SYSTEMIC INHIBITION OF mTOR KINASE VIA RAPAMYCIN DISRUPTS CONSOLIDATION AND RECONSOLIDATION OF AUDITORY FEAR MEMORY

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Systemic Inhibition of mTOR Kinase via Rapamycin Disrupts Consolidation and Reconsolidation of Auditory Fear Memory

by

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Abstract

The mammalian target of rapamycin (mTOR) kinase is a critical regulator of mRNA translation and is known to be involved in various long lasting forms of synaptic and behavioural plasticity. However, information concerning the temporal pattern of mTOR activation and susceptibility to pharmacological intervention during both consolidation and reconsolidation of long-term memory (LTM) remains scant. Male C57BL/6 mice were injected systemically with rapamycin at various time points following conditioning or retrieval in an auditory fear conditioning paradigm, and compared to vehicle (and/or anisomycin) controls for subsequent memory recall. Systemic blockade of mTOR with rapamycin immediately or 12 hours after training or reactivation impaired both consolidation and reconsolidation of an auditory fear memory. Further behavioural analysis revealed that the enduring effects of rapamycin on reconsolidation were dependent upon reactivation of the memory trace. Rapamycin, however, had no effect on short-term memory or the ability to retrieve an established fear memory. Collectively, these data suggest that biphasic mTOR signalling is essential for both consolidation and reconsolidation-like activities that contribute to the formation, re-stabilization, and persistence of long term auditory-fear memories, while not influencing other aspects of the memory trace. These findings also provide cogent evidence for a treatment model for acquired anxiety disorders such as posttraumatic stress disorder (PTSD) and specific phobias, through pharmacologic blockade of mTOR using systemic rapamycin following reactivation.

Keywords: mTOR; Reconsolidation; Consolidation; Auditory fear memory; Posttraumatic stress disorder; Rapamycin

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AMPA = Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid

αCAMKII = Alpha Calcium/Calmodulin-Dependent Protein Kinase Kinase

APA = American Psychiatric Association

ANOVA = Analysis of Variance

ANISO = Anisomycin

BDNF = Brain Derived Neurotrophic Factor

CS = Conditioned Stimulus

4EBP1 = Eukaryotic Initiation Factor 4E-Binding Protein 1

ERK2 = Extracellular Signal-Regulated Kinase 2

LSD = Least Significant Difference

LTM = Long-Term Memory

mTOR = Mammalian Target of Rapamycin

MAP2 = Microtubule-Associated Protein 2

NMDA = N-Methyl D-Aspartate

NS = No Shock

p70s6K = Phosphorylated70-kDa Ribosomal s6 Kinase

PTSD = Post Traumatic Stress Disorder

PKA = Protein Kinase A

RAP = Rapamycin

VEH = Vehicle

STM = Short-Term Memory

SEM = Standard Error of the Mean

TS = Tone-Shock

US = Unconditioned Stimulus

1.0 Introduction

1.1 Molecular Mechanisms and Temporal Kinetics of Consolidation and Reconsolidation

Evidence that protein synthesis is necessary for memory consolidation has been demonstrated primarily through the use of protein synthesis inhibitors given around the time of, or in the first few hours following, training (Cohen et al., 2006; Davis & Squire, 1984, Desgranges, Lévy, & Ferreira, 2008; Meiri & Rosenblum, 1998; Milekic, Pollonini, & Alberini, 2007). Importantly, support for this conclusion has been very consistent across a variety of behavioural paradigms and species (Davis & Squire, 1984, McGaugh, 2000). However, while protein synthesis dependency for long-term memory (LTM) formation has been well documented, more recent evidence suggests that there is at least a second wave of protein synthesis that is required for persistence of the engram (the postulated biochemical changes in neural tissue that represent a memory) under certain conditions (Bekinschtein et al., 2007a; Bourtchouladze et al., 1998; Freeman, Rose, & Scholey, 1995; Grechsch & Matthies, 1980; Quevedo et al., 1999). In these studies at least two time periods of sensitivity to the amnestic effects of the global protein synthesis inhibitor anisomycin (ANISO) were confirmed, first around the time of training, and the second 3-5 (Bourtchouladze et al., 1998; Freeman, Rose, & Scholey, 1995; Grechsch & Matthies, 1980; Quevedo et al., 1999) or 12 hours (Bekinschtein et al., 2007a) post-training.

Elucidation of events upstream, related to the expression and regulation of protein synthesis, including signalling cascades and mRNA synthesis, have become critical in furthering our understanding of the neurobiology of learning and memory. Similar to protein synthesis, many of these substrates and cascades are amenable to pharmacological disruption (Bernabeu et al., 1997; Lee, Everitt, & Thomas, 2004; Trifilieff et al., 2006). Moreover, these upstream events, such as increased mRNA expression, or the activation of signalling cascades and growth factors, for example protein kinase A (PKA) or brain derived neurotrophic factor (BDNF) appear to parallel the same biphasic kinetic pattern and susceptibility to pharmacological interventions as *de novo* protein synthesis in LTM formation and persistence (Bekinschtein et al., 2007a; Bernabeu et al., 1997; Bourtchouladze et al., 1998; Igaz, Vianna, Medina, & Izquierdo, 2002; Trifilieff et al., 2006; Trifilieff, Calandreau, Herry, Mons, & Micheau, 2007). However, the temporal kinetics of certain signalling pathways that contribute to LTM formation and persistence, such as those involved in translational regulation, remain poorly understood.

Once consolidated, memories are not impervious to disruption. Following reactivation, memories again appear to be susceptible to disruption by protein synthesis inhibitors and other types of pharmacological interference (Blundell, Kouser, & Powell, 2008; Debiec & LeDoux, 2004; Duvarci, Nader, & LeDoux, 2008; Nader, Schafe, & LeDoux, 2000). This period of vulnerability following reactivation requires *de novo* protein synthesis to re-stabilize the engram, empirically defining the reconsolidation phase of memory (Nader et al., 2000). Although this property of *de novo* protein synthesis-dependency is congruent with consolidation, it is unknown whether

reconsolidation recapitulates other mechanisms that constitute consolidation (Duvarci et al., 2008; Lee et al., 2004; Parsons, Gafford, & Helmstetter, 2006). Further, it is important to investigate whether time-sensitive properties of signalling events upstream of protein synthesis are also required for the re-stabilization of the memory trace after reactivation. Moreover, elucidation of the temporal parameters and molecular mechanisms of reconsolidation has significant clinical implications for identifying novel treatments for acquired anxiety disorders including post-traumatic stress disorder (PTSD) (Blundell et al., 2008; Debiec & LeDoux, 2006).

1.2 PTSD and Fear Conditioning as a Model of PTSD

PTSD is a functionally impairing anxiety disorder following exposure to an extreme traumatic stressor (event), and is characterized by the development of three clusters of symptoms (American Psychiatric Association (APA), 2000). The first cluster of symptoms is characterized by the persistent re-experiencing of the traumatic event, which results in severe psychological distress and physiological reactivity. This commonly occurs through recurrent intrusive recollections of the event, exposure to cues or symbols associated with the event, distressing dreams (nightmares), and more rarely through dissociative reliving of the event (flashbacks). The second cluster is marked by persistent avoidance of stimuli associated with the trauma and general emotional numbing in responsiveness to the external world. In this sense, individuals suffering from PTSD will often experience anhedonia, emotional detachment, avolition, and deliberately avoid any event or symbol that can trigger recollection of the traumatic event. The final cluster of symptoms is represented by chronic hyperarousal that was absent prior to the trauma;

which results in hypervigilance, irritability, an exaggerated startle response, and difficulty concentrating.

Exposure to events that would be qualified as extremely traumatic in a community-based setting appears relatively high, with estimates ranging from 39.1% (Breslau, Davis, Andreski, & Peterson, 1991) to 81.3% (Stein, Walker, Hazen, & Forde, 1997). From this population, a substantial subset of individuals will go on to develop the clinical symptoms of PTSD (Van Ameringen, Mancini, Patterson, & Boyle, 2008). Epidemiological studies have revealed the lifetime prevalence of PTSD to range from 6.8% to 14% for the general adult population in the United States (APA, 2000; Kessler et al., 2005). Similar in magnitude to the prevalence rates referenced above for the United States, albeit with a clear-cut paucity of and a need for more epidemiological studies in the Canadian context, the lifetime prevalence for PTSD is currently estimated to be 9.2 % for the general population in Canada (Van Ameringen et al., 2008). While most individuals that present with the symptoms of PTSD actively seek out some sort of psychotherapy, pharmacotherapy, or both, only about 60 % of patients respond to these interventions (Onder, Tural, & Aker, 2006; Zohar et al., 2002), with only 20-30 % of patients achieving full remission (Berger et al., 2009).

Such an inequality in achieving full remission, with only partial improvements and high refractoriness to treatment, underscores the need for novel therapeutic approaches. One such novel contemporary approach is a type of medication-enhanced psychotherapy that uses exposure therapy, the most efficacious type of psychotherapy for PTSD (Ballenger et al., 2000; Frances, 1999), paired before or after with medication, with

the intention of disrupting reconsolidation or manipulating other psychological processes amenable to drug interference (Dunlop, Mansson, & Gerardi, 2012). Clinical trials for this type of therapy have mostly focused on alterations to the glucocorticoid and adrenergic signalling systems, however, further randomized, double-blind clinical studies delineating the precise effectiveness of such interventions are still required (de Quervain & Margraf, 2008; Dunlop et al., 2012; Poundja, Sanche, Tremblay, & Brunet, 2012).

Much of what has influenced this pharmacological adjunct to exposure therapy emanates from pre-clinical studies examining the neurobiology of learning and memory (Dunlop et al., 2012). Investigations concerned with the neurobiology of reconsolidation through Pavlovian fear conditioning have discovered important information about the molecular mechanisms of memory, in addition to providing putative clinical therapeutic targets. Moreover, the most efficacious psychotherapy for PTSD, exposure therapy, is grounded in the principles of Pavlovian fear conditioning. However, while fear conditioning accurately models the exaggerated fear response that accompanies recollection of the traumatic event, it does not model the other cardinal, non-associative features of PTSD such as increased startle or hypervigilant behaviours (Siegmund & Wotjak, 2006). Despite these shortcomings, fear conditioning facilitates the systematic examination of the molecular mechanisms of memory, and the development and testing of pharmacological agents that act on reconsolidation. Thus, fear conditioning provides robust translational potential for identifying novel clinical pharmacological opportunities to couple with reactivation of the traumatic memory (through exposure therapy) to potentially reduce the core symptoms of PTSD, while also providing insight into the

neurobiology of learning and memory, such as examining the role of the translational regulator mammalian target of rapamycin (mTOR) in the consolidation and reconsolidation of memory.

1.3 Translational Regulation by mTOR in Long-lasting Forms of Behavioural and Synaptic Plasticity

MTOR, a serine/threonine protein kinase, controls the initiation and capacity of a subset of mRNA translation in neurons primarily through phosphorylation of two downstream targets, phosphorylated70-kDa ribosomal s6 kinase (p70s6K) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1) (Hay & Sonenberg, 2004; Raught, Gingras & Sonenberg, 2001). Previous research using rapamycin (RAP), a selective inhibitor of the mTOR kinase, has found that mTOR is a critical constituent of activity-dependent synaptic plasticity in a range of preparations (Casadio et al., 1999; Gong et al., 2006; Tang et al., 2002). Further, the mTOR pathway appears to be engaged following a variety of fear-motivated and non-fear-motivated learning paradigms, exhibiting increased p70s6K expression in hippocampo-amygdalar nuclei (Bekinschtein et al., 2007b; Dash, Orsi, & Moore, 2006; Glover, Ressler, & Davis, 2010; Parsons et al., 2006). Akin to the effects on activity-dependent synaptic plasticity, which are the electrophysiological correlates of memory, treatment with RAP around the time of training has elucidated a fundamental role for the mTOR pathway in consolidation of several forms of memory (Bekinschtein et al., 2007b; Blundell et al., 2008; Dash et al., 2006; Glover et al., 2010; Jobim et al., 2012a). As well, translational regulation through the mTOR pathway also appears to be essential in reconsolidation of the engram following retrieval. Trained

animals exhibit increased levels of p70s6K following retrieval (Gafford, Parsons, & Helmstetter, 2011), while mTOR blockade using RAP following reactivation disrupts subsequent retention under certain conditions (Blundell et al., 2008; Gafford et al., 2011; Glover et al., 2010; Jobim et al., 2012a; Jobim et al., 2012b; Myskiw et al., 2008; Parsons et al., 2006).

While it is well known that RAP blocks consolidation and reconsolidation of contextual fear memory (Bekinschtein et al., 2007b; Blundell et al., 2008; Gafford et al., 2011; Glover et al., 2010; Jobim et al., 2012a; Parsons et al., 2006), its effects on cue-based fear memory are far less consistent (Gafford et al., 2011; Glover et al., 2010; Parsons et al., 2006). When RAP is directly infused into the amygdala (bilaterally at a volume of 0.5 μl/side, using a dose of 5 μg/μl for consolidation, and 1 μg/μl for reconsolidation) it negatively affects cue-based fear memory formation and reconsolidation (Parsons et al., 2006). Conversely, direct infusion into the hippocampus (bilaterally at a volume of 1 μl/side, using a dose of 5 μg/μl) fails to disrupt consolidation (Gafford et al., 2011), while systemic RAP treatment (40 mg/kg) fails to attenuate both consolidation and reconsolidation of fear-potentiated startle to an odour-conditioned stimulus, which is a cued-based Pavlovian fear memory (Glover et al., 2010). As a result, it is unclear whether systemic RAP treatment would attenuate auditory fear memory – a cue-based fear memory. Moreover, with the exception of only one other published study to date (Slipczuk et al., 2009), the time course for mTOR regulation of translation in consolidation is not known. Slipczuk et al. (2009) described two distinct windows of mTOR activation at the moment of, or three hours post-training, in an inhibitory

avoidance task, and that intra-hippocampal treatment with RAP at these distinct time periods effaced subsequent retention. In contrast, to the best of my knowledge, no published studies have examined the temporal pattern of activation and susceptibility of mTOR in reconsolidation of memory.

1.4 Goals and Aims

The aim of the present study was to determine if a single systemic injection of RAP would disrupt consolidation and reconsolidation of an *auditory* fear memory in a time-dependent manner after training or retrieval, and whether these effects would be enduring. Herein, it was demonstrated that systemic inhibition of mTOR with RAP, during two critical periods of sensitivity, blocked both consolidation and reconsolidation-like activities that contribute to the formation, retention, and maintenance of LTM, while sparing retrieval and short-term memory (STM). Overall, the data suggest that biphasic translational control through the mTOR pathway is normally required during the long-term formation and stabilization of memory through recurrent consolidation and reconsolidation-like events.

2.0 Materials and Methods

2.1 Animals

A total of 390 male, approximately 7 to 8 week-old C57BL/6 mice (Charles River Laboratories, St Constant, QC, Canada), were used for these experiments. Mice were group housed with 4 mice per cage, and had *ad libitum* access to food and water in standard laboratory conditions on a 12 hour light-dark cycle (lights on at 7 a.m., lights off

at 7 p.m.). Prior to and during the course of experiments, all animals were handled daily for identification marking with non-toxic markers and routine husbandry duties during the light-phase of their cycle. Mice were transported from the animal housing facility to an anteroom, adjacent to the testing room and allowed to habituate to the new location for a minimum of one hour prior to training and testing. All behavioural testing and experimental manipulations were conducted during the light-phase of their cycle unless stated otherwise.

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

2.2 Behaviour

All mice from a particular cage were run simultaneously in separate chambers for training and testing. Each fear conditioning chamber contained a 26 grid shock floor, with transparent Plexiglas front and rear walls, situated within a sound attenuating isolation cubicle (Habitest, Coulbourn Instruments, Whitehall, Pennsylvania). For all training sessions, mice were placed in the conditioning chamber for a 120-s habituation period before the two identical conditioning trials began (except for Experiment 2, see below for additional details). This habituation period was followed by a continuous 30-s, 80 dB tone. During the last 2 seconds of tone presentation, a 0.6 mA foot shock was delivered and co-terminated with the auditory cue. An inter-trial interval of 60-s preceded a second identical trial. Mice remained in the chamber for an additional 120-s after the second

shock before being returned to their home cages. To test for cued-fear memory after varying intervals (determined by the specific experiment below), animals were placed in conditioning chambers with different tactile, visual and olfactory configurations than the original conditioning context (wooden floor over grid platform, cardboard and electrical tape along the walls, and vanilla extract wiped over the chamber) and their freezing behaviour in response to the auditory cue was measured. Mice were habituated to the altered test chambers for the first 3 minutes, after which the same continuous tone from training was presented for an additional 3 minutes before the mice were removed from the testing chambers. All probes of cued-fear memory for both retrieval and reactivation sessions were identical in procedure, with freezing behaviour –the absence of movement, except for respiration –being measured throughout using automated software (FreezeFrame, Coulbourn Instruments, Whitehall, Pennsylvania). Chambers were cleaned with 10% ethanol and allowed to air-dry between mice.

2.3 Experiments

2.3.1 Experiment 1 – Evaluating the associability of an auditory fear conditioning protocol. Two groups of mice were trained as described above. However, only one group received the two-trial, tone-shock, conditioning paradigm (tone-shock (TS), n = 12). The control group was presented with just the tone, omitting the co-terminating footshock for both conditioning trials (no shock (NS), n = 4). To determine the associability for this cued-learning protocol, animals were testing for memory to the auditory tone in a novel chamber forty-eight hours following training.

2.3.2 Experiment 2 – Determining the optimal frequency of tone-shock trials to maximize the conditioned response. Four groups of mice received single session auditory fear training, with each group receiving a different number of discrete tone-shock pairing trials. All training sessions followed the parameters and sequence pattern established in the previously described training protocol for time allotted to habituate, duration and termination of stimuli used to condition, inter-trial interval duration, and time allotted at the end of the training session to return behaviour to baseline. However, the number of tone-shock trials and hence total time within the conditioning chambers for each group of animals was systematically altered. Mice received 2 (2-TS, n =8), 3 (3-TS, n = 8), 4 (4-TS, n = 8), or 5 (5-TS, n = 8) tone-shock trials, for a total training session of 360, 450, 540, or 630-s respectively. Forty-eight hours post training, freezing behaviour was measured in a novel chamber to assess the magnitude of the cued-fear memory, as described above.

2.3.3 Experiment 3 – Effects of post-training systemic rapamycin on auditory fear memory consolidation. Upon removal from each chamber following training, mice were immediately injected with either RAP (40.0 mg/kg, n = 11) or vehicle (VEH) (n = 11). Forty-eight hours later, cued-fear memory was assessed as described above.

2.3.4 Experiment 4 – Short-term memory response to post-training mTOR blockade. Upon removal from each chamber following training, mice were injected with either RAP (40.0 mg/kg, n = 12) or VEH (n = 10). One hour later, STM for the conditioned-tone was examined in a novel chamber (as described above, 3 min

habituation followed by 3 min presentation of the conditioned tone). Forty-eight hours later, memory for the conditioned-tone was again vetted in the same chamber.

2.3.5 Experiment 5 – Effects of mTOR blockade immediately prior to retrieval of an established fear memory. Two days following training, 30 minutes prior to retrieval mice were injected with either RAP (40.0 mg/kg, n = 12) or VEH (n = 12). Mice were then re-exposed to the conditioned-tone in a novel chamber to assess whether RAP disrupts retrieval of an established fear memory.

2.3.6 Experiment 6 – Determining whether the effects of rapamycin on auditory fear memory consolidation are enduring. Immediately following training, animals were injected with either RAP (40.0 mg/kg, n = 12), ANISO (150.0 mg/kg, n =12), or VEH (n =12). Three weeks later, memory for the conditioned-tone was tested in a novel chamber to determine the long-term effects of RAP on fear memory.

2.3.7 Experiment 7 – Effects of mTOR blockade at varying intervals post-acquisition on long-term memory. Mice received injections of RAP (40.0 mg/kg) or VEH at 3 hours (RAP-3, n = 12; VEH-3, n = 12), 12 hours (RAP-12, n = 12; VEH-12, n = 11), or 24 hours (RAP-24, n = 12; VEH-24, n = 12) post-training. Injections at the 12 hour delay occurred during the dark phase of the light-dark cycle. Forty-eight hours after training, memory for the conditioned-tone was assessed for all mice in a novel chamber.

2.3.8 Experiment 8 – Long-term memory response to rapamycin treatment 18-hours following training. During the dark phase of their light-dark cycle, mice either received a single injection of RAP (40.0 mg/kg, n = 11) or VEH (n = 11) 18 hours after

training. Forty-eight hours after training, freezing behaviour to the conditioned-tone was measured in a novel chamber to examine any general interaction effects from changes in circadian rhythms and treatment with RAP on memory consolidation.

2.3.9 Experiment 9 – Effects of post-retrieval systemic rapamycin on auditory fear memory reconsolidation. Two days after training, mice were re-exposed to the conditioned-tone in a novel chamber. Upon removal from the chambers after reactivation, mice received either RAP (40.0 mg/kg, n = 15) or VEH (n = 14). One day later, cued-fear memory was assessed to determine if RAP blocks reconsolidation.

2.3.10 Experiment 10 – Effects of mTOR blockade in the absence of retrieval on subsequent recall. Two days following training, animals were injected with either RAP (40.0 mg/kg, n = 12) or VEH (n = 11). However, unlike Experiment 9, mice were not re-exposed to the conditioned-tone, but simply returned to their home cages immediately after injections. Twenty-four hours after injections, mice were assessed for cued-fear memory to the conditioned-tone in a novel chamber.

2.3.11 Experiment 11 – Evaluating the long-lasting effects of post-retrieval mTOR blockade on an established memory trace. Memory for the conditioned-tone was reactivated in a novel chamber 48 hours after training. Immediately after reactivation, mice received an injection of RAP (40.0 mg/kg, n = 12), ANISO (150 mg/kg, n = 12), or VEH (n =12). Three weeks after reactivation, mice were measured for freezing to determine the long-term effects of RAP on reconsolidation of cued-fear memory.

2.3.12 Experiment 12 – Effects of rapamycin treatment at varying intervals post-retrieval to long-term memory. Two days post-training, mice were re-exposed to the conditioned-tone in a novel chamber. Mice were then injected with RAP (40.0 mg/kg) or VEH at either 12 hours (RAP-12, n = 12; VEH-12, n = 11), or 24 hours (RAP-24, n = 12; VEH-24, n = 12) post-reactivation of the cued-fear memory. Injections at the twelve hour delay occurred during the dark phase of the light-dark cycle. Forty-eight hours after reactivation, freezing was measured to assess the effect of RAP on the reconsolidation of auditory-fear memory.

2.3.13 Experiment 13 – Effects of mTOR blockade 18-hours after retrieval to an established fear memory. Fear memory for the conditioned-tone was reactivated in a novel chamber 48 hours after training. Mice were then injected with RAP (40.0 mg/kg, RAP-18, n = 12) or VEH (VEH-18, n = 12) 18 hours after reactivation during the dark phase of their light-dark cycle. Forty-eight hours after reactivation, fear memory for the tone was assessed to determine any non-specific interaction effects from circadian rhythm changes and RAP treatment on reconsolidation of the engram.

2.4 Drug Preparation/Administration

Immediately prior to experimentation fresh solution of drug was made by dissolving RAP or ANISO in a vehicle of 5% ethanol, 4% PEG 400, and 4% Tween 80 in distilled water. Mice received intraperitoneal injections of VEH, RAP, or ANISO in volumes ranging from 0.2 to 0.3 ml based on their weight. The RAP dosage of 40 mg/kg and ANISO dosage of 150 mg/kg are based on previous studies that demonstrated 40

mg/kg and 150 mg/kg respectively, to have the most efficacy at disrupting contextual fear-memory, while conserving normal locomotion and nociception (Blundell et al., 2008, Cai, Blundell, Han, Greene, & Powell, 2006).

2.5 Statistics

Mixed Analysis of Variance (ANOVA) and post hoc Fisher's Least Significant Difference (LSD) tests were used for experiments with multiple groups or requiring multiple comparisons. A priori t- tests were used for follow up two-group comparisons. Freezing data for statistical analysis were obtained from fear memory probes by taking the difference in percent freezing during tone activation (latter 3 minutes of test) from percent freezing during no tone presentation (first 3 minutes of test), to obtain a measure of freezing to the conditioned tone that accounts for any non-specific freezing behaviour. Freezing data were also obtained from training sessions (first 2 minutes of training; time immediately prior to the first conditioning trial), thus, allowing for within group comparisons of freezing behaviour across training and memory probe days (within subjects main effect of day in the mixed ANOVA design). Significance was taken as $p < 0.05$.

3.0 Results

3.1 Two-Trial, Cued Fear Conditioning Optimizes Associability (Exp. 1) and Maximizes the Auditory Fear Memory Response (Exp. 2)

Prior to examining the effects of RAP on cued-fear memory, the robustness of the chosen behavioural procedure to produce fear conditioning was first confirmed. To

determine this, two groups of mice were trained in a single session, two-trial auditory fear conditioning task, and tested for retention 48 hours later. Both groups received an equal number of trials and exposure to the conditioning tone during training, however, only one group received the co-terminating shock with each trial (TS group), while the control group was spared exposure to the foot-shocks (NS group). A mixed ANOVA revealed a significant change in freezing behaviour following re-exposure to the conditioning tone in the novel chambers [main effect of condition, day, and interaction effect of condition and day, all Fs $(1, 14) > 20.386$, $p < .001$]. Follow-up t-tests on freezing behaviour observed during memory testing showed that only those animals that received the tone-shock pairing during training demonstrated successful associative learning relative to their NS training counterparts [Fig 1a; $t(14) = -4.515$, $p < .001$]. It should be noted that while statistical analyses are principally based on percent freezing to the conditioned tone during test, corrected for any spontaneous freezing, the baseline level of freezing (freezing during the first 3 minutes in the testing chamber prior to tone activation) was also separately analyzed to evaluate any change in spontaneous freezing behaviour for each experiment, as well as for any differences in percent freezing during training. Importantly, no differences in freezing behaviour were observed during training (2 min habituation period or 2 min post shock) or during the baseline period(s) for fear-memory testing (first 3 min without tone present) across groups in all experiments (1-13), indicating unaltered spontaneous behaviour or differences during training (data not shown, all $p > .05$).

To assess whether a greater magnitude freezing response could be elicited, mice were trained using the above procedure, but instead of just 2 trials, animals received 2, 3, 4, or 5 trials of the tone-shock pairing, and were tested for fear memory to the auditory cue 48 hours later. A mixed ANOVA revealed a significant main effect of day [$F(1, 28) = 347.433$, $p < .001$], indicating successful learning in all groups; but no significant main effect of number of trials, or interaction effect of number of trials and day [all $Fs(3, 28) < 1.447$, $p > .250$]. Although the data indicate an initial trend of increased freezing to the conditioned tone as a function of increasing the number of conditioning trials, this tendency was not significant and diminished after the four-trial training procedure (Fig 1b). As a result, the two-trial, conditioning procedure was employed for all subsequent experiments, since this procedure was sufficient at achieving maximal freezing to the conditioned tone.

3.2 Systemic Rapamycin Blocks Auditory Fear Memory Consolidation (Exp. 3), but Not Short-Term Memory (Exp. 4) or Retrieval (Exp. 5)

To determine whether systemic administration of RAP would affect auditory fear memory consolidation, mice were trained in the auditory fear conditioning task. Immediately after training, animals were injected with either RAP or VEH and tested for auditory fear memory 48 hours later. A mixed ANOVA revealed significant dampening of fear expression (decreased freezing) upon re-exposure to the conditioned-tone [main effect of drug $F(1, 20) = 4.553$, $p = .045$, main effect of day $F(1, 20) = 67.807$, $p < .001$, interaction effect of drug and day $F(1, 20) = 4.553$, $p = .045$]. Follow up t-test revealed that RAP-treated animals exhibited significantly less freezing than VEH-treated animals

when prompted by the conditioned-tone during memory testing [Fig 2a; $t(20) = -2.134$, $p = .045$].

To determine if the effects of RAP on auditory-fear memory were indeed the result of disrupting consolidation and not from alterations to other aspects of the memory trace, mice were injected immediately after training with either RAP or VEH. One hour later, memory for the cued-tone was evaluated in a novel chamber, and again 48 hours after training. A mixed ANOVA of a three time points (training, reactivation, and test) revealed a significant main effect of day [$F(2, 40) = 27.240$, $p < .001$], and an interaction effect of drug and day [$F(2, 40) = 4.098$, $p = .024$]. In follow up t-tests, memory in both groups were comparable when tested one-hour following training – indicative of normal acquisition and STM [Fig.2b left panel; $t(20) = 0.054$, $p = .958$]. However, while short-term memory was spared, long-term memory was significantly decreased in RAP-treated animals relative to controls when tested 48 hours after training [Fig. 2; $t(20) = 2.225$, $p = .038$], consistent with data from Experiment 3 (See Fig. 2a).

The half-life of RAP in circulation of mice is between 4 and 6 hours (Baker, Sidorowicz, Sehgal, & Vezina, 1978), thus, the drug has been fully excreted when the fear-memory is challenged during recall 48 hours after training and drug injection (See Figs. 2a & b). Accordingly, the observed disruption to long-term memory from RAP treatment should not be the result of a direct effect of RAP resulting in an inability to retrieve the memory trace when prompted. To be certain that RAP was not impinging on the ability to retrieve the memory trace, mice were treated with RAP or vehicle 30 minutes prior to a fear-memory probe, 48 hours after training. A mixed ANOVA revealed

that RAP infusion immediately before recall does not significantly affect retrieval [Fig.2c; main effect of day $F(1, 22) = 130.023$, $p < .001$, but no significant main effect of drug or drug by day interaction, all $Fs(1, 22) < 0.816$, all $p > .05$].

3.3 The Effects of Rapamycin on Auditory Fear Memory Consolidation are Not Long-Lasting (Exp. 6)

To determine if block of consolidation by RAP is long-lasting (comparable to that of ANISO, Lattal & Abel, 2004), mice were injected with RAP, ANISO, or VEH immediately after training, and tested for auditory fear-memory 21 days later. A mixed ANOVA revealed a main effect of drug [$F(2, 33) = 16.056$, $p < .001$], main effect of day [$F(1, 33) = 84.533$, $p < .001$], and interaction effect of drug and day [$F(1, 33) = 14.915$, $p < .001$]. Post hoc Fisher's LSD tests revealed that auditory fear memory was significantly effaced in ANISO-treated animals relative to all other groups, which showed normal amounts of freezing [Fig.3; ANISO vs. RAP, $p < .001$; ANISO vs. VEH, $p < .001$; RAP vs. VEH, $p = .529$].

3.4 Long-Term Memory is Susceptible to Rapamycin at Twelve Hours After Acquisition (Exp. 7 and 8)

It has previously been demonstrated that the consolidative process for a variety of learning tasks has a varied and often multi-phasic temporal sensitivity depending on the molecular or cellular substrate being investigated (Bekinschtein et al., 2007a; Trifilieff et al; 2007). However, characterization of the temporal dynamics of mTOR signalling in auditory fear-memory consolidation has not been fully examined. To determine if the

consolidative process is temporally susceptible to mTOR blockade, the effects of systemic RAP treatment were examined at various times following training. Mice received single intraperitoneal injections of RAP or VEH at 3, 12, or 24 hours post-training, and were subsequently tested for auditory fear memory 48 hours after training. Results of a mixed ANOVA revealed a significant main effect of drug [$F(1, 65) = 6.338$, $p = .014$] and main effect of day [$F(1, 65) = 158.254$, $p < .001$], but no main effect of time injected [$F(2, 65) = 4.12$, $p = .664$]. Follow-up t-tests showed that RAP attenuated memory for the conditioned-tone at 12, but not at 3 or 24 hours post-training [Fig.4; $t(21) = 2.253$, $p = .035$; $t(22) = 0.645$, $p = .526$; $t(22) = 1.419$, $p = .170$, 12 hours, 3 hours, and 24 hours respectively]. Moreover, it is important to note that the effects of RAP on consolidation 12 hours post-training did not result from non-specific changes in circadian rhythms interacting with RAP, as there were no effects of RAP treatment 18 hours post-training [mixed ANOVA: main effect of day $F(1, 20) = 97.464$, $p < .001$, but no main effect of drug $F(1, 20) = 3.256$, $p = .086$, and no interaction effect of day and drug $F(1, 20) = 3.192$, $p = .089$]. Thus, these findings illustrate that the formation and persistence of LTM storage require the mTOR signalling pathway.

3.5 Systemic Blockade of mTOR Disrupts Auditory Fear Memory Reconsolidation (Exp. 9 and 10)

To determine if the mTOR pathway is required for the stability of the memory trace in an auditory tone-shock association, mice were treated with RAP or VEH immediately following memory reactivation and tested 24 hours later (i.e.72 hours after training). A mixed ANOVA revealed a significant main effect of day [$F(2, 54) = 45.573$,

$p < .001$], and a significant interaction effect of drug and day [$F(2, 54) = 4.206$, $p = .020$], while the main effect of drug approached, but did not reach significance [$F(1, 27) = 3.659$, $p = .066$]. As expected, forty-eight hours after training, re-exposure to the conditioned-tone resulted in robust freezing in both groups, indicative of successful learning [Fig.5a; t-test: $t(27) = 0.151$, $p = .881$]. However, animals given RAP immediately after recall expressed a significantly diminished fear response compared to VEH-treated controls 24 hours later [Fig. 5a; t-test: $t(27) = -2.817$, $p = .009$]. Importantly, this attenuation of an established fear memory was not merely an effect of RAP treatment alone. In the absence of reactivating the auditory fear memory, RAP alone had no effect on subsequent memory recall when tested 24 hours after drug treatment, 72 hours after training [Fig.5b; mixed ANOVA: main effect of day $F(1, 22) = 142.387$, $p < .001$, but no significant main effect of drug or drug by day interaction [all $Fs(1, 22) < 1.661$, all $p > .05$].

3.6 Rapamycin has Enduring Effects on an Established Memory Trace (Exp. 11)

A single systemic post-reactivation injection of RAP can attenuate contextual fear memory in a long-lasting manner that is significantly stronger than ANISO (Blundell et al., 2008). Thus, it was next determined whether these effects could be extended to auditory fear-memory. To do this, mice were trained, and 48 hours later memory was reactivated. Immediately after reactivation, mice received a single systemic injection of RAP, ANISO, or VEH. Twenty-one days later, mice were tested for cue fear memory recall. Vehicle-treated animals exhibited normal freezing behaviour, while RAP and ANISO-treated animals showed significantly reduced freezing to the conditioned-tone

[Fig.6; mixed ANOVA: main effect of drug $F(2, 33) = 3.609$, $p = .038$, main effect of day $F(2, 66) = 45.396$, $p < .001$, interaction effect of drug and day $F(4, 66) = 1.853$, $p = .129$; Post hoc Fisher's LSD tests: VEH vs. RAP, $p = .018$, VEH vs. ANISO, $p = .042$, RAP vs. ANISO, $p = .710$]. Collectively, these findings delineate a role for mTOR signalling in the reconsolidation and the long-term persistence of an auditory fear memory trace.

3.7 Established fear memory is labile to rapamycin at twelve hours post-reactivation (Exp. 12 and 13)

Consolidation and reconsolidation are fairly congruent with regards to molecular signalling cascades. Thus, the next experiment attempted to elucidate whether reconsolidation recapitulates the same temporal pattern of susceptibility to systemic RAP as consolidation (See Fig.4). To assess for critical periods of mTOR activity following reactivation of an established memory, animals were injected with RAP or VEH at either 12 hours or 24 hours (negative comparison group) post-reactivation. Forty-eight hours after re-exposure, animals were tested again for recall. As expected, there were no significant differences between groups during reactivation of the memory trace (no data shown, t-tests, all $p > .05$). However, animals treated with RAP at 12 hours post-reactivation displayed significantly decreased memory when tested 48 hours later, while no effect was observed in the 24 hour post-reactivation injection group during test [Fig.7A & B respectively; mixed ANOVA: main effect of drug $F(1, 43) = 4.078$, $p = .050$, main effect of day $F(2, 86) = 89.903$, $p < .001$, main effect of time injected $F(1, 43) = .003$, $p = .960$; follow up t-tests: $t(21) = 2.460$, $p = .023$, $t(22) = 0.190$, $p = .851$, 12 delay and 24 hour delay, respectively]. Importantly, the effects of RAP treatment on

reconsolidation 12 hours post-reactivation were not artefacts of RAP interacting with circadian rhythm changes of the animal, as RAP treatment 18 hours post-recall did not alter subsequent memory [mixed ANOVA: main effect of day $F(2, 44) = 76.34$, $p < .001$, no significant main effect of drug $F(1, 22) = 3.272$, $p = .084$, or interaction effect of day and drug $F(2, 44) = 2.435$, $p = .094$]. Again, there was no significant difference between groups during reactivation of the memory trace (data not shown, t-test, $p > .05$). Thus, following reactivation, initial re-stabilization and persistence of long-term memory storage requires the mammalian target of rapamycin.

4.0 Discussion

Single, systemic administration of RAP, either immediately following or 12 hours post-training or post-memory reactivation impairs both consolidation and reconsolidation of an auditory fear memory. Importantly, RAP effects on memory are not artefacts of RAP interacting with changes in circadian rhythms, as treatment at 18 hours post-training or reactivation did not disrupt subsequent memory recall (See sections 3.4 & 3.7). Thus, this data indicates that at the very least, biphasic mTOR signalling within 24 hours of post-training or post-retrieval is critical for both the consolidation and reconsolidation of LTM.

4.1 Consolidation of Auditory Fear Memory

These results add to a growing volume of evidence indicating that mTOR-mediated regulation of protein translation is a critical mechanism in facilitating consolidation. Parsons and colleagues (2006) were the first to demonstrate impaired

auditory fear memory retention following intra-amygdalar infusion of RAP (5 μg/μl) immediately following training in rats. These results reported here are in concert with their findings. However, the present findings demonstrate that RAP affects consolidation and not other aspects (e.g. acquisition & retrieval) of the auditory fear memory trace (See section 3.2); since immediate post-training blockade of mTOR fails to disrupt acquisition of auditory fear, as evidenced by the amount freezing 1 hour after training, while still impairing memory 48 hours after training. Furthermore, RAP treatment prior to recall does not impair the ability of the animal to retrieve the memory trace. Finally, these findings provide evidence of systemic efficacy at a dose that does not appear to cause noticeable effects on pain sensitivity, anxiety, or locomotor activity (Blundell et al., 2008). Thus, these results confirm and extend previous findings indicating RAP disrupts auditory fear memory formation, but not other aspects of the memory trace.

It was also demonstrated that RAP infusion at 12 hours post-training has deleterious effects on fear memory when probed 48 hours after learning. This is in direct contrast to recent findings that RAP treatment (bilaterally into the dorsal hippocampi in a volume of 0.5 μl/side, using a dose of 5 pg/ μl) at 12 hours post-training does not alter fear memory consolidation and expression (Bekinschtein et al., 2008; Slipczuk et al., 2009). Rather, Slipczuk and colleagues (2009) have illustrated two distinct time-restricted windows for RAP-sensitivity in the consolidation of fear memory, immediately around the time of conditioning and again 3 hours post-training. Those results support a role for mTOR signalling in initial formation, but not the protracted cellular consolidation-like events that contribute to memory persistence observed 12 to 24 hours post-training, such

as increased expression of extracellular signal-regulated kinase 2 (ERK2), c-FOS, Akt, Homer 1a, and α calcium/calmodulin-dependent protein kinase kinase (αCamKII) (Bekinschtein et al., 2010). However, there are a number of explanations for the differences in results between these studies and the findings presented here. First, in the aforementioned studies, RAP infusion was restricted to the dorsal hippocampus; here RAP was administered systemically, allowing for wider distribution of the drug. Also, while the dorsal hippocampus is integral to fear memory formation, evidence suggests it is not necessary for the unconditioned stimulus - conditioned stimulus (US-CS) association in fear learning (Matus-Amat, Higgins, Barrientos, & Rudy, 2004). Secondly, different types of fear learning paradigms were examined. In these other studies (Bekinschtein et al., 2008; Slipczuk et al., 2009), contextual fear conditioning was examined using an inhibitory avoidance task, while the present experiments investigated a cued-based fear memory, which appears not to be mediated by the hippocampus, as direct infusion of RAP into the dorsal hippocampus immediately after training failed to impair auditory fear memory retention (Gafford et al., 2011).

RAP was also assessed for whether it has long-lasting effects on fear memory consolidation. In agreement with Lattal and Abel (2004), it was found that ANISO treatment effaced memory when probed 21 days after training and drug injection. Surprisingly, post-training RAP treatment had no enduring effect on memory when tested 21 days later. Thus, it appears that the effects of RAP on memory consolidation gradually decay over time. While unlikely, the dosage of RAP (40 mg/kg) used to disrupt consolidation may not have been optimal to efface long-term retention. Nevertheless, the

concentration of RAP used in this study has been shown to optimally decrease memory, while sparing any patent changes to locomotion, anxiety, or nocieception (Blundell et al., 2008). RAP is also a potent immunosuppressant, thus, increasing the dosage may raise the risk of injury and disease due to an elevated immunocompromised state. Further, while RAP did not completely block retention, the effect sizes in these experiments (both consolidation and reconsolidation) are comparable to other published studies using RAP and other protein synthesis inhibitors, with memory seldom ever being completely effaced by these agents (Bourtchouladze et al., 1998; Debiec & LeDoux, 2004; Parsons et al., 2006).

4.2 Reconsolidation of Auditory Fear Memory

As with consolidation, the reconsolidation experiments confirm and augment the findings of Parsons et al. (2006) for an effect of RAP on auditory cued fear memory. Indeed a one-trial memory reactivation, combined with systemic RAP-treatment dampens subsequent fear memory expression. Moreover, the amnestic effects of RAP occur only in conjunction with re-exposure to the cue, as treatment in the absence of reactivation does not hinder memory. Nevertheless, these systemic effects on reconsolidation are in contrast to another study that found no effect from systemic RAP treatment (40 mg/kg) on either consolidation or reconsolidation of a cued fear memory (Glover et al., 2010). However, the discrepancies between this study and Glover et al. (2010) might be due the use of different modalities of CS (auditory vs. olfactory), and a different measure of fear (freezing vs. fear potentiated startle).

Unlike the case of consolidation, a single systemic injection of RAP paired with memory reactivation blocked subsequent recall when measured 21 days after retrieval. Moreover, these effects were of equal magnitude to ANISO, a drug that empirically defines reconsolidation (Nader et al., 2000). Numerous reports have revealed dissociative recruitment of various substrates in either the consolidation or reconsolidation of memory, indicating that reconsolidation is not a complete recapitulation of the mechanisms of consolidation (Duvarci et al., 2008; Lee et al., 2004; Parsons et al., 2006). This possibility cannot be overlooked in the present data. Indeed, these results parallel those of Blundell et al. (2008), exhibiting a lasting effect on reconsolidation, and expanding the generality of RAP efficacy on reconsolidation from contextual fear memory to cued, auditory fear memory. As such, it should be noted that unlike this present study, Blundell et al. (2008) did not characterize the long-term effects of RAP on memory consolidation, only examining retention 24 hours post-training. Thus, it is unclear whether mTOR is necessary in the enduring expression of contextual memory.

Having established that mTOR blockade immediately after memory retrieval hinders subsequent memory retention similar to the motif established in the consolidation experiments, albeit having a longer-lasting effect, subsequent experiments sought to determine whether reconsolidation recapitulates late phase sensitivity to RAP treatment. To the best of my knowledge, this is the first study to investigate whether any protracted molecular events important for retention occur hours after memory reactivation. Analogous to consolidation, reconsolidation of auditory fear memory demonstrates two time-windows of RAP-sensitivity to subsequent memory recall, first immediately after

training and 12 hours thereafter. These findings suggest recurrent waves of RAP-sensitivity in the reconsolidation of auditory fear memory, with the latter phase possibly contributing to protracted cellular events required for persistence of the re-stabilized memory trace. It is unknown whether other molecular substrates germane to learning and memory, especially those that exhibit biphasic patterns of activation and susceptibility to pharmacological intervention during consolidation, demonstrate similar temporal patterns as mTOR in reconsolidation; as evidenced through RAP inhibition of this translational regulator. Further research will be needed to address these questions.

4.3 Putative mTOR Signal-Transduction Pathways in Long-Lasting Forms of Plasticity

In neurons, mTOR is present post-synaptically in dendrites where it governs local protein synthesis, essential for long-lasting forms of synaptic potentiation and memory (Jourdi et al., 2009; Takei et al., 2004; Vickers, Dickson, & Wyllie, 2005). Recent studies have begun to elucidate the signal-transduction pathways that activate the mTOR pathway in response to conditioning, and the concomitant proteins thought to stabilize long-lasting forms of plasticity. BDNF is regarded as a facilitator of enduring plastic changes (Bekinschtein et al 2007a; Patterson et al., 1996; Tyler, Alonso, Bramham, & Pozzo-Miller, 2002). Both *in vitro* and *in vivo* studies have shown that BDNF affects behavioural and synaptic plasticity through RAP-sensitive signalling (Spliczuk et al., 2009; Tang et al., 2002). This is most likely achieved through local dendritic up-regulation of translational machinery and protein synthesis, as BDNF mediates these alterations in an mTOR-sensitive manner (Takei et al., 2004). Specifically, BDNF-mTOR

signalling increases GluR1 expression. GluR1 is a subunit of glutamatergic *a*-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, necessary for memory formation and other synaptic plastic changes (Fortin et al., 2012; Schratt, Nigh, Chen, Hu, & Greenberg, 2004; Slpiczuk et al., 2009). Similarly to BDNF, glutamatergic activation of *N*-methyl-D-aspartate (NMDA) receptors post-synaptically appears to be another upstream mediator, intimately coupled to mTOR-dependency, for activity-dependent plasticity and dendritic protein synthesis (Gong et al., 2006; Takei et al 2004; Vickers et al., 2005). MTOR also regulates the translation of other transcripts triggered by conditioning, essential to behavioural and synaptic plasticity, such as microtubule-associated protein 2 (Map2), and αCamKII (Gong et al., 2006; Miller et al., 2002). However, determination of whether these mTOR-dependent proteins are even translated in response to the same or different extracellular or intrinsic signals during protracted consolidation-like events hours after training, or during the biphasic RAP-sensitive time windows during reconsolidation established here still needs to be addressed.

4.4 Experimental Conditioning Protocol as a Cogent Model of PTSD and Tool to Study the Neurobiology of Learning and Memory

Experiments 1 and 2 demonstrated that the single session, two-trial, auditory fear conditioning procedure was efficacious for establishing a strong learned cue association, comparable to other studies using similar protocols (Gamache, Pitman, & Nader, 2012; Kishioka, Uemura, Fukushima, & Mishina, 2013; Tang et al., 2001). The two-trial learning protocol was also revealed to maximize the magnitude of the learned response. Additionally, the stimulus intensity (footshock amperage) used did not induce a state of

generalized fear in any experiment, as evidenced by the lack of any significant freezing behaviour in all control and experimental groups upon exposure to the novel chamber prior to tone presentation for memory testing. The generalization phenomenon is a potential source of contamination to the conditioned auditory fear response, often observed with higher range footshock intensities (Baldi, Lorenzini, & Bucherelli, 2004). Altogether, these data establish a confidence level that the conditioning procedure is sufficient and robust in inducing a specific conditioned (auditory) fear response which can be used to adequately and consistently examine the neurobiology of consolidation and reconsolidation.

Even though no animal model is plenary in simulating PTSD, specific symptoms can be mimicked in diverse models of PTSD (Adamec, Head, Soreq, & Blundell, 2008; Blundell et al., 2008; Cohen, Kaplan, Matar, Loewenthal, Kozlovsky, & Zohar, 2006; Jobim et al., 2012a). Auditory fear conditioning is a valid heuristic model of PTSD, accurately capturing the fear that accompanies reminders of the traumatic event (Siegmund & Wotjak, 2006; Zovkic & Sweatt, 2013). Moreover, while the information gleaned here and from similar studies provides important insights into the neurobiology of learning and memory, it also supports a cogent model for treatment of PTSD, and other learned emotional disorders through pharmacological exploitation of unstable reactivated memories. These experiments provide important pre-clinical information concerning the pharmacological treatment of acquired traumatic memories. RAP was demonstrated to effectively reduce conditioned responding in a long-lasting manner after a one trial reactivation session coupled with drug treatment. Additionally, a second possible

therapeutic time-point for RAP treatment 12 hours after retrieval was demonstrated. Importantly, like the effects of RAP to the reconsolidation of contextual fear memory (Blundell et al. 2008), reactivation of the memory prior to treatment is necessary, as retrograde amnesia was not observed when RAP was administered in the absence of reactivation, an important aspect for treatment specificity in reducing the emotional intensity of the traumatic memory without interfering with other cognitive processes.

4.5 General Conclusions

Collectively, the evidence presented here agrees with other published studies that mRNA translational regulation by mTOR is critically involved in the consolidation and reconsolidation of memory. Furthermore, this study is the first to demonstrate recurrent, protracted consolidation and reconsolidation-like RAP-sensitive events that contribute to the formation and reconsolidation of fear memory. Finally, these reconsolidation experiments provide a tenable model for a therapeutic approach to decreasing the emotional valence of traumatic memories using an FDA-approved drug that could potentially be used in patients suffering from PTSD.

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Figure Captions

Figure 1. Single session, two-trial auditory fear procedure optimizes associability and maximizes the conditioned response. (A) Only animals that received the tone-shock (TS; n = 12) pairing with each trial during training exhibited the conditioned fear response during 48 hours later; controls (NS; n = 4) [mixed ANOVA: main effect of condition, day, and interaction effect of condition and day, all Fs (1, 14) > 20.386, $p < .001$; follow-up t-test: t (14) = -4.515, $p < .001$]. (B) Increasing the number of trials in a single training session does not increase the conditioned freezing response [mixed ANOVA: main effect of day F (1, 28) = 347.433, $p < .001$, but no main effect of number of trials or interaction effect of number of trials and day, both F (3, 28) = 1.447, p = .250] when tested for 48 hours later. The number for before each TS group indicates the number of TS pairings. N = 8 for all groups. Data are expressed as mean (±SEM) of percent freezing for fear memory probe sessions (and training where applicable) in all figures. Asterisks represent $p < .05$ for all figures.

Figure 2. Systemic rapamycin impairs auditory fear consolidation. (A) Rapamycin injected immediately following training disrupts recall 48 hours later [mixed ANOVA: main effect of drug F (1, 20) = 4.553, p = .045, main effect of day F (1, 20) = 67.807, $p < .001$, interaction effect of drug and day F (1, 20) = 4.553, p = .045]. Post hoc comparison (t-test) of vehicle (VEH) and rapamycin-treated (RAP) animals for freezing behaviour at test [t (20) = -2.134, p = .045]. N = 11 for each group. (B) Rapamycin treatment

immediately after training does not impair recall when probed 1 hour later (STM, t-test: t (20) = 0.054, p = .958), but still attenuates fear expression 48 hours after training (LTM, t-test: t (20) = 2.225, p = .038). N = 10 (VEH), 12 (RAP). (C) Injection of rapamycin 30 minutes prior to testing does not alter fear memory retrieval when probed 48 hours post-training [mixed ANOVA: main effect of day F (1, 22) = 130.023, p < .001, but no significant main effect of drug or drug by day interaction, all Fs (1, 22) < 0.816, all p > .05]. N = 12 for each group.

Figure 3. The effects of systemic rapamycin following conditioning are not enduring. Systemic injection of anisomycin (ANISO), but not rapamycin (RAP) or vehicle (VEH), immediately after training significantly reduced fear memory retention when probed 21 days later in the absence of drug [mixed ANOVA: main effect of drug F (2, 33) = 16.056, p < .001, main effect of day F (1, 33) = 84.533, p < .001, and interaction effect of drug and day F (1, 33) = 14.915, p < .001; post-hoc Fisher's LSD ANISO vs. RAP, p < .001; ANISO vs. VEH, p < .001; RAP vs. VEH, p = .529]. N = 12 for all groups.

Figure 4. Systemic rapamycin 12 hours after acquisition impairs fear memory formation. Injection of rapamycin 12 hours (B), but not 3 (A) or 24 (C) hours following auditory fear conditioning significantly hinders retention when probed 48 hours post-training [mixed ANOVA: main effect of drug F (1, 65) = 6.338, p = .014 and main effect of day F (1, 65) = 158.254, p < .001]. Post hoc comparisons (t-tests) for freezing behaviour at probe of

vehicle (VEH0 versus rapamycin (RAP) for post-training treatment at 3, 12, or 24 hours [t (22) = 0.645, p = .526; t (21) = 2.253, p = .035; t (22) = 1.419, p = .170] respectively. N = 12 for all groups, except for VEH-12, n = 11.

Figure 5. Systemic rapamycin impairs fear memory reconsolidation. (A) Single trial post-retrieval rapamycin dampens subsequent fear recall. Post-reactivation rapamycin inhibits subsequent fear memory 24 hours after retrieval, 72 hours post-training. A mixed ANOVA revealed a main effect of day [F (2, 54) = 45.573, p < .001], and a significant interaction effect of drug and day [F (2, 54) = 4.206, p = .020], while the main effect of drug approached, but did not reach significance [F (1, 27) = 3.659, p = .066]. Post hoc comparisons (t-tests) of vehicle (VEH) and rapamycin (RAP) treated animals for freezing behaviour on test days 2 (48 hours post-training) and 3 (72 hours post-training) [t (27) = 0.151, p = .881; t (27) = -2.817, p = .009, respectively]. Hour zero delineates the training session (first 2 minutes in conditioning chambers; no difference between groups during training, t-test, p > .05). N = 10 (VEH), 12 (RAP). (B) Reactivation of the fear memory is necessary for the effects of rapamycin upon subsequent recall. Rapamycin treatment in the absence of reactivation does not alter fear memory expression 24 hours after drug treatment, 72 hours post-training (VEH vs. RAP, p > .05). N = 12 for both groups.

Figure 6. Administration of rapamycin following fear memory reactivation has long-lasting effects, equivalent to anisomycin. A single trial fear memory reactivation paired

with rapamycin (RAP) or anisomycin (ANISO) diminished subsequent recall when probed 21 days later in the absence of drug [mixed ANOVA: main effect of drug $F(2, 33) = 3.609$, $p = .038$, main effect of day $F(2, 66) = 45.396$, $p < .001$, interaction effect of drug and day $F(4, 66) = 1.853$, $p = .129$; Post hoc Fisher's LSD tests: VEH vs. RAP, $p = .018$, VEH vs. ANISO, $p = .042$, RAP vs. ANISO, $p = .710$]. Day zero delineates the training session (first 2 minutes in conditioning chambers; no significant difference between groups during training or reactivation (Day 2), Post hoc Fisher's LSD tests (data not shown): all $p > .05$). $N = 12$ for all groups.

Figure 7. Systemic rapamycin treatment twelve hours after reactivation impairs fear memory reconsolidation. Mice receiving a single rapamycin (RAP-12) injection at 12 hours (A), but not at 24 hours (B) post-reactivation (RAP-24), show significantly decreased recall 48 hours after retrieval, 96 hour post-training, relative to vehicle (VEH-12, 24) counterparts [mixed ANOVA: main effect of drug $F(1, 43)$ 4.078, $p = .050$, and main effect of day $F(2, 86) = 89.903$, $p < .001$]. Post hoc comparisons (t-tests) of vehicle versus rapamycin-treated mice for 12 and 24 hour delay groups [$t(21) = 2.460$, $p = .023$, $t(22) = 0.190$, $p = .851$, respectively] on final day of testing. $N = 12$ for all groups, except for RAP-12, $n = 11$.

Figure 1.

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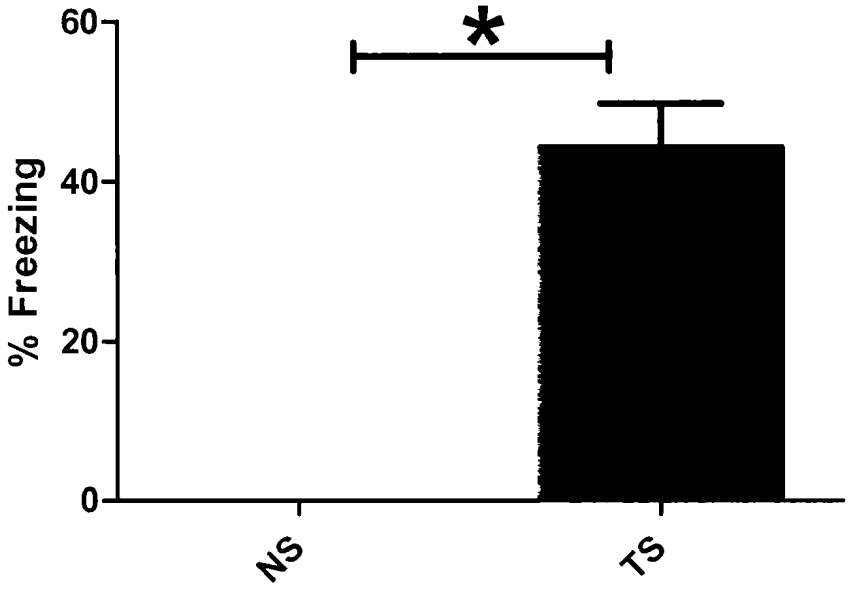

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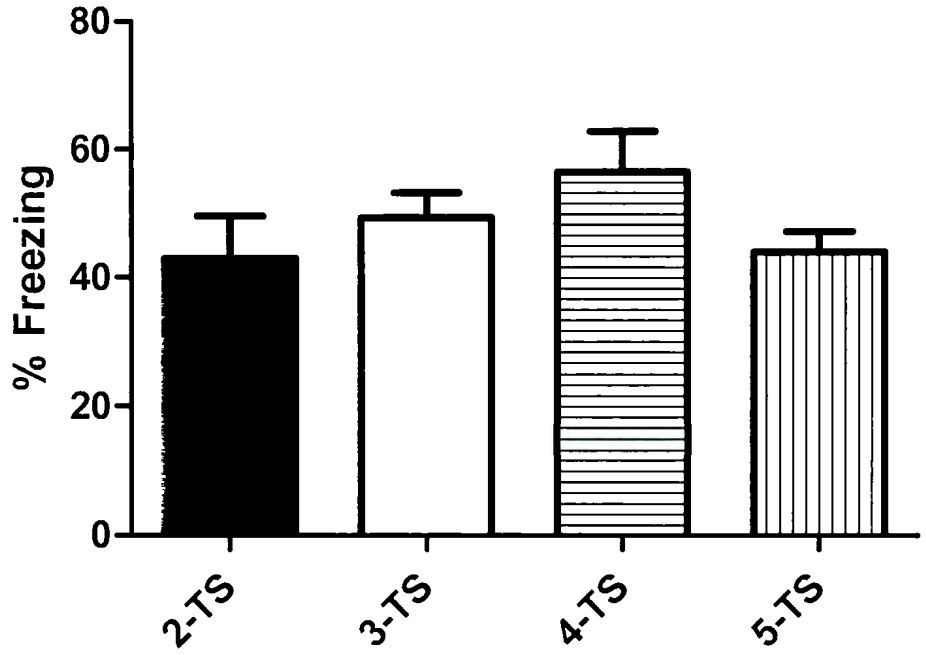

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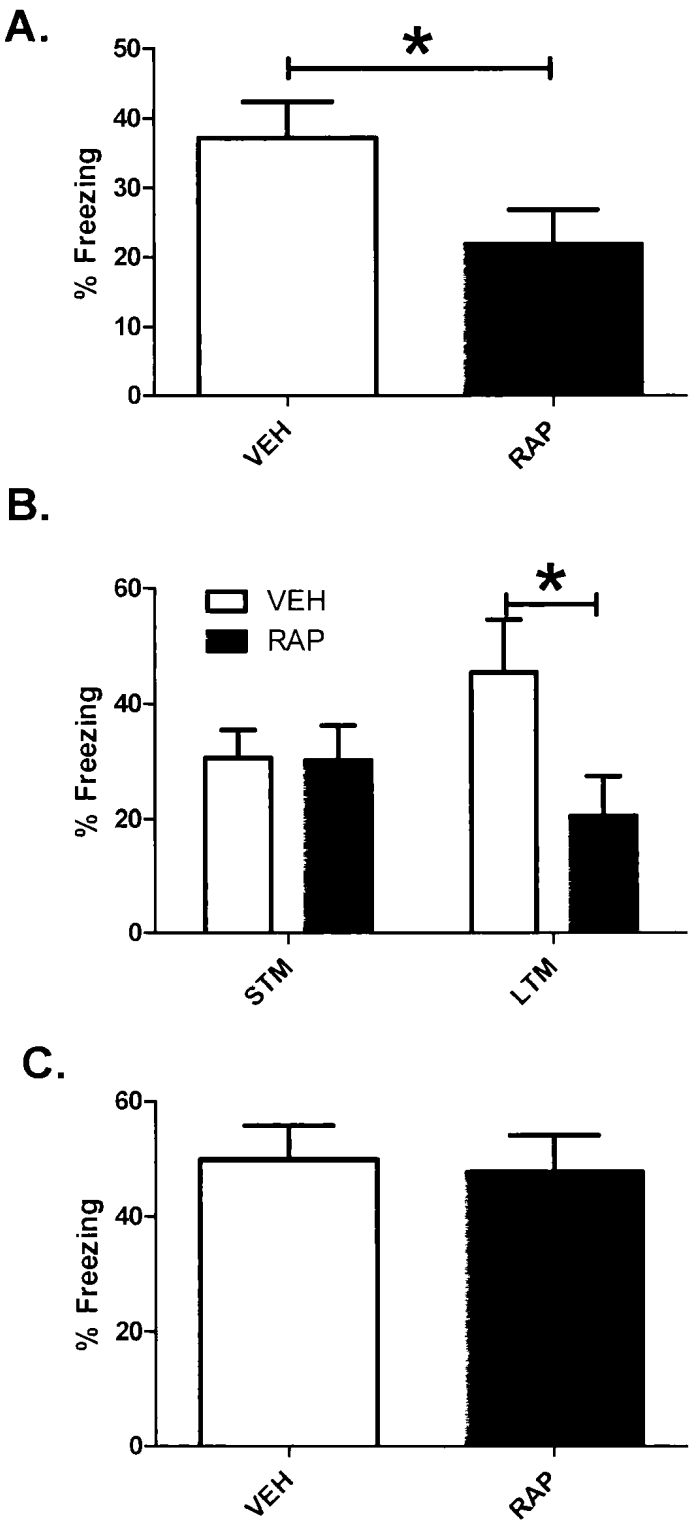
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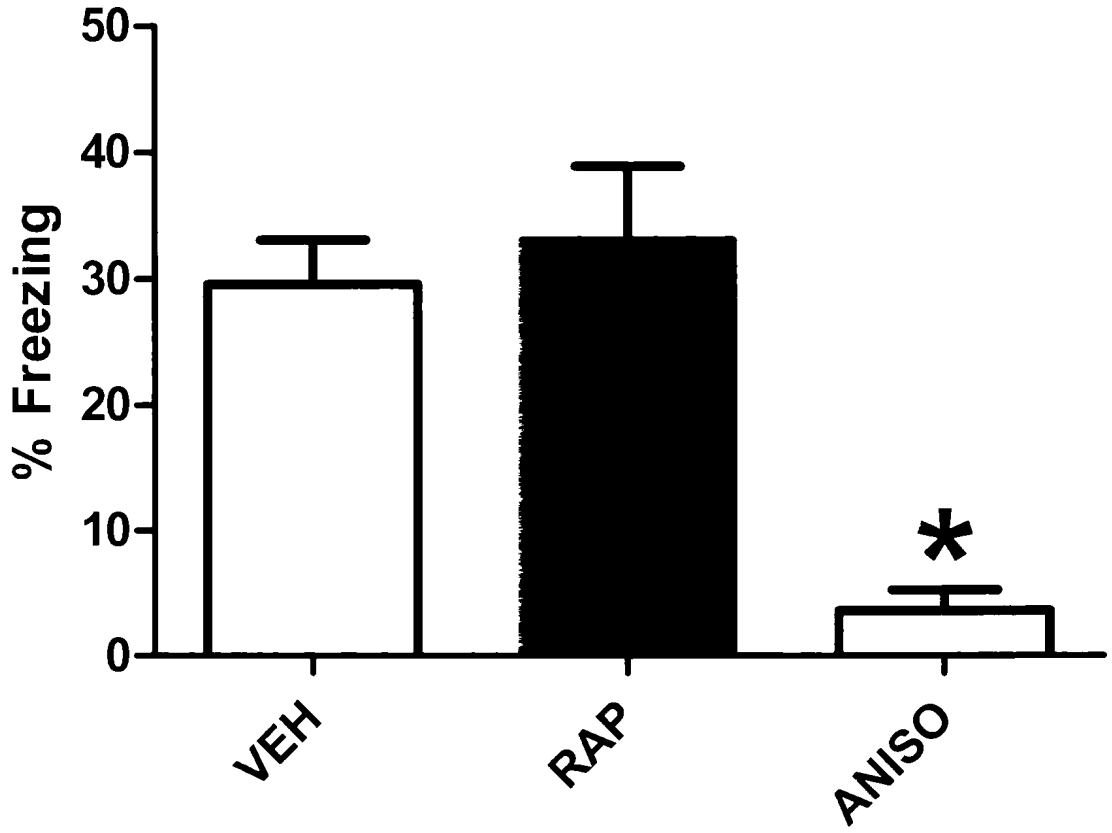

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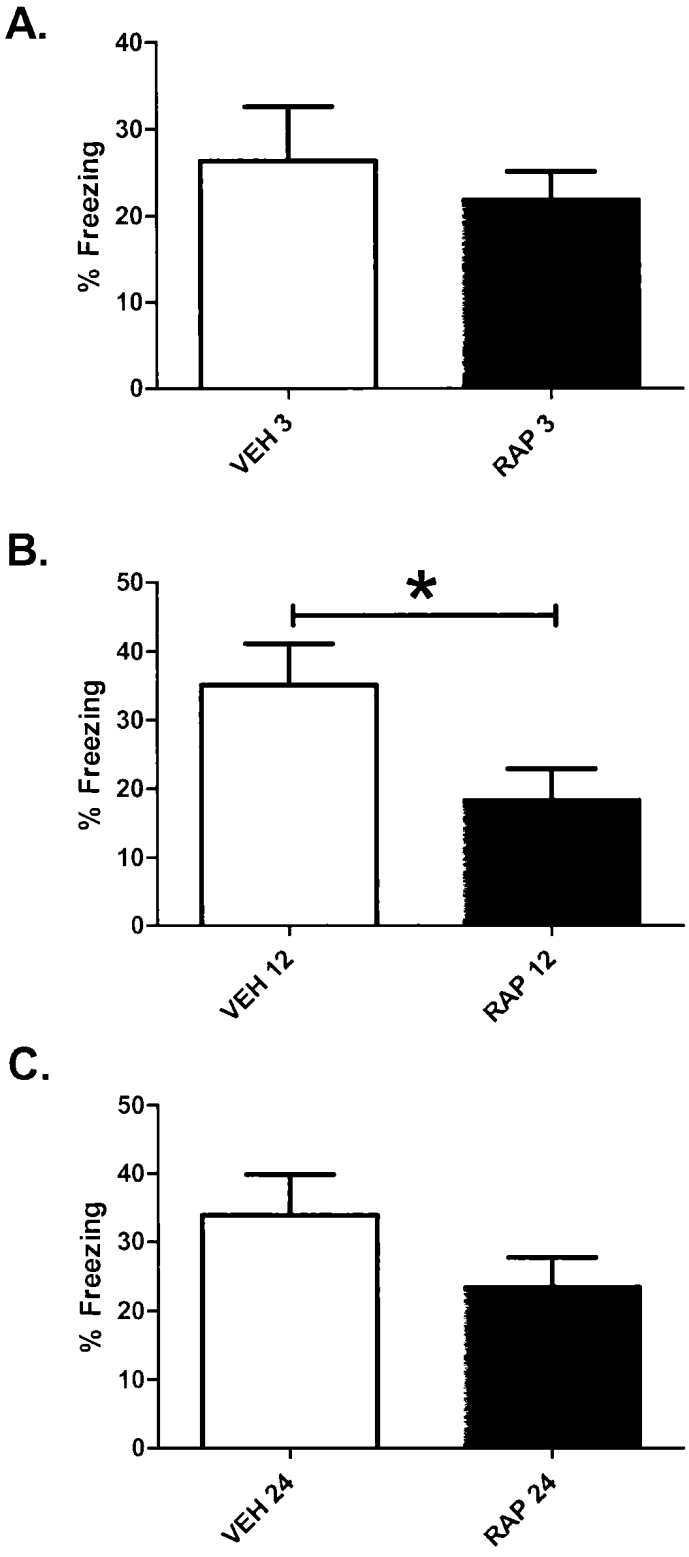
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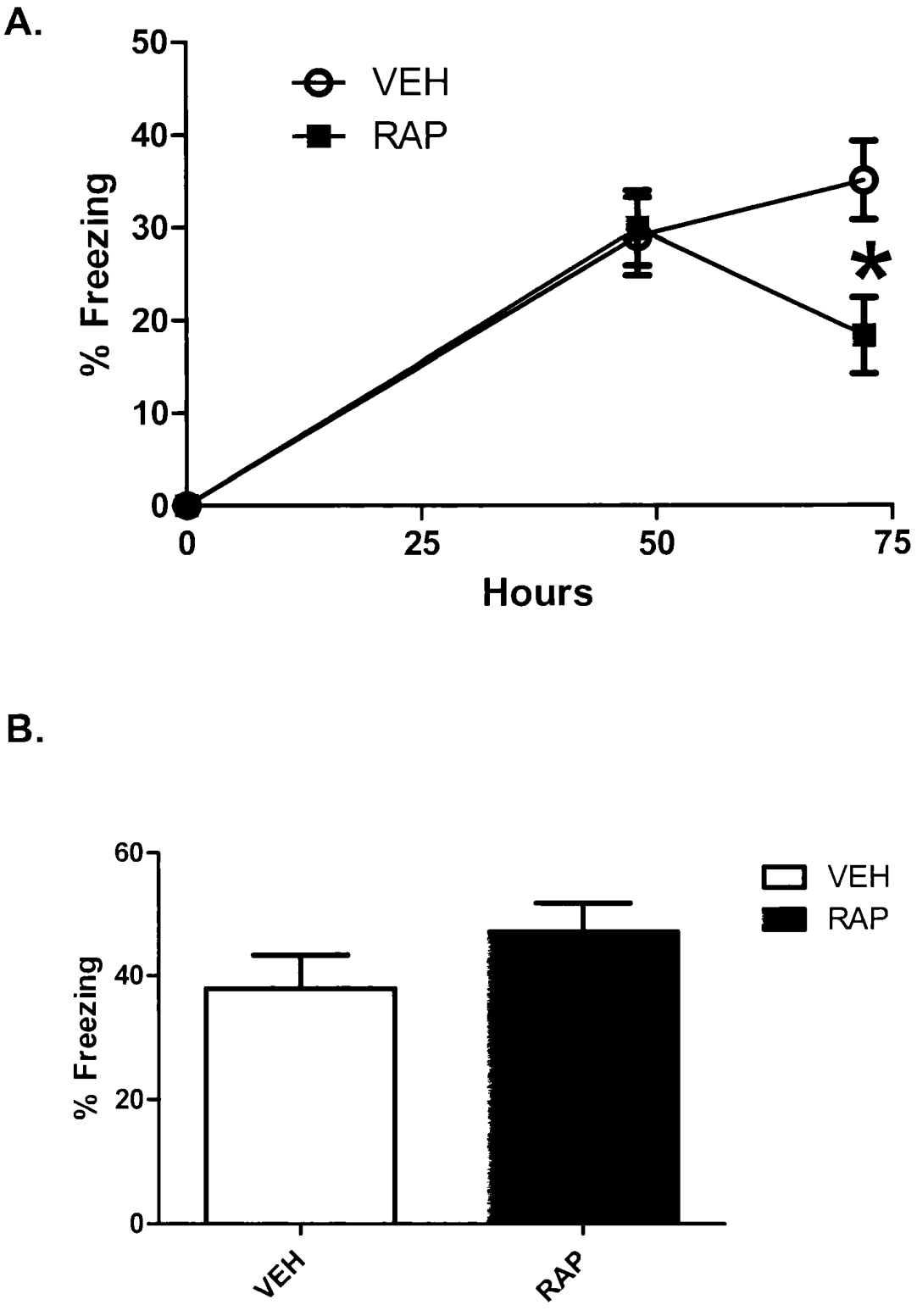
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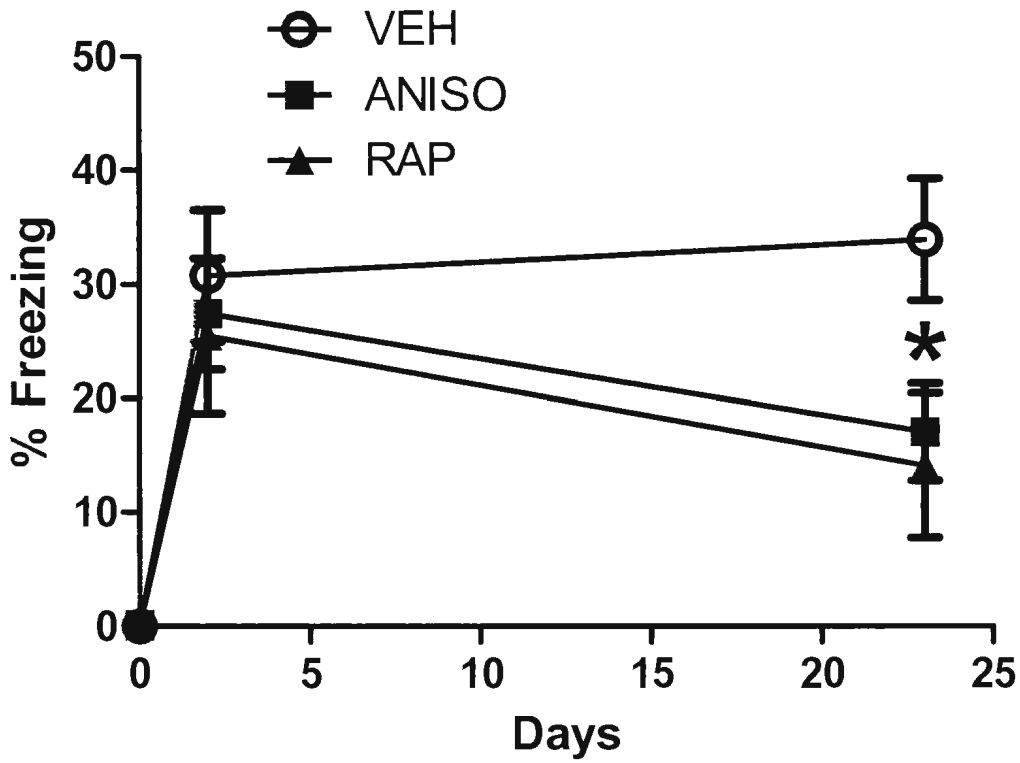

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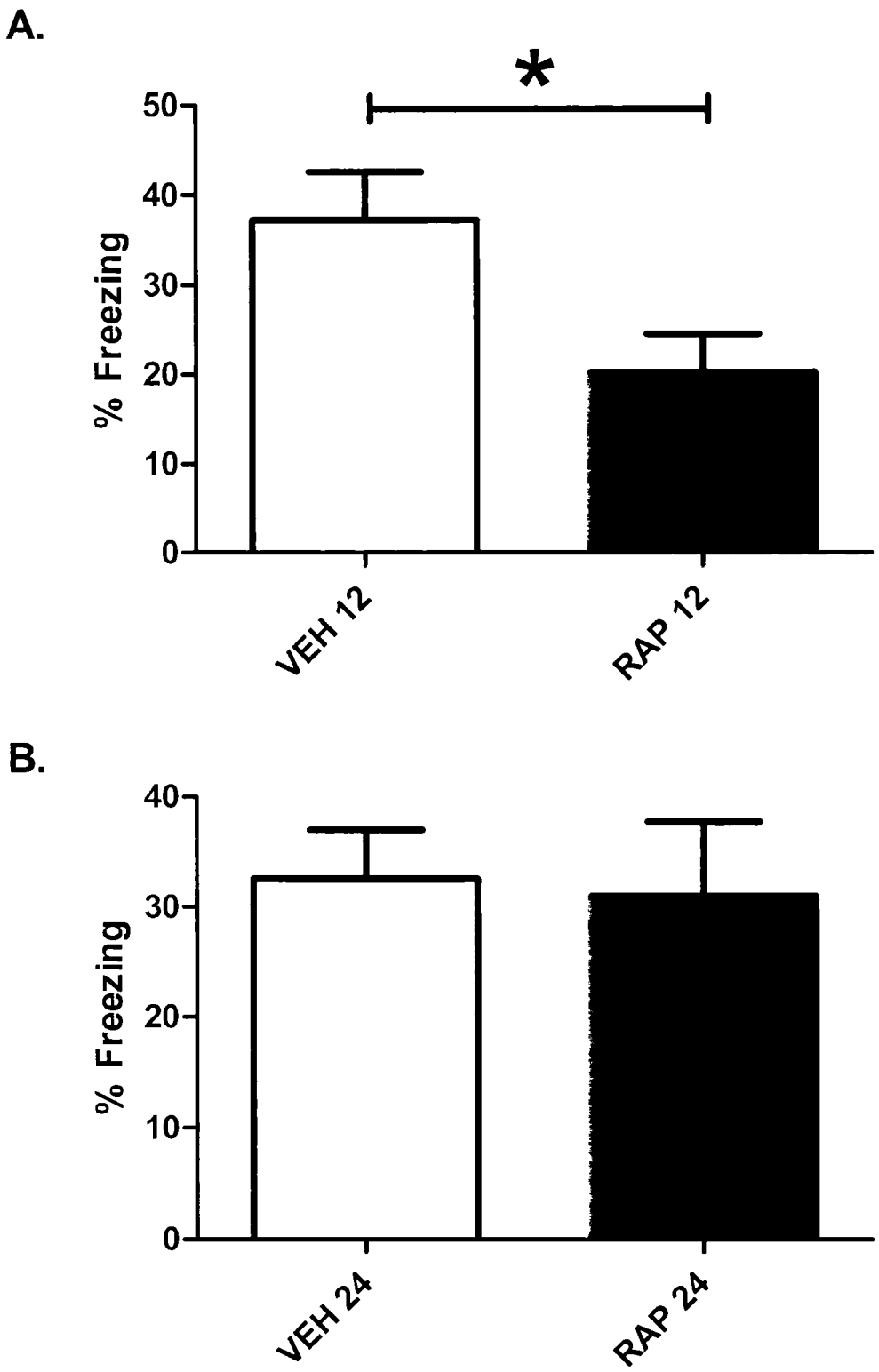
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