RAPAMYCIN ATTENUATES RECONSOLIDATION OF A BACKWARD CONDITIONED FEAR MEMORY

by © Jared Trask

A thesis submitted

to the School of Graduate Studies in partial fulfillment of the

requirements of the degree of

Master of Science, Department of Psychology, Faculty of Science

Memorial University of Newfoundland

May 2024

St. John's, Newfoundland and Labrador

Abstract

The mechanistic target of rapamycin (mTOR) kinase has been implicated in the consolidation and reconsolidation of aversive memories. Most studies in this area employ a forward conditioning (FW) paradigm (Pavlovian or auditory fear conditioning) which consists of a conditioned stimulus (CS) that precedes the unconditioned stimulus (US). Little is known, however, about the neurobiological underpinnings of the reverse, backward (BW) conditioning paradigms, particularly in female mice. In BW conditioning, the CS does not become directly associated with the US; it instead evokes conditioned fear by reactivating a memory of the conditioning context and indirectly retrieving a memory of the aversive US. Our studies confirm and extend the findings on BW-conditioned fear memory processes to female mice. We show that conditioned freezing to a BW CS is mediated by fear to the conditioning context. Furthermore, the mTOR inhibitor rapamycin (RAPA), when given immediately following BW conditioning, impairs consolidation of both cued and contextual fear memory. Similarly, RAPA given following retrieval of a BW CS blocks context recall and CS retrieval is necessary to see the effects of RAPA on context memory recall. In sum, our study provides novel evidence that indirect retrieval cues are sensitive to RAPA in female mice.

Keywords: Backward conditioning; Females; Reconsolidation; Consolidation; Fear memory; Post-traumatic stress disorder; Rapamycin; mTOR

General Summary

Generally, studies in this field use a forward conditioning procedure, in which the tone precedes the shock to induce fear. Less is known about the how the reverse, or backward conditioning, paradigms work. In backward conditioning, the cue does not become directly associated with the shock, but instead fear occurs by reactivating a memory of the conditioning context and then indirectly retrieving a memory of the shock. Our work extends the findings on backward conditioned fear memory processes to female mice. We show that fear, as measured by freezing behaviour to a backward cue pairing (shock then tone), is caused by fear to the conditioning context. Furthermore, the FDA approved drug rapamycin, when given immediately following the backward pairing, impairs memory formation of both the cue and the context. Overall, our study provides new evidence that indirect cues are sensitive to the drug rapamycin in female mice.

Co-Authorship Statements

Dr. Evan Preisser helped run the three-way interactions statistics and co-authored the results section of this thesis. Dr. Jacqueline Blundell aided in figure design using Prism, experimental design and proofreads/editing. Haley Rideout helped run experiments. All other aspects including practical work, other analyses and authorship of other sections were completed by Jared Trask.

Prior Publication of Material

Parts of this thesis and its data have been previously published in the journal Psychopharmacology. The introduction and discussion sections are greatly modified from the published versions. The materials/methodology and results sections have some minor changes made, mainly shortening the length of them.

Trask, J., MacCallum, P. E., Rideout, H., Preisser, E. L., & Blundell, J. J. (2024). Rapamycin attenuates reconsolidation of a backwards-conditioned aversive stimuli in female mice. Psychopharmacology, 241(3), 601–612. https://doi.org/10.1007/s00213-024-06544-6

Acknowledgements

l would like to wholeheartedly thank the following, for which this thesis would have not been possible. Firstly, I would like to thank my supervisor Dr. Jacqueline Blundell, for her expertise and advice in the development and completion of this thesis, as well as helping me grow both as an academic and a person. To Phillip MacCallum, without his expertise and troubleshooting, no experiment in our lab would come to fruition, and his help in implementing and conducting these experiments is greatly appreciated. I would also like to sincerely thank my mother Cheryl Trask for always providing meals for me, along with my father Doug Trask for always being there when I need it and my partner, Laura Dawe for her unwavering support and patience while I have pursued my academic endeavours. Furthermore, I acknowledge that the lands on which Memorial University's campuses are situated are in the traditional territories of diverse Indigenous groups and I acknowledge and respect the diverse histories and cultures of the Beothuk, Mi'kmaq, Innu, and Inuit of this province. Lastly, I would like to thank Drs. Darlene Skinner and Francis Bambico for agreeing to be on my thesis supervisory committee, and the granting agencies, Natural Science and Engineering Research Council, and Memorial University School of Graduate Studies for financially supporting these experiments.

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List of Abbreviations

4EBP1	Eukaryotic Initiation Factor 4E-Binding Protein I
AMPA	Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
APA	American Psychiatric Association
APV	D, L-2-amino-5-phosphonovalerate
ANOVA	Analysis of Variance
ANISO	Anisomycin
ASR	Acoustic Startle Response
BDNF	Brain Derived Neurotrophic Factor
BLA	Basolateral Amygdala
BNST	Bed Nucleus of the Stria Terminalis
BW	Backward (Backward Conditioning)
C	Control
CAMKII	Alpha Calcium/Calmodulin-Dependent Protein Kinase
CeA	Central Nucleus of the Amygdala
CHD	Chronic Heart Disease
CORT	Corticosterone
CS	Conditioned Stimulus
DH	Dorsal Hippocampus
DNA	Deoxyribonucleic Acid
DREADDS	Designer Receptors Exclusively Activated by Designer Drugs

eEFK2	Eukaryotic Elongation Factor-2 Kinase
e-GRASP	Electronic GFP Reconstitution Across Synaptic Partners
ERK2	Extracellular Signal-Regulated Kinase 2
FKBP5	FK506 Binding Protein 5
FST	Forced Swim Test
FW	Forward (Forward Conditioning)
GC	Glucocorticoid
GFP	Green Fluorescent Protein
GR	Glucocorticoid Receptor
HPA	Hypothalamic-Pituitary-Axis
IHC	Immunohistochemistry
ISI	Interstimulus interval
KLK8	Kallikrein-8
PE	Prolonged Exposure Therapy
PET	Positron Emission Tomography
LSD	Least Significant Difference
LTM	Long-Term Memory
LTD	Long-Term Depression
LTP	Long Term Potentiation
MAP2	Microtubule-Associated Protein 2
MAPK	Mitogen-Activated Protein Kinase
MDD	Major Depressive Disorder
mRNA	Messenger RNA

mTOR	Mechanistic (Mammalian) Target of Rapamycin
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NMDA	N-Methyl D-Aspartate
NR3C1	Nuclear Receptor Subfamily 3 Group C Member 1
NS	No Shock
OCD	Obsessive Compulsive Disorder
p70s6K/S6K1	Ribosomal Protein S6 Kinase Beta-1
PE	Prolonged Exposure
PET	Positron Emission Tomography
PFC	Pre-frontal Cortex
PKA	Protein Kinase A
PDCD4	Programmed Cell Death Protein 4
PTSD	Post-Traumatic Stress Disorder
RAPA	Rapamycin
SSRIs	Selective Serotonin Reuptake Inhibitors
SNRI	Selective Norepinephrine Reuptake Inhibitor
STM	Short-Term Memory
TTX	Tetrodotoxin
TSC	Tuberous Sclerosis Complex
US	Unconditioned Stimulus
VEH	Vehicle
VTA	Ventral Tegmental Area

Theses Canada Participation

I, Jared Trask, authorize Theses Canada to publish this thesis.

1.0 Introduction

1.1 Post-Traumatic Stress Disorder (PTSD)

The symptomology of post-traumatic stress disorder (PTSD) is hallmarked by the experience of a traumatic or stressful event which triggers a cascade of neurobiological events culminating into a characteristic cluster of symptoms persisting for a minimum of four weeks. These symptoms may include intrusive thoughts or feelings, the avoidance of triggers, hyperactivity, cognitive or mood alternations, and dissociation, either depersonalization or derealization (American Psychiatric Association, 2017). A traumatic event is defined as any perceived or real threat, death, severe injury or act of sexual violence experienced via direct exposure, witnessing, or even learning of the event (Courtois & Brown, 2019). Sociodemographic surveys estimate traumatic events to impact nearly 90% of the general population at least once during their lifetime (Kessler et al., 2005a).

On a societal level, the occurrence of traumatic events translates into an overall prevalence rate of PTSD of 5.7% for males and 12.8% for females, with any typical U.S citizen having approximately a nine percent chance of developing PTSD by the age of 75 (Breslau et al., 2000; Kessler et al., 2005a; Kessler et al., 2005b; Kilpatrick et al., 2013). Comparable prevalence rates have been observed in Canada and it is estimated that the average Canadian experiences about 2.5 traumatic events over the course of their lifetime (Kessler, 2000). These rates may be increased in developing countries due to higher occurrences of political or ethnic violence (Van Ameringen et al., 2008). Historically, it was seemingly random whether a traumatic event would cause the onset and development

of PTSD in an individual, but through the detailed study of trauma survivors and combat veterans, coupled with extensive animal model work, research has begun to unravel the risk factors associated with its pathophysiology (Bailey et al.,2013).

1.1.1 Risk Factors

Previous epidemiological studies have demonstrated stress exposure to be insufficient as the sole etiological factor required to cause the onset and development of PTSD (Coley et al., 2019). In humans, a variety of social factors, genetic polymorphisms, and epigenetic changes interact and contribute to the heterogeneous manifestation of PTSD (Franklin et al., 2010). This is especially so for individuals who have experienced early life adversity, as these effects are magnified (Almli et al., 2013; Kappeler & Meaney, 2010; Mehta & Binder, 2012). In both Holocaust survivors and survivors of the Rwandan genocide, adult offspring are more likely to have psychiatric conditions such as anxiety, depression and mood disorders (Yehuda et al., 1998). Interestingly, low cortisol has been associated with PTSD in both offspring and their parents (Yehuda et al., 2000). In fact, follow-up studies by Vukojevic and colleagues (2014) have shown NR3C1 (glucocorticoid receptor) methylation in males to be linked to a less intrusive memory of a traumatic event and decreased PTSD risk. In a meta-analysis, Ozer et al. (2008) identified that other than dissociation occurring immediately following a traumatic event (peritraumatic dissociation), having a low-level of social support, as well as a familial history of psychological disorders and prior trauma are the strongest predictors of PTSD onset. This underlying pre-existing vulnerability is exacerbated by the number and nature of stressful exposures as for example, repeated sexual and physical assaults result in a

more debilitating condition (Kilpatrick et al., 2013). Furthermore, differences in neurobiology between sexes may help explain the differences in rate of onset, symptomology and efficacy of treatment seen at the clinical level of PTSD diagnosis and treatment (Shansky, 2015). For example, in normally cycling premenopausal women, high estrogen levels are correlated with an attenuation of the brain deactivation and typical negative mood responses to psychosocial stress (Albert et al., 2015). These results suggest that the menstrual cycle-related fluctuations in stress vulnerability may be associated with the greater risk of developing PTSD for women. Additionally, despite males having a higher incidence of lifetime traumatic events, females are still more likely to develop PTSD following a traumatic event, illuminating the apparent sex differences in its prognosis (Shansky, 2015). It is also important to note that sex and gender expression have multiple ways of affecting trauma and PTSD (Christiansen & Berke, 2020). Specifically, gender roles have been found to influence hormone levels and thus, the expressions of sex (Johnson et al., 2009). But more broadly, the impact of sex and gender in PTSD include combinations of genetic predisposition and hormonal influences along with individual gender roles and whether or not these are at odds with what is generally recognised by the social surroundings as being acceptable masculine or feminine behaviour.

1.1.2 Impacts

For those diagnosed with PTSD, traumatic events can evoke a maladaptive stress response producing a multitude of further negative sequelae such as reoccurring nightmares, isolation and irritability rooted in underlying neurobiological disruptions

(American Psychiatric Association, 2017). These symptoms are only the tip of the iceberg for patients suffering with PTSD as they are often accompanied by the onset of additional psychological co-morbidities such as major depressive disorder (MDD), obsessive compulsive disorder (OCD) and substance abuse disorder (SUD; Breslau, 2002; Kessler, et al., 2005a). Collectively, the aftermath of a traumatic event can lead to a long-term impairment in life trajectory, illustrated by patients who are more likely to quit high school, have teen childbirth and be unemployed, translating into potentially life-altering experiences such as suicide ideation or attempts (Kessler, 2000). The presence of any of these circumstances, in conjunction with PTSD symptomology, can impair work productivity and lead to unhealthy behaviours such as smoking, overeating, substance abuse and unsafe sex, ultimately resulting in higher rates of homelessness, divorce and poor child-parent relationships (Tanielian, 2009). Furthermore, longitudinal studies show how the psychological afflictions of PTSD create lasting physical health problems which include impaired sleep initiation and maintenance, impaired extinction of fearful memories and an increased risk for chronic heart disease (CHD), especially in women (Edmondson et al., 2013; Kobayashi et al., 2012; Polta et al., 2013). On a societal level, the impacts of PTSD are colossal, effectuating an overall loss of productivity estimated by Breslau et al. (1998) to equate to 3.6 days of lost work time per month and three billion dollars annually in lost revenue in the U.S. alone (see also Graves, 2020). Moreover, the suffering originating from trauma exposure creates a significant burden on quality of life for patients, highlighting the critical need to identify potential biomarkers, advance the knowledge of PTSD pathophysiology, and improve targeted therapeutic modalities (Pitman et al., 2012; Tanielian, 2009).

1.1.3 Current Treatments for PTSD

Due to the heterogeneous nature of the disorder, a wide spectrum of treatments are employed including psychological interventions such as cognitive-behavioural therapy, eye movement desensitization and reprocessing therapy and prolonged exposure (PE) therapy, as well as pharmacological treatments such as selective serotonin reuptake inhibitors (SSRIs) and the selective norepinephrine reuptake inhibitor (SNRI), Venlafaxine (Bufka et al., 2020; Martin et al., 2021). Current treatments tend to focus on cognitive disruptions and reducing the patients' maladaptive fight-or-flight response (Courtois & Brown, 2019). The 'gold' standard treatment of PTSD is PE therapy, which has proven quite effective in reducing negative cognitions about the self and diminishing symptom severity for PTSD patients (Kumpula et al., 2017; Raut et al., 2022). Despite the effectiveness of this treatment, patients must endure nine to fifteen sessions, one and a half to two hours long, with some patients unable to complete it due to discomfort and others still retaining residual PTSD symptoms (Powers et al., 2010). To overcome this, preclinical research applying biological agents to augment therapeutic gains using rodent models is beginning to be applied to psychotherapies to increase efficacy and effectiveness (Zoellner et al., 2017). In fact, a meta-analysis revealed that compared to PE therapy alone, a therapeutic modality including a pharmacological intervention led to lower PTSD symptoms and a marginally lower dropout rate following the treatment (Zhou et al., 2020). Another newly developed therapeutic strategy is targeting reconsolidation of the fear memory. Targeting reconsolidation to produce long-lasting disruption to memory may be a beneficial therapeutic strategy for memory or fear-based psychiatric disorders (Chen et al., 2021; Raut et al., 2022).

1.2 Animal Models of Fear Memories and PTSD

Animal models enable researchers to decipher the complicated biological principles underlying development, behavior, and health (Phillips & Roth, 2019). With respect to PTSD research, animal models are useful because they permit 1) exposure to a severe stressor in a controlled fashion; 2) study of the effect of stress on affect as it develops; and 3) study of pharmacological and other treatments which may be difficult to test in humans but can be easily evaluated in animals. Although it is not possible to model all aspects of PTSD in animals, several experimental paradigms have been developed which demonstrate PTSD-like symptoms.

The fear conditioning paradigm is most commonly used to model the intrusive fear memories associated with PTSD (Norrholm & Jovanovic, 2018). Fear conditioning occurs when a neutral stimulus (i.e., tone or context) elicits defensive behaviours (i.e., freezing, conditioned response, CR) when the neutral stimulus (conditioned stimulus, CS) was previously paired with an aversive stimulus (i.e., shock; unconditioned stimulus, US) (Dexter & Merrill, 1969; Maren, 2001). The learned association is reflected in the subjects' behavior upon subsequent re-exposure to the previously neutral stimulus or the training environment. By using biochemical assays in tandem with behavioral analysis, investigators can obtain a large amount of data that describe multiple aspects of learning and memory (McGuire et al., 2017). In rodents, fear memory is assessed based on the duration of freezing behaviour during re-exposure to the cue/context for a set duration of time (Daviu et al., 2012). Both cued (auditory) and contextual fear conditioning have been used to model aspects of PTSD. Fear conditioning is an appropriate model of PTSD

because not only does it demonstrate a learned fear association as seen in PTSD patients, but it also demonstrates a long-lasting persistence of those fear memories (Orr et al., 1993; Orr et al., 2000; Rothbaum & Davis, 2003).

1.3 The mTOR Pathway

A pathway critical to fear memory processes is the mechanistic (formerly named mammalian) target of rapamycin (mTOR). mTOR is a highly conserved kinase present in nearly all cells and vital to cell growth, metabolism and protein synthesis (Switon et al., 2017). mTOR activity is regulated via a variety of mechanisms including N-methyl-D-aspartate receptors (NMDA-R), a-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid receptors (AMPA-R), brain-derived neurotropic factor (BDNF), dopaminergic receptors and metabotropic glutamate receptors (Lipton & Sahin, 2014). The highlighted receptors are some of the most important effectors for long-term potentiation (LTP) and long-term depression (LTD), both of which are important features of long-term memory storage (Mafei, 2018). mTOR is comprised of two signaling complexes, mTORC1 and mTORC2. While mTORC1 is sensitive to environmental stimuli, such as amino acids but also glucose and oxygen, it controls protein translation, autophagy and numerous other cellular processes (Switon et al., 2017). mTORC2, first thought to be RAPA-insensitive but can be indirectly inhibited by the compound, regulates organization of the actin cytoskeleton and is an effector of insulin downstream (Fu & Hall, 2020; Szwed et al., 2021).

mTORC1 is closely associated with the extracellular signal-regulated kinase 2 (ERK2) in its role in protein synthesis with ERK2 phosphorylating and inhibiting the

tuberous sclerosis complex (TSC), the molecular brake on mTORC1 (Gebauer & Hentze, 2004). Downstream mTORC1 phosphorylates eukaryotic initiation factor 4E-binding protein I (4E-BP), which promotes the formation of cap-binding complex elF4E-H, and ribosomal protein S6 kinase beta-1 (S6K1), which in turn phosphorylates programmed cell death protein 4 (PDCD4) (Switon et al., 2017). This molecular cascade sets the stage for S6K1 and ERK2 to phosphorylate and inhibit the eukaryotic elongation factor-2 kinase (eEFK2), releasing the molecular brake on the factor eEF2, required in the elongation step of protein synthesis (Shrestha & Klann, 2022).

The protein synthesis inhibitor, RAPA, alters rodent behavior through the inhibition of the mTOR pathway. Studies have investigated its impact on various behavioral paradigms in rodents, highlighting its multifaceted influence on neurochemistry and behavior. For instance, Hoeffer & Klann (2010) delved into RAPA's role in synaptic plasticity and memory formation, showcasing its capacity to alter learning behaviors in rodents. Moreover, the work by Neasta et al. (2014) underscored RAPA's involvement in reward-related behaviors and addiction processes, indicating its impact on motivational aspects in rodent behavior. The varying effects of mTOR inhibitors, especially RAPA, on the brain and behavior depend on several factors, including species, subject age, administration route, and dosage. Studies using rats treated with a higher dose of RAPA (10 mg/kg) did not exhibit anxiety-related behavior under red light, but under white light conditions, moderate doses (1 & 3 mg/kg) resulted in increased anxiety-related behaviors in the open field (Lu et al., 2015). However, others have reported opposite effects, as the chronic inhibition of mTOR via oral RAPA administration (2.24

mg/kg) exhibited positive effects on learning, memory, and demonstrated anxiolytic and antidepressant effects in older C57BL/6J mice (Halloran et al., 2012). It is clear that RAPA's behavioural effects need to be better delineated through further examination of different species, sex and test conditions The following sections describes fear memory processes (e.g., consolidation, reconsolidation, extinction) and the role of the mTOR pathway following forward and backward conditioning.

1.4 Fear Memory Processes: Consolidation

Memory can be operationalized as the ability of an organism to capture, retain and recall information (Davis & Squire, 1986; Prerana & Klann, 2022). Memory consolidation is the process of stabilizing a labile short-term memory (STM) and has two distinct stages (McKenzie & Eichenbaum, 2011; Silva et al., 1998). The first, cellular consolidation, takes places within the first few hours to one day afterward and requires new gene expression and *de novo* protein synthesis leading to changes in neural circuitry (Barry & Commins, 2017; Genzel & Wixted, 2017). The second, systems consolidation, takes much longer (three to four weeks in rodents) with the memory becoming less hippocampal dependent and more reliant on cerebral cortices (Dudai, 2012). According to the unified engram complex theory, engrams of a specific memory are distributed among multiple functionally connected brain regions, becoming more diffuse over time (Omid et al., 2021; Roy et al., 2022).

1.4.1 Consolidation, mTOR and Forward Conditioning

Classical or forward conditioning (FW) is well-known as one of the leading learning paradigms uncovered by Ivan Pavlov while he was studying the gastrointestinal

reflexes of the dog (Pavlov et al., 1928). Pavlov noticed that dogs salivated in response to stimuli that were consistently present *before* the food arrived, such as the sound of a food cart arriving. He conducted an experiment in which he sounded a metronome just before providing food to the dogs to test his idea and the dogs gradually began to salivate just by hearing it. In aversive conditioning, the US is one to which the animal has an intrinsic and reflexive fear response usually manifesting as freezing or tonic immobility in both rodents and humans (Maren, 2001). A conditioned stimulus (CS = tone) is a stimulus that can eventually trigger a conditioned response (CR = freezing to the tone) when re-exposed to the CS (in the absence of the US). In FW (e.g., cued auditory fear), the CS must be presented before the US (co-terminated). In first-order conditioning, a CS (e.g., tone) is paired with the delivery of a foot shock. In higher-order conditioning, pairings of the higher-order stimulus (e.g., light) with the first-order stimulus (e.g., tone) can occur either prior to (i.e., sensory preconditioning) or after (i.e., second-order conditioning) first-order conditioning (Gostolupce et al., 2021). Generally, fear memory in rodents is assessed based on the duration of freezing behaviour during re-exposure to the cue/context for a set duration of time (Daviu et al., 2012; Rehman et al., 2022). With contextual fear conditioning, the US (e.g., a foot shock) is paired with a neutral context and, because of this pairing, exposure to the previously neutral context elicits a fear response indexed by freezing behaviour (Maren et al., 2013; Phillips & LeDoux, 1992). Impairments in fear learning during and following trauma may be involved in the etiology of PTSD by contributing to the inappropriate recall of traumatic memories (Acheson et al., 2012; Liberzon & Abelson, 2016).

Targeting consolidation, while potentially less ethologically relevant due to the inability of clinicians to determine who will (or will not) develop PTSD, still provides valuable information about the way trauma-dependent fear memories are stored. A variety of agents targeting different steps in the protein synthesis 3-stage process have been used to interrupt consolidation. For example, the protein synthesis inhibitor cycloheximide immobilizes ribosomes in the elongation step, impairing long-term memory (LTM) consolidation, while RAPA inhibits the mTORC1 complex affecting initiation and elongation, impairing LTM consolidation (Jobim et al., 2012; MacCallum et al., 2014; Lana et al., 2017; Nikitin et al., 2019). Surprisingly, there are reported differential vulnerabilities of cued and contextual fear memories to RAPA. While studies have shown that RAPA disrupts the consolidation of hippocampal-dependent fear memories (contextual fear memories; Gafford et al., 2013; Han et al., 2016), others have reported that hippocampal-independent forms (cued) are unaffected (Glover et al., 2010; Luyten et al., 2021). However, our lab has repeatedly shown that consolidation of both context and cue memories are RAPA sensitive (MacCallum et al., 2014; MacCallum et al., 2020). The consolidation of contextual fear memories appears to require mTOR-dependent translation in the dorsal hippocampus, while cued fear memories may rely more on cortical areas such as the entorhinal cortex, offering a possible explanation for why local injections into the hippocampus fail to produce such an effect (Glover et al., 2010). Within the basolateral amygdala (BLA), the consolidation of first-order fear memories requires signaling from the Extracellular Signal-Regulated Kinase/Mitogen-Activated Protein Kinase (ERK/MAPK) pathway, the PKC pathway and *de novo* protein synthesis (Lay et al., 2018; Schafe et al., 2000; Schafe & LeDoux, 2000). Specifically, the

activation of both mTOR and ERK/MAPK pathways initiate protein translation and contribute to synaptic plasticity to support new memories with the disruption of these pathways in the amygdala resulting in decreased protein expression in the hippocampus and auditory regions which receive inputs from the amygdala (Lonergan, 2012). Overall, the ERK/MAPK and mTOR signaling pathways, particularly in the amygdala and hippocampus, are critical following fear conditioning in the consolidation of contextual and auditory fear memories.

Many rodent studies have investigated sex differences in Pavlovian fear conditioning and memory retrieval showing that overall, females have a reduced contextual fear conditioning response as indicated by decreased freezing during the retrieval of contextual fear memory (Barker & Galea, 2010; Chang et al., 2009; Daviu et al., 2014; Gresack et al., 2009; Gupta et al., 2001; Maren et al., 1994; Ribeiro et al., 2010; Wiltgen et al., 2001). In some of this research, females show more locomotor activity than males, raising the possibility that the reduction in contextual fear shown by females simply reflects the fact that they are more active (Aguilar et al., 2003; Day et al., 2016). The literature is more mixed for cued fear conditioned with inconsistent effects and even several studies citing no sex differences (Baran et al., 2009; Chen et al., 2014; Clark et al., 2019; Fenton et al., 2016; Fenton et al., 2014; Maren et al., 1994; Markus & Zecevic, 1997). It is thought that the estrous cycle may impact how the fear memory is consolidated leading to enhanced fear learning as increased estradiol results in increased freezing compared to controls in mice (Matsumoto et al., 2018). Interestingly, the activation of the dorsal hippocampal mTOR signaling pathway is necessary for estradiol

(E2) to enhance object recognition memory consolidation and that E2-induced mTOR activation is dependent on the upstream activation of ERK signaling (Florido et al., 2021). In addition, dorsal hippocampal ERK and mTOR activation appears necessary for progesterone to facilitate memory consolidation, as suggested by the fact that inhibitors of both pathways infused into the dorsal hippocampus immediately after training blocked the progesterone-induced enhancement of object recognition (Orr et al., 2012). Collectively, these studies provide a solid basis for further investigation of females, and hormone levels, on the consolidation of fear memories.

1.4.2 Consolidation and Backward Conditioning

Following Pavlov's initial discovery of the phenomenon involving cued conditioning to a stimulus, researchers began investigating the timing of CS/US cues leading to a backward conditioning (BW) procedure, where the US is followed by a CS (Pavlov et al., 1928). In BW conditioning, the CS does not become directly associated with the US but evokes conditioned fear by reactivating a memory of the conditioning context and indirectly retrieving a memory of the aversive US (Chang et al., 2004; Wagner & Terry, 1975). BW conditioning paradigms (US: shock, CS: light) have the capability to be as biologically relevant as forward ones, capable of acting as a conditioned inhibitor of analgesia in rats, giving support that they follow similar Pavlovian mechanisms as classical FW (Wiertelak et al., 1992). Interestingly, taste aversion studies have found that while age is a powerful variable for the saliency of forward pairings, with rats becoming more susceptible to pairings as they grow older, it plays no substantial role in BW conditioning paradigms (Minnier et al., 2007; Misanin et

al., 2002). BW associations also occur in many species; however, they are generally less salient than forward associations. For instance, bees receiving 15 second excitatory BW pairings show reduced performance on an olfactory association task after a subsequent single forward pairing trial (Dacher & Smith, 2008). In humans, BW excitatory conditioning has also been observed, demonstrating that the pairing can subsequently be used in conditioned reinforcement paradigms (Prével et al., 2016).

Although few studies have examined the neural targets associated with BW conditioning paradigms, recently, Sietz et al. (2022) showed that a BW reward-cue conditioning procedure produced both excitatory and inhibitory associations that can be abolished by ventral tegmental area (VTA) dopamine neuron inhibition. Furthermore, the expression of BW fear conditioning, requires the bed nucleus of the stria terminalis (BNST). Using male and female rats, Ressler et al. (2020) showed NMDA receptor antagonism in the BNST prior to fear conditioning significantly reduced freezing to an unpredictable BW CS, but not a predictable FW CS. This finding highlights that NMDA receptors can act differentially in the acquisition of conditioned fear depending on whether the threat is predictable or unpredictable (Ressler et al., 2020). To our knowledge, the role of mTOR on consolidation of BW conditioning is not known.

1.5 Fear Memory Processes: Reconsolidation

Memory reconsolidation is the process in which retrieval causes a previously consolidated memory to return to a labile or malleable state (Forcato et al., 2009; Lee et al., 2017). Once the memory is in a labile state, it is possible to be modified or even erased following the administration of protein synthesis inhibitors (Haubrich & Nader,

2018). In addition to modifying the original memory trace, retrieval can strengthen or weaken existing memories (Alberini & Ledoux, 2013). Below is a description of reconsolidation in FW and BW conditioning procedures.

1.5.1 Reconsolidation, mTOR and FW Conditioning

Over two decades ago, Nader et al. (2000) showed that the conditioned fear response in rats could be eliminated by administering a protein synthesis inhibitor in the lateral amygdala immediately after the reactivation of a previously consolidated FW fear memory. Since this landmark study, which reintroduced similar notions of reconsolidation from the 70s and 80s (Lewis, 1979), reports have suggested that its underlying mechanisms are similar to consolidation (Alberini, 2005: Bang et al., 2018), although others have reported differences between the two processes (Gisquet-Verrier & Riccio, 2018). Reconsolidation results in the modification of stored information, specifically memory strength (Forcato et al., 2014; Sara, 2000).

Contextual fear memory reconsolidation appears to be reliant on mRNA synthesis as studies showing local injections of mRNA inhibitors in the hippocampus impair subsequent recall (De Oliveira Alvares et al., 2008: Lee et al., 2004). In addition, the genetic disruption of cAMP responsive element-binding protein (CREB)-mediated transcription blocks the reconsolidation of a contextual fear memory (Mamiya et al. 2009). Furthermore, the inhibition of gene expression (and henceforth protein synthesis) during memory reconsolidation results in the disruption of memory suggesting that retrieved memories are in a labile state similar to STM (Kida, 2018; Nader et al., 2000). Reconsolidation appears to rely upon β-adrenergic signalling via NMDA receptors and

protein kinase A (PKA) signalling that increase neuronal membrane excitability when a contextual fear memory is reactivated (Finnie & Nader, 2012; Lim et al., 2018). Our lab, and others have reported that reconsolidation can be blocked with a systemic or intra-hippocampal injection of RAPA (Blundell et al., 2008; Gafford et al., 2011; MacCallum et al., 2014). Finally, de la Fuente et al. (2019) used positron emission tomography (PET) imaging alongside designer receptors exclusively activated by designer drugs (DREADDS) to direct neuronal inhibition during a contextual fear conditioning paradigm. They demonstrated the involvement of key brain region such as the hippocampus, lateral neocortex and their projections to the amygdala during reconsolidation (de la Fuente et al., 2019).

As with contextual fear conditioning, reconsolidation of a cued fear memory is dependent on the mTORC1 pathway, specifically requiring the simultaneous association of eIF4E to eIF4G, as well as S6K1 activity (Blundell et al., 2008; Huynh et al., 2014; MacCallum et al., 2014). Furthermore, the medial prefrontal cortex (mPFC) also appears to be involved in cued fear memory reconsolidation with older more remote memories being more reliant on cortical association areas than newer memories. (Mamiya et al., 2009; Stern et al., 2014).

Limited research exists on reconsolidation in females. Flint et al. (2007) compared male and female rats given cycloheximide following reconsolidation of a long-term spatial memory. During the probe trial, males had shorter latencies to the platform than females, indicating that females were more susceptible to memory impairment. In humans, Drexler et al. (2016) concluded that the stress hormone cortisol, in combination

with reactivation of the memory, does not enhance fear memory reconsolidation in women, contrast to their previous work from their group which showed cortisol to enhance fear reconsolidation in men (Drexler et al., 2015; Drexler et al., 2016). Overall, it is apparent more work must be undertaken to delineate whether there are sex differences in reconsolidation processes. Specifically, determining the roles (if any) that hormones such as progesterone, cortisol/corticosterone and estrogen play will increase the translatability of reconsolidation-based therapies from animal work to clinical applications.

1.5.2 Reconsolidation and BW Conditioning

There is also little research examining reconsolidation of a BW conditioned stimuli. Recently, Ressler et al. (2021), using a combination of sophisticated behavioral approaches, engram tagging and chemogenic manipulations in male rats, showed that presentation of the CS following BW conditioning reactivates the memory of the CS→context association. In turn, this indirectly reactivates the memory of the context→US association, leading to activation of the hippocampus. Furthermore, a viral-based approach was employed to label the contextual memory engram with mCherry, and the subsequent presentation of the cue in a memory-reactivation session led to activation of the same engram, yielding molecular evidence to support the indirect reactivation of the memory trace. In their final experiment, they tested whether indirectly reactivating the contextual fear memory – through re-exposure to the cue alone – would be sufficient to make the fear memory vulnerable to disruption. This experiment showed a post-retrieval RAPA infusion into the dorsal hippocampus impaired contextual freezing in BW, but not

FW conditioned rats (Ressler et al., 2021). This confirmed that BW conditioned memories in male rats are likely to undergo subsequent destabilization, and henceforth protein synthesis, which can be then blocked by RAPA to attenuate fear/freezing behaviour (Ressler et al., 2021). However, this line of research has not been explored in females, nor whether RAPA acts the same in both sexes to disrupt the reconsolidation of a BW conditioned stimulus.

1.6 Fear Memory Processes: Extinction

Memory extinction is a process in which a conditioned response gradually diminishes with presentations of the CS without the US as an animal learns to separate a response from a stimulus (Myers & Davis, 2007; Velasco et al., 2019). Extinction is considered a new memory, one in which the context/cue is no longer associated with the US (Dunsmoor et al., 2015). In the case of contextual fear memories, extinction occurs when the mouse is placed into the context without shock after training (Concoran & Maren, 2001). While for cued fear memories, extinction occurs when the mouse is exposed to the cue without shock after training (Kida, 2013).

1.6.1 Extinction, mTOR and FW conditioning

Extinction is context-specific, such that fear responses (i.e., freezing) that diminish in one context because of extinction training will renew in a novel context (Hermann et al., 2017). Corcoran and Maren (2001) reported that rats repeatedly exposed to the conditioning context following training (extinction) exhibited low levels of freezing, whereas those repeatedly exposed to a novel context (no extinction) showed high levels of freezing. New research using electronic green fluorescent protein (GFP)

reconstitution across synaptic partners (e-GRASP) *in-vivo* imaging technology has shown that some of the new synapses formed during a fearful experience are eliminated over the course of the memory extinction process, specifically correlating with the disappearance of CA3 engram to CA1 engram synapses (Lee et al., 2023). The inhibition of protein synthesis in the CA1 region of the dorsal hippocampus following extinction training in rats impedes further extinction from occurring without affecting the original memory trace, suggesting extinction memories can undergo processes that closely resemble reconsolidation in the hippocampus (Rossato et al., 2010). For example, when male rats are subjected to a fear-motivated step-down inhibitory avoidance protocol, consisting of a apparatus with two compartments: a lighted compartment and a darkened compartment, such that if the animal steps down to the darkened component, it receives an aversive stimulus (Borba Filho et al., 2015). These animals show impaired extinction memory when a variety of agents are administered post-extinction, including nicotinic and muscarinic cholinergic receptor antagonists, mecamylamine and scopolamine, and mTOR inhibitor, RAPA, each respectively, into the CA1 region of the dorsal hippocampus (Rosa et al., 2023). Moya et al. (2020) observed that intracerebral-ventricular administration of the mTOR inhibitor RAPA reduced the immunoreactivity of phosphorylated S6K1, a downstream target of mTOR, in brain regions involved in fear extinction. They also found RAPA to eliminate the fear extinction enhancing effects produced by acute exercise, hypothesizing the augmentation of fear extinction via exercise involves central mTOR signaling (Moya et al., 2020). Additionally, Radiske et al. (2021) found that the recall of an extinction memory activates mTOR in the dorsal CA1, and that post-recall inhibition of this kinase hinders avoidance extinction memory persistence and recovers

the learned aversive response. Importantly, they determined that coadministration of BDNF impedes the behavioral effect of hippocampal mTOR inhibition, suggesting that BDNF acts downstream on mTOR in a protein synthesis-independent manner maintaining the reactivated extinction memory trace (Radiske et al., 2021).

Since the first studies of Pavlov, it has been well known that extinction does not result from the erasure of a previous memory associated with the CS but is due to new learning taking place (Pavlov et al., 1927). This is supported by three key observations in extinction. First, a learned fear response to a CS can reappear after some time (spontaneous recovery). Second, the conditioned response returns when the CS is presented in a context different from the one in which extinction training originally took place (renewal). Third, if the US is presented unexpectedly following extinction, this can lead to can lead to the response to the CS being restored (reinstatement). Wicking et al. (2016) reported that PTSD patients are more vulnerable to the return of fear when being confronted with a CS after extinction and they exhibit higher amygdala activity in a novel context compared to controls. This finding may be explained by the reactivation of the conditioned response occurring due to the change in context after extinction (renewal) or spontaneous recovery (Milad et al., 2005).

Sex differences have been identified in the extinction literature. For example, female mice and rats express more learned fear during extinction training and/or subsequent extinction memory testing, which may suggest that females show resistance to extinction (Baker-Andresen et al., 2013; Baran et al., 2009; Clark et al., 2019; Fenton et al., 2014; Greiner et al., 2019). Less research has been reported on contextual fear

extinction in females, and what is published, is mixed. Matsuda et al. (2015) found reduced extinction of contextual fear in female mice, compared to males. Daviu et al. (2014) reported enhanced contextual fear extinction in female rats, although the interpretation of this finding was complicated by the decrease in contextual fear shown by females at the start of extinction training. Baker-Andresen et al. (2013) found female mice to be more resistant to fear extinction and freezing behaviour to be correlated to an increase in DNA methylation around the brain – derived neurotrophic factor (BDNF) exon IV within the infralimbic PFC compared to male mice. A retrospective analysis of behavior during fear conditioning and extinction determined the neural processes underlying successful or failed extinction maintenance may be sex specific (Gruene et al., 2014). Two subsequent studies found similar results; despite no sex differences in the magnitude of the immediate extinction deficit, there were sex differences in the renewal of fear when the extinguished CS was presented outside the extinction context with males exhibiting significantly greater renewal than female rats (Binette et al., 2022; Schoenberg et al., 2022). In one of these studies, the effect was found in the retrieval and/or extinction of remote, but not recent fear memories (Schoenberg et al., 2022). The need to account for sex and hormonal status when conducting fear conditioning research is foreshadowed by studies demonstrating that women and female rodents seem to be generally hyper responsive to threats during low estrogen/estradiol hormonal phases, while presenting impairments in fear extinction (Velasco et al., 2019).

Brain regions such as the amygdala, prefrontal cortex, and hippocampus also show sexually dimorphic activation patterns during conditioning tasks (Le, 2023;

Méndez-López et al., 2009). These sex specific neurobiological responses may contribute to the variability in learning and memory outcomes observed between males and females in BW conditioning paradigms. This is corroborated by human imaging studies that show that while no sex differences appear within the trauma-exposed healthy control group, both psychophysiological and neural activation patterns within the PTSD group indicated deficient recall of extinction memory among men and not among women, which correlated with increased activation in the left rostral dACC during extinction recall (Shvil et al., 2014). There have been differing reports of the influence of the estrous cycle upon extinction as one study reported females that underwent extinction during low estrogen estrous phases (estrus/metaestrus/diestrus) froze more during extinction retrieval than those in the high-estrogen phase (proestrus; Rey et al., 2014). Others have reported that high plasma estradiol levels impair extinction for adolescent females, a finding consistent with the prevalence of anxiety disorders observed in females (Perry et al., 2020). Overall, although sex hormones may modulate fear extinction, the exact underlying molecular mechanisms remain largely unknown.

1.6.2 Extinction and BW Conditioning

Few studies have examined the effects of extinction on BW conditioning. Chang et al. (2003) have demonstrated as little as three US-CS pairings are necessary to make an excitatory BW cue association and that extinction to the training context attenuated this effect. In addition, they showed that these effects of context extinction were specific to BW-trained cues conditioned in the extinguished context (Chang et al., 2003). More recently, Ressler et al. (2021) showed that contextual extinction had no effect upon FW

animals (as the cue is the better predictor of shock and overshadows the context), whereas contextual extinction impaired those who were BW conditioned (where the context is an essential component of the cue→context→shock association). These findings suggest that fear to a BW CS is driven by the retrieval of a contextual fear memory. However, this effect has only been examined using males and not yet to been expanded to females.

1.7 Rationale for Current Study and Study Objectives

In a field where replication success is quite low (10 – 30%), the current study attempted to replicate the previous findings of Ressler et al. (2022) using a different species and sex, while filling in important gaps in the literature surrounding BW conditioning (Carneiro et al., 2022; Ressler et al., 2022). We chose to focus our research questions solely on females, excluding male animals, as this was performed in Ressler et al., 2022 and would have required a substantially greater number of animals. However, future studies could include both sexes of animals. Likewise, we investigated whether similar effects could be found using a systemic injection compared to a local one as this method is more clinically relevant with the currently available treatment methods.

The objectives of this study were to 1) determine whether a BW US-CS paradigm would be sufficient to elicit a persistent fear memory and examine the effects of extinction upon the shock-tone pairing following BW conditioning in female mice; 2) investigate whether a single systemic injection of RAPA is capable of diminishing fear of a reactivated BW conditioned memory in female mice; and 3) extend the known conditions under which RAPA interferes with protein synthesis dependent processes.

In line with the current literature, I hypothesize that the BW US-CS paradigm will induce a lasting fear memory and that subsequent extinction to the context will have differential effects upon the shock-tone (or vice-versa) protocols, affecting BW but not FW in female mice. I also hypothesize that a single systemic injection of RAPA will be sufficient to abate reconsolidation and mitigate conditioned fear (marked by a decrease in freezing behaviour) to a reactivated BW conditioned memory in female mice. Finally, I hypothesize that the conditions under which RAPA influences memory will extend to other protein synthesis reliant processes such as consolidation.

Overall, this research is imperative to bring reconsolidation-based therapies from pre-clinical animal models to clinical treatments for PTSD, as currently the reactivation of the memory involves indirect re-exposure (i.e., only some of the cues associated with the trauma are presented) or even imaginal exposure (where the person is asked to imagine the trauma or trauma-related cues). The capacity to indirectly reactivate memories and render them susceptible to disruption overcomes a major potential hurdle in the translation of reconsolidation-based approaches to the clinic. It is also quite possible that other Pavlovian memories, such as cue–drug memories that promote relapse in addicted patients trying to remain abstinent, could also be indirectly reactivated, potentially extending the impact of this study to other mental health disorders.

2.0 Materials and Methods

2.1 Animals

A total of 210 female, approximately 8- to 10-week-old C57BL/6 mice (Charles River Laboratories, St. Constant, Quebec, Canada) were used for these experiments. Mice were group housed with three mice per cage and had *ad Libitum* access to food and water in standard laboratory conditions on a 12-hour light-dark cycle (lights on at 7 a.m., lights off at 7 p.m.). Prior to and over the course of all experiments, animals were handled daily with routine husbandry duties during the light-phase of their cycle and before starting were marked for identification with non-toxic markers. Before habituation on test days, mice were vaginally swabbed using a sterile Q-tip ® to check the phase of estrous and rinsed using a 0.9% saline solution to prevent infection. Swabs were rolled on a glass slide and verified using a microscope (Photo Makroskop M400 (1.25x, 32x); WILD Heerbrugg, Switzerland) and checked against standard estrous phase images for mice (Byers et al., 2012; Cora et al., 2015; Queens University, 2018). Mice were also weighed each test day, including the three days prior to any injection to get a three-day baseline for the calculation of injection volumes.

Before conditioning or testing sessions, all mice were transported in their home cages from the animal housing room to a small hallway adjacent to the testing room and left undisturbed for at least 30 minutes (min). Mice from the same cage were trained and tested simultaneously in separate conditioning chambers, with all equipment cleaned using Prevail sanitizing spray between animals. Following training/testing, all mice were

placed back into their home cage and returned to the animal housing room. Freezing behaviour – the absence of movement, except for respiration – was measured throughout training and testing using automated software (FreezeFrame, Coulbourn Instruments, Whitehall, Pennsylvania, USA) as an index of fear memory.

All procedures and protocols for experiments, animal care and housing were followed according to the guidelines of the Canadian Counsel on Animal Care and Memorial University of Newfoundland's Animal Care Committee.

2.2 Apparatus and Context Descriptions

Each conditioning chamber contained a shockable floor consisting of 26 stainless steel parallel rods, a drop pan placed underneath the floor, transparent Plexiglas rear and front walls, stainless steel ceiling and side walls, a speaker, and a house light for illumination, situated within a sound attenuating isolation cubicle (Habitest, Coulbourn Instruments, Holliston, Massachusetts, USA). These conditions represented context A for all experiments. Modifications to context A were made for context B and context C. Specifically, in context B, white wooden boards were placed over the grid floor, colourful cardboard inserts were placed on the walls along with strips of coloured tape, and the context was scented with vanilla. Mice were placed in an adjacent anteroom, with their home cages partially covered by a towel during their pre-test habituation period. In context C, white paper was placed on the walls, a tray with unused bedding covered the floors, and the context was scented with banana. These scents were chosen as they are close to equivalent and both non-aversive to mice (Arbuckle et al., 2015). Mice were

placed in a different adjacent anteroom (still in their home cages uncovered) during their pre-test habituation period.

2.3 Drug Preparation/Administration

Previously made stock solution 4% PEG 400, and 4% Tween 80 was added to 5% ethanol to make up the vehicle (VEH) solution. Immediately prior to experimentation, fresh solution of the drug was made by dissolving RAPA in the same volume of 5% ethanol and adding it the same volume of stock solution. Mice received intraperitoneal injections of VEH or RAPA in volumes ranging from 0.15 to 0.27 ml based on their weight. The RAPA dosage of 40 mg/kg is based on previous studies that demonstrated 40 mg/kg to have the most efficacy at disrupting contextual fear-memory, while conserving normal locomotion and nociception (Cai et al., 2006; MacCallum & Blundell, 2020).

2.4 Conditioning Procedures

For all training sessions, mice were placed in the conditioning chamber for a five-minute habituation period before the conditioning trials began. For the ***forward conditioning (FW)*** procedure, mice received 12 conditioned stimulus-unconditioned stimulus (CS-US) pairings with an intertrial interval of 58 seconds (s). The CS was an auditory tone (80 dB, 2kHz, 10 s) that co-terminated with the onset of US, which was a scrambled foot shock (2 s, 0.6mA) delivered via the grid floor. Mice remained in the chamber for an additional 60 s after the shock before being returned to their home cages. The ***backward conditioning (BW)*** procedure was identical to the FW procedure except the order of the CS and US was reversed (US-CS). Following the five-minute habituation

period, the US occurred (2 s, 0.6mA foot shock), terminated at the time of CS onset (80 dB, 2kHz, 10 s) (Ressler et al., 2021).

2.5 Experimental Descriptions

Experiment 1 – Validate the BW Procedure

Thirty mice were randomly assigned to either the BW (n = 15) or the control (C, n = 15) condition. The BW group underwent the conditioning procedure, as described above, while the C group underwent the same procedure except without the presentation of the shock (US). Twenty-four hours following conditioning, mice began the first of six extinction trials (once a day/6 days; days 2 - 7). Extinction trials consisted of a tone recall session in context B that consisted of five presentations of the CS (5 min baseline, 60 s). Mice remained in the testing chamber for one minute before they were returned to their home cages. On day eight, all mice were returned to context A for 20 min and freezing behavior was measured. The procedure can be found in Figure 1A.

Experiment 2 – Effects of Extinguishing the Context on Cued Responding in BW or FW Female Mice

Sixty mice were randomly assigned to one of four groups: BW-NoExt (n = 15), BW-Ext (n = 15), FW-NoExt (n = 15) and FW-Ext (n = 15). Mice received either FW or BW conditioning procedures in context A on day one, as described above. On day two and three, mice were either returned to the conditioning context (context A, extinction group - Ext) or exposed to a novel context (context C, no extinction group - NoExt) for 30 min per day, and then returned to their home cages. The CS was not presented during

these sessions. On day four, mice were placed in a novel context (context B) and received five presentations of the CS (in the absence of the US) after a five-minute baseline. Each CS presentation was separated by a 60 s interstimulus interval (ISI). Mice remained in the chamber for one min after the last CS presentation and were then returned to their home cages. Two weeks later mice were returned to the same context B and underwent the same CS recall session. The procedure can be found in Figure 2A.

Experiment 3 – The Effects of RAPA on Reconsolidation of FW Fear Memories in Female Mice

Thirty mice were randomly assigned to either RAPA (n = 15) or VEH (n = 15) groups. All mice received FW conditioning on day one (as described above), and 24 hours (hrs) later underwent CS reactivation procedure in context B which consisted of a single presentation of the CS (3-minute baseline). Mice remained in the testing apparatus for one minute after CS presentation and immediately received a systemic intraperitoneal (i.p.) injection of either RAPA (40 mg/kg) or VEH. Following injection, mice were returned to their home cage. Forty-eight hours after the injection, mice were placed back into context A (no CS) for a 20-minute context test and then returned to their home cage. The procedure can be found in Figure 3A.

Experiment 4 – The Effects of Adding a pre-Reactivation Habituation Session

Thirty mice were randomly assigned to either FW-VEH (n = 15) or FW-RAPA (n = 15) groups. All mice received FW conditioning (as described above) in context A on day one. This experiment was similar to Experiment 3 (with the addition of a CS test),

except 24 hours after conditioning (on day 2), mice were given a 20-min exposure session to the novel context B in the absence of the CS or the US. This exposure session was conducted to reduce any fear that may have generalized across contexts. Three hours later, mice were returned to context B and presented with a single CS (10 s) after a three-min baseline period. The mice remained in the chamber for one min (250 s for the entire session). Immediately thereafter, mice received an i.p. injection of RAPA (40mg/kg) or VEH, and then returned to their home cages. Two days later, all mice were returned to the conditioning context A for a 20-min context test (without the CS or US), and then returned to their home cages. The following day, all mice were returned to context B and presented with a three-minute CS after a three-minute baseline period, and then returned to their home cages. The procedure can be found in Figure 4A.

Experiment 5 – The Effects of RAPA on Reconsolidation of a BW Conditioned Fear Memory in Female Mice

Thirty mice were randomly assigned to either the BW-VEH or BW-RAPA group (n = 15/group). All mice received BW conditioning (as described above) in context A on day one. Twenty-four hours after conditioning (on day two), mice were exposed for 20 min to the novel context B in the absence of the CS or the US. This exposure session was conducted to reduce any fear that may have generalized across contexts. Three hours later, mice were returned to the context B and presented with a single 10-second CS after a three-min baseline period. The mice remained in the chamber for one min (250 s for the entire session). Mice were then injected with either RAPA (40mg/kg) or VEH and returned to their home cages. Two days later, all mice were exposed to the conditioning

context A for a 20-min context test (without the CS or US), then returned to their home cages. The following day, all mice were returned to context B, presented with a three-minute CS after a three-minute baseline period, then returned to their home cages. Two weeks later, mice were placed back into context A (no tone) for 20 min. AThe procedure can be found in Figure 5A.

Experiment 6 – The Effects of RAPA on Memory Recall if the Memory is Not Reactivated in BW Conditioned Female Mice

Thirty mice were split into two groups (n = 15): RAPAno-react and VEHno-react. On day one, mice underwent BW conditioning. Twenty-four hours later, mice were habituated to the novel context (context B) for 20 minutes. Three hours later mice were injected with RAPA or VEH (depending on group) in an adjacent anteroom. Forty-eight hours later (day four), mice were placed back into context A (no tone or shock) for 20 minutes. Twenty-four hours later, mice were placed back into context B for a three-minute baseline followed by three minutes with the tone present. Two weeks later, mice were placed back into context A (no tone) for 20 min. The procedure can be found in Figure 6A.

Experiment 7 – The Effects of RAPA on Consolidation of BW Conditioned Fear Memories in Female Mice

Thirty mice were split into two groups (n = 15): RAPA and VEH. On day one, mice first underwent BW conditioning and were then injected with either RAPA or VEH. Forty-eight hours later (day three), mice were placed back into context A (no tone or

shock) for 20 minutes. Twenty-four hours later, mice were habituated to the novel context (context B, without the CS) for 20 minutes and three hours later, mice were placed back into context B for a three-minute baseline followed by three minutes with the tone present. Two weeks later, mice were placed back into context A (no tone) for 20 min. Twenty-four hours later, mice were placed back into context B for a three-minute baseline followed by three minutes with the tone present. The procedure can be found in Figure 7A.

2.6 Statistics

Mixed Analysis of Variance (ANOVA) and post hoc Tukey tests were used for experiments with multiple groups or requiring multiple comparisons. A priori t-tests were used for follow-up two-group comparisons. Freezing data for statistical analysis were obtained from fear memory tone probes by taking the difference in percent freezing during tone activation (latter three minutes of test) from the percent freezing during no tone presentation (first three minutes of test), to obtain a measure of freezing to the conditioned tone that accounts for any non-specific freezing behaviour. Freezing data from contextual retrieval probes included the total freezing, first three minutes, last minute and freezing during the 10 s presentation of the CS. Significance was taken as $p < 0.05$ for all experiments. Data from experiments two through four were analyzed using either ANOVA (main effects of group [RAPA or VEH], estrus phase, and the group*estrus phase interaction) or a repeated measures ANOVA (rm-ANOVA;main effects of group, estrus phase, block or day, and all interactions). All analyses were conducted using JMP 17.1.0 (SAS 2023).

3.0 Results

As expected, the five min baseline period prior to conditioning (FW or BW conditioning) did not differ between groups (all $F < 2.47$, all $p > 0.13$) across all experiments. The number of diestrus and estrus mice in each analysis averaged 16.6 $\pm$ 0.61 SE and 12.7 $\pm$ 0.68, respectively. Importantly, in only three of the 21 analyses that included estrous phase did the number of mice in estrus dip below 10 (6, 8, and 8); the fewest number of mice in the diestrus phase was 11.

3.1 BW Conditioning Procedure is Sufficient to Elicit a Fear Response (Exp. 1)

To ensure that the BW conditioning procedure produced robust and lasting cued and contextual fear memories in female mice (measured as freezing), animals underwent BW or C (same protocol without the shock) conditioning followed by extinction of the CS (days 2-7), and then re-exposure to the conditioning context (day 8; Fig 1A). During conditioning, as expected, all mice exhibited low freezing before the first block but showed increased freezing across the conditioning blocks (rm-ANOVA: main effect of block [$F(4, 112) = 240$, $p < .001$]; main effect of group [$F(1, 28) = 1237$, $p < .001$] and group*block interaction [$F(4, 112) = 201$, $p < .001$]; Fig. 1B). During CS extinction trials (days 2-7), BW mice froze more than C mice and freezing was decreased across extinction days in the BW, but not the C mice (rm ANOVA: main effect of group [$F(1, 24) = 55.5$, $p < .001$], main effect of block [$F(5, 120) = 1.39$, $p = 0.233$], and group*block interaction [$F(5, 120) = 3,83$, $p = 0.003$]; Fig 1c). These data suggest that the BW conditioning produces memory of the CS and that the memory was extinguished in

female mice. During context recall, BW mice froze more than C mice (1-way ANOVA, $F(1, 28) = 26.1$, $p < 0.001$; Fig 1d). These data suggest that the BW conditioning produces fear memory to the CS in female mice. Together, these data confirm that BW conditioning paradigm results in fear memory that is both robust and long lasting and can be extinguished in female mice.

3.2 Extinguishing the Context Blocked Recall of the CS in BW but Not FW Conditioned Female Mice (Exp. 2)

Next, we tested whether freezing to a BW CS is mediated by fear of the conditioning context in female mice (Fig. 2a). We hypothesized that extinction of the context would reduce freezing to the CS in BW but not FW conditioned female mice. All mice exhibited low freezing during baseline (BL) before the first trial; freezing increased across trials (rm-ANOVA: trial $F[3, 52] = 89.1$, $p < 0.001$; training $F[1, 54] = 4.03$, p = 0.05; trial*training $F[3, 52] = 0.169$, $p = 0.9$; Fig. 2b). Following training, half of the mice were exposed to the conditioning context (context A, extinction: Ext) while the remainder were exposed to a novel context (context C, no extinction: no-Ext) across two days (Fig 2c). As expected, freezing behavior in mice exposed to the conditioning context was elevated initially and decreased across sessions; mice exposed to the neutral context showed low levels of freezing behavior on both sessions (rm-ANOVA: training $F[1, 51] = 4.76$, $p = 0.034$; context extinction $F[1, 51] = 147$, $p < 0.001$; day $F[1, 51] = 0.85$, $p = 0.36$; day*context extinction $F[1, 51] = 28.8$, $p < 0.001$; all other interactions $p > 0.05$). Freezing during session one was 4.9x higher in the context extinction group than in the no-extinction group, and freezing in the context extinction group decreased 22% from

session one to session two ($F[1, 26] = 18.7$, $p < 0.001$). The following day, freezing behavior to the CS in a novel context (context B) was assessed in all mice (Fig. 2d). During presentation of the CS (five trials following BL), a rm-ANOVA of freezing to the CS revealed a significant effect of training ($F[1, 52] = 69.9$, $p < 0.001$) and context extinction ($F[1, 52] = 6.42$, $p = 0.014$), but not of trial ($F[4, 49] = 1.66$, $p = 0.18$) or any interactions (all $p > 0.05$). Context extinction had an effect in the BW training groups: freezing behavior in the BW-EXT group was significantly lower than freezing in the BW-noEXT group ($F[1, 28] = 6.35$, $p = 0.018$). Context extinction had no effect, however, on freezing to the CS in FW training groups ($F[1, 24] = 1.04$, $p = 0.32$). Together these data support the hypothesis that the expression of fear to a BW CS is mediated by the retrieval of a contextual fear memory. Two weeks later, there were no differences in the five-minute baseline [$F(1, 27) = 0.02$, $p = 0.88$] or during exposure to the CS [$F(1, 27) = 0.26$, $p = 0.61$].

3.3 Systemic RAPA Following Reactivation of a FW CS Alters Freezing Behavior to the Conditioning Context

To our knowledge, little is known about the effects of RAPA on reconsolidation of a FW conditioned fear memory in female mice. We hypothesized that RAPA given following CS recall would leave context recall unaltered (Fig. 3a). During FW conditioning, all mice exhibited low freezing before the first block but showed increased freezing across the conditioning blocks (Fig. 3b). A rm-ANOVA revealed a significant main effect of block [$F(3, 24) = 67.8$, $p < 0.001$]; but no significant main effects of group [$F(1, 26) = 0.001$, $p = 0.99$]; estrus [$F(1, 26) = 0.92$, $p = 0.35$]; or two- or three-way

interactions (all $p > 0.05$). The next day, mice were placed in a novel context (context B) and the CS was presented. As expected, one-way ANOVAs revealed no significant differences in freezing behaviour before [$F(1, 26) = 2.14$, $p = 0.15$], during [$F(1, 26) = 0.23$, $p = 0.64$] or after re-exposure to the conditioning tone in the novel context [$F(1, 26) = 0.17$, $p = 0.68$]. There was no effect of estrus or interactions at any of the time points (all $p > 0.10$). Immediately following CS reactivation, half of the mice were injected with RAPA while the remaining mice were injected with VEH. Two days later, when mice were re-exposed to the original conditioning context, RAPA-treated mice showed less freezing across the 20 min exposure than VEH-treated controls (Fig. 3c). A rm-ANOVA revealed a main effect of group [$F(1 ,24) = 9.63$, $p = 0.005$], no main effect of bin [$F(19, 6) = 2.41$, $p = 0.14$] or estrus [$F(1, 24) = 0.46$, $p = 0.50$] and no significant two- or three-way interactions [all $p > 0.4$]. These results were likely due to a similarities of context A and context B. Hence, in experiment four, we repeated the experiment but included a habituation session to the novel context (context B), to reduce any possible fear from generalization to context A (like that used in Ressler et al, 2021; Fig. 4a.) During FW conditioning, all mice exhibited low freezing before the first block but showed increased freezing across the conditioning blocks (Fig. 4b). A rm-ANOVA revealed a main effect of block [$F(3, 22) = 24.6$, $p < 0.001$]. There was no significant effect of group [$F(1, 24) = 0.43$, $p = 0.51$) and no significant effect of estrus or any interactions (both $p > 0.05$). Two days later, mice were habituated to the novel context (context B) without the conditioned CS, and as expected, a two-way ANOVA revealed no significant main effect of group [$F(1, 26) = 0.40$, $p = 0.53$] or estrus [$F(1, 26) = 2.63$, $p = 0.12$] or interactions (all $p > 0.05$). Three hours later, mice were placed back in context B and the CS was presented

(Fig. 4d). As expected, one-way ANOVAs revealed no significant drug-related differences in freezing behaviour before [$F(1, 26) = 2.04$, $p = 0.17$], during [$F(1, 26) = 1.21$, $p = 0.28$] or after re-exposure [$F(1, 26) = 1.52$, $p = 0.23$] to the conditioning tone in the novel context. There was no effect of estrus or any interaction at any of the times (all $p > 0.05$). Immediately following CS reactivation, half of the mice were injected with RAPA while the remaining mice were injected with VEH. Two days later when mice were re-exposed to the original conditioning context (context A), RAPA-treated mice froze less than VEH-treated mice (Fig. 4c. A rm-ANOVA revealed a main effect of bin [$F(19, 8) = 3.38$, $p = 0.045$] and group [$F(1, 26) = 5.57$, $p = 0.026$] but not of estrus [$F(1 ,26) = 1.75$, $p = 0.19$] or any two- or three-way interactions (all $p < 0.05$). The following day, we assessed memory of the CS in context B (Fig. 4e). A one-way ANOVA (tone on – tone off) revealed a main effect of group [$F(1, 26) = 4.55$, $p = 0.043$] and no significant effect of estrus [$F(1, 26) = 0.05$, $p = 0.82$] or interaction [$F(1,26) = 0.079$, $p = 0.78$]. Overall, these data suggest that RAPA, given following reactivation of a FW conditioned CS, blocks subsequent recall of both the context and CS memory in female mice.

3.4 Systemic RAPA Following Reactivation of a BW CS Attenuates Freezing to the Conditioning Context (Exp. 5) but Without the RAPA-Reactivation Pairing, This Effect is Lost (Exp. 6)

We next tested whether contextual fear memory could be indirectly reactivated and attenuated without exposure to the conditioning context by injecting RAPA following reactivation of a BW CS and measuring subsequent freezing to the conditioning context in female mice (Fig. 5a). During BW conditioning, all mice exhibited low freezing before

the first trial but showed increased freezing across the conditioning trials (Fig. 5b; rm-ANOVA: group $F[1, 26] = 0.60$, $p = 0.45$; estrous phase $F[1, 26] = 0.05$, $p = 0.83$; trial $F[3, 24] = 131$, $p < 0.001$; all interactions $p > 0.05$). Two days later, mice were habituated to the novel context (context B) without the conditioned CS; as expected, there were no between-group differences (ANOVA: group $F[1, 26] = 0.99$, $p = 0.33$; estrous phase $F[1, 26] = 0.007$, $p = 0.93$; group* estrous phase $F[1, 26] = 0.45$, $p = 0.51$, data not shown). Three hours later, mice were placed back in context B and the CS was presented. Surprisingly, during the three min baseline period, there was a significant group difference ($F[1, 26] = 7.85$, $p = 0.010$) but not during presentation of the CS ($F[1, 26] = 1.45$, $p = 0.24$) or during the one min after the CS ($F[1, 26] = 2.84$, $p = 0.10$; Fig. 5c). There were no significant effects of estrous phase or any interactions on any of the three measurements (all $p > 0.05$). Following CS reactivation, half of the mice were injected with RAP while the remaining mice were injected with VEH. The next day, when mice were re-exposed to the original conditioning context, RAPA-treated mice froze less than VEH-treated mice; this suggests that blocking a reactivated CS memory impaired context recall (Fig 5d; rm-ANOVA: group $F[1, 26] = 7.49$, $p = 0.011$; estrous phase $F[1, 26] = 2.80$, $p = 0.11$; time $F[19, 8] = 1.57$, $p = 0.022$; all interactions $p > 0.05$). The following day we confirmed that block of the reactivated CS memory with RAPA attenuated subsequent CS recall (Fig. 5e; ANOVA: group $F[1,24] = 5.88$, $p = 0.023$; estrous phase $F[1, 24] = 1.48$, $p = 0.24$; group* estrous phase $F[1, 24] = 1.35$, $p = 0.26$). Two weeks later, when returned to the conditioning context (context A), there was no difference between groups (Fig. 5f; rm-ANOVA: group $F[1, 26] = 0.06$, $p = 0.82$; estrous phase $F[1, 26] = 3.04$, $p = 0.093$; time $F[19, 8] = 1.63$, $p = 0.244$; all interactions $p > 0.05$). These

data suggest that presentation of the BW CS covertly retrieves a contextual fear memory which is, at least in the short term, sensitive to RAPA in female mice.

The attenuation of an established contextual fear memory from a single systemic RAPA injection after reactivation of a BW CS was not merely an effect of RAPA treatment (Fig. 6a). During BW conditioning, all mice exhibited low freezing before the first trial but showed increased freezing across the conditioning trials (Fig. 6b; rm-ANOVA: group $F[1, 26] = 2.96$, $p = 0.098$; estrous phase $F[1, 26] = 7.22$, $p = 0.012$; trial $F[3, 24] = 236$, $p < 0.001$; trial*estrous phase $F[3, 24] = 3.56$, $p = 0.029$; all other interactions $p > 0.05$). Because this was the only significant effect of estrous phase, it is unlikely that estrous phase plays a substantial role in these experiments. Two days later, mice were habituated to the novel context (context B) without the conditioned CS; as expected, there was no difference between groups (ANOVA: group $F[1, 26] = 0.006$, $p = 0.94$; estrous phase $F[1, 26] = 0.05$, $p = 0.88$; group*estrous phase $F[1, 26] = 0.11$, $p = 0.75$, data not shown). Three hours later, half of the mice were injected with RAPA while the remaining mice were injected with VEH. When mice were re-exposed to the original conditioning context (context A) the next day, freezing behavior in RAPA- and VEH-treated mice did not differ (Fig. 6c; rm-ANOVA: group $F[1, 25] = 0.001$, $p = 0.98$; estrous phase $F[1, 25] = 0.09$, $p = 0.76$; time $F[19, 7] = 0.89$, $p = 0.61$; all interactions $p > 0.05$). When mice were presented with the CS in a novel context, freezing behavior in RAPA- and VEH-treated mice again did not differ (Fig. 6d; rm-ANOVA: group $F[1, 26] = 0.001$, $p = 0.99$; estrous phase $F[1, 26] = 0.13$, $p = 0.93$; group*estrous phase $F[1, 26] = 0.061$, $p = 0.81$). When mice were reassessed in the original conditioning context (context

A) after two weeks, there was still no difference between groups (rm-ANOVA: group $F[1, 26] = 0.196$, $p = 0.66$; estrous phase $F[1, 26] = 0.05$, $p = 0.82$; time $F[19, 8] = 1.76$, $p = 0.21$; group*estrous phase $F[1, 26] = 5.53$, $p = 0.027$; all other interactions $p > 0.05$).3.5

3.5 Systemic RAPA Blocks Consolidation of a BW Conditioned Fear Memory (Exp. 7)

Finally, we tested whether systemic administration of RAPA following BW conditioning blocked consolidation of both context and cued fear memory recall in female mice (Fig. 7a). During BW conditioning, as expected, all mice exhibited low freezing before the first trial but showed increased freezing across the conditioning trials (Fig. 7b; rm-ANOVA: group $F[1, 26] = 0.25$, $p = 0.62$; trial $F[3, 24] = 114$, $p < 0.001$; estrous phase $F[1, 26] = 0.17$, $p = 0.68$; all interactions $p > 0.05$). Following conditioning, half of the mice were injected with RAPA while the remaining half were injected with VEH. Two days later, when mice were re-exposed to the original conditioning context (context A), RAPA-treated mice froze less than VEH-treated mice suggesting impaired memory of the conditioning context (Fig. 7c; rm-ANOVA: group $F[1, 26] = 5.96$, $p = 0.022$; time $F[3, 24] = 25.0$, $p < 0.001$; estrous phase $F[1, 26] = 0.85$, $p = 0.37$; all interactions $p > 0.05$). The next day, when habituated to a novel context (context B), freezing behavior did not differ between groups (ANOVA: group $F[1, 23] = 1.88$, $p = 0.18$; estrous phase $F[1, 23] = 0.26$, $p = 0.62$; group* estrous phase $F[1, 23] = 0.91$, $p = 0.35$, data not shown). Three hours later, freezing to the CS (tone on – tone off) in context B differed across groups; RAPA-treated mice froze less than VEH-treated mice suggesting impaired cued memory (Fig. 7d; ANOVA: group $F[1, 25] = 5.41$, $p = 0.028$; estrous phase $F[1, 25] = 0.03$, $p =$

0.86; group* estrous phase $F[1, 25] = 2.00$, $p = 0.17$). Two weeks later, when mice were reassessed in the original conditioning context (context A), freezing behavior did not differ across groups (Fig. 7e; rm-ANOVA: group $F[1, 23] = 1.90$, $p = 0.18$; estrous phase $F[1, 23] = 0.06$, $p = 0.81$; time $F[19, 5] = 1.37$, $p = 0.39$; all interactions $p > 0.05$). The following day, freezing to the CS (tone on-tone off) did not differ across groups (Fig. 7f; ANOVA: group $F[1, 26] = 0.57$, $p = 0.46$; estrous phase $F[1, 26] = 0.13$, $p = 0.73$; group* estrous phase $F[1, 26] = 0.81$, $p = 0.38$). These data suggest that RAPA attenuates at least short-term consolidation of BW-conditioned fear memories.

4.0 Discussion

We investigated whether indirectly retrieved aversive fear memories could be manipulated in female mice. We showed that a BW CS generates a fear response that is driven by the conditioning context, unlike the fear response to a FW CS. We also demonstrated that the mTOR inhibitor, RAPA attenuates consolidation of a BW CS. In addition, our results revealed that RAPA disrupts reconsolidation of a contextual fear memory retrieved covertly by the BW CS in female mice. RAPA is impairing reconsolidation, as RAPA without memory reactivation did not alter subsequent recall. However, the difference between the RAPA- and VEH-treated groups did not persist when memory was probed two weeks post injection. Our results indicate that indirectly retrieved memories in female mice are impaired by systemic injection of an FDA-approved drug that could also be used by patients suffering from PTSD or other fear-related disorders.

4.1 Effects of Extinction on Conditioning Procedure

There are numerous studies describing extinction of FW fear conditioning paradigms (Concoran & Maren, 2001; Milad et al., 2005), yet few have examined extinction of a BW fear conditioning paradigm (Chang, et al., 2003; Gould & Steinmetz, 1996). Hence, the goal of the first set of experiments (Exp 1 and 2), was to better understand extinction of a BW conditioning paradigm. We chose to use females as similar research had been performed only in males and primarily wished to see if we could replicate these findings using females (Ressler et al., 2021). In our pilot experiment (Exp 1), we showed that repeated exposure to the context (without the shock) results in extinction of the BW fear memory. The next step was to test the hypothesis that a BW CS causes a fear response that is mediated via the conditioning context. However, prior to that, we had to confirm that the BW conditioning paradigm would cause a lasting fear memory that could be extinguished in female mice. Thus, we subjected females to 12 presentations of the unconditioned stimulus-conditioned stimulus (US-CS), identical to the procedure used by Ressler et al. (2021). Freezing behaviour in all groups increased over the course of the training and when re-exposed to the conditioning context the following day (EXT groups). In contrast, neither BW- nor FW-conditioned females froze more in response to the novel context (no-EXT groups). Extinction to the context was then examined in both FW- and BW-conditioned females, revealing that freezing to the conditioned context in both groups decreased with the second context exposure session. Lastly, we investigated whether extinction of the contextual memory would alter the memory of the CS. When the FW conditioned group was exposed to the CS in a novel

context, freezing was high regardless of previous exposure to the context. In contrast, BW conditioned females that underwent the context extinction trials froze less in response to the CS than BW conditioned females that did not undergo the context extinction trials. This response is consistent with those reported in male rats (Ressler et al. 2021) and suggests that the expression of fear memory to a BW CS is mediated by retrieval of a contextual fear memory in both sexes and across species. Taken together, our data highlight the differential effects of extinction on FW and BW conditioning procedures and support the hypothesis that the expression of fear to a BW CS is mediated by the retrieval of a contextual fear memory.

4.2 Effects of RAPA on Consolidation of a BW CS

We tested whether systemic administration of RAPA following BW conditioning blocks consolidation of both context and cued fear memory recall in female mice. Systemic RAPA given immediately following BW conditioning impaired memory of both the context and CS in female mice. When re-exposed to the context, our data showed freezing behaviour was higher and thus fear to the CS was still present in the VEH group. However, two weeks later, the RAPA and VEH groups did not differ. This might suggest that RAPA's effect on consolidation is not long lasting. This would be surprising as RAPA, given following consolidation using a FW conditioning paradigm, results in long-lasting impairments in fear recall (Bekinschtein et al., 2007; Blundell et al., 2008; Gafford et al., 2011; Glover et al., 2010; Jobim et al. 2012; Lana et al. 2017; MacCallum & Blundell, 2020). Unlike the current work, however, these experiments were run in males and used a FW conditioning paradigm. It may be that the response to systemic injection

of RAPA following a BW CS in females is different than in males. However, it seems more likely that the lack of a group-level difference is due to extinction. VEH-treated mice froze more at the start of the first context recall session than the end. They froze even less during the second recall session (at two weeks), indicating mice were forgetting the shock-context pairing. If VEH-treated mice no longer feared the context, this may explain why there was no between-group difference in context recall at two weeks. As with the context recall, there was also no difference between RAPA- and VEH-treated mice during recall of the CS at two weeks. Experiment one showed that, unlike in FW conditioning, extinction to the context in BW conditioning reduced subsequent CS recall. It is likely that the two 20 min context recall sessions were sufficient to extinguish the CS recall at two weeks. Future experiments that only test exposure to the context and to the CS once, at a later time point (e.g., 2 weeks post training), are necessary to determine if RAPA injection yields a long-lasting (or permanent) block of BW conditioned consolidation in female mice.

4.3 Reconsolidation of a FW Fear Memory

Consistent with studies from our lab (Blundell et al., 2008; MacCallum et al., 2014; MacCallum & Blundell, 2020) and others (Gafford et al., 2011; Huynh et al., 2014), we showed that RAPA, when given following reactivation of a FW CS, attenuates subsequent recall of the CS memory. Surprisingly, RAPA also impaired recall of the context. This finding is not consistent with the literature, as blocking reconsolidation of a CS does not alter memory of the conditioning context (Debiec et al., 2006; Doyère et al., 2007; Gafford, Parsons & Helmstetter, 2011; Ressler et al., 2021). One possible

explanation for our results is the similarities of context A and context B, which may have caused the animals to recall the conditioning context (context A) when exposed to context B. Hence, we repeated the experiment but included a habituation session to the novel context (context B), to reduce any generalization fear to context B (as in the procedure used by Ressler et al., 2021). Despite this change, our results again showed attenuated memory to the CS. Our results may differ from Ressler et al. (2021) because we used a systemic injection of RAPA in females, as opposed to an intra-cerebral injection (e.g., hippocampus) in males. While we used a 40mg/kg i.p. injection, Ressler et al. 2021 used bilateral infusions into the hippocampus using a guide cannula (0.3 µl /hemisphere) and although rapamycin can penetrate the blood brain barrier, research is yet to uncover the specifics as to how much reaches specific targets within the brain (Zhang et al., 2022). Future studies should assess intracerebral injection of RAPA following CS reactivation on CS and context fear memory. Overall, our findings indicate that systemic injection of RAPA given following CS reactivation blocks reconsolidation of both CS and context fear memory in FW conditioned female mice.

4.4 Reconsolidation of a BW Conditioned Fear Memory

Consistent with Ressler et al. (2021), we showed that RAPA given following reactivation of a BW CS impairs contextual memory in female mice. This suggests that indirectly reactivated memories are sensitive to amnesic agents during reconsolidation, either via RAPA producing a retrieval deficit or blocking reconsolidation (Lee et al., 2017; Nader, 2015). The effects of RAPA were transient, however, since there were no between-group differences when context recall was tested two weeks later. Since the

injection we used was given systemically, our results may reflect an incomplete attenuation of protein synthesis; however, other studies using the same systemic dose report lasting impairments in contextual memory (Blundell et al., 2008). It is more likely that extinction occurred (as discussed above) in the VEH group during the first 20 min recall session leading to a lack of group differences. Future experiments that only test exposure to the context once, at a later time-point (e.g., two weeks post training), are necessary to determine if RAPA has a long-lasting (or permanent) effect on reconsolidation in female mice. Finally, to test if RAPA's effects are on retrieval or reconsolidation, we injected RAPA or VEH without CS reactivation and subsequently tested context recall. Consistent with previous effects on reconsolidation, RAPA injected without CS retrieval did not affect fear to the context (Forcato et al., 2009). This also supports the necessity of a reminder cue for reconsolidation to take place.

4.5 Fear Memory in Females

Despite its importance, there is a dearth of research on fear memory in females. Thus, the main goal of these experiments was to examine BW conditioned fear memory processes in female mice, and whether variations in hormone levels (specifically estrogen and progesterone) affect the outcomes. Our findings in females illustrate that 1) fear response to a BW conditioned stimulus (CS) is mediated through the conditioning context, and 2) RAPA disrupts reconsolidation of a contextual fear memory retrieved covertly by the BW CS, consistent with work in males (Ressler et al., 2021). Although the estrous cycle phase can influence the acquisition, consolidation, and extinction of fear memories (Chang et al., 2009; Milad et al., 2009; Rocks & Kundakovic, 2023), little is

known about their involvement in BW conditioned learning. Estrogen and progesterone are primary sex hormones that fluctuate throughout the menstrual cycle in females, in response to various stimuli, modulating even hippocampal neurophysiology and processing (Rocks & Kundakovic, 2023). Furthermore, estrogen has been linked to enhancing hippocampal-dependent memory and promoting consolidation processes (Frick et al., 2018). On the other hand, it is also well known that testosterone and its more active metabolite, dihydrotestosterone attenuate mild cognitive impairment in men, suggesting a role of androgens in sustaining synaptic memory (Hogervorst et al., 2004; Kang et al., 2014). Therefore, future studies could include investigating whether androgens play in BW conditioning. Interestingly, across all our four experiments, we found only one significant effect of the estrous cycle on one measure (during BW conditioning in experiment 4) of freezing behavior. Thus, it is unlikely that estrous cycle phase has a substantial effect on BW conditioned memory processes. There are other studies where estrous does not seem to effect conditioning or reaction (Voulo & Parsons, 2019; Zhao et al., 2018). In a meta-analysis of nearly 300 published neuroscience articles that used rats as research subjects, the evaluation of variability of data collected from female rats — regardless of the estrous cycle — did not vary more than that from males, and in some instances data from males varied more than female data (Becker et al., 2016). To rule out the possibility of estrous influence, it would be best to have animals undergo conditioning and treatment once identified at a particular stage in the cycle. However, this approach is both extremely time (and resource) intensive and where there was no major evidence of estrous having an effect in the first few experiments, we did not proceed with this line of experimentation.

It is also important to note that studies show female rodents, when tested using traditional models assessing fear and anxiety, generally exhibit lower levels of anxiety compared to males (Lovick et al., 2021). However, since most animal tests rely heavily on locomotor activity, the naturally higher activity levels in females might skew these findings. A recent investigation into locomotor activity across three anxiety tests—EPM, open field, and social interaction—did not reveal a significant influence of sex (Scholl et al., 2019). Therefore, it is plausible that rather than displaying reduced anxiety, female rats might express distinct forms of anxiety-like behaviors not effectively captured by testing protocols developed primarily using male rodents. Consequently, the readouts of numerous standard behavioral tests, originally validated in male animals, may need recalibration to effectively capture the wider range of behavioural response to gauge similar emotional states in females. For instance, in a classic fear conditioning setup where animals freeze in response to conditioned stimuli or a context, males overall exhibited more freezing behavior than females. However, a subset of females tended to engage in darting behavior, a response not attributable to overall hyperactivity (Colom-Lapetina et al., 2019; Gruene et al., 2015). Interestingly, in scenarios more aligned with natural behaviors, like navigating a confined space or dwelling in an open field with cover, females seem to manifest higher anxiety levels and risk aversion compared to males (Pellman et al., 2017; Shepherd et al., 1992). Moreover, studies exploring auditory fear conditioning and the stress-induced freezing paradigm observed that female rats exhibit a broader range of coping behaviors than males, indicating greater behavioral diversity (Colom-Lapetina et al., 2019). Understanding these behavioral nuances between sexes is crucial as different environmental pressures might render active or passive

responses more advantageous for each sex. Females, for instance, might benefit more from escaping threats, while males might conserve energy by adopting more passive strategies. This aligns with observations in behavioral studies such as the forced swim test (FST), where males showed a sex-specific learned helplessness effect over two days, unlike females (Colom-Lapetina et al., 2017). Studies in stress literature also highlight that exposure to inescapable shocks impairs active behaviors in males but not females in various test scenarios (Steenbergen et al., 1989, 1991). The existing focus on measuring freezing behavior might be insufficient to fully unravel the differences in how males and females respond to fear conditioning paradigms. A more comprehensive behavioral analysis could shed light on these sex-specific responses, potentially contributing to a deeper understanding of the etiology of PTSD.

4.6 Neural Mechanisms Underlying BW Conditioning

Others have begun to tease apart the neural mechanisms underlying indirect retrieval processes. In male rats, intra-hippocampal RAPA disrupted the reconsolidation of a contextual fear memory retrieved covertly by the BW CS (Ressler et al., 2021). This is consistent with previous work showing the role of the dorsal hippocampus in reconsolidation of contextual fear (Kheirbek et al., 2013; Saxe et al., 2006) . Other brain areas (e.g., the amygdala) that work with the hippocampus to mediate contextual fear memory retrieved covertly by a BW CS will be the subject of future experiments. Whether these same brain areas influence memory processes in females also warrants investigation.

Researchers have demonstrated that the acquisition of contextual fear requires NMDA receptors in both the central nucleus of the amygdala (CeA) and bed nucleus of the stria terminalis (BNST), showing that contextual freezing can be disrupted by bilateral intra-CeA & BNST infusions of the NDMA receptor antagonist D, L-2-amino-5-phosphonovalerate (APV) (Ressler et al., 2020). Additionally, contextual fear conditioning induces the selective strengthening of a subset of the ventral CA1 hippocampal projections to the basal amygdala (BA) (Kim & Cho, 2020). As such, it would be of interest to investigate these areas using immunohistochemistry (IHC) to visualize and quantify key receptors such as NR3C1, NDMA, BDNF and S6K1. These receptors are highly expressed in the BA and CA1 and are involved in modulating memory formation, synaptic plasticity and consolidation/reconsolidation to shape fear-related memories (Revest et al., 2013). Furthermore, we suspect there may be sex differences in these underlying neural mechanisms. Research performed by Tuscher and co-workers (2016) demonstrated that inhibition of ERK and mTOR activation in the dorsal hippocampus prevented estradiol (E2) from increasing dorsal hippocampus and mPFC spines, implicating that dorsal hippocampal ERK and mTOR activation is necessary for the formation of neuronal spines caused by E2 in the dorsal hippocampus and mPFC. Additionally, areas of the cortex such as the anterior cingulate cortex (ACC), medial prefrontal cortex (mPFC) & the sexually dimorphic bed nucleus of the stria terminalis (BNST) are of importance to conditioning paradigms and their dysfunction have been previously implicated in multiple psychiatric disorders (Asok et al., 2019; Lebow & Chen, 2016; Ravenelle, 2021). In particular, researchers have shown that NMDA receptors are disrupted, inhibiting the formation, consolidation and

reconsolidation of recent and remote contextual fear memories (Einarsson & Nader, 2012). Furthermore, genetic deletions or knockouts of upstream and downstream components of mTOR affect fear memory (Huynh et al., 2014). For example, the genetic inhibition of S6K1 impairs contextual fear memory, while knocking out 4E-BP2 impairs spatial and associative learning and a heterozygous deletion of tuberous sclerosis complex (TSC) leads to reduced protein synthesis and an impaired spatial memory (Antion et al., 2008; Banko et al., 2007; Ehringer et al., 2008). These findings indicate that any alterations to the ERK/mTORC1 molecular cascade involved in protein synthesis dependent processes will influence memory retention and reconsolidation.

4.7 Boundary Conditions of Reactivation-Dependant Amnesia

A growing number of studies have failed to find evidence of reactivation-dependent amnesia – the alternative nomenclature for blocking a previously reactivated memory, highlighting that the effect may be more specific and have more stringent boundary conditions than previously thought (Bierdenkapp & Rudy, 2004; Carneiro et al., 2022; Cassini et al., 2017; Luyten et al., 2021; Schroyens et al., 2017; Schroyens et al., 2019; Schroyens et al., 2020). Throughout the course of our experiments this has become quite evident in needing to add an additional habituation period, requiring the contexts to differ as much as possible and the effect being semi-transient (no differences in freezing behaviour two weeks later). A recent meta-analysis revealed that there are still robust effects of protein synthesis inhibitors upon consolidation and reconsolidation, with injection timing and re-exposure duration as moderators for this effect (Carneiro et al., 2022). In fact, four key criteria have been identified for reconsolidation to occur. First, a

previously consolidated memory must be triggered or reactivated by a reminder cue (Forcato et al., 2009). Second, the manipulation targeting reconsolidation should ideally be post-reactivation (Reichelt & Lee, 2013). Third, reconsolidation is time-dependent process (Nader et al., 2000). This implies the animal is computing a time interval and without proper CS-offset memory, the memory will not become malleable again and reconsolidation will not occur (Forcato et al., 2009). Fourth, there are other conditions such as the necessity of sleep, behaviours such as avoidance (Nitta et al., 2020) and the permanence of the effect (i.e., the memory should not spontaneously recover; Alfei et al, 2020; Kindt & Soeter, 2018). In a key study confirming and further examining the boundary conditions under which reconsolidation in humans occurs, Forcato et al. (2007) found that both the context reminder and the cue reminder could retrieve the target memory. They also showed that the reactivated memory was impaired by new learning only when the memory was labile, and omission of one of the reminder components also prevents the memory from becoming labile (Forcato et al., 2009). It is important to note, not every memory retrieval results in reactivation and flexibility. For example, brief reminders trigger reconsolidation whereas longer or repeat exposures typically result in extinction, although this may depend upon the strength and age of the memory as well (Eisenberg et al., 2003; Lee, 2009; Suzuki et al., 2004). An older, more remote fear memory may require a longer reactivation period to destabilize it and higher dosing to interfere with reconsolidation (Bustos et al., 2008). Hippocampal dependent memories such as contextual and episodic memories may become more rigid and less sensitive to reactivation-dependent manipulations as they become more reliant on cortical regions

(Kroes et al., 2017). However, long context re-exposures may render even remote contextual fear memories labile and hippocampus-dependent (Ishikawa et al., 2016).

On one hand, the reoccurring retrieval of traumatic memories may destabilize, strengthen and henceforth update those memories with added emotional impact. On the other hand, it is possible these memories lack destabilization mechanisms to undergo reconsolidation and become update resistant. An overly reconsolidated memory would require interference to prevent further reconsolidation, whereas destabilization-resistant memories would be unaffected by such treatment and require destabilization promotors. Providing support for the dichotomous nature of labile memories, genetic studies using auditory fear conditioning in mice looked at the hippocampus to determine whether memory will reconsolidate or extinguish and identified two related transcriptional factors working as a molecular switch – NF-kB + calcineurin activation in reconsolidation and NF-kB blockade in extinction (de la Fuente et al., 2011). These two conflicting scenarios could help explain the dichotomous nature of memory and patients' differential response to PTSD treatments (Lee et al., 2017). Overall, the identification of individuals' specific reactivation conditions that lead to the most optimal treatment outcome will be critical to overcome this potential double dissociation. Sex may also be a determining factor for memory flexibility, or it could be due to differences in metabolic rate or dose sensitivity (Kroes et al., 2017). Regardless, it is evident that specific conditions must coincide for reactivation-dependent amnesia, namely the blocking of a previously reactivated fear memory to occur and more exploration must be done is this area.

4.8 General Conclusions

In sum, we show that indirect retrieval of a contextual fear memory results in a labile memory trace that is vulnerable to disruption in female mice. This process may contribute to the efficacy of clinical interventions, such as imaginal exposure, that rely on indirect retrieval and manipulation of traumatic memories.

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Figures and Captions

Figure 1. BW Conditioning Procedure is Sufficient to Elicit a Fear Response. Panel A: Schematic of the experimental design. Panels B-D: Mean + Standard Error of the Mean (SEM) plotted over two groups: BW and C (no shock). Panel B: During conditioning mice exhibited low freezing before the first block and increased freezing across the conditioning blocks. Panel C: During CS extinction trials (days 2-7), BW mice froze more than C mice and freezing was decreased across extinction days in the BW, but not the C mice. Panel D: During context recall BW mice froze more than C mice.

Figure 2. Extinction of the Context Blocks Cue Recall in BW, but Not FW. Panel A: Schematic of the experimental design. Panels B-D: Mean + SEM plotted over four groups: BW conditioned + extinction (BW-EXT), BW conditioned + no extinction (BW-noEXT), FW + extinction (FW-EXT), FW + no extinction (FW-noEXT). During conditioning, all mice exhibited low freezing before the first trial but showed increased freezing across the conditioning trials (Panel B). On day two and three, mice were either returned to the conditioning context (context A, extinction group) or exposed to a novel context (context C, no extinction group). Unlike the BW-noEXT and FW-noEXT groups, BW-EXT and FW-EXT groups showed higher levels of freezing in session 1, which decreased on session 2 (Panel C). During CS retrieval, FW-EXT and FW-no-EXT mice showed high levels of freezing while BW-EXT mice showed lower levels, below that of BW-noEXT mice (Panel D). BL: baseline, CS: conditioned stimulus, %: percent, * $p < 0.05$.

Figure 3. Systemic RAPA Following Reactivation of a FW CS Alters Freezing Behavior to the Conditioning Context. Panel A: Schematic of the experimental design. Panels B-C: Mean + SEM plotted over two groups: RAPA and VEH. Panel B: During FW conditioning, all mice exhibited low freezing before the first block but showed increased freezing across the conditioning blocks. A rm-ANOVA revealed a significant main effect of block but no significant main effects of group, estrus or two- or three-way interactions (all $p > 0.05$). Panel C: Two days later when mice were re-exposed to the original conditioning context, RAPA-treated mice showed less freezing across the 20 min exposure than VEH-treated controls. A rm-ANOVA revealed a main effect of group ($p < .05$), no main effect of bin or estrus and no significant two- or three-way interactions [all $ps > 0.4$].

Figure 4. The Effects of Adding a pre-Reactivation Habituation Session Panel A: Schematic of the experimental design. Panels B-D: Mean + SEM plotted over two groups: FW-VEH and FW-RAPA. Panel B: During FW conditioning, all mice exhibited low freezing before the first block but showed increased freezing across the conditioning. A rm-ANOVA revealed a main effect of block. There was no significant effect of group and no significant effect of estrus or any interactions. Panel D: One-way ANOVAs revealed no significant drug-related differences in freezing behaviour before, during or after re-exposure to the conditioning tone in the novel context. There was no effect of estrus or any interaction at any of the times (all $p > 0.05$). Panel C: The next day, when mice were re-exposed to the original conditioning context, RAPA-treated mice froze less than VEH-treated mice. A rm-ANOVA revealed a main effect of bin and group but not of estrus or

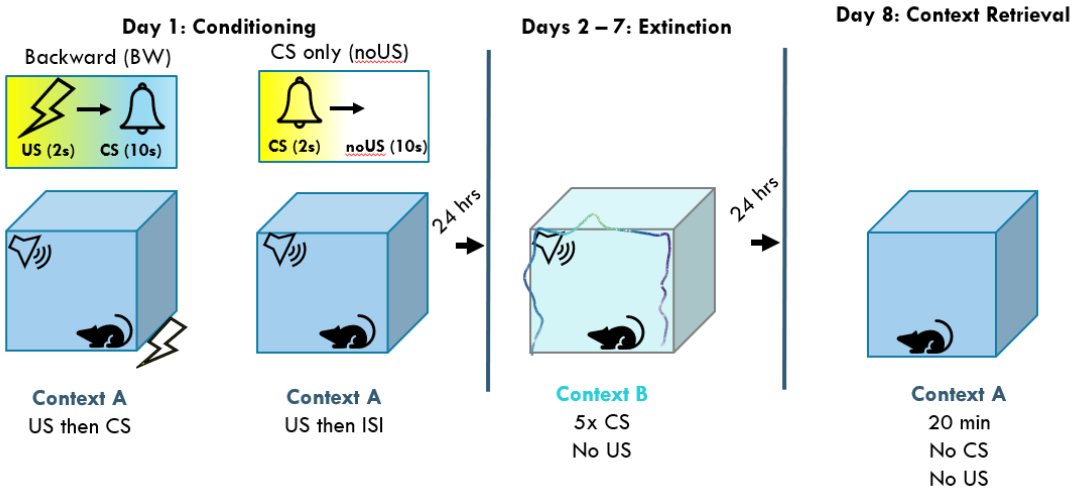
any two- or three-way interactions (all $p < 0.05$). Panel E: The following day, we assessed memory of the CS in context B. A one-way ANOVA (tone on – tone off) revealed a main effect of group and no significant effect of estrus or interaction.

Figure 5: Systemic RAPA Following Reactivation of a BW Conditioned Stimulus Attenuates Freezing to the Conditioning Context. Panel A: Schematic of the reconsolidation procedure. Panels B-E: Mean + SEM plotted over two groups: RAPA and VEH. Panel B: During conditioning, all mice exhibited low freezing before the first trial but showed increased freezing across the conditioning trials. Panel C: RAPA- and VEH-treated mice differed during baseline (BL) testing; there was no difference between groups during the time when the tone (CS) was on or immediately after. RAPA-treated mice displayed lower levels of freezing during context recall (Panel D) and CS recall (Panel E) than VEH-treated mice. Panel F: Two weeks later, there was no difference in freezing levels between groups during context recall. BW: backward, CS: conditioned stimulus, %: percent, * $p < 0.05$.

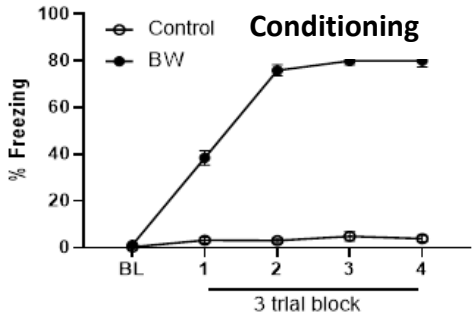
Figure 6. In the Absence of the Conditioned Stimulus Reactivation, RAPA had No Effect on Context Recall. Panel A: Schematic of the procedure. Panels B-D: Mean + SEM plotted over two groups: RAPA and VEH. Panel B: During conditioning, all mice exhibited low freezing before the first trial but showed increased freezing across the conditioning trials. There was no difference between groups during context recall (Panel C) or CS recall (Panel D) and VEH-treated mice. BW: backward, CS: conditioned stimulus, %: percent, * $p < .05$.

Figure 7. Systemic RAPA Blocks Consolidation of a BW Conditioned Fear Memory. Panel A: Schematic of the consolidation procedure. Panels B-D: Mean + SEM plotted over two groups: RAPA and VEH. During conditioning, all mice exhibited low freezing before the first trial but showed increased freezing across the conditioning trials (Panel B). RAPA-treated mice displayed lower levels of freezing during context recall (Panel C) and CS recall (Panel D) than VEH-treated mice. Two weeks later, there was no difference in freezing levels between groups during context recall (Panel E) or CS recall (Panel F). BL: baseline, CS: conditioned stimulus, %: percent, * $p < .05$.

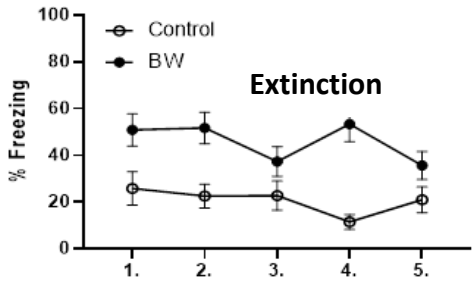
1 A.


Day 1: Conditioning
Backward (BW)
US (2s)
CS (10s)
CS only (noUS)
CS (2s)
noUS (10s)
Days 2 – 7: Extinction
Day 8: Context Retrieval
24 hrs
24 hrs
Context A
US then CS
Context A
US then ISI
Context B
5x CS
No US
Context A
20 min
No CS
No US

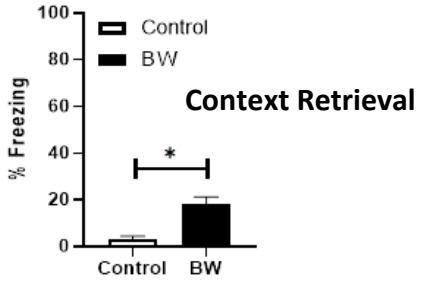
B.

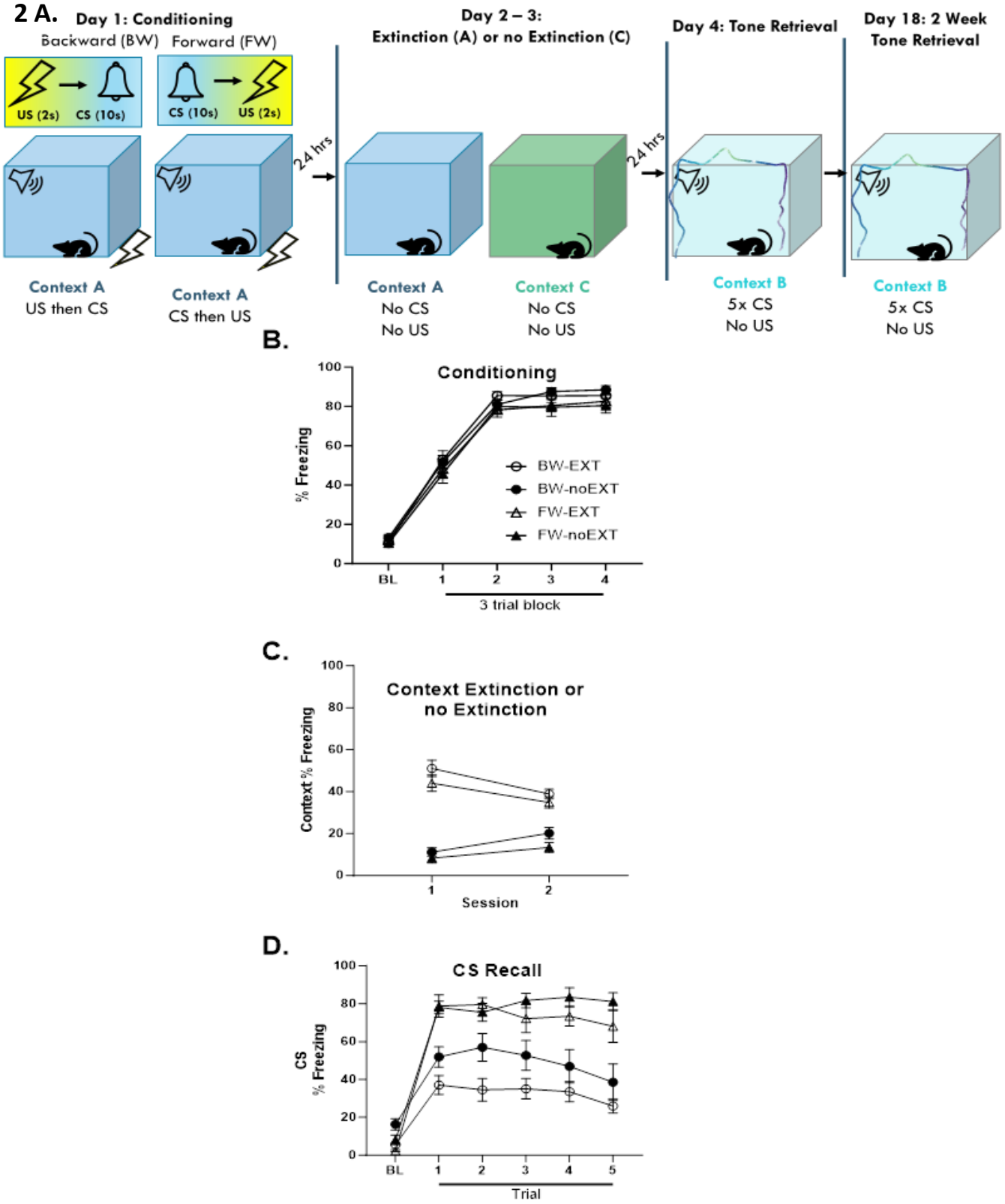

Conditioning
Control
BW
% Freezing
100
80
60
40
20
0
BL
1
2
3
4
3 trial block

C.

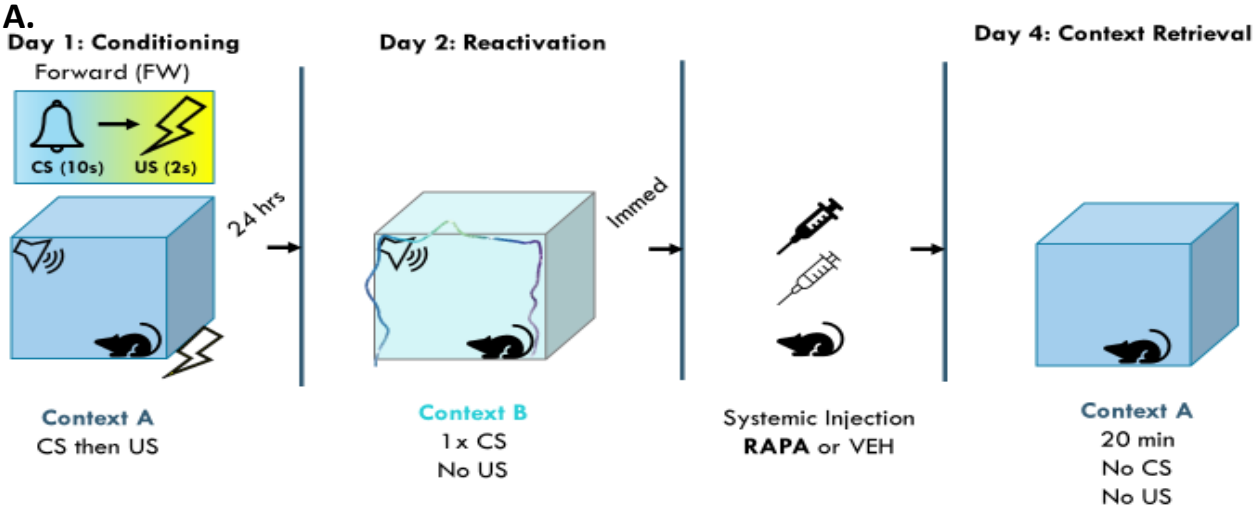

Extinction
Control
BW
% Freezing
100
80
60
40
20
0
1.
2.
3.
4.
5.

D.

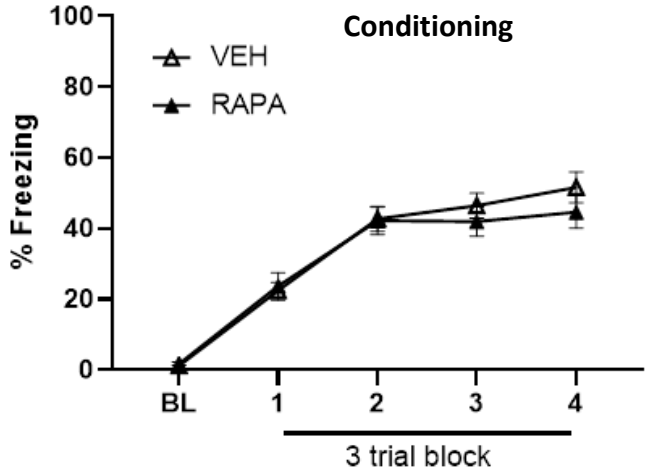

Context Retrieval
Control
BW
% Freezing
100
80
60
40
20
0
*
Control
BW


2 A.
Day 1: Conditioning
Backward (BW)
Forward (FW)
US (2s)
CS (10s)
CS (10s)
US (2s)
24 hrs
Day 2 – 3:
Extinction (A) or no Extinction (C)
24 hrs
Day 4: Tone Retrieval
Day 18: 2 Week
Tone Retrieval
Context A
US then CS
Context A
CS then US
Context A
No CS
No US
Context C
No CS
No US
Context B
5x CS
No US
Context B
5x CS
No US
B.
Conditioning
% Freezing
BW-EXT
BW-noEXT
FW-EXT
FW-noEXT
BL
1
2
3
4
3 trial block
C.
Context Extinction or
no Extinction
Context % Freezing
Session
D.
CS Recall
CS
% Freezing
Trial

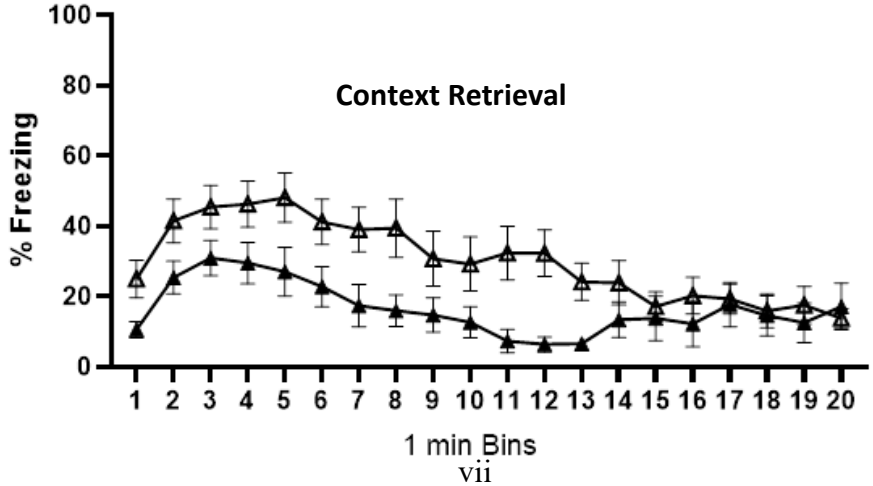
3 A.


Day 1: Conditioning
Forward (FW)
CS (10s)
US (2s)
24 hrs
Day 2: Reactivation
Immed
Day 4: Context Retrieval
Context A
CS then US
Context B
1 x CS
No US
Systemic Injection
RAPA or VEH
Context A
20 min
No CS
No US

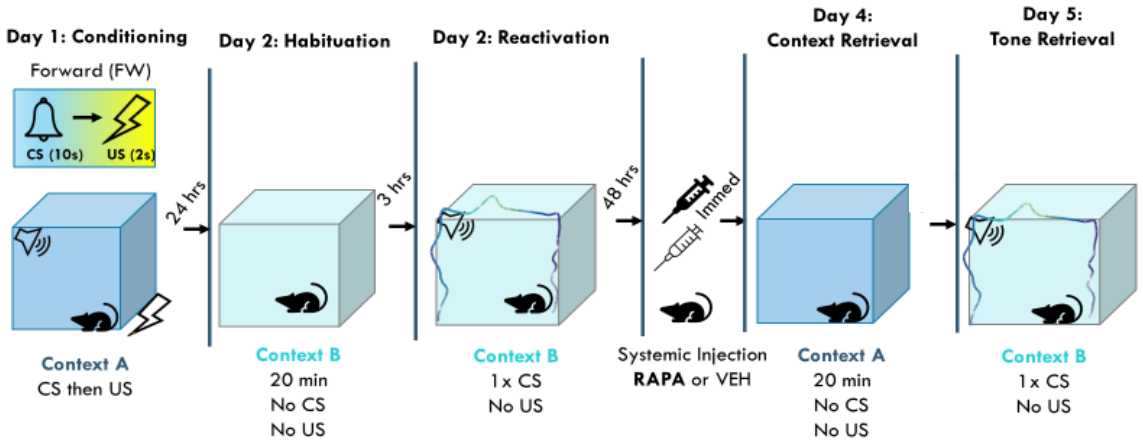
B.


Conditioning
VEH
RAPA
% Freezing
100
80
60
40
20
0
BL
1
2
3
4
3 trial block

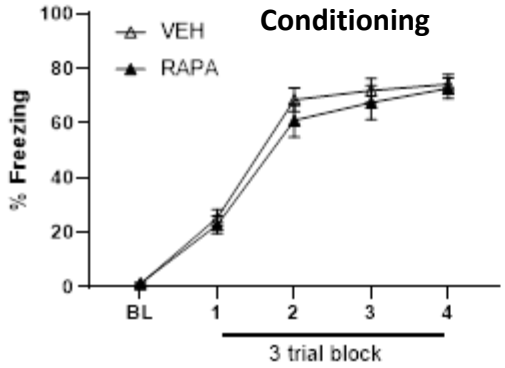
C.


Context Retrieval
% Freezing
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1 min Bins

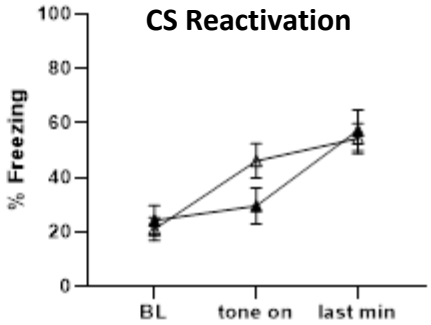
4 A.


Day 1: Conditioning
Forward (FW)
CS (10s)
US (2s)
Context A
CS then US
24 hrs
Day 2: Habituation
Context B
20 min
No CS
No US
3 hrs
Day 2: Reactivation
Context B
1 x CS
No US
48 hrs
Immed
Systemic Injection
RAPA or VEH
Day 4:
Context Retrieval
Context A
20 min
No CS
No US
Day 5:
Tone Retrieval
Context B
1 x CS
No US

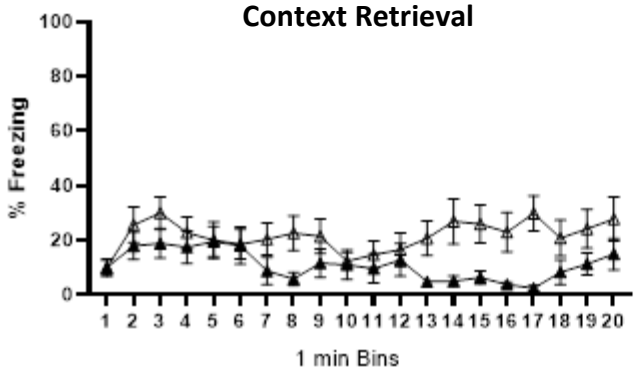
B.


Conditioning
VEH
RAPA
% Freezing
100
80
60
40
20
0
BL
1
2
3
4
3 trial block

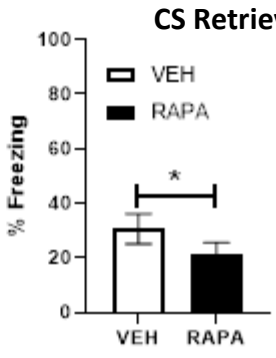
D.


CS Reactivation
% Freezing
100
80
60
40
20
0
BL
tone on
last min

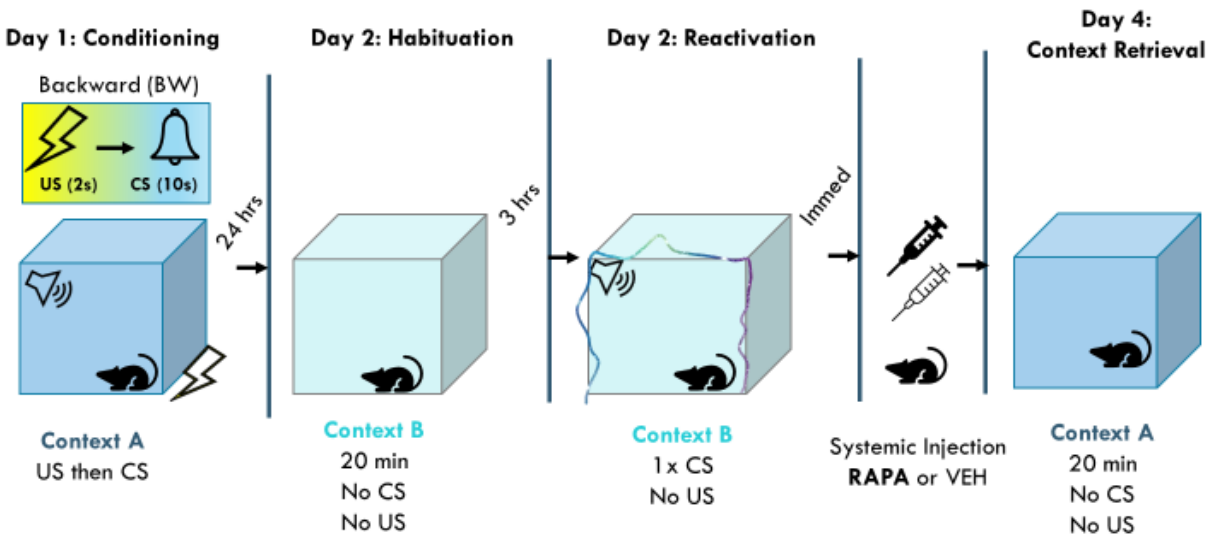
C.

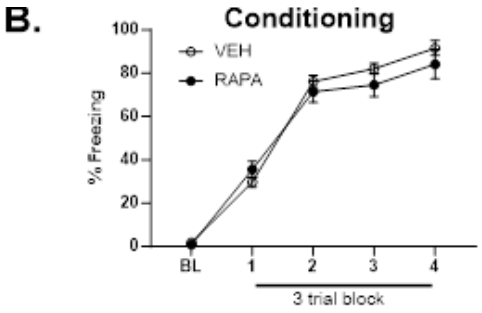

Context Retrieval
% Freezing
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1 min Bins

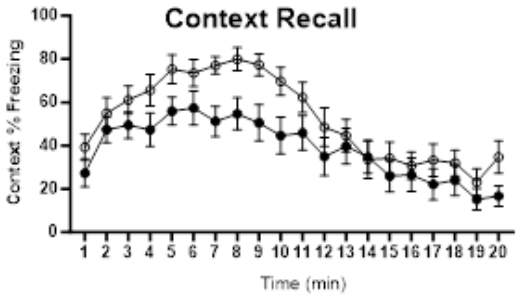
E.

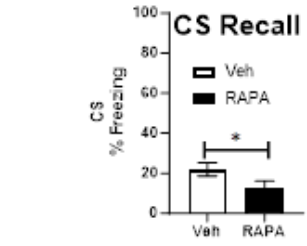

CS Retrieval
VEH
RAPA
% Freezing
100
80
60
40
20
0
*
VEH
RAPA

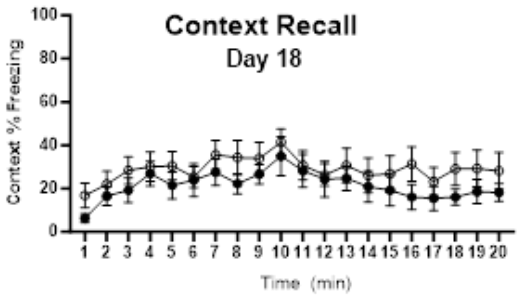
5 A.

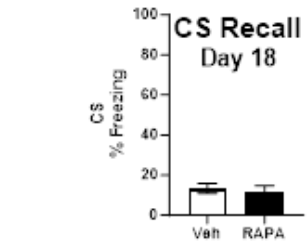

Day 1: Conditioning
Day 2: Habituation
Day 2: Reactivation
Day 4:
Context Retrieval
Backward (BW)
US (2s)
CS (10s)
24 hrs
3 hrs
Immed
Context A
US then CS
Context B
20 min
No CS
No US
Context B
1 x CS
No US
Systemic Injection
RAPA or VEH
Context A
20 min
No CS
No US


B.
Conditioning
VEH
RAPA
% Freezing
BL
1
2
3
4
3 trial block

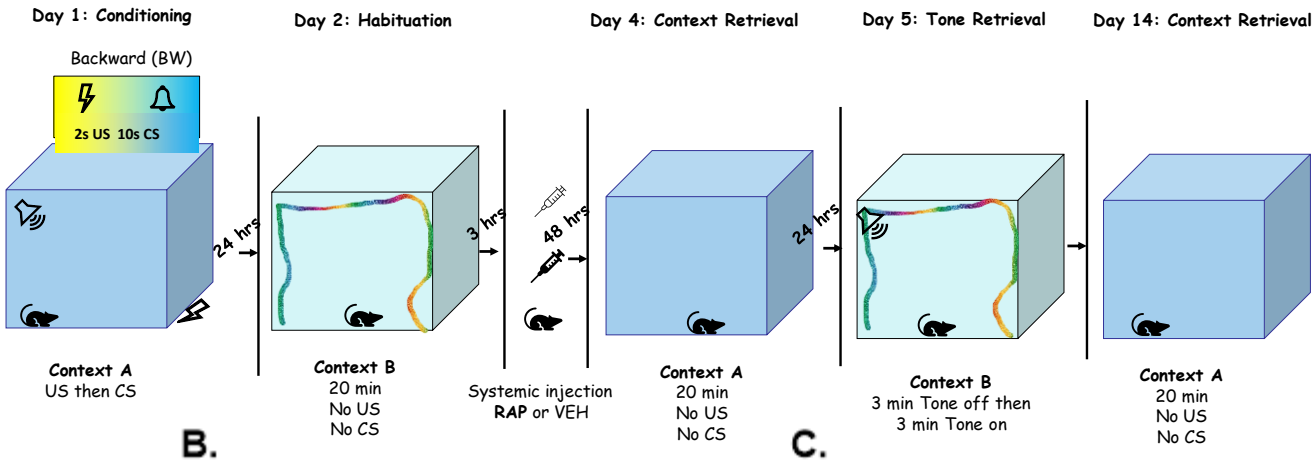

C.
Context Recall
Context % Freezing
Time (min)


D.
CS Recall
Veh
RAPA
CS
% Freezing
*
Veh
RAPA

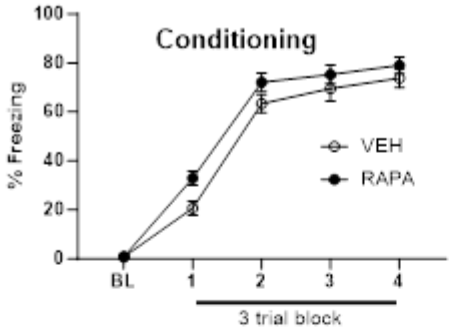

E.
Context Recall
Day 18
Context % Freezing
Time (min)


F.
CS Recall
Day 18
CS
% Freezing
Veh
RAPA

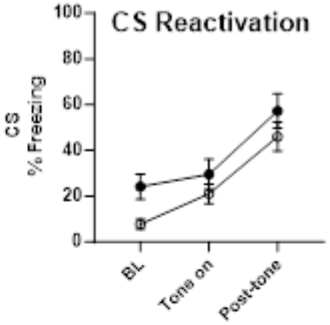
6 A.


Day 1: Conditioning
Day 2: Habituation
Day 4: Context Retrieval
Day 5: Tone Retrieval
Day 14: Context Retrieval
Backward (BW)
2s US 10s CS
24 hrs
3 hrs
48 hrs
24 hrs
Context A
US then CS
Context B
20 min
No US
No CS
Systemic injection
RAP or VEH
Context A
20 min
No US
No CS
Context B
3 min Tone off then
3 min Tone on
Context A
20 min
No US
No CS

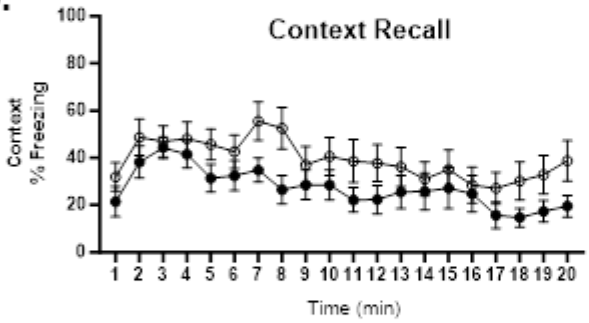
B.


Conditioning
% Freezing
VEH
RAPA
BL
1
2
3
4
3 trial block

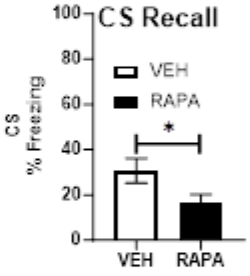
C.


CS Reactivation
CS
% Freezing
BL
Tone on
Post-tone

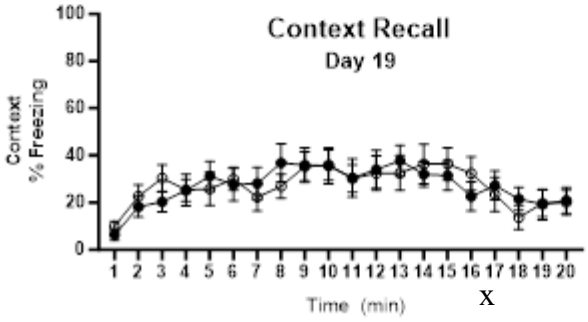
D.


Context Recall
Context
% Freezing
Time (min)

E.

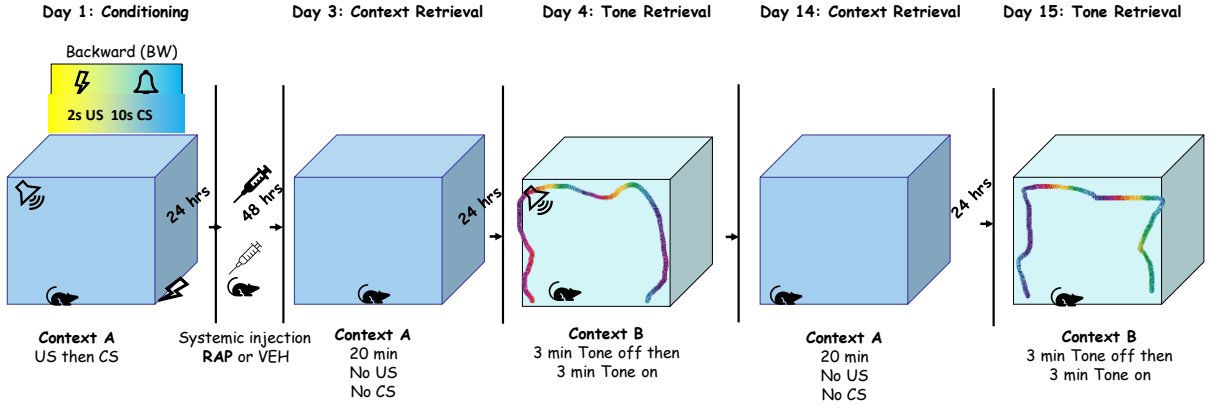

CS Recall
CS
% Freezing
VEH
RAPA
*
VEH
RAPA

F.

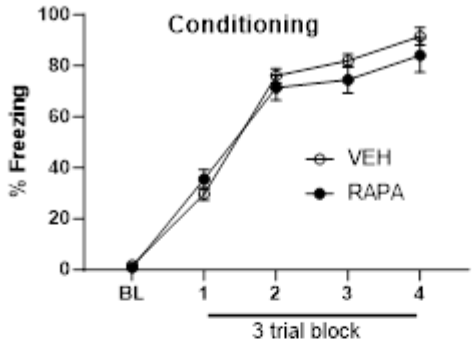

Context Recall
Day 19
Context
% Freezing
Time (min)

X

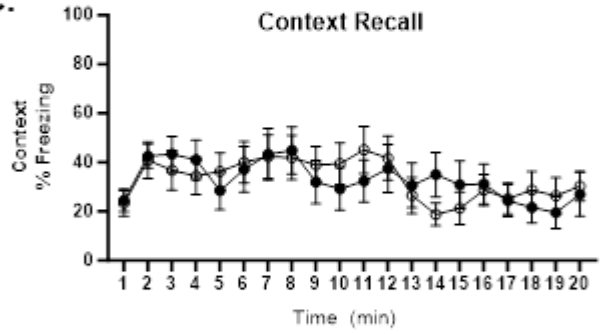
7 A.


Day 1: Conditioning
Day 3: Context Retrieval
Day 4: Tone Retrieval
Day 14: Context Retrieval
Day 15: Tone Retrieval
Backward (BW)
2s US 10s CS
24 hrs
48 hrs
24 hrs
24 hrs
Context A
US then CS
Systemic injection
RAP or VEH
Context A
20 min
No US
No CS
Context B
3 min Tone off then
3 min Tone on
Context A
20 min
No US
No CS
Context B
3 min Tone off then
3 min Tone on

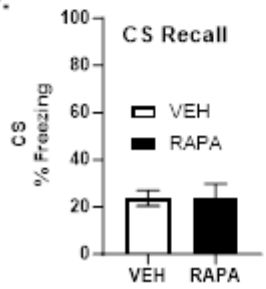
B.


Conditioning
% Freezing
VEH
RAPA
BL
1
2
3
4
3 trial block

C.


Context Recall
Context % Freezing
Time (min)

D.


CS Recall
CS % Freezing
VEH
RAPA
VEH
RAPA