Protein SUMOylation is a Sex-Specific Regulator of Fear Memory Formation in the Amygdala

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ACADEMIC ABSTRACT

SUMOylation is a type of post-translational protein modification similar to ubiquitination and it involves the covalent attachment of a small ubiquitin-like modifier (SUMO) protein to the lysine residue of a target substrate. While there is strong evidence for the role of protein ubiquitination in the formation of fear-based memories, few studies have been conducted examining the role that SUMOylation plays in this same process. The amygdala is the main site of storage for emotional memories and there is strong evidence that protein ubiquitination is critical for fear memory formation in this region. However, it has not previously been studied whether protein SUMOylation in the amygdala is also involved in fear memory formation. Additionally, although there is evidence to support sex differences in ubiquitin signaling during fear memory formation in the amygdala, whether males and females differ in their need for protein SUMOylation during fear memory formation has not been investigated. We have found significant sex differences in protein SUMOylation in the amygdala both at baseline (rest) and during fear memory formation. Western blot analysis revealed higher resting levels of SUMOylated proteins in females when compared to males, though both sexes showed global increases following fear conditioning. A SUMOylation-specific proteomic analysis discovered that only females had increased protein targeting with SUMO following fear conditioning, with four proteins being identified that gained SUMOylation modifications, the main target being a heat shock protein. One heat shock protein in males was identified as having lower SUMOylation levels following fear

conditioning. This suggests sex differences in the interaction and targeting of proteins by SUMOylation following fear conditioning. We also inhibited the function of the only E2 conjugase for SUMOylation, *Ube2i*, via siRNA in the amygdala and found impaired fear memory in males but enhanced fear memory in females, though the latter only occurred under high siRNA concentrations. Interestingly, western blot analysis revealed that knockdown of *Ube2i* caused an increase in protein SUMOylation levels in females but a decrease in males, indicating that compensation is likely occurring in females. This suggests that in females, protein SUMOylation may be critical for basal cellular functioning, which precludes us from directly determining its role in fear memory formation. Collectively, these data reveal a novel, sex-specific role for protein SUMOylation in the amygdala during fear memory formation and expand our understanding of how ubiquitin-like signaling regulates memory formation.

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GENERAL AUDIENCE ABSTRACT

SUMOylation is a modification of protein which plays a key role in various biological processes and is similar to the protein modification process called ubiquitination, which has been implicated in the formation of fear-based memories for traumatic events. Despite this and the established role of SUMOylation in genomic stability, cell proliferation, and migration, less is known about its role in the process of memory formation. Importantly, ubiquitination and SUMOylation of proteins often work in tandem to regulate cell signaling and recent evidence suggests that SUMOylation may also be involved in fear memory formation. However, the role of protein SUMOylation in regulating fear memory formation in the amygdala, the primary site of storage for emotional memories, has never been directly examined. Additionally, there is also a significant gap in the literature regarding whether sex differences exist for the requirement of protein SUMOylation in fear memory formation. We have found that there are significant differences between the sexes regarding protein SUMOylation during fear memory formation in the amygdala. Western blot analysis showed that females have higher resting (baseline) levels of SUMOylated proteins in the amygdala compared to males, though both sexes showed global increases in protein SUMOylation following fear conditioning. In addition, a proteomic analysis revealed that four proteins in females gained a SUMOylation modification following fear conditioning. In contrast, one protein was identified in males which lost a SUMOylation modification, together suggesting unique targeting of proteins by SUMOylation across sexes

during fear memory formation. Further, when the function of an essential enzyme for protein SUMOylation was inhibited *in vivo*, fear memory in males was impaired but enhanced in females. Collectively, these data reveal a novel, sex-specific role for protein SUMOylation in the amygdala during fear memory formation and expand our understanding of how ubiquitin-like signaling regulates memory formation.

DEDICATION

I would like to dedicate this thesis to my family, my PI, and my lab mates for always supporting me and offering words of encouragement.

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LIST OF ABBREVIATIONS

ACTG2	Actin gamma 2, smooth muscle
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Aβ	Amyloid beta
BLA	Basolateral amygdala
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CH25H	Cholesterol 25-hydroxylase
CREB	cAMP response element binding protein
CREB-1	Cre-response element binding protein 1
CRISPR	Clustered regularly interspaced short palindromic repeats
dCas9	Nuclease-dead CRISPR-associated protein 9
DMEM	Dulbecco's Modified Eagle's Medium
HR	Hour
HSP	Heat shock protein
HSP10	Heat shock protein 10
HSP60	Heat shock protein 60
HSPE1	Heat shock protein family E (HSP10)
IS	Immediate shock
LC/MS	Liquid chromatography mass spectrometry
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase

mRNA	Messenger RNA
N	Number
PCR	Polymerase chain reaction
PKA	Protein kinase A
PTSD	Post-traumatic stress disorder
RNA	Ribonucleic acid
SENP	Sentrin-specific protease
siRNA	Small interfering RNA
SMAD4	SMAD family member 4
SUMO	Small ubiquitin-like modifier
UBC9	Ubiquitin conjugating enzyme 9
Ube2i	Ubiquitin conjugating enzyme E2 I
UPS	Ubiquitin-proteasome system

CHAPTER ONE: LITERATURE REVIEW

1.1. Abstract

The process of memory formation is complex and involves multiple molecular signaling pathways. The role of some pathways, such as the protein ubiquitination process, are well established to be involved as a critical regulator of memory formation by controlling protein degradation and other processes necessary for memory consolidation in cells. Similarly, recent evidence has suggested protein SUMOylation, which is similar in many ways to the ubiquitination process, may also play a role in the memory formation process and synaptic plasticity in the brain. However, much still remains unknown about the role of protein SUMOylation in memory formation.

1.2. Molecular mechanisms of memory formation

When new memories are formed, they are unstable and can be disrupted. The process by which unstable short-term memories are converted to stable long-term memories is called consolidation (McGaugh, 2000). Strong evidence suggests that memory consolidation requires specific neurochemical mechanisms important in synaptic plasticity (Asok, Leroy, Rayman, and Kandel, 2019; Bang et al., 2018). Synaptic plasticity, the strengthening or weakening of the synapses based on activity levels, enables neurons to communicate and thus plays a critical role in the formation of memories (Hegde, 2017; Jarome and Helmstetter, 2013). Important neurochemical mechanisms in the memory formation process include *de novo* gene transcription and protein synthesis (Bailey et al., 1999; Schafe and LeDoux, 2000). For example, a role for intracellular signaling molecules mitogen-activated protein kinase (MAPK) and protein kinase A (PKA) in nuclear Cre-response element binding protein 1 (CREB-1) activation have been reported as critical in the regulation of protein synthesis and synaptic growth that are essential for memory

consolidation to occur (Asok et al., 2019; Kandel, 2009). Importantly, various transcriptional control pathways, particularly that of the cAMP cascade, mediate mRNA and protein synthesis along with synaptic growth. These pathways are central to regulating transcriptional and translational processes essential for memory consolidation via alterations in synaptic plasticity.

1.3. The importance of sex in memory formation

Sex is an important variable when studying the molecular mechanisms of memory formation (Andreano and Cahill, 2009). However, despite this, the majority of past studies have focused solely on males, leaving questions as to whether the neurochemical mechanisms involved in memory formation are the same for both sexes. Importantly, recent studies have found that there are sex differences in hippocampal synaptic plasticity as well as in normal learning and memory processes (Hyer, Phillips, and Neigh, 2018; Safari et al., 2021; Wang et al., 2018). Male rats outperformed female rats in spatial memory tasks in one study while females outperformed males in trace fear memory retention and had a higher density of new hippocampal neurons after learning in another (Dalla, Papachristos, Whetstone, and Shors, 2009; Safari et al., 2021). Some of these performance differences on spatial memory tasks could be attributed to males and females using unique strategies to learn the task (Dalla and Shors, 2009). In addition, before memory formation even takes place, there are differences between the sexes in the number of synapses in neuronal circuits known to be involved in the memory consolidation process. As the memory formation process relies on these neuronal circuits, whether an existing memory for a task or situation is enhanced or weakened can depend on how many synapses are engaged during training (Mizuno and Giese, 2010). Another interesting factor worth noting is that stress seems to affect memory retention in males and females in opposite manners due to differential regulation of synapse number (Leuner and Shors, 2004). Collectively, this recent evidence has highlighted the

importance of sex as a biological variable in memory formation, and can influence everything from synaptic signaling mechanisms to the activation of transcription factors (Mizuno and Giese, 2010), although this research has been largely focused in the hippocampus and other brain regions important in the formation of certain types of memories, such as the amygdala, have received little attention.

1.4. The ubiquitin-proteasome system and its role in memory formation

In addition to traditional intracellular signaling molecules, recent evidence suggests a role for protein degradation mediated by the ubiquitin-proteasome system (UPS) in memory formation (Jarome and Helmstetter, 2013; 2014; Musaus, Navabpour, and Jarome, 2020). The UPS is a complex pathway involving multiple enzymes that is primarily involved in protein degradation in cells. Ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2), and ubiquitin ligases (E3) all play central roles. The small protein ubiquitin is first activated by ATP-dependent thioester bond formation between the E1 active site and ubiquitin C-terminus. Once the ubiquitin is activated and bound, it is transported to the E2 ligase via the E1. The E1 is able to transport two activated ubiquitin molecules at a time. The primary function of the E2 is to transport the ubiquitin molecule to the E3 where it undergoes further changes. Both the E2 and E3 enzymes have high specificity. Isopeptide bonds are formed between the C-terminus of the activated ubiquitin and the lysine residues of the target substrate. This process is repeated, and each ubiquitin attaches to the one before it, forming a polyubiquitin chain attached to the target protein. The chain is identified, and the target protein destroyed by the 26S proteasome. (Finley, Ulrich, Sommer, and Kaiser, 2012; Gong, Radulovic, Figueiredo-Pereira, and Cardozo, 2016; Musaus et al., 2020)

The memory consolidation process relies heavily on the ubiquitin proteasome system as not only is an increase in gene transcription and protein synthesis necessary for memory formation,

but protein degradation is as well. Specifically, the UPS has been shown to regulate the levels of synaptic proteins such as presynaptic regulators, postsynaptic receptors, and scaffold proteins during synaptic plasticity (Ehlers, 2003). Additionally, several studies have been conducted demonstrating protein ubiquitination and 26S proteasome activity increased after learning and that proteasome inhibitors impaired memory for a variety of tasks (Devulapalli et al., 2019; Fioravante and Byrne, 2011; Jarome, Ferrara, Kwapis, and Helmstetter, 2016; Jarome, Kwapis, Ruenzel, and Helmstetter, 2013; Jarome, Werner, Kwapis, and Helmstetter, 2011; Lopez-Salon et al., 2001; Orsi et al., 2019; Reis, Jarome, and Helmstetter, 2013).

Sex as a biological variable has also become a recent focus in ubiquitin processes related to memory formation. It has been shown that while both sexes require protein degradation, they differ in their regulation of this process within the amygdala (Devulapalli et al., 2021; Devulapalli et al., 2019). In addition, it has been found that proteasome-independent linear polyubiquitination plays a critical role in synaptic plasticity and fear memory formation in the amygdala, though the protein targets of this ubiquitin modification varied by sex (Musaus et al., 2021). Male rats were found to have increased protein degradation in the amygdala following learning, while females had increased baseline levels of ubiquitin-proteasome activity and K48 polyubiquitin protein targeting (Devulapalli et al., 2021). A recent study identified 106 protein degradation targets following fear conditioning, with only 3 shared between the sexes in the amygdala (Farrell et al., 2021). This suggests sex-specific roles in the function and regulation of both degradation-dependent and -independent ubiquitin processes in fear memory formation in the amygdala.

1.5. Protein SUMOylation

While much attention has been paid to the UPS, to date, little is known about the role of protein SUMOylation in synaptic plasticity and memory formation. The protein SUMOylation

pathway involves the conjugation of the small ubiquitin-like modifying enzyme (SUMO) to a target protein and is similar to the ubiquitin pathway in many respects. It too is reversible and has an activating E1 enzyme, E2 conjugase, and E3 ligases. However, it is also different in many respects. For example, while there are many E2 conjugase enzymes involved in the ubiquitin pathway, there is only one, UBC9, involved in SUMOylation.

There are 4 types of SUMO proteins. SUMO 1 is distinct from SUMO 2 and 3, which are 95% structurally homologous and differ only by 3 N-terminal residues and have not yet been differentiated functionally (Lee et al., 2014). SUMO 4 has been proposed to be a pseudogene and little is known about its function in cells (Wilkinson and Henley, 2010). These SUMO proteins are initially inactive precursors until they mature via C-terminal cleavage by SENP protease. They are then activated by ATP-dependent thioester bond formation between an E1 enzyme active site and SUMO C-terminal glycine residue. After activation, the SUMO attaches to the only known E2 enzyme, UBC9. Then, an E3 enzyme brings the E2 enzyme-SUMO into contact with the target protein. The SUMO covalently attaches to a lysine residue of the target protein. This process is reversible, and the SUMO can be cleaved from the target protein by a SENP protease (deSUMOylation). SUMOylation can have at least 3 known effects on a target protein: It can inhibit binding site interactions, change binding site interactions by recruiting new proteins to a new binding site, and/or create a conformational change. SUMOylation works in tandem with ubiquitination to regulate the substrate protein by blocking ubiquitin from attaching to and degrading certain proteins, promoting the substrate stability. DeSUMOylation can also occur to allow ubiquitination of a target protein for degradation.

Recently, protein SUMOylation has also been implicated in fear memory formation and synaptic plasticity as a regulator of long-term potentiation (LTP) (Lee et al., 2014). Elevated

amyloid beta (Aβ) peptide levels have been shown to impair activity-dependent SUMOylation, which causes LTP deficits. In contrast, increasing levels of the Ubc9 E2 enzyme have shown to increase SUMOylation and restore LTP deficits. Mice with overexpressed neuronal SUMO1 have reduced basal synaptic transmission and impaired contextual fear memory, whereas a neuron-specific deletion of SUMOylation impaired multiple forms of fear memories and increased anxiety behaviors (Matsuzaki et al., 2015; Wang et al., 2014). Importantly, previous studies have mostly used brain-wide manipulation of SUMOylation throughout development and no study has specifically examined the role of protein SUMOylation in fear memory formation in the amygdala, the primary site of storage for all emotional memories and where the majority of evidence has implicated the importance of ubiquitin-proteasome activity in the memory consolidation process. Additionally, despite compelling evidence from our lab of dramatic sex differences in the engagement and regulation of, but not requirement for, UPS-mediated protein degradation in fear memory formation in the amygdala (Devulapalli et al., 2021; Devulapalli et al., 2019), it is currently unknown if males and females differ in their need for protein SUMOylation during fear memory consolidation.

1.6. Significance

Understanding how protein SUMOylation differentially contributes to fear memory formation in males and females is critical in the effort to develop effective sex-specific therapeutic strategies for the treatment of PTSD and other anxiety disorders. Importantly, understanding the role of sex in this process is imperative because despite overwhelming evidence of differences in how traumatic events affect males and females, there are still large gaps in the literature as to why this sex difference exists. Filling these gaps in the field could allow for more effective treatment options for PTSD in both males and females.

CHAPTER TWO: PROTEIN SUMOYLATION IN THE AMYGDALA

2.1. Abstract

Strong evidence has implicated ubiquitin signaling in the process of fear memory formation. While less abundant than ubiquitination, evidence suggests that protein SUMOylation may also be involved in fear memory formation in neurons. However, the importance of amygdala protein SUMOylation in fear memory formation has never been directly examined. Furthermore, while recent evidence indicates that males and females differ significantly in the requirement for ubiquitin signaling during fear memory formation, whether sex differences also exist in the importance of protein SUMOylation to this process remains unknown. Here we found that males and females differ in the requirement for protein SUMOylation in the amygdala during fear memory formation. Western blot analysis revealed that while females had higher resting levels of SUMOylation, both sexes showed global increases following fear conditioning. However, SUMOylation-specific proteomic analysis revealed that only females have increased targeting of individual proteins by SUMOylation following fear conditioning, some of which were heat shock proteins. This suggests that protein SUMOylation is more robustly engaged in the amygdala of females following fear conditioning. *In vivo* siRNA mediated knockdown of *Ube2i*, the coding gene for the essential E2 ligase for SUMOylation conjugation, in the amygdala impaired fear memory in males without any effect in females. Importantly, higher siRNA concentrations than what was needed to impair memory in males reduced *Ube2i* levels in the amygdala of females but resulted in an increase in SUMOylation levels, suggesting a compensatory effect in females that was not observed in males. Collectively, these data reveal a novel, sex-specific role for protein SUMOylation in the amygdala during fear memory formation and expand our understanding of how ubiquitin-like signaling regulates memory formation.

2.2. Introduction

Post-traumatic stress disorder, or PTSD, is a common anxiety disorder that affects roughly 6% of the U.S. population at some point in their lives. Interestingly, despite that men are reported to experience a greater number of traumatic events, women have higher rates of diagnosed PTSD. Importantly, women are almost 3 times more likely to develop PTSD than men (Breslau et al., 1997; Inslicht et al., 2013). However, little is known about why women are more prone to developing PTSD than men. Understanding the neurobiology of PTSD is critical then for developing sex-specific therapeutic strategies for treatment of this major anxiety disorder.

The ubiquitin proteasome system (UPS) is primarily involved in the regulation of protein degradation in cells (Hegde, 2017; Hershko and Ciechanover, 1998; Jarome and Helmstetter, 2013). This pathway utilizes three main types of enzymes (E1, E2 and E3) with the ubiquitin ligases (E3) having substrate specificity. Once one ubiquitin binds to a target protein, each subsequent ubiquitin then attaches to the one before it, forming a polyubiquitin chain that allows some, but not all, target substrates to be destroyed by the 26S proteasome complex (Akutsu, Dikic, and Bremm, 2016; Musaus et al., 2020). While initial studies focused on the role of ubiquitin-proteasome mediated protein degradation in activity-dependent synaptic plasticity, recent evidence has strongly implicated this process in memory formation in the brain (Artinian et al., 2008; Cullen, Ferrara, Pullins, and Helmstetter, 2017; Figueiredo et al., 2015; Furini et al., 2015; Jarome et al., 2011; Lee et al., 2008; Lopez-Salon et al., 2001; Reis et al., 2013; Rodriguez-Ortiz et al., 2011; Rosenberg, Elkobi, Dieterich, and Rosenblum, 2016; Rosenberg, Elkobi, and Rosenblum, 2016). Of note, we recently found that while both male and female rats need degradation-dependent and independent UPS signaling to regulate fear memory formation in the amygdala (Devulapalli et al., 2021; Musaus et al., 2021), they differ significantly in the protein targets of ubiquitin signaling

following learning (Farrell et al., 2021). Furthermore, these sex differences in degradation-specific UPS extend into other brain regions involved in fear memory formation, including the hippocampus (Martin et al., 2021). These surprising data suggest that sex differences exist in the functional role of ubiquitin-proteasome signaling in fear memory formation in the brain. However, much still remains unknown about the importance of sex as a biological variable when studying UPS signaling in the brain and whether other ubiquitin-like modifications also have a sex-specific role in fear memory formation.

While less studied, especially within the brain, the protein SUMOylation pathway is similar in many ways to the UPS (Geiss-Friedlander and Melchior, 2007). The small ubiquitin-like modifier, SUMO, of which there are three functionally redundant isoforms (1-3), is initially an inactive precursor until it matures via C-terminal cleavage by the SENP protease. It is then activated by ATP-dependent thioester bond formation between an E1 enzyme active site and SUMO C-terminal glycine residue, similar to the UPS. After activation, the SUMO attaches to the only known E2 enzyme, UBC9. Then, an E3 enzyme brings the E2 enzyme-SUMO pair into contact with the target protein and SUMO covalently attaches to a lysine residue of the target protein. This process is reversible, and the SUMO can be cleaved from the target protein by a SENP protease. Unlike ubiquitination that heavily focuses on degradation, SUMOylation differs in terms of the effect on the target substrate, including inhibiting binding site interactions, recruiting new binding partners, causing a conformational change in the substrate protein to alter the function of the protein, promoting protein stability or altering substrate subcellular localization (Celen and Sahin, 2020).

Recently, evidence has emerged implicating protein SUMOylation in synaptic plasticity and memory formation. Inhibition of SUMOylation impairs long-term potentiation (LTP) and

contextual fear and Morris water maze memories (Lee et al., 2014). Mice overexpressing SUMO1 in neurons throughout the brain have reduced basal synaptic transmission, decreased dendritic spine density and impaired contextual fear memory (Matsuzaki et al., 2015). Conversely, neuron-specific deletion of SUMOylation throughout the brain increased anxiety behaviors and impaired multiple forms of fear memories in mice (Wang et al., 2014). Furthermore, the formation of spatial memories requires SUMOylation of SMAD4 and the transcription factor CREB in the hippocampus (Chen et al., 2014; Hsu, Ma, Liu, and Lee, 2017). Together, these studies point to an emerging role for protein SUMOylation in memory formation, particularly those memories that are fear based. However, while it is clear that protein SUMOylation is likely involved in fear memory formation, prior studies have primarily used brain-wide approaches to manipulate the SUMOylation process throughout all of development. As a result, it is currently unknown whether SUMOylation regulates fear memory formation in the amygdala, a brain region critical for the storage of emotional memories, specifically in adulthood. Additionally, prior behavioral work has been conducted in only male rodents, leaving unanswered questions about whether females have a similar need for protein SUMOylation in fear memory formation. Considering the recently reported sex differences in the ubiquitination process (Devulapalli et al., 2021; Martin et al., 2021; Musaus et al., 2021), a better understanding of how protein SUMOylation regulates fear memory formation in the amygdala of males and females is needed.

In this study, our goal was to determine if protein SUMOylation is involved in fear memory formation in the amygdala and if this varied by sex. To do this, we used western blotting and unbiased proteomic analyses to compare SUMOylated protein levels in the amygdala of male and female rats after contextual fear conditioning. Additionally, to determine the importance of protein SUMOylation for fear memory formation, we knocked down the expression of the E2 enzyme,

Ube2i (codes for UBC9), via siRNA in the amygdala and tested the effect on behavior. Together, we found that protein SUMOylation differentially regulates fear memory formation in the amygdala of males and females.

2.3. Experimental Procedures

2.3.1. Subjects

All experiments used 8-9 week old male or female Sprague Dawley rats obtained from Envigo (Frederick, MA). Animals were housed two per cage, with one per treatment group, with free access to water and rat chow. The colony was maintained under a 12:12 hr light/dark cycle. All experiments took place during the light portion of the cycle. All animal procedures were approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (protocol #18-019 and #20-233) and conducted with the ethical guidelines of the National Institutes of Health.

2.3.2. Cell Culture

Rat B35 neuroblastoma cell line (#CRL-2754; ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (#30-2002; ATCC, Manassa, VA, USA) supplemented with 10% Fetal Bovine serum (#35-016-CV; Corning, Tewsbury, MA, USA) and 0.1% Penicillin/Streptomycin (#15070063; Gibco, Gaitherburg, MD, USA). One day prior to transfection, cells at 70–90% confluency in a 100 mm dish were treated with 0.05% Trypsin-EDTA (1X) (#25300054; Gibco, Gaitherburg, MD, USA). Cells were then placed into a 96-well dish with a 1:56 ratio of cells going into each well containing 100 µL of DMEM-based media. Transfection was conducted using Accell siRNAs following manufacturer's instructions. Briefly, on the day of transfection, DMEM-based media was removed and cells were washed with DPBS

(#14190144; Gibco, Gaithersburg, MD, USA). In a separate tube, 7.5 µL of the 100 µM siRNA was mixed with 750 µL Accell Delivery Media (Cat #B-005000, Dharmacon, Lafayette, CO, USA). The final concentration was 1 µM Accell siRNA per well in a 96-well plate. Sixteen wells were transfected with the scrambled siRNA (control) and sixteen wells were transfected with Ube2i-siRNA. Cells were cultured in a NAPCO series 8000 Water Jacket CO_2 incubator (model 3586, Thermo Fisher,Waltham, MA, USA) for 48 hr post transfection. Every 4 wells of each group were combined together to make N = 4/group. Then, RNA was isolated from the cells by TRIzol (#15596018; Ambion, Austin, TX, USA) following the manufacturer's instructions. cDNA synthesis and real-time qPCR were performed as described below.

2.3.3. siRNA Preparation

Fresh Accell (Horizon, Lafayette, CO) SMARTpool Rat Ube2i (#E-089522-00-0005) and nontargeting control (#D-001910-10-05) siRNA stocks (100 µM) were resuspended in Accell siRNA delivery media to a concentration of ~ 10 or 20 µM on the day of surgery.

2.3.4. Surgery

Rats underwent stereotaxic surgeries where Accell siRNAs were injected into the basolateral amygdala (BLA) using coordinates relative to Bregma (A/P: -3.0, M/L: +/- 5.0, D/V: -7.7). Animals were anesthetized with 1.5-4% isoflurane and received bilateral injections into the amygdala using a 26-gauge Hamilton syringe connected to an automated pump (Harvard Apparatus, Cambridge, MA) at a rate of 0.1 µl per minute for a total of 0.5 µl per hemisphere. Animals received a subcutaneous injection of carprofen (5 mg/kg) and topical lidocaine on the day of surgery and oral carprofen tablets the day following surgery.

2.3.5. Apparatus

The 2 identical Habitest chambers used for contextual fear conditioning have been previously described in detail (Orsi et al., 2019). Habitest chamber consisted of a steel test cage with front and back Plexiglas walls and a grid shock floor above a plastic drop pan. The right wall of the chamber consisted of a house light in the top back corner, which remained on during the behavioral procedures, and an infrared light in the top middle, which was not illuminated during this project. The left wall of the chamber consisted of a high-bright light, which was not illuminated during this project. All remaining slots of both walls were filled with blank metal panels. A USB camera was mounted on a steel panel outside the back Plexiglas wall of the chamber, angled at ~45 degrees. The entire chamber was housed in an isolation cubicle with an acoustic liner and a house fan, which remained active during all behavioral procedures. The shock was delivered through the grid floor via a Precision Animal Shocker under the control of FreezeFrame 4 software, which also analyzed animal behavior in real-time. A freezing threshold of 2.0 was used as the scoring parameter for all animals, which we found matches hand-scoring procedures. All video was recorded and stored for later analysis. The chamber walls were wiped with 70% isopropanol before use.

2.3.6. Behavioral Procedures

Rats underwent contextual fear conditioning training and testing as described previously (Devulapalli et al., 2019; Orsi et al., 2019) in a Habitest chamber. Animals were handled for 4 days prior to behavioral training; the first two days occurred in the animal housing room and the second two days occurred in an adjacent room where behavioral training was to occur. Following this, animals were placed into the fear conditioning apparatus and after a 1 minute baseline, received 4 unsignaled footshock (1.0 mA, 1 second) presentations. After a 1 minute post-shock period, the animals were returned to their home cages. Importantly, we recently reported that male and female

Sprague Dawley rats perform similar on this task and do not differ in shock reactivity (Devulapalli et al., 2021), eliminating concerns of any biochemical effects seen between sexes being due to differences in behavioral performance or sensory processing. For the immediate shock procedure, animals were placed into the chamber and immediately received 4 consecutive unsignaled shock presentations (1.0 mA, 1 second) after which they remained in the chamber for an additional 5 minutes. We previously showed that this procedure does not result in the formation of a contextual fear memory (Orsi et al., 2019). For testing, which occurred 24 hr after training, animals were placed back into the training context for 5 minutes in the absence of shock. Male and female animals underwent identical procedures and were ran at the same time, in a counterbalanced manner, unless otherwise noted below.

2.3.7. Specific Experimental Procedures

EXPERIMENT 1: Male (N = 8) and female (N = 4) rats were trained to contextual fear conditioning and brain tissue was collected 1 hr later. Separate groups of male (N = 8) and female (N = 4) rats were not exposed to the context training and were used as controls. Amygdala lysates were compared using a western blot analysis. As the western blot analyses for each sex were completed at different times, sex was not used a variable in this experiment. Due to limited tissue quantity, free SUMO2/3 levels were analyzed at n = 4 per group for both males and females.

EXPERIMENT 2: Male (N = 8) and female (N = 8) rats experienced an immediate footshock stimulus in the behavior chamber and brain tissue was collected 1 hr later. Separate groups of male (N = 8) and female (N = 8) rats were not exposed to the context training and were used as controls. Amygdala lysates were compared using western blot analysis with both sexes included on the same membrane to allow direct comparisons.

EXPERIMENT 3: Male (N = 6) and female (N = 5) rats were trained to contextual fear conditioning and brain tissue was collected 1 hr later. Separate groups of male (N = 6) and female (N = 5) rats were not exposed to the training context and were used as controls. Collected amygdala tissue was used for SUMO capture-based mass spectrometry analysis. Due to differences in streptavidin signal (noted below), male and female samples could not be directly compared for differences in fold change.

EXPERIMENT 4: Male and female rats were stereotaxically injected with Accell siRNA targeting *Ube2i* (10 μM) or control (N = 6 per group males, N = 7-8 per group females) into the basolateral amygdala. Five days later, animals were trained to contextual fear conditioning and the following day re-exposed to the training context to assess memory retention. For male animals, amygdala tissue was collected 1 hr after the test session for RNA and protein analysis.

EXPERIMENT 5: Female rats were stereotaxically injected with a high concentration of Accell siRNA targeting *Ube2i* (20 μM) or control (N = 5-6 per group) into the basolateral amygdala. Four days later, animals were trained to contextual fear conditioning and the following day re-exposed to the training context to assess memory retention. Amygdala tissue was then collected 1 hr after the test session for RNA and protein analysis.

EXPERIMENT 6: Female rats were stereotaxically injected with Accell siRNA targeting *Ube2i* (10 μM) or control (N = 4 per group) into the basolateral amygdala. Five days later, animals were trained to contextual fear conditioning and amygdala tissue collected 1 hr later for protein analysis.

2.3.8. Tissue Collection

Rats were overdosed on isoflurane in a necrosis chamber and the brain rapidly was removed and immediately frozen on dry ice. Tissue containing the basolateral amygdala (BLA)

was then dissected out by blocking the brain in a rat brain matrix (Harvard Apparatus, Holliston, MA) incubated with dry ice. All dissected tissue was frozen at -80^{o}C until needed.

2.3.9. Whole Cell Lysate Preparation

For whole-cell tissue collection samples were lysed in buffer (500 mM HEPES, 1 M MgCl, 1 M KCl, 1 M DTT, 10% SDS, 10 µl/ml protease inhibitor cocktail, and 10 µl/ml phosphatase inhibitor cocktail, 10% NP-40, 125 mM NEM), centrifuged for 10 minutes at 10,000 x g at 4^{o}C, the supernatant collected and protein concentration was determined by using the Bio-Rad DC protein assay.

2.3.10. Antibodies

Antibodies included mouse monoclonal against SUMO 2/3 (1:500; #ASM23, Cytoskeleton Inc, Denver, CO), rabbit monoclonal against Actin (1:1000, #4967, Cell Signaling Technology, Canvers, MA), rabbit monoclonal against HSP60 (1:1000, #12165S, Cell Signaling Technology), rabbit polyclonal against H3 (1:1000, #ab1791, Abcam, Waltham, MA), rabbit polyclonal against UBC9 (1:1000, #4918S, Cell Signaling Technology) and rabbit monoclonal against K48 polyubiquitin (1:1000, #ab140601, Abcam).

2.3.11. Protein Immunoprecipitation

Nuclear extracts were collected using a previously described procedure (Devulapalli et al., 2021). Normalized nuclear protein extracts (10 µg) were diluted in PBS with 0.01% Tween-20 and incubated with primary antibody (SUMO2/3, 5 µg) or control (no antibody) overnight at 4°C. Pierce Magnetic Protein A/G beads (Thermo Fisher) were washed and added to the protein-antibody complex for over-end mixing at 4°C for 1 hr. After incubating, samples were washed three times in PBS with 0.01% Tween-20, then immunoprecipitates were eluted by heating

samples at 95°C for 5 minutes at 800 rpm in 1X sample buffer. Eluted precipitates were loaded on 7-20% SDS-PAGE, exposed to primary antibody, and imaged as described below in the **Western Blot** section.

2.3.12. Western Blots

Western blots were performed with 7, 12, or 20% Acrylamide gels using 10µg of protein and transferred to a PBDF membrane with a Turbo Transfer System (Biorad) as described previously (Orsi et al., 2019). Membranes were incubated in a 50:50 blocking buffer (50% Licor TBS blocking buffer and 50% TBS + 0.1% Tween-20) for 1 h at room temperature, followed by an overnight incubation in primary antibody in 50:50 blocking buffer at 4^{o}C. Membranes were then washed 3 times for 10 minutes with TBS + 0.1% Tween-20 (TBSt) and incubated in secondary antibody (1:20,000, goat anti-mouse IgG2a 800CW or 1:40,000, goat anti-rabbit 800CW) in 50:50 blocking buffer for 45 minutes. After two 10 minutes washes in TBSt, the membranes were washed in TBS, imaged using the Odyssey Fc (LI-COR, Lincoln, NE) and visualized proteins were analyzed using Image Studio Ver 5.2. SUMOylation images were normalized to Coomassie blue, except in Figure 4 (Experiment 5) due to noise in the 700 channel that prevented accurate quantification of the Coomassie stain. In this case Actin was used in the 800 channel. The Coomassie blue procedure consisted of staining membranes for 10 sec, washing extensively in 50% methanol and imaging at 700CW using the Odyssey Fc. All optical densities were taken from the entire length of the molecular standards ladder for the SUMOylation and Coomassie blue developments. HSP60 was normalized to Actin. Free (unconjugated) SUMO2/3 was normalized to histone H3.

2.3.13. SUMO Capture Assay

BLA tissue was homogenized in a whole cell buffer (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.5% IGEPAL, 0.02% SDS, 70 mM NEM, 10 µl/ml protease inhibitor cocktail, and 10 µl/ml phosphatase inhibitor cocktail). Then a SUMO capture assay was performed. MagnaLink streptavidin magnetic beads (#M-1003-010; Vector Laboratories, Burlingame, CA) were aliquoted (100 µl), washed thoroughly with Wash Buffer (100 mM Tris-HCL, 150 mM NaCl, 5 mM EDTA, 0.08% NP-40) and 4 µl of Biotin labeled SUMO protein capture reagent (#SM-0101-0200; Life Sensors, Malvern, PA) was added, followed by incubation for 2 hr on rotator at 4ºC. Beads were then washed and a 500 µl mixture of protein (300 µg for all samples and both sexes), protease inhibitor (10 µg/µl), and Wash Buffer and was added to each tube, followed by incubation for 2 hr on rotator at 4ºC. Samples were then washed twice and incubated at 96ºC for 5 minutes at 800 rpm in 1X sample buffer. After cooling at room temperature, the supernatant was collected and stored at -80ºC for mass spectrometry analysis.

2.3.14. Liquid Chromatography Mass Spectrometry (LC/MS)

Optima™ LC/MS grade solvents, Pierce™ trypsin protease (MS grade), were from Fisher Scientific (Waltham, MA). S-Trap™ micro columns were from ProtiFi (Farmingdale, NY). Triethylammonium bicarbonate, pH 8.5 (TEAB), *o*-phosphoric acid, and formic acid were from MilliporeSigma (St. Louis, MO).

Protein samples were acidified by the addition of 11.1 µl 12% (v/v) *o*-phosphoric acid then protein precipitated by the addition of 725 µl LC/MS grade methanol and incubation at -80°C overnight. Precipitated protein was collected at the bottom of the sample tubes by centrifugation at room temperature for 15 minutes at 13000 x g. All but approximately 150 µl of the liquid from each sample was removed and discarded. Precipitated protein in each sample tube was then

homogenized in the remaining liquid by scraping the sides of the tube with a pipette tip and repeated pipetting. The protein homogenate from each sample was then loaded onto an S-Trap™ micro column by centrifugation at room temperature for one minute at 1000 x g. Each S-Trap™ micro column was washed four times with 150 µl LC/MS grade methanol at room temperature for one minute at 1000 x g. Pierce™ trypsin protease (0.8 µg in 25 µl 50 mM TEAB) was loaded on top of each S-Trap™ micro column and incubated for 4 hr at 37°C. A second aliquot of trypsin (0.8 µg in 25 µl 50 mM TEAB) was loaded on top of each S-Trap™ micro column and incubated overnight at 37°C.

Peptides were recovered by sequential washing of the spin column with 25 µl 50 mM TEAB, 25 µl solvent A (2:98 LC/MS grade acetonitrile: LC/MS grade water supplemented with 0.1% (v/v) formic acid), and 25 µl solvent B (80:20 LC-MS grade acetonitrile: LC-MS grade water supplemented with 0.1% (v/v) formic acid). Acetonitrile was removed using a centrifugal vacuum concentrator, then peptide concentration was determined by measuring the absorbance at 215 nm using a DS-11 FX+ spectrophotometer/fluorometer (DeNovix, Wilmington, DE). Samples were diluted to 0.5 mg/ml using solvent A and 2 µl (1 µg, females) or 4 µl (2 µg, males) were analyzed using LC-MS/MS and each sample was analyzed twice yielding technical duplicates. The higher concentration used for males was due to a greater streptavidin signal than seen in females. As noted below, final values are normalized to streptavidin to account for this difference.

Samples were first loaded onto a precolumn (Acclaim PepMap 100 (Thermo Scientific, Waltham, MA), 100 µm x 2 cm) after which flow was diverted to an analytical column (50 cm µPAC (PharmaFluidics, Woburn, MA). The UPLC/autosampler utilized was an Easy-nLC 1200 (Thermo Scientific, Waltham, MA). Flow rate was maintained at 150 nl/min and peptides were eluted utilizing a 2 to 45% gradient of solvent B in solvent A over 88 minutes. The mass

spectrometer utilized was an Orbitrap Fusion Lumos Tribid™ from Thermo Scientific (Waltham, MA). Spray voltage on the µPAC compatible Easy-Spray emitter (PharmaFluidics, Woburn, MA) was 1300 volts, the ion transfer tube was maintained at 275°C, the RF lens was set to 30% and the default charge state was set to 3.

MS data for the m/z range of 400-1500 was collected using the orbitrap at 120000 resolution in positive profile mode with an AGC target of 4.0e5 and a maximum injection time of 50 ms. Peaks were filtered for MS/MS analysis based on having isotopic peak distribution expected of a peptide with an intensity above 2.0e4 and a charge state of 2-5. Peaks were excluded dynamically for 15 seconds after 1 scan with the MS/MS set to be collected at 45% of a chromatographic peak width with an expected peak width (FWHM) of 15 seconds. MS/MS data starting at m/z of 150 was collected using the orbitrap at 15000 resolution in positive centroid mode with an AGC target of 1.0e5 and a maximum injection time of 200 ms. Activation type was HCD stepped from 27 to 33.

Data were analyzed utilizing Proteome Discoverer 2.5 (Thermo Scientific, Waltham, MA) combining a Sequest HT and Mascot 2.7 (Matrix Science, Boston, MA) search into one result summary for each sample. Both searches utilized the UniProt reference *R. norvegicus* proteome database (downloaded July 28, 2020) and a common protein contaminant database provided with the Proteome Discoverer (PD) software package. Each search assumed trypsin-specific peptides with the possibility of 2 missed cleavages, a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.1 Da. Sequest HT searches also included the PD software precursor detector node to identify MSMS spectra containing peaks from more than one precursor. Sequest HT searches included a fixed modification of carbamidomethyl at Cys and the variable modifications of oxidation at Met and loss of Met at the N-terminus of a protein (required for using the INFERYS

rescoring node). Peptide matches identified by Sequest HT were subjected to INFERYS rescoring to further optimize the number of peptides identified with high confidence.

Mascot searches included the following dynamic modifications: oxidation of Met, acetylation of the protein N-terminus, cyclization of a peptide N-terminal Gln to pyro-Glu, N-ethylmaleimide at Cys, DeStreak (β-mercaptoethanol) at Cys, GlyGly at Lys, and deamidation of Asn/Gln residues.

Protein identifications were reported at a 1% false discovery rate (high confidence) or at a 5% false discovery rate (medium confidence) based on searches of decoy databases utilizing the same parameters as above. The software matched peptide peaks across all runs and protein quantities are the sum of all peptide intensities associated with the protein. Values were normalized to Streptavidin. Technical duplicates were averaged then biological replicates were averaged before determination of the trained to naïve ratio. A simple t-test was used to determine p-values comparing the 6 trained males to the 6 naïve males and the 5 trained females to the 5 naïve females. All data and related files were submitted to the ProteomeXchange Consortium via the PRIDE partner repository with accession number PXD029825 and 10.6019/PXD029825.

2.3.15. RNA Extractions

RNA was extracted from BLA lysates using the Qiagen (Germantown, MD) RNA kit, according to the manufacturer's instructions. RNA concentration was measured on the Take3 (BioTek, Winooski, VT), normalized (200 ng) and converted to cDNA using the iScript cDNA synthesis kit (Bio-rad).

2.3.16. Quantitative Real-Time PCR

Real-time PCR amplifications of the cDNA were performed on the Bio-rad CFX96 Real-Time System using the following protocol: 95.0°C for 3 minutes, then 95.0°C for 10 seconds, followed by 60°C for 30 seconds (39 repeats), 55–95°C for 0.5°C/cycle, followed by a melt curve starting at 55.0°C for 10 seconds (81 repeats), and then held at 4.0°C. Primers were *Ube2i* (F: GGGTCCTCACAACACCTCAG; R: TGTCTATCCAGGCCATCCCA), and *Gapdh* (F: ACCTTTGATGCTGGGGCTGGC; R: GGGCTGAGTTGGGATGGGGACT) was used as an internal control and data was analyzed using the comparative Ct method.

2.3.17. Statistical Analyses

All data are presented as mean with standard error, with scatter plots to identify individual samples (except in line graphs). Molecular data with two groups were analyzed with two-tailed *t*-tests. Molecular data with more than two groups were analyzed with 2-way ANOVA with Sex and Condition as variables, followed by Fisher LSD post hoc tests (Experiment 2). Behavioral data in Experiment 4 were analyzed with 2-way ANOVA with Sex and Manipulation as variables, followed by Fisher LSD posthoc tests. Behavioral data in Experiment 5 were analyzed with One-way ANOVA for training and *t*-test for testing since only one sex was used. Data in Experiment 1 (males, SUMOylation levels) and Experiment 2 were log transformed prior to analysis. Statistical outliers were defined as those samples that were two or more standard deviations from the mean and were determined by the outlier function in Prism. Figures shown are with outliers excluded.

2.4. Results

2.4.1. Protein SUMOylation is increased globally in the amygdala of male and female rats following fear conditioning

We first wanted to test if protein SUMOylation is increased in the amygdala of male and female rats following fear conditioning. All animals were trained to contextual fear conditioning and BLA whole cell lysates were collected 1 hr later for western blot analysis using a SUMO2/3 antibody. We choose to focus on SUMO2/3 because in pilot studies we were unable to find an antibody that could consistently visualize SUMO1 targeted proteins in rat brain tissue. As western blot analyses were done at different times for each sex, no direct comparisons between males and females could be completed in this study. We found significant increases in global protein SUMOylation in the BLA of male (U = 11, P = 0.0281; **Figure 1A**) and female (t_6 = 3.349, P = 0.0154; **Figure 1C**) rats following fear conditioning. These increases in SUMOylated proteins were not associated with significant changes in free SUMO2/3 levels in both males (t_6 = 1.723, P = 0.1357; **Figure 1B**) and females (t_6 = 1.269, P = 0.2514; **Figure 1D**). Together, these results suggest that protein SUMOylation is increased in the amygdala in a sex-independent manner following fear conditioning.

Next, we tested if these increases in protein SUMOylation were learning-specific. For this experiment, we trained rats to an immediate shock (IS) procedure that allows exposure to both the training context and the footshock stimulus in a non associative manner (Orsi et al., 2019). BLA whole cell lysates were collected 1 hr after training and compared with naïve animals. For this experiment, western blot analyses on BLA tissue for both sexes were completed at the same time to allow direct comparison between males and females (**Figure 1E**). We found a main effect for Sex ($F_{(1,28)}$ = 4.297, P = 0.0475), but not Training ($F_{(1,28)}$ = 0.4798, P = 0.4942) and there was not a Sex by Training Interaction ($F_{(1,28)}$ = 0.1943, P = 0.6628). These data suggest that exposure to the shock or context stimuli alone were not sufficient to increase protein SUMOylation in the amygdala of either sex. However, females have significantly elevated baseline levels of protein

SUMOylation in the amygdala in comparison to males. This is consistent with our previous report showing higher resting levels of protein ubiquitination in the amygdala of females relative to males (Devulapalli et al., 2021).

2.4.2. Heat shock proteins are targeted by SUMOylation in females following fear conditioning

We wanted to identify the potential protein targets of SUMOylation in the amygdala following fear conditioning, which could be used to help infer function. BLA tissue was collected from both naïve and fear conditioned male and female rats and purified with a SUMO capture reagent that binds SUMOylated proteins regardless of the SUMO isoform present. We confirmed that this capture reagent is able to isolate SUMOylated proteins from samples without cross-reactivity to ubiquitinated proteins (K48 ubiquitin) or SUMO ligases (UBC9; **Figure 2A**). We then isolated these captured SUMOylated proteins from the rest of the lysate and identified them using liquid chromatography mass spectrometry (LC/MS). For this experiment, LC/MS analyses on BLA tissue were completed separately for each sex due to differences in streptavidin signal; therefore no direct comparisons on fold change could be completed. In total, we identified 345 and 429 SUMOylated proteins in females and males, respectively, with 279 (56%) common to both sexes (**Figure 2B**). In females, four significant SUMO positive targets, those proteins that had greater abundance in our purified sample following fear conditioning, were identified: Cholesterol 25-hydroxylase, Actin gamma-enteric smooth muscle, Erythrocyte membrane protein band 4.1-like 2, and 60 kDa heat shock protein mitochondrial (**Figure 2C**). There were no negative targets, or those that had reduced abundance in our purified sample, identified in the females. Conversely, one significant SUMO negative target was identified in the males (HSPE1; **Figure 2D**), but no positive targets were found. For males this suggests that the significant increase in global

SUMOylation levels observed in the western blot analysis (**Figure 1A**) was likely due to moderate changes in a large number of proteins as opposed to significant targeting of a few select proteins. Interestingly, of the 4 significant proteins identified as positive targets in females the target with the highest fold change was a heat shock protein. Similarly, in males the one negative target of SUMOylation was also a heat shock protein. A comparison of the identified SUMOylation targets following fear conditioning in both sexes is shown in **Table 1**. Next, using co-immunoprecipitation we confirmed that Heat Shock Protein 60 (HSP60) was a target of SUMOylation in the female amygdala (**Figure 2E**). Additionally, cytoplasmic levels of HSP60 remained unchanged 1 hr after fear conditioning (t_6 = 0.4748, P = 0.6517; **Figure 2F**), which is consistent with this mark not being associated with protein degradation. Together, these data suggest that an important target of SUMOylation following fear conditioning are heat shock proteins, which are known to be critically involved in fear memory formation (Porto et al., 2018).

2.4.3. Protein SUMOylation differentially regulates fear memory formation in males and females

To test the importance of protein SUMOylation in the amygdala to fear memory formation in both sexes, we injected male and female rats with Accell siRNA targeting *Ube2i*, the gene coding for the E2 ligase (UBC9) essential for protein SUMOylation in mammals, or control in the amygdala. The concentration of the siRNA used in this experiment (10 μM) was consistent with our prior work using this methodology (Navabpour, Rogers, McFadden, and Jarome, 2020). Animals received the siRNA injection into the BLA 5 days prior to training and were tested 24 hrs later (**Figure 3A**). For males, animals were euthanized and BLA tissue collected 1 hr after the test session. We confirmed that the siRNA was able to effectively reduce *Ube2i* expression *in vitro* (t_6 = 11.33, P < 0.0001; **Figure 3B**) and in the BLA of males *in vivo* (t_9 = 2.772, P = 0.0217; **Figure**

3C), the latter of which was associated with a reduction in SUMOylated proteins (t_9 = 2.047, P = 0.0355; **Figure 3D**). There were no significant effects for siRNA manipulation or Sex during training, but there was an effect for Time (siRNA: $F_{(1,24)}$ = 1.859, P = 0.1854; Sex: $F_{(1,24)}$ = 1.022, P = 0.3221; Time: $F_{(4,96)}$ = 74.20, P < 0.0001; **Figure 3E**). Importantly, there was a Sex x Time Interaction ($F_{(4,96)}$ = 2.490, P = 0.0483) where male control animals (black dotted line) had greater freezing behavior at the end of the test session than both female groups (solid black and red lines). This result is consistent with what we have previously shown during contextual fear conditioning training with male and female rats [19]. During the testing session we found a significant Group by Sex Interaction ($F_{(1,22)}$ = 4.349, P = 0.0489) with no main effect for Sex ($F_{(1,22)}$ = 1.076, P = 0.3108) or Group ($F_{(1,22)}$ = 1.184, P = 0.2884; **Figure 3F**). These surprising data reveal divergent results from *Ube2i* knockdown across sexes, with a significant decrease in fear memory in males without any effect in females.

To confirm that protein SUMOylation was not necessary for fear memory formation in the amygdala of females, we repeated the previous experiment using a higher concentration of siRNA (**Figure 4A**). Additionally, as all prior work using this approach had verified the concentration and timing of peak knockdown in only male rodents, we performed the training session 4 days after siRNA injection to better target the peak time point of gene knockdown with this approach (Nakajima et al., 2012). Similar to above, we collected amygdala tissue 1 hr after the testing session and confirmed *Ube2i* knockdown (t_9 = 2.457, P = 0.0363; **Figure 4B**). Surprisingly, this resulted in an increase in SUMOylation levels (t_9 = 2.909, P = 0.0173; **Figure 4C**), suggesting that in the female amygdala there was a compensatory effect for loss of *Ube2i.* Consistent with this, we found that injection of standard concentration *Ube2i* siRNA (10μM) into the amygdala of females increased SUMOylation levels 1 hr after fear conditioning (**Figure 5**). Next, we assessed how the

higher concentration siRNA would affect memory in female rats. There was not a significant effect for siRNA manipulation during training (**Figure 4D**). Similar to our previous experiment, found that *Ube2i* knockdown did not significantly alter memory in females (t_9 = 1.638, P = 0.1358; **Figure 4E**), though all animal performance, including in controls, was lower than our previous experiment, which was likely due to the high concentration of siRNA used. Together with our previous data showing higher baseline levels of SUMOylation in females, these results suggest that protein SUMOylation differentially regulates fear memory formation in the amygdala of males and females and that, in general, SUMOylation may have a more critical function in normal cellular functioning in females.

2.5. Discussion

Recently, strong evidence has emerged that sex differences exist in the role of proteasome-dependent and independent ubiquitin signaling in fear memory formation and storage (Devulapalli et al., 2021; Devulapalli et al., 2019; Dulka, Trask, and Helmstetter, 2021; Farrell et al., 2021; Martin et al., 2021; Musaus et al., 2021). However, whether such sex differences also exist for the ubiquitin-like modification SUMOylation is unknown. Here, we found that while male and female rats both have increased global levels of protein SUMOylation in the amygdala following fear conditioning, loss of the critical SUMO E2 ligase *Ube2i* impaired memory in males without any effect in females, though the latter of which was associated with a compensatory increase in SUMOylated proteins. These data suggest that protein SUMOylation may be a sex-specific regulator of fear memory formation in the amygdala.

A number of recent studies have shown a critical role for protein SUMOylation in activity- and learning-dependent synaptic plasticity in the brain (Craig et al., 2012; Jaafari et al., 2013; Lee et al., 2014; Matsuzaki et al., 2015; Wang et al., 2014), particularly in neurons. Our study adds to

this growing literature by showing an important role for protein SUMOylation in the amygdala during fear memory formation. While our siRNA approach was not neuron-selective, it did avoid potential confounds of altered SUMOylation levels across multiple brain regions simultaneously and throughout development, where it has been suggested to have an important role in neuronal development (Josa-Prado et al., 2019; Rocca, Wilkinson, and Henley, 2017). Importantly, our data are the first to examine the role of protein SUMOylation in memory formation in females, which could potentially open a new avenue for study in this rapidly emerging area.

Related, one surprising finding from our study was that while both males and females had increased levels of protein SUMOylation following fear conditioning, inhibition of this process only impaired memory in males. Furthermore, concentrations of siRNA greater than what was needed to impair memory in males resulted in a compensatory increase in SUMOylated proteins in females, an effect that was not seen in males. This would suggest that protein SUMOylation is critical for fear memory formation in the amygdala of both sexes but that in females the role it plays in normal cellular functioning (i.e., at baseline) precludes the importance of it during the memory consolidation process. This likely compensatory increase in SUMOylation could be related to the elevated resting levels we observed of this modification in the amygdala of females, a pattern we previously observed with protein ubiquitination (Devulapalli et al., 2021). Importantly, in our prior work we did not observe a compensatory increase in ubiquitination following manipulation of ubiquitin coding genes, though this was likely due to the sustained manipulation (CRISPR-dCas9) used as opposed to the more rapid knockdown approach employed in the present study. Unfortunately, due to the high similarity in DNA sequence for the *Ube2i* promoter with other regions in the genome, the CRISPR-dCas9 approach could not be used here. Furthermore, temporally controlled manipulations of SUMOylation are currently an area of

technical limitation in the field. As a result, this compensatory increase prevents us from fully elucidating the importance of protein SUMOylation to fear memory formation in the female amygdala. Future studies will need to develop more temporally controlled manipulations of protein SUMOylation to better examine the role of this protein modification in fear memory formation in females.

Recently, we have begun to identify the protein targets of degradation-dependent and independent ubiquitin signaling in the amygdala during fear memory formation (Farrell et al., 2021; Musaus et al., 2021). While these analyses have revealed a number of different classes of proteins targeted by ubiquitin following learning, one consistent pattern has been the general lack of overlap among these targets across sexes. However, in our present study we found that both males and females had altered SUMOylation of heat shock proteins following fear conditioning. While the significance of this similar targeting of protein class across sexes is unknown, it does suggest that males and females may have some common protein targets for specific forms of ubiquitin modifications during fear memory formation. Future studies will aim to better understand the relationship between protein SUMOylation and degradation-dependent and independent ubiquitin modifications during fear memory formation and how this differs between sexes.

While our study identifies a novel sex difference in the role of protein SUMOylation in fear memory formation, some important limitations exist. First, we did not track the estrous cycle of female rats, an approach that is consistent with our previous examination of sex differences in ubiquitin signaling during fear memory formation in free cycling animals (Devulapalli et al., 2021; Devulapalli et al., 2019; Farrell et al., 2021; Martin et al., 2021; Musaus et al., 2021). While it is unknown if SUMOylation levels in the brain vary across the estrous cycle, some evidence suggests that this does occur in the mouse uterus (Liu et al., 2020). Thus, it is possible that the importance

of protein SUMOylation to fear memory formation in females may vary across the estrous cycle and is of importance to examine in more detail in future studies. Second, as noted above, our siRNA approach was not cell-type specific, leaving unanswered questions about the sex-specific role of protein SUMOylation in fear memory formation across different cell types. Finally, while we did identify some targets of SUMOylation following fear conditioning, the functional significance of SUMO targeting these proteins remains unknown. Despite this, our data clearly reveal that a sex difference exists in the role of protein SUMOylation in the amygdala during fear memory formation.

2.6. Conclusion

In conclusion, we report a novel sex difference in the role of protein SUMOylation in the amygdala during fear memory formation. This information adds to the growing literature on a sex-specific role for ubiquitin signaling in fear memory formation and expands our understanding of the molecular mechanisms supporting memory formation. Additionally, these data could have important implications for understanding the molecular mechanisms controlling sex differences in fear memories that underly post-traumatic stress disorder.

Table 1: Fear conditioning-induced SUMOylation targets in males and females

Protein	Female Log Change (Trained/Naïve)	Female p Value	Male Log Change (Trained/Naïve)	Male p Value
CH25H	0.87	0.0016*	N/A	N/A
ACTG2	0.89	0.0072*	N/A	N/A
EBP41I2	1.45	0.0425*	0.34	0.3504
HSP60	2.09	0.0455*	-0.52	0.3037
HSP10	1.15	0.1450	-0.72	0.0272*

*Denotes significant difference between Trained and Naïve rats

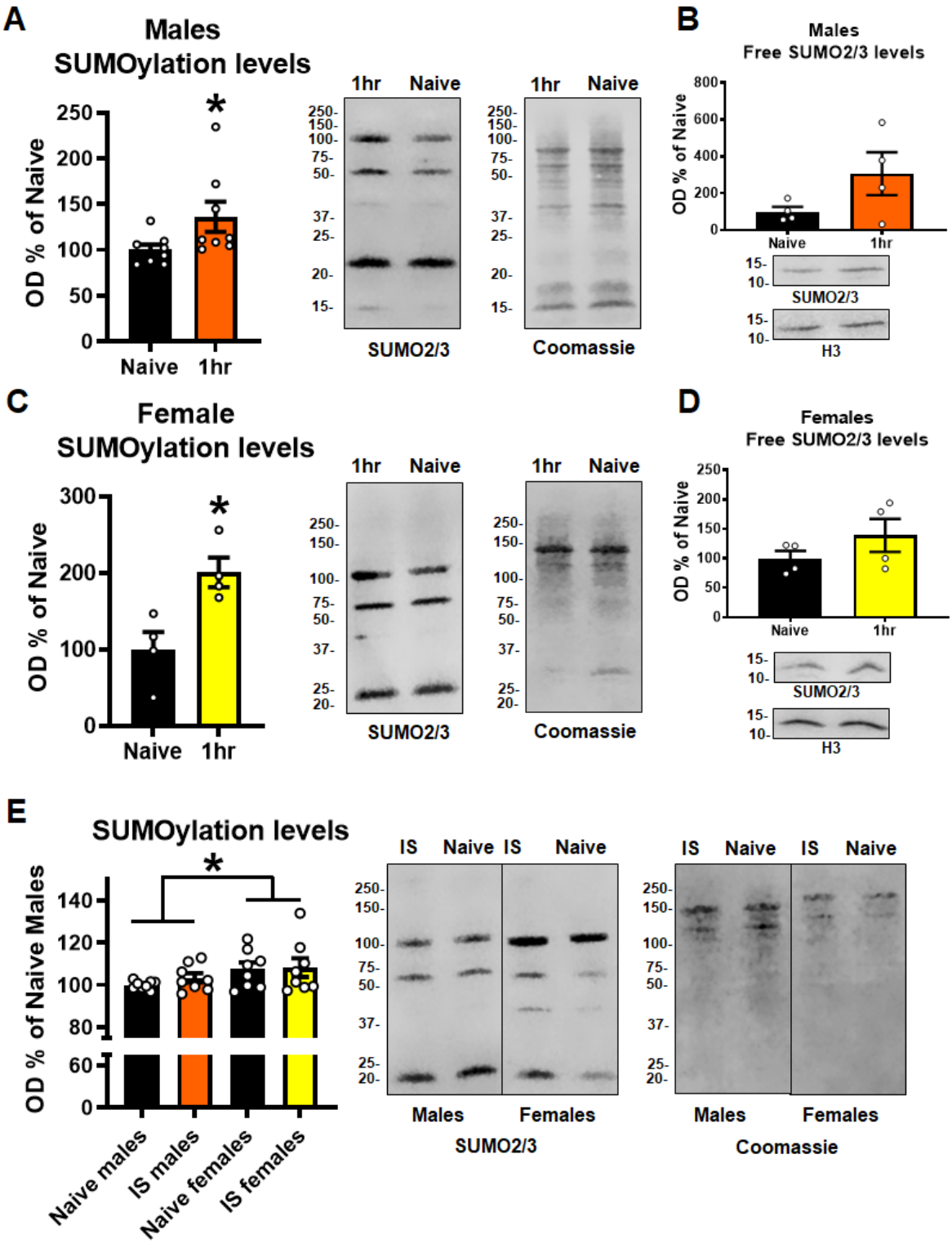

Figure 1. Global levels of protein SUMOylation are increased in the amygdala of male and female rats following fear conditioning. Male and female rats were trained to contextual fear

conditioning and amygdala tissue collected 1 hr later for western blot analysis of protein SUMOylation (2/3) levels. (**A-D**) Global levels of protein SUMOylation were increased in the amygdala of male (**A**) and female (**C**) rats following fear conditioning (N = 8 per group for males, 4 per group for females). However, free SUMO2/3 levels were not significantly changed in males (**B**) or females (**D**; N = 4 per group per sex). (**E**) Male and female rats were trained to an immediate shock (IS) procedure in which the footshock and training context were presented in a non-associative manner. Amygdala tissue was collected 1 hr later for western blot analysis of protein SUMOylation (2/3) levels. Global levels of protein SUMOylation were not altered as a function of training in either sex (black vs colored bars). However, females had higher resting levels of protein SUMOylation in the amygdala in comparison to males (black bars). N = 8 per group. Western blot images were spliced from the same gel. Coomassie blue was used as a loading control for SUMOylation levels and H3 for free SUMO2/3. For SUMOylation levels (A, C, E), quantification was performed for the entire column spanning the molecular weights ladder. Specific bands identified within a given column represent SUMOylated proteins of unknown identify. * $P < 0.05$ denotes significant difference from Naïve (**A, C**) or a main effect of Sex (**E**).

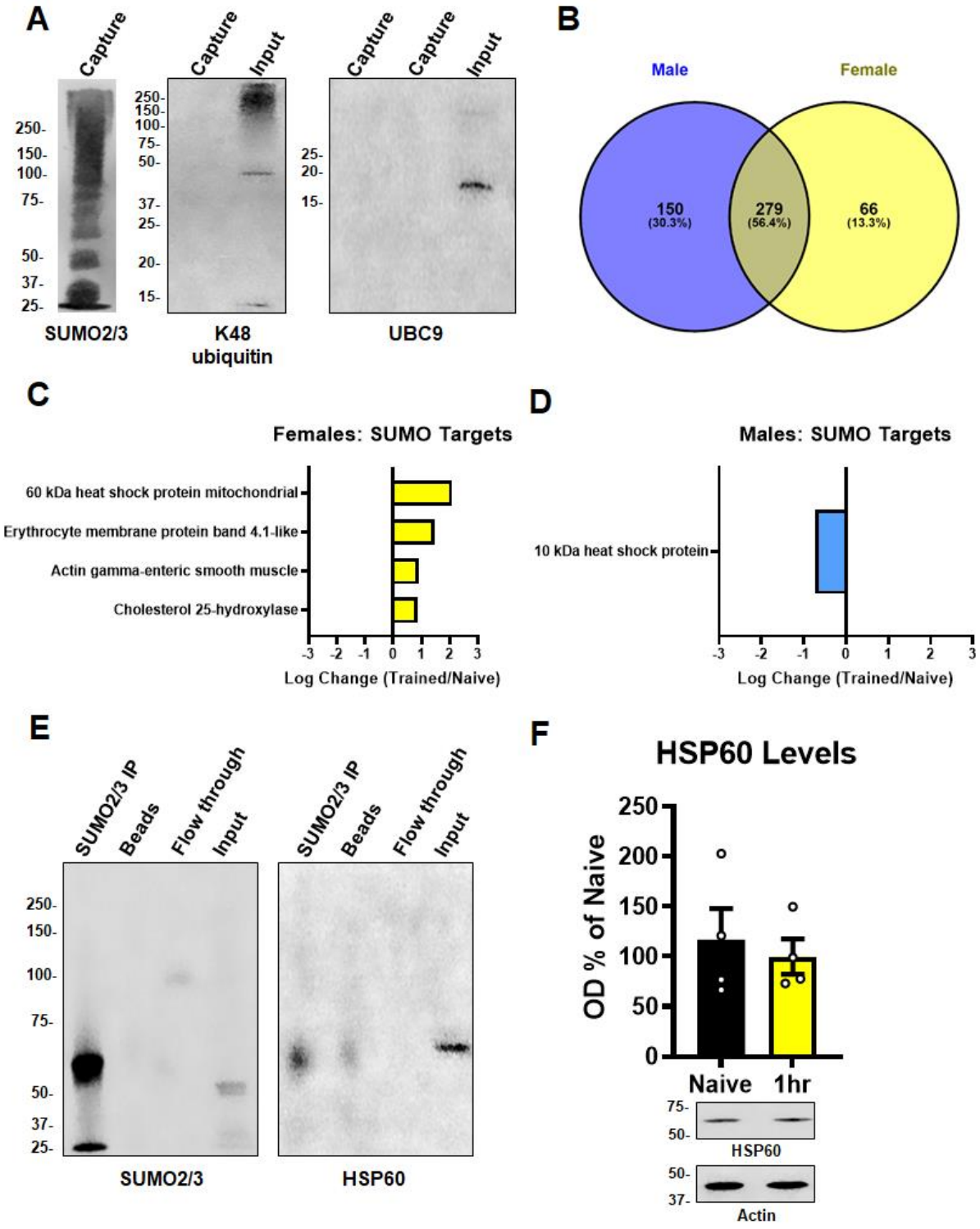

Figure 2. Increases in protein SUMOylation occur on specific proteins in the amygdala following fear conditioning. Male and female rats were trained to contextual fear conditioning

and amygdala tissue collected 1 hr later for SUMO-specific liquid chromatography mass spectrometry (LC/MS) analysis. (**A**) Confirmation of SUMO capture reagent specificity. Western blot analysis of SUMO-captured sample revealed enrichment of SUMOylated proteins, with no cross-reactivity to ubiquitination (K48 ubiquitin) or SUMO ligases (UBC9). (**B**) The total number of SUMOylated proteins identified in LC/MS analysis, separated by sex. (C-D) The graph shows proteins that had a significant change in abundance in our SUMO capture assay with fear conditioning (relative to Naïve controls) in female (**C**) and male (**D**) rats (N = 6 per group for males, 5 per group for females). Log change greater than zero indicates greater abundance (more SUMOylation) in our capture assay, while those below zero had reduced abundance (less SUMOylation). (**E**) Confirmation of LC/MS analysis via co-immunoprecipiation of SUMO2/3 and Heat Shock Protein 60 (HSP) in the female amygdala. (**F**) HSP60 levels do not change in the amygdala of female rats following fear conditioning (N = 4 per group).

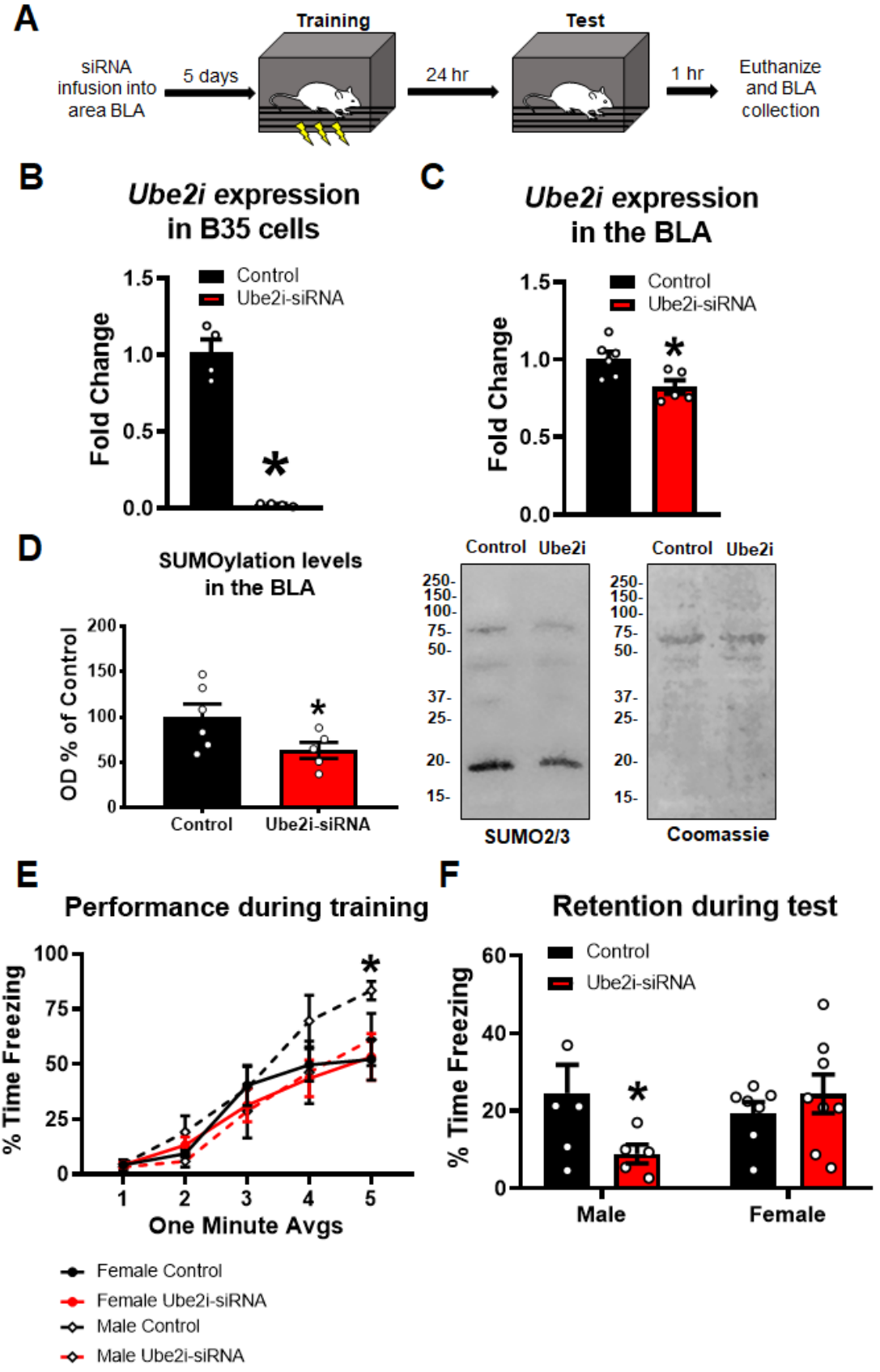
A
Training
Test
siRNA infusion into area BLA
5 days
24 hr
1 hr
Euthanize and BLA collection
B
Ube2i expression in B35 cells
Control
Ube2i-siRNA
Fold Change
1.5
1.0
0.5
0.0
*
C
Ube2i expression in the BLA
Control
Ube2i-siRNA
Fold Change
1.5
1.0
0.5
0.0
*
D
SUMOylation levels in the BLA
OD % of Control
200
150
100
50
0
*
Control
Ube2i-siRNA
Control
Ube2i
250-
150-
100-
75-
50-
37-
25-
20-
15-
SUMO2/3
Control
Ube2i
250-
150-
100-
75-
50-
37-
25-
20-
15-
Coomassie
E
Performance during training
% Time Freezing
100
75
50
25
0
*
1
2
3
4
5
One Minute Avgs
Female Control
Female Ube2i-siRNA
Male Control
Male Ube2i-siRNA
F
Retention during test
Control
Ube2i-siRNA
% Time Freezing
60
40
20
0
*
Male
Female

Figure 3. Protein SUMOylation selectively regulates fear memory formation in the amygdala of males. (**A**) Male and female rats were injected with *Ube2i* siRNA or Control (10 μM each) into the amygdala and 5 days later trained to contextual fear conditioning. Twenty four hours later animals were tested for retention to the context cue. Amygdala (BLA) tissue was collected 1 hr after the test session to confirm successful *Ube2i* knockdown. (**B-D**) Successful knockdown of *Ube2i*, the gene coding for the essential SUMO E2 ligase, in rat B35 cells *in vitro* (**B**; N = 4 per group) and amygdala *in vivo* (**C**; N = 5-6 per group, males), which was associated with a reduction in SUMOylation levels in the amygdala (**D**; N = 5-6 per group, males). Coomassie blue was used as a loading control for SUMOylation levels. (**E**) There were no effects of siRNA or Sex during training, but there was a Sex x Time Interaction. Males are dotted lines; females are solid lines. (**F**) During test *Ube2i* knockdown impaired memory in males but had no effect in females (N = 6 per group for males, 7-8 per group for females). * $P < 0.05$ from Control.

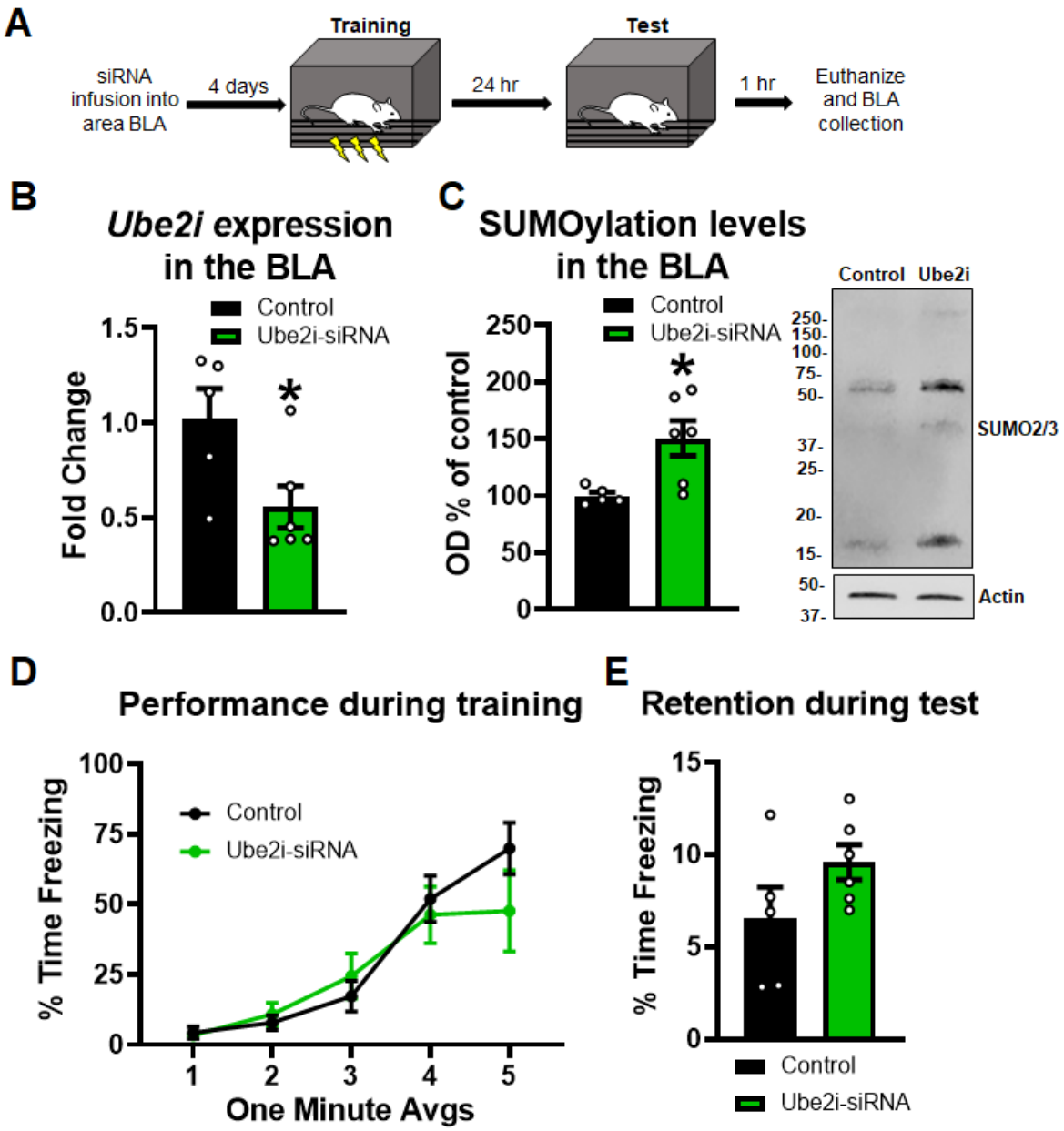

Figure 4. Inhibition of protein SUMOylation via high concentration of siRNA enhances fear memory formation in the amygdala of females. (**A**) Female rats were injected with a high concentration (20 μM) of *Ube2i* siRNA or Control into the amygdala and 4 days later trained to contextual fear conditioning. Twenty-four hours later animals were tested for retention to the context cue. Amygdala (BLA) tissue was collected 1 hr after the test session to confirm successful *Ube2i* knockdown. (**B-C**) Successful knockdown of *Ube2i*, the gene coding for the essential

SUMO E2 ligase, in the female amygdala (**B**; N = 5-6 per group), which was associated with an increase in SUMOylation levels (**C**; N = 5-6 per group). Actin was used as a loading control for SUMOylation levels. (**D**) There were no effects of siRNA during training. (**E**) During test *Ube2i* knockdown enhanced memory in females (N = 5-6 per group). * $P < 0.05$ from Control.

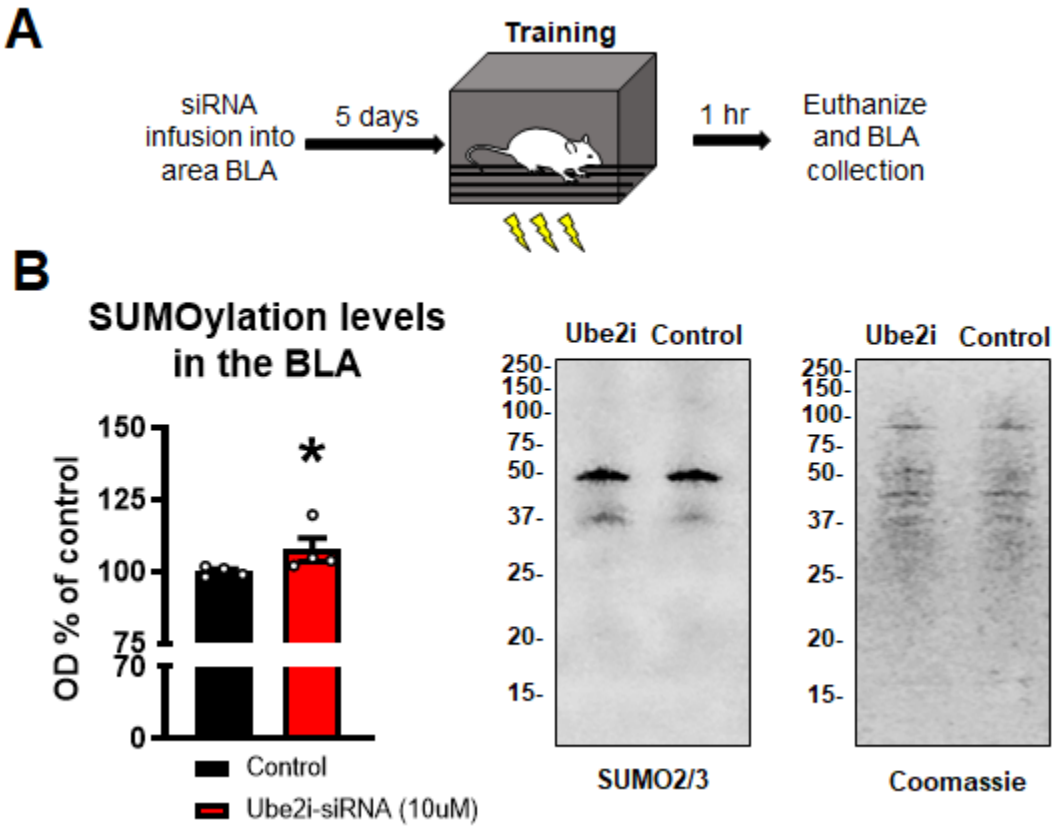

Figure 5. Enhancement in SUMOylation in the female amygdala following fear conditioning with *Ube2i* knockdown. (**A**) Female rats received an injection of standard concentration (10μM) *Ube2i* siRNA or Control into the amygdala. Five days later animals were trained to contextual fear conditioning and euthanized 1 hr later for western blot analysis (N = 4 per group). (**B**) Knockdown of *Ube2i* resulted in an increase in SUMOylation levels following fear conditioning. * $P < 0.05$ from Controls.

CHAPTER THREE: OVERALL CONCLUSIONS AND FUTURE WORK

3.1 Overall Conclusions

We found novel sex differences in the requirement for and role of protein SUMOylation in fear memory formation in the amygdala. While both sexes showed global increases in SUMOylated proteins following fear conditioning, females were found to have higher resting levels in the amygdala. SUMOylation-specific proteomic analysis revealed that only females have increased targeting of individual proteins by SUMOylation following fear conditioning, though males did show a loss of SUMOylation at one protein after training. For both sexes different heat shock proteins were targeted by SUMOylation, which previously had been implicated in fear memory formation, suggesting a potential common functional role of SUMOylation across the sexes. We further determined that protein SUMOylation differentially regulates fear memory formation in males and females by reducing *Ube2i* expression in the amygdala *in vivo* in both sexes. The result of this was decreased fear memory in males but enhanced fear memory in females (when a higher concentration of siRNA was used). Further investigation revealed a surprising increase in SUMOylation levels in females following our siRNA manipulation, suggesting there was a compensatory effect for loss of *Ube2i*. This suggests that SUMOylation may play a more critical role in normal cellular functioning in the amygdala of females than males. Collectively, these data reveal novel, sex-specific differences in the requirement and role of protein SUMOylation in the amygdala during fear memory formation and enhance our understanding of the molecular mechanisms involved in fear memory formation.

3.2. Future Work

While our work has produced interesting, novel data, it has also opened up future avenues of research. For example, future studies may benefit from tracking the estrous cycle in females as it

is possible that the sex differences in basal and learning-related SUMOylation may be related to estrogen signaling in females. Important here is that we did not track the estrous cycle because such procedures can be stressful for the rats, and could interact with anxiety produced by our fear conditioning procedure. Thus, it would be a beneficial avenue of future investigation to test if SUMOylation levels vary in the amygdala of females across the estrous cycle. Further, identifying the functional significance of SUMO targeting the identified proteins and any sex differences therein would also be a valid query for future work. We were able to identify a few of the targets of SUMOylation following learning, however this raises unanswered questions as to the functional significance of these targets, i.e., what does SUMOylation do to these proteins to regulate the formation of fear memories. In addition, cell-type specific sex differences could be investigated. While our studies were specific to the amygdala and our siRNA knockdown was neuron-preferring, it was not cell-type specific. It is currently unknown if non-neuronal SUMOylation plays an important role in the fear memory formation process and if there are any cell-type specific difference between the sexes. Together, such studies would help to provide a more detailed understanding of the sex-specific role of protein SUMOylation in fear memory formation.

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