Evaluating the effects of systemic mTOR inhibition on fear memory processes in rodents with relevance to ameliorating PTSD-like symptoms

A Thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Psychology

Memorial University of Newfoundland

May 2025

St. John's, Newfoundland and Labrador

Abstract

In this dissertation, I use physical and psychogenic contextual fear conditioning procedures to study the role of the mechanistic target of rapamycin (mTOR) in memory processes and investigate its potential as a target for treating post-traumatic stress disorder (PTSD)-like symptoms in rodents. I determined that inhibiting both mTOR complex 1 and 2 with AZD2014 impairs the consolidation of a foot-shock induced contextual fear memory in mice similar to the mTORC1 inhibitor rapamycin, while inhibition of the mTORC1 downstream effector S6K1 with PF-4708671 does not. Using the same conditioning procedure, I found that rapamycin treatment three-hours, but not 12-hours, post-conditioning impairs the consolidation and persistence of contextual fear memory in mice. In support of earlier published work showing rapamycin impairs the consolidation of long-lasting contextual fear, hyperarousal, and anxiety-like behaviour in rats following a brief unprotected exposure to a cat, I show here, using immunohistochemistry, increased mTORC1 activation in the hippocampus and periaqueductal grey of rats shortly after this type of exposure. I also investigated the effects of psychogenic-only predator stress at inducing long-lasting behavioural changes via a model of mouse defensive behaviour previously unexplored for this purpose. Following a fully protected exposure to a rat, mice exhibited associative contextual fear and other non-associative fear and anxiety-like behaviours, with many of these behaviours weakened from post-exposure rapamycin treatment. In a different set of experiments using foot-shock conditioning, I reveal that rapamycin injected three- or 12-hours post-reactivation impairs the persistence of contextual fear memory, while the same treatment appears to induce a gradient effect against reconsolidation of the memory. Additionally, I demonstrate that two consecutive days of reactivation and rapamycin treatment maximizes abatement to recall foot-shock associated contextual memory through impaired

reconsolidation in mice. Lastly, I show that the effects of rapamycin to consolidation and reconsolidation on contextual fear memory in mice does not interfere with the ability to subsequently learn and recall new auditory fear associations but protects against fear generalization. Collectively, these findings advance our neurobiological understanding of mTOR in memory processes and provide preclinical evidence on how to pharmacologically treat PTSD-like symptoms.

General Summary

The goals of my PhD are to better our understanding of the neurobiology of memory and to help combat PTSD symptoms. My research focused on a molecule called mTOR and a drug named rapamycin, which blocks its activity. To model the abnormal and maladaptive fear memories found in PTSD, I used rodent contextual fear conditioning procedures, which although not maladaptive, taps into the same underlying memory processes precipitated by a fearful event. Following fear learning or fear memory reactivation, rodents received a placebo or rapamycin treatment. The placebo did not affect fear memory towards the training context. Yet, rapamycin decreased the strength of this contextual fear memory. By adding an extra day of memory reactivation and rapamycin, I was able to maximize the effects of the drug, whereas a third day of pairing reactivation and rapamycin did not have any additive value. When I delayed drug treatment hours after training or reactivation, rapamycin still hindered memory, but these effects appeared to show a diminishing return as treatment moved away training or memory retrieval. The effects of rapamycin on the memory it interfered with, importantly, did not encroach on the ability to learn and remember new information and allowed the rodents to be less frightened when entering new contexts and situations. Overall, my research adds to our biological understanding of memory processes and provides insights on how to potentially treat some PTSD symptoms.

Acknowledgments

I would like to thank the following, for which this dissertation would not have been possible.

Foremost, I want to express my utmost gratitude to my supervisor Dr. Jacqueline Blundell. Throughout my academic journey you have shown me infinite patience and unparalleled support, providing countless opportunities to learn, grow, and become a better and more independent researcher – it has been an honour to be supervised by you.

Dr. Charles Malsbury, thank you for your impeccable feedback, insights, and mentorship as an examiner on my comprehensive and as one of my teaching assistant supervisors. I also want to express my gratitude to Dr. Gerard Martin for his thoughtful advice and guidance as a committee member for both my comprehensive exam and thesis. Rounding out my committees and another former teaching assistant supervisor of mine is Dr. Susan Walling. Sue, I have greatly appreciated your continuous instruction, expertise, especially with my histology and densitometry work on deer mice brains, and for always making time to chat and give me advice.

A huge thank you to all the dedicated and enthusiastic people, past and present, in the Blundell Lab, all the PIs and students in the other Behavioural Neuroscience labs, Steve Milway, and the other Behavioural Neuroscience lab instructors. Without their efforts, respect, coordination, and collegiality, my experiments would not have run so smoothly. I would be remiss if I did not also acknowledge the Psychology Head, Dr. Christina Thrope, the Department of Psychology Office staff, and the Psychology Graduate Officers, for all their hard work in getting people like me to finish and submit their theses – thank you for keeping the pressure on me to complete this.

Financial and in-kind support towards my dissertation research (e.g., purchase of materials, animals, and equipment; access to research software, computing resources, and technical services; care and veterinary services for my research animals; and covering article processing charges) were generously made by Memorial University and the Natural Science and Engineering Research Council of Canada. Routledge, Springer Nature, and Elsevier published my dissertation work and agreed to their republication herein, for which I greatly appreciate.

My friends, coworkers, and extended family have all been instrumental in keeping my morale high throughout this process. From kitchen parties, beer festivals, trekking along the East Coast Trail, potluck brunches and dinners, trivia nights at The Ship, concerts, late night poker games, trips to Miquelon and Fogo Islands to run in races, playing intramural sports with the BGSA, to having afternoon ‘tea’, I am truly grateful for these incredible moments and distractions outside of my thesis work. Throughout the course of my program, I have also had the privilege of having a menagerie of foster and rescue cats and dogs, backyard chickens, and even a hermit crab come into my home life. Taking care of these creatures and having their companionship has been incredibly rewarding and important to my physical and mental wellbeing, especially with completing this thesis. To my friends and family that have passed on during this time, I wish you were able to celebrate this achievement with me; I love and miss you.

Not to be forgotten are my parents, Jean and Roger, and my sister, Lisa, who have been with me through it all. Thank you for your unconditional love, encouragement, support, and belief in me as I have pursued my academic dreams. Lastly, to my incredible partner, Emilie, I want to let

you know how proud I am of what you have accomplished in your studies, your unwavering support of my work, and for the life we have built together in Newfoundland – I couldn't imagine being on this journey with anyone else.

Co-authorship Statement

The contents of Chapters 2, 3, and 4 of this *manuscript-style dissertation* are from three different coauthored publications of original research. Each chapter corresponds to a separate publication, respectively. I, **Phillip MacCallum**, the sole author of this dissertation, am the first and primary author for each coauthored publication presented herein.

The publications are reproduced verbatim in each chapter except for alternations where warranted. Minor revisions include typographical error corrections, insertion of omitted words, and adaption of headings and figures to correspond to each chapter. Significant updates to figures and figure captions were required for Chapter 3. The original publication of this work contains multiple figure caption errors and several incorrect figures but with correct figure captions for which I have revised or replaced accordingly for this dissertation. For Chapter 4, I changed the original in-text citations used in publication to align with all other chapters of the dissertation (from a numeric style, where citations are numbered in square brackets in the order of appearance (i.e., Institute of Electrical and Electronics Engineers style), to the author, date in parentheses American Psychological Association (APA) Publication Manual style used in the other two publications and throughout this dissertation). Lastly, I have omitted the individual reference sections from each publication in favor of using a single unified Reference Chapter (in APA style).

Contributor Roles Taxonomy (CRediT) is a high-level classification system, which includes 14 roles to describe accurately and transparently individual contributions to multi-authored works. Many publishers (e.g., Elsevier, Wiley, and Sage) are adopting CRediT and requiring author

contribution statements using CRediT, for which my coauthors and I did for the work presented in Chapter 4. As such, the CRediT author statement for the publication corresponding to Chapter 4 is included below. Since there are no author contribution statements for the other two publications used in this dissertation, I have created them here using CRediT to provide a consistent, accurate, and transparent description of contributions made by me and my coauthors. The below statements are to the best of my knowledge, accurate, and have been corroborated by my supervisor and the corresponding author of each of these works, Dr. Jacqueline Blundell.

Chapter 2 (publication type: peer-reviewed article; publisher: Springer Nature):

MacCallum, P. E**.**, & Blundell, J. (2020). The mTORC1 inhibitor rapamycin and the mTORC1/2 inhibitor AZD2014 impair the consolidation and persistence of contextual fear memory. *Psychopharmacology*, 237(9), 2795–2808. https://doi.org/10.1007/s00213-020-05573-1

CRediT authorship contribution statement: Phillip MacCallum: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Resources, Writing - original draft, Writing – review & editing, Project administration; Jaqueline Blundell: Conceptualization, Methodology, Visualization, Writing - review & editing, Supervision, Funding acquisition, Project administration, Resources.

Chapter 3 (publication type: edited book chapter; publisher: Routledge, an imprint of Taylor & Francis Group):

MacCallum, P., Whiteman, J., Kenny, T., Fallon, K., Bhattacharya, S., Drover, J., & Blundell, J. (2021). Developing a reliable animal model of PTSD in order to test potential pharmacological treatments: Predator stress and the mechanistic Target of Rapamycin. In R. Ricciardelli., S. Bornstein, A. Hall, & R. N. Carleton (eds), *Handbook of Posttraumatic Stress: Psychosocial, Cultural, and Biological Perspectives* (1st ed.) (pp. 369–399). New York: Routledge. https://doi.org/10.4324/9781351134637

CRediT authorship contribution statement: Phillip MacCallum: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Supervision, Resources, Writing - original draft, Writing – review & editing, Project administration; Jesse Whiteman: Conceptualization, Methodology, Investigation, Writing - original draft; Katelyn Fallon: Conceptualization, Methodology, Investigation, Writing – review & editing; Therese Kenny: Investigation, Writing – review & editing; Sriya Bhattacharya: Investigation, Writing – review & editing; James Drover: Conceptualization, Writing – review & editing; Jacqueline Blundell: Conceptualization, Methodology, Visualization, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition, Project administration, Resources.

Chapter 4 (publication type: peer-reviewed article; publisher: Elsevier):

MacCallum, P. E., Cooze, J. B., Ward, J, Moore, K.A.M., & Blundell, J. (2024). Evaluating the effects of single, multiple, and delayed systemic rapamycin injections to contextual fear reconsolidation: Implications for the neurobiology of memory and the treatment of

PTSD-like re-experiencing. *Behavioural Brain Research*, 461, 114855. https://doi.org/10.1016/j.bbr.2024.114855

CRediT authorship contribution statement: Phillip MacCallum: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Supervision, Resources, Writing - original draft, Writing – review & editing; Jane Cooze: Investigation, Formal analysis; Joshua Ward: Investigation, Formal analysis; Kelsey Moore: Investigation, Formal analysis; Jaqueline Blundell: Conceptualization, Methodology, Visualization, Writing - review & editing, Supervision, Funding acquisition, Project administration, Resources.

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Chapter 1: Introduction

1.1 Background

In Pavlovian (respondent/classical) fear conditioning paradigms, animals, typically mice or rats, are trained to fear an initially neutral stimulus such as a tone or a particular context through association after pairing the neutral stimulus with a noxious, aversive stimulus, typically a foot-shock. When encountering the formerly neutral stimulus again, it now elicits a conditioned defensive response in anticipation of a perceived or real aversive threat. Learning in these procedures is passive, occurs quickly (usually in just one trial or session), and the memory is enduring. Furthermore, the stimuli used to condition and test fear memory are controlled by the experimenter, while the conditioned response produced by the conditioned stimuli, for example freezing, is a clearly defined species-specific and easily quantifiable behaviour, serving as an indicative measure of memory strength (Curzon et al., 2009; Rodrigues et al., 2004; Sanders et al., 2003). These characteristics make Pavlovian fear conditioning methods and techniques incredibly valuable and well suited for studying the neuroanatomical, synaptic, cellular, and molecular mechanisms underlying learning and memory. Moreover, since learned fear and maladaptive, aberrant, fear memories are at the core of a variety of stress, trauma, and anxiety disorders such as post-traumatic stress disorder (PTSD), Pavlovian fear conditioning procedures, albeit likely to induce adaptive memories and behaviours important for the survival of a model organism, also provides us with the opportunity to model the experience and symptoms of PTSD, while also investigating potential treatment advances in controlled laboratory settings (Beckers et al., 2023; Fanselow & Sterlace, 2014; Izquierdo et al., 2016).

The mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that forms the core of two distinct molecular units, mTOR complex 1 (mTORC1) and 2 (mTORC2). Rapamycin (RAPA) and other RAPA-related drugs called rapalogues inhibit mTORC1 activity, whereas mTORC2 is largely insensitive to these drugs and lacks, at this time, any specific inhibitors (although, there are dual mTORC1/2 ATP competitive inhibitors available such as AZD2014 and nanomedicine in development to inhibit mTORC2) (Benavides-Serrato et al. 2017; Murray & Cameron 2017; Pike et al., 2013; Sabatini, 2017; Saxton & Sabatini 2017; Werfel et al., 2018). Consequently, very little is known about the role of mTORC2 in memory processes (except through genetic deletion studies; for examples, see Angliker & Ruegg 2013; Huang et al. 2013; Sun et al. 2019; Zhu et al. 2018), while fear conditioning experiments using RAPA have considerably increased our understanding of the protein translational regulator, mTORC1, in memory processes. Specifically, these studies have given further credence to the requirement of de novo protein synthesis in memory consolidation and reconsolidation, provided evidence for one of the specific molecular mechanisms underlying these processes, and posited RAPA as a potential treatment option for addressing PTSD symptoms (Blundell et al., 2008; Fifield et al., 2013; Gafford et al., 2011; Glover et al., 2010; Huynh et al., 2014; Jobim et al., 2012a; Lana et al. 2017; Mac Callum et al., 2014; Parsons et al., 2006b; Pedroso et al., 2013; Slipczuk et al. 2009).

Despite these findings, there are still gaps in fully characterizing the contribution of mTORC1 signaling to memory processes and further establishing the preclinical efficacy of RAPA and other rapalogues for treating PTSD-like symptoms. For instance, findings indicate other general and more specific molecular mechanisms are involved in late phase consolidation- and

reconsolidation-like events, which contribute to the persistence of memory; however, the contribution of mTORC1 to these protracted cellular events remains scant, especially for contextual fear memory (Bekinschtein et al. 2007a; Bekinschtein et al. 2010; Bourtchouladze et al., 1998; Freeman et al., 1995; Grechsch & Matthies 1980; Igaz, et al., 2002; Krawczyk et al., 2016; Mac Callum et al., 2014; Martinez-Moreno et al. 2011; Nakayama et al., 2013; Nakayama et al., 2016; Ou et al., 2009; Pena et al., 2014; Pereira et al., 2019; Quevedo et al. 1999; Rossato et al. 2007; Trifilieff, et al., 2006; Wanisch et al. 2008). Moreover, while preclinical findings of impaired reconsolidation to associative electric foot-shock memories from RAPA provide a compelling rationale for the drug as a PTSD treatment, these findings are only from single treatments. To offer more thorough preclinical strategies, it is important to investigate if additional treatments of combining RAPA and reactivation maximizes the decrease in the negative emotional valance of the fear memory or if the single treatment is sufficient. Another valuable aspect for RAPA for it to be considered an optimal treatment option is to determine if the effects to consolidation and reconsolidation create any permanent changes in the ability to learn and remember anew, as such treatments should be specific and not interfere with other processes (Gamache et al., 2012). Additionally, most of what we know about the contribution of mTORC1 to fear memory processes (and for many other mechanisms) draws from studies that used a physical stressor (i.e., electrical foot-shock) and to a lesser extent, a combination of physical and psychogenic stress (i.e., an unprotected predator exposure). It has yet to be determined whether the effects of RAPA to associative fear memories will hold true if the unconditioned stressor stimulus was wholly psychogenic, such as through a fully protected predator exposure. Conditioning rodents with an ecologically relevant psychogenic predator stimulus also provides the opportunity to explore the effects of RAPA on non-associative

memory changes that closely mimic other PTSD symptoms (e.g., hyperarousal). Interestingly, such non-associative fear memories often emerge in predator stress paradigms but are seldom tested for in foot-shock models or when they are, typically require high amperage during conditioning to achieve such behavioural changes in these non-associative memory domains (Bali & Jaggi, 2015; Flandreau & Toth, 2018; Török et al., 2019; Verbitsky et al., 2020).

1.2 Dissertation Research Aims, Sub-objectives, and Questions

Motivated by these knowledge gaps, the primary aims of my dissertation research are to i) advance our biopsychological understanding of mTORC1 in memory processes, ii) glean insights into the possible underlying mechanisms of PTSD-like fear memories, and iii) test if pharmacological mTOR inhibition has translational relevance for treating PTSD-like symptoms. With Pavlovian fear learning (via physical and psychogenic stressors) and memory procedures at the fulcrum, my primary goals interweave around this methodology to shape the specific sub-objectives and corresponding research questions for each research chapter delineated below.

1.2.1 Chapter 2 sub-objectives and questions

Sub-objective a: Investigate whether the disruptive effects of pharmacological mTORC1 inhibition immediately after contextual fear conditioning to consolidation are long lasting and not likely due to circulating levels of drug or its metabolites interfering with the ability to retrieve the fear memory.

Research question a: Does rapamycin (RAPA, mTORC1 inhibitor) injected immediately after contextual fear conditioning (via electric foot-shock) significantly diminish context-dependent fear recall in mice one hour, two days, seven days, and 21 days post-conditioning?

Sub-objective b: Determine any deleterious latent time-dependent windows of susceptibility to pharmacological mTORC1 inhibition post-conditioning on contextual fear memory consolidation and persistence.

Research question b: Will a single injection of RAPA administered three- or 12-hours after contextual fear conditioning (via electric foot-shock) impair the consolidation and persistence of the associative context memory in mice?

Sub-objective c: Explore the effects of systemic S6K1 (downstream effector of mTORC1) and dual mTORC1/2 inhibition to contextual fear memory consolidation and persistence.

Research question c: Will systemically administering PF-470867, an inhibitor of S6K1, or concomitantly inhibiting both mTORC1/2 activity with AZD2014 immediately after contextual fear conditioning (via electric foot-shock) impair the consolidation and persistence of the associative context fear memory in mice similar to RAPA?

1.2.2 Chapter 3 sub-objectives and questions

Sub-objective d: Characterize the immunohistochemical distribution of mTORC1 activation in specific brain areas of prey animals both shortly and long after an unprotected, non-lethal

predator-stress exposure using a paradigm that results in both associative and non-associative predator-stress memories.

Research question d: What is the pattern of phosphorylated-mTORC1 immunostaining in the rat periaqueductal grey and hippocampus one-hour and seven-days after rats experience a 10-minute unprotected inescapable cat (rat-cat dyad) exposure?

Sub-objective e: Pilot a modified mouse-rat dyadic non-lethal, non-physical, predator-prey exposure paradigm, the Rat Exposure Test, otherwise used to assay mouse defensive behaviours to instead study long-term conditioned associative and non-associative fear in mice.

Research question e: What are the conditioned associative and non-associative consequences to mouse behaviour after a five-minute protected exposure to a rat?

Objective f: To test, whether the learned behavioural outcomes in mice, if any, following a brief rat exposure are susceptible to changes due to mTORC1 inhibition.

Research question f: Does RAPA administered systemically to mice immediately following a five-minute rat exposure disrupt subsequent conditioned mouse behaviour?

1.2.3 Chapter 4 sub-objectives and questions

Sub-objective g: Examine whether mTORC1 inhibition at various timepoints hours after contextual fear memory retrieval affect the reconsolidation and persistence of said memory.

Research question g: If rapamycin is administered to mice three- or 12-hours after context fear memory (conditioned via electric foot-shock) reactivation, what will the effect be to recall at two- and seven-days post-retrieval?

Sub-objective h: Assess for any additive effects from consecutive pairings of memory retrieval with mTOR inhibition immediately post-reactivation to disrupting subsequent recall, and if so, determine the behavioural process underlying it (i.e., enhanced extinction vs. impaired reconsolidation).

Research question h: When a contextual fear memory (conditioned via electric foot-shock) is reactivated and paired with a systemic injection of RAPA, and this process is repeated for two more consecutive days, for a total of three treatments, what will be the result to contextual fear recall in mice and what will occur if a subthreshold 'reminder' shock is administered prior to recall testing?

Sub-objective i: Investigate whether impaired consolidation and reconsolidation from mTOR inhibition interferes with the ability to later learn and recall new memories.

Research question i: How will the administration of RAPA immediately after context fear conditioning (either via electric foot-shock or rat predator-stress exposure) or context fear retrieval (via an electric foot-shock) influence the ability of mice to subsequently learn and recall a new fear association (via electric foot-shock auditory fear conditioning and recall procedures)?

1.3 Dissertation Structure and Description of Chapters

Together with this introductory chapter, this *manuscript-style* dissertation consists of three research chapters and a concluding chapter. Each research chapter is represented by a standalone publication of original research, of which I am the first and primary author of each piece, with the contributions made by me and my coauthors towards these publications described in the Co-authorship Statement for this dissertation. Chapters 2 and 4 are peer-reviewed journal articles and Chapter 3 comes from a chapter in an edited handbook on PTSD. Given that each of these publications can be read independent of one another but are conceptually related, it should be noted that there is unavoidably some methodological and background overlap between research chapters. The respective publishers of these works have permitted republication herein, with copies of the permission agreements found in the Appendix.

Although the sequence of research chapters follows the same chronological order of publication for these literary works, this is not the reason why I arranged the content in this order. Rather, to me the chapter order of these publications provides the most coherent flow and cohesive narrative for my dissertation's research and aims. One could also certainly make the case that despite containing original experimental research, Chapter 3 is from an edited volume with much more background exposition than the two peer-reviewed research articles that constitute Chapters 2 and 4, respectively, and as such would be better positioned in front of the peer-reviewed pieces instead of in the middle. However, I argue Chapter 3 best anchors and bridges the other research chapters together, starting with the basic experimental work on consolidation in Chapter 2, then moving on to exploring consolidation and the nature of PTSD in Chapter 3, and finally investigating reconsolidation and the treatment of PTSD in Chapter 4.

In Chapter 2, “The mTORC1 inhibitor rapamycin and the mTORC1/2 inhibitor AZD2014 impair the consolidation and persistence of contextual fear memory”, I demonstrate that a single injection of AZD2014 to mice immediately after electric foot-shock induced contextual fear conditioning weakens the associative strength for this context memory similar to RAPA treatment, whereas the S6K1 inhibitor PF-4708671 does not. Additionally, I show that RAPA systemically injected into mice immediately or three-hours, but not 12-hours after fear conditioning, enduringly diminishes the long-term strength of this contextual fear memory.

In Chapter 3, “Developing a Reliable Animal Model of PTSD in Order to Test Potential Pharmacological Treatments: Predator Stress and the Mechanistic Target of Rapamycin”, I characterize the pattern of mTORC1-phosphorylation in the hippocampus and periaqueductal grey of rats one-hour and seven-days after they experienced a brief unprotected encounter with a cat; a behavioural paradigm which has previously been shown to induce RAPA-sensitive long-lasting associative and non-associative fear memory-related behavioural changes (Fifield et al., 2013). Using a modified version of the Rat Exposure Test, I also show that mice in this psychogenic predator stress paradigm exhibit a PTSD-like sequalae of associative and non-associative fear memory-related behaviours, of which I found a subset of these behaviours to be weakened when RAPA is delivered immediately after the five-minute non-injurious physically-protected predator encounter.

In Chapter 4, “Evaluating the effects of single, multiple, and delayed systemic rapamycin injections to contextual fear reconsolidation: Implications for the neurobiology of memory and

the treatment of PTSD-like re-experiencing", I report that RAPA administered to mice three- or 12-hours post-retrieval of a context fear memory (learned via an electric foot-shock) impairs the persistence of the memory. Moreover, I reveal that two consecutive days of memory reactivation paired with RAPA is sufficient to maximize the attenuation of context fear-memory recall, with this effect displaying resistance to reinstatement from a subthreshold 'reminder' shock. Additionally, I show that post-learning (via rat exposure or foot-shock) and post-retrieval (via foot-shock) RAPA treatment to mice does not impinge on the ability to learn and recall a new electric foot-shock induced auditory-cued fear memory while also protecting against fear generalization.

Lastly, Chapter 5, "Thesis Conclusions", summarizes and discusses the significance of the results from the preceding research chapters through the lens of my overall dissertation objectives. In this final chapter, I also remark on the limitations of my findings, discuss methodological issues and potential solutions, and comment on future directions of basic rodent memory and preclinical PTSD research.

Chapter 2: The mTORC1 inhibitor rapamycin and the mTORC1/2 inhibitor AZD2014 impair the consolidation and persistence of contextual fear memory[1]

2.1 Abstract

Rationale The mechanistic target of rapamycin (mTOR) kinase mediates various long-lasting forms of synaptic and behavioural plasticity. However, there is little information concerning the temporal pattern of mTOR activation and susceptibility to pharmacological intervention during consolidation of contextual fear memory. Moreover, the contribution of both mTOR complex 1 and 2 together or the mTOR complex 1 downstream effector p70S6K (S6K1) to consolidation of contextual fear memory is unknown.

Objective Here, we tested whether different timepoints of vulnerability to rapamycin, a first generation mTOR complex 1 inhibitor, exist for contextual fear memory consolidation and persistence. We also sought to characterize the effects of dually inhibiting mTORC1/2 as well as S6K1 on fear memory formation and persistence.

Methods Rapamycin was injected systemically to mice immediately, 3 h, or 12 h after contextual fear conditioning, and retention was measured at different timepoints thereafter. To determine the effects of a single injection of the dual mTROC1/2 inhibitor AZD2014 after learning on memory consolidation and persistence, a dose-response experiment was carried out.

[1] Republished with permission from Springer Nature. Co-author: Blundell, J.

Memory formation and persistence was also assessed in response to the S6K1 inhibitor PF-4708671.

Results A single systemic injection of rapamycin immediately or 3 h, but not 12 h, after learning impaired the formation and persistence of contextual fear memory. AZD2014 was found, with limitations, to dose-dependently attenuate memory consolidation and persistence at the highest dose tested (50 mg/kg). In contrast, PF-4708671 had no effect on consolidation or persistence.

Conclusion Our results indicate the need to further understand the role of mTORC1/2 kinase activity in the molecular mechanisms underlying memory processing and also demonstrate that the effects of mTORC1 inhibition at different timepoints well after learning on memory consolidation and persistence.

2.2 Introduction

Newly learned information is at first susceptible to disruption but gradually matures and consolidates over time into a more sound, stable, and relatively impervious long-lasting representation. This enduring quality of consolidated memory is a defining characteristic of long-term memory (LTM), which can last many hours, days, weeks, years, or even a lifetime compared with short-term instantiations that decay quickly and only last from seconds to several hours. However, whether short-term memory (STM) and LTM traces are processed in serial (continuous) or parallel is still a matter of debate (Abel & Lattal 2001; Babayan et al. 2012; McGaugh, 2000; Rodriguez-Ortiz & Bermudez-Rattoni 2007; Sossin, 2008).

Molecularly, there are two key differences between STM and LTM. Whereas LTM formation requires de novo mRNA and protein synthesis (although there are exceptions to this, please see Lay et al., 2018; Ryan et al., 2015; Zhao et al., 2019 for recent examples), short term representation of memory is considered mRNA and protein synthesis independent (McGaugh, 2000). Louis and Josefa Flexner's seminal work in the 1960s first elucidated this conclusion by showing that global protein synthesis inhibitors disrupt LTM but not STM when given around the time of, or shortly after training (Flexner et al. 1967; Izquierdo & McGaugh 2000; Hernandez & Abel 2008). Importantly, these findings have been supported in many subsequent studies using an assortment of learning paradigms across a variety of taxa (Davis & Squire, 1984, Desgranges, Lévy, & Ferreira, 2008; McGaugh, 2000; Meiri & Rosenblum, 1998; Milekic et al., 2007).

As a result of these findings, memory updating (i.e., reconsolidation) notwithstanding, it was largely assumed that as time elapses, a memory became consolidated and invulnerable to insult from protein synthesis inhibitors at least 1–2 h post-learning. However, recent evidence suggests that there is at least a second wave of protein synthesis that is required for the formation and persistence of memory under certain learning experiences (Bekinschtein et al. 2007a; Bekinschtein et al. 2010; Bourtchouladze et al., 1998; Freeman et al., 1995; Grechsch & Matthies, 1980; Martinez-Moreno et al. 2011; Pena et al. 2014; Quevedo et al. 1999; Rossato et al., 2007; Wanisch et al., 2008). In these studies at least two timepoints of sensitivity to the amnestic effects of the global protein synthesis inhibitor anisomycin were confirmed, first around the time of training, and the second 3–7 h (Bourtchouladze et al., 1998; Freeman et al., 1995; Grechsch & Matthies, 1980; Martínez-Moreno et al. 2011; Pena et al., 2014; Quevedo et

al., 1999; Rossato et al., 2007) or 9–15 h (Bekinschtein et al., 2007a; Bekinschtein et al., 2010; Wanischa et al., 2008) post-acquisition.

In the process of synthesizing de novo proteins required for memory formation, translational control (regulation of mRNA translated into proteins) has often been held as a secondary passive factor due, in part, to studies that mainly focused on transcriptional control (regulation of DNA copied into mRNA; Banko & Klann, 2008; Bekinschtein et al., 2007b; Kelleher et al., 2004). However, research now posits a much more salient role for translational regulation in consolidation, especially for the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1). In addition to regulating cellular metabolism and protein turnover, mTORC1 acts as the central regulator of translation for a subset of transcripts (5′ cap-dependent) through phosphorylation of two downstream substrates, p70S6 kinase (S6K1) and eukaryotic initiation factor 4E-binding protein (4EBP1). Although the catalytic subunit of mTORC1 is the same serine/threonine protein kinase that nucleates mTOR complex 2 (mTORC2), unlike mTORC2, substrate selectivity for mTORC1 is conferred by its constituent component regulatory-associated protein of mTOR (raptor). Sequestering of substrates to active mTOR sites by raptor, however, is narrowed and partially blocked by the mTORC1 allosteric inhibitor rapamycin (RAPA; Sabatini, 2017; Saxton & Sabatini 2017; Sengupta et al., 2010).

Acute inhibition of mTORC1 activity using RAPA shortly before or after learning has highlighted the significance of translational regulation in memory consolidation. For instance, RAPA administered systemically or intracerebrally to brain areas important to certain types of associative memories prevents learning-induced mTORC1 phosphorylation of S6K1 in these

brain areas (Bekinschtein et al., 2007b; Glover et al., 2010; Lana et al., 2017; Parsons et al., 2006b; Slipczuk et al., 2009). In concert with these physiological effects, several studies have shown that RAPA disrupts LTM formation of the newly learned information (Bekinschtein et al., 2007b; Blundell et al., 2008; Gafford et al., 2011; Glover et al., 2010; Jobim et al., 2012a, b; Parsons et al., 2006b; Slipczuk et al., 2009). Furthermore, mTOR heterozygous (mTOR+/−) mice show greater sensitivity to RAPA impairment of associative memory consolidation compared with heterozygous wildtype mice (Stoica et al., 2011). Oppositely, mice genetically engineered to be insensitive to RAPA have greater basal mTOR-raptor interactions and display enhanced contextual fear memory compared with their wildtype counterparts (Hoeffer et al., 2008).

As the life of a memory eclipses the turnover rate of the synaptic proteins that underwrites its consolidation, there needs to be a mechanism to confer persistence against gradual decay (Aslam et al., 2009; Bekinschtein et al., 2008). Although it appears that a second de novo protein synthesis window is required for memory persistence, the precise role of mTORC1 in delayed consolidation-like molecular events are still being deciphered. For instance, RAPA administered systemically immediately or 12 h after training, but not at several other timepoints, has negative effects on amygdala-dependent auditory fear memory when tested 48 h after training (Mac Callum et al., 2014). In contrast, intrahippocampal RAPA infusion 15 min before or 3 h after learning, but not at other timepoints, including 12 h, diminishes hippocampal-dependent fear memory formation and persistence for inhibitory avoidance (Bekinschtein et al., 2008; Slipczuk et al., 2009). This is noteworthy since Bekinschtein et al. (2010) found that ansiomycin injected into the hippocampus 12 h after learning decayed the strength of inhibitory avoidance memory

gradually over a week and abolished the expression of delayed learning-evoked increases in several immediate early genes. These differences are likely due to RAPA only inhibiting the translation of a subset of transcripts, anisomycin inhibiting almost all protein synthesis, and different mnemonic processes underlying each type of fear learning used (Lattal & Abel 2004; Parsons et al., 2006b). It is unknown, however, whether abatement of memory from time-dependent post-training mTOR blockade would be consistent for contextual fear conditioning, which is procedurally like amygdala-dependent cued-fear conditioning, but requires the hippocampus, like inhibitory avoidance.

Unlike mTORC1, mTOR complex 2 (mTORC2) lacks raptor and instead has the analogous protein RAPA insensitive companion of mTOR (rictor) as a constituent component. As a result, mTORC2 is not susceptible to acute RAPA treatment, but if given chronically, RAPA indirectly blocks the assembly of mTORC2 (Sarbassov et al., 2006). At the cellular level, mTORC2 primarily controls survival, proliferation, ion transport, glucose metabolism, and cytoskeletal rearrangement through regulation of downstream serine/threonine protein kinase 1, protein kinase B, and C (Lamming, 2016; Sabatini, 2017; Saxton & Sabatini, 2017). The study of mTORC2 function in the neurobiology of behaviour and biomedical research in general, however, has been limited by the lack of specific mTORC2 inhibitors, although some inhibitors are currently in development (Benavides-Serrato et al., 2017; Murray & Cameron 2017; Werfel et al., 2018). Nonetheless, conditional knockout studies have shown that drosophila lacking rictor have impaired spatial memory, while mice lacking rictor were likewise found to have impaired consolidation of long-term fear and non-fear associative memories due to deficient actin polymerization (Angliker & Ruegg, 2013; Huang et al., 2013; Sun et al., 2019; Zhu et al., 2018).

Interestingly, pharmacologically restoring actin polymerization in the hippocampus of conditional knockout rictor mice rescued contextual fear memory deficits, but not hippocampus-independent auditory fear memory, while enhancing contextual fear memory after weak training in wildtypes (Huang et al., 2013).

Although there are no specific mTORC2 inhibitors, there are now second-generation mTOR inhibitors that dually inhibit mTORC1 and mTORC2 kinase activity by competing for the ATP catalytic site on mTOR (Sabatini, 2017; Saxton & Sabatini, 2017). Interestingly, there is no published research to date that examines the effects of these dual inhibitors on the neurobiology of behaviour. This is surprising, however, since research now indicates RAPA only blocks the phosphorylation of some downstream mTORC1 targets. Specifically, phosphorylation of 4EBP1 is RAPA-insensitive in mammalian lines, while dual mTORC1/2 inhibitors robustly inhibit phosphorylation of all mTORC1 substrates (Choo et al., 2008; Feldman et al., 2009; Sabatini, 2017; Thoreen et al., 2009; Yu et al., 2009).

The phosphorylation of S6K1 is the hallmark readout of mTORC1 kinase activity, while the opposite is an indication of mTORC1 inhibition by RAPA. Indeed, soon after fear or non-fear conditioning, S6K1 activity is increased in regions of the brain germane to memory consolidation, while RAPA treatment prevents this effect and impairs the memory associated with the learning event (Dash et al., 2006; Glover et al., 2010; James et al., 2016; Jobim et al., 2012a, b; Lana et al., 2017; Neasta et al., 2014; Parsons et al., 2006b; Slipczuk et al., 2009). Genetic studies of S6K1 knockout mice have shown deficient spatial and taste learning and impaired consolidation of object recognition and contextual fear memory (Antion et al., 2008;

Bhattacharya et al., 2012). Surprisingly, however, these knockout mice show normal acquisition and consolidation of cued auditory fear memory and are resistant to cued fear extinction (Antion et al., 2008; Huynh et al., 2018). Auditory fear memory extinction is also blocked by pharmacological inhibition of S6K1 when mice are injected 1 h before extinction training with the first reported S6K1 specific inhibitor PF-4708671 (Huynh et al., 2018; Pearce et al., 2010). Interestingly, when PF- 4708671 is administered immediately after auditory fear memory retrieval, the persistence of reconsolidated memory becomes compromised, but not the initial reconsolidation (Huynh et al., 2014). In contrast to the effects on reconsolidation and extinction, there is no published data reporting the effects of pharmacological inhibition of S6K1 to consolidation of a conditioned associative memory.

As such, one aim of this project is to evaluate the effects of S6K1 inhibition to contextual fear memory consolidation and persistence. Likewise, we also assess whether there are any additive effects to disturbing contextual fear memory formation and persistence by simultaneously blocking mTORC1/2 activity. Further, established time-dependent windows of susceptibility to RAPA for cued fear and inhibitory avoidance memory consolidation are tested against associative contextual fear memory using a single systemic injection of RAPA.

2.3 Methods

Memorial University of Newfoundland's (MUN's) Animal Care Committee approved all animal procedures and experimental protocols with husbandry and regulatory oversight of animal care

and use provided by MUN's Animal Care Services pursuant to the standards and guidelines of the Canadian Council on Animal Care.

2.3.1 Animals

Male C57BL/6NCrl mice (Charles River Laboratories, St. Constant, QC, CA) were used as subjects for all experiments described herein. Mice, 3–4 weeks old upon arrival, were group housed with 2–3 conspecifics per cage and given ad libitum access to food and water in standard laboratory conditions (i.e., temperature and humidity) on a 12 h light-dark cycle (lights on at 7:00 AM). Behavioural procedures began at 5–6 weeks of age. All husbandry duties, recording of body weight, tail marking using non-toxic markers for identification purposes, and experimental procedures occurred during the light phase of the light-dark cycle unless stated otherwise.

2.3.2 Pharmacological treatments

The mTORC1 inhibitor rapamycin (RAPA, 40 mg/kg of body weight, LC Laboratories, Woburn, MA, US), the S6K1 inhibitor PF-4708671 (50 mg/kg, Toronto Research Chemicals, Toronto, ON, CA), and the dual mTOR complex 1/2 inhibitor AZD2014 (1, 10, and 50 mg/kg, Toronto Research Chemicals) were each prepared using the same procedure. Close to the time of injections, the specific drug was first dissolved in ethanol (5% of total vehicle solution), then in a vehicle (VEH) solution of 5% Tween 80 and 5% PEG 400 in distilled water through sonication and vortex mixing. Drug was administered to mice systemically through single intraperitoneal

(i.p.) injections at a volume of 10 ml/kg of body weight. Control mice received a single i.p. injection of the VEH solution (5% ethanol, 5% Tween 80, and 5% PEG 400 in distilled water) at the same volume as drug treated animals (10 ml/kg). In experiments with a delayed drug injection following fear conditioning (see below for specific timetable of events for each experiment), injections were performed in the animal housing room rather than the behavioural training and testing room. Further, for mice that received drug treatment in the dark phase of the light-dark cycle, injections were administered under overhead red-lights to minimize circadian rhythm disruption.

The RAPA dosage of 40 mg/kg of body weight was selected based on evidence that it effectively disrupts memory without changing locomotor, anxiety, or nociceptive behaviour (Blundell et al. 2008), while the PF-4708671 dosage of 50 mg/kg significantly reduces brain S6K1 phosphorylation without varying motor behaviour (Huynh et al., 2014). Preclinical and phase I and II clinical trials have focused on the efficacy of AZD2014 at treating certain forms of cancers, with fatigue, nausea, and mucositis found to be the most common side effects from intermittent or continuous dosing (Basu et al., 2015; Guichard et al., 2015; Jones et al., 2019; Kahn et al., 2014; Powles et al., 2016; Teh et al., 2018; Zhong et al., 2014). Moreover, although a 50 mg/kg oral dose of AZD2014 crosses the blood-brain barrier and inhibits mTOR kinase activity in intracerebral tumour xenografts (Kahn et al., 2014), to our knowledge, there is no published research to date examining the cognitive effects of AZD2014. As such, a dose-response relationship was used to examine the effects of a single systemic injection of AZD2014 (1, 10, and 50 mg/kg) on memory consolidation and persistence.

2.3.3 Contextual fear conditioning and associative memory testing

Before training and testing sessions, mice were carted in their home cages from the animal housing room to a room adjacent to the training and testing room for a minimum of 1 h. Mice from the same cage were trained and tested simultaneously in separate conditioning chambers, with all equipment cleaned using 40% ethanol and air-dried between each animal's usage. At the conclusion of any procedure mice were promptly placed back into their home cage and returned to the animal housing room.

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, MA, US). To condition mice to fear the training context, mice were given a single 338 s training session in the conditioning chambers. Ninety seconds after being placed into the conditioning chambers, mice received four, 2 s, 0.7 mA foot shocks (physical unconditioned stimuli), with an average 50 s variable interval between shocks (Precision Animal Shocker, Coulbourn Instruments). Following the last foot shock, mice remained in the conditioning chambers for an additional 90 s before being removed.

The strength of contextual fear memory associability from pairing foot shock with the conditioning chamber was tested at various frequencies and after varying intervals of time (see below for specific timetable of events for each experiment) by returning mice to the original

training environment (the conditioned stimulus) for 240 s and measuring freezing behaviour. Importantly, no foot shocks were administered during any recall session and all retention tests of contextual fear memory were identical in procedure. Freezing behaviour — a species specific behaviour to a threat is defined as the absence of movement, except for those movements associated with respiration — was measured throughout recall tests and during the first and last 90 s of training using automated software (FreezeFrame, Coulbourn Instruments) and expressed as a percentage of total time (s) per recall session or training interval. Conditioning and testing protocols were adapted from Blundell et al. (2008), Cai et al. (2006), Curzon et al. (2009) and tested through a preliminary study (data not shown).

2.3.4 Experiments

Experiment 1. Immediately following contextual fear conditioning, mice received a single i.p. injection of either VEH (control group, n = 12) or RAPA (40 mg/kg of body weight, n = 12). Mice were then tested for contextual fear memory recall, as described above, 1 h and 48 h after training.

Experiment 2. Mice were fear-conditioned to the training context, treated with either VEH (n = 15) or RAPA (40 mg/kg, n = 15) immediately following training, then tested for contextual fear memory recall 7 days later.

Experiment 3. Like experiment 1 and 2, mice were conditioned to fear the context and then treated with either VEH (n = 11) or RAPA (40 mg/kg, n = 12) immediately following training. Twenty-one days later, contextual fear memory was tested.

Experiment 4. Mice received either an i.p. injection of VEH (n = 12) or RAPA (40 mg/kg, n = 12) 3 h after context fear conditioning. Contextual fear memory was then measured 48 h and 21 days after training.

Experiment 5. Like experiment 4, mice were injected with either VEH (n = 15) or RAPA (40 mg/kg, n = 15) 3 h after contextual fear conditioning. Contextual fear memory was then tested 7 days after training.

Experiment 6. Vehicle (n = 12) or RAPA (40 mg/kg, n = 12) was administered systemically to mice 12 h after contextual fear conditioning during the dark phase of the light-dark cycle. Recall for contextual fear memory was then measured 48 h after training.

Experiment 7. Like experiment 6, VEH (n = 15) or RAPA (40 mg/kg, n = 16) was administered systemically to mice 12 h after contextual fear conditioning during the dark phase of the light-dark cycle. Recall for contextual fear memory was then measured 7 days after training.

Experiment 8. Immediately following contextual fear training, mice received a single i.p. injected of either VEH (n = 12), RAPA (40 mg/kg, n = 12), PF-4708671 (50 mg/kg, n = 12), or

AZD2014 at a dose of 1 mg/kg (n = 10), 10 mg/kg (n = 11), or 50 mg/kg (n = 11). Recall for contextual fear memory was then measured 48 h, 7 days, and 21 days after training.

2.3.5 Statistics

Independent samples *t* tests were used for two group comparisons of freezing behaviour for single recall events (retention tests). For experiments with two or more recall events mixed analysis of variance (ANOVA) tests were used to examine the between-subjects factor of treatment condition and the within-subjects factor of time on freezing behaviour. In addition to using a mixed ANOVA, linear mixed model procedures (restricted maximum likelihood method, Sattherwaite approximation for degrees of freedom) were also used to fit to our data from the dose-response experiment with three recall events to change and compare the variance-covariance structure of freezing over time and to accommodate missing data, both not adequately compensated for by classical repeated measures analyses. Where appropriate, significant main effects or interactions were followed up with planned contrasts or multiple comparisons using Bonferroni's post hoc tests. A mixed ANOVA was also employed to evaluate learning acquisition by comparing pre-learning (90 s before first foot shock) and post-learning (90 s following last foot shock) freezing between and within groups for all experiments. Data organization and statistical analyses were made using SPSS (Version 26, IMB, Armonk, NY, US) and Excel (Microsoft, Redmond, WA, US), while figures were made using Prism (GraphPad Software, San Diego, CA, US). Group data for freezing percentage is reported as mean ± standard error, with significance taken at $p < 0.05$. Please note that because of mortality in home

cages (health related or from fighting) or recording issues during testing some experiments have unequal sample sizes between groups.

2.4 Results

For all experiments, as expected, naïve mice froze significantly more in the 90 s period following the last foot shock compared with the 90 s preceding the first foot shock, indicating acquisition of contextual fear learning had taken place (data not shown for mixed ANOVA tests, but all experiments found a significant main effect of time, *ps* < .05). Moreover, since all mice were naïve to the conditioning chambers and had not yet received pharmacological treatment, as anticipated, there were no between subjects differences found in freezing behaviour during either of these periods (first 90 s and last 90 s) of the contextual fear conditioning procedure for any experiment (data not shown, all *ps* > .05 for main effect of treatment).

2.4.1 The consolidation and persistence of contextual fear memory is susceptible to mTORC1 blockade immediately after learning

We and others have previously shown that RAPA blockade of mTORC1 around the time of learning impairs the strength of long-term memory but spares short-term memory recall for cues, contextual contingencies, and familiar objects (Bekinschtein et al., 2007b; Jobim et al., 2012a, b; Lana et al., 2017; Mac Callum et al., 2014; Stoica et al. 2011; Sui et al., 2008). To replicate these findings, we gave mice a single i.p. injection of RAPA or VEH immediately after fear conditioning and tested for context memory retention 1 h and 48 h after training. Results of a

mixed ANOVA revealed a significant main effect of time (F (1, 22) = 24.511, p < .001) and an interaction effect of time X treatment (F (1, 22) = 10.399, p = .004), but no main effect of treatment (F (1, 22) = 1.246, p = .276). Indeed, follow-up comparisons of each recall event were found to be consistent with previous reports, as mice treated systemically with RAPA froze equally as much as their VEH counterparts 1 h after training (Fig. 2.1a; t (22) = − .452, p = .655), but showed significantly less freezing towards the conditioning context when tested again 48 h after training compared with VEH-treated controls (Fig. 2.1a; t (22) = 2.135, p = .044).

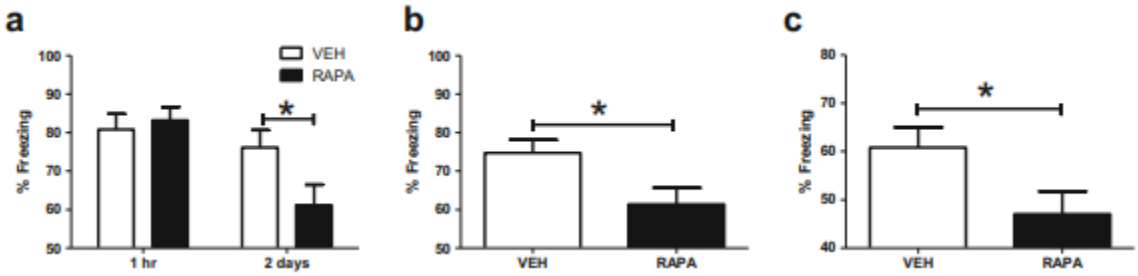

Figure 2.1 Systemic RAPA immediately after learning impairs the consolidation and persistence of contextual fear memory. **a** Mice treated with RAPA immediately after learning show normal recall 1 h thereafter (t (22) = − .452, p = .655) but have poor memory retention when tested 2 days later (t (22) = 2.135, p = .044). **b**, **c** Injection of RAPA immediately after training diminished conditioned freezing to the context when tested (**b**) 7 days (t (28) = 2.348, p = .026); n (VEH) = 15, (RAPA) = 15) or (**c**) 21 days later (t (21) = 2.166, p = .042). n = 12 (VEH), 12 (RAPA) for Fig. 2.1a; n = 15 (VEH), 15 (RAPA) for Fig. 2.1b; and n = 11 (VEH), 12 (RAPA) for Fig. 2.1c

The results from the above experiment has several limitations in delineating the function of mTOR in memory consolidation. First, there is the potential interference from retrieval of the

learned behaviour 1 h after training on memory processing and drug action. Second, since RAPA is reported to have a long terminal half-life (Arriola Apelo & Lamming, 2016; Bottiger et al., 2001; Drion et al., 2016; Honcharik et al., 1992; Supko & Malspeis, 1994), there is the probability, albeit low, that the drug is impinging on the ability to retrieve the information about the context during testing 48 h after training. To control for these confounds, we conditioned mice, treated them with RAPA or VEH immediately afterwards, then tested for contextual fear memory retention 7 days after training. This protocol allowed for a long drug washout period, while also limiting any interference from retrieval on drug action to memory consolidation. Freezing data from the recall session was analysed using an independent samples *t* test. Here we found that mice treated with RAPA immediately after conditioning froze significantly less to the fear conditioning context than VEH-treated mice when tested 7 days after training (Fig. 2.1b; $t(28) = 2.348$, $p = .026$). Importantly, this result was consistent with our results on long-term memory from the first experiment.

Anisomysin, the global protein synthesis inhibitor, when administered immediately after learning results in long-lasting consolidation deficits for at least 21 days for either cued or contextual fear memories (Lattal & Abel, 2004; Mac Callum et al., 2014). Conversely, while RAPA disrupts the consolidation of cued-fear memory (Mac Callum et al., 2014; Parsons et al., 2006b), this effect appears to be ephemeral as retention is comparable with controls when tested 21 days after training (Mac Callum et al., 2014). As a result, we tested whether the contextual fear memory retention deficit observed 7 days after RAPA treatment immediately post-acquisition in the prior experiment would persist if tested at 21 days instead or whether the effects on consolidation would diminish over time like cued-fear memory. An independent samples t test found a

significant decrease in freezing behaviour of mice injected systemically with RAPA after training relative to VEH-treated controls when tested 21 days later (Fig. 2.1c; t (21) = 2.166, p = .042). Collectively, these results indicate RAPA treatment immediately after conditioning interferes with consolidation of contextual fear memory and that these effects are long-lasting.

2.4.2 Systemic RAPA 3 h after learning, but not 12 h, impairs contextual fear memory consolidation and persistence

As previously mentioned, the consolidation and persistence of inhibitory avoidance memory is RAPA-sensitive 15 min before and 3 h after learning, whereas cued fear memory consolidation is susceptible to RAPA immediately after and 12 h post-training, but not at different time points for either type of learning (Mac Callum et al., 2014; Slipczuk et al., 2009). Consequently, we investigated whether Pavlovian contextual fear memory consolidation and persistence demonstrated either of these time-dependent susceptibilities to systemic RAPA treatment at 3 h or 12 h post-training through several experiments.

To first test for time-dependent susceptibility of mTOR blockade to contextual fear consolidation, mice were given a single systemic injection of RAPA or VEH 3 h following conditioning, then tested for contextual fear memory retention 48 h and 21 days after training. A mixed ANOVA of the freezing data from recall tests indicated a significant main effect of time (F (1, 22) = 14.19, p < .001), main effect of treatment (F 1, 22) = 14.867, p < .001), but no interaction effect of time X treatment (F (1, 22) = .997, p = .334). Planned a priori contrasts of each retention test showed that fear memory was significantly attenuated in RAPA-treated

animals compared with VEH-treated controls at both 48 h (Fig. 2.2a; t (22) = 2.674, p = .014) and 21 days (Fig. 2.2a; t (22) = 4.253, p < .001) after training.

To control for interference from multiple recall tests and any residual effects of circulating levels of RAPA at the time of the first recall test, 48 h after training, and 45 h after drug treatment, we conducted a follow-up experiment. Here, like the previous experiment, mice received a single systemic injection of RAPA or VEH 3 h after contextual fear conditioning. But unlike the previous experiment, mice were only tested for contextual fear memory retention once, 7 days after training instead of twice, first 48 h, and second 21 days after training. Consistent with the findings from the previous experiment, an independent t test showed that mice treated with RAPA 3 h after conditioning stymied the persistence of fear memory as these animals froze significantly less to the shock-paired context than their VEH-treated counterparts 7 days after training (Fig. 2.2b; t (28) = 4.543, p < .001).

Next, we sought to examine the effects of systemic RAPA 12 h after training on memory consolidation and persistence. In two separate but similar experiments, mice received a single i.p. injection of RAPA or VEH 12 h after contextual fear conditioning and were tested either 48 h (experiment # 6) or 7 days (experiment # 7) after the learning event. Statistically for both experiments, there were no significant differences as systemic blockade of mTOR through RAPA at 12 h after conditioning failed to change the strength of contextual fear memory relative to VEH-treated controls either 48 h or 7 days post-training (Fig. 2.2 c and d; data not shown, all p > .05). Taken together, these results indicate that contextual fear memory consolidation is

susceptible to mTOR blockade from systemic RAPA treatment 3 h after, but not 12 h after learning.

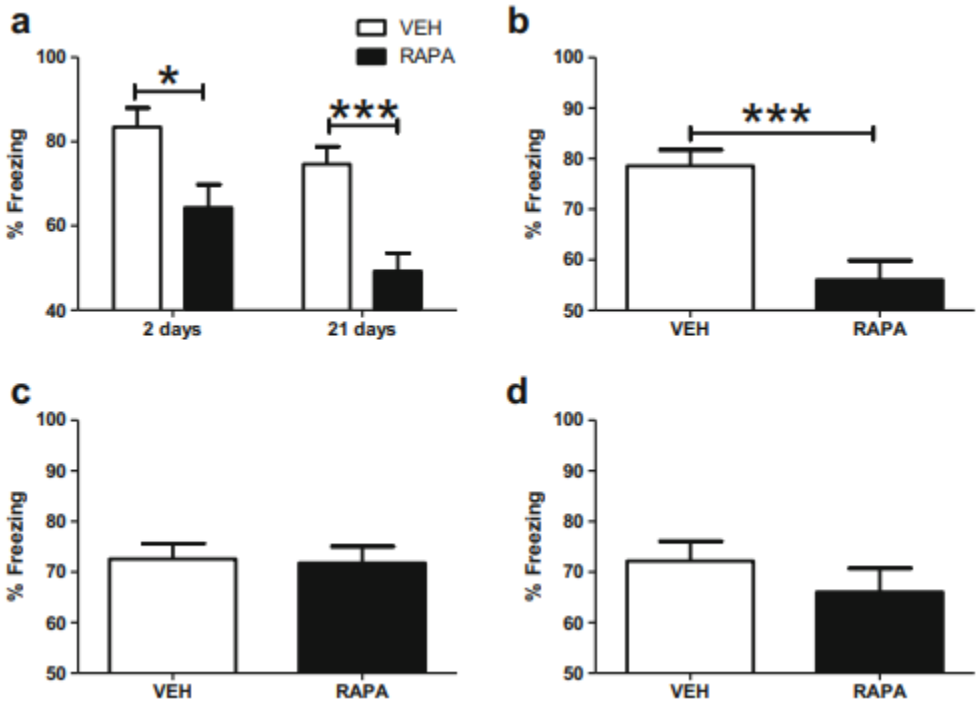

Figure 2.2 Delayed RAPA treatment 3 h after learning impairs contextual fear memory formation and persistence. **a** Injection of RAPA 3 h following acquisition significantly hinders contextual fear memory when tested 2 days (t (22) = 2.674, p = .014) and 21 days after training (*t* (22) = 4.253, $p < .001$). **b** Mice that receive a single systemic injection of RAPA 3 h after conditioning display reduced freezing when tested 7 days after training (*t* (28) = 4.543, $p < .001$). **c, d** RAPA injected 12 h after learning does not alter contextual fear memory retention when tested either (**c**) 2 days or (**d**) 7 days post-training (data not shown, all $p > .05$). n = 12 (VEH), 12 (RAPA) for Fig. 2.2a; n = 15 (VEH), 15 (RAPA) for Fig. 2.2b; n = 12 (VEH), 12 (RAPA) for Fig. 2.2c, and n = 15 (VEH), 16 (RAPA) for Fig. 2.2d

2.4.3 The dual mTORC1/2 inhibitor AZD2014 impairs contextual fear memory

There is evidence that the inhibitor of mTORC1 downstream target S6K1, PF-470867, when administered immediately after fear memory retrieval has no effect on reconsolidated memory 24 h after reactivation but impairs the persistence of reconsolidated memory when tested a second time, 10 days after reactivation (Huynh et al., 2014). Here, we wanted to test whether S6K1 inhibition using PF-470867 would confer similar effects against the consolidation and persistence of contextual fear memory. Further, we also wanted to evaluate the effects of the dual mTORC1/2 inhibitor AZD2014 on memory consolidation and persistence using a dose-response relationship as there is no evidence to date about the cognitive effects of this compound. A RAPA treatment group was used as a positive control.

Following contextual fear conditioning, mice received a single i.p. injection of either 50 mg/kg of PF-470867, 1 mg/kg of AZD2014, 10 mg/kg of AZD2014, 50 mg/kg of AZD2014, 40 mg/kg of RAPA, or VEH. Contextual fear memory was then evaluated 48 h, 7 days, and 21 days after training (Fig. 2.3a). A mixed ANOVA of the freezing data from recall events only found a significant main effect of time (F (2, 124) = 47.124, $p < .001$). The main effect of treatment approached but was not statistically significant (F (5, 62) = 2.913, $p = .066$), while neither was the interaction effect of time X treatment (F (10, 124) = .916, $p = .521$). Follow-up polynomial contrasts for the effect of time revealed significant linear (F (1, 62) = 69.174, $p < .001$) and quadratic (F (1, 62) = 14.922, $p < .001$) trends in freezing behaviour from the mice over the three recall events, but no significant linear or quadratic time X treatment trends (data not shown, all $p > .05$). Moreover, in regard to the linear and quadratic trends for time, post hoc pairwise

comparisons showed that time spent freezing diminished significantly following the first recall event 48 h after training when compared with the second, 7 days after training, and third, 21 days after training (Fig. 2.3b; Bonferroni analysis for both comparisons $p < .001$), whereas there was no significant difference between the second and third recall events ($p = .357$).

Although the sphericity assumption was not violated here (Mauchly's test, $p = .335$), given the significant trends in freezing over time we also tested our data using linear mixed model procedures to compare two different covariance structures for the repeated measures. The first structure used is a stricter but closely related characteristic of the sphericity assumption called compound symmetry, which forces equal covariances across all trials. The second structure employed, heterogenous autoregressive covariance, assumes that adjacent ordered measurements are more highly correlated than measurements further apart akin to the actual covariances for time in our data (48 h and 7 days post learning recall were moderately correlated, r (69) = 0.549, $p < .001$; 48 h and 21 days post learning recall were moderately correlated, (69) = 0.466, $p < .001$; and 7 days and 21 days post learning recall were strongly correlated, r (66) = 0.685, $p < .001$). The versatility of the linear mixed model approach allowed us to test model quality, evaluate whether significance levels would be sustained, and treat time as a continuous rather than a categorical variable. Moreover, this model allowed us to include subjects otherwise excluded in an ANOVA because of at least one missing data point.

Predictably, when the model was fitted with a compound symmetry structure to balanced data, results for the main and interaction effects were identical to that of the mixed ANOVA. A Schwarz's Bayesian Information Criterion (BIC) value of 1586.585 was also calculated for this

model, which would later be used to compare model quality (where smaller-is-better). When the covariance was changed to a heterogenous autocorrelated structure, the results were very similar to those found in the compound symmetry model. Time was still the only significant effect (F (2, 86.67) = 43.455, $p < .001$), while treatment again approached but was not statistically significant (F (5, 63.547) = 2.261, $p = .059$), and nor was the interaction effect of time X treatment (F (10, 86.67) = .994, $p = .455$). Nevertheless, the lower BIC estimation for this model (BIC = 1582.813) indicates that this model better contributes to the balance between model sensitivity (complexity) and specificity (goodness of fit) compared with the compound symmetry model (difference = 3.772). We next ran the same linear mixed model procedures as above but with unbalanced data to include three subjects with missing data for the final recall event due to recording issues or mortality before the third recall test (one subject each from each of the AZD2014 groups). Consistent with our previous models, the time X treatment interaction effect was not found to be significant under either covariance structure (data not shown, all $p > .05$). However, with the additional data included both the main effects of time and treatment were found to be significant under both covariance structures (compound symmetry: time, F (2, 127.809) = 50.155, $p < .001$; treatment, F (5, 65.217) = 2.46, $p = .042$; heterogenous autoregressive: time, F (2, 89.021) = 46.993, $p > 001$; treatment, F (5, 67.068) = 2.52, $p = .038$). BIC comparisons of the two models again indicated that the heterogenous autoregressive covariance structure (BIC = 1631.462) was the better quality model (recall smaller-is-better) than the compound symmetry structure (BIC = 1634.947, difference = 3.485).

To further examine the significant main effect of treatment from the unbalanced dataset, post-hoc Bonferroni comparisons were made from the estimated marginal means of the heterogenous

autoregressive covariance model with the VEH-treated control group (Mean = 80.078 (± 3.977)) used as the reference group against each of the five treatment conditions (Fig. 2.3c). Consistent with our earlier experiments, RAPA-treated mice (Mean = 62.931 (± 3.977)) froze significantly less to the training context compared with VEH-treated controls (Mean difference = 17.147; p = .016). In contrast, animals treated with PF-470867 (Mean = 69.403 (± 3.977)) were not statistically different from VEH-treated mice (Mean difference = 10.675; p = .31). Likewise, neither groups of mice treated with either of the lower doses of AZD2014 were significantly different from their VEH counterparts (Mean = 68.42 (± 4.181) and 68.765 (± 4.001), Mean difference = 11.657 and 11.313, p = .237 and .245 for 1 and 10 mg/kg AZD2014, respectively). However, unlike the lower doses of AZD2014, mice treated with 50 mg/kg of AZD2014 (Mean = 62.666 (± 4.001)), like RAPA, froze significantly less overall than VEH-treated controls (Mean difference = 17.412, p = .015). As such, our results for the highest dose of AZD2014 posits the possibility that other mTOR inhibitors might have the capacity to interfere with memory processing like RAPA. However, we must stress that this inference needs to be approached with an abundance of caution as the main effect of treatment from the mixed ANOVA was not statistically significant but became significant when data discounted from the repeated measures ANOVA was included in a linear mixed model. Moreover, a lack of an interaction effect or any a priori predictions limited our examination of any simple main effects. Regarding the main effect of time, follow-up comparisons of time were slightly different from the mixed ANOVA but followed the same pattern of significance for all contrasts and pairwise comparisons as shown in Fig. 2.3b (data not shown).

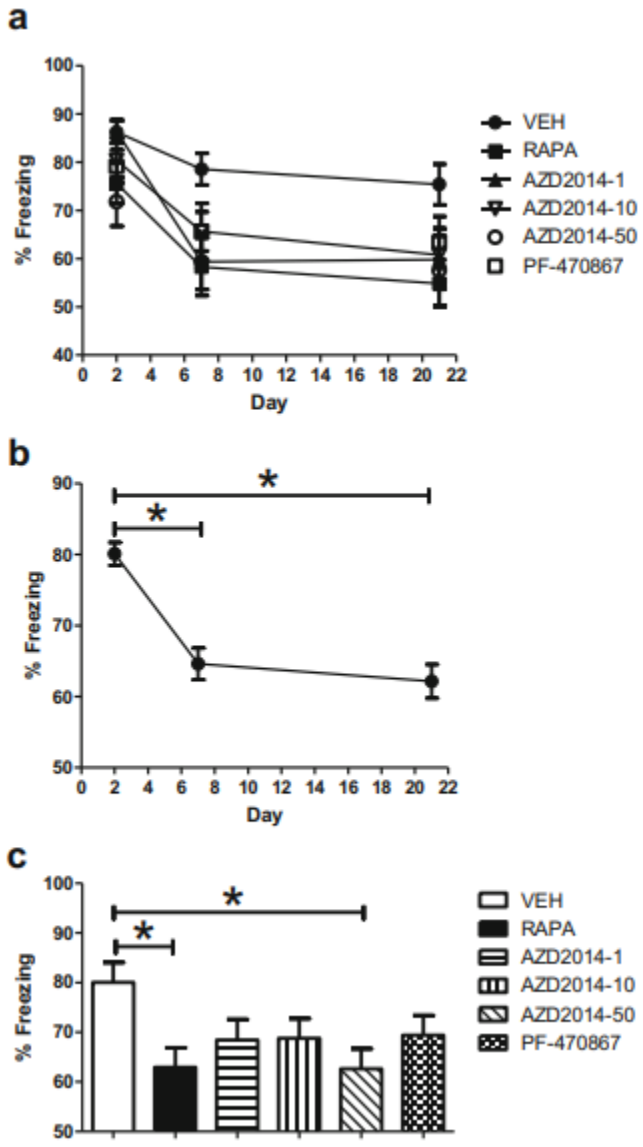

Figure 2.3 The effects of the dual mTORC1/2 inhibitor AZD2014 on contextual fear memory formation and persistence. **a** Mice received a single systemic injection of either VEH, 40 mg/kg of RAPA, 50 mg/kg of PF-470867, 1 mg/kg of AZD2014, 10 mg/kg of AZD2014, or 50 mg/kg of AZD2014 immediately after learning and were tested for contextual fear memory recall 2, 7, and 21 days after training in the absence of any foot shocks. **b** Overall, freezing behaviour significantly diminished when tested at 7 and 21 days after training compared with the first recall test that occurred 2 days after contextual fear conditioning (Bonferroni post hoc tests for the main effect of time: 2 days v. 7 days, $p < .001$; 2 days v. 21 days, $p < .001$; 7 days v. 21 days, $p = .357$). **c** Estimated marginal means for each group was compared against VEH using Bonferroni post hoc tests. Freezing behaviour for RAPA-treated mice was significantly decreased compared with VEH controls ($p = .016$). Likewise, freezing from animals given the highest dosage of AZD2014 at 50 mg/kg was found to be significantly less than their VEH counterparts ($p = .015$). Conversely, mice given either of the

smaller doses of AZD2014 (1 or 10 mg/kg) or the S6K1 inhibitor, PF-4708671 at 50 mg/kg were all found to be not statistically different from VEH-treated mice (all $p > .05$). n = 12 (VEH), n = 12 (RAPA at 40 mg/kg)), n = 12 (PF-4708671 50 mg/kg), n = 11 (AZD2014 at 1 mg/kg), n = 12 (AZD2014 at 10 mg/kg), n = 12 (AZD2014 at 50 mg/kg)

2.5 Discussion

Our results confirm and expand upon earlier work by showing that a single, 40 mg/kg, systemic injection of RAPA immediately after associative learning significantly weakens the consolidation and persistence of contextual fear LTM without encroaching upon STM. Further, we demonstrate that fear memory formation and persistence is still susceptible to systemic mTORC1 blockade 3 h, but not 12 h, after learning. As a result, the present findings indicate mTORC1 activation immediately after and in the hours shortly after learning strongly contribute to the molecular mechanisms required for contextual fear memory formation and persistence. We also show, with limitations, that a single systemic application of the ATP-competitive mTOR kinase inhibitor AZD2014 immediately after learning dose-dependently impairs contextual fear memory consolidation and persistence. Although it is tempting to suggest a fundamental role for both mTORC1/2 kinase activity in the molecular mechanisms underlying memory processing from these findings, it does underscore the need to better understand the function of mTORC2 in memory processing. Further, we found that systemically inhibiting the mTORC1 downstream effector S6K1 with PF-4708671 immediately after learning does not significantly alter contextual fear memory formation or persistence, perhaps due to other mTORC1 downstream targets not being simultaneously inhibited.

The results from our experiments on consolidation are aligned with previous studies using systemic or intracerebral RAPA treatment shortly before or after learning and testing for fear memory recall strength 24–48 h later (Bekinschtein et al., 2007b; Blundell et al., 2008; Lana et al., 2017; Mac Callum et al., 2014; Parsons et al., 2006b). Nonetheless, our findings further differentiate the function of mTORC1 in memory processing through systemic blockade using RAPA. Indeed, past studies have shown that infusion of RAPA directly into the brain or systemically impairs consolidation of hippocampal-dependent recognition and inhibitory avoidance LTM without affecting STM (Bekinschtein et al., 2007b; Jobim et al., 2012a, b; Lana et al., 2017; Myskiw et al., 2008; Stoica et al., 2011). However, in many of these studies, STM and LTM were tested in separate experiments with separate animals. Importantly here, like our previous research with auditory fear memory, systemic RAPA likewise did not affect the expression of contextual fear shortly after the learning event occurred, but attenuated LTM when tested 48 h after training and drug treatment, 47 h after STM testing. An unfortunate drawback of this type of testing, however, is that the conclusions drawn from LTM testing are susceptible to interference from STM recall but at the same time illustrates the juxtaposition of mTORC1 function in memory processes through a single experiment.

We also show that RAPA administered immediately after learning diminishes contextual fear memory retention when tested 1 week later. As such, these results help strengthen our earlier conclusions and allays doubt from our first experiment by removing the influence of STM retrieval while isolating the effects of systemic drug action on LTM formation processes evoked by learning. Moreover, our data confirm and extend earlier findings that concluded systemic RAPA administered around the time of learning disrupts contextual fear memory consolidation

(Blundell et al., 2008). Although this earlier work revealed decreased freezing 24 h after training and treatment, RAPA has a relatively long half-life in mammalian systems and could have potentially been interrupting retrieval at this testing timepoint, disguising the actual effects of RAPA on consolidation. Indeed, RAPA has blood levels detectable up to 3 days after i.p. injections in mice capable of inhibiting mTORC1 signalling in cell cultures (Arriola Apelo et al., 2016; Sarbassov et al., 2006). By waiting 1 week, we allowed for a much larger washout period for RAPA to be metabolized and removed before testing memory retention, thus, lifting any uncertainty that residual levels of the drug are altering retrieval but instead strongly implicating impaired consolidation. Another study similarly found that systemic RAPA administered immediately after paired odour-shock fear learning diminishes fear-potentiated startle to the training context, but interestingly not to the odour cue in a new context when both were tested 1 week later (Glover et al., 2010). Additionally, several other studies have shown that intrahippocampal infusion of RAPA impairs hippocampal-dependent fear memory for at least a week (Bekinschtein et al., 2007b; Slipczuk et al., 2009).

The memory deficits reported here at 48 h and 7 days from systemically inhibiting mTORC1 activity immediately after learning were also found to persist much longer, up to 21 days later. These findings are in contrast with our past work, which showed the effects of RAPA on auditory fear memory consolidation decayed over time and absent at 21 days post-training (Mac Callum et al., 2014). Instead, our current findings are congruent, as previously mentioned, with the deleterious effects of the global protein synthesis inhibitor anisomycin to both contextual and cued fear memory consolidation and persistence (Lattal & Abel, 2004; Mac Callum et al., 2014). Furthermore, we established that contextual fear memory consolidation and persistence is

vulnerable to RAPA at least at a second timepoint 3 h, but not 12 h after learning, paralleling prior work examining the effects of dorsal intrahippocampal RAPA treatment to one-trial inhibitory avoidance LTM (Bekinschtein et al., 2008; Slipczuk et al., 2009). In contrast, we have previously shown that the consolidation of cued fear memory is negatively affected by RAPA immediately or 12 h, but not 3 h, after training using a learning task that is procedurally akin to contextual fear learning (Mac Callum et al., 2014). Note that we assessed the effects of RAPA at these time points (immediate, 3 h, 12 h) on cued fear memory in different experiments so a direct comparison across time points cannot be made.

The above discrepancies between types of memories likely reflect the different molecular cascades evoked by each unique training event in brain loci subserving each type of learning (Izquierdo et al., 2006). Indeed, inhibitory avoidance and contextual fear memory depend on the hippocampus for spatial processing, while cued fear memory does not require the hippocampus but rather the amygdala to form associations with learned cues (Curzon et al., 2009). For mTOR, RAPA infused into the hippocampus immediately after learning has no effect on cued fear memory but impairs the formation of contextual fear memory, while RAPA infused into the amygdala impairs both contextual and cued fear formation (Gafford et al., 2011; Parsons et al., 2006b). Further, although the effects of immediate post-learning RAPA treatment to cued fear memory observed 24 h or 48 h thereafter are absent when tested 7 days or 21 days after learning (Glover et al., 2010; MacCallum et al., 2014; Parsons et al., 2006b), the effects of RAPA 12 h post-learning to cued memory persistence have not been evaluated. It is possible that delayed RAPA treatment 12 h after learning hinders the persistence of newly learned cued fear memory,

unlike that observed for contextual fear or inhibitory avoidance memory (Bekinschtein et al., 2008; Slipczuk et al., 2009).

Regardless of memory type, our current findings illustrate two timepoints of RAPA sensitivity and provide some credence to the idea that consolidation is not necessarily a continuous process, but that it might require multiple, recurrent consolidation-like events to help support the permanence of the memory trace. Nonetheless, it is worth acknowledging that our timepoints chosen for pharmacological intervention were far from exhaustive and limit this interpretation. Rather, the timepoints used for pharmacological interference around the time of learning, then again at 3 h and 12 h post-learning were chosen based on established time-dependent RAPA-sensitivities for the consolidation of inhibitory avoidance and cued fear memories (Mac Callum et al., 2014; Slipczuk et al., 2009). Slipczuk et al. (2009) established that inhibitory avoidance LTM was sensitive to RAPA around the time of learning and at 3 h thereafter, but also tested the effects on consolidation from RAPA treatment at other timepoints post-learning, including 1 h, a point relatively in between the two time periods of RAPA sensitivity. As a result, an important caveat is that our findings do not discount the possibility that sensitivity to RAPA is unitary between the two timepoints of susceptibility for contextual fear memory formation and persistence since we did not test any intermediary timepoints. This possibility cannot be overlooked and will need to be addressed further.

Second-generation mTOR inhibitors, such as AZD2014, curb all mTOR kinase activity regardless of the protein complex by binding to the ATP-catalytic site of mTOR (Sabatini, 2017). Using a dose-response experiment, we also found that AZD2014 dose-dependently impaired the

consolidation and persistence of contextual fear memory when administered immediately after learning, albeit with strict caveats to this statement as our statistical analyses appears to be more exploratory than confirmatory in nature as the results from the mixed ANOVA motivated further examination using linear mixed model procedures. Likewise, the experimental design we used was perhaps too cumbersome for the questions we attempted to answer, and we would have perhaps been better served by separating certain components of this study into different experiments (e.g., testing AZD2014 and PF-4708671 separately). Nonetheless, to the best of our knowledge, we are the first to illustrate the behavioural effects of acutely inhibiting both mTORC1/2 kinase activity per se, with our highest dose tested of 50 mg/kg most effectively disrupting LTM. Although these effects were in concert with our data for the 40 mg/kg dosage of RAPA in that experiment, we did not test for any non-specific changes to behaviour from AZD2014, which could potentially confound our results and will need to be addressed in the future, while also using a more succinct study design to better evaluate if any, simple main effects. Nevertheless, with the realization that mTORC1 phosphorylation of mammalian 4EBP1 is insensitive to RAPA and the absence of any mTORC2 specific inhibitors, the ability to temporally and acutely inhibit both complexes will be advantageous in uncovering the neurobiology of learning and memory (Choo et al., 2008; Saxton & Sabatini, 2017). Certainly, behavioural pharmacogenetic studies would benefit from this approach since, for example, torin1, a different ATP-competitive mTOR inhibitor, prevents mTORC1 cellular functioning to a much greater degree than RAPA in cells lacking rictor (Thoreen et al., 2009). Employing such a strategy behaviourally could thus better tease apart specific mTOR complex function (Stoica et al., 2011). Additionally, for our data, although not directly compared, we did not observe any additive effect from using the dual mTORC1/2 inhibitor over RAPA. It is possible that the

inhibition of mTORC2 has limited effect on memory consolidation; however, research from knockout studies of rictor suggest otherwise (Huang et al., 2013; Sun et al., 2019; Zhu et al., 2018). It is far more likely that we observed a floor effect for each at the dosages used, especially since pharmacological interference seldom if ever completely excises or prevents a new memory from marginally taking root.

In contrast to RAPA or AZD2014, the S6K1 inhibitor PF-4708671 only slightly diminished memory over multiple recall events compared with controls. Perhaps a higher dose of PF-4708671 would have had a greater impact on the consolidation of memory. However, the selected dosage of the S6K1 inhibitor was chosen based on research that showed the persistence of reconsolidated auditory fear memory or extinction consolidation was susceptible to the 50 mg/kg (Huynh et al., 2014; Huynh et al., 2018). Nevertheless, it is more likely that the lack of effect underscores the concomitant need to inhibit other downstream mTORC1 targets to achieve a desired level of change in memory, but this remains unconfirmed.

Overall, our findings are the first to show, although with some reservations, the effects of acute, systemic dual pharmacological inhibition of mTORC1/2 to contextual fear memory consolidation and persistence. Moreover, we also revealed that mTORC1 likely confers a greater contribution compared with its downstream target S6K1 to consolidation, as S6K1 inhibition alone was insufficient to significantly disrupt LTM. Lastly, we demonstrated that contextual fear memory is at least RAPA-sensitive at two timepoints, first immediately and second 3 h thereafter contextual fear learning, indicating that the molecular events underwriting a memory trace extend much longer after the learning event has finished.

Chapter 3: Developing a reliable animal model of PTSD in order to test potential pharmacological treatments: Predator stress and the mechanistic Target of Rapamycin[2]

3.1 Posttraumatic Stress Disorder (PTSD)

Several current models in psychology and neuroscience describe post- traumatic stress disorder (PTSD) as a condition of disturbed emotional learning and memory processes in which the *consolidation* of **traumatic fear memories** is enhanced, fear cues are generalized, and the extinction of fear memories is impaired (Mahan & Ressler, 2012; Murray et al., 2014). Bailey and Balsam (2013) describe PTSD as a syndrome where "old memories evoke responses ill-suited to current circumstances" (p. 245). Bailey and Balsam's description of PTSD reflects the plight of a traumatized combat veteran induced to panic, terror, or rage by the gunshot-like sounds of holiday firecrackers or an otherwise innocuous car backfire. Such vividness ensures that understanding trauma is not just another research goal in behavioural science, but that the quality of life and well-being of these individuals drives our interest in understanding the mechanisms that contribute to the development and maintenance of PTSD. The considerable suffering experienced by people with PTSD and the relatively high prevalence of PTSD (i.e., lifetime prevalence is estimated to be 6.1% and 9.2% among the general population in the United States and Canada, respectively; Goldstein et al., 2016; Kessler & Wang, 2008; Van Ameringen et al., 2008) contribute to the urgency for research on the brain mechanisms underlying the disorder. Clarification of brain mechanisms will help clinicians and other scientists understand

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the aetiology of PTSD, which may ultimately help identify candidate drug treatments (Hauger et al., 2012; Reul & Nutt, 2008; Steckler & Risbrough, 2012).

3.2 The Dynamic Nature of Memory

Research linking basic neuroscience to behavioural models of learning and memory has primarily focused on "simple system" learning models using invertebrates and rodents (for comprehensive reviews, see Kandel et al., 2014; Mayford et al., 2012; Sweatt, 2010; Squire & Kandel, 2008). The simple system approach distinguishes between **associative** and **non-associative** learning. Associative learning is typified by **Pavlovian fear conditioning**, a laboratory paradigm in which the pairing of a neutral stimulus (the *Conditioned Stimulus*) with an aversive stimulus (the *Unconditioned Stimulus*) allows the animal to learn to respond to the previously neutral stimulus with fear on subsequent encounters. In the Pavlovian model, fear is usually defined as the visible performance of species-typical defence behaviours, such as freezing or fleeing, which have evolved to allow evasion of environmental dangers like predators (Bolles, 1975; Ledoux, 1995, Panksepp, 1998). Associative processes allow an animal to learn about relations between environmental events and how to respond appropriately to such events (Gallistel, 2003; Rescorla, 1988; Timberlake, 1994). For example, the prey animal that learns to freeze in the presence of a predator is more likely to survive and transmit its genes to the next generation. Non-associative learning includes the contrasting phenomena of **habituation** and **sensitization**. Habituation and sensitization typically occur with reflexive behaviours (e.g., when you jump in response to a loud noise), the intensity of which can be attenuated (habituation) or increased (sensitization) by repeated exposure to the triggering stimulus. Habituation is a decreased response to a stimulus, whereas sensitization is an increased response to a stimulus.

While much of our knowledge of the physiology of non-associative learning comes from studies of invertebrates (e.g., Aplysia (a sea slug); Byrne, 2012; Carew & Kandel, 1973; Kandel, 2001; Lau et al., 2013); habituation and sensitization are conserved across phyla and studied in rodents and humans as well (Davis, 1970, 1972; Davis et al., 2008; Leaton & Supple, 1986; Lissek & van Meurs, 2015; Orr et al., 2002; Piltz & Schnitzler, 1996).

Distinguishing associative and non-associative learning is useful in the context of PTSD because symptoms can be split into those shaped by associative learning (i.e., re-experiencing and avoidance symptoms) and those shaped by non-associative learning (i.e., hyperarousal symptoms and negative alterations in cognitions and mood). Thus, clarification of the respective neural substrates for associative and non-associative learning processes is crucial to understanding how these symptoms emerge and are maintained in PTSD. Any animal model designed to study the neural mechanisms of PTSD should produce as many symptom clusters analogues of the human disorder as possible by eliciting species-specific responses to species- relevant threats in the animal of choice (Adamec, 1997; Goswami et al., 2013; Skolnick & Paul, 1983). Associative and non-associative fear memories both follow the standard stages of processing for any memory trace; specifically, fear memories are subject to acquisition and consolidation, and can be altered by intrinsic updating processes like reconsolidation and extinction. Consolidation is the critical inflection point in the survival of new fear memories and the molecular biological processes underlying consolidation is mostly well understood; as such, consolidation is the aspect of a potentially psychologically traumatic event (PPTE) memory perhaps most amenable to development of therapeutic drugs.

3.3 Consolidation

Neuroscientists define *learning* two ways: either as a relatively permanent change in behaviour as a result of experience (Bouton, 2007; Pearce & Bouton, 2001; Smock, 1999) or as the acquisition of information as a result of experience (Squire, 1987; Tulving, 2000). Accordingly, a *memory* is an experience-dependent internal representation (Dudai, 2004) formed by the capacity of the brain to retain learned information (Alberini, 2009). Researchers describing one particular memory are typically referring to a neural pattern carrying information about an aspect of the experienced world; however, such a memory is not a static entity within the brain. Five decades of research on the pharmacological manipulation of learning and memory in animals have led to the broadly accepted proposal that there are two stages of memory formation: **acquisition** and **consolidation** (Alberini & LeDoux, 2013; McGaugh & Izquierdo, 2000; Nader & Hardt, 2009; Squire, 1987). The learning experience is the crux of memory acquisition. In simplest terms, acquisition can be considered as the *generation of a memory trace*. Once information is learned and a memory is acquired (i.e., a *memory trace* is generated in the brain), the memory trace remains in a labile state where its strength (and perhaps existence) is acutely sensitive to pharmacological manipulations. The lability of memory is evidenced by several drug interventions, but most comprehensively with protein synthesis inhibitors (e.g., Anisomycin; Davis & Squire, 1984; Klann & Sweatt, 2008; McGaugh, 1966, 2000; McGaugh & Herz, 1972).

Protein synthesis inhibitors given after memory acquisition have been shown to block the formation of certain types of memories, but not others. For example, a “long-term memory” (i.e., memories evident when tested hours to years following training) can be blocked with protein synthesis inhibitors; however, a “short-term memory” (i.e., memories evident minutes to hours

following training) cannot be blocked with protein synthesis inhibitors.[3] The difference indicates a time-limited neural process where the initial memory trace must be "laid down" in the brain in order to be transferred to a long-term/permanent storage format. The process of "laying down" the memory is referred to as **consolidation** (Dudai, 1996; 2004; Kandel et al., 2014; McGaugh, 2000; Müller & Pilzecker, 1900; Squire & Bayley, 2007) and occurs on a timescale of minutes to hours.

Consolidation requires new protein synthesis that provides a physical basis for changes in synaptic efficacy. These physical changes are thought to represent the instantiation of the new memory or the engram (Dudai, 2004; Kandel, 2001; Nader, 2003;). Synaptic changes embed the memory within patterns of neuronal connections over broad timescales, which allows consolidation to be formally defined as a "time-dependent stabilization process leading eventually to permanent storage of a new memory" (Nader & Hardt, 2009, p. 224). In other words, consolidation is the process that transfers the new trace from a nebulous entity in short-term memory to a stable component of long-term memory.

3.4 From Molecule to Memory

Much of what we presently know about how brains create, maintain, and modify memory traces comes from invertebrate models of non-associative learning and rodent models of associative learning (particularly Pavlovian fear conditioning). Beginning with the insights of McGill's Donald O. Hebb (1949), behavioural neuroscientists have searched for a physiological process in

[3] Note these neuroscientific definitions of long- and short-term memory differ from how the terms are used in cognitive psychology; cf. Atkinson & Schiffrin, 1968.

neural connections that parallels the nature of the behavioural associative learning process (i.e., a process where a **synapse** linking two neurons is strengthened when both of these neurons are active at the same time; Bliss & Collingridge, 1993). Bliss and Lømo (1973) discovered such a process, which they termed *long-term potentiation*. Briefly, long-term potentiation refers to the enhancement of neural transmission at a given synapse (i.e., a communicative juncture between brain cells or neurons) by repeated, high-frequency stimulation of presynaptic neural inputs. For example, when both a weak and a strong input arrive at a synapse *at the same time* and are intended for the *same postsynaptic cell*, the weak input becomes *potentiated* through association, and the weak pathway consequently responds to the tetanic electrical stimulation with greater depolarization than would have previously occurred (Dudek & Bear, 1992; Nicoll & Roche, 2013). The phenomena of long-term potentiation was anticipated in 1949 by Hebb's groundbreaking theorem, which is commonly paraphrased as "neurons that fire together, wire together". Hebb's rule can be illustrated in a population of neurons (typically CA1 neurons) with stimulating electrodes, recording electrodes, and an oscilloscope. In this setup, a weak pulse of electrical stimulation will only produce a weak electrophysiological response (weak depolarization). But if a weak pulse is followed up in short succession with a pulse of strong stimulation, then subsequent responses to weak stimulation on the oscilloscope are shown to be enhanced or potentiated, demonstrating the "fire together, wire together" logic across a neural population (Rudy, 2014; Sweatt, 2010).

Researchers have resolved in detail the molecular mechanisms underlying long-term potentiation (see Frankland & Josslyn, 2016; Malenka & Bear, 2004). Consistent with the hypothesis of long-term potentiation as a mechanism for memory consolidation, long-lasting long-term

potentiation—like behavioural consolidation—requires protein synthesis. At the synaptic level, researchers have shown the N-methyl-D-aspartate (NMDA) glutamate receptor to have the properties needed to underlie a synaptic potentiation process. Despite many of the identified synaptic elements (e.g., NMDA receptors) that participate in long-term potentiation (Mayford et al., 2012; Panja & Branham, 2014), how action at the synapse contributes to protein synthesis, and ultimately memory consolidation, remains relatively unresolved. Thus, researchers have recently focused on the cellular signalling cascades (second-messenger pathways) that mediate synaptic changes by driving protein synthesis in neurons. Brain-Derived Neurotrophic Factor (BDNF) has emerged as a key molecule in synaptic plasticity and long-term potentiation as related to learning and memory (Panja & Branham, 2014). The BDNF molecule provides a mechanistic link between learning and consolidation of a fear memory (Monfils et al., 2007). Research suggests the sub-cortical **amygdala** is a key neural hub for plasticity in fear learning-related processes (Blair et al., 2001; Rogan et al., 1997); therefore, researchers have focused on the role of amygdalar BDNF activity in fear memory consolidation. Many such studies report BDNF transcription (synthesis of RNA from DNA) during fear memory consolidation (Reviewed in Rudy, 2014). Based on these and other results, there is now a large and growing body of literature on the role of the *mechanistic (formerly mammalian) Target of Rapamycin (mTOR*) *kinase* pathway in memory processes. The literature specifically addresses how upstream signals from the synapse (e.g., the BDNF receptor tyrosine receptor kinase B (TrkB)) activate mTOR, and how mTOR, in turn, drives translation (synthesis of protein from RNA) of molecular products needed for the ongoing plasticity underlying fear memory acquisition and consolidation. The molecular and pharmacological aspects of mTOR can further inform Pavlovian and predator stress models of fear learning.

3.5 mTOR and Rapamycin

mTOR is a kinase (i.e., enzyme) at the centre of a signalling pathway that is strongly conserved from bacteria to humans (Hay & Sonenberg, 2004; Li et al., 2014). In the brain, mTOR contributes to synaptic plasticity by controlling a subset of protein synthesis (translation) through downstream target effector proteins (e.g., p70s6k and 4E-BP1). The mTOR kinase also responds to signals initiated by receptors (e.g., NMDA, TrkB) that are crucial to synaptic plasticity required in long-term potentiation (Graber et al., 2013; Hoeffer & Klann, 2010). The mTOR molecule is found in cells as a component of two distinct molecular complexes, complex 1 (mTORC1) and complex 2 (mTORC2; Hay & Sonenberg, 2004; Hoeffer & Klann, 2010). The mTORC1 structure and function is well characterized with much of what we understand about the biochemistry of mTORC1 coming from research using the bacterium-derived mTORC1 allosteric inhibitor drug **rapamycin**. The drug specifically inhibits the complex by first binding with FK506-binding protein 12 (FKBP12), which then binds to the FKB12 rapamycin-binding (FRB) domain of mTORC1 forming a ternary complex preventing mTORC1 from carrying out its kinase activities to its substrates (Yang et al., 2013). mTORC2 is considered rapamycin-insensitive and is only mildly responsive to rapamycin treatment following chronic administration (Hay & Sonenberg, 2004; Sarbassov et al., 2006). Lacking any specific inhibitor, much less is known about the characteristics of mTORC2 biochemically, with even less know about its function in the biology of behaviour (Bockaert & Martin, 2015; Hoeffer & Klann, 2010; Huang et al., 2013). In the current chapter, we focus exclusively on mTORC1, referred to as mTOR hereafter.

3.6 Animal Models of PTSD: From Pavlov to Predation

Human psychobiology research on PTSD is large and includes neuroendocrine, psychophysiological, and neuroimaging approaches (e.g., Acheson et al., 2014; Bryant et al., 2005; Etkin & Wager, 2007; Pole, 2007; Rasmusson et al., 2003; Yehuda, 2009). The approaches are largely non-invasive for practical and ethical reasons, limiting the bases of most results to correlational data. Direct manipulation of the brain to identify causal mechanisms requires the use of animal models. Substantial research efforts have gone into modelling PTSD symptoms in rodents to investigate underlying cellular and molecular mechanisms of PTSD (especially the mechanisms that underlie consolidation, extinction, and reconsolidation of traumatic memories). An understanding of these mechanisms offers opportunities to identify potential pharmacological treatment targets. However, no particular animal model of PTSD ideally recapitulates all symptoms of the disorder, with Pavlovian fear conditioning paradigms and predator stress paradigms being the major approaches used by researchers to date. Pavlovian fear conditioning effectively models the re-experiencing and cue-related symptoms of PTSD. Predator stress models capture re-experiencing and cue-related symptoms as well, but also produce hyperarousal and anxiety-like behaviour, making predator stress models arguably more comprehensive animal models of PTSD (Deslauriers et al., 2017).

3.7 Pavlovian Fear Conditioning

The fear memories produced by Pavlovian paradigms involve an organism learning that a previously innocuous or neutral cue (the conditioned stimulus; e.g., a light or buzzer) predicts the

onset of a naturally fear-producing stimulus (the unconditional stimulus; e.g., a mildly painful foot shock). The unconditioned stimulus is a stimulus to which the animal has an innate and reflexive behavioural fear response (i.e., the unconditioned response). Unconditioned responses usually manifest as freezing or *tonic immobility* in both rodents and humans (Ledoux, 2003; Maren, 2001). Little experience is required for animals to associate the conditioned and unconditioned stimuli in memory, and the conditioned stimulus quickly comes to elicit the fear response. When the conditioned stimulus elicits the fear response without the presence of the unconditioned stimulus, the fear response is then referred to as the conditioned response (Pearce & Bouton, 2001). Pavlovian conditioning involves the animal learning relations between events in the world and fear has been powerfully shaped as a threat-avoidance mechanism over evolutionary time (Cosmides & Tooby, 2000; Ledoux, 2012; Öhman & Mineka, 2001). For example, a rat that freezes or hides in response to a hawk's cry is demonstrating an adaptive behaviour. Little experience is needed for objects and contexts predictive of danger or pain to prime the animal to respond with fear when these cues are encountered again. Pavlovian paradigms have been successful in modelling one set of PTSD symptoms, namely associative fear memories. The fear learning mechanisms activated in these protocols appear dramatically recalibrated in people with PTSD, where fear memories seem "over- consolidated". The process of extinction learning that allows an animal to update predictive relationships also appears compromised in PTSD (Mahan & Ressler, 2012; Morgan et al., 1993; Wessa & Flor, 2007), so cues and contexts reminiscent of the original PPTE exposure continue to generate powerful fear responses long after the predictive value has disappeared (Bailey & Balsam, 2013).

3.7.1 mTOR and Pavlovian Fear Conditioning

The role of mTOR in the formation and maintenance of associative fear memories as tested in Pavlovian fear conditioning models is well established. As described above consolidation and other aspects of memory can be disrupted via global protein synthesis inhibitors. Unlike other inhibitors that arrest all cellular machinery related to protein synthesis, rapamycin only selectively inhibits mTOR, which allows for the precise investigation of the mTOR pathway contribution to memory consolidation. There is a discrete period of increased activation of p70S6K (a downstream target of mTOR) in two brain areas underlying fear memory after fear memory acquisition: specifically, the hippocampus (Bekinschtein et al., 2007b; Gafford et al., 2011) and the amygdala (Parsons et al., 2006b). The increase in p70S6K activation is thought to drive consolidation of the fear memory. Concordantly, inhibition of mTOR in the hippocampus by rapamycin blocks both consolidation of a shock-induced Pavlovian fear memory and the associated increase in p70S6K activation (Bekinschtein et al., 2007b). Similar inhibitory effects follow direct rapamycin administration into the amygdala (Parsons et al., 2006b), with systemic rapamycin following Pavlovian training inhibiting consolidation of associative fear memories (Mac Callum et al., 2014; Bekinschtein et al., 2007b; Blundell, et al., 2008; Tishmeyer et al., 2003). Together, the available results suggest that consolidation of a Pavlovian fear memory depends on mTOR, which highlights a role for mTOR in the development of persistent fear memories in PTSD. The data point to the importance of mTOR in context or stimulus-specific fear memories, but do not address hyperarousal or generalized anxiety symptoms.

3.7.2 Limitations of Pavlovian Fear Conditioning as a PTSD Model

Fear conditioning defensibly captures the associative aspects of PTSD symptoms by generating strong fear memories for contexts and cues. Notable disadvantages of Pavlovian fear conditioning include not exposing the organism to a truly life-threatening event and the inability to robustly mimic other PTSD symptoms (e.g., persistent hyperarousal; increased anxiety-like behaviour; Pitman, 1997; Pitman et al., 1993). Experiments in our lab have shown that while animals display normal freezing behaviour to the training context following contextual fear conditioning, other behaviours germane to PTSD remain relatively unbridled. Conditioned animals showed no difference from their untrained counterparts in arousal state or in levels of anxiety (as measured in the elevated plus maze and dark/light box). Fear conditioning has allowed us to learn a great deal about the neural basis of associative fear and stress, from critical structures (i.e., hippocampus, amygdala) to molecular components (i.e., mTOR, BDNF); but to truly model and understand the neurobiology of PTSD, an animal model is needed that recapitulates a more comprehensive set of symptoms of the disorder. A prominent candidate for such a model is the predator stress paradigm.

3.8 Predator Stress

3.8.1 Cat Exposure as a Paradigmatic Predator Stress Model

Predator stress typically involves acute exposure of a prey species (e.g., mouse, rat) to a predator (e.g., cat, rat, ferret). The classic predator stress paradigm developed by Adamec and Shallow (1993) involves a single, unprotected exposure of a rodent to an adult male domestic cat. Cat

exposures last for 10 minutes and are videotaped to capture the activities of both the rodent and the cat. When the 10-minute test is completed, the rodent is gently removed from the cat exposure room and returned to its home cage. Video recordings can later be examined to determine locomotor activity of the rodent and cat, approaches to and flights from the cat by the rodent, the number of cat/rodent interactions, and the number of vocal calls made by the cat. Control groups for studies using the predator stress model are handled by experimenters rather than exposed to a predator (hereafter referred to as "handled control"). Cat exposures consistently generate high levels of associative fear, non-associative fear, and anxiety-like behaviour (Adamec & Shallow, 1993; Adamec, Shallow, & Budgell, 1997; Adamec, 1998; Adamec et al., 1999; Adamec, 2001; Adamec, Bartoszyk, & Burton, 2004; Adamec, Walling, & Burton, 2004; Adamec, Blundell, & Burton, 2005; Blundell, Adamec, & Burton, 2005; Adamec et al., 2008; Fifield et al., 2013; Fifield et al., 2015; Lau, Whiteman, & Blundell, 2015) therefore, unprotected domestic cat exposure may be an ecologically valid and comprehensive rodent model of PTSD. The ethological vividness of the feline predator stress model is attractive, but the effects can be highly variable (Adamec, Walling, & Burton, 2004; Fifield et al., 2013; 2015).

3.8.2 Predator Stress, Consolidation, and Protein Synthesis

Ample evidence demonstrates that protein synthesis is necessary for consolidation of predator stress-induced non-associative fear memories, such as hyperarousal and anxiety-like behaviour, paralleling associative fear memories from the fear conditioning literature (Adamec et al., 2006; Blundell et al., 2005; Cohen et al., 2006; Kozlovsky et al., 2008). Systemic injection of anisomycin (i.e., a global protein synthesis inhibitor) following predator exposure can block the increase in anxiety-like behaviour and exaggerated response to acoustic startle (a measure of

hyperarousal) when measured one week later (Adamec et al., 2006). Similarly, infusion of anisomycin either before or after predator scent stress (i.e., exposure to predator odour stimulus, such as urine or fur) reduced anxiety and startle responses (Cohen et al., 2006). Collectively this work from the Adamec and Cohen labs has supported the hypothesis that the synthesis of novel proteins is necessary for consolidation of non-associative fear memories in predator stress paradigms as evidenced through the effects of protein synthesis inhibitors on non-associative memories. It follows that these memories likely require translation regulation in order to be consolidated.

Anisomycin is a global protein synthesis inhibitor as it inhibits ribosomal activity, which reduces protein synthesis by as much as 60–80%. In contrast, rapamycin selectively inhibits only mTOR activity and reduces protein synthesis by a mere 10% (Parsons et al., 2006b). The selectivity of rapamycin makes it ideal for investigating the role of mTOR in predator stress. Work from the Blundell lab has demonstrated that the mTOR pathway plays a role in predator stress-induced associative and non- associative fear memories. Rats exposed to a cat and then injected with systemic rapamycin showed decreased freezing when re-exposed to the cat room context, suggesting reduced associative fear memories. Moreover, predator stressed rats given rapamycin showed lower anxiety-like behaviour in the elevated plus maze and decreased hyperarousal in the acoustic startle test compared with controls injected with a physiologically inert vehicle solution, suggesting reduced non-associative fear memories. In all cases, animals exposed to predator stress and treated with rapamycin exhibited similar behaviour to that of handled control animals (Fifield et al., 2013), suggesting that rapamycin blocks consolidation of the predator stress- induced associative *and* non-associative fear memories. As the results indicate a potential

role for mTOR in both types of fear memory induced by predator stress, we were interested in examining the distribution of mTOR activity in the brain during consolidation of such memories.

3.8.3 Experimental Approaches to Predator Stress Models: Molecules, Drugs, and Behaviour

3.8.3.1 Experiment 1: What is the Neuroanatomical Distribution of mTOR Activation Underlying Predator Stress-Induced Fear Memories?

Data from the Blundell lab suggested that like shock-induced Pavlovian fear memories, predator stress-induced fear memories are regulated by mTOR activity. Unlike shock-induced fear memory, the localization of mTOR activation in the brain following predator stress is unknown. Thus, we intended to identify brain areas that could mediate consolidation of mTOR-dependent predator stress-induced fear memories. Rats were randomly assigned to predator stress or handled control conditions and were euthanized 1 h or one week following cat exposure or control handling. Brains were extracted from rats of both groups and frozen for tissue analysis by immunohistochemistry. Immunohistochemistry was done using an antibody designed to detect "phospho-mTOR" (noted as p-mTOR) as a proxy of mTOR activity. p-mTOR expression was analysed using densitometry with respect to two brain areas commonly implicated in memory, stress, and anxiety: the hippocampus and the midbrain periaqueductal grey.

Decades of research results implicate the hippocampal complex as a critical brain structure in the formation of fear memories (Foster & Burman, 2010; Kim & Fansewlow, 1992; Phillips & LeDoux, 1992; Saxe et al., 2006). The role of the hippocampus as a critical structure in the

formation of many types of memories in general is supported by a very diverse body of evidence (For a review, see Andersen et al., 2006).

The hippocampus has a highly complex internal structure containing the CA1 and CA3 regions, hilus, and associated dentate gyrus amongst other subregions (Amaral & Lavenex, 2006). The CA1 pyramidal cell layer of the dorsal hippocampus is of particular relevance to the consolidation of fear memory. Optogenetic work has demonstrated that selective inhibition of excitatory CA1 neurons during fear conditioning sufficiently abolishes the acquisition and recall of contextual fear memory (Goshen et al., 2011). Moreover, a study using immunohistochemistry identified increased neuronal activation (e.g., c-Fos staining) in the CA1 region of the hippocampus in chronically stressed and fear conditioned rats (Hoffman et al., 2014). In addition to the CA1 pyramidal cell layer, the dentate gyrus is a subregion of the dorsal hippocampus that has been implicated in the encoding of contextual fear memories (Hernandez-Rabaza et al., 2008; Khierbek et al., 2013). An analysis by Kheirbek et al. (2013) demonstrated that inhibition of the dorsal dentate gyrus during fear conditioning significantly reduced freezing behaviour to the learning context. Experimental lesions of the dentate gyrus also appear to impair fear conditioning in rats when compared to “sham-lesion” animals (Hernandez-Rabaza et al., 2008).

The periaqueductal grey, a prominent feature of the midbrain, is an evolutionarily older structure than the hippocampus. Anatomists have divided the periaqueductal grey into three longitudinal columns that are termed dorsal, lateral, and ventral subregions (Bandler et al., 1991). The periaqueductal grey has been strongly implicated in cardiovascular functioning, nociception (pain), and vocalization (Jürgens, 1991; Kim et al., 2013). Evidence also indicates that the

periaqueductal grey modulates fear memories and defensive behaviours through its connections with the structures of the limbic system (the emotional brain; e.g., hippocampus, amygdala; Adamec et al., 2001). Experimental evidence demonstrates increased activity in the periaqueductal grey in response to several different types of stress (Adamec et al., 2012; Blundell & Adamec, 2006; Canteras & Goto, 1999). For example, Adamec and colleagues (2012) showed that c-Fos (a ubiquitous marker of neuron activity) was elevated in the *dorsolateral* periaqueductal grey after predator stress and the *ventrolateral* periaqueductal grey after water submersion stress. Phosphorylated cAMP response element binding protein (pCREB), the activated form of a transcription factor (DNA binding protein that controls the rate of transcription) involved in memory formation, was also elevated in the lateral periaqueductal grey after predator stress (Blundell & Adamec, 2006). Further, electrical stimulation of the periaqueductal grey elicits fear in rodents (Di Scala et al., 1987; Kim et al., 2013). Specifically, in fear conditioning experiments, stimulation of the periaqueductal grey acts as an aversive stimulus, which elicits a fear state that is paired with a conditioned stimulus resulting in robust conditioned emotional responding to the conditioned stimulus in the absence of periaqueductal grey stimulation (Di Scala et al., 1987; Fanselow, 1991). Periaqueductal grey stimulation also provokes defensive behaviours, including bursts of activity, vocalizations, and robust freezing (Kim et al., 2013). The freezing behaviour is blocked by ventral periaqueductal grey lesions, which also attenuate the learned fear conditioned response (Amorapanth et al., 1999).

Little is known about the long-term effects of mTOR activation. Therefore, we were interested in mTOR activation differences between the two brain areas at different time points. Our research has shown that anxiety-like behaviours in rodents can last up to seven days following exposure

to a predator (Adamec & Shallow, 1993; Blundell & Ademec, 2006; Adamec et al., 2006). Accordingly, we wanted to determine whether changes in mTOR activation also persist at this approximate time point. If such changes persist, they could be a candidate for the expression of sustained associative and non-associative fear memories. We compared levels of p-mTOR expression in rats sacrificed one-hour following predator stress to those sacrificed one-week following predator stress. To our knowledge, the current study is the first study to examine mTOR activation patterns in the brain following predator stress. Based on behavioural-pharmacological data (Fifield et al., 2013) and previous molecular work using Pavlovian models (Parsons et al., 2006b; Gafford et al., 2011), we hypothesized that p- mTOR levels would be elevated one hour following predator stress relative to handled control animals in both the hippocampus and periaqueductal grey. The current study was also designed to investigate whether altered levels of mTOR expression remained one week following predator stress.

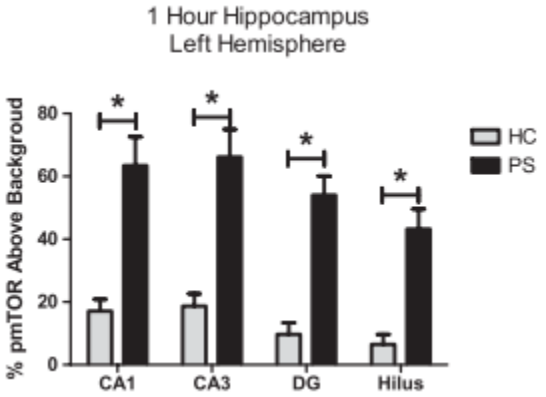

Figure 3.1 mTOR activation in control and stressed groups across subregions of the hippocampus in the left hemisphere.

As predicted, p-mTOR expression was significantly higher in the hippocampus of predator stress rats in comparison to handled control rats one-hour post-predator stress. This was the case bilaterally (i.e., in both right and left hemispheres) and across the major anatomical subregions of

the hippocampus. p-mTOR expression was higher in predator stress rats in the CA1, CA3, dentate gyrus, and Hilus subregions in both left and right hemispheres (Figures 3.1 and 3.2). These results are consistent with previous Pavlovian research showing that the mTOR pathway is activated in the dorsal hippocampus in response to contextual fear conditioning (e.g., Gafford et al., 2011). The results also support the hypothesis that mTOR activation in the dorsal hippocampus is essential for the consolidation of predator stress-induced fear memories as well. Upon separating the periaqueductal grey into the three columnar regions (i.e., dorsal, ventral, and lateral periaqueductal grey), differences were identified across regions. Specifically, p-mTOR expression was significantly higher in the dorsal periaqueductal grey in predator stress rats compared to handled controls, with a *trend* towards an increase in p-mTOR expression in the lateral periaqueductal grey of predator stress animals (Figure 3.3).

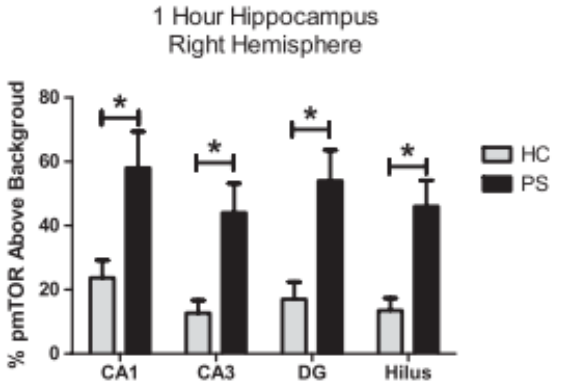

Figure 3.2 mTOR activation in control and stressed groups across subregions of the hippocampus in the right hemisphere.

There were no significant differences in p-mTOR expression between predator stress and handled control groups in the ventral periaqueductal grey (Figure 3.3). The increase of p-mTOR expression in the dorsal and lateral regions of the periaqueductal grey is consistent with our hypothesis and previous literature implicating the periaqueductal grey in stress and fear

processes. Following predator stress, pCREB expression increases in both the dorsal and lateral periaqueductal grey columns (Adamec et al., 2011), suggesting that increases in activity in these regions are important in the formation of fear memories. The results dovetail with our observed increases in mTOR activation. The parallel increase of p-mTOR and pCREB indicated activation of dorsal and lateral regions of the periaqueductal grey following predator stress provide compelling evidence for (and a clean picture of) dorsolateral periaqueductal grey activity during fear memory consolidation. The current results are also consistent with evidence demonstrating that the stimulation of the dorsal periaqueductal grey elicits anxiety-like and natural defensive behaviours in rodents (i.e., panic-like behaviour), implying that activity in the dorsal periaqueductal grey is a crucial substrate for expression of fear and anxiety (Borelli et al., 2013; Panksepp, 1998; Pinto de Almeida et al., 2006). No significant differences were found between predator stress and handled control groups in p-mTOR levels in either the hippocampus or periaqueductal grey at one week following exposure.

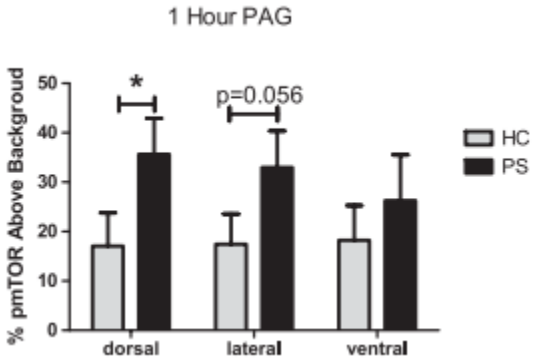

Figure 3.3 mTOR activation by subregion of the periaqueductal grey.

Research has shown that activity in the ventral region of the periaqueductal grey is increased in response to stress, as c-Fos was shown to be elevated in the ventrolateral periaqueductal grey

following submersion stress (Adamec et al., 2012) and in all areas of the periaqueductal grey following predator stress (Cantera & Gota, 1999). Therefore, we expected alterations in mTOR activation in the ventral periaqueductal grey following predator stress. In reference to our negative results, there may have been insufficient statistical power to detect changes in mTOR activity in the periaqueductal grey region. Alternatively, changes in mTOR activation may occur on a different timescale in the ventral periaqueductal grey in comparison with other regions and molecular cascades with changes not detectable at the one-hour time point. Another consideration is that the ventral periaqueductal grey may not be involved in the processes of fear learning and memory *per se*, but may instead be an output nucleus responsible for mediating defensive behaviours and conditioned emotional fear responses since lesions to this area inhibit these behaviours typically evoked by electrical stimulation of the dorsal periaqueductal grey (Amorapanth et al., 1999). If this is the case, it would explain why increases in p-mTOR expression were not seen in the ventral region following predator stress. Perhaps the ventral area is involved in the production of a conditioned response but would not be activated simply upon the *formation* or consolidation of a fearful memory. Our experiment described here only examines the molecular basis of the consolidation of a putative fear memory; the roles of mTOR in behavioural expression of a predator stress-induced fear memory remain indirectly shown by rapamycin injection studies (Fifield et al., 2013).

We did not observe any differences between predator stress and handled control groups in p-mTOR levels in either the hippocampus or periaqueductal grey at one week following exposure. Accordingly, mTOR activation may be limited to the initial consolidation phase of memory formation and other changes (perhaps downstream of mTOR) may maintain the memory over the

long term. In any event, the results suggest mTOR activation in the dorsal hippocampus and dorsal and lateral periaqueductal grey are important in mediating consolidation of predator stress-induced fear memories. We also demonstrated that changes in mTOR activation do not persist at one-week post-exposure, suggesting transient mTOR activation underlies changes in synaptic efficacy via protein synthesis in order to lay down the nascent memory.

3.8.3.2 Experiment 2A: The Rat-Exposure Test and Fear Memory

Despite research success, the feline predator stress model is associated with high economic costs (i.e., maintaining experimental felines), reliability issues (i.e., variability across studies) and threats to its validity (i.e., occasional docile cat behaviour). These important caveats coupled with benefits of mouse-rat models (i.e., lower cost, clear external validity, increased reliability) led us to examine another mammalian exposure model; The Rat-Exposure Test (Yang et al., 2004). The Blanchard group, based at the University of Hawaii, first developed the rat-exposure test to evaluate mouse defensive behaviours. Defensive behaviours are innate, unconditioned responses that are elicited in response to a perceived threatening situation (Blanchard & Blanchard, 1988). Defensive behaviours are the outputs of brain systems evolved to increase chances of escape and survival when an animal is exposed to a predator. Examples of defensive behaviours include freezing, defensive burying, and avoidance (Yang et al, 2004). The defensive behaviours are indicators that the animal perceives a situation as potentially life-threatening and therefore contributes to the construct validity of a predator stress model. For the initial study, Yang et al. (2004) used amphetamine-injected Long-Evans rats (to ensure mobility across trials) for predators and BALB/C or C57BL/6 strain mice as prey. On the exposure day, a mouse was placed in the exposure cage and either a live rat or a stuffed toy rat control (instead of the

handled control condition) was immediately introduced behind the wire mesh screen. The mouse could investigate the rat in the exposure cage or return to its home cage through a tunnel connecting the two boxes. In response to a live rat, mice subsequently demonstrated high levels of defensive behaviours, including freezing and avoidance. The results from Yang et al. (2004) have been successfully replicated (Amaral et al., 2010; Campos et al., 2013; Furuya-da-Cunha et al., 2016), supporting the rat-exposure test as an effective way to induce predator stress in mice.

We modified the rat-exposure test to a simplified exposure model with the mouse on one side of a perforated Plexiglas wall-divided cage and the rat (or stuffed control rat) on the other side. Instead of amphetamine, the live rats were calorie-restricted to increase activity and motivation during exposures, but importantly were never able to physically interact with mice during interactions.

During initial exposure, C57BL/6 mice exposed to a live rat froze significantly more than mice exposed to a stuffed control (Figure 3.4), suggesting that mice in the predator stress condition did indeed perceive the stimulus of the live rat to be stressful.

Upon re-exposure to the predator stress context, mice previously exposed to a live rat also froze significantly more than those who had been exposed to a stuffed control rat (Figure 3.5), suggesting that a five-minute protected exposure of a mouse to a rat is sufficient to produce associative (contextual) fear memories upon re-exposure to the stressful context.

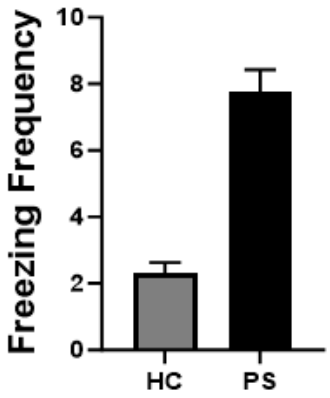

Figure 3.4 Exposure freezing for control and stressed animals during the rat-exposure test.

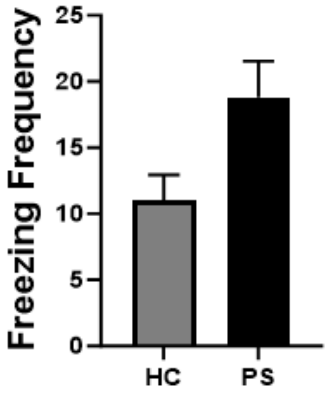

Figure 3.5 Re-exposure freezing for control and stressed animals following the rat-exposure test.

Non-associative memory testing was conducted over successive days with a different test carried out each day. Both groups of mice were first run through a battery of tests for anxiety-like behaviours using the elevated plus maze (EPM) on the first day, open field on the second day, and light-dark box on the third day, with acoustic startle testing on the final day to examine for hyperarousal (for a full description of these tests see 3.10 Appendix: Methods). The results indicated control mice spent proportionately more time in the open arms of the EPM than did

predator stress mice (Figure 3.6). Predator stress mice made fewer entries into the light side of the light–dark box (Figures 3.7). No differences were observed between groups in the time spent in the centre of the open field or acoustic startle response (data not shown).

Excluding the last results, the overall data suggest that a five-minute protected exposure of a mouse to a rat produces both associative and non-associative fear memories in the mouse. Subsequent experiments in the Blundell lab have demonstrated increased startle behaviour following mouse exposure to a live rat. The overall results align with research indicating predator stress models produce both associative and non-associative fear memories in a prey animal following an acute non-lethal exposure to a predator (Adamec et al., 1998; Fifield et al., 2013).

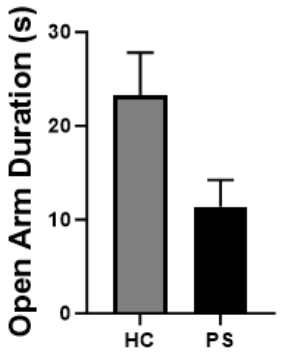

Figure 3.6 Time spent on open arms of elevated plus maze for control and stressed animals following the rat-exposure test.

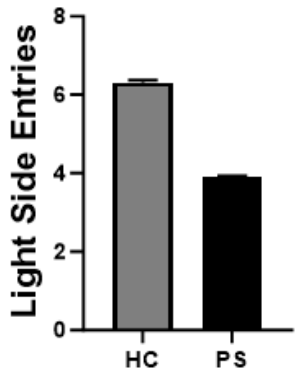

Figure 3.7 Number of entries into light side of light–dark box for control and stressed animals following the rat-exposure test.

3.8.3.3 Experiment 2B: Etho-Pharmacological Exploration of a Candidate PTSD Therapeutic—Rat-Exposure Test and Rapamycin

Initial results supporting the rat-exposure test as a reasonably robust model of PTSD-like fear memories led us to question whether the associative and non-associative fear memories elicited by the rat-exposure test were mTOR dependent. Mice were randomly assigned to predator or stuffed rat control groups and injected with either rapamycin (40 mg/kg) or an inert vehicle (VEH) immediately following exposure to the live or stuffed rat. The results were somewhat consistent with the previous experiment, with no differences in the open field or startle across the drug and stress conditions (data not shown).

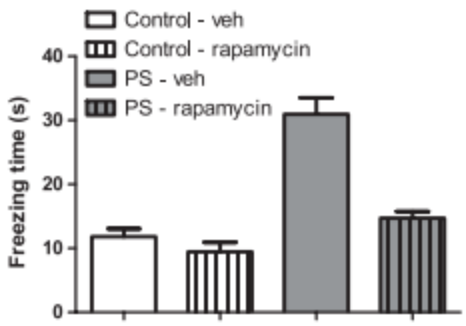

Figure 3.8 Freezing for the four Experiment 2B groups during re-exposure to the rat-exposure test context.

A strong context effect was demonstrated, and memory consolidation was shown to be at least partially mTOR dependent. Predator stress+VEH mice froze more on re-exposure compared to controls (Stuffed Control+VEH, Stuffed Control + rapamycin), while freezing in predator stress+rapamycin mice was comparable to control group levels (Figure 3.8). In the EPM, a similar pattern was seen for ratio time, with rapamycin increasing the proportion of time predator stress animals spent on the open arms as compared to VEH-treated mice. Unlike freezing, predator stress+rapamycin mice ratio times in the EPM were not as closely aligned with control levels (Figure 3.9). The "core" of a predator stress animal model of PTSD is sometimes reduced to contextual/cued fear, anxiety-like behaviour, and hyperarousal, as measured in predator context re-exposure, EPM, and startle, respectively (e.g., Cohen et al., 2006). Our results captured the first two components of the predator stress model, but without evidence of a startle effect as the curves over the course of the 30 trials did not differ (data not shown).

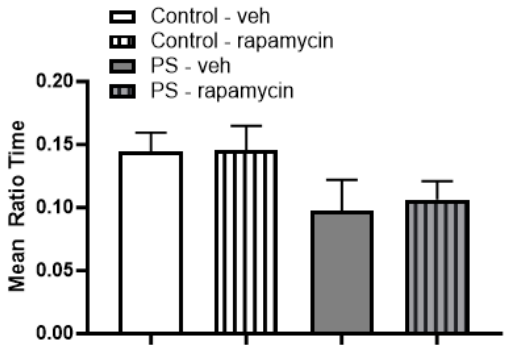

Figure 3.9 Ratio time (time in open arms/time in all arms) in the elevated plus maze for the four groups in Experiment 2B.

The results suggest that further research is needed to examine the involvement of mTOR in hyperarousal memory. Hyperarousal induced by the rat-exposure test may be impervious to rapamycin or might simply be more variable than the context or EPM effects. Given that consolidation of hyperarousal memory appears to be under mTOR control in the cat exposure test (Fifield et al., 2013), and that effects in other non-associative tests (light–dark box, open field) are somewhat variable, the latter is more likely the case. The contributions of mTOR to consolidation of hyperarousal may also be more nuanced than currently understood. Research using cat exposure indicated time-dependent effects of rapamycin on hyperarousal (Fifield et al., 2015). The ubiquity of mTOR in neurons (and all other cells) introduces complexity. Research on ingestive behaviour has come to the consensus that the effects of the mTOR pathway on eating and body weight are highly dependent on several factors (i.e., signalling stimulus, cell population, behavioural context; Haissaguerre et al., 2014). Therefore, the effects of mTOR on learning and memory may be similarly complex. For example, amygdalar and hippocampal mTOR pathway activity promotes fear memory acquisition and consolidation, while prefrontal mTOR pathway activity promotes extinction or other forms of modulation to such memories

(Bekinschtein et al., 2007b; Gafford et al., 2011; Glover al., 2010; Jobim et al., 2012a; Levin et al., 2017; Mac Callum et al., 2014). Such a scenario would explain the variable nature of results following systemic rapamycin reported here and, in some experiments reported in Fifield et al. (2013, 2015). It will be critical for future research using the rat-exposure test to cannulate rapamycin into specific brain regions (e.g., hippocampus, periaqueductal grey) to measure its effects on contextual and non-associative fear memories. Doing so will allow the dissociation of effects of the mTOR pathway in different regions in parallel to work showing site-specific effects of mTOR in fear conditioning (Helmstetter et al., 2008).

The slightly more modest results from experiment 2B suggest that the rat-exposure test generates contextual fear and speak to a role for mTOR in consolidation of fear memory. In contrast, the EPM results suggest that the non-associative fear memory/anxiety-like behaviour is also at least partially mTOR-dependent. The current results align with results using cat exposure (Fifield et al., 2013), but unlike cat exposure results, the rat-exposure test from Experiments 2A and B did not produce a startle effect or show that startle effect was subject to modification by mTOR blockade with rapamycin. Given that the unprotected cat exposure induces unwanted variability and can be complicated to execute, the rat-exposure test provides a more controlled alternative producing similar associative and non-associative fear behaviours. Thus, despite limitations and opportunities for refinement, the rat-exposure test represents a novel predator stress paradigm that holds much promise for elucidating the mechanisms underlying fear memories. As a final point, the still varying nature of the effects seen in these models reflect two important facts about real PPTE: (1) not every PPTE will produce the same constellation of behavioural and brain changes; and (2) the nature of symptoms appearing in humans with PTSD is itself highly

variable, suggesting that models capturing such variability may be more accurate than appears intuitively.

3.9 Summary

The experimental results discussed above provide information relevant to PTSD. First, immunohistochemistry work using traditional cat exposure models provided the first evidence of region-specific mTOR activation underlying predator stress-induced memory formation, advancing our understanding of fear memory and potential treatments. Second, the rat-exposure test is a useful model for studying the modulation of predator stress-induced fear memories and a helpful tool in translational research aimed at modelling and developing treatments for PTSD symptoms. Indeed, the results of experiment 2B add to evidence that the blockade of mTOR with rapamycin may be a useful pharmacological treatment, given rapamycin lead to an attenuation of contextual fear memory and some anxiety-like behaviour. Finally, inconsistent results with respect to hyperarousal symptoms mean that future research will help to fully tease apart the complex contribution of mTOR to fear memory formation and modulation and clarify the best uses of rapamycin as a PTSD treatment.

3.10 Methods

3.10.1 Drug Administration

For experiment 2B, mice received an intraperitoneal (i.p.) injection of rapamycin (40 mg/kg dose, injection volumes of 10 ml/kg, volume dependent on mouse weight) or vehicle (5% ethanol, 4% PEG400, and 4% Tween 80 in sterile water, volume dependent on mouse weight).

3.10.2 Behavioural Testing

3.10.2.1 Elevated Plus Maze

The elevated plus maze consisted of four arms arranged in the shape of a plus sign, with two opposite arms uncovered and two covered. For the rat-sized apparatus, each arm was 10 cm wide, 50 cm long and elevated 50 cm above the ground. The four arms were joined at the centre by a 10-cm square platform. Two of the arms opposite each other had no sides while the other two arms had walls 40 cm high and were open at the top. For the mouse-sized elevated plus maze, each arm was 5.1 cm wide, 29.2 cm long and the maze was elevated 45.7 cm above the ground. The four arms were joined at the centre by a 6.4-cm square platform. The animal was placed in the centre of the elevated plus maze and behaviour was recorded for five minutes. Rodents were then returned to their home cages. Behavioural measures included time spent in the open arms, time spent in the closed arms, frequency in the open, frequency in the closed arms, and ratio measurements of these variables. Ratio time is defined as time in open arms/(time in open)+(time in closed). Ratio frequency follows the same formula.

3.10.2.2 Open Field

The open field is a square Plexiglas box (rat-sized apparatus: 60 cm long x 60 cm wide x 35 cm high; mouse-sized apparatus: 48 cm x 48 cm x 48 cm) painted with grey enamel. Rodents were placed in the centre of the floor at the beginning of each trial. The rodents were then videotaped for five-minute trials. Behaviours measured included time in the centre of the box and number of rears. Rears were defined as any instance where the mouse or rat raised itself up on its hind legs, with its forepaws leaving the ground (with the exception of obvious grooming behaviour). Rodents were considered in the centre when the full body was within the centre area defined by white masking tape, and near the wall when all four feet were between the masking tape and the wall.

3.10.2.3 Light/Dark Box

The light/dark box was a single alley apparatus constructed of Plexiglas, divided into two chambers of equal size. For the rat light–dark box, each chamber was 31.75 cm long, 10.48 cm wide and 14.6 cm high. Both chambers were covered by a transparent Plexiglas top, hinged so it could not be opened. Both tops had centre pieces cut out to provide ventilation. One chamber had a solid wooden floor and was painted white. The other chamber had a metal mesh floor and its walls were painted black. The chamber painted black had its Plexiglas top rendered opaque with a black plastic covering. In addition, a 100-Watt LED light was positioned 66 cm above the white chamber. Testing took place in a darkened room illuminated only by the lamp over the white chamber. This produced a light intensity at the centre of the floor of the white chamber of 55 foot candles (fc), and an intensity of two fc at the centre of the floor of the dark chamber.

The mouse light dark box was a 50 cm long, 15 cm high structure with two square-shaped boxes (20 x 20 cm) connected by a short (10cm) tunnel. The dark side was covered by a removable lid, while the light side had a hinged Plexiglas lid with air holes to provide proper ventilation. Illumination and light intensity were the same as for the rat apparatus. Behaviour in the testing apparatus was videotaped for later analysis with a video camera mounted directly over the apparatus. Rodents were placed in the light chamber at the start of the test and their activity was videotaped for five minutes. Rodents were then returned to their home cages. Behavioural measures included time spent in each chamber, number of entries into each chamber (defined as having all four paws in the chamber) and number of faecal boli in each chamber.

3.10.2.4 Acoustic Startle Testing

Startle testing took place in a San Diego Instruments standard startle chamber. During testing, rodents were placed in the chamber in a cylindrical small animal enclosure. The animal enclosure sat atop a piezo-electric transducer that produced an electrical signal sampled by a computer, providing a measure of rodent movement. Startle testing was done in a dark chamber. This involved acclimating rodents to the startle apparatus with a background of 60dB white noise for 5 minutes. Then the rodents were exposed to 30 pulses of 50 msec bursts of white noise of 120dB amplitude rising out of a background of 60dB of white noise with a 30 second inter-trial interval. Startle response was measured over a 250 msec recording window.

Chapter 4: Evaluating the effects of single, multiple, and delayed systemic rapamycin injections to contextual fear reconsolidation: Implications for the neurobiology of memory and the treatment of PTSD-like re-experiencing[4]

4.1 Abstract

The mechanistic target of rapamycin (mTOR) kinase is known to mediate the formation and persistence of aversive memories. Rapamycin, an mTOR inhibitor, administered around the time of reactivation blocks retrieval-induced mTOR activity and de novo protein synthesis in the brains of rodents, while correspondingly diminishing subsequent fear memory. The goal of the current experiments was to further explore rapamycin's effects on fear memory persistence. First, we examined whether mTOR blockade at different time-points after reactivation attenuates subsequent contextual fear memory. We show that rapamycin treatment 3 or 12 hours post-reactivation disrupts memory persistence. Second, we examined whether consecutive days of reactivation paired with rapamycin had additive effects over a single pairing at disrupting a contextual fear memory. We show that additional reactivation-rapamycin pairings exacerbates the reconsolidation impairment. Finally, we examined if impaired reconsolidation of a contextual fear memory from rapamycin treatment had any after- effects on learning and recalling a new fear association. We show that rapamycin- impaired reconsolidation does not affect new learning or recall and protects against fear generalization. Our findings improve our understanding of mTOR-dependent fear memory processes, as well as provide insight into potentially novel treatment options for stress-related psychopathologies such as posttraumatic stress disorder.

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4.2 Introduction

In the aftermath of a highly traumatic experience, a survivor can develop post-traumatic stress disorder (PTSD). Symptoms can include re-experiencing the trauma, avoidance of cues related to the trauma, alterations in arousal/reactivity, and negative changes in mood/cognition. For a PTSD diagnosis, symptoms must be present for at least a month, not be present before the trauma, and lead to significant distress or functional impairment (American Psychiatric Association, 2022). Trauma-focused psychotherapies and several selective serotonin reuptake inhibitors (SSRIs, i.e., sertraline and paroxetine) are recommended first-line treatment options for PTSD (Martin et al., 2021; Veterans Health Administration and Department of Defense, 2018; Watkins et al., 2018).

A strong body of evidence supports the efficacy of these interventions at meaningfully reducing PTSD symptoms, but these treatment options are not without limitations (Belsher et al., 2019; Cusack et al., 2016; Watts et al., 2013). Several systematic reviews and meta-analyses have shown that treatment response rates for trauma-focused psychotherapies vary significantly, with the proportion of non-responders often exceeding half (Bradley et al., 2005; Green, 2013; Kar, 2011; Schottenbauer et al., 2008; Steenkamp et al., 2015). Treatment response rates for SSRI pharmacotherapies on the other hand are relatively higher and more consistent (~60-80% response rate), but also appear to require long-term use of these drugs as discontinuation of SSRIs is correlated with PTSD symptom relapse regardless of the length of the drug trial (Asnis, et al., 2004; Berger et al., 2009; Green, 2013; Lancaster et al., 2016). For those that do respond to these treatment types, however, many are still thought to be subsyndromal or only partially

recovered (Berger et al., 2009; Bradley et al., 2005). Moreover, nearly all PTSD clinical trials for SSRIs and trauma-focused therapies suffer from high rates of participant dropout (Lurie & Levine, 2010; Steenkamp et al., 2015; Stein et al., 2006; Sullivan & Neria, 2009; Zhou et al., 2020). Consequently, studying new, augmented, and varied approaches that may potentially afford enhanced treatment effects remains a focus for therapy and research.

One treatment method that has gained interest over the last decade is pharmaceutically augmented or assisted trauma-focused psychotherapy. Under this plan, patients take medication shortly before or after therapy to ostensibly exploit or make more amenable the underlying neurobiology of therapy evoked psychological processes (Dunlop et al., 2012; Hoskins et al., 2021; Marchetta et al., 2023; Meister et al., 2023). Since aberrant emotional learning and pathological fear memories are considered to be at the core of PTSD, a key component of most PTSD trauma-focused therapies, especially pharmaceutically augmented ones, is repeated exposure to stimuli associated with the fearful experience but with no actual risk of harm (Dunlop et al., 2012; Hoskins et al., 2021; Marchetta et al., 2023; Meister et al., 2023). Conceptually and procedurally, the traumatic event that leads to PTSD and therapeutic exposure to trauma-relevant stimuli parallel rodent models of Pavlovian fear memory extinction and reconsolidation (Careaga et al., 2016; Dunlop et al., 2012; Siegmund & Wotjak, 2006; VanElzakker et al., 2013). Fear conditioning and re-exposure to associated cues and contexts elicit responses that mimic the trauma and re-experiencing of fear found in PTSD. Through extinction, which is the underlying framework for exposure therapy, a conditioned fear response is gradually *inhibited* by a competitive, *newly formed*, associative memory after repeated exposure to non-reinforced stimuli. Alternatively, conditioned fear can be *diminished* following

memory retrieval, as reactivation can, under certain parameters (e.g., memory age, memory strength, and length of retrieval), bring the established (consolidated), *original*, long-term fear memory into a temporarily labile state susceptible to attenuating influences before being stabilized once again (reconsolidated), albeit, in a weaker form (de Oliveria Alvares & Do-Monte, 2021; Jardine et al., 2022; Kida, 2020). Unlike extinction alone, however, impaired reconsolidation does not result in a return of fear (e.g., reinstatement, renewal, spontaneous recovery) (Duvarci & Nader, 2004; Ferrara et al., 2023; Nader & Hardt, 2009). Other than analogous behaviour, rodent models of Pavlovian fear also hold considerable translational importance for pharmaceutically augmented exposure therapy. In general, fear paradigms are powerful procedures for investigating the neurobiology of learning and memory, but when leveraged for translational work, findings gleaned from these procedures have had exceptional utility in identifying new candidate drugs and establishing the neurobiological rationale for exposure augmentation targeting extinction or reconsolidation (Baker et al., 2015; Hoskins et al., 2021; Meister et al., 2023; Singewald et al., 2015; Stojek et al., 2018).

Most drugs of interest identified through preclinical rodent studies for PTSD exposure therapy augmentation have two or less published clinical trials except for SSRIs and D-cycloserine (a partial NMDA receptor agonist). Studies for these drugs have focused on enhancing extinction but results have been mixed (Astill Wright et al., 2021; Baker et al., 2015; Hoskins et al., 2021; Meister et al., 2023). Another putative drug treatment, rapamycin (RAPA) – a specific inhibitor of the mechanistic target of rapamycin complex 1 (mTORC1), which controls 5’ cap-dependent translation of mRNAs into proteins, has shown some promise in a single trial. Suris et al. (2013) demonstrated that a single dose of RAPA paired with traumatic memory reactivation reduced

PTSD symptomology at follow-up in war veterans with more recent combat trauma. This effect was consistent with, and likely due to, impaired reconsolidation by RAPA obstructing *de novo* protein synthesis in the brain *necessary (under most scenarios)* for reconsolidation after reactivation. Indeed, rodent studies using more toxic global protein synthesis inhibitors, such as anisomycin and cycloheximide, were the first to demonstrate this requirement of *de novo* protein synthesis in reconsolidation (Debiec et al., 2002; Duvarci et al., 2005; Nader et al., 2000), but an abundance of work with RAPA – an FDA approved drug – has identified mTORC1 translational regulation of protein synthesis as an essential component of reconsolidation. Specifically, these studies have illustrated that RAPA administered around the time of reactivation decreases retrieval-induced mTORC1 activity and *de novo* protein synthesis in the brains of rodents, while correspondingly diminishing subsequent behaviour associated with the reactivated memory (Blundell et al., 2008; Gafford et al., 2011; Glover et al., 2010; Huynh et al., 2014; Jobim et al., 2012a, b; Mac Callum, Hebert, Adamec, & Blundell, 2014; Parsons et al., 2006b; Pedroso et al., 2013). Moreover, these effects are absent with non-reactivation and are resistant to reinstatement (Blundell et al., 2008; Jobim et al., 2012a, b; Mac Callum et al., 2014).

Although the evidence for the role of mTORC1 in reconsolidation is strong, to better inform our understanding of this mRNA translational regulator in normal, and possibly abnormal, fear learning and memory, several details still need to be addressed. For instance, several recent reports have shown that anisomycin administered ~12 h after memory retrieval impairs subsequent recall 7 days but not 2 days after retrieval (Nakayama et al., 2013; Nakayama et al., 2016). This suggests that additional, delayed, protein synthesis-dependent events after initial reconsolidation contribute to the persistence of memory; however, the temporal properties of

mTORC1 towards such persistence have not fully been explored. Targeting molecular cascades such as mTORC1 at delayed time points after reactivation also represents an opportunity to engender long-lasting impairments to fear memories while also providing a buffer between fear-related cue and context re-exposure and drug administration when it is not feasible or desirable immediately around the time thereof (e.g., self-administration of a medication might not be feasible during an assigned in vivo session such as driving by the location of a trauma reminder). Thus, one aim of this project was to examine whether systemic mTORC1 blockade with RAPA at different time points after reactivation alters the reconsolidation and persistence of a foot-shock-induced contextual fear memory. Our second aim was to assess whether consecutive days of reactivation paired with RAPA had additive effects over a single pairing at disrupting a context (foot-shock-induced) memory, and to confirm that such effects were blocking reconsolidation and not enhancing extinction. RAPA-induced impairments of reconsolidated memory should not affect the capacity to later learn and form new memories. Therefore, our third aim was to examine if impaired reconsolidation of a contextual fear memory from RAPA treatment immediately after reactivation had any after-effects to learning and recalling a new fear association. Related to this aim, we also explored whether the impairing effects to consolidation from RAPA treatment immediately after contextual fear conditioning (via foot-shock or predator exposure) resulted in any carryover effects to learning and recalling a new subsequent fear association (shock-tone). Ultimately, our findings from investigating these aims will improve our basic understanding of mTOR-dependent fear memory processes, as well as provide insight into potentially novel treatment options for stress-related psychopathologies such as PTSD.

4.3 Methods

All procedures related to the care and use of animals for this research were approved by Memorial University's Animal Care Committee and adhered to the guidelines and regulations of the Canadian Council on Animal Care. Animals for this research were housed, cared for, and used only within the Memorial University's Biotechnology Building once they were received from the supplier (Charles River Laboratories, St. Constant, QC, CA).

4.3.1 Subjects

Two hundred sixty-three naïve male C57BL/6NCrl mice were used for all nine experiments. Mice, 4 weeks of age upon arrival, were group housed with 2-3 conspecifics per individually ventilated cage (Tecniplast, Buguggiate, VA, IT) in a housing room under standard laboratory conditions (i.e., temperature and humidity) and a 12-h light-dark cycle (lights on at 7:00 AM) with *ad libitum* access to food and water. Tails were coloured with non-toxic markers every second day for identification purposes. All procedures related to the care and use of mice occurred during the light phase of the light-dark cycle unless stated otherwise. Behavioural procedures started one week after arrival when mice were approximately 5 weeks of age. At the end of any procedure, mice were promptly returned to their home cages.

4.3.2 Predator Stimuli

Naïve male Long-Evans rats (N = 2) were used as nonlethal predator stimuli towards mice as part of Expt. 9 described below. When received, rats were approximately 6 weeks old and

weighed between 150-200-g. They were housed singly in individually ventilated cages in a separate housing room from mice on a *reverse* 12-h light-dark cycle (lights off at 7:00AM) under standard laboratory conditions with *ad libitum* access to food and water. Each day until used as predator stimuli, rats were handled for several minutes and their daily food intake was measured and recorded. Handling of rats and food intake measurements happened at the same time everyday during the dark phase of the reverse light-dark cycle under overhead red lights to minimize circadian rhythm disruptions. Three days before the rats were used in predator exposures, *ad libitum* access to food was removed and rats were food restricted to 80-85% of their average daily intake based on four days of baseline measurements. Food restriction and reverse light cycle procedures were used to increase predator activity on the day of exposure events to prey (i.e., mice), removing the need for pharmacological stimulants as used elsewhere (Campos et al., 2013; Yang et al., 2004). Immediately following predator exposure sessions *ad libitum* access to food was restored.

4.3.3 Drug Preparation and Delivery

Close to the time of use (~ 1-h prior), RAPA (LC Laboratories, Woburn, MA, US) was dissolved in ethanol (5% of total volume), then in a stock solution of 5% Tween 80 and 5% PEG 400 in distilled water using a vortex mixer (MaxiMix, Thermo Scientific, Waltham, MA, US). Vehicle (VEH) was prepared identically to RAPA but with the drug omitted. Mice that received VEH acted as controls. RAPA (40 mg/kg) or VEH was delivered intraperitoneally (i.p.) to mice at a volume of 10 ml/kg according to their bodyweight on a scheduled drug treatment day. For experiments with a delayed drug injection following a behavioural procedure (see below for

specific timetable of events), injections were performed in the mouse housing room rather than the specific behavioural procedure space. Further, for mice that received drug treatment in the dark phase of the light-dark cycle, injections were administered under overhead red lights to minimize circadian rhythm disruptions. The RAPA dosage of 40 mg/kg of body weight was selected based on evidence that it effectively disrupts memory without changing locomotor, anxiety, or nociceptive behaviour (Blundell et al., 2008).

4.3.4 Behavioural Procedures

Mice were carted in their home cages to the anteroom of the specific behavioural procedure room to be used and left undisturbed for a minimum of 1 h before the start of any behavioural session. Mice from the same home cage were run simultaneously, but individually in separate conditioning chamber systems for electric foot shock (physically stressed)-induced contextual and cued associative fear learning and memory procedures. For the single experiment (Expt. 9) with a predator exposure (psychogenic stress) component, mice were run one-by-one for each procedural step related to predator exposure and associative predator context re-exposure recall testing. Behavioural study apparatuses were cleaned using a 40% ethanol solution and air-dried between each animals' usage for all procedures.

4.3.4.1 Electric Foot Shock Induced Contextual Fear Conditioning and Associative Memory Testing

Each basic conditioning system featured a chamber with a shockable floor (26 stainless steel parallel rods), a drop pan underneath the shockable floor, a camera mounted to a stainless-steel ceiling, a transparent Plexiglas front entrance wall, a transparent Plexiglass rear wall, and stainless-steel side panels equipped with a speaker on one side and a house light on the other (Context A). Sound attenuating isolation cubicles housed the chambers to complete each conditioning system (Habitest, Coulbourn Instruments, Holliston, MA, US). To condition mice to fear the chambers (the to be conditioned contextual stimuli), a single 338-s contextual fear training protocol was used (MacCallum & Blundell, 2020). For this training procedure, mice were individually placed into separate chambers and after 90-s subjected to four, 2-s, 0.7-mA electric foot shocks (unconditioned stimuli), with an average 50-s variable interval between shocks (Precision Animal Shocker, Coulbourn Instruments). Following the last foot shock, mice remained in the chambers for an additional 90-s before being removed.

The strength of the contextual fear memory was tested by returning mice to the original training environment (the conditioned stimulus), individually in separate chambers, for 240-s and measuring their conditioned emotional response over this period. The number of memory recall tests and the latency between training and recall tests or successive recall tests varied according to the specific experiment (see below for exact schedules). Importantly, no foot shocks were administered during any recall test apart from Expt. 6 (see below) which used a 1-s, 0.2 mA ‘reminder shock’ halfway through a re-exposure session. This level of electrical stimulation used

for the reminder shock is insufficient to fear condition naïve mice but can otherwise recover (reinstate) a conditioned fear response following extinction but not impaired reconsolidation (Baldi et al., 2004; Blundell et al., 2008; Cai et al., 2006).

Freezing is a species-specific response to a threat characterized by the absence of movement except for respiration. This behaviour was used as a measure of fear memory retention (conditioned emotional fear response) and scored throughout for all electric foot shock induced contextual fear recall tests and during the first and last 90-s of conditioning using automated software (FreezeFrame, Coulbourn Instruments) as a percentage of total time (s) for each.

4.3.4.2 Electric Foot Shock Induced Auditory Fear Conditioning and Associative Memory Testing

Auditory fear conditioning and memory retention tests were conducted in modified conditioning chambers. For auditory fear conditioning, chambers had the drop pans underneath the shockable floor filled with corncob bedding, the walls covered with white printer paper, and lemon extract solution wiped over the chamber surfaces and reapplied after each animals' usage (Context B). To condition mice to fear a tone in this setting, mice were placed individually into separate chambers and after 120-s subjected to two identical pairings of a 30-s, 80-dB tone (to be conditioned stimulus) co-terminating with a 2-s, 0.7-mA foot shock with the first and second pairings separated by a 60-s interval. Following the co-termination of the second tone-shock pairing, mice remained in the chambers for an additional 120-s before being removed for a total training time of 360-s (Mac Callum et al., 2014).

Auditory fear memory retention tests were conducted 48-h and 10-days after training in chambers again altered to best limit the influences of past experiences of mice within them. For both recall tests wooden platforms were placed over the shockable floors, brown cardboard inserts were used to cover the side walls, strips of different coloured electrical (insulating) tape were placed across the front and back walls, and vanilla extract was wiped over chamber surfaces and reapplied after each animals' usage (Context C). For each of the two identical 240-s recall tests, mice were individually placed into separate chambers and after 120-s the same auditory tone used during conditioning was played for the last 120-s of the retention test. No foot shocks were administered during any auditory fear recall test. Freezing behaviour during the first 120-s of recall was used as a measure of generalized fear, whereas, freezing elicited by the conditioned auditory tone during playback was used as a measure of cued fear memory strength. In addition, freezing response was measured for the 120-s period before the first tone was activated and the in the 120-s following the co-termination of the last tone-shock pairing during training. All freezing data for auditory-cued fear conditioning and memory probes were obtained using automated software (FreezeFrame, Coulbourn Instruments) as a percentage of total time (s) for each.

4.3.4.3 Psychogenic Predator Exposure and Associative Contextual Memory Testing

Two identical predator exposure arenas were assembled with each arena consisting of a clear polycarbonate cage (47-cm x 26-cm x 20-cm) and a Plexiglass partition to divide the width of the cage into two equal compartments. The bottom half of each transparent Plexiglas divider was

perforated to allow for unobstructed olfactory, visual, and auditory stimuli of the opposite compartment. The floor of one compartment was-covered with corncob bedding (rat compartment), while the floor of the other compartment was-left bare (mouse compartment). To prevent animals from escaping during use, a piece of transparent perforated Plexiglass was placed on the top of the arena. Behaviour was recorded using a digital camcorder mounted to a miniature tripod facing the mouse compartment of the cage for predator exposures and arena re-exposures. All predator stress procedures occurred in a different room from electric foot shock-induced associative learning and testing procedures.

Mice and rats were habituated to one of the identical arenas by placing each mouse or rat into their respective compartments without the presence of the other in the opposing compartment for 300-s once a day for five consecutive days. Despite cages being sterilized between each animals' usage, one arena was used exclusively for all five-days of habituations for mice, while the second identical cage was used exclusively for all habituation sessions for rats to minimize odour contamination between species. A day after the last habituation session, mice were placed into the compartment with no bedding, but instead of an unoccupied second compartment, there was a rat (the psychogenic unconditioned stimulus) in the opposite bedded compartment for the inescapable, but protected predator exposure event for 300-s. One week later, each mouse was returned to the predator exposure arena (conditioned context) without the rat for a single 300-s re-exposure session to test the associative memory strength of pairing an ecologically relevant psychogenic stressor with a specific context (see Expt. 9 below for further details of experimental timeline). Recorded video from each mouse's rat exposure and context re-exposure were scored using automated software (EthoVision XT, Noldus, Wageningen, GE, NL) for total

time (s) immobile (used as a proxy for freezing behaviour, set at a threshold of 5% change in pixels of the subject independent of their spatial displacement as per Pham et al., 2009).

4.3.5 Experiments

Please see Figures for schematics of experimental timelines.

Experiment 1. Mice were re-exposed to the conditioning chambers two days after contextual fear training (Day 3). Three hours after memory retrieval, mice received a single i.p. injection of VEH (n = 14) or RAPA (n = 14). Post-retrieval context memory was then tested two days after the initial re-exposure (four days after conditioning, Day 5).

Experiment 2. The same procedure was repeated from experiment 1, but VEH (n = 14) and RAPA (n = 14) treated mice were tested for reconsolidated memory seven days instead of at two days post-retrieval (Day 10).

Experiment 3. Two days after contextual fear training (Day 3), mice were re-exposed to the conditioning chambers, then injected with either VEH (n = 13) or RAPA (n = 14) 12 h later. Post-retrieval memory was tested two days following re-exposure (Day 5).

Experiment 4. This experiment was identical to experiment 3, but VEH (n = 14) and RAPA (n = 14) treated mice were tested for reconsolidated memory seven days instead of two days post-retrieval (Day 10).

Experiment 5. Starting one day after contextual fear training, mice were re-exposed to the conditioning chambers once a day for three consecutive days and received an injection of VEH (n = 15) or RAPA (n = 15) immediately following each re-exposure (Days 2-4). Memory for the fear learning context was assessed for recall across these three days, then probed one (Day 5) and eight days (Day 12) after the last re-exposure and drug treatment.

Experiment 6. The experiment described above was repeated, but with an extra re-exposure session that included a "reminder shock" interposed into the experimental timeline 4-h after the last re-exposure and drug treatment (VEH, n = 15; RAPA, n = 15). Like the previous experiment, recall tests were carried out one (Day 5) and eight days (Day 12) after the last re-exposure and drug treatment.

Experiment 7. Two days after contextual fear conditioning, mice were re-exposed to the training context, then treated with VEH (n = 15) or RAPA (n = 15) immediately thereafter (Day 3). Post-retrieval contextual fear memory was assessed seven days later in the same learning environment (Day 10). Four days later, mice received auditory (cued) fear training (Day 14). Memory to the fear conditioned tone was then tested two (Day 16) and ten days (Day 24) after auditory fear conditioning.

Experiment 8. Immediately after contextual fear conditioning (Day 1), mice were treated with VEH (n = 15) or RAPA (n = 15) and were tested for contextual fear recall seven days later (Day 8). Four days after contextual fear recall, mice were conditioned to fear an auditory tone (Day

12). Memory to the fear conditioned tone was then tested two (Day 14) and ten days (Day 22) after auditory fear conditioning.

Experiment 9. VEH (n = 15) or RAPA (n = 16) was administered to mice immediately following predator exposure (Day 1). Mice were then evaluated seven days later for contextual fear memory to the psychogenic stress event (Day 8). Four days after context re-exposure, mice were conditioned to fear an auditory tone (Day 12). Memory to the fear conditioned tone was then tested two (Day 14) and ten days (Day 22) after auditory fear conditioning.

4.3.6 Statistics

Independent t-tests were used to evaluate differences for certain events prior to drug manipulation where appropriate (e.g., first re-exposure to the conditioning context) and when only a single recall test followed drug treatment (Huynh et al., 2014). Mixed analysis of variance (ANOVA) tests with drug group, experimental day, and procedural phases (i.e., the time period before tone activation and the time period during tone activation) as main factors were used to evaluate freezing behaviour in experiments with multiple recall events following drug treatment. Mixed ANOVA tests were also used to assess foot shock induced changes in freezing during training sessions to ensure there were no learning differences between groups (pre-learning (90-s period before the first foot shock for contextual fear conditioning or the 120-s period before the first tone was played during auditory fear conditioning) and post-learning (90-s or 120-s period after the last foot shock for contextual or auditory fear conditioning, respectively). Where appropriate, Bonferroni post hoc contrasts or *a priori* t-tests were used for follow-up between or

within group comparisons. Data organization and statistical analyzes were made using SPSS (IMB, Armonk, NY, US) and Excel (Microsoft, Redmond, WA, US), while figures were made using Prism (GraphPad Software, San Diego, CA, US). Group data is reported as mean ± standard error, with significance taken at $p < 0.05$.

4.4 Results

For all experiments using a foot-shock context conditioning protocol, as expected, naïve mice froze significantly more in the 90-s period following the last foot shock compared to the 90-s preceding the first foot shock during conditioning, indicating acquisition of contextual fear learning had taken place (data not shown for mixed ANOVA tests, but all experiments found a significant main effect of time, $p < .05$). Moreover, since all mice were naïve to the conditioning chambers and had not yet received pharmacological treatment, as anticipated, there were no between-subjects differences found in freezing behaviour during either of these periods (first 90-s and last 90-s) of the contextual fear conditioning procedure [data not shown, all $p > .05$ for main effect of treatment]. Likewise, for all reconsolidation experiments, expectedly there were no differences in freezing between groups when mice were first re-exposed to the chambers they received contextual fear conditioning in since no drug manipulation had yet occurred (see results below for more details; all t-tests, $p > .05$).

4.4.1 Systemic rapamycin 3-h or 12-h post-retrieval impairs the reconsolidation and persistence of contextual fear memory

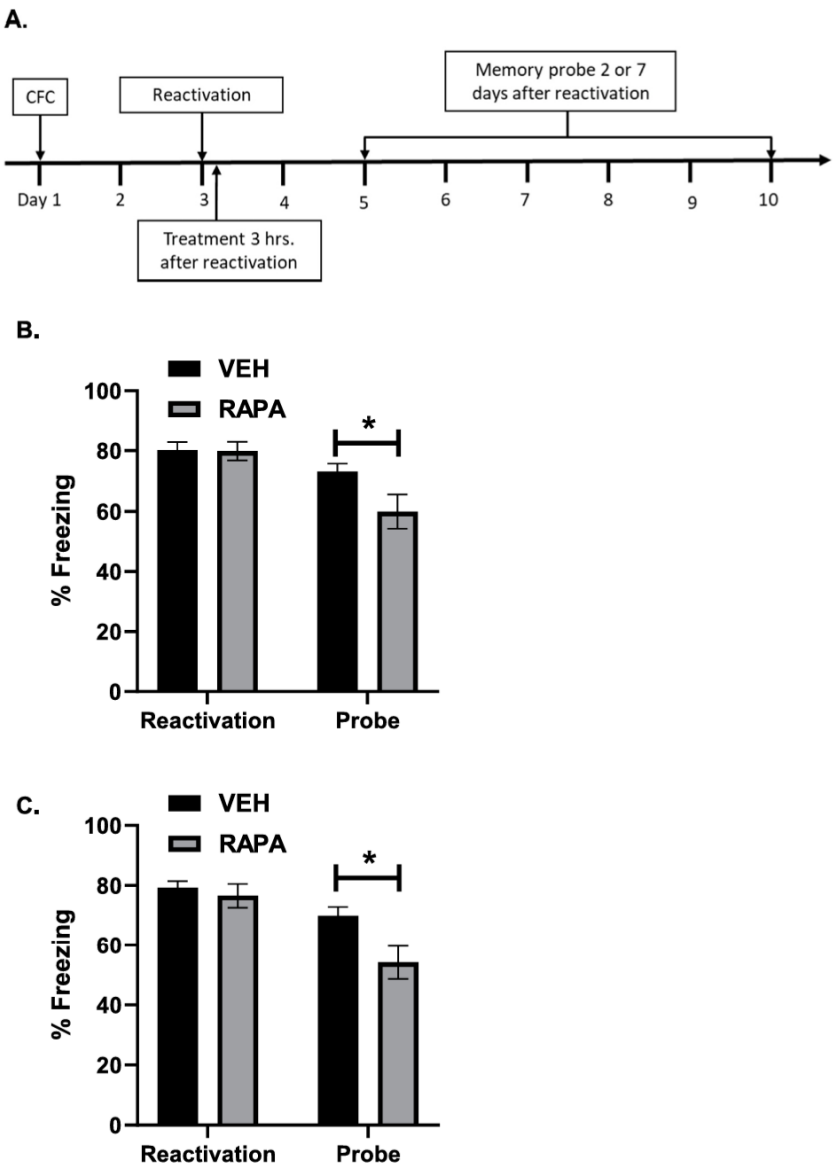

Figure 4.1 RAPA administered three hours after reactivation attenuates the reconsolidation and persistence of shock-induced contextual fear memory. (A) Timeline of Expt. 1 and 2. Both experiments were identical, except the post-reactivation memory probe was on Day 5 for Expt. 1 and Day 10 for Expt. 2. Mice that received RAPA three hours post-reactivation froze significantly less than their VEH counterparts when probed for subsequent memory retention two (B) or seven (C) days after reactivation. Data represented as mean and ±SEM. Statistical significance denoted by * ($p < 0.05$). CFC: Contextual Fear Conditioning, VEH: Vehicle, RAPA: Rapamycin.

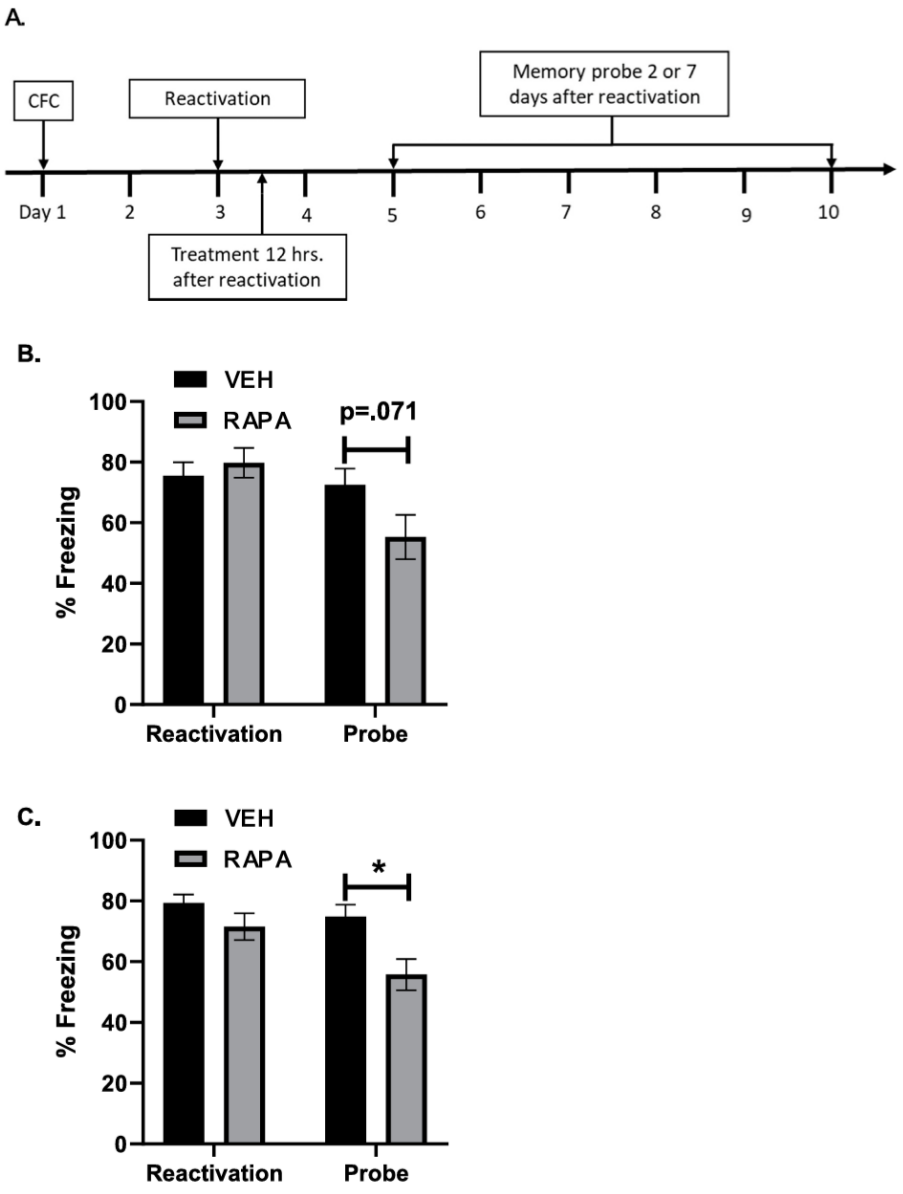

Figure 4.2 Systemic RAPA treatment 12 hours post-reactivation disrupts memory persistence. (A) Timeline of Expt. 3 and 4. Both experiments were identical, except the post-reactivation memory probe was on Day 5 for Expt. 3 and Day 10 for Expt. 4. (B) Mice treated with RAPA 12 hours post-reactivation froze less than VEH-treated controls when probed for retention two days after reactivation but this was not significantly different. (C) In contrast, when deferring the memory probe by a week after reactivation, RAPA-treated mice froze significantly less than their VEH-treated counterparts did. Data represented as mean and ±SEM. Statistical significance denoted by * ($p < 0.05$). CFC: Contextual Fear Conditioning, VEH: Vehicle, RAPA: Rapamycin.

In our first set of experiments (Expt. 1-4), we examined whether RAPA administered several hours after retrieval would interfere with the reconsolidation and persistence of a fear memory (Fig. 4.1A & 4.2A). When injected 3-h post-reactivation, RAPA-treated mice froze significantly less than their VEH counterparts when probed for fear memory retention two (Day 5) ($t(26) = 2.074$, $p = .048$; Expt. 1, Fig. 4.1B) or seven days later (Day 10) ($t(26) = 2.513$, $p = .019$; Expt. 2, Fig. 4.1C). When injected 12-h post-reactivation, RAPA and VEH treated mice did not differ when tested for retention two days after reactivation (Day 5) (although a possible trend appeared, $t(25) = 1.884$, $p = .071$; Expt. 3, Fig 4.2B) but differed when tested seven days later (Day 10) ($t(26) = 2.972$, $p = .006$, Expt. 4, Fig. 4.2C).

4.4.2 Two consecutive days of post-retrieval rapamycin treatment maximizes memory impairment through disrupted reconsolidation

We next sought to determine whether we could maximize fear memory impairment in mice by repeating the procedure of administering RAPA immediately after reactivation for three consecutive days (Expt. 5, Fig 4.3A). Expectedly, mice randomized to either RAPA or VEH treatment groups froze comparably during the first reactivation session prior to receiving any treatment on Day 2 (data not shown, $p > .05$). A mixed ANOVA of the freezing data for all other reactivation sessions after the first drug treatment on Day 2 (i.e., reactivation two (Day 3) and three (Day 4), each paired with drug treatment) and the recall tests 24-h (Day 5) and eight days (Day 12) after the last drug treatment revealed a significant main effect of drug treatment [$F(1, 28) = 11.149$, $p = .002$] and main effect of day [$F(3, 84) = 21.101$, $p < .001$], but no interaction

effect of day X drug treatment [F (3, 84) = .648, p = .587]. RAPA-treated mice showed significantly reduced freezing behaviour during the second reactivation session compared to the first reactivation session immediately prior to the mice receiving their first drug treatment (RAPA, Day 2 vs. Day 3; t (14) = 5.344, p < .001). Bonferroni corrected paired sample t-tests indicated significantly further reduced freezing in RAPA-treated mice from the second reactivation session-drug treatment to the third reactivation recall session (Day 3 vs. Day 4; p = .003), but with no additional significant reductions yielded from the third reactivation-treatment (Day 4 vs Day 5; p = 1.00) or across the two recall tests (Day 5 vs. Day 12; p = .066; Fig. 4.3B). VEH treated mice showed a trend of diminished freezing over time but none of the repeated within group contrasts were significantly different (all p > .05). Between group comparisons (using Bonferroni corrections) after the first reactivation session and first drug treatment revealed that RAPA-treated mice froze significantly less than their VEH counterparts during the second (Day 3; p = .036) and third (Day 4; p = 0.016) reactivation sessions, and eight days after the last drug treatment (Day 12; p = .024) but not 24-h after the last treatment (Day 5; p = .116; Fig. 4.3B).

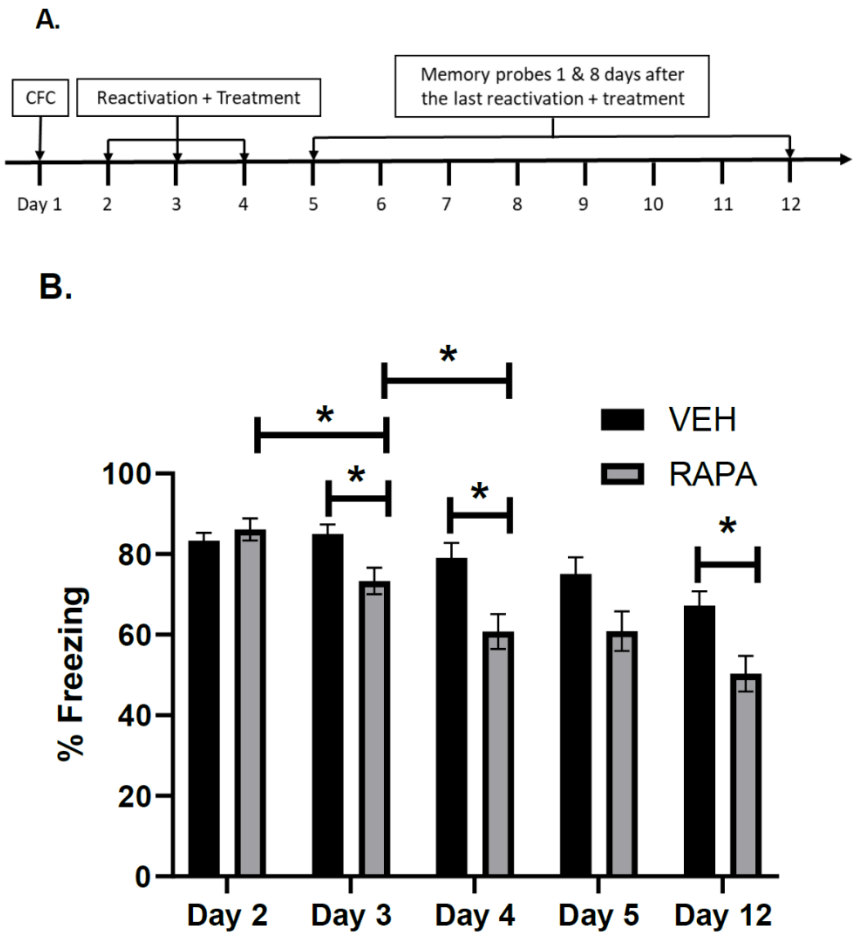

Figure 4.3 Additional RAPA-paired reactivation sessions further diminishes the associative strength of a shock-induced contextual fear memory. (A) Timeline for Expt. 5. (B) Two post-reactivation treatments were sufficient to maximize the reduction in freezing within the RAPA treatment group (Reactivation 1 v. 2 and Reactivation 2 v. 3). Moreover, following the first reactivation and drug treatment on Day 2, RAPA-treated mice froze significantly less than VEH-treated mice on Day 3 (Reactivation + Treatment 2), Day 4 (Reactivation + Treatment 3), and Day 12 (Probe, seven days after the last reactivation and drug treatment). Data represented as mean and ±SEM. Statistical significance denoted by * ($p < 0.05$). CFC: Contextual Fear Conditioning, VEH: Vehicle, RAPA: Rapamycin.

While we demonstrated in the previous experiment that consecutive days of reactivation paired with RAPA could further diminish subsequent post-reactivation memory, we wanted to know whether this effect was due to augmented extinction or reconsolidation interference. To try to

tease this a part, we repeated the previous experiment but interposed a 0.2 mA, 1-s "reminder shock" 4-h after the last context re-exposure and drug treatment into the experimental timeline (Expt. 6, Fig. 4.4A). A mixed ANOVA of the freezing data for all reactivation and recall sessions after the first drug treatment (i.e., reactivation two (Day 3) and three (Day 4), each paired with drug treatment, the 'reminder shock' re-exposure session (Day 4'), and the recall tests 24-h (Day 5) and eight days (Day 12) after the last drug treatment) revealed a significant main effect of drug treatment [$F(1, 28) = 16.076$, $p = .001$] and day [$F(4, 112) = 2.953$, $p < .023$], but no interaction effect of day X drug treatment [$F(4, 112) = 1.235$, $p = .300$]. Like the previous experiment, RAPA treatment immediately after the first and second reactivation significantly reduced subsequent post-reactivation conditioned responding (RAPA, Bonferroni corrected: reactivation 1 vs. 2 (Day 2 vs. 3); $p = .001$; 2 vs. 3 (Day 3 vs. 4); $p = .04$; Fig. 4B). There were also no significant changes from the third reactivation-RAPA pairing to the recall test 24-h later (Day 4 vs. 5) or between the recall tests one week apart (Day 5 vs. 12; all $p > .05$) despite the addition of the 'reminder shock' after the last RAPA treatment. Freezing from VEH-treated mice did not significantly change over time from session to session and remained very stable across the two recall sessions after receiving the 'reminder shock' (all $p > .05$). Moreover, RAPA-treated mice froze significantly less than VEH mice at all recall sessions following the first reactivation and drug treatment (Bonferroni corrected; reactivation two (Day 3), $p = .032$; reactivation three (Day 4), $p < .001$; recall test one (Day 5), $p = .028$; and recall test two (Day 12), $p = .020$; Fig. 4.4B).

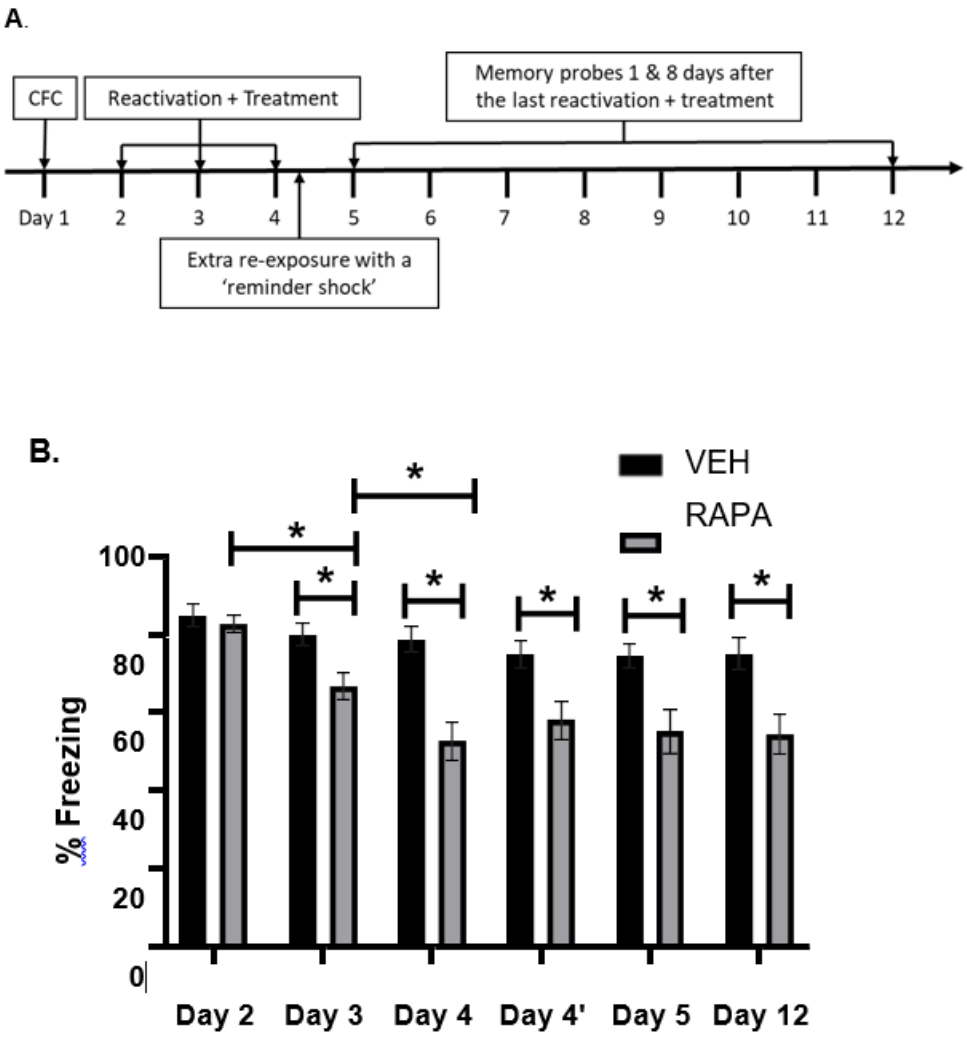

Figure 4.4 Effects of multiple RAPA-paired reactivation sessions to contextual fear memory are due to impaired reconsolidation. (A) Timeline for Expt. 6 is identical to Exp. 5 (See Fig. 3A), but includes an additional re-exposure (reactivation) "reminder shock" session four hours after the last reactivation and drug treatment. (B) Two post-reactivation treatments were again sufficient to maximize the reduction in freezing within the RAPA treatment group alone (Reactivation 1 v. 2 and Reactivation 2 v. 3). Following the first post-reactivation drug treatment on Day 2, RAPA-treated mice froze significantly less than VEH-treated mice during each of the next two reactivation sessions (Days 3 and 4), during the "reminder shock" session (Day 4'), and during each recall probe (Day 5 and 12) despite the addition of the "reminder shock" session. Data represented as mean and ±SEM. Statistical

significance denoted by * ($p < 0.05$). CFC: Contextual Fear Conditioning, VEH: Vehicle, RAPA: Rapamycin.

4.4.3 Post-reactivation and post-conditioning rapamycin treatment attenuates subsequent recall but does not impair the ability to learn a new Pavlovian association and protects against generalized fear

We then turned our focus to examining whether RAPA-impaired reconsolidation for a specific memory interferes with the ability to learn and recall a new associative memory (Expt. 7, Fig. 4.5A). We found that RAPA-treated mice showed significantly reduced freezing compared to VEH controls when probed for fear memory recall to Context A on Day 10 ($t (28) = 3.505$, $p = .002$; Fig. 4.5B). Differences in freezing behaviour were punctuated further during auditory fear conditioning in Context B on Day 14 (Mixed ANOVA: main effect of treatment, $F (1, 28) = 9.402$, $p = .005$; main effect of time $(1, 28) = 146.042$, $p < .001$; but no interaction effect, $F (1, 28) = 2.848$, $p = .103$). Indeed, each group demonstrating learning by freezing significantly more in the 120 s period following the last tone-shock paring compared to freezing during the equal period of time preceding when the first tone was played (Bonferroni post hoc tests: RAPA, $p < .001$; VEH, $p < .001$; Fig. 4.5C). However, RAPA-treated mice still froze significantly less than their VEH counterparts did during either of these time periods (Bonferroni post hoc tests: first 120 sec of training, $p = .022$; last 120 s of training, $p = .024$; Fig. 4.5C). Freezing behaviour was also significantly different during auditory fear recall tests in Context C on Day 16 and 24 (Mixed ANOVA: main effect of treatment, $F (1, 28) = 19.053$, $p < .001$; main effect of time $(3, 84) = 41.061$, $p < .001$; and an interaction effect of treatment x time $F (3, 84) = 10.853$, $p < .001$;

Fig. 4.5D). Specifically, RAPA-treated mice demonstrated standard recall for an auditory fear memory procedure by freezing significantly more during the second-half of each recall test when the conditioning tone was replayed (last 120 s) compared to the first-half (first 120 s) when no auditory stimuli were activated (Bonferroni post hoc tests: both $p < .001$; Day 16 & 24, respectively). VEH-treated mice, however, only showed this pattern during the second recall test on Day 24 ($p = .028$), and not during the first recall test on Day 16 where freezing was comparably high during their first encounter with Context C and when they first re-encountered the conditioning tone ($p = .884$). Between subjects contrasts of both auditory recall sessions revealed that RAPA-treated mice froze significantly less than VEH mice during the first halves (no auditory stimuli) of both recall sessions and the second half (conditioning auditory cue replayed) of the first recall test but not the second test (Bonferroni post hoc tests: Day 16, first 120 s, $p > .001$, last 120 s, $p = .044$; Day 24, first 120 s, $p = .016$, last 120 s, $p = 1.00$; Fig. 4.5D).

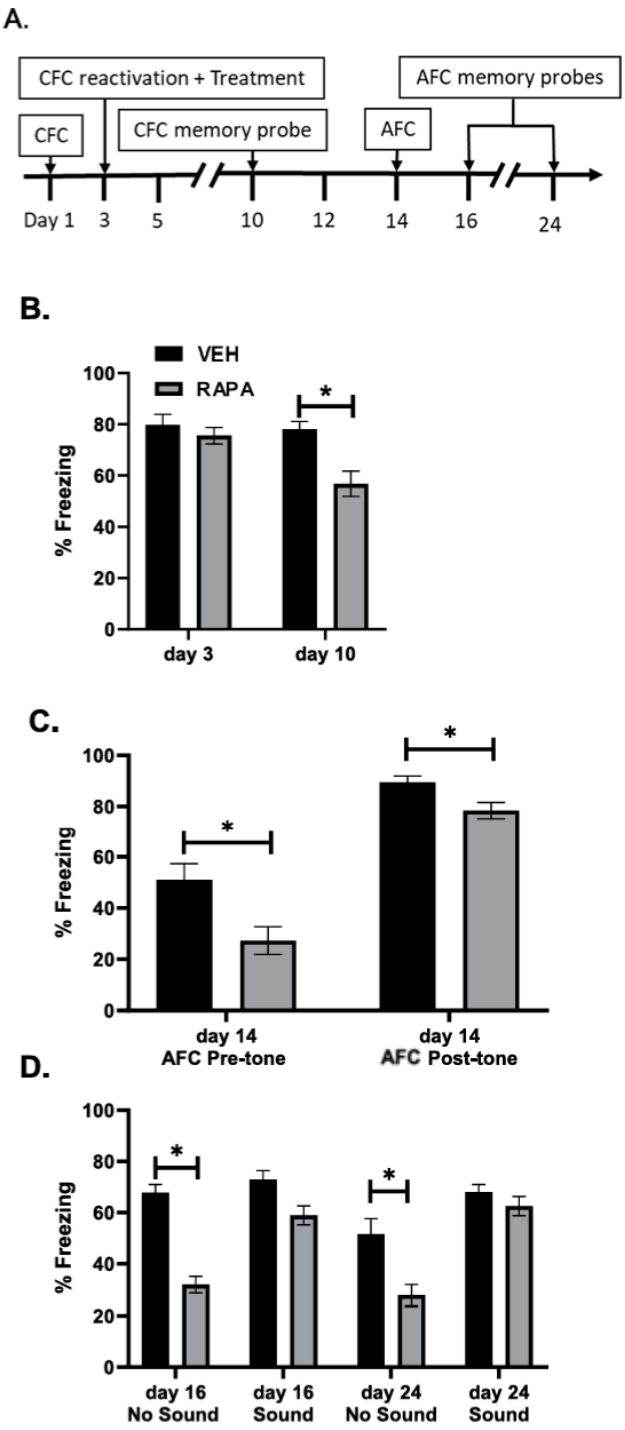

Figure 4.5 RAPA-impaired reconsolidation does not affect the ability to subsequently learn and recall a new fear association but does confer protection against fear generalization to new contexts. (A) Timeline for Expt. 7. (B) Mice that received RAPA treatment immediately after CFC reactivation for Context A on Day 3 froze significantly less than VEH-treated mice when probed for post-reactivation CFC memory to Context A on Day 10. (C) In Context B on Day 14, both groups of mice demonstrated AFC acquisition, but RAPA-treated mice still froze significantly less than VEH-treated mice during both the 120 s pre tone and post tone periods of the AFC session. (D) During AFC recall probes in Context C on Days 16 and 24, RAPA-treated mice froze very little to the context in general but did freeze when the conditioning tone replayed. In contrast, VEH-treated mice froze highly both to the new context and the conditioning tone on Day 16, then behaved in a similar manner to RAPA-treated mice during the second recall test on Day 24. Regardless, RAPA-treated mice froze significantly less

than VEH-treated mice during both the first (no tone) and second (tone) halves of the first recall probe (Day 16) and during the first half of the second the probe (Day 24). Data represented as mean and ±SEM. Statistical significance denoted by * ($p < 0.05$). AFC: Auditory Fear Conditioning, CFC: Contextual Fear Conditioning, VEH: Vehicle, RAPA: Rapamycin.

Since mTORC1 also contributes to the memory consolidation (Gafford et al., 2011; Jobim et al., 2012a, b; Mac Callum et al., 2014; Parsons et al., 2006b), we were curious to test whether RAPA-induced impairment of fear memory consolidation for one specific context would also protect mice against fear generalization to new contexts without interfering with the ability to learn and remember a new aversive association (Expt. 8, Fig. 4.6A). An independent t-test of freezing data from the context recall test on Day 8 revealed that mice treated with RAPA immediately after training froze significantly less than VEH-treated mice when returned to Context A ($t (28) = 2.467$, $p = .020$). When we introduced the mice to Context B for auditory fear training four days later on Day 12, RAPA-treated mice first showed reduced freezing compared to VEH-treated mice, but overall we found no differences between groups, with both VEH and RAPA-treated mice demonstrating increased freezing after the final tone-shock pairing (Mixed ANOVA: main effect of time, $F (1, 28) = 133.713$, $p < .001$, but no main effect of treatment, $F (1, 28) = 2.688$, $p = .112$ or interaction effect, $F (1, 28) = 3.826$, $p = .061$; Fig. 4.6B). A mixed ANOVA of the auditory fear recall tests in Context C on Day 14 and 22, however, revealed significant differences between groups (main effect of treatment, $F (1, 28) = 15.331$, $p < .001$; main effect of time, $F (3, 84) = 10.803$, $p < .001$; but no interaction effect, $F (3, 84) = 2.274$, $p = .086$). Although both groups froze comparably during the second half of each test when the conditioning tone was replayed (Bonferroni post hoc tests: $p > .05$, for both Day 14

and 22, respectively), RAPA-treated mice froze significantly less during the first half of each test, prior to the auditory stimuli being activated (Bonferroni post hoc tests: Day 14, $p = .032$; Day 22, $p > .001$, Fig. 4.6C).

Based on the results of Expt. 8, we were interested in whether a similar pattern of findings would follow if we used a psychogenic stressor instead of a physical stressor during contextual fear conditioning (Expt. 9, Fig 4.6A). We found no differences between groups during predator exposure, as both sets of mice demonstrated high levels of immobility (freezing) within the protected half of the arena (data not shown, all $p > .05$). During re-exposure to the conditioning arena seven days later (Day 8) there were again no differences in immobility between groups ($t (28) = .148$, $p = .884$). However, when mice were subjected to auditory fear conditioning in Context B on Day 12, a mixed ANOVA of freezing data revealed a significant main effect of treatment ($F (1, 28) = 11.858$, $p = .002$) and a significant main effect of time ($F (1, 28) = 92.299$, $p = < .001$), but no interaction effect ($F (1, 28) = 1.670$, $p = .207$). Both groups froze significantly more during the time period following the last tone-shock pairing compared to freezing during the time period immediately prior to the to-be-conditioned tone being played (Bonferroni post hoc tests: both $p < .05$ for VEH and RAPA, respectively). RAPA-treated mice, however, froze significantly less during both of these two time periods than VEH-treated mice (Bonferroni post-hocs: pre-tone, $p = .038$; post last tone-shock pairing, $p = .024$; Fig. 4.6D). Auditory fear recall probes on Day 14 and 22 in Context C also revealed significant differences in freezing behaviour (mixed ANOVA: main effect of treatment, $F (1, 28) = 5.756$, $p = .023$, main effect of time, $F (3, 84) = 69.867$, $p = < .001$, while an interaction effect approached significance, $F (3, 84) = 2.703$, $p = .051$). Each group froze significantly more during the time

the tone was replayed than when it was off for each probe in Context C (all $p < .05$ for both VEH and RAPA). Likewise, both groups of mice demonstrated similar levels of conditioned responding when the tone was replayed in the second half of each recall probe (Bonferroni post hoc tests: all $p > .05$ for both Day 14 and 22). Nevertheless, RAPA-treated mice froze significantly less than VEH mice in the first half of each probe before the conditioned tone was activated (Day 14, $p = .04$; Day 22, $p = .048$; Fig. 4.6E).

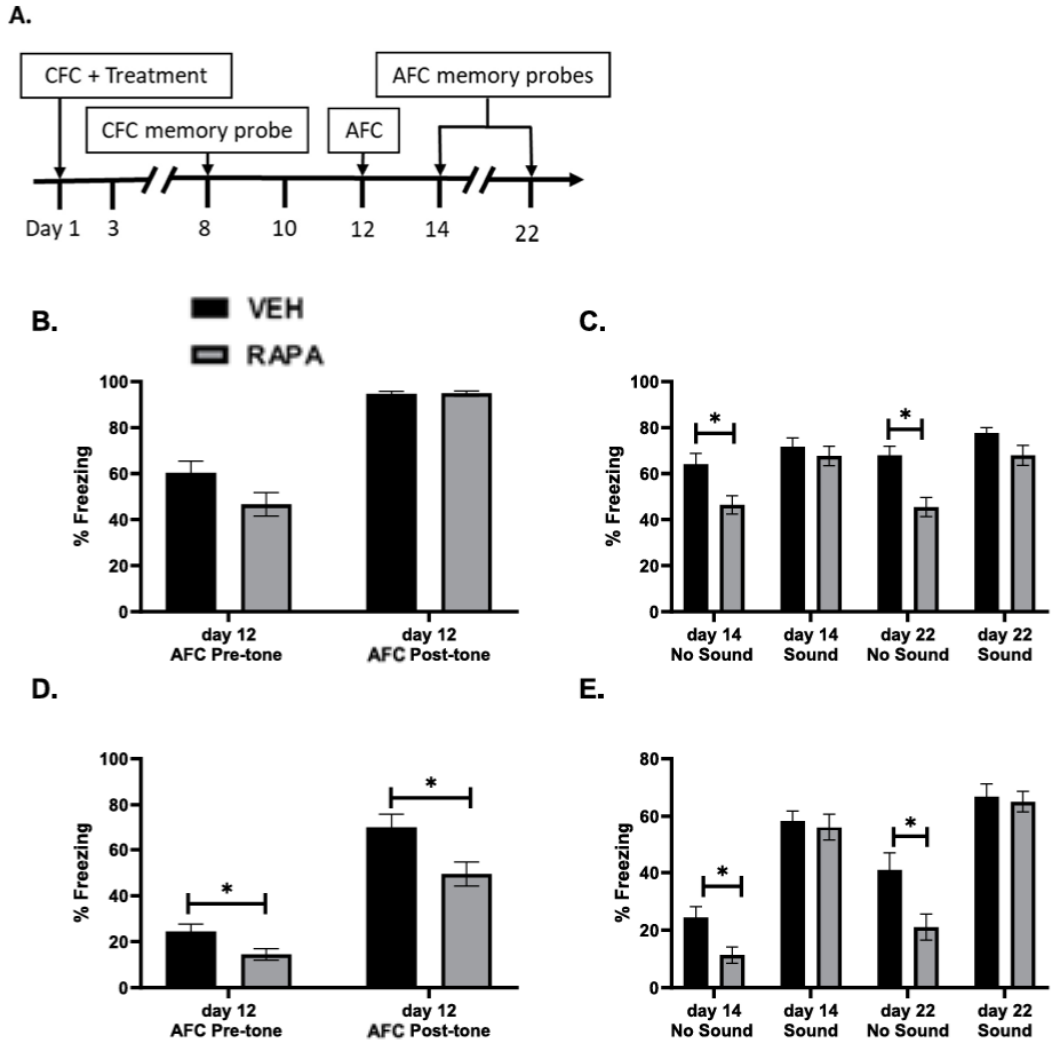

Figure 4.6 Post-conditioning RAPA treatment does not interfere with the capacity to subsequently learn and recall a new fear association and protects against fear generalization to new contexts. (A) Timeline of Expt. 8 and 9. Expt. 8 used a physical, foot-shock, stressor,

whereas, Expt. 9, used a psychogenic, predator-exposure, stress during contextual fear conditioning, immediately prior to drug treatment. (B) Impaired CFC consolidation to the shock-associated Context A by RAPA did not alter AFC acquisition in Context B on Day 12. (C) Both RAPA and VEH-treated mice froze comparably to the AFC tone replay during probes in Context C on Days 14 and 22, but prior to the tone being activated RAPA-treated mice froze significantly less than their VEH counterparts. (D) Post-predator stress RAPA-treatment did not alter immobility (freezing) to Context A upon re-exposure but it did decrease freezing in the period immediately before and after AFC in Context B on Day 12 compared to VEH-treated mice, although both groups demonstrated AFC acquisition. (E) Both groups of predator-stressed mice showed similar levels of freezing to the conditioning tone during AFC memory probes in Context C on Days 14 and 22, but RAPA-treated mice froze significantly less when the AFC tone was not turned on. Data represented as mean and ±SEM. Statistical significance denoted by * ($p < 0.05$). AFC: Auditory Fear Conditioning, CFC: Contextual Fear Conditioning, VEH: Vehicle, RAPA: Rapamycin.

4.5 Discussion

We investigated basic mTORC1-dependent fear memory processes to better understand the therapeutic potential of rapamycin (RAPA). We demonstrate that RAPA injected three or 12 hours after contextual fear retrieval diminishes memory persistence. RAPA acts via blocking reconsolidation as repeated reactivation with RAPA impairs recall with or without a reminder shock. Further, while RAPA impairs the consolidation or reconsolidation of a specific memory it does not interfere with the ability to learn a new fear association. Moreover, RAPA-treated mice

show an absence of fear generalization to new contexts. Overall, our results suggest that RAPA has persistent effects on reconsolidated fear memory, does not alter the formation of new memories, and reduces generalized fear to novel contexts.

Several studies have established a time-limited role for *de novo* protein synthesis, and in particular mTORC1 translational control of protein synthesis, for reconsolidation to occur (Jobim et al., 2012a b; Nader et al., 2000; Yan et al., 2020; Zhang et al., 2021). In these studies, RAPA or anisomycin delivered immediately after reactivation, but not 6 h later, impaired recall the next day. Interestingly, recent reports have shown anisomycin injected ~12 hours, but not at 5 or 24 hours, post-reactivation for a contextual fear memory impairs subsequent recall when tested seven days but not two days after retrieval (Nakayama et al., 2013; Nakayama et al., 2016). The effects of delayed protein-synthesis inhibition implies an additional time-window of susceptibility after the initial reactivation window, which appears to contribute to the persistence of a reconsolidated memory. Indeed, this interpretation aligns with our findings as we showed RAPA given three or 12 hours after reactivation impaired memory persistence (at 7 days) but only RAPA given three hours post-reactivation (not 12 hours) impaired reconsolidation (at 2 days).

Overall, our current and past work indicates contextual fear memory reconsolidation and persistence is vulnerable to RAPA at three time points post-reactivation (Blundell et al., 2008). First, immediately after reactivation, second, 3 h post-reactivation, and lastly, 12 h post-reactivation. Although our results imply the possibility of discrete, recurrent RAPA-sensitive events that contribute to the reconsolidation and persistence of memory, the time points we used

to inject RAPA are far from exhaustive and limit our interpretations. It is possible that RAPA-sensitivity immediately after and 3-h post-reactivation to contextual fear reconsolidation and persistence is unitary between these time points and part of the initial time window of vulnerability before re-stabilization as we did not test any intermediary time points between 0 and 3 h post-reactivation. Likewise, we did not test any intermediary points between the 3 and 12 h injections unlike Nakayama et al. (2013), which indicated at the very least a break point between periods of post-reactivation susceptibility to anisomycin on subsequent memory recall. Nonetheless, our temporal information on the susceptibility to RAPA provides some initial insights into how mTORC1-signalling might be contributing to the reconsolidation and persistence of reactivated memory. Moreover, therapeutically, our data imply that if RAPA treatment has to be delayed post-reactivation, there is still the prospect of the drug contributing long-lasting impairments to the trauma-associated memory.

We also assessed whether additional pairings of context re-exposure with RAPA would further impair fear memory retention. Consistent with our earlier work (Blundell et al., 2008), we demonstrated that memory recall was significantly diminished in RAPA-treated mice compared to VEH-treated mice when probed eight days after the last of several reactivation-drug treatment pairings. Moreover, a key reason for conducting these experiments was to determine if there were any additive effects of repeating the reactivation and drug treatment procedure *within* the RAPA group alone. We found two consecutive days of reactivation, each followed by systemic RAPA treatment (i.e., reactivation 1 v. 2 and reactivation 2 v. 3), maximized the impairment incurred to the associative strength of the contextual fear memory. While a third RAPA treatment did not provide any further significant reductions to conditioned responding, the

effects of three consecutive treatments remained consistent post-treatment and deterred any rebound in fear responding (reinstatement) via the reminder-shock. Importantly, this insensitivity to the reminder-shock is consistent with our earlier work with just a single reactivation and RAPA treatment (Blundell et al., 2008), and posits that under these circumstances post-reactivation mTORC1 inhibition is weakening reconsolidation rather than enhancing fear memory extinction. Therapeutically this would be advantageous, as this implies that the return or relapse of the emotional fear response is unlikely, which would not necessarily occur under altered extinction.

We would be remiss, however, if we neglected to discuss the support for mTORC1 signalling in fear extinction processes (de Carvalho Myskiw et al., 2014; Girgenti et al., 2016; Huynh et al., 2018; Moya et al., 2020; Penha Farias et al., 2019; Radiske et al., 2021; Rosa et al., 2023; Song et al., 2018). In these studies, RAPA administration around the time of re-exposure to a conditioned stimulus or context increased subsequent retention of the conditioned response. Ostensibly, these studies parallel reconsolidation experiments but with opposite expected behavioural outcomes from RAPA treatment (i.e., impaired extinction). However, in general, most of these studies used much longer re-exposures to the conditioned stimulus or context during reactivation; which is conducive for activating extinction instead of reconsolidation (Inaba et al., 2015; Lee et al., 2006; Lunardi et al., 2018; Pedreira & Maldonado, 2003; Suzuki et al., 2004). Nevertheless, these reconsolidation and extinction studies highlight the dynamic role of mTORC1 in two opposing memory processes elicited by retrieval but also underscores the need to understand the parameters that are the most auspicious for RAPA to influence these processes.

Taking this into consideration, another important boundary condition between reconsolidation and extinction worth mentioning is the interval between acquisition and first retrieval. Reactivated, recent memories, following a short interval between acquisition and first retrieval, such as the intervals we used in our first seven experiments (24-48-h), are receptive to engaging reconsolidation processes. With longer latencies, memories become more remote, less likely to reconsolidate, and more likely to extinguish, but paradoxically, can be destabilized with longer reminder durations (Frankland et al., 2006; Inda et al., 2011; Ishikawa et al., 2016; Suzuki et al., 2004). As such, it will be important for future studies to investigate if our findings are replicable using much longer intervals between acquisition and retrieval (e.g., 3 or 8 weeks). Likewise, although the context re-exposure duration (240-s) we used was sufficient for RAPA to interfere with reconsolidation, it will be interesting to find out if this holds true for remote memories or whether longer re-exposure durations are required. Studying remote memories will also be therapeutically relevant for PTSD, since traumatic memories are enduring, cues and contexts associated with the trauma are actively avoided, there are pervasive delays in making initial treatment contact, and early interventions are often not readily available (Goldberg et al., 2019; Kida, 2019; Tsai & Gräff, 2014; Wang et al., 2005).

In our final three experiments, we found that a single systemic injection of RAPA after contextual fear conditioning or reactivation does not subsequently interfere with the ability to learn and recall a new auditory-shock association. In contrast to learning new associations, two previous studies have shown relearning or retraining of the same associations after initial RAPA-induced impairment of consolidation (Bekinschtein et al., 2007b; Tischmeyer et al., 2002). Levin et al. (2017) reported that RAPA-impaired reconsolidation for a shock-associated memory did

not affect subsequent object recognition memory and enhanced object location memory. However, in that experiment those tasks focused on short-term, working memory changes, whereas our work focused on changes to new Pavlovian learning and long-term memory expression. We also found RAPA treatment protected against fear generalization to the new environments where auditory fear conditioning and recall took place. Levin et al. (2017) similarly showed in the same experiment mentioned above that post-reactivation RAPA normalized shock-induced freezing enhancement to a novel open field environment. Together, these works and ours, augurs well for potential clinical use of RAPA, as it indicates no permanent learning or memory impairments and a potential reduction in fear overgeneralization, which occurs in many trauma and anxiety-related disorders such as PTSD (Cooper et al., 2022; Morey et al., 2015). An important caveat worth mentioning about our findings, however, is we only used male mice subjects. This limitation will need to be addressed for our work to have broader applicability, especially considering there are sex and gender based differences in PTSD (APA, 2022; Careaga et al., 2016; Kar, 2011; Maren & Holmes, 2016).

Despite our consolidation experiments having similar findings for auditory fear and fear generalization, we did not discover any changes in freezing (immobility) of RAPA-treated mice returned to the predator exposure context seven days after training and drug treatment unlike what we found for shock-conditioned mice. It is possible that our predator exposure procedure is not amenable to inducing conditioned immobility but perhaps other behaviours related to predator defense. Indeed, this interpretation has credence as Philbert and colleagues (2015) showed increased locomotor activity and escape attempts in mice re-exposed to the context where they experienced the mouse defense test battery, which is a very systematic and sequenced

exposure to a rat to test mouse defensive repertoire (Blanchard et al., 2003). Homiack et al. (2017) similarly reported increased hyperactivity and decreased freezing in rats re-exposed to a context where they previously experienced a predator odor. To address this, future experiments by us using this paradigm will need to include a context only exposure control group and include a wider ethogram of behaviours beyond just immobility to screen for contextual conditioning associability. Nevertheless, we view this work as important first steps in establishing an associative psychogenic, predator, stress model that can be used to investigate not just consolidation but also reconsolidation of conditioned responding as this been long overlooked in predator stress models.

In conclusion, our findings demonstrate that contextual fear memory reconsolidation and persistence is susceptible to RAPA injected systemically at 3-h post-reactivation, whereas RAPA delivered 12 h after reactivation impairs the persistence of the post-reactivated memory. Moreover, we show that two consecutive days of reactivation paired with immediate RAPA treatment maximizes memory impairment likely through disrupted reconsolidation. Lastly, we found that RAPA-impaired consolidation and reconsolidation does not interferes with the ability to further learn and remember a new fear association, but provides protection against fear generalization to new contexts. Collectively, our reconsolidation findings provide preclinical evidence of RAPA as a putative treatment enhancer to combat PTSD-like re-experiencing.

Chapter 5: Thesis Conclusions

In this dissertation, I focused on further elucidating the contribution of mTOR (predominately mTORC1) to fear memory consolidation, reconsolidation, and persistence in rodents, relating how these mTOR-dependent memory processes might be contributing to PTSD-like symptoms, and determining if pharmacological inhibition of mTORC1 with RAPA is effective at disrupting PTSD-like symptoms.

5.1 Overview of Findings

Chapter 2. I found that RAPA administered immediately after foot-shock contextual fear conditioning impairs the consolidation and persistence of the associative contextual fear memory in mice without interfering with the short-term expression of the memory. When I delayed RAPA treatment by three-hours post-conditioning, the consolidation and persistence of the contextual fear memory suffered in mice, whereas delaying treatment by 12-hours post-conditioning did not alter consolidation or persistence of the memory. In a dose-response experiment, I demonstrated that the dual mTORC1/2 inhibitor AZD2014 dose-dependently impairs the consolidation and persistence of the context foot-shock memory similar to RAPA. In contrast, when I targeted S6K1, often used as a readout of mTORC1 activity, with the S6K1 inhibitor PF-4708671, this only revealed a slight diminishment in the strength of the fear memory in mice over time.

Chapter 3. Complementing an earlier study that found RAPA impairs the consolidation of associative and non-associative fear memories in rats following a brief unprotected exposure to a

cat (Fifield et al., 2013); my immunohistochemistry work revealed an increase in mTORC1 activity in brain areas important to memory and elicited predator responses (i.e., the hippocampus and periaqueductal grey) of rats shortly after this type of predator stress. Additionally, I piloted a wholly psychogenic mouse-rat exposure paradigm to determine if it could elicit conditioned responses in mice following predator stress, as hitherto this, the Rat Exposure Test was only used to assay innate mouse defensive behaviours during and immediately after said exposures. The brief protected exposure to a rat induced conditioned associative and non-associative fear responding from mice, with the negative valence of some of these behaviours weakened from post-exposure RAPA treatment.

Chapter 4. By delaying RAPA treatment three- and 12-hours post-reactivation of a context foot-shock memory in mice, I discovered that this resulted in impaired memory persistence, with a diminishing effect against memory reconsolidation as the time between the reactivation and RAPA administration increased. When I subjected mice to three consecutive days of memory retrieval paired immediately thereafter with RAPA, this resulted in maximal impairment of reconsolidation after two consecutive days of reactivation and treatment, with the third reactivation and treatment not providing any additional significant changes to freezing. Moreover, this effect from multiple reactivation and RAPA treatments was resistant to reinstatement from a subthreshold 'reminder' shock. Lastly, my research revealed that post-conditioning (via a mice exposed to a rat or foot-shock) and post-retrieval (via foot-shock to mice) RAPA treatment does not result in any permanent impairments in the ability to learn and recall a new foot-shock induced auditory fear memory, while also conferring protection against fear generalization to new contexts.

5.2 Consolidation, Reconsolidation, (and) Persistence, and Time-Dependent Effects of Rapamycin

My findings from delaying drug treatment following conditioning and reactivation add to a growing body of evidence supporting the postulation that delayed molecular events long after learning or recall largely contribute to the longevity of an engram (Bekinschtein et al., 2007a; Bekinschtein et al., 2010; Bourtchouladze et al., 1998; Freeman et al., 1995; Grechsch & Matthies, 1980; Igaz, et al., 2002; Katche et al., 2016; Krawczyk et al., 2016; Mac Callum et al., 2014; Martinez-Moreno et al., 2011; Nakayama et al., 2013; Nakayama et al., 2016; Ou et al., 2009; Pena et al., 2014; Pereira et al., 2019; Quevedo et al., 1999; Rossato et al., 2007; Trifilieff, et al., 2006; Wanisch et al., 2008; Yang et al., 2013). As such, it might be tempting to suggest that my findings and those by others demonstrating the effects of delayed pharmacological or other manipulations are evidence against the notion of single restricted time-limited windows of vulnerability immediately around the time of learning and reactivation to interfere with consolidation and reconsolidation, respectively. However, I argue rather, that these results are complimentary insofar as they provide a plausible link between initial memory formation and re-stabilization with the persistence of a memory through recurrent consolidation- and reconsolidation-like events.

In studies that have shown a limited time-window of susceptibility to pharmacological manipulations (i.e., a lack of effect from delayed treatment) against memory consolidation and reconsolidation, they typically only tested for memory changes 24 to 48-hours post-training or reactivation (Jobim et al., 2012a, b; Nader et al., 2000; Yan et al., 2020; Zhang et al., 2021). Conversely, my work, like the other studies that have shown protracted timepoints of

susceptibility to interference, distinguished between changes to consolidation and reconsolidation versus changes to persistence by testing separate cohorts of rodents at 24 to 48-hours and seven-days post-training or reactivation, respectively. Doing this allows for the establishment of events that are likely acting on consolidation and reconsolidation in the near-term of a newly established or recently reactivated long-term memory (~24 to 48-hours), while highlighting those events that appear to only be essential for the long-term maintenance of a memory trace (~ a week). It should be noted, however, that although such protracted molecular events following learning or reactivation appear to have a strong proclivity towards influencing memory persistence (and vice versa for earlier events towards consolidation and reconsolidation), this is not a steadfast rule. Targeting these later (or earlier) timepoints does not necessarily guarantee, assuming a drug effect, which memory process will be affected. For instance, some drugs administered close to the time of reactivation have been shown to not affect initial reconsolidation but only persistence (Da Silva et al., 2013; Huynh et al., 2013), whereas delayed drug administration has been shown to diminish memory at much earlier recall tests than would be expected if only acting on persistence (Barrientos et al., 2002; Mac Callum et al., 2014; Nazari et al., 2023). Regardless of these exceptions, overall, testing recall in one cohort in the days immediately after post-training or reactivation and another cohort at more distant time point offers a simple but effective way to analyze the effects of drugs and concomitantly the molecular pathways potentially underlying memory consolidation, reconsolidation, and persistence.

My results from administering RAPA immediately after and three-hours post-training and post-reactivation, respectively, were similar, with subsequent recall to the foot-shock associated context stunted in all these experiments. As I moved to the later 12-hour RAPA treatment delay,

the effects to recall became more disparate between the consolidation (Chapter 2) and reconsolidation experiments (Chapter 4). The systemic injections of RAPA to mice 12-hours post-conditioning had no effect on consolidation or persistence, whereas this delayed treatment following reactivation had a partial effect on reconsolidation while impairing persistence of the reconsolidated contextual (or post-reactivated) fear memory. Nevertheless, this split between post-conditioning and post-reactivation temporal RAPA-sensitivity was not entirely unexpected. While many of the same molecular mechanisms are required for consolidation and reconsolidation in the immediate aftermath of learning and reactivation, it is important to remember that reconsolidation is not a full recapitulation of consolidation and vice versa (Hall et al., 2001; Lee et al., 2004; Parsons et al., 2006a; von Hertzen & Giese, 2005). It is, therefore, not unreasonable to expect such consolidation- and reconsolidation-events to not necessarily match up temporally or perhaps occur at all for certain phases. Moreover, RAPA has been shown to have differential effects based on the type of memory (e.g., cued versus context), mnemonics of a particular memory (e.g., cue and contextual freezing versus cued and contextual fear potentiated startle), and the specific brain loci being targeted (Gafford et al., 2011; Glover et al., 2010; Mac Callum et al., 2014; Parsons et al., 2006b).

In terms of brain loci, it will be fruitful to replicate my experiments using cannulated injections to determine which brain structures germane to consolidation, reconsolidation, and persistence are RAPA-sensitive at these identified points of susceptibility from systemic injections (e.g., amygdala, hippocampus, prefrontal cortex). To help corroborate my findings and to better guide which brain areas to cannulate it will also be important to conduct immunostaining work (e.g., western blot, immunohistochemistry) to determine the areas that show changes in

phosphorylated mTORC1 or for activation of its downstream effector SK61 at these later timepoints of systemic RAPA-sensitivity post-conditioning and post-reactivation (three and/or 12-hours). Moreover, as the timepoints I chose to investigate are largely motivated from the work done by the Hiroshi Nomura Lab (reconsolidation) and Jorge H. Medina and the late Ivan Izquierdo's shared research group (consolidation) using the global protein synthesis inhibitor anisomycin, they are relatively limited in scope (Bekinschtein et al., 2007a; Bekinschtein et al., 2010; Nakayama et al., 2013; Nakayama et al., 2016). Performing such immunostaining work will, therefore, also effectuate the exploration of changes to mTOR activity at different timepoints following learning and reactivation, which could potentially reveal other points of vulnerability to RAPA.

5.3 Effects of Rat-Induced Psychogenic Predator Stress in Mice

The adapted Rat Exposure Test apparatus I used to investigate long-lasting changes to mouse behaviour in Chapters 3 and 4 did not include the adjacent mouse home chamber and interconnecting tunnel between the home chamber and the mouse side of the exposure chamber like the original version of the apparatus created by Caroline and the late Bob Blanchard's lab. Instead, I used a simplified version of the apparatus with only the partitioned exposure chamber, with one side for a prey mouse and the other for the predator rat. My version also differed from the original version of the arena in that I used a perforated Plexiglas separator that ran lengthwise instead of a wire mesh barrier widthwise (see Campos et al., 2013 for a schematic of the original version of the exposure chamber). In the original Rat Exposure Test work by Yang et al. (2004), they used the complete apparatus to assay a repertoire of spatiotemporal and ethological relevant predator-induced mouse defensive behaviours, including defensive burying, risk assessment,

freezing, and avoidance of the rat-predator stimulus. I only measured freezing behaviour during exposure and re-exposure to the arena. One last difference between my work and the Blanchard's is the rats used by the Blanchard lab during exposures were amphetamine treated to maintain movement of these predators, while the rats I used were calorie-restricted to ostensibly increase activity and motivation during exposures to prey.

I was unable to replicate my finding from Chapter 3 in Chapter 4 showing decreased freezing in mice returned to the predator exposure context following post-conditioning RAPA treatment. As such, I am not opposed to revisiting these experiments using the original configuration used by the Blanchards to study the effects of RAPA to freezing under this scenario. Utilizing this configuration would also allow for the use of an expanded ethogram to evaluate whether other non-freezing predator-elicited behaviours in this paradigm are amenable to associative conditioning. Likewise, it would be important to compare the effects of caloric-restricted versus amphetamine treated predator rats, as there are no direct or indirect comparisons on whether one is more effective at eliciting innate prey defensive behaviours, entraining conditioned responding in prey, or what the variance in movement and other behaviours of these predators are throughout the course of running exposures. While it is important to report these null effects when they occur to overcome the 'file-drawer problem/ positive publication bias", it is unlikely any of these details would have necessarily corrected the inconsistencies in Chapter 3 and 4. Addressing these points will, however, improve the robustness and ethological relevance of this paradigm at examining PTSD. Moreover, with the exception of a few predator scent studies (Arluk et al., 2022; Aykac et al., 2020; Oliver et al., 2016; Robinson et al., 2019), fully characterizing the associability of this live psychogenic predator exposure model will provide an

excellent opportunity to examine the underlying mechanisms of fear reconsolidation from this mode of stress instead of with the typical foot-shock methods.

5.4 Foot-Shock and Predator Exposure Stress Fear Models of PTSD

For this dissertation, I largely conducted experiments using contextual electric foot-shock fear conditioning procedures to investigate the contribution of mTOR to memory and changes to freezing (or PTSD-like re-experiencing) in rodents from the application of RAPA. In fear conditioning and PTSD literature, the words electric foot-shock are typically omitted. Instead, just contextual fear conditioning (or CFC) is used to refer to these learning and memory procedures and PTSD models. In this, the introductory chapter, and Chapter 4, however, I have deliberately included electric foot-shock in my descriptions of these procedures, as I place the predator stress models I utilized for a fraction of my experiments under the same larger umbrella of contextual fear conditioning. While electric foot-shock and predator stress models are often categorized as physical or psychogenic (or hybrid in the case of unprotected exposures), respectively, they are largely on the same side of the coin procedurally. That is, a specific context or environment is paired with exposure to an unconditioned stressor, which, depending on the parameters of the experiment (e.g., unconditioned stimulus duration, frequency, specific use and timing of non-contextual to be conditioned cues), will subsequently elicit an array of associative and non-associative fear behaviours that map onto certain PTSD-like symptom clusters (Flandreau & Toth, 2018; Siegmund & Wotjak, 2006, 2007; Török et al., 2019; Verbitsky et al., 2020).

Beyond these procedural similarities, predator stress models are not up to par with electric foot-shock conditioning in terms of reliability as a model to study the neurobiology of memory or PTSD. As described above in the previous section and in the proceeding chapters, the adapted Rat Exposure Test I used still is very much incipient and not fully characterized or optimized yet with regards to conditioning and capturing fear memory changes. Moreover, while the Rat Exposure Test controls for physical interaction between the prey, a mouse, and the predator, a rat, it, like the other predator stress paradigm I employed for my dissertation, exposing rats to a brief, non-lethal, but unprotected, cat encounter, and those used by others (e.g., predator scent, single prolonged stress), cannot control for the level of predator intensity. Likewise, the inability to control the intensity of the trauma is also endemic to all other non-foot-shock PTSD models, such as water submersion, restraint/immobilization, social defeat by a conspecific, and single prolonged stress without a predator component (Verbitsky et al., 2020).

Since electric foot-shock reliably induces long-lasting behavioural consequences in the vast majority of subjects, a common critique of this model is that it does not create individual variability or the ability to differentiate between vulnerable and resilient subjects. In contrast, predator stress and the other PTSD models are often characterized by significant inter-individual variability of subjects based on post-trauma behaviours (Flandreau & Toth, 2018; Török et al., 2019; Verbitsky et al., 2020). Subjects in these models are categorized as resilient or vulnerable through behavioural cutoff criteria or median splits following assessment of elevated plus maze and acoustic startle data, as these behaviours are partially representative of Cluster C (avoidance) and Cluster E (arousal) symptoms, respectively (Adamec et al., 2012; Cohen et al., 2006; Schwendt et al., 2018). Such responses to these stressors are thought to mimic human responses

to traumatic events, where many individuals are found to be resilient to traumatic stress, while others go on to develop PTSD. Nevertheless, while the ability to focus on and identify the extreme ends of stress responses might be useful in understanding neurobiological factors for adaptive and maladaptive responding, the inability to control the intensity of the trauma in these models should give pause to the veracity of these group classifications. It is unclear from these models whether the resilient and susceptible groups identified are indicative of variability in the ability to cope with the fallout from the stressor experienced or whether the uncontrollable intensity inherent in these stressors confers different post-trauma behaviour. All models, including foot-shock, however, would largely benefit from the development of pre-trauma behavioural screening to potentially identify resilient and susceptible groups to make more informed *a priori* predictions about responses to trauma and treatments before post-trauma behavioural testing. For instance, evidence indicates individuals with PTSD likely have pre-existing deficits in cued spatial processing and navigating complex spatial environments prior to developing PTSD (Gilbertson et al., 2007; Marlatte et al., 2022; Smith et al., 2015). It might, therefore, be worthwhile to investigate for deficits in allocentric navigation strategies of rodents in the Morris Water Maze prior to trauma exposure as a potential predictor of susceptibility and resilience to the traumatic stress they will experience.

Unlike foot-shock stress, predator stress constitutes a real-life ethologically and ecologically relevant threat to a prey species akin to severe trauma a human might experience. Findings from predator stress PTSD models, however, can potentially be interpreted as mere artifacts from not just the artificial lab environment, but also the laboratory rodents used themselves. Through generations of domestication, laboratory rodents, although maintaining many of the behavioural,

anatomical, and physiological characteristics of their ancestors, are not necessarily the same as their wild counterparts. Artificial selection pressures have produced, to name a few examples, laboratory rodents that reach sexual maturity quicker, have larger litters, possess distinct molar morphology, explore less in unfamiliar spaces, show less interspecific aggression, have smaller brain areas related to wariness, and show reduced defensive behaviours compared to their wild cousins (Bárdos et al., 2024; Blanchard et al., 1986, 1998; Brown & Bronson, 1992; Himmler et al., 2013; Holmes et al., 2000; Koizumi et al., 2018; Savriama et al., 2022; Vogt et al., 2024). Moreover, there was traditionally a longstanding assumption that predator stress is ephemeral in wild animals with no long-lasting repercussions from non-lethal interactions with predators (Clinchy, et al., 2013; Matar et al., 2013). In other words, this premise postulated wild 'less cognitively complex' animals do not experience long-lasting stress-induced psychopathologies but rather just return to homeostasis following fleeing, fighting, or freezing from traumatic non-consumptive predator encounters. This reasoning also underlies Standford University biologist Robert Sapolsky's popular mainstream book Why Zebras Don't Get Ulcers: A Guide to Stress, Stress-Related Diseases, and Coping. Ecological work over the last two decades, however, has challenged and disconfirmed this perspective, revealing that this assumption is likely not tenable.

Specifically, demographic experiments and observational studies have shown reduced number and survival of offspring in free-living birds and mammalian prey species in response to predator and predator cues (Reviewed in Zanette & Clinchy, 2020). Beyond population-based studies, experimental work from Liana Zanette's lab has highlighted the long-lasting physiological, neurohistological, and behavioural consequences of sustained psychological predator stress in wild animals (Epperly et al., 2021; Widén et al., 2022; Zanette et al., 2019). Through a

collaboration between my supervisor, Jacqueline Blundell, and the Sheriff & Zanette Labs, I got to participate in a one such study focused on the ecology of fear in wild, free-living, deer mice during my PhD program. This research involved broadcasting predator playbacks or non-predator playbacks over loudspeakers for 20 days on a four day on /off cycle in four different trapping grids (two control grids, two stressed grids) on an island off the coast of British Columbia. Feeding trials were conducted on the last two days of playbacks, then after the twentieth day a subset of deer mice were captured and sacrificed for their blood and brains. Deer mice exposed to predator playbacks were found to forage less (higher giving up density), have higher stress hormone levels (plasma corticosterone), and exhibit alterations in hippocampal mTOR activity compared to controls in this field experiment (MacCallum et al., 2018).

This deer mice work provides a direct empirical link between mammalian predator stress-induced psychopathology models and the ecology of fear, which will be important for advancing interdisciplinary work in these fields and at the minimum improves our confidence that findings from lab animals and settings are externally valid. Fittingly, Hagit Cohen's lab recently published converging evidence supporting the argument that wild rodents are susceptible to predator stress induced psychopathology. But instead of conducting field experiments, they tested the effects of predator playbacks on three different types of captured wild rodent species in a laboratory set-up. Predator calls elicited a PTSD-like sequalae of behaviours and physiological changes in all these wild species tested paralleling results from lab rodents (Cohen et al., 2023).

Individually, no animal model of PTSD fully recapitulates PTSD as the disorder is highly heterogeneous with twenty possible symptoms across the four symptom clusters in the DSM-5

(Clusters B: re-experiencing, C: avoidance, D: negative changes in mood and cognition, and E: arousal and reactivity). Despite this large challenge, animal models capture important facets of the disorder by exposing rodents to a variety of stressors that precipitate PTSD-like symptoms as revealed through testing behavioural analogs for these symptom clusters (see Verbitsky et al., 2020 for a comprehensive list). Notably, no symptoms in PTSD or the behavioural analogs used to test for PTSD-like symptoms are unique to PTSD or PTSD models (Flandreau & Toth, 2018; Schöner et al., 2017; Török et al., 2019; Verbitsky et al., 2020). For instance, anhedonia is found in both PTSD and depression, while the sucrose preference test is used to measure anhedonia in both models of PTSD and depression. The considerable overlap in symptoms and the high level of comorbidity between PTSD, depression, and anxiety disorders, might make these disorders difficult to distinguish between or determine the validity of models designed to replicate these disorders. Under these circumstances, when distinguishing or evaluating models for goodness of fit to a particular disorder, it is therefore important to recognize the etiology or mechanism to induce such symptoms (Schöner et al., 2017). Yehuda and Anetelman (1993) proposed that for a PTSD model to be useful the stressor imposed needs to be brief and intense, whereas more chronic and mild stressors, such as learned helplessness protocols, are more appropriate at developing models of depression. With this in mind, the lab-based models I used would pass this criterion, but the deer mice field experiment I contributed to (described above) would fail as a model of PTSD as the predator playbacks were played on and off over a 20-day period. It will be interesting to determine if the acute effects of such playbacks in a similar field experiment would result in comparable or different sequalae of symptoms (findings) from the chronic exposure to predator playbacks since such chronic predator exposure might be more indicative of a depression-like phenotype and not PTSD.

Foot-shock lab models of PTSD studies often only focus on changes to associative freezing, whereas lab-based predator stress models largely focus on non-associative memory changes. Although foot-shock and predator stress are perhaps specifically better suited to accentuate associative and non-associative memory changes, respectively, expanding the test batteries for each will provide better science in general. Moreover, to model properly or more comprehensively, expanded test batteries should be utilized. For my foot-shock experiments, I predominantly just examined associative recall tests, which covers Cluster B, re-experiencing of the trauma, while also modelling exposure-like therapy, which allowed me to investigate if RAPA could enhance the exposure-like therapy process through impaired reconsolidation. Several of my experiments also highlighted changes to generalized anxiety-like behaviour, which replicates elements of Cluster C, avoidance (Flandreau & Toth, 2018; Verbitsky et al., 2020). This still leaves Cluster D and E outstanding from my foot-shock work. As such, it will be prudent for future work to determine if exposure-like therapy, pharmacological enhanced or not, has any beneficial effects to the other non-associative memory related symptom clusters. Unlike most predator models that focus on Clusters C-E, my paradigm specifically included returning mice to the context where they encountered a rat to mimic Cluster B. To me this is an important first step towards incorporating exposure-like therapy, which is strongly rooted in associative learning and memory, but often overlooked in predator-based fear models. By increasing this practice, it will likely bring predator stress models closer to parallel with the capabilities of foot-shock paradigms.

Overall, foot-shock and predator stress models replicate important features of PTSD and confer us the ability to test theories and study the neurobiology of fear learning and memory otherwise inaccessible to human research. Moreover, these paradigms and methods provide an avenue to investigate potential drugs alone or through pairing them with re-exposure to trauma related cues and contexts as I did with RAPA. Preclinical data from these studies will thereby inform important translational efforts at potentially pharmacologically enhancing exposure therapy through enhanced extinction or disrupted reconsolidation, creating expanded treatment options for PTSD and other stress-related psychopathologies.

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