are more susceptible to stress-enhanced fear learning and HDAC3 in the amygdala is a critical negative regulator of stress-enhanced fear learning, at least in male mice. The first experiment demonstrated the basic stress-enhanced fear learning (SEFL) paradigm, showing that trauma exposure via 10 shocks predisposes male and female mice to show exaggerated fear learning upon exposure to mild fear conditioning in a new context. The second experiment showed that a weak trauma event via 2 shocks sensitizes only female mice to subsequent mild fear learning, suggesting that this mild trauma event is encoded much more strongly in females. However, we found that inhibiting HDAC3 activity in the amygdala of male mice was able to transform this weak, acute fear event into one that sensitized them to future fear learning. HDAC3 inhibition, in other words, made male mice show the full SEFL response (just like female mice) to the subthreshold 2-shock trauma. These results indicate that there is a sex-dependent sensitivity to trauma and differences in histone acetylation in the amygdala may determine whether a stressful event drives persistent changes in subsequent fear learning.

The second experiment is especially significant because it suggests that histone acetylation primes the genome to strongly encode trauma events in a manner that impacts future fear conditioning. The full, 10-shock SEFL protocol consistently caused an exaggerated fear response in both male and female mice. However, when subjected to a lower stress event (fewer and less intense shocks), only female mice showed evidence of SEFL. Male mice showed no lasting response to this mild trauma; freezing levels during the Context Test was similar to those seen in mice that received no initial trauma in Context A, indicating that this subthreshold trauma

was not sufficiently intense to create a persistent memory in male mice. When administered an HDAC3 inhibitor in the amygdala, however, the fear response in male mice was restored to a level similar to that seen in female mice, suggesting that HDAC3 may modulate whether a stressful experience is encoded in a manner that drives persistent subsequent responding. These results suggest that in female mice, even relatively weak stress events may drive persistent changes in histone acetylation that enable rapid, robust gene expression in response to subsequent fear learning. In male mice, this weak trauma event is not sufficient to change histone acetylation or form a long-term memory. When weak trauma is combined with HDAC3 inhibition, however, the male mice show lasting sensitization, similar to the female control mice.

The Stress-Enhanced Fear Learning paradigm is thought to model PTSD in rodents, with trauma exposure driving exaggerated subsequent fear responding to mild stressors, similar to what is seen in PTSD (Rau et al., 2005). Especially in military cases, veterans may react violently or excessively to a combat-similar but less severe stimulus, such as a balloon popping or fireworks. In addition, SEFL is effective in creating a context-dependent fear memory that sensitizes subsequent fear learning, rather than a generalized fear to all contexts, even those that have never been presented with shocks (Rau et al., 2005; Rajbhandari et al., 2018). This can be seen from the baseline responses during Context Conditioning; mice in Context B are not anticipating a shock during the baseline period and it is only after shocks are delivered that these mice show exaggerated responding. Despite its utility, however, SEFL is not a perfect model for all aspects of PTSD. PTSD is characterized by not only an exaggerated fear response, but also by avoidance behaviors and negative alterations in mood (American Psychiatric Association [APA], 2022) that are not apparent, or cannot be assessed, in rodents subjected to SEFL. It is also interesting to note that female mice appear to form fear memories

despite the weaker trauma event, indicating that the 10 0.7 mA shocks in the original SEFL paradigm may be excessive, at least in female mice. This implies that SEFL, while useful for creating a context-dependent fear memory, may be an imperfect model for PTSD.

Despite this, the epigenetic changes that occur in the amygdala that evoke a fear response may still be similar between rodents and humans. More research will be needed to investigate where in the genome those changes are taking place, if epigenetic mechanisms other than histone acetylation are active, and the differences in the various genes and proteins synthesized in response to the different trauma protocols. By discovering these "fear proteins" and the pathways and structures on which they operate, treatments for anxiety disorders and other fear-causing disorders such as PTSD can be developed. An additional avenue of research is determining if this interaction between HDAC3 and fear-memory formation is specific to the amygdala. Our lab is currently conducting preliminary research to analyze the effect of HDAC3 inhibition in the hippocampus during a subthreshold trauma event. The hippocampus plays a specific role in context during fear conditioning, something that may be encoded differently by male and female mice (Keiser et al., 2017). Another potential direction for further study is to investigate how extinction impacts fear learning for a weakly stressful stimulus. In the original SEFL paradigm, animals in the trauma condition that were given an exposure to a single shock in Context B after fear extinction in Context A continued to show enhancement of a fearful response, indicating a lasting change that was not persistently reversed by the extinction (Rau et al., 2005). However, extinction and its effects on a weak trauma event were not investigated in this experiment. Lastly, further research can be done to investigate the sex differences seen in this study. Females have already been shown to be more sensitive towards PTSD (Klabunde et al., 2017), which is

corroborated by the results from this study. As such, it is possible that sex hormones play a role in regulating fear memory formation.

In conclusion, this study demonstrated that HDAC3 activity, and therefore histone acetylation, affected whether or not male mice showed a lasting sensitization to fear conditioning following a traumatic event. Blocking HDAC3 activity in male mice and exposing them to a weak trauma event led to an exaggerated fear response during fear conditioning. This response is similar to what was seen in female mice, which consistently showed an exaggerated fear response following the 2-shock weak trauma regardless of if HDAC3 was inhibited. This indicates that HDAC3 is a negative regulator of stress-enhanced fear learning.

Exposure to trauma can cause epigenetic mechanisms such as histone acetylation that create a persistent genomic change that sensitizes an organism to future, similar stimuli. Even weak stimuli can potentially cause this; for females, it does so consistently, while for males, it would occur if amygdalar HDAC3 is inhibited. This provides an avenue of research for treatments for PTSD; treatments that target histone acetylation in the amygdala could potentially reduce the exaggerated fear behavior that occurs when exposed to future, yet more mild, stimuli.

References

Agranoff, B. W., Davis, R. E., Casola, L., & Lim, R. (1967). Actinomycin D blocks formation of memory of shock-avoidance in goldfish. *Science*, *158*(3808), 1600-1601. https://doi.org/10.1126/science.158.3808.1600

American Psychiatric Association. (2022). Trauma- and Stressor-Related Disorders. In *Diagnostic and statistical manual of mental disorders* (5th ed., text rev.). https://doi-org.ezaccess.libraries.psu.edu/10.1176/appi.books.9780890425787.x07_Trauma_and_Stressor_Related_Disorders

Broadbent, N. J., Squire, L. R., & Clark, R. E. (2004). Spatial Memory, Recognition Memory, and the Hippocampus. *Proceedings of the National Academy of Sciences*, *101*(40), 14515-14520. https://doi.org/10.1073/pnas.0406344101

Christiansen, D. M., & Elklit, A. (2012). Sex differences in PTSD. In E. Ovuga (Ed.), *Posttraumatic Stress Disorder in a Global Context* (pp. 113-142). InTech.

Davis, M. (1992). The role of the amygdala in fear and anxiety. *Annual Review of Neuroscience*, *15*(1), 353-375.

Hassien, A. M., Shue, F., Bernier, B. E., & Drew, M. R. (2020). A mouse model of stress-enhanced fear learning demonstrates extinction-sensitive and extinction-resistant effects of footshock stress. *Behavioural brain research*, *379*, 112391. https://doi.org/10.1016/j.bbr.2019.112391

Hayakawa, T., & Nakayama, J. I. (2011). Physiological roles of class I HDAC complex and histone demethylase. *Journal of Biomedicine and Biotechnology*, 2011, 129383. https://doi.org/10.1155/2011/129383

Izquierdo, I., Furini, C. R., & Myskiw, J. C. (2016). Fear memory. *Physiological Reviews*, *96*(2), 695-750. https://doi.org/10.1152/physrev.00018.2015

Jakovljević, M., Brajković, L., Lončar, M., & Čima, A. (2012). Postraumatic stress disorders (PTSD) between fallacy and facts: What we know and what we don't know? *Psychiatria Danubina*, *24*(3.), 241-245.

Keiser, A. A., Turnbull, L. M., Darian, M. A., Feldman, D. E., Song, I., & Tronson, N. C.

(2017). Sex Differences in Context Fear Generalization and Recruitment of Hippocampus and Amygdala during Retrieval. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology, 42*(2), 397–407. https://doi.org/10.1038/npp.2016.174

Kessler, R. C., Sonnega, A., Bromet, E., Hughes, M., & Nelson, C. B. (1995). Posttraumatic stress disorder in the National Comorbidity Survey. *Archives of General Psychiatry, 52*(12), 1048-1060. doi:10.1001/archpsyc.1995.03950240066012

Kilpatrick, D. G., Resnick, H. S., Milanak, M. E., Miller, M. W., Keyes, K. M., & Friedman, M. J. (2013). National estimates of exposure to traumatic events and PTSD prevalence using DSM-IV and DSM-5 criteria. *Journal of traumatic stress*, *26*(5), 537-547. https://doi.org/10.1002/jts.21848

Klabunde, M., Weems, C. F., Raman, M., & Carrion, V. G. (2017). The Moderating Effects of Sex on Insula Subdivision Structure in Youth with Posttraumatic Stress Symptoms. *Depression and Anxiety*, *34*(1), 51-58. https://doi.org/10.1002/da.22577

Kwapis, J. L., Alaghband, Y., López, A. J., White, A. O., Campbell, R. R., Dang, R. T., Rhee, D., Tran, A. V., Carl, A. E., Matheos, D. P., & Wood, M. A. (2017). Context and Auditory Fear are Differentially Regulated by HDAC3 Activity in the Lateral and Basal Subnuclei of the Amygdala. *Neuropsychopharmacology*, *42*(6), 1284-1294. https://doi.org/10.1038/npp.2016.274

Lahm, A., Paolini, C., Pallaoro, M., Nardi, M. C., Jones, P., Neddermann, P., Sambucini, S., Bottomley, M. J., Surdo, P. L., Carfi, A., Koch, U., De Francesco, R., Steinkühler, C., & Gallinari, P. (2007). Unraveling the hidden catalytic activity of vertebrate class iIa histone deacetylases. *Proceedings of the National Academy of Sciences*, *104*(44), 17335-17340. https://doi.org/10.1073/pnas.0706487104

Lubin, F. D., Gupta, S., Parrish, R. R., Grissom, N. M., & Davis, R. L. (2011). Epigenetic mechanisms: critical contributors to long-term memory formation. *The Neuroscientist*, *17*(6), 616-632. https://doi.org/10.1177%2F1073858410386967

McQuown, S. C., Barrett, R. M., Matheos, D. P., Post, R. J., Rogge, G. A., Alenghat, T., Mullican, S. E., Jones, S., Rusche, J. R., Lazar, M. A., & Wood, M. A. (2011). HDAC3 is a critical negative regulator of long-term memory formation. *Journal of Neuroscience*, *31*(2), 764-774. https://doi.org/10.1523/JNEUROSCI.5052-10.2011

Nadel, L., & Moscovitch, M. (1997). Memory consolidation, retrograde amnesia and the hippocampal complex. *Current Opinion in Neurobiology*, *7*(2), 217-227. https://doi.org/10.1016/S0959-4388(97)80010-4

Ponomarev, I., Rau, V., Eger, E. I., Harris, R. A., & Fanselow, M. S. (2010). Amygdala transcriptome and cellular mechanisms underlying stress-enhanced fear learning in a rat model of posttraumatic stress disorder. *Neuropsychopharmacology*, *35*(6), 1402-1411. https://doi.org/10.1038/npp.2010.10

O'Keefe, J., & Nadel, L. (1979). Précis of O'Keefe & Nadel's The Hippocampus as a Cognitive Map. *Behavioral and Brain Sciences*, *2*(4), 487-494. doi:10.1017/S0140525X00063949

Phelps, E. A., & Anderson, A. K. (1997). Emotional memory: what does the amygdala do?. Current biology, *7*(5), R311-R314. https://doi.org/10.1016/S0960-9822(06)00146-1

Ramikie, T. S., & Ressler, K. J. (2018). Mechanisms of Sex Differences in Fear and Posttraumatic Stress Disorder. *Biological Psychiatry*, *83*(10), 876-885. https://doi.org/10.1016/j.biopsych.2017.11.016

Rau, V., DeCola, J. P., & Fanselow, M. S. (2005). Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. *Neuroscience & Biobehavioral Reviews*, *29*(8), 1207-1223. https://doi.org/10.1016/j.neubiorev.2005.04.010

Rajbhandari, A. K., Gonzalez, S. T., & Fanselow, M. S. (2018). Stress-enhanced fear learning, a robust rodent model of post-traumatic stress disorder. *Journal of Visualized Experiments: JoVE*, (140). https://doi.org/10.3791%2F58306

Squire, L. R., Genzel, L., Wixted, J. T., & Morris, R. G. (2015). Memory consolidation. *Cold Spring Harbor Perspectives in Biology*, *7*(8), a021766. 10.1101/cshperspect.a021766.

Stankiewicz, A. M., Swiergiel, A. H., & Lisowski, P. (2013). Epigenetics of stress adaptations in the brain. *Brain Research Bulletin*, *98*, 76-92. https://doi.org/10.1016/j.brainresbull.2013.07.003

Street, A. E., & Dardis, C. M. (2018). Using a social construction of gender lens to understand gender differences in posttraumatic stress disorder. *Clinical Psychology Review*, *66*, 97-105. https://doi.org/10.1016/j.cpr.2018.03.001

Tobias, B. A., Kihlstrom, J. F., & Schacter, D. L. (1992). Emotion and implicit memory. The handbook of emotion and memory: *Research and theory*, *1*, 67-92.

Urban, M. (2022, September 13). *Sex-specific Epigenetic Regulation of Context Fear Memory;*

CMIND – Fall 2022.

ACADEMIC VITA

MARC LUSSIER
mjl6332@psu.edu
marclussier013@gmail.com

Education

Pennsylvania State University, University Park, PA, 16802
Major: B.S. Premedicine
Major: B.S. Psychology, Neuroscience
Schreyer Honors College
Graduation: Spring 2023

Research Experience

Kwapis Lab, Penn State University
Undergraduate Researcher January 2020 – present

- Investigate the epigenetic changes that contribute to fear memory formation
- Assist with stereotaxic surgeries and fear conditioning behavioral experiments

---

Volunteering/Community Service/Philanthropy

Red Cross Club, Penn State University
Volunteer September 2019 – present

- Promote awareness for and volunteer at Red Cross blood drives
- Train new volunteers

Penn State THON Penn State University
Club Tennis September 2019 – present

- Raise money for pediatric cancer research in affiliation with Club Tennis
- Dancer in THON 2023, Foster the Magic

Phoenixville Area Community Services, Phoenixville, PA
Volunteer June 2020, February 2021 – August 2021, May 2022 – July 2022

- Assist in the running of a suburban food bank
- Participate in food drives, bagging orders, taking inventory, and accepting deliveries

Paoli Hospital, Paoli, PA
Junior Volunteer 2017 – 2019

- Assist in transporting patients to and from their rooms for various tests
- Discharge patients

Teaching and Mentoring Experience

Penn State Learning, — Penn State University
Guided Study Group Leader — August 2021 – present

- Tutor students taking CHEM 112 (Inorganic Chemistry II)
- Mentor new Guided Study Group Leaders on how to best run review sessions

Alpha Epsilon Delta, Health Preprofessional Honor Society — Penn State University
Peer Mentor — August 2022 – present

- Mentor younger students on the health preprofessional track
- Give advice on classes, applications, and interviews

Penn State, — Penn State University
Learning Assistant — January 2020 – May 2021

- Tutor students taking CHEM 112 (Inorganic Chemistry II) by answering questions, holding study groups, and organizing practice exams

Honors and Awards

Penn State University, — Penn State University
Dean's List — Fall 2019 – present

Phi Sigma Pi National Honor Fraternity — Penn State University
April 2022 – present

Alpha Epsilon Delta, Health Preprofessional Honor Society — Penn State University
General Member/Peer Mentor — August 2022 – present

National Honor Society — 2018 – 2019

Skills/Other Interests

Laboratory/Technical

- Mice handling and husbandry
- Cryostat
- Microsoft, basic JAVA

Language

- Fluent English, Intermediate French

Other Interests

- Tennis, Basketball, Golf, Skiing