INHIBITION OF mTOR KINASE VIA RAPAMYCIN BLOCKS PERSISTENT PREDATOR STRESS-INDUCED HYPERAROUSAL

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Inhibition of mTOR Kinase via Rapamycin Blocks Persistent Predator Stress-Induced Hyperarousal

by

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Abstract

Traumatic, stressful life events are thought to trigger acquired anxiety disorders such as post-traumatic stress disorder (PTSD). PTSD is characterised by several symptoms including both associative and non-associative fear memories. It has been previously established that the mammalian target of rapamycin (mTOR) pathway plays a key role in associative fear memories; however, it is unknown whether this pathway attenuates non-associative fear memories (or fear sensitization). Thus, the goal of these experiments was to examine the role of mTOR in non-associative fear memories. In the current set of experiments, non-associative fear memories were produced by predator stress. Predator stress involves an acute, unprotected exposure of a rat to a cat which causes long-lasting non-associative fear memories expressed as generalized hyperarousal (manifested as increased startle response and anxiety-like behavior and measured in the elevated plus maze, hole board and light/dark box). Here, we show that rapamycin, when given before (Experiment 1) or after (Experiment 2) stress, attenuated predator stress-induced hyperarousal, lasting at least three weeks. In addition, rapamycin blocked a subset of anxiety-like behaviors. Furthermore, when re-exposed to the predator stress context, rapamycin-treated predator stressed rats showed increased activity compared to vehicle controls. These data suggest that rapamycin blocks consolidation of predator stress-induced non-associative and associative fear memories. In a second set of experiments, we examined the effects of rapamycin following reactivation (Experiment 3) and without reactivation (Experiment 4) of predator stress-induced fear memories on non-associative fear memories. A single, 10 minute re-exposure to the predator stress context

was sufficient to extinguish predator stress-induced hyperarousal (Experiments 3, 4). Rapamycin blocked this extinction (Experiment 3). We also show that, consistent with previous data, rapamycin significantly reduced weight gain lasting at least four weeks (Experiments 1-4). Taken together with past research, our results indicate that mTOR regulation of protein translation is required for consolidation of both associative and non-associative fear memories. Overall, these data suggest that rapamycin, a drug already in clinical trials, may be a novel treatment for patients suffering from acquired anxiety disorders such as PTSD.

Keywords: Rapamycin, mTOR, predator stress, anxiety, acquisition, consolidation, reconsolidation, extinction

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1.0 Introduction

1.1 Post-traumatic Stress Disorder

Post-traumatic stress disorder (PTSD) is a debilitating condition characterized by intense moments of fear related to a prior traumatic experience (American Psychiatric Association, 2000). Classification of the disorder involves several criteria including (1) re-experience of the traumatic event triggered by conditioned stimuli or cues symbolizing the distressing experience. This typically occurs through intrusive recollection of the event or through reccurring dreams. (2) Cues related to the event are persistently avoided and a general unresponsiveness or emotional numbing to the person's surroundings ensues. Detachment from others and important activities emerge and the person may be unwilling to discuss the event. (3) Increased arousal, indicated by an exaggerated startle response, is also seen (American Psychiatric Association, 2000). These symptoms can be so severe and persistent that they significantly impair patients' ability to function. The prevalence of developing PTSD after experiencing a traumatic event is between 6.8- 15% in North America (Kessler, Chiu, Demler, Merikangas, & Walters, 2005). However, traumatic events such as the terrorist attacks on the World Trade Center in 2001 have increased the prevalence of PTSD (Galea, Ahern, Tracy, Hubbard, Cerda, Goldmann, & Vlahov, 2002; Kessler & Wang., 2008).

Acquired anxiety disorders, such as PTSD, can be characterized as disorders involving disturbed emotional learning and memory processes resulting in enhanced fear response acquisition and maintenance. Identification of the neural mechanisms underlying such processes, therefore, may aid in the treatment of acquired anxiety disorders. Thus,

the goal of this set of experiments is to use an animal model of PTSD to identify factors that modulate fear memory.

1.2 Animal Models of PTSD

Animal models are useful because they allow the opportunity to simulate a human condition in a controlled setting; the disease can be studied as it develops; and pharmacological and other treatments that may be difficult to test in humans can be easily evaluated in animals. Although no animal model is yet available to reproduce PTSD fully, several experimental paradigms have been developed which produce PTSD-like symptoms. The two discussed here are fear conditioning and predator stress paradigms. Fear conditioning models the associative fear memories (e.g. cued memories) while predator stress models the non-associative fear memories (e.g. hyperarousal) associated with the disorder.

1.2.1 Fear conditioning

Classical fear conditioning links the trauma with the symptoms of PTSD. It has been suggested that the feeling of fear and extreme anxiety the victim experiences at the time of the trauma can become conditioned to a variety of stimuli present at the time of the trauma (Blair, Schafe, Bauer, Rodrigues, & Ledoux, 2001; Johansen et al., 2010; Maren, De Oca, & Fanselow, 1994; Rogan, Stäubli, & Ledoux, 1997; Schafe, Nader, Blair, & Ledoux, 2001). This can be modeled in animals whereby a neutral stimulus (tone or context) can elicit fearful behaviors (freezing) if the tone (or context) was previously paired with an aversive stimulus (shock). This is an appropriate model of PTSD because not only does it demonstrate a learned fear association (associative fear memories) as

seen in PTSD patients, but it also demonstrates a long lasting persistence of these fear memories (Orr et al., 1993; 2000; Rothbaum & Davis, 2003).

1.2.1.1 Fear Conditioning, Consolidation, and Protein Synthesis

Consolidation of a memory is the process by which a labile short-term memory trace is transferred into a fixed long-term memory (de Quervain et al., 2009). During short-term memory, modification of previously synthesized proteins modulates existing synaptic connections (Goelet et al., 1986). Substrate proteins are phosphorylated by protein kinases that have been activated by second messengers. Continuation of this modulation of synaptic connections depends on activity of the substrate proteins and the second-messenger cascade (Goelet et al., 1986). Transition to long-term memories involves novel protein synthesis and mRNA transcription possibly activated by the same extracellular signals and second messenger systems used in short term memory (Bailey & Kandel, 1996; Goelet et al., 1986). Pharmacological inhibition of protein synthesis disrupts long-term memory development in behavioural experiments, supporting the view that long-term memory formation requires intracellular translation of proteins (Cohen et al., 2006; Davis & Squire, 1984; Kandel, 2001; McGaugh & Izquierdo, 2000). Specifically, several studies have shown that anisomycin, a global protein synthesis inhibitor, blocks consolidation of shock-induced fear memories (Huff & Rudy, 2004; Kwapis et al., 2011; Maren et al., 2003; Rudy & Matus-Amat, 2005; Schafe & LeDoux, 2000; Schafe et al., 2001; Schafe, Nadel, Sullivan, Harris & LeDoux, 1999; Wanisch et al., 2005). Protein synthesis within the amygdala and hippocampus is necessary for consolidation of associative fear memories (Bekinschtein et al., 2007; Gafford et al., 2011; Parsons et al., 2006) as anisomycin injected into these areas following training

blocks subsequent fear memory recall (Huff & Rudy, 2004; Kwapis et al., 2011; Maren et al., 2003; Schafe & LeDoux, 2000; Vianna et al., 2001).

1.2.1.2 Fear Conditioning, Reconsolidation, and Protein Synthesis

In addition to consolidation, growing evidence suggests that fear memories have a selective sensitivity to pharmacologic interventions. For instance, protein synthesis inhibitors given after reactivation of fear memories negatively affect subsequent memory (Nader et al., 2000; Pedreira & Maldonado, 2003; Przybyslawski & Sara, 1997; Sara, 2000; Schneider & Sherman, 1968; Suzuki, Josselyn, Frankland, Masushige, Silva, & Kida, 2004; Tronel and Alberini, 2007). Pharmacologic vulnerability to the protein synthesis inhibitor anisomycin following reactivation empirically defines the "reconsolidation" phase of memory (Abel & Lattal, 2001; Dudai, 2004; Duvarci & Nader, 2004; Lattal & Abel, 2004; Nader et al., 2000; Mamiya et al., 2009; Nader, Schafe & Le Doux, 2000; Rudy et al., 2006; von Hertzen & Giese, 2005). Nader et al. (2000) have shown similar results with infusion of anisomycin into the lateral and basal nuclei of the amygdala following reactivation highlighting the role of the amygdala in reconsolidation of fear memories. Similarly, intra-hippocampal administration of anisomycin prior to reactivation of context conditioning reduced the initial shock-induced fear memory (Debiec, LeDoux & Nader, 2002; Stafford & Lattal, 2009). However, blocking reconsolidation via intra-hippocampal administration of anisomycin is not consistently reported and may depend on the duration of re-exposure to the context (Biedenkapp & Rudy, 2004; McGaugh, 2004; Power et al., 2006).

1.2.1.3 Fear Conditioning, Extinction, and Protein Synthesis

Established fear memories may also be affected during reactivation through extinction, another process amenable to pharmacologic manipulation (Bouton, 1993; Cai, Blundell, Han, Greene, and Powell, 2006; Myers and Davis, 2002). Extinction is defined as a reduction in conditioned fear response(s) when the conditioned stimulus is repeatedly presented in the absence of the unconditioned stimulus (Quirk & Mueller, 2008). Following fear conditioning training, animals returned to the training context without shock exhibit increased freezing when compared to non-shocked controls, indicating fear memory. However, when repeatedly exposed to the fear conditioning chamber (in the absence of the shock) freezing to the context decreases, suggesting a decrease in fear of the context or extinction (Milad et al., 2009; Rescorla, 1996). Extinction is not only the result of forgetting or memory erasure but also involves the formation of new associations which compete with prior fear-conditioned associations (Falls & Davis, 1995; Maren & Quirk, 2004; Myers & Davis, 2002; Rescorla, 1996). Like consolidation and reconsolidation, consolidation of extinction memories is protein synthesis dependent. Anisomycin infused into the medial prefrontal cortex (Santini et al., 2004) or amygdala (Lin et al., 2003) blocks consolidation of extinction memory. These data highlight the importance of protein synthesis in consolidation, reconsolidation, and extinction of associative fear memories. However, the identity of these proteins is largely unknown. Recent studies indicate that proteins activated by the mammalian target of rapamycin (mTOR) pathway may be involved in consolidation and reconsolidation of associative fear memories (Gafford et al., 2011; Parsons et al., 2006; Slipczuk et al, 2009; Sui et al., 2008).

1.2.1.4 Associative Fear Memories and the Mammalian Target of Rapamycin (mTOR)

As described above, consolidation, reconsolidation, and extinction of associative fear memories can be disrupted via protein synthesis inhibitors such as anisomycin. However, anisomycin interrupts all protein synthesis in the cell by inhibiting a component of the ribosome, the molecular machine responsible for protein synthesis (Grollman, 1967). Given that anisomycin inhibits the ribosome itself, downstream elements in this cascade, if any, are unknown. Consequently, anisomycin provides a narrow insight as to which specific synthesis pathways are necessary for memory consolidation, reconsolidation, and extinction. Therefore, it is important to identify protein synthesis inhibitors that disrupt specific intracellular cascades in order to provide a more specific idea of the molecular pathways contributing to fear memory formation.

A candidate for such a pathway is mTOR. This intracellular signalling molecule is active in all cells of the body, regulating protein synthesis and growth in response to the cell's environment, trophic signalling, and stress (Hartford & Ratain, 2007). mTOR is a serine/threonine kinase that belongs to the phosphoinositide-3-kinase family (PI3K) and is composed of two distinct complexes: the mTOR complex 1 (mTORC1) and the mTOR complex 2 (mTORC2). It is known that mTORC1 can be inhibited by rapamycin, while mTORC2 is rapamycin insensitive under most conditions. More specifically, rapamycin inhibits mTORC1's ability to phosphorylate its substrates-- S6 Kinase 1 (p70S6K) and eIF4e-binding protein 1 (4E-BP1) -- both of which are known to regulate important aspects of mRNA translation (Zoncu et al., 2011, Gingras, Raught, & Sonenberg, 2001).

Recent data have shown that a downstream target of mTOR, p70S6K, increases in the hippocampus (Bekinschtein et al., 2007; Gafford et al., 2011) and amygdala (Parsons

et al., 2006) during a discrete time period after acquisition of fear memory, which leads to consolidation. Concordantly, inhibition of mTOR by rapamycin blocks both consolidation of a shock-induced fear memory and this increase in p70S6K (Bekinschtein et al., 2007). Similarly, Parsons et al. (2006) demonstrated that formation of associative fear memories, and p70S6K, are inhibited following rapamycin administration into the amygdala. Furthermore, systemic rapamycin following fear conditioning training inhibits consolidation of associative fear memories (Bekinschtein et al., 2007; Blundell et al., 2008; Tishmeyer et al. 2003).

Several studies have examined the role of mTOR in reconsolidation of associative fear memories (Blundell et al., 2008; Gafford et al., 2011; Glover et al., 2010; Parsons et al., 2006; Stoica et al., 2011). Systemic administration of rapamycin following memory reactivation blocks reconsolidation of a shock-induced fear memory (Blundell et al., 2008; Glover et al., 2010; Stoica et al., 2011). Furthermore, rapamycin's block was persistent, lasting at least 21 days Inhibition of reconsolidation of an associative fear memory is also seen with administration of rapamycin directly into the amygdala (Parsons et al., 2006) or hippocampus (Gafford et al., 2011). These studies suggest that reconsolidation of an associative fear memory is mTOR dependent. Although the effects of rapamycin on consolidation and reconsolidation of associative fear memories have been identified, the effects of rapamycin on extinction have not been assessed.

While these data highlight the importance of mTOR in context-specific fear memories, they do not address another core symptom of PTSD, hyperarousal. Nor do they

address the associated symptom of generalized anxiety. Thus, the role of mTOR in non-associative fear memories using an alternative model of PTSD must be examined.

1.2.1.5 Limitations of Fear Conditioning as a Model of PTSD

To date, preclinical models of PTSD have focused on fear conditioning due to its methodological simplicity and demonstration of robust, persistent fear memories, a PTSD-like symptom. Despite the merits of fear conditioning as a model of PTSD, there are several concerns. For instance, it has been argued that conditioning does not account for the sensitized fearfulness which is also a key feature of PTSD manifested as hyperarousal and generalized anxiety (Pitman, 1997). Stress-induced fear sensitization, or non-associative fear memories, appears in novel situations unrelated to the initial trauma (Adamec et al., 2006). In contrast to fear conditioning models, exposure to a predator or predator odours results in long-lasting hyperarousal and anxiety-like behaviour (ALB) (Adamec et al., 2006; Cohen et al., 2006).

1.2.2 Predator Stress

Predator stress is an ecologically relevant animal model of PTSD in that it presents animals with a traumatic event (exposure to a predator or predator cues) that they may encounter in nature (Adamec and Shallow, 1993; Cohen and Zohar, 2004; Munoz-Abellan, Andero, Nadal, and Armario, 2008). Predator stress paradigms reliably induce hyperarousal (enhanced acoustic startle response) and ALB. The predator stress paradigm allows us to determine if pharmacologically targeting fear memory processes (e.g., consolidation, reconsolidation and extinction) not only affects subsequent context/cue-specific symptoms *(i.e.*, persistent trauma-associative fear memories), but also more generalized context/cue-independent symptoms of hyperarousal and anxiety (non-

associative fear memories). Elucidating the molecular factors contributing to both associative and non-associative fear memories will provide valuable insight into the nature of pathological fear disorders such as PTSD and specific phobias.

Predator stress is both fear provoking and stressful (Adamec et al., 1998; Blanchard, et al., 1998; Dielenberg, Carrive, & McGregor, 2001; McGregor et al., 2002). Predator stress typically involves a short (5-10 min) unprotected exposure of a rodent to a predator (i.e. cat) or predator odor (Adamec & Shallow, 1993; Cohen & Zohar, 2004; Adamec, Walling & Burton 2004; Mũnoz-Abellán et al., 2008; Mũnoz-Abellán, Armaraio & Nadal, 2009). This "traumatic" event is ecologically valid as it presents the animal with an event (exposure to a predator or predator cues) that it could possibly encounter in nature (Adamec & Shallow 1993; Cohen & Zohar, 2004; Mũnoz-Abellán et al., 2008). Also, predator stress paradigms reliably induce hyperarousal (enhanced acoustic startle response) which closely parallels symptoms seen in patients with PTSD (Adamec, Blundell & Burton, 2003; Adamec et al., 2006a; Adamec, Head, Soreq & Blundell, 2008; Cohen & Zohar, 2004). In addition, predator stress causes a long-lasting increase in ALB as measured in the elevated plus maze, light/dark box, and hole board (Adamec & Shallow, 1993; Adamec et al., 2004; Adamec, Head, Soreq & Blundell, 2008; Cohen & Zohar, 2004). Increased generalized anxiety is co-morbid with PTSD (Pitman, Orr & Shalev, 1993). Importantly, common pharmacological treatments for PTSD (e.g., sertraline) are efficacious in reducing ALB and hyperarousal following predator stress (Adamec et al., 2004; Adamec et al., 2007; Matar et al., 2006; Zohar et al., 2008). Furthermore, predator stress also produces associative (context-dependent) fear memories, similar to those produced by fear conditioning (Clay et al., 2011). Finally,

elevations in stress hormones (cortisol in humans, corticosterone in animals) have been found in PTSD patients (Jovanovic et al., 2011), and following predator stress in rodents (Adamec, et al., 2006; Cohen et al., 2008).

1.2.2.1 Predator Stress, Consolidation, Reconsolidation, Extinction, and Protein Synthesis

Like shock-induced associative fear memories, protein synthesis is necessary for consolidation of predator stress-induced non-associative fear memories (i.e., hyperarousal and ALB) (Adamec et al., 2006; Cohen et al., 2006; Kozlovsky et al., 2008). Specifically, Adamec et al. (2006) have shown that a systemic injection of anisomycin immediately after exposure to a predator blocked ALB and response to acoustic startle measured 7- 8 days later. Similarly, infusion of anisomycin into the lateral ventricle, before and after predator stress, reduced ALB and startle (Cohen et al., 2006). While the identity of the proteins is unknown, these data confirm that the synthesis of novel proteins is necessary for consolidation of non-associative fear memories.

To our knowledge, only two studies have examined the effects of a protein synthesis inhibitor following contextual reactivation of a predator stress memory (Adamec et al., 2006; Cohen et al., 2006). Anisomycin given after a single reactivation of the predator scent memory (re-exposed to the context in which the rat was previously exposed to the cat odor but void of cat odor, Cohen et al., 2006) or following re-exposure to the cat (Adamec et al., 2006) did not affect subsequent ALB or startle. These data suggest that reconsolidation may not occur following reactivation of a predator stress memory. While the majority of research in humans and other animals supports a reconsolidation process following fear memory reactivation (Flavell et al., 2011;

Johansen et al., 2011; Martijena & Molina, 2012; Schiller et al., 2010), there are at least two reports to suggest that reconsolidation does not occur (McKenzie & Eichenbaum, 2011; Monfils et al., 2009). However, it may be premature to suggest that reconsolidation does not occur following predator stress. It may be that methodological parameters necessary to interrupt reconsolidation of predator stress-induced fear memories were not achieved. For instance, Debiec et al. (2002) suggest that a higher dose of anisomycin within the hippocampus is required to block reconsolidation than that which would block consolidation. Furthermore, in the study by Adamec et al. (2006) anisomycin was given after a second exposure to the cat, not following contextual reminders only, which may have confounded the results. Thus, future research assessing reconsolidation following predator stress is warranted.

Recently our lab has shown that predator stress-induced fear memories undergo extinction (Clay et al., 2011). Predator stressed animals repeatedly exposed to the predator stress context (without the cat present) extinguished both associative and non-associative fear memories (Clay et al., 2011). Like extinction of shock-induced fear memories, extinction of predator stress-induced fear memories is also protein synthesis-dependent (Sandusky et al., 2012). In this study, predator scent stressed animals were repeatedly exposed to clean litter (1, 2 or 4 extinction trials) in the presence of cycloheximide and ALB was assessed 72 hrs later. Cycloheximide prevented extinction of predator stress-induced ALB as measured in the elevated plus maze. While the identity of the proteins is unknown, these data confirm that protein synthesis is necessary for extinction of non-associative fear memories.

1.3 Goals and Aims

While the studies described above indicate that protein synthesis is necessary for the consolidation of stress-induced increases in ALB and hyperarousal, the identity of the substrates and, more broadly, the molecular pathway mediating these effects is unknown. Given that the mTOR pathway mediates associative fear memory consolidation, it is likely that this pathway also mediates consolidation of non-associative fear memories (e.g., consolidation of stress-induced fear sensitization). Thus, the first goal of these experiments was to determine if consolidation of predator stress-induced fear memories (non-associative fear memories) is mTOR-dependent. Pharmacologic modulation of the reactivation process to alter subsequent recall either through extinction or reconsolidation has not been fully characterized despite its potential as a feasible therapeutic target. Thus, the second goal of these experiments was to examine the role of the mTOR pathway following reactivation of predator-stress induced fear memories.

Elucidating the molecular factors contributing to associative and non-associative fear memories will provide valuable insight into the nature of pathological fear disorders such as PTSD and specific phobias. Ultimately this knowledge will aid in the development of novel therapeutic agents to treat these disorders. If rapamycin decreases both associative and non-associative fear memories, it may be a successful therapeutic agent to treat PTSD. Moreover, rapamycin and its analogues are already FDA approved, used clinically to treat PTSD, and well-tolerated (Abizaid, 2007; Elit, 2002; Eto & Naito, 2006).

2.0 Methods

2.1 Experiment 1- The role of mTOR in predator stress-induced fear memories

2.1.1 Subjects

A total of 80 male Long Evans rats (Charles River, Canada) were used in Experiment 1. Rats were individually housed in clear plastic cages with wire tops (42 cm X 25 cm X 20 cm). Food and water were available ad libitum and rats were habituated to the housing room for two weeks on a 12 hour light/dark reverse light cycle (lights off at 7 am). Animals were handled for five consecutive days prior to experimentation; handling consisted of petting and lifting rats for approximately 30 sec to 1 min under a red lamp in the colony room. The colony rooms for the rats were at the point farthest possible from the room where the cats were housed to ensure isolation from olfactory cues. After exposure to the cat, predator stressed rats were housed in a different room away from handled control rats. Residual olfactory cues from the cat exposure may have been present on predator stressed rats; therefore housing these rats away from handled controls would eliminate the effect of any olfactory cues on unstressed rats. These basic procedures were followed for Experiments 1-4.

Procedures for Experiments 1-4 adhered to the guidelines of the Canadian Council on Animal care, and were approved by the Institutional Animal Care committee of Memorial University.

2.1.2 Groups and Procedures

Rats were randomly assigned to one of four groups (n=20): handled controls (HC), predator stressed animals (PS), predator stressed plus rapamycin injection (PSR) or predator stressed plus vehicle (PSV). Rats in the handled control (HC) group were not

exposed to a cat. Instead they were only handled on predator exposure day, and then remained undisturbed in their home cage until behavioral testing. Predator stressed animals (rats in PS, PSR, and PSV groups) received a 10 min unprotected exposure to a cat. Full details of the cat exposure can be found in the section 2.6.1 entitled ***Cat exposures and behavioral measures.*** Thirty minutes prior to cat exposure, rats in the PSV and PSR groups received an intraperitoneal (i.p.) injection of vehicle or rapamycin, respectively. Refer to section 2.5 entitled ***Drug Administration*** for drug dose. Rats were returned to their home cage in the housing room immediately after cat exposure and left undisturbed until behavioral testing.

Seven days after the predator exposure or handling, all rats underwent several tests of anxiety and hyperarousal including elevated plus maze (EPM), hole board (HB), light/dark (LD) box, and response to acoustic startle. Behavioral tests were run across three days with HB and EPM on the first testing day, LD box on the second day, and acoustic startle response on the third. To determine if the effects of rapamycin on predator stress-induced hyperarousal were long-lasting, acoustic startle response was measured again three weeks after the initial predator exposure. The following day, rats in the PS, PSV and PSR groups were re-exposed to the predator stress room without the cat present to test for contextual fear memory. Refer to section 2.6.2 for a complete description of the room re-exposure and behavioral tests.

The rats' initial body weight was measured immediately after predator exposure. To determine the effect of rapamycin on body weight, weight was measured immediately after startle testing (nine days after predator exposure) and again three weeks later (following the second startle test).

2.2 Experiment 2 – The role of mTOR in consolidation of predator stress-induced fear memories.

2.2.1 Subjects

A total of 80 male Long Evans rats (Charles River, Canada) were used in Experiment 2. Housing conditions and handling were the same as in Experiment 1.

2.2.2 Groups and Procedures

Rats were randomly assigned to one of four groups (n=20): handled controls (HC), predator stressed only (PS), predator stressed plus vehicle (PSV) and predator stressed plus rapamycin (PSR). As described in Experiment 1, rats in the HC group were handled on predator exposure day and remained undisturbed in their home cage until behavioral testing. Predator stressed animals (rats in PSR, PS, and PSV groups) received a 10 min unprotected exposure to a cat. Full details of the cat exposure can be found in the section 2.6.1 entitled ***Cat exposures and behavioral measures***. Immediately following cat exposure, rats in the PSV and PSR groups received an i.p. injection of vehicle or rapamycin, respectively. Refer to section 2.5 entitled ***Drug Administration*** for drug doses. Rats were returned to the housing room immediately after cat exposure and left undisturbed until behavioral testing.

Seven days after cat exposure or handling, all rats underwent several tests of anxiety and hyperarousal including EPM, HB, LD box, and response to acoustic startle. As in Experiment 1, behavioral tests were run over three days with HB and EPM on the first testing day, LD box on the second day and acoustic startle response on the third. A detailed description of the behavioral tests can be found below in the section 2.6.

Body weight was also measured throughout the experiment: four days prior to predator exposure, the day of predator exposure and days seven, nine and 23 after predator exposure.

2.3 Experiment 3- The effects of post-retrieval rapamycin on predator stress-induced anxiety and hyperarousal.

2.3.1 Subjects

A total of 80 male Long Evans rats (Charles River, Canada) were used in Experiment 3. Housing conditions and handling were the same as in Experiments 1 and 2.

2.3.2 Groups and Procedures

Rats were randomly assigned to one of four groups (n=20): handled control (HC), predator stressed only (PS), predator stressed plus room re-exposure (PSR) plus rapamycin, and predator stressed plus room re-exposure plus vehicle (PSV). HC rats were handled only on predator exposure day, and returned to their home cages until behavioral testing. Predator stressed rats (rats in the PS, PSR, and PSV groups) received a 10 min unprotected exposure to a cat. Full description of the cat exposure can be found in the section 2.6.1 entitled ***Cat exposures and behavioral measures.*** Two days after cat exposure, PSR and PSV rats were returned to the exposure room without the cat for 10 minutes. A full description of the room re-exposure can be found in section 2.6.2 entitled ***Room Re-exposures and behavioral measures.*** Immediately following re-exposure, rats were given an i.p. injection of either rapamycin (PSR) or vehicle (PSV). Refer to section 2.5 entitled ***Drug Administration*** for drug dose. Following injection, PSR and PSV rats were returned to the housing room and left undisturbed until behavioral testing

Seven days after re-exposure to the room (a total of nine days after predator exposure or handling), all rats underwent several tests of anxiety and hyperarousal including EPM, HB, LD box, and response to acoustic startle. Behavioral tests were run over three days with HB and EPM on the first testing day, LD box on the second day and acoustic startle response on the third. A detailed description of the behavioral tests can be found below in the section 2.6. Body weight was measured immediately after the room re-exposure and nine days later (after startle testing).

2.4 Experiment 4: The role of mTOR in extinction of predator stress-induced fear memories

2.4.1 Subjects

A total of 80 male Long Evans rats (Charles River, Canada) were used in Experiment 4. Housing conditions and handling were the same as in Experiments 1-3.

2.4.2 Groups and Procedures

Rats were randomly assigned into four groups: Handled controls (HC), predator stressed only animals (PS), predator stressed animals plus an injection of rapamycin (PSR) and predator stressed animals plus a vehicle injection (PSV). As in the previous experiments, HC rats were handled only on cat exposure day. Predator stressed rats (PS, PSR, PSV) were exposed to a cat for a 10 min period. To ensure that extinction was occurring in Experiment 3, two days later, PSR and PSV rats were given an i.p. injection of rapamycin or vehicle, respectively (without re-exposure to the predator stress context). Refer to section 2.5 entitled ***Drug Administration*** for drug doses.

Seven days after rapamycin or vehicle injection (a total of nine days after predator exposure or handling), all rats underwent several tests of anxiety and

hyperarousal including EPM, HB, LD box, and response to acoustic startle. Behavioral tests were run over three days with HB and EPM on the first testing day, LD box on the second day and acoustic startle response on the third. A detailed description of the behavioral tests can be found below in the section 2.6. Body weight was measured immediately before rapamycin or vehicle injection and nine days later (after startle testing).

2.5 Drug administration

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

2.6 Behavioral Testing

Groups were counterbalanced for time of day tested and time of day exposed to a predator. This was done to control for possible variability due to circadian rhythms. Testing for cat exposures, all ALB tests, and startle were conducted between 8:00 am and 4:00 pm.

2.6.1 Cat exposures and behavioral measures

Predator stressed rats received a 10 min unprotected exposure with a male cat. The exposure room was approximately 2 m by 1.3 m and 3.5 m in height with no windows. Thirty minutes prior to testing, the cat was transported to the exposure room via a small animal carrier. Food, water, and a litter box were provided in between trials. Rats were singly placed into the room through a small grey plastic container 18.5 cm high, 19 cm long and 14.5 cm wide. The container consisted of a sliding door with a moving plate that forced the rat into the exposure room when pushed. Each exposure was videotaped for a

10 minute period with a camera mounted on the wall of the room. After 10 minutes the rat was put back into the container and was brought back into the housing room. Rats were exposed to the same male cat.

Rat behavioral measures included the frequency of approaches to the cat and the frequency of flights away from the cat. Cat behavioral measures included the frequency of approaches to the rat, the frequency of sniffs, bites and physical contact of the cat's paw to the rat. The number of cat vocalizations was also measured. The total time the cat and rat were in close proximity of one another was also measured. Close proximity was defined as either the rat or cat being one foot from one another. Masking tape was used to divide the floor of the exposure room into 1 foot squares.

2.6.2 Room re-exposures and behavioral measures

For the room re-exposures, rats were placed into the cat exposure room without the cat for 10 min. Locomotor activity was measured by the number of lines crossed by the rat. Video-tracking software (Ethovision by Noldus) recorded the distance the rat moved and the immobility (s) and mobility (s) of the rat.

2.6.3 Hole Board (HB)

The HB test was used as described previously (Adamec et al., 2006). The room was illuminated with red overhead lights to permit videotaping. Illumination levels were 44 foot candles (fc) at the light bulb and a very low light intensity at the floor of the testing apparatuses. The hole board consisted of an opened top square wooden box (60 cm long X 60 cm wide X 35 cm high) painted with grey enamel. The floor of the apparatus was elevated 12 cm above the floor. There were four evenly spaced holes (1 cm in diameter) located in each corner, 9 cm from the wall, in the floor of the box. The holes

formed a square and white masking tape outlined the center of the box which included the holes. At the beginning of each trial a rat was placed in the center of the open field and behavior was videotaped for 5 min.

Behavioral measures included the frequency of head dips into the holes, the frequency of rears, the number of faecal boli and the amount of time spent in the center of the box as well as in the area near the walls. Head dips were scored manually and were operationally defined as extending of the rat's head into one of the holes. Rears, also scored manually, were defined as any instance where the rat raised itself on its hind legs with forepaws leaving the ground, with the exception of grooming behavior. Using Ethovision, rats were recorded as in the center of the open field when the full body was within the center area defined by white masking tape. Rats were recorded as near the wall when all four feet were between the masking tape and the wall.

2.6.4 Elevated Plus Maze (EPM)

The EPM test was used as described previously (Adamec et al., 2006). Immediately after the HB test, rats were placed into the EPM. The room was illuminated with red light as previously described in the HB test. The EPM consisted of four arms in the shape of a plus sign. Each arm was 10 cm wide, 50 cm long and was elevated 50 cm above the floor. The four arms were joined at the center 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 open at the top. The walls did not extend into the center of the maze and the maze was painted with flat grey enamel paint. At the beginning of each 5 min trial, rats were singly placed in the center of the apparatus facing the same open arm.

Behavioral measures included the frequency and time of risk assessment, the number of entries and time spent in the open and closed arms and the number of center head dips. Rats were considered to have entered the arm if all four legs were on the arm. Risk assessment behavior was defined as having at least two hind paws in a closed arm with the nose pointed toward one of the open arms. The frequency and time of relative risk assessment behavior were recorded and defined as the ratio of time spent in the closed arms. For the ratio time measurement, the ratios were calculated as the total time in the open arms divided by the total time in any arm. For the ratio entry measurement, the ratios were calculated as the number of entries into the open arms divided by the number of entries into any arm.

2.6.5 Light*/Dark Box (LD box)*

The LD box test was used as described previously (Adamec et al., 2006). The apparatus consisted of a single alley constructed of 0.5 inch Plywood. The box was divided into two chambers of equal size; each chamber was 31.75 cm long, 10.48 cm wide and 14.6 cm high. The chambers were covered by a transparent Plexiglas top, hinged to open. The center pieces of each chamber top were cut to allow ventilation. One chamber had a solid wooden floor with the walls and floor painted white, while the other had a metal mesh floor with the walls painted black. The black chamber had a Plexiglas opaque top and half of the top was covered with black plastic. The apparatus was illuminated with a 100 W lamp positioned 66 cm above the white chamber. The light intensity at the center of the white chamber floor was 55 fc, whereas the intensity at the center of the dark chamber floor was 2 fc. Behavior was videotaped with a video camera mounted over the apparatus for later analysis. At the beginning of each 5 min trial, rats

were singly placed in the light chamber and allowed to move freely between the two chambers.

Behavioral measures included the total time spent in each chamber, the number of entries into each chamber and the number of faecal boli in each chamber. A rat was considered in the compartment when all four paws were in the chamber.

2.6.6 Acoustic Startle Testing

The acoustic startle response was measured as previously described (Adamec et al., 2006). Startle testing took place in a San Diego Instruments standard startle chamber. Within the startle chamber, rats were singly placed in a cylindrical small animal enclosure measuring 12.7 cm long and 3.7 cm in diameter. The enclosure was mounted on top of a piezo electric transducer, which produces electrical signals sampled by a computer. This provided a measure of rodent movement. Rats were acclimated to the startle apparatus for 5 min. The chamber was completely dark inside and emitted a background of 60 db white noise during this 5 min acclimation period. Immediately following acclimation, rats were exposed to 30 pulses of 50 ms bursts of white noise of 120 db amplitude rising out of a background of 60 db of white noise. There was a 30 s inter trial interval between noise bursts. The startle response was measured over a 250 ms recording period via a computer. Analysis included the maximal output of the transducer (Vmax) within the 250 ms recording window and Vstart was measured before the pulse. For each trial, peak startle amplitude was calculated as Vmax – Vstart and divided by rat body weight in kg giving peak startle amplitude in volts/kg.

3.0 Results

3.1 Experiment 1- The role of mTOR in predator stress-induced fear memories

3.1.1 Cat-rat interaction during predator exposure

There were no differences in the behavior of the cat or rat across all groups during predator exposure (all $p > 0.05$). Thus, any subsequent differences across groups can be attributed to the treatment effects and not to variation in predator exposure. See Table 1 for complete statistical analysis.

3.1.2 Rapamycin blocks predator stress-induced hyperarousal

Response to acoustic startle was measured in the HC, PS, PSR and PSV groups. The non-normality of the data (Omnibus test = 2046.0, $p < 0.0001$) required the use of the Kruskal-Wallis non parametric chi square test of median differences across groups. Median peak startle amplitude across 30 trials revealed a main effect of group ($X^2(3) = 8.81$, $p < 0.032$: Figure 1 A). Consistent with previous studies (Adamec et al., 2006; Blundell et al., 2005; Cohen et al., 2004), PS rats showed enhanced peak startle amplitude compared to HC rats (Kruskal-Wallis Multiple-Comparison Z-test, $p < 0.05$, Figure 1 A). Rapamycin injected 30 minutes prior to predator stress (PSR group) reduced peak startle amplitude to control levels (PSR vs. PS, Kruskal-Wallis Multiple-Comparison Z-test, $p < 0.05$, PSR vs. PSV, Kruskal-Wallis Multiple-Comparison Z-test, $p < 0.05$, PSR vs. HC, Kruskal-Wallis Multiple-Comparison Z-test, $p > 0.05$, Figure 1 A). Vehicle injection had no effect on peak startle amplitude (PS vs. PSV, Kruskal-Wallis Multiple-Comparison Z-test, $p > 0.05$, Figure 1 A).

Examination of the mean peak startle amplitude for all four groups revealed a decline in startle response (habituation) over trials. A slower rate of habituation of the

startle response occurs in predator-stressed mice and rats also showing enhanced startle amplitudes (Adamec et al., 2006; Adamec et al., 2008; Adamec, Fougere, & Risbrough, 2009). Rate of habituation to the tone was measured by the trial constant (Tau) estimated from fits of the exponential decay function

$$Y = Y_o e^{-1/Tau}$$

to mean peak startle amplitude over trials for each of the three groups (all df adjusted $r^2 > 0.61 - 0.98$, all exponential fits $F(2, 27) > 8.60$, $p < 0.01$, all Tau > 0, t tests $p < 0.001$). Y and Y_o in the function are mean peak startle amplitude, e is startle trial and the parameter Tau is the number of startle trials required for startle amplitude to decline to 37% of maximum. The program fitting the functions (Jandel Table Curve V4) also estimates standard error (SE) of each Tau value and these SE were used to calculate t tests of Tau differences between groups. Rats in the HC and PSR groups habituated faster (smaller Tau values) than those in the PS and PSV groups (Bonferroni protected t tests, HC vs. PSR, $p > 0.05$, HC vs. PS, $p < 0.05$, PSR vs. PS, $p < 0.05$, PSR vs. PSV, $p < 0.05$; Figure 1 B). Vehicle injection did not affect habituation (PS vs. PSV, Bonferroni protected post hoc comparisons, $p > 0.05$, Figure 1 B). Thus, rapamycin given prior to predator exposure reduced the peak startle amplitude and increased the rate of habituation to the tone to control levels, suggesting that rapamycin blocks consolidation of predator stress-induced hyperarousal. Refer to Table 1 for statistical analysis.

3.1.3 Rapamycin blocks persistent predator stress-induced hyperarousal.

Startle response was tested again three weeks after predator stress (two weeks after the initial startle test) to determine the persistence of the rapamycin effect on predator stress-induced hyperarousal. The non-normality of the data (Omnibus test =

1724.3, $p < 0.0001$) required the use of the Kruskal-Wallis non parametric chi square test of median differences across groups. Median peak startle amplitude across 30 trials revealed a main effect of group ($X^2(3)= 210.41$, $p < 0.001$: Figure 2 A). The predator stress-induced increase in peak startle amplitude was persistent, lasting at least three weeks (PS vs. HC, Kruskal-Wallis Multiple-Comparison Z-test, $p < 0.01$, Figure 2 A). Interestingly, peak startle amplitude in the PSR remained at control levels (PSR vs. PS, Kruskal-Wallis Multiple-Comparison Z-test, $p < 0.01$, PSR vs. PSV, Kruskal-Wallis Multiple-Comparison Z-test, $p < 0.05$, PSR vs. HC, Kruskal-Wallis Multiple-Comparison Z-test, $p > 0.05$, Figure 2 A). The PSV group did not differ from the PS group (PSV vs. PS, Kruskal-Wallis Multiple-Comparison Z-test, $p > 0.05$, Figure 2 A).

Group differences were also seen in startle habituation (all df adjusted $r^2 > 0.877$, all exponential fits $F(2, 27) > 108.73$, $p < 0.001$, all Tau > 0, t tests $p < 0.001$). When tested three weeks post-treatment, HC rats habituated faster (smaller Tau values) in comparison to PS (Bonferroni protected t tests, $p < 0.01$; Figure 2 B). Importantly, rate of habituation in the PSR group remained similar to that of HC rats (Bonferroni protected post hoc comparisons, PSR vs. PS, $p < 0.001$, PSR vs. PSV, $p < 0.05$, PSR vs. HC, $p > 0.05$, Figure 2 B). Vehicle injection did not affect habituation to the tone and startle habituation was comparable to predator stressed animals (PS vs. PSV, Bonferroni protected post hoc comparisons, $p > 0.05$, Figure 2 B). Overall, these data suggest that rapamycin's effect on predator stress-induced hyperarousal is long-lasting.

3.1.4 Elevated Plus Maze, Hole Board, and Light/Dark Box

ALB and activity were assessed in the EPM, HB, and LD box. Group differences were found in two measures taken from the EPM, namely ratio time ($F(3,75)= 5.310$, $p <$

0.01: Figure 3 A) and the frequency of risk assessment ($F(3,75)= 5.93$, $p < 0.001$: Figure 3 B). HC rats spent more time in the open arms compared to all arms of the EPM (ratio time) than PS rats suggesting increased ALB in the PS rats. Importantly, rapamycin blocked the predator stress-induced decrease, increasing ratio time to that of control levels (Bonferroni protected post hoc comparisons, PSR vs. HC, $p > 0.05$, PSR vs. PS, $p < 0.05$, PSR vs. PSV, $p < 0.05$; Figure 3 A). There were no differences between PS and PSV groups (PS vs. PSV, Bonferroni post hoc comparisons, $p > 0.05$). In addition, HC rats engaged in risk assessment more often than PS rats (Bonferroni protected post hoc comparisons, HC vs. PS, $p < 0.05$). Importantly, previous studies have shown that an increased frequency of risk assessment implies a lower level of ALB in rodents (Adamec & Shallow, 1993). Similar to ratio time, rapamycin blocked the predator stress-induced decrease in risk assessment, increasing the frequency of risk assessment to that of control levels (Bonferroni protected post hoc comparisons, PSR vs. HC, $p > 0.05$, PSR vs. PS, $p < 0.05$, PSR vs. PSV, $p < 0.05$). There were no differences between PS and PSV rats in frequency of risk assessment (Bonferroni protected post hoc comparisons, PS vs. PSV, $p > 0.05$). There were no other group differences between groups in the EPM (all $p > 0.05$).

Significant differences were also seen in the ratio time measure (time in center/time in periphery, Figure 3 C) and frequency of rears in the HB (Figure 3 D). PS rats displayed decreased ratio time compared to both HC and PSR groups ($F(3,75)= 4.918$, $p < 0.05$, Bonferroni protected post hoc comparisons HC vs. PS, PSR vs. PS, all $p < 0.05$) with PSV rats showing intermediate levels of ratio time (Bonferroni protected post hoc comparisons HC vs. PSV, PSR vs. PSV, all $p > 0.05$). In addition, HC rats reared more than PS and PSV rats ($F(3,75)= 4.98$, $p < 0.01$, Bonferroni protected post

hoc comparisons HC vs. PS, HC vs. PSV, all $p < 0.05$). The predator stress-induced suppression of rears was partially reversed with rapamycin (Bonferroni protected post hoc comparisons HC vs. PSR, PSR vs. PSV, PSR vs. PS, all $p > 0.05$). To determine whether frequency of rears was a measure of activity or anxiety, an analysis of covariance with ratio time in the HB as a covariate of rears was conducted. The ANCOVA revealed that decreased rearing did not reflect increased anxiety ($F(1,72)= 6.184$, $p < 0.05$). There were no other differences in the HB (all $p > 0.05$). Furthermore, there were no differences between groups in the LD box ($p > 0.05$). Overall, our data suggest that rapamycin, when given prior to predator exposure, reduces ALB, as measured in the EPM and HB. See Table 1 for statistical analyses.

3.1.5 Activity during room re-exposure measured three weeks after predator stress

To assess predator stress-induced associative (contextual) fear memory, rats were placed back in the predator stress room (without the cat present) three weeks after the initial predator stress exposure. A one-way ANOVA revealed significant differences among PS, PSR and PSV groups on total distance moved (cm) ($F(2,54)= 7.50$, $p < 0.001$, Figure 4 A), total time mobile (s) ($F(2,54)= 7.47$, $p < 0.001$, Figure 4 B) and total time immobile (s) ($F(2,54)= 7.47$, $p < 0.001$, Figure 4 C). Bonferroni post hoc comparisons demonstrated that PSR rats were more mobile, traveled more distance, and were less immobile in comparison to PS and PSV groups (PSR vs. PS, $p < 0.05$, PSR vs. PSV, $p < 0.05$), which did not differ (PS vs. PSV, $p > 0.05$). Consistent with shock-induced fear memories (Bekinschtein et al., 2007; Blundell et al., 2008; Tishmeyer et al. 2003), our data suggest that rapamycin blocks consolidation of predator stress-induced associative fear memories.

3.1.6 Weight Measurement

A single injection of rapamycin decreased body weight measured throughout the experiment (Figure 5). A mixed ANOVA revealed a main effect of group [$F(3,219)= 9.14$, $p < 0.0001$], a main effect of day [$F(3,219)= 1747.92$, $p < 0.0001$], and an interaction of group x day [$F(9,219)= 20.05$, $p < 0.0001$]. Following injection, body weight was significantly lower in the PSR group than in all other groups across days (all $p < 0.05$).

3.2 Experiment 2 – The role of mTOR in consolidation of predator stress-induced fear memories.

3.2.1 Cat-rat interaction during predator exposure

There were no differences in the behavior of the cat and rat across all groups and measures during predator exposure (all $p > 0.05$). Thus, any subsequent differences across groups can be attributed to the treatment effects and not to variation in predator exposure. See Table 2 for complete statistical analyses.

3.2.2 Rapamycin given after predator exposure blocks predator stress-induced hyperarousal

Startle response was measured in HC, PS, PSR and PSV groups. Similar to Experiment 1, the non-normality of the data (Omnibus test = 1213.2, $p < 0.0001$) required the use of the Kruskal-Wallis non parametric chi square test of median differences across groups. Median peak startle amplitude across 30 trials revealed a main effect of group (X^2 (3) = 75.94, $p < 0.001$: Figure 6 A). Consistent with Experiment 1, and previous studies (Adamec et al., 2006; Blundell et al., 2005; Cohen et al., 2004), PS rats displayed increased peak startle amplitude compared to HC (Kruskal-Wallis

Multiple-Comparison Z-test, $p < 0.001$, Figure 6 A). As expected, an injection of rapamycin immediately following predator exposure reduced peak startle amplitude to control levels (PSR vs. PS, Kruskal-Wallis Multiple-Comparison Z-test, $p < 0.001$, PSR vs. PSV, Kruskal-Wallis Multiple-Comparison Z-test, $p < 0.05$; PSR vs. HC, Kruskal-Wallis Multiple-Comparison Z-test, $p > 0.05$, Figure 6 A). Startle amplitude did not differ in PS and PSV groups (PS vs. PSV, Kruskal-Wallis Multiple-Comparison Z-test, $p > 0.05$, Figure 6 A).

Similar to Experiment 1, rate of habituation to the tone was measured by the trial constant (Tau) estimated from fits of the exponential decay function

$$Y = Y_o e^{-1/Tau}$$

to mean peak startle amplitude over trials for each of the three groups (all df adjusted $r^2 > 0.82$, all exponential fits $F(2, 27) > 75.2$, $p < 0.001$, all Tau > 0, t tests $p < 0.01$). As expected, HC rats habituated faster (smaller Tau values) in comparison to PS rats (Bonferroni protected t tests, $p < 0.01$; Figure 6 B). Rapamycin reduced the predator stress-induced delay of habituation to that of HC levels (Bonferroni protected post hoc comparisons, PSR vs. PS, $p < 0.01$, PSR vs. PSV, $p < 0.05$, PSR vs. HC, $p > 0.05$, Figure 6 B). Vehicle injection did not affect habituation to the tone (PS vs. PSV, Bonferroni protected post hoc comparisons, $p > 0.05$, Figure 6 B).

3.2.3 Elevated Plus Maze, Hole Board, and Light/Dark Box

ALB and activity were assessed in the EPM, HB, and LD box. Overall, there were no group differences on any measure in the EPM and HB (all $p > 0.05$). However, PS rats entered the light side of the LD box less often than HC rats ($F(3,76) = 5.97$, $p < 0.001$, Bonferroni protected post hoc comparisons, PS vs. HC, $p < 0.05$, PSV vs. HC, $p < 0.05$:

Figure 7), indicating increased ALB (Adamec et al., 2006). Surprisingly, rapamycin had no effect on this measure (Bonferroni post hoc comparisons, PSR vs. HC, $p < 0.05$, PSR vs. PS, $p > 0.05$, PSR vs. PSV, $p > 0.05$). There were no differences between PS and PSV groups (PS vs. PSV, Bonferroni post hoc comparisons, $p > 0.05$). There were no other group differences in the LD box. Refer to Table 2 for complete statistical analysis. These data suggest that rapamycin, when given immediately following predator stress, does not block predator stress-induced ALB as measured in the LD box.

3.2.4 Weight Measurement

As seen in Experiment 1, a single injection of rapamycin decreased body weight gain (Figure 8). A mixed ANOVA revealed a main effect of group ($F(3,42) = 3.39$, $p < 0.05$), a main effect of day ($F(4, 172) = 262.1$, $p < 0.000001$), and an interaction of group x day ($F(4, 172) = 8.94$, $p < 0.0000001$). Weight of rats in the PSR group was significantly lower than other groups from day 7 to day 23 (all $p < 0.05$).

3.3 Experiment 3- The effects of post-retrieval rapamycin on predator stress-induced anxiety and hyperarousal

3.3.1 Cat- rat interaction during predator exposure

Once again, there were no differences in the behavior of the cat and rat across all groups and measures during predator exposure ($p > 0.05$). See Table 3 for complete statistical analyses.

3.3.2 Re-exposure to the predator stress context

Measures of activity were taken during the room re-exposure tested two days after the initial predator exposure in the PSV and PSR groups. Surprisingly, mixed ANOVAs revealed significant main effects in the total distance moved (cm) ($F(1,342)= 7.27$, $p <$

0.01, Figure 9 A), the total time mobile (s) ($F(1,342)= 7.57$, $p < 0.01$, Figure 9 B) and the total time immobile (s) ($F(1,38)= 12.925$, $p < 0.001$, Figure 9 C) during the room re-exposure for PSR and PSV groups. Rats in the PSR group moved less than rats in the PSV group. The room re-exposure was conducted prior to injection of rapamycin or vehicle and there were no group differences during the initial cat exposure, therefore, it is surprising that significant group differences were present.

3.3.3 Rapamycin given after re-exposure to the predator stress context potentiates hyperarousal

Startle response was measured in the HC, PS, PSR and PSV groups nine days after re-exposure to the predator stress room. Similar to Experiments 1 and 2, the non-normality of the data (Omnibus test = 2457.5, $p < 0.0001$) required the use of the Kruskal-Wallis non parametric chi square test of median differences across groups. Median peak startle amplitude across 30 trials revealed a main effect of group (X^2 (3) = 69.89, $p < 0.0001$: Figure 10 A). Consistent with Experiments 1 and 2, PS rats displayed increased peak startle amplitude compared to HC rats (Kruskal-Wallis Multiple-Comparison Z-test, $p < 0.05$, Figure 10 A). Re-exposure to the room (with a vehicle injection – PSV group) decreased peak startle amplitude to that of HC rats (Kruskal-Wallis Multiple-Comparison Z-test, $p > 0.05$, Figure 10 A) and this reduction was blocked by rapamycin (PSR group) (Kruskal-Wallis Multiple-Comparison Z-test, $p < 0.05$, Figure 10 A). It appears that a single room exposure (lasting 10 minutes) two days after predator stress extinguishes hyperarousal and this extinction is blocked by rapamycin.

Similar results were seen in rate of habituation to the tone. As previously described, rate of habituation to the tone was measured by the trial constant (Tau) estimated from fits of the exponential decay function

$$Y = Y_o e^{-1/Tau}$$

to mean peak startle amplitude over trials for each of the four groups (all df adjusted $r^2 > 0.87$, all exponential fits $F(3, 27) > 61.27$, $p < 0.01$, all Tau > 0, t tests $p < 0.01$). Handled control (HC) rats habituated faster (smaller Tau values) in comparison to predator stressed rats (PS) showing that rats exposed to a cat display an increased hyperarousal (Bonferroni protected t tests, $p < 0.01$; Figure 10 B). Vehicle injection following re-exposure in predator stressed rats increased habituation (smaller Tau values) to the tone comparable to handled control rats (HC vs. PSV, Bonferroni protected post hoc comparisons, $p > 0.05$, PS vs. PSV, $p < 0.05$, Figure 10 B). The suppression of hyperarousal suggests that re-exposure to the predator stress context extinguishes predator stress memory. An injection of rapamycin immediately after room re-exposure decreased habituation to predator stressed levels (Bonferroni protected post hoc comparisons, PSR vs. PS, $p > 0.05$, PSR vs. PSV, $p < 0.05$, PSR vs. HC, $p < 0.01$, Figure 10 B). It appears that rapamycin following re-exposure to the predator stress context blocks extinction of hyperarousal.

3.3.4 Elevated Plus Maze, Hole board, and Light/Dark Box

Surprisingly, there were no differences in ALB or activity measures across groups in the EPM, HB or LD box. Refer to Table 3 for statistical analyses.

3.3.5 Weight measurement

A single injection of rapamycin, given immediately after re-exposure to the predator stress context, significantly reduced body weight across days (Figure 11). A mixed ANOVA revealed a main effect of group [$F(3,148)= 37.24$, $p < 0.0001$], a main effect of day [$F(2,148)= 1452.62$, $p < 0.0001$], and an interaction of group x day [$F(6,148)= 52.31$, $p < 0.0001$].

3. 4 Experiment 4: The role of mTOR in extinction of predator stress-induced fear memories

3.4.1 Cat-rat interaction during predator exposure

There were no differences in the behavior of the cat and rat across all groups and measures during predator exposure (all $p > 0.05$). Thus, any subsequent differences across groups can be attributed to the treatment effects and not to variation in predator exposure. See Table 4 for complete statistical analyses.

3.4.2 Rapamycin without room re-exposure sensitizes startle response

Startle response was measured in the HC, PS, PSR and PSV groups. The non-normality of the data (Omnibus test = 1327.81, $p < 0.0001$) required the use of the Kruskal-Wallis non parametric chi square test of median differences across groups. Median peak startle amplitude across 20 trials revealed a main effect of group (X^2 (3) = 178.92, $p < 0.0001$: Figure 12 A). Consistent with Experiments 1-3, PS rats displayed increased peak startle amplitude compared to HC rats (Kruskal-Wallis Multiple-Comparison Z-test, $p < 0.05$, Figure 12 A). PSV rats (predator stressed rats given an injection of vehicle two days after predator exposure) exhibited startle amplitude levels equal to that of the PS group (Kruskal-Wallis Multiple-Comparison Z-test, $p > 0.05$,

Figure 12 A) and above that of the HC group (Kruskal-Wallis Multiple-Comparison Z-test, $p < 0.05$, Figure 12 A). These data suggest that in the absence of room re-exposure, rats do not show extinction of predator stress-induced hyperarousal. Surprisingly, PSR rats showed potentiated startle compared to all groups (Kruskal-Wallis Multiple-Comparison Z-test, all $p < 0.05$, Figure 12 A).

Similar to Experiments 1-3, rate of habituation to the tone was measured by the trial constant (Tau) estimated from fits of the exponential decay function

$$Y = Y_o e^{-1/Tau}$$

to mean peak startle amplitude over trials for each of the four groups (all df adjusted $r^2 > 0.81$, all exponential fits $F(3, 27) > 65.17$, $p < 0.01$, all Tau > 0, t tests $p < 0.01$). As expected, HC rats habituated faster (smaller Tau values) in comparison to PS rats (Bonferroni protected t tests, $p < 0.01$; Figure 12 B). PSV rats exhibited intermediate habituation levels (Bonferroni protected t tests, $p < 0.01$), while PSR rats showed a decreased rate of habituation compared to all groups (Bonferroni protected t tests, $p < 0.01$; Figure 12 B)

3.4.3 Elevated Plus Maze, Hole Board and Light-Dark Box

ALB and activity were measured in the EPM, HB and LD box for all four groups. Group differences were found in frequency of risk assessment in the EPM ($F(3,76) = 10.28$, $p < 0.01$: Figure 13 A). Consistent with previous data, rats in the HC group engaged in risk assessment more frequently than those in the PS and PSV groups, which did not differ (Bonferroni protected post hoc comparisons, HC vs. PS, $p < 0.05$, HC vs. PSV, $p < 0.05$, PS vs. PSV, $p > 0.05$, Figure 13 A). Rapamycin blocked the predator stress-induced decrease in frequency of risk assessment (PSR vs. PSV, $p < 0.05$, PSR vs.

HC, $p > 0.05$, Figure 13 A). However, PSR rats did not differ from PS rats (PSR vs. PS, $p > 0.05$, Figure 13 A). A group difference was also found in ratio time in the HB ($F(3,75)= 3.122$, $p < 0.05$: Figure 13 B). While there was no difference between handled controls and predator stressed rats (Bonferroni protected post hoc comparisons, PS vs. HC, $p > 0.05$, Figure 13 B), the PSR group displayed decreased ratio time in comparison to PSV rats (Bonferroni protected post hoc comparisons, PSR vs. PSV, $p < 0.05$, Figure 13 B). There were no significant differences in the LD box. Refer to Table 4 for statistical analysis.

3.4.4 Weight measurement

Similar to Experiments 1-3, body weight was significantly reduced seven days following rapamycin injection (Figure 14). A one-way ANOVA on the startle day revealed that rapamycin significantly reduced body weight in comparison to HC, PSV and PS groups ($F(3, 80)= 24.4$, $p < 0.001$, mean contrasts Tukey Kramer test all $p < 0.05$).

4.0 Discussion

While it has been established that the mTOR pathway plays a key role in associative fear memories (Bekinschtein et al, 2007; Blundell et al., 2008; Parsons et al, 2006), it is unknown whether this pathway mediates non-associative fear memories. Both fear conditioning and predator stress paradigms produce associative, context-dependent fear memories. However, predator stress also produces non-associative fear memories that are context-independent, such as hyperarousal and ALB. The goal of the present set of experiments was to determine the role of the mTOR pathway in predator stress-

induced non-associative and associative fear memories. Systemic administration of rapamycin, a selective inhibitor of mTOR, 30 minutes before (Experiment 1) or immediately following (Experiment 2) predator stress inhibits consolidation of associative and non-associative fear memories. Furthermore, rapamycin may block extinction of predator stress-induced non-associative fear memories (Experiments 3 and 4). Our data suggest that administration of the FDA-approved drug rapamycin, depending on time of administration, may have therapeutic relevance for the treatment of acquired anxiety disorders such as posttraumatic stress disorder (PTSD).

4.1 Consolidation of predator stress-induced fear memories

Consistent with previous studies (Adamec, Blundell & Burton, 2003; Adamec et al. 2006; Adamec, Head, Soreq & Blundell, 2008; Cohen & Zohar, 2004), predator stress lastingly increased hyperarousal, measured as increased startle response to an acoustic stimulus (Figures 1, 2, 6, 10). Increased startle response appeared as increased peak startle amplitude and decreased rate of habituation of peak startle amplitude (delayed habituation) following exposure to a cat. Similar to shock-induced associative fear memory, consolidation of predator stress-induced non-associative fear memories is mTOR-dependent. Rapamycin injected 30 minutes prior to (Experiment 1) or immediately after (Experiment 2) stress attenuated predator stress-induced hyperarousal (Figures 1 A, B, 6 A, B). Specifically, a reduction in startle amplitude and startle habituation to the tone was observed in stressed rats administered rapamycin. This is consistent with previous data which showed that consolidation of predator stress-induced hyperarousal is protein synthesis-dependent (Adamec et al., 2006; Cohen et al., 2006). Administration of anisomycin before or after exposure to a cat or to the scent of a cat

reduced startle amplitude and habituation measured seven days later (Adamec et al., 2006; Cohen et al., 2006). Given that the magnitude of the amnesic effect seen with rapamycin is quite similar to that found with anisomycin, and that rapamycin decreases protein synthesis only by 10–15% instead of 70–95% as seen with anisomycin (Morris, 2006; Parsons et al., 2006), the subset of transcripts whose translation is affected by rapamycin seems to be critical for predator stress-induced fear memory formation. Identification of these transcripts may aid in the development of novel, more effective treatment of acquired anxiety disorders such as PTSD. Future studies will be aimed at the identification of rapamycin-sensitive proteins following predator stress. Candidate upstream and downstream proteins are discussed in the section entitled "Potential Mechanisms of Action of mTOR in Non-associative Fear Memories".

When tested three weeks after predator stress, rapamycin-treated rats (rapamycin given 30 minutes prior to predator stress) showed startle amplitude and habituation levels similar to that of handled controls (Figure 2 A, B). These data are consistent with previous findings showing the persistent effect of an acute exposure to a predator (Adamec et al., 1993). Furthermore, it suggests that rapamycin's effects on predator stress-induced non-associative fear memories (in this case, hyperarousal) are long-lasting. The long-lasting effect of rapamycin on predator stress-induced non-associative fear memory is consistent with its lasting effect on shock-induced associative fear memories (Blundell et al., 2008). The current study only assessed hyperarousal three weeks post-predator stress; future studies will examine the long-lasting effects of rapamycin on predator stress-induced associative fear memories as well.

Consistent with previous studies (Adamec et al., 2006; Cohen et al., 2006), an increase in ALB was observed seven days following predator stress. Specifically, predator stressed rats exhibited a decrease in both ratio time (time spent in the open arms compared to time spent in all arms) and frequency of risk assessment in the EPM (Figure 3 A, B). In addition, predator stressed rats exhibited decreased ratio time (time in center compared to time in periphery) in the HB (Figure 3 C). Importantly, rapamycin given 30 minutes prior to predator stress blocked the predator stress-induced ALB in the HB and EPM (Experiment 1, Figure 3). These data suggest that the mTOR pathway mediates predator stress induced ALB, as measured in the HB and EPM. However, predator stress-induced ALB as measured in the LD box was not sensitive to rapamycin (Experiment 2, Figure 7). When rapamycin was injected immediately after predator stress, there were no differences in the LD box between predator stressed animals given rapamycin or vehicle. These data are consistent with the view that different neural substrates likely mediate different aspects of ALB (Adamec, 2001; Adamec et al., 2001; Adamec et al., 2006; Adamec, Blundell & Burton, 2006). It must be pointed out, however, that predator stress-induced ALB was not found in the LD box in Experiment 1. Thus, it is not known if rapamycin given 30 minutes prior to predator stress would affect subsequent ALB in the LD box.

Note that changes in all measures of ALB (as measured in the EPM, HB, LD) following predator stress were not consistently found across experiments. For example, predator stress-induced ALB was evident in the LD box, but not the EPM and HB in Experiment 2 while in Experiment 4, predator stress-induced ALB was evident in the EPM, but not HB and LD. Furthermore, predator stress did not affect any measure of

ALB in Experiment 3. Given that the same cat and cat exposure protocol were used across experiments, it is unclear why this variability in ALB exists. However, not all studies have reported changes in all measures of ALB following predator stress (Adamec, 2001; Adamec et al., 2001; Adamec et al., 2006; Adamec, Blundell & Burton, 2006; Adamec, Walling & Burton, 2004). Adamec, Blundell and Burton (2006) found significant increases in ALB following predator stress in the EPM, but no effect in the LD box. Similarly, an increase in ALB was observed in some measures in the EPM, namely an increase in risk assessment, while measures in the HB and LD box were unaffected (Adamec, Walling and Burton, 2004). Unlike ALB, hyperarousal is consistently shown following exposure to a predator (or predator odors) (in current experiments, and Adamec, Blundell & Burton, 2003; Adamec et al., 2006; Adamec, Head, Soreq & Blundell, 2008; Cohen & Zohar, 2004). Given that hyperarousal, and not ALB, is a core symptom of PTSD, future studies will focus on the long-lasting changes in hyperarousal following cat exposure.

In addition to its effects on non-associative fear memories, rapamycin inhibited predator stress-induced associative fear memories. When re-exposed to the predator stress context without the presence of a cat, rapamycin-treated rats were more active (travelled more distance, more mobile, less immobile) in comparison to predator stressed rats and predator stressed rats given a vehicle injection (Experiment 1, Figure 4). Blundell et al. (2008) have shown similar results with shock-induced fear memories wherein, mice treated with rapamycin froze less in comparison to vehicle controls when re-exposed to the context that was previously paired with a shock. Thus, similar mechanisms mediating associative fear memories appear to be present across paradigms.

4.2 Extinction of non-associative fear memories

To our knowledge, the role of mTOR following reactivation of predator stress-induced fear memory is unknown. Thus, the second goal of these experiments was to examine the effects of rapamycin following reactivation of a predator stress-induced associative fear memory on subsequent hyperarousal and ALB. Predator stressed rats re-exposed to the predator stress context (without the cat present) for 10 minutes (and given vehicle) exhibited decreased hyperarousal compared to predator stressed only rats (Experiment 3, Figure 10). In fact, startle amplitude and habituation equalled that of handled control rats suggesting that a single, 10 minute re-exposure to the context was sufficient to abolish predator stress-induced hyperarousal. It is important to note that extinction to the predator stress context was evident during the room re-exposure in the predator stressed vehicle rats. Distance moved and time mobile increased, while time immobile decreased across the 10 minute re-exposure (Figure 9). To confirm that re-exposure to the predator stress context was sufficient to produce extinction of hyperarousal, predator stressed rats were given an injection of vehicle or rapamycin two days following cat exposure but *not* re-exposed to the predator stress context (Experiment 4). Without re-exposure to the predator stress context, one would expect no extinction in the predator stressed rats given vehicle. This was what was seen, as predator stressed rats given vehicle (without re-exposure to the predator stress context) exhibited hyperarousal levels equalling that of predator stressed alone rats (Experiment 4, Figure 12). Thus, it appears that a single 10 minute re-exposure to the predator stress context is sufficient to cause extinction of both startle amplitude and habituation. This is consistent with previous work from our laboratory (Clay et al., 2011). Overall, our findings suggest that

extinction of a context-dependent, predator stress-induced fear memory may also reduce the generalized, persistent, PTSD-like symptom of hyperarousal.

Rapamycin following reactivation of the predator stress-induced contextual fear memory blocked consolidation of extinction of predator stress-induced hyperarousal. Indeed, rapamycin-treated rats show startle amplitude and startle habituation equal to that of predator stressed rats not re-exposed to the predator stress context (Experiment 3, Figure 10). These data are somewhat surprising given that rapamycin following fear memory reactivation blocks subsequent recall of the shock-induced, contextual fear memory (Blundell et al., 2008). In that case, the authors clearly distinguished between an effect of rapamycin on reconsolidation rather than on extinction. In particular, the effect of rapamycin was not reversed by a reminder shock which is known to overcome effects of both standard extinction and extinction augmented pharmacologically. Furthermore, the effect of rapamycin did not show spontaneous recovery which can occur following extinction. It is not surprising that the mechanisms underlying predator stress-induced fear memory and shock-induced fear memory are different. Indeed, we have previously shown that glucocorticoids mediate extinction of shock-induced contextual fear memories (Blundell et al., 2011) but not predator stress-induced contextual fear memoires (Clay et al., 2011). Our data are consistent with recent data showing that the protein synthesis inhibitor, cycloheximide, given following reactivation of a predator stress-induced contextual fear memory blocks extinction of non-associative fear memories (in this case, ALB measured in the EPM (Sandusky et al., 2012)). As mentioned above, there was no effect of predator stress in the EPM in Experiment 3 (or on any measure of ALB); however, rapamycin following reactivation of a predator stress-induced contextual fear

memory did block extinction of another non-associative fear memory, hyperarousal. Thus, mTOR-dependent protein synthesis facilitates extinction of predator stress-induced non-associative fear memories. Identity of these specific proteins will be the focus of future studies.

While our data support an effect of rapamycin on extinction, it must be noted that prior to treatment (with rapamycin or vehicle), rapamycin-treated rats showed less activity (and more time immobile) during the re-exposure to the cat room than vehicle controls (Experiment 3, Figure 9). We would have expected these groups to be identical during the room re-exposure given that they had yet to receive treatment (injections) and that there were no group differences during the initial cat exposure. Nevertheless, there were group differences in time immobile, time mobile, and distance travelled. While both rapamycin- and vehicle-treated rats showed extinction during the room re-exposure (increased activity over the 10 min re-exposure), the rapamycin-treated rats showed less extinction than vehicle-treated rats during the room re-exposure. Less extinction in the rapamycin-treated rats during room re-exposure may have contributed to the elevated startle response.

Surprisingly, rapamycin injected two days after predator stress (without re-exposure to the predator stress room) potentiated startle (Experiment 4, Figure 12). In fact, rapamycin-treated predator stressed rats show increased peak startle amplitude and delayed habituation in comparison to both predator stress and predator stress rats given vehicle. This effect of rapamycin was opposite to that seen when rapamycin was injected 30 min prior to or immediately after predator stress (Experiments 1 and 2, Figures 1, 6) thus it is not simply a drug effect. Rather, these data suggest that rapamycin given two

days post stress may be interfering with post predator stress processes that reduce startle. The identity and length of these processes is yet unknown. Thus, future studies will examine the effects of rapamycin at various time points post predator stress on subsequent hyperarousal.

Rapamycin given two days following predator stress produced inconsistent effects across tests of ALB. In the EPM, rapamycin produced a slight anxiolytic effect on risk assessment (Figure 13 A) while in the HB, rapamycin reduced ratio time (in comparison to vehicle controls) indicating an anxiogenic effect. The reason behind these differences is unknown. As mentioned above, given that predator stress-induced effects on ALB are not consistently found, future studies will focus on the long-lasting changes in hyperarousal following cat exposure.

4.3 Neuroanatomy, mTOR, and fear memories

Consolidation of predator stress-induced fear memories is dependent on amygdala circuitry. In particular, predator stress-induced fear memories involve potentiation of ventral hippocampal inputs to the basolateral amygdala and central amygdala outputs to the periaqueductal gray following consolidation (Adamec, Blundell & Burton, 2006). Potentiation in both pathways positively correlates with the severity of negative affective changes (Adamec, Blundell & Burton, 2006). In addition, inhibition of the prefrontal cortex (PFC) follows predator stress. In particular, cFos expression of medial prefrontal cortex (mPFC) cells is reduced in highly anxious rats following predator stress exposure (Adamec et al., 2012). As well, reduced suppression of phosphorylated calcium/calmodulin-dependent protein kinase II (p-CaMKII), a kinase involved in LTP, is seen in the mPFC following predator stress (Zoladz et al., 2012). This suggests that the

mPFC may play a protective role to inhibit emotional responses following traumatic stress (Adamec et al., 2012).

Importantly, recent studies reveal that mTOR regulation of protein synthesis in the amygdala (Parsons, Gafford, & Helmstetter, 2006) and hippocampus (Bekinschtein et al., 2007), as well as the medial prefrontal cortex (Sui, Wang, & Li, 2008) are necessary for the consolidation of shock-induced associative fear memories. In particular, p70s6K and 4E-BPs (downstream targets of mTOR) levels were elevated in the hippocampus, amygdala, or PFC during consolidation of associative fear memories (Gafford et al., 2011; Parsons et al., 2006; Slipczuk et al, 2009; Sui et al., 2008). Furthermore, when rapamycin was injected into the amygdala, hippocampus, or PFC during training, fear memory recall and p70S6K levels were inhibited (Gafford et al., 2011; Parsons et al., 2006; Slipczuk et al, 2009; Sui et al., 2008). These studies suggest that mTOR activity within the amygdala, hippocampus and mPFC is required for associative fear learning. Although no previous studies have examined mTOR regulation of predator stress-induced fear memories, it is likely that consolidation of both associative and non-associative fear memories share common brain areas and neural mechanisms. Future studies will examine mTOR activation in these brain areas during consolidation of predator stress-induced fear memories.

Presently, brain areas involved in extinction of non-associative fear memories produced through predator stress are unknown. However, the functional neuroanatomy involved in extinction of associative fear memories has been well documented. Given that the neural circuitry underlying consolidation of associative and non-associative fear memories is similar, it is likely that the neural circuitry underlying extinction of both

types of fear memories is also similar. Like consolidation, several studies have implicated the amygdala (Pare et al., 2004; Davis, 2006; Pare & Smith, 1998; Chatwal et al., 2005; Markram et al., 2007) the medial prefrontal cortex (mPFC) (Barrett et al., 2003; Phelps et al., 2004; Santini et al., 2004; Milad, et al., 2005; Morgan et al., 1993; Quirk et al., 2000; Milad & Quirk, 2002) and the hippocampus (Duvcarci & Pare, 2007; Corcoran et al., 2005) in extinction of shock-induced associative fear memories. Specifically, the infralimbic region of the mPFC inhibits the central nucleus of the amygdala, an area involved in mediating fear responses, through intercalated cells (Pare et al., 2004). Indeed, studies have shown that extinction of conditioned fear is inhibited with lesion of the infralimbic region of the mPFC (Morgan et al., 2003; Quirk et al. 2000). Metabolic mapping of brain activity following extinction of conditioned fear shows increased activity in the prefrontal cortex (Barrett et al., 2003). Increased activation in the ventral mPFC following extinction of a conditioned response is also seen in human subjects (Phelps et al., 2004). This supports the view that the mPFC inhibits the amygdala and, correspondingly, inhibits conditioned emotional responses. In addition to the amygdala and prefrontal cortex, previous studies have implicated the hippocampus in extinction of shock-induced associative fear memories (Corcoran et al., 2005; Fiorenza et al., 2012; Maren & Hobin, 2007; Orsini et al., 2011). Specifically, pharmacological inhibition via muscimol, a GABAA receptor agonist, of the dorsal hippocampus disrupts extinction of conditioned fear (Corcoran et al., 2005; Maren & Hobin, 2007). Input from both the ventral hippocampus (VH) and the PFC to the amygdala (amygdaloid basal nuclei (BA)) is involved in renewal of a fear response after extinction learning, while disconnecting projections from the VH to the BA impedes renewal of fear learning (Orsini et al., 2011).

Since these brain areas are involved in extinction of associative fear memories, it is likely that these brain areas are involved in extinction of non-associative fear memories as well. Given that rapamycin blocks extinction of predator stress-induced non-associative fear memoires, future studies will examine mTOR activation in these brain areas.

4.4 Potential mechanisms of action of mTOR in non-associative fear memories

Given current and previous data (Cai et al., 2006; Clay et al., 2011, Blundell et al., 2011), we can speculate as to a possible mechanism underlying the effects of rapamycin on predator stress-induced fear memories. For instance, rapamycin may act by inhibiting glucocorticoid release (corticosterone in animals). Previous studies have shown that animals exposed to a predator or predator odor display increased levels of corticosterone (CORT) (Blanchard et al., 1998; Wang et al., 2012) while block of the mineralcorticoid receptor (a CORT receptor) prevents consolidation of predator stress-induced hyperarousal and ALB (Adamec et al., 2007). Incidentally, the mineralcorticoid antagonist blocked all predator stress-induced behaviors excluding ALB in the LD, which was also rapamycin-insensitive (Experiment 2, Figure 7). It may be that rapamycin reduces predator stress-induced CORT release and as a result, prevents predator stress-induced hyperarousal and most ALB. In addition to CORT's effect on consolidation, we have previously shown that blocking CORT following reactivation of a predator stress-induced contextual fear memory prevents extinction of hyperarousal (Clay et al., 2011). Thus, if rapamycin reduces CORT, then rapamycin given following reactivation of a predator stress memory should potentiate startle. Indeed, this is what we found (Experiment 3, Figure 10). Thus, our data suggest that rapamycin may act by inhibiting CORT release. Future studies will assess CORT levels following rapamycin treatment

before predator stress or after reactivation. If rapamycin does indeed block CORT, future studies will begin to determine the mechanism by which mTOR modulates glucocorticoid release.

Specific upstream and downstream targets of mTOR in the hippocampus have been identified that may play a role in consolidation of associative and non-associative fear memories. It is well known that rapamycin inhibits mTOR function by preventing the phosphorylation of its downstream targets, p70S6K and 4E-BP and thus, interfering with the initiation of translation of a subset of mRNAs rather than general translation (Kim et al., 2002). Rapamycin blocks long term memory formation in several learning tasks (Deli et al., 2012; Jobim et al., 2012; Stoica et al., 2011; Qi et al., 2010), including predator stress (Figures 1, 2, 6). While little is known about the extracellular signals triggered by training that are essential to activate mTOR for regulation of protein synthesis during memory consolidation, a recent report suggests that brain-derived neurotrophic factor (BDNF) may be one critical factor (Slipczuk et al., 2009). BDNF, a member of the neurotrophins, has been implicated in synaptic plasticity (Garcia et al., 2010; Lessmann & Brigadski, 2009; Nanobashvili et al., 2005) and memory formation (Monfils et al., 2007; Ou & Gean, 2006; Ou & Gean, 2007; Rattiner et al., 2004a; Rattiner et al., 2004b; Slipczuk et al., 2009). With respect to mTOR, BDNF induces rapamycin-sensitive synaptic potentiation (Tang et al., 2002) and regulates translation of dendritic proteins through an mTOR-dependent pathway (Takei et al., 2004). Importantly, blocking BDNF in the dorsal hippocampus prior to or three hours after fear conditioning abolishes mTOR activation and p70S6K phosphorylation, as well as inhibits associative, shock-induced fear memory consolidation (Slipczuk et al., 2009). Changes in BDNF expression

following predator stress have been reported (Kozlovsky et al., 2007; Kozlovsky et al., 2008), however, the effect of blocking BDNF prior to or following predator stress on mTOR expression and predator stress-induced hyperarousal have not been examined. Given that rapamycin blocks consolidation of both predator stress- and shock-induced fear memories, it is likely that BDNF expression in the hippocampus and amygdala mediates predator stress-induced, mTOR-dependent hyperarousal. Thus, future studies will examine the effects of blocking BDNF on consolidation of predator stress-induced mTOR expression and hyperarousal.

Interestingly, recent data suggests that mTOR may mediate GluR1 expression through its downstream targets, p70S6K and 4E-BP. Previous studies have shown that GluR1-containing AMPA receptors in CA3-CA1 synapses (Mitsushima et al., 2011; Takahashi, 2011) and lateral amygdala synapses (Nedelescu et al., 2010) are required for associative, shock-induced fear learning. Blocking BDNF before or after inhibitory avoidance training inhibits subsequent mTOR activity, p70S6K phosphorylation and GluR1 expression, as well as consolidation of the associative fear memory (Slipczuk et al, 2009). Given that consolidation of associative and non-associative fear memories are mTOR-dependent; future studies will examine the effect of rapamycin on GluR1 expression following consolidation of predator stress-induced fear memories.

4.5 Rapamycin reduces body weight

We demonstrated that a single systemic injection of rapamycin before or after predator stress exposure inhibits body weight gain, lasting at least 23 days (Figures 5, 8, 11, 14). Although rats were exposed to predator stress in the current set of experiments, rapamycin's suppression of body weight gain has been reported in the absence of stress

(Chang et al., 2009; Cota, 2009; Cybulski et al., 2009; Deblon et al., 2012; Krebs et al., 2007; Polak et al., 2008). In contrast to previous studies which examined the effects of multiple injections of rapamycin, our laboratory has recently shown that a single injection of rapamycin (systemic) dose-dependently decreases food intake (lasting about five days), body weight gain (lasting at least 60 days), and food efficiency (lasting about three days) without compensatory rebounds in any of these measures (Hebert et al., submitted). In addition, total visceral fat and fat cell size were decreased in rapamycin-treated rats. It is important to note that the effect of rapamycin was not due to malaise, as rapamycin-treated rats do not show conditioned taste avoidance. Finally, centrally administered rapamycin (i.c.v.) produced a similar pattern of results, suggesting that at least some of the systemic effects may be mediated by a central action of rapamycin. Our findings are consistent with others that have shown that knockout of mTOR substrates produces a leaner phenotype. For example, knockout of raptor, a component of mammalian TOR complex 1 (mTORC1), results in lean mice with reduced adipose tissue despite a fixed caloric intake and normal physical activity (Polak et al., 2008). As described above, mTORC1 activates downstream targets p70S6K and 4EBPs, targets involved in cell growth and division (Hay & Sonenberg, 2004). Knockout of the downstream target of mTORC1, S6K1, in mice also results in a lean phenotype which is resistant to diet-induced obesity (Shima et al., 1998; Um et al., 2004). Taken together, our results indicate that rapamycin has potent, consistent and persistent effects on food intake and body weight regulation which cannot be explained by the presence of malaise or illness. In light of these data, rapamycin may be a viable treatment option for obese individuals.

4.6 Implications for PTSD

We demonstrated that a systemic injection of rapamycin inhibits consolidation of associative and non-associative fear memories (Figures 1, 2, 6). This finding has clinical relevance, as individuals with PTSD display intrusive traumatic memories and heightened hyperarousal (Kamkwalala et al., 2012). The data suggest that the mTOR pathway is involved in the formation and prolonged sustainability of traumatic memories. Therefore, rapamycin may block memory of the traumatic event in patients suffering from PTSD. However, timing of rapamycin administration appears critical as we now show that rapamycin given 48 hours following stress potentiates stress-induced hyperarousal. Elucidating the molecular factors contributing to both associative and non-associative fear memories will provide understanding into the nature of pathological fear disorders such as PTSD. This will aid in the development of novel therapeutic agents to treat these disorders.

4.7 General conclusions

Consolidation of predator stress-induced fear memories (both associative and non-associative) is mTOR-dependent. This is consistent with studies showing that consolidation of shock-induced fear memories is also mTOR-dependent (Bekinschtein et al., 2007; Blundell et al., 2008; Tishmeyer et al. 2003). Unlike shock-induced fear memories, however, it appears that mTOR facilitates extinction of predator stress-induced fear memories. We also show that a single, systemic injection of rapamycin causes a persistent reduction in body weight. Overall, these data suggest that the mTOR inhibitor, rapamycin, under specific conditions, may be a novel treatment for patients suffering from acquired anxiety disorders such as PTSD.

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Table 1

Experiment 1 – The role of mTOR in predator stress-induced fear memories.

Test Variant	**Parameter**	**Comparison**	**Mean and Standard Deviation**	**n of each group**	**Results**
Predator Exposure	Frequency of rat approaches to cat	PS vs. PSR vs. PSV	**PS:** Mean= 0.5 SD=1.15 **PSR:** Mean= 0.7 SD=0.979 **PSV:** Mean=1.00 SD=1.53	20	1-way ANOVA: group $F(2,54)=1.96$, $p=0.151$
	Frequency of rat flights from the cat	PS vs. PSR vs. PSV	**PS:** Mean= 0.05 SD=0.224 **PSR:** Mean=0.05 SD= 0.224 **PSV:** Mean= 0 SD= 0	20	1-way ANOVA: group $F(2,54)=0.534$, $p=0.589$
	Frequency of cat approaches to rat	PS vs. PSR vs. PSV	**PS:** Mean= 0.2 SD=0.523 **PSR:** Mean= 0.3 SD=0.733 **PSV:** Mean=0.1053 SD=0.315	20	1-way ANOVA: group $F(2,54)=0.067$, $p=0.935$
	Frequency of cat physically contacting the rat with paw	PS vs. PSR vs. PSV	**PS:** Mean= 0 SD= 0 **PSR:** Mean=0.05 SD= 0.224 **PSV:** Mean= 0 SD= 0	20	1-way ANOVA: group $F(2,54)=0$, $p=1.0$
	Total time cat and rat were within one square of each other (s)	PS vs. PSR vs. PSV	**PS:** Mean=11.83 SD=32.82 **PSR:** Mean=7.98 SD=12.40 **PSV:** Mean=12.46 SD=19.80	20	1-way ANOVA: group $F(2,54)=0.855$, $p=0.431$

Table 1 (Continued)

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Room Re-exposure	Total distance moved (cm)	PS vs. PSR vs. PSV	**PS:** Mean=1567.75 SD=1020.12 **PSR:** Mean=2725.76 SD=907.40 **PSV:** Mean=1903.23 SD=958.61	20	1-way ANOVA: group F(2,54)=7.50, p=0.001* Bonferroni post hoc comparisons PSR vs. PS, $p < 0.05$, PSR vs. PSV, $p < 0.05$
	Total time Immobile (s)	PS vs. PSR vs. PSV	**PS:** Mean=506.48 SD=68.19 **PSR:** Mean=433.60 SD=55.73 **PSV:** Mean=487.84 SD=59.37	20	1-way ANOVA: group F(2,54)=7.47, p=0.001* Bonferroni post hoc comparisons PSR vs. PS, $p < 0.05$, PSR vs. PSV, $p < 0.05$
	Total time mobile (s)	PS vs. PSR vs. PSV	**PS:** Mean=93.53 SD=68.20 **PSR:** Mean=166.41 SD=55.74 **PSV:** Mean=112.17 SD=59.36	20	1-way ANOVA: group F(2,54)=7.47, p=0.001* Bonferroni post hoc comparisons PSR vs. PS, $p < 0.05$, PSR vs. PSV, $p < 0.05$
Acoustic Startle Response	Median peak startle Amplitude (Volts per kg)	HC vs. PS vs. PSR vs. PSV	**HC:** Median= 5.85 SEMd=0.120 **PS:** Median=6.64 SEMd=0.310 **PSR:** Median=5.85 SEMd=0.190 **PSV:** Median=6.29 SEMd=0.241	20	Kruskal Wallis $X^2(3)=8.81, p<0.032$* Median contrasts Kruskal Wallis multiple z test $p<0.05$.

Table 1 (Continued)

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Acoustic Startle Response	Habituation (Tau)	HC vs. PS vs. PSR vs. PSV	**HC:** Median=2.13 SE=1.23 **PS:** Median=5.56 SE=0.267 **PSR:** Median=1.86 SE=0.100 **PSV:** Median=4.47 SE=0.135	20	All fit $F(2,27)=8.60, p<0.01$* All Tau > 0 $t(27)=9.58, p<0.04$* Tau contrasts all $t(58)=2.109, p<0.04$*
Second Acoustic Startle Response	Median peak startle amplitude (Volts per kg)	HC vs. PS vs. PSR vs. PSV	**HC:** Median=3.77 SEMd=0.17 **PS:** Median=7.01 SEMd=0.30 **PSR:** Median=3.78 SEMd=0.12 **PSV:** Median=6.09 SEMd=0.22	20	Kruskal Wallis $X^2(3)=210.41, p<0.001$* Kruskal Wallis multiple z test $p<0.01$*
	Habituation (Tau)	HC vs. PS vs. PSR vs. PSV	**HC:** Median=2.11 SE=0.22 **PS:** Median=4.51 SE=0.26 **PSR:** Median=1.55 SE=0.13 **PSV:** Median=3.82 SE=0.30	20	All $F(2,27)=108.73, p<0.001$* All tau > 0 $t(29)=7.01, p<0.001$ Tau comparisons all differences $t(58)=4.58, p<0.001$*
Hole Board	Frequency of head dips	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=14.55 SD=4.22 **PS:** Mean=12.40 SD=4.71 **PSR:** Mean=13.05 SD=3.87 **PSV:** Mean=13.79 SD=2.80	20	1-way ANOVA: group $F(3,75)=1.09$, $p=0.359$

Table 1 (Continued)

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Hole Board	Frequency of rears	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=21.70 SD=6.25 **PS:** Mean=15.20 SD=5.45 **PSR:** Mean=18.55 SD=7.20 **PSV:** Mean=15.16 SD=5.94	20	1-way ANOVA: group $F(3,75)=4.98$, $p=0.003$* Bonferroni protected post hoc comparisons HC vs. PS, $p < 0.05$, HC vs. PSV, $p < 0.05$
	Ratio Time	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=0.321 SD=0.125 **PS:** Mean=0.180 SD=0.119 **PSR:** Mean=0.274 SD=0.131 **PSV:** Mean=0.237 SD=0.106	20	1-way ANOVA: group $F(3,75)=4.918$, $p=0.004$* Bonferroni protected post hoc comparisons HC vs. PS, $p < 0.05$, PSR vs. PS, $p < 0.05$
Elevated Plus Maze	Frequency of risk assessment	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=10.10 SD=3.07 **PS:** Mean=6.80 SD=4.22 **PSR:** Mean=10.15 SD=2.35 **PSV:** Mean=7.21 SD=3.30	20	1-way ANOVA: group $F(3,75)=5.93$, $p=0.001$* Bonferroni protected post hoc comparisons HC vs. PSR, $p > 0.05$, HC vs. PS, $p < 0.05$, HC vs. PSV, $p < 0.05$, PSR vs. PS, $p < 0.05$, PSR vs. PSV, $p < 0.05$
	Total time risk assessment (s)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=35.51 SD=17.71 **PS:** Mean=31.21 SD=22.90 **PSR:** Mean=37.67 SD=13.61 **PSV:** Mean=31.15 SD=18.26	20	1-way ANOVA: group $F(3,75)=0.613$, $p=0.608$

Table 1 (Continued)

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Elevated Plus Maze	Total distance moved (cm)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=4065.11 SD=1720.84 **PS:** Mean=3323.11 SD=1706.00 **PSR:** Mean=4309.11 SD=1634.22 **PSV:** Mean=3561.21 SD=1576.85	20	1-way ANOVA: group F(3,75)=1.47, p=0.229
	Ratio Time	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=0.3227 SD=0.2370 **PS:** Mean=0.1138 SD=0.1342 **PSR:** Mean=0.3377 SD=0.1940 **PSV:** Mean=0.2086 SD=0.2345	20	1-way ANOVA: group F(3,75)=5.310, p=0.002* Bonferroni protected post hoc comparisons HC vs. PS, $p < 0.05$, HC vs. PSV, $p < 0.05$, PSR vs. PS, $p < 0.05$, PSR vs. PSV, $p < 0.05$
	Ratio Frequency	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=0.2802 SD=0.1786 **PS:** Mean=0.1928 SD=0.1166 **PSR:** Mean=0.2804 SD=0.1535 **PSV:** Mean=0.1737 SD=0.1588	20	1-way ANOVA: group F(3,71)=2.507, p=0.066
Light/Dark Box	Frequency to enter light	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=14.63 SD=5.31 **PS:** Mean=13.3 SD=5.06 **PSR:** Mean=14.74 SD=10.52 **PSV:** Mean=14.95 SD=6.30	20	1-way ANOVA: group F(3,73)=0.217,p=0.884

Table 1 (Continued)

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Light/Dark Box	Total time in light (s)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=96.40 SD=25.59 **PS:** Mean=84.41 SD=36.13 **PSR:** Mean=71.41 SD=23.10 **PSV:** Mean=82.44 SD=27.06	20	1-way ANOVA: group $F(3,73)=2.45, p=0.071$

Table 2

Experiment 2 – The role of mTOR in consolidation of predator stress-induced fear memories.

Test Variant	**Parameter**	**Comparison**	**Mean and Standard Deviation**	**n of each group**	**Results**
Predator Exposure	Frequency of rat approaches to the cat	PS vs. PSR vs. PSV	**PS:** Mean= 0.5 SD=1.15 **PSR:** Mean= 0.7 SD=0.979 **PSV:** Mean=1.00 SD=1.53	20	1-way ANOVA: group $F(2,56)=0.809$, $p=0.451$
	Frequency of rat flights from the cat	PS vs. PSR vs. PSV	**PS:** Mean= 0.05 SD=0.224 **PSR:** Mean=0.05 SD= 0.224 **PSV:** Mean= 0 SD= 0	20	1-way ANOVA: group $F(2,56)=0.475$, $p=0.625$
	Frequency of cat approaches to rat	PS vs. PSR vs. PSV	**PS:** Mean= 0.2 SD=0.523 **PSR:** Mean= 0.3 SD=0.733 **PSV:** Mean=0.1053 SD=0.315	20	1-way ANOVA: group $F(2,56)=0.602$, $p=0.551$
	Frequency of cat physically contacting the rat with paw	PS vs. PSR vs. PSV	**PS:** Mean= 0 SD= 0 **PSR:** Mean=0.05 SD= 0.224 **PSV:** Mean= 0 SD= 0	20	1-way ANOVA: group $F(2,56)=0.974$, $p=0.384$
	Total time cat and rat were within one square of each other (s)	PS vs. PSR vs. PSV	**PS:** Mean=11.83 SD=32.82 **PSR:** Mean=7.98 SD=12.40 **PSV:** Mean=12.46 SD=19.80	20	1-way ANOVA: group $F(2,56)=0.214$, $p=0.808$

Table 2 (Continued)

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Acoustic Startle Response	Median peak startle Amplitude (Volts per kg)	HC vs. PS vs. PSR vs. PSV	**HC:** Median=5.90 SEMd=0.20 **PS:** Median=8.40 SEMd=0.30 **PSR:** Median=8.50 SEMd=0.25 **PSV:** Median=5.80 SEMd=0.25	20	Kruskal Wallis $X^2(3) = 75.94$, $p < .001$* Kruskal-Wallis Multiple-Comparison Z-test, $p < .001$*
	Habituation (Tau)	HC vs. PS vs. PSR vs. PSV	**HC:** Median=3.00 SE=0.35 **PS:** Median=4.75 SE=0.45 **PSR:** Median=4.70 SE=0.40 **PSV:** Median=2.80 SE=0.35	20	All fit $F(2,27)=75.2$, $p<0.001$, all Tau>0, t tests $p<0.01$*
Hole Board	Total distance moved (cm)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=2756.50 SD=314.03 **PS:** Mean=2691.47 SD=486.61 **PSR:** Mean=2842.47 SD=637.91 **PSV:** Mean=2865.54 SD=515.02	20	1-way ANOVA: group $F(3,75)=0.511$, $p=0.676$
	Frequency of entries into center	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=22.90 SD=9.57 **PS:** Mean=27.70 SD=12.01 **PSR:** Mean=31.42 SD=16.48 **PSV:** Mean=26.35 SD=16.96	20	1-way ANOVA: group $F(3,75)=1.22$, $p=0.307$

Table 2 (Continued)

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Hole Board	Total time in center (s)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=65.96 SD=26.65 **PS:** Mean=73.86 SD=30.74 **PSR:** Mean=62.00 SD=23.90 **PSV:** Mean=54.79 SD=21.96	20	1-way ANOVA: group $F(3,75)=1.86$, $p=0.143$
	Total time in periphery (s)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=229.33 SD=29.19 **PS:** Mean=220.98 SD=31.43 **PSR:** Mean=233.35 SD=23.16 **PSV:** Mean=241.33 SD=22.07	20	1-way ANOVA: group $F(3,75)=1.996$, $p=0.122$
	Ratio Time	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=0.3087 SD=0.1781 **PS:** Mean=0.3627 SD=0.2132 **PSR:** Mean=0.2779 SD=0.1302 **PSV:** Mean=0.2373 SD=0.1186	20	1-way ANOVA: group $F(3,75)=2.05$, $p=0.114$
Elevated Plus Maze	Frequency of risk assessment	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=15.00 SD=3.15 **PS:** Mean=9.90 SD=3.95 **PSR:** Mean=13.4 SD=4.10 **PSV:** Mean=10.9 SD=4.27	20	1-way ANOVA: group $F(3,76)=7.11$, $p=0.001$*

Table 2 (Continued)

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Elevated Plus Maze	Total time risk assessment (s)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=53.71 SD=14.42 **PS:** Mean=77.68 SD=57.25 **PSR:** Mean=63.98 SD=19.46 **PSV:** Mean=52.89 SD=20.70	20	1-way ANOVA: group $F(3,76)=2.49$, $p=0.066$
	Ratio time	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=0.2743 SD=0.1668 **PS:** Mean=0.2556 SD=0.2766 **PSR:** Mean=0.2821 SD=0.1535 **PSV:** Mean=0.1739 SD=0.1800	20	1-way ANOVA: group $F(3,66)=1.17$, $p=0.329$
	Ratio frequency	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=21.34 SD=14.64 **PS:** Mean=12.15 SD=16.91 **PSR:** Mean=17.91 SD=14.21 **PSV:** Mean=14.76 SD=12.55	20	1-way ANOVA: group $F(3,75)=1.46$, $p=0.233$
Light/Dark Box	Frequency to enter dark	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=8.90 SD=2.32 **PS:** Mean=6.70 SD=1.84 **PSR:** Mean=7.80 SD=2.26 **PSV:** Mean=7.25 SD=2.75	20	1-way ANOVA: group $F(3,76)=3.30$, $p=0.025$*

Table 2 (Continued)

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Light/Dark Box	Total time in dark (s)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=142.21 SD=29.38 **PS:** Mean=151.08 SD=48.08 **PSR:** Mean=156.83 SD=29.27 **PSV:** Mean=157.40 SD=38.60	20	1-way ANOVA: group $F(3,76)=0.719$, $p=0.544$
	Frequency to enter light	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=8.10 SD=2.34 **PS:** Mean=5.90 SD=1.74 **PSR:** Mean=6.20 SD=1.44 **PSV:** Mean=5.85 SD=2.18	20	1-way ANOVA: group $F(3,76)=5.97$, $p=0.001$* Bonferroni protected post hoc comparisons, HC vs. PS, $p < 0.05$; HC vs. PSR, $p < 0.05$; HC vs. PSV, $p < 0.05$
	Total time in light (s)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=112.53 SD=28.98 **PS:** Mean=110.67 SD=41.63 **PSR:** Mean=105.15 SD=34.40 **PSV:** Mean=97.51 SD=40.21	20	1-way ANOVA: group $F(3,76)=0.677$, $p=0.569$

Table 3

Experiment 3 – The effects of post-retrieval rapamycin on predator stress-induced anxiety and hyperarousal.

Variant Test	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Predator Exposure	Frequency of rat approaches to the cat	PS vs. PSR vs. PSV	**PS:** Mean= 0.7 SD=0.923 **PSR:** Mean=0.7 SD=1.22 **PSV:** Mean=0.631 SD=1.07	20	1-way ANOVA: group $F(2,56)=0.026$, $p=0.974$
	Frequency of rat flights from the cat	PS vs. PSR vs. PSV	**PS:** Mean= 1.25 SD=1.55 **PSR:** Mean=1.45 SD=1.47 **PSV:** Mean=1.53 SD=1.17	20	1-way ANOVA: group $F(2,56)=0.201$, $p=0.818$
	Frequency of cat approaches to rat	PS vs. PSR vs. PSV	**PS:** Mean= 0.65 SD=1.50 **PSR:** Mean=0.200 SD=0.616 **PSV:** Mean=0.263 SD=0.562	20	1-way ANOVA: group $F(2,56)=1.193$, $p=0.311$
	Frequency of cat physically contacting the rat with paw	PS vs. PSR vs. PSV	**PS:** Mean= 0 SD=0 **PSR:** Mean=0 SD=0 **PSV:** Mean=0.105 SD=0.0339	20	1-way ANOVA: group $F(2,56)=1.055$, $p=0.355$
	Total time cat and rat were within one square of each other (s)	PS vs. PSR vs. PSV	**PS:** Mean= 19.35 SD=49.5 **PSR:** Mean=8.30 SD=16.5 **PSV:** Mean=11.01 SD=15.5	20	1-way ANOVA: group $F(2,56)=0.660$, $p=0.521$

Table 3 (Continued)

Variant Test	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Re-exposures	Distance moved (cm)	PSR vs. PSV	**PSR:** Mean=3686.77 SD=1384.19 **PSV:** Mean=4892.75 SD=1444.95	20	1-way ANOVA: group $F(1,38)=7.265$, $p=0.010$*
	Time immobile (s)	PSR vs. PSV	**PSR:** Mean=441.45 SD=53.46 **PSV:** Mean=379.55 SD=55.41	20	1-way ANOVA: group $F(1,38)=12.925$, $p=0.001$*
	Time mobile (s)	PSR vs. PSV	**PSR:** Mean=158.56 SD=53.47 **PSV:** Mean=220.42 SD=55.33	20	1-way ANOVA: group $F(1,38)=12.922$, $p=0.001$*
Acoustic Startle Response	Median peak startle Amplitude (Volts per kg)	HC vs. PS vs. PSR vs. PSV	**HC:** Median=4.66 SE=0.49 **PS:** Median=5.90 SE=0.60 **PSR:** Median=10.12 SE=1.02 **PSV:** Median=6.99 SE=0.61	20	Kruskal-Wallis: $X^2(3)= 178.92$, $p<0.0001$.* Median amplitude contrasts with the Kruskal-Wallis multiple z-test revealed that PSR was different from HC, PS and PSV. PS and PSV did not differ from each other ($p>0.05$).
	Habituation (Tau)	HC vs. PS vs. PSR vs. PSV	**HC:** Tau=1.92 SE=0.168 **PS:** Tau=2.55 SE=0.169 **PSR:** Tau=3.73 SE=0.699 **PSV:** Mean=2.17 SE=0.128	20	Fit of exponential decay: All exponential fits $F(2,27)>65.17$, $p<0.001$. * All Tau >0, $t(27)>5.33$, $p<0.01$. Tau contrasts all $t(58)>=2.38$ all $p<.01$

Table 3 (Continued)

Variant Test	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Hole Board	Frequency of head dips	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=12.40 SD=2.76 **PS:** Mean=14.35 SD=2.76 **PSR:** Mean=14.1 SD=3.61 **PSV:** Mean=14.8 SD=2.91	20	1-way ANOVA: group $F(3,76)=2.396$, $p=0.075$
	Frequency of rears	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=19.45 SD=4.36 **PS:** Mean=18.00 SD=5.48 **PSR:** Mean=19.8 SD=6.4286 **PSV:** Mean=18.05 SD=7.23	20	1-way ANOVA: group $F(3,76)=0.490$, $p=0.690$
	Ratio time	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=0.29 SD=0.16 **PS:** Mean=0.23 SD=0.11 **PSR:** Mean=0.22 SD=0.081 **PSV:** Mean=0.26 SD=0.15	20	1-way ANOVA: group $F(3,76)=1.07$, $p=0.367$
Elevated Plus Maze	Frequency of risk assessment	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=10.15 SD=3.80 **PS:** Mean=10.35 SD=2.23 **PSR:** Mean=11.00 SD=3.87 **PSV:** Mean=9.85 SD=2.18	20	1-way ANOVA: group $F(3,76)=0.49$, $p=0.69$

Table 3 (Continued)

Variant Test	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Elevated Plus Maze	Total time risk assessment (s)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=40.96 SD=21.34 **PS:** Mean=57.71 SD=22.45 **PSR:** Mean=48.29 SD=19.50 **PSV:** Mean=52.71 SD=14.39	20	1-way ANOVA: group $F(3,76)=2.61$, $p=0.058$
	Ratio Time	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=0.2877 SD=0.1731 **PS:** Mean=0.2132 SD=0.1424 **PSR:** Mean=0.3059 SD=0.1456 **PSV:** Mean=0.3166 SD=0.1456	20	1-way ANOVA: group $F(3,76)=1.88$, $p=0.140$
	Ratio Frequency	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=30.87 SD=15.90 **PS:** Mean=23.80 SD=12.40 **PSR:** Mean=29.31 SD=10.84 **PSV:** Mean=27.24 SD=10.79	20	1-way ANOVA: group $F(3,76)=1.17$, $p=0.328$
Light/Dark Box	Frequency to enter dark	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=7.7 SD=2.03 **PS:** Mean=7.45 SD=1.67 **PSR:** Mean=7.4 SD=0.995 **PSV:** Mean=6.85 SD=1.50	20	1-way ANOVA: group $F(3,76)=1.01$, $p=0.39$

Table 3 (Continued)

Variant Test	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Light/Dark Box	Total time in dark (s)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=159.95 SD=35.62 **PS:** Mean=167.8 SD=27.94 **PSR:** Mean=166.0 SD=21.52 **PSV**: Mean=159.05 SD=29.46	20	1-way ANOVA: group $F(3,76)=0.5$, $p=0.683$
	Frequency to enter light	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=7.45 SD=2.09 **PS:** Mean=6.30 SD=2.81 **PSR:** Mean=6.20 SD=1.40 **PSV:** Mean=6.35 SD=1.60	20	1-way ANOVA: group $F(3,76)=1.64$, $p=0.187$
	Total time in light (s)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=96.55 SD=29.33 **PS:** Mean=85.8 SD=28.6 **PSR:** Mean=95.35 SD=19.31 **PSV:** Mean=104.75 SD=29.79	20	1-way ANOVA: group $F(3,76)=1.64$, $p=0.187$

Table 4

Experiment 4 – The role of mTOR in extinction of predator stress-induced fear memories.

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Predator Exposure	Frequency of rat approaches to cat	PS vs. PSR vs. PSV	**PS:** Mean=0.40 SD=0.60 **PSR:** Mean=0.79 SD=0.71 **PSV:** Mean=0.85 SD=0.90	20	1-way ANOVA: group $F(2,56)=1.93$, $p=0.155$
	Frequency of rat flights from the cat	PS vs. PSR vs. PSV	**PS:** Mean=0.10 SD=0.31 **PSR:** Mean=0.16 SD=0.38 **PSV:** Mean=0.45 SD=0.95	20	1-way ANOVA: group $F(2,56)=1.85$, $p=0.168$
	Frequency of cat approaches to rat	PS vs. PSR vs. PSV	**PS:** Mean=0.25 SD=0.55 **PSR:** Mean=0.32 SD=0.67 **PSV:** Mean=0.50 SD=1.05	20	1-way ANOVA: group $F(2,56)=0.538$, $p=0.587$
	Frequency of cat physically contacting the rat with paw	PS vs. PSR vs. PSV	**PS:** Mean=0.00 SD=0.00 **PSR:** Mean=0.16 SD=0.69 **PSV:** Mean=0.00 SD=0.00	20	1-way ANOVA: group $F(2,56)=1.06$, $p=0.355$
	Total time cat and rat were within one square of each other (s)	PS vs. PSR vs. PSV	**PS:** Mean=4.66 SD=6.42 **PSR:** Mean=13.56 SD=24.80 **PSV:** Mean=11.87 SD=22.61	20	1-way ANOVA: group $F(2,56)=1.14$, $p=0.327$

Table 4 (Continued)

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Acoustic Startle Response	Median peak startle amplitude	HC vs. PS vs. PSR vs. PSV	**HC:** Median=4.66 SEMd=0.49 **PS:** Median=5.90 SEMd=0.60 **PSR:** Median=10.12 SD=1.02 **PSV:** Median=6.99 SD=0.61	20	Kruskal Wallis $X^2(3)=178.92, p<0.0001$* Median contrasts Kruskal Wallis Multiple z test $p<0.05$*
	Habituation	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=1.92 SE=0.168 **PS:** Mean=2.55 SE=0.169 **PSR:** Mean=3.73 SE=0.699 **PSV:** Mean=2.168 SE=0.128	20	All fit $F(2,27)>65.17, p<0.01$* All Tau>0, $t(27)>5.33, p<0.01$* Tau contrasts all $t(58)=2.38, p<0.01$*
Hole Board	Frequency of head dips	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=13.73 SD=3.70 **PS:** Mean=13.80 SD=6.18 **PSR:** Mean=13.15 SD=3.72 **PSV:** Mean=12.80 SD=5.33	20	1-way ANOVA: group $F(3,75)=0.194$, $p=0.901$
	Frequency of rears	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=17.47 SD=5.15 **PS:** Mean=16.35 SD=6.62 **PSR:** Mean=18.25 SD=5.46 **PSV:** Mean=16.60 SD=7.71	20	1-way ANOVA: group $F(3,75)=0.375$, $p=0.771$

Table 4 (Continued)

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Hole Board	Total Distance Moved (cm)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=2994.45 SD=344.05 **PS:** Mean=2645.62 SD=508.77 **PSR:** Mean=3056.97 SD=318.46 **PSV:** Mean=2722.67 SD=616.42	20	1-way ANOVA: group $F(3,75)=3.723$, $p=0.015$*
	Ratio Time	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=0.283 SD=0.153 **PS:** Mean=0.301 SD=0.166 **PSR:** Mean=0.250 SD=0.109 **PSV:** Mean=0.388 SD=0.161	20	1-way ANOVA: group $F(3,75)=3.122$, $p=0.031$* Bonferroni protected post hoc comparisons, PSR vs. PSV, $p < 0.05$
Elevated Plus Maze	Frequency of risk assessment	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=15.10 SD=3.81 **PS:** Mean=10.55 SD=4.21 **PSR:** Mean=12.20 SD=3.05 **PSV:** Mean=8.75 SD=3.88	20	1-way ANOVA: group $F(3,76)=10.28$, $p=0.00$* Bonferroni protected post hoc comparisons, HC vs. PS, $p < 0.05$, HC vs. PSV, $p < 0.05$
	Total time risk assessment (s)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=54.29 SD=14.26 **PS:** Mean=48.28 SD=25.38 **PSR:** Mean=54.53 SD=17.57 **PSV:** Mean=47.46 SD=25.55	20	1-way ANOVA: group $F(3,76)=0.636$, $p=0.594$

Table 4 (Continued)

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Elevated Plus Maze	Total distance moved (cm)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=2752.84 SD=505.07 **PS:** Mean=2323.64 SD=643.32 **PSR:** Mean=2742.61 SD=639.37 **PSV:** Mean=2191.90 SD=735.29	20	1-way ANOVA: group $F(3,76)=4.10$, $p=0.009$*
	Ratio Time	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=0.2598 SD=0.1505 **PS:** Mean=0.1913 SD=0.2017 **PSR:** Mean=0.2302 SD=0.1687 **PSV:** Mean=0.1844 SD=0.1754	20	1-way ANOVA: group $F(3,76)=0.802$, $p=0.497$
	Ratio Frequency	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=26.31 SD=13.55 **PS:** Mean=18.20 SD=16.88 **PSR:** Mean=20.13 SD=12.18 **PSV:** Mean=24.43 SD=19.57	20	1-way ANOVA: group $F(3,75)=1.125$, $p=0.345$
Light/Dark Box	Total time in dark (s)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=146.02 SD=30.06 **PS:** Mean=155.03 SD=41.03 **PSR:** Mean=162.24 SD=32.14 **PSV:** Mean=157.66 SD=39.02	20	1-way ANOVA: group $F(3,76)=0.726$, $p=0.540$

Table 4 (Continued)

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Light/Dark Box	Total time in light (s)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=91.64 SD=27.47 **PS:** Mean=87.51 SD=41.39 **PSR:** Mean=84.46 SD=26.80 **PSV:** Mean=85.44 SD=37.71	20	1-way ANOVA: group $F(3,76)=0.176$, $p=0.913$
	Total time in between light and dark side (s)	HC vs. PS vs. PSR vs. PSV	**HC:** Mean=62.35 SD=14.63 **PS:** Mean=57.47 SD=18.35 **PSR:** Mean=53.31 SD=13.41 **PSV:** Mean=56.90 SD=15.31	20	1-way ANOVA: group $F(3,76)=1.15$, $p=0.336$

Figure Captions

Figure 1: *Rapamycin blocks predator stress-induced hyperarousal*. **A.** Median + SEMd peak startle amplitude (Volts/kg) plotted over four groups: predator stressed (PS), predator stressed + vehicle (PSV), predator stressed + rapamycin (PSR) and handled control (HC). Medians marked with the same letter do not differ; medians marked with different letters differ. Rats in the HC and PSR groups show lower startle amplitude than those in the PS and PSV groups. **B.** Trial constants (*Tau*) + SE plotted over four groups: predator stressed (PS), predator stressed + vehicle (PSV), predator stressed + rapamycin (PSR) and handled control (HC). Tau values marked with the same letter do not differ, Tau's marked with different letters differ. Rats in the HC and PSR groups habituated faster (smaller *Tau* values) than those in the PS and PSV groups.

Figure 2: *Rapamycin blocks persistent predator stress-induced hyperarousal*. **A.** Median + SEMd peak startle amplitude (Volts/kg) measured three weeks post-treatment plotted over four groups: predator stressed (PS), predator stressed + vehicle (PSV), predator stressed + rapamycin (PSR) and handled control (HC). Medians marked with the same letter do not differ; medians marked with different letters differ. Rats in the HC and PSR groups show lower startle amplitude than those in the PS and PSV groups when measured three weeks post stressor. **B.** Trial constants (*Tau*) + SE measured three weeks post-treatment plotted over four groups: PS, PSV, PSR and HC. Tau values marked with the same letter do not differ, Tau's marked with different letters differ. Rats in the HC and PSR groups habituated faster (smaller *Tau* values) than those in the PS and PSV groups when measured three weeks post stressor.

Figure 3: *Rapamycin blocks predator stress-induced anxiety-like behavior*. **A.** Mean + SEM of ratio time in the elevated plus maze (EPM) plotted over four groups: predator stressed (PS), predator stressed + vehicle (PSV), predator stressed + rapamycin (PSR) and handled control (HC). For panels A-D, mean values marked with the same letter do not differ; means marked with different letters differ. Rats in the HC and PSR groups exhibited greater ratio time (time in open arms/time in all arms) than rats in the PS and PSV groups. **B.** Mean + SEM frequency of risk assessment in the elevated plus maze (EPM) plotted over the four groups: PS, PSV, PSR and HC. Rats in the HC and PSR groups engaged in risk assessment more often than rats in the PS and PSV groups. **C.** Mean + SEM of ratio time (time in

center/time in periphery) in the hole board (HB) plotted over four groups: PS, PSV, PSR, and HC. PS rats displayed decreased ratio time compared to both HC and PSR groups, while PSV rats showed intermediate levels of ratio time. **D.** Mean + SEM of frequency of rears in the hole board (HB) plotted over four groups: PS, PSV, PSR, and HC. HC rats reared more than PS and PSV rats ($p < 0.05$). Rapamycin (PSR) partially reversed predator stress-induced suppression of rears.

Figure 4: *Rapamycin reduces contextual fear measured three weeks after predator stress*. **A.** Mean + SEM of the total distance moved (cm) in the room re-exposure measured three weeks post-treatment plotted over three groups: predator stressed (PS), predator stressed + vehicle (PSV), and predator stressed + rapamycin (PSR). For panels A-C, means marked with the same letter do not differ; means marked with different letters differ. PSR rats traveled more distance in comparison to PS and PSV groups ($p < 0.05$). **B.** Mean + SEM of the total time mobile (s) in the room re-exposure measured three weeks post-treatment plotted over three groups: PS, PSV, and PSR. PSR rats were more mobile in comparison to PS and PSV groups ($p < 0.05$). **C.** Mean + SEM of the total time immobile (s) in the room re-exposure measured three weeks post-treatment plotted over three groups: PS, PSV, and PSR. PSR rats were less immobile in comparison to PS and PSV groups ($p < 0.05$).

Figure 5: *Rapamycin decreases body weight*. Mean + SEM of body weight (g) measured across days plotted over four groups: predator stressed (PS), predator stressed + vehicle (PSV), predator stressed + rapamycin (PSR) and handled control (HC). Following injection, body weight was significantly lower in the PSR group than PS, PSV and HC groups across days (* indicates significant difference, all $p < 0.05$).

Figure 6: *Rapamycin given after predator exposure blocks predator stress-induced hyperarousal.* **A.** Median + SEMd of peak startle amplitude (Volts/kg) plotted over four groups: predator stressed (PS), predator stressed + vehicle (PSV), predator stressed + rapamycin (PSR) and handled control (HC). Medians marked with the same letter do not differ; medians marked with different letters differ. Rapamycin (PSR group) significantly reduced median peak startle amplitude to HC levels ($p < 0.05$). In comparison to HC and PSR groups, PS and PSV groups displayed an increase in startle amplitude. **B.** Trial constants (*Tau*) + SE plotted over four groups: PS, PSV, PSR, and HC. Tau values marked with the same letter do not differ,

Tau's marked with different letters differ. Rapamycin (PSR group) significantly reduced habituation (*Tau*) to HC levels ($p > 0.05$). Rats in the HC and PSR groups habituated faster (smaller *Tau* values) than those in the PS and PSV groups ($p < 0.05$).

Figure 7: *Rapamycin does not block predator stress-induced anxiety-like behavior in LD box.* Mean + SEM of the frequency to enter the light side in the light/dark (LD) box plotted over four groups: predator stressed (PS), predator stressed + vehicle (PSV), predator stressed + rapamycin (PSR) and handled control (HC). Means marked with the same letter do not differ; means marked with different letters differ. All predator stressed rats (PS, PSR, and PSV) entered the light side of the LD box less often than HC rats indicating increased ALB.

Figure 8: *Rapamycin decreases body weight.* Mean + SEM of body weight (g) measured across days plotted over four groups: predator stressed (PS), predator stressed + vehicle (PSV), predator stressed + rapamycin (PSR) and handled control (HC). Following injection, body weight was significantly lower in the PSR group than PS, PSV and HC groups across 23 days (* indicates significant differences, all $p < 0.05$).

Figure 9: *Decreased activity during re-exposure to the predator stress context.* **A.** Mean + SEM of total distance moved (cm) across time (divided into bins) during the room re-exposure plotted over two groups: predator stressed + rapamycin (PSR), and predator stressed + vehicle (PSV). Prior to injection, both PSR and PSV groups show increased distance travelled across bins, however, PSR rats travelled less distance than PSV rats when re-exposed to the predator stress context. **B.** Mean + SEM of total time mobile (s) across time (divided into bins) during the room re-exposure plotted over two groups: PSR, and PSV. Prior to injection, both PSR and PSV rats increased mobility across bins, however, PSR rats were less mobile than PSV rats **C.** Mean + SEM of total time immobile (s) across time (divided into bins) during the room re-exposure plotted over two groups: PSR, and PSV. Prior to injection, PSR and PSV rats showed a decrease in time immobile across bins, however, PSR rats were more immobile than PSV rats.

Figure 10 *Rapamycin given after re-exposure to the predator stress context blocks extinction.* **A.** Median + SEMd of peak startle amplitude (Volts/kg) plotted over four groups: predator stressed (PS), predator stressed +

vehicle (PSV), predator stressed + rapamycin (PSR) and handled control (HC). Medians marked with the same letter do not differ; medians marked with different letters differ. PS rats displayed increased peak startle amplitude compared to HC rats. PSV rats showed decreased peak startle amplitude equivalent to that of HC rats, and this reduction was blocked by rapamycin (PSR). Room exposure (lasting 10 minutes) two days after predator stress extinguishes hyperarousal which is blocked by rapamycin. **B.** Trial constants (*Tau*) + SE plotted over four groups: PS, PSV, PSR, and HC. Tau values marked with the same letter do not differ, Tau's marked with different letters differ. HC rats habituated faster (smaller *Tau* values) in comparison to PS rats. PSV rats increased habituation (smaller Tau values) to the tone comparable to HC rats and this increase was blocked with rapamycin (PSR).

Figure 11: *Rapamycin decreases body weight*. Mean + SEM of body weight (g) measured across days plotted over four groups: predator stressed (PS), predator stressed + vehicle (PSV), predator stressed + rapamycin (PSR) and handled control (HC). Following injection, body weight was significantly lower in the PSR group than PS, PSV and HC groups across days (* indicates significant differences, all $p < 0.05$).

Figure 12: *Rapamycin injection two days after stress potentiates startle response.* **A.** Median + SEMd of peak startle amplitude (Volts/kg) plotted over four groups: predator stressed (PS), predator stressed + vehicle (PSV), predator stressed + rapamycin (PSR) and handled control (HC). Medians marked with the same letter do not differ; medians marked with different letters differ. PS and PSV rats displayed increased peak startle amplitude compared to HC rats, while PSR rats showed potentiated startle compared to PS, PSV and HC groups. **B.** Trial constants (*Tau*) + SE plotted over four groups: PS, PSV, PSR, and HC. Tau values marked with the same letter do not differ, Tau's marked with different letters differ. HC rats habituated faster (smaller *Tau* values) in comparison to PS rats. PSV rats exhibited intermediate habituation levels, while PSR rats showed a decreased rate of habituation compared to all groups.

Figure 13: *Rapamycin affects predator stress-induced anxiety-like behavior*. **A.** Mean + SEM frequency of risk assessment in the elevated plus maze (EPM) plotted over four groups: predator stressed (PS), predator stressed + vehicle (PSV), predator stressed + rapamycin (PSR) and handled control

(HC). For panels A and B, means marked with the same letter do not differ; means marked with different letters differ. HC group engaged in risk assessment more frequently than those in the PS and PSV groups, which did not differ. PSR rats show elevated frequency of risk assessment compared to PSV rats, but do not differ from PS rats. **B.** Mean + SEM of ratio time in the hole board (HB) plotted over four groups: PS, PSV, PSR, and HC. PSR group displayed a decreased ratio time in comparison to PSV rats.

Figure 14: *Rapamycin decreases body weight*. Mean + SEM body weight (g) plotted over four groups: predator stressed (PS), predator stressed + vehicle (PSV), predator stressed + rapamycin (PSR) and handled control (HC). Rapamycin (PSR) significantly reduced body weight in comparison to HC, PSV and PS groups (* indicates significant difference, $p < 0.05$).

Figure 1

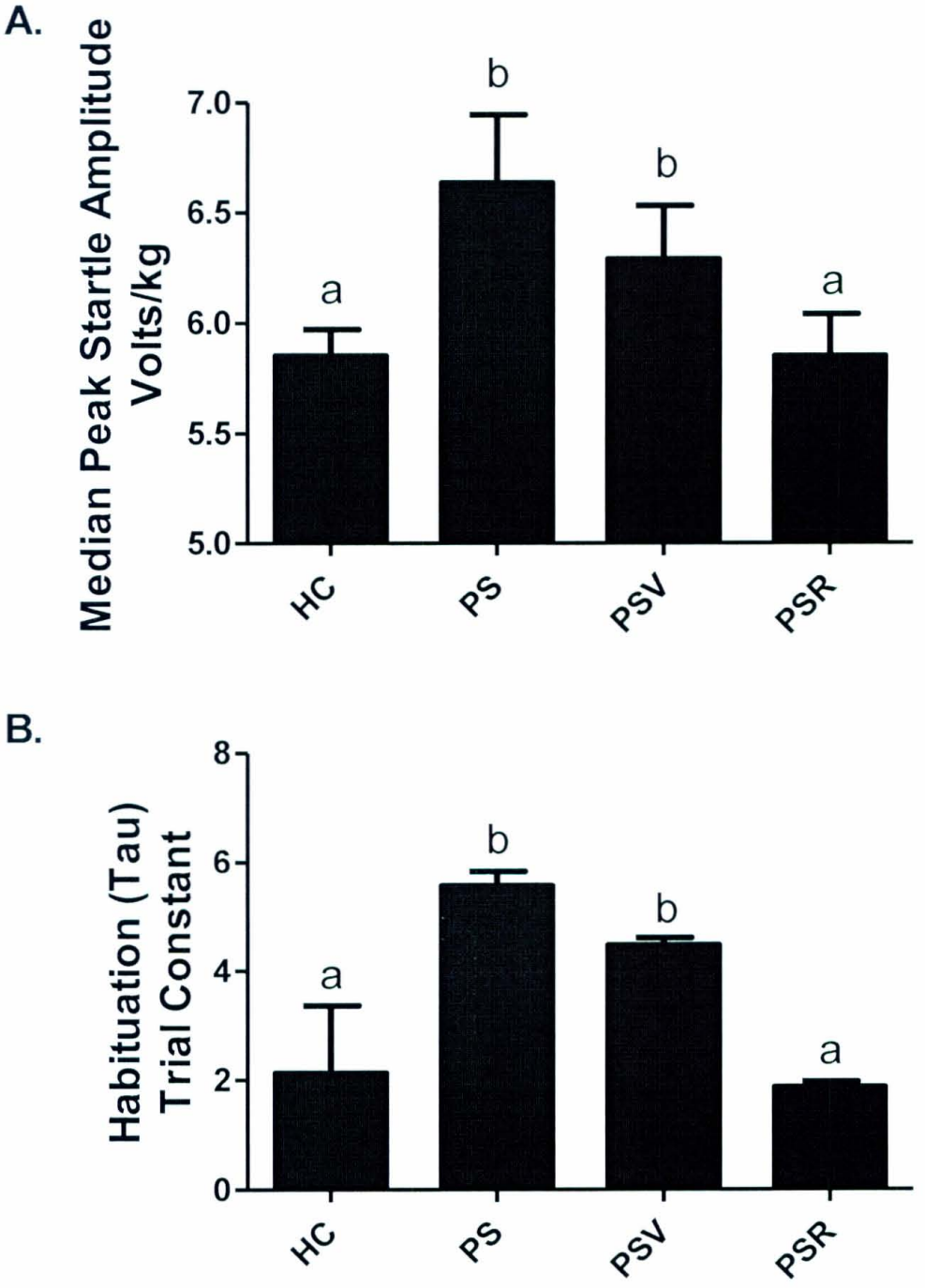

A.
Median Peak Startle Amplitude
Volts/kg
7.0
6.5
6.0
5.5
5.0
a
b
b
a
HC
PS
PSV
PSR
B.
Habituation (Tau)
Trial Constant
8
6
4
2
0
a
b
b
a
HC
PS
PSV
PSR

Figure 2

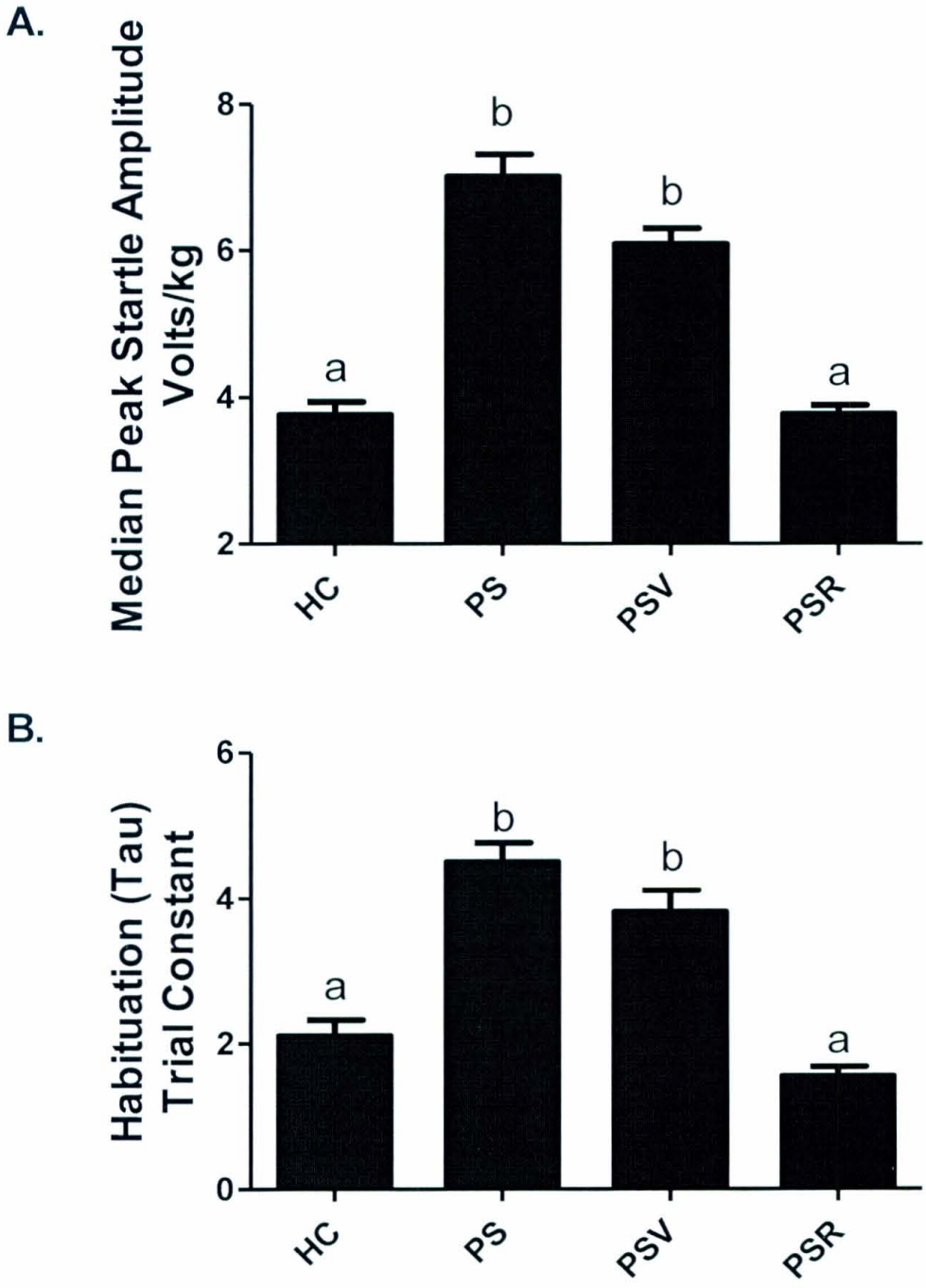

A.
Median Peak Startle Amplitude
Volts/kg
8
6
4
2
a
b
b
a
HC
PS
PSV
PSR
B.
Habituation (Tau)
Trial Constant
6
4
2
0
a
b
b
a
HC
PS
PSV
PSR

Figure 3.

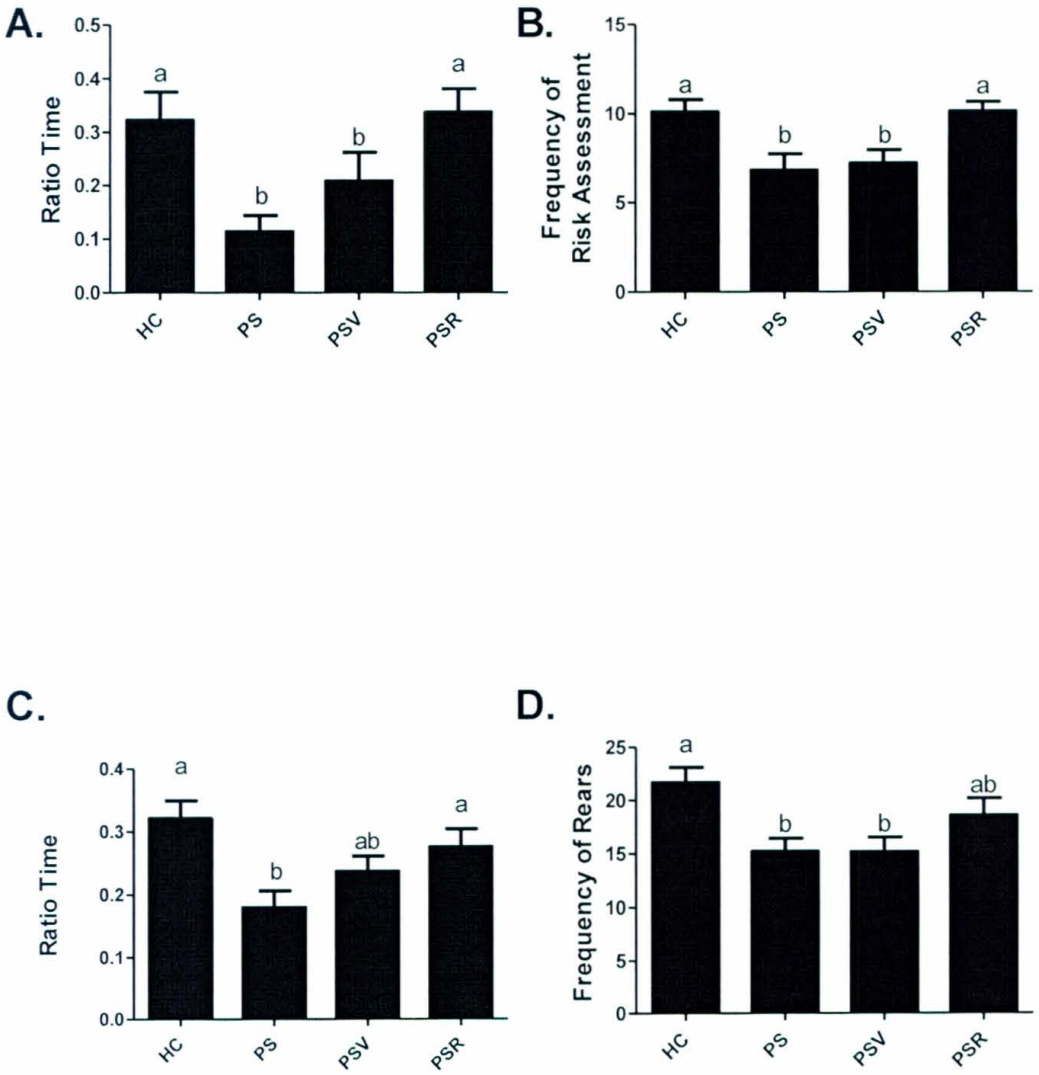

A.
Ratio Time
0.5
0.4
0.3
0.2
0.1
0.0
a
b
b
a
HC
PS
PSV
PSR
B.
Frequency of Risk Assessment
15
10
5
0
a
b
b
a
HC
PS
PSV
PSR
C.
Ratio Time
0.4
0.3
0.2
0.1
0.0
a
b
ab
a
HC
PS
PSV
PSR
D.
Frequency of Rears
25
20
15
10
5
0
a
b
b
ab
HC
PS
PSV
PSR

Figure 4.

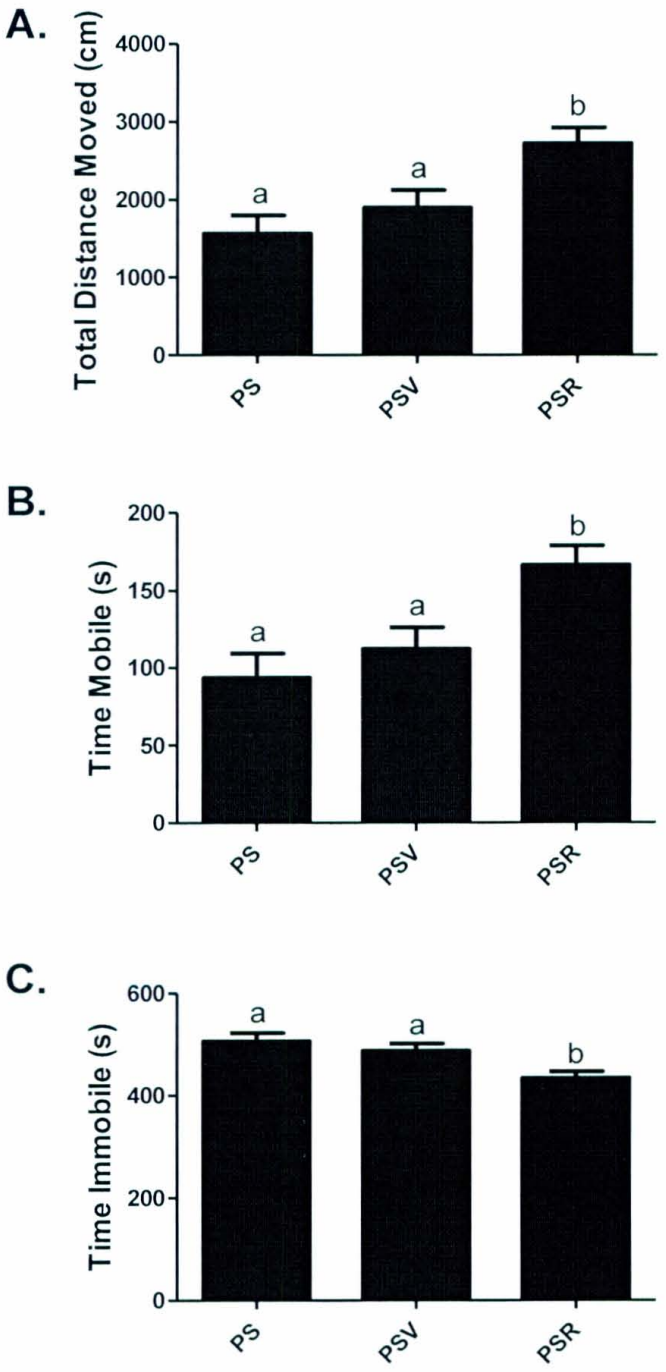

A.
Total Distance Moved (cm)
4000
3000
2000
1000
0
a
a
b
PS
PSV
PSR
B.
Time Mobile (s)
200
150
100
50
0
a
a
b
PS
PSV
PSR
C.
Time Immobile (s)
600
400
200
0
a
a
b
PS
PSV
PSR

Figure 5.

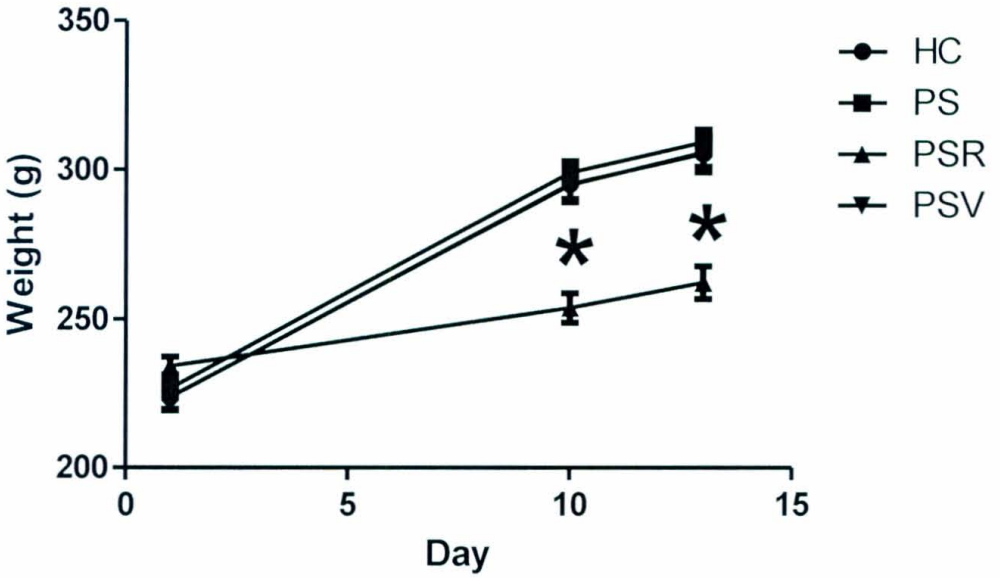


Figure 6.

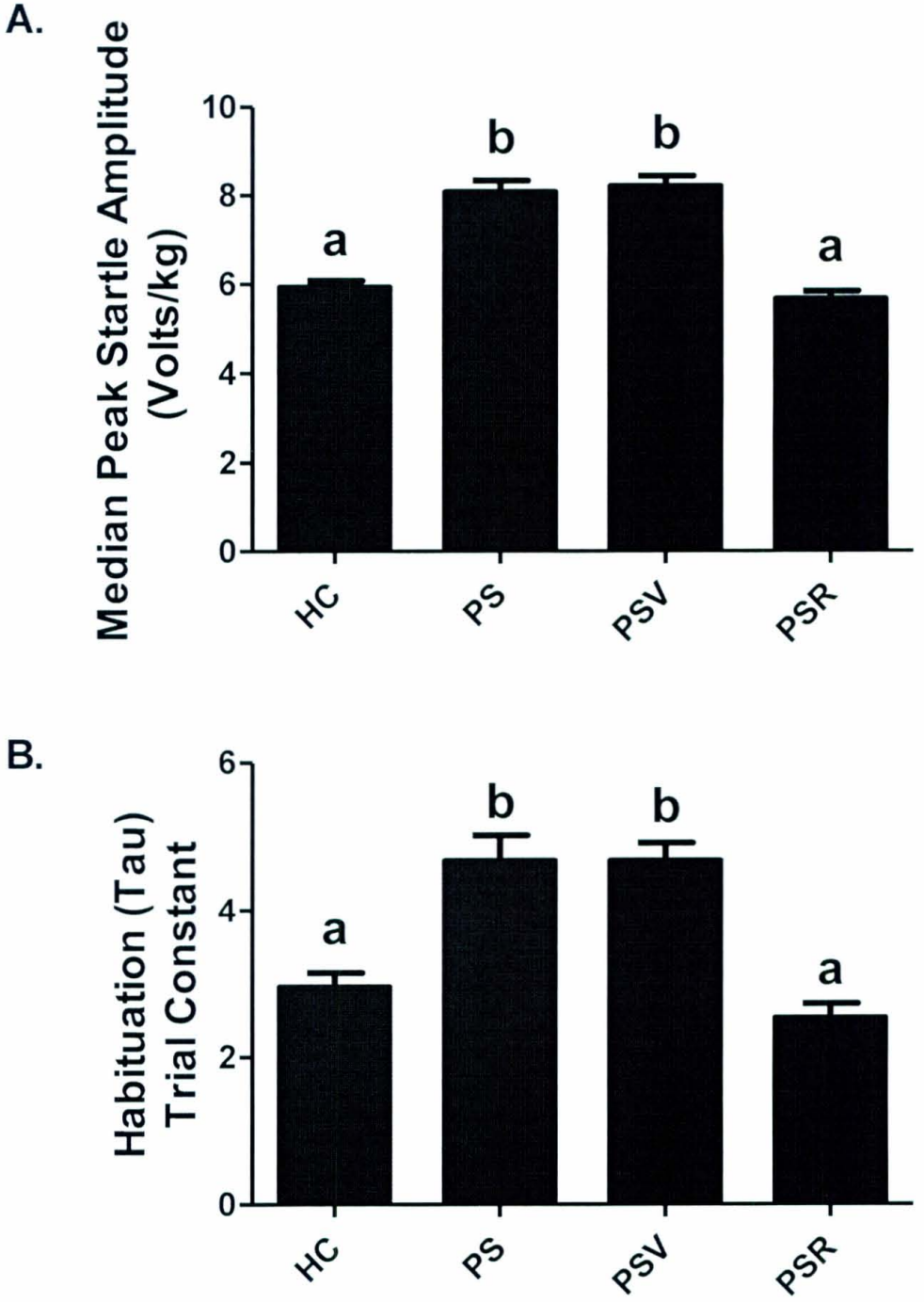

A.
Median Peak Startle Amplitude (Volts/kg)
10
8
6
4
2
0
a
b
b
a
HC
PS
PSV
PSR
B.
Habituation (Tau) Trial Constant
6
4
2
0
a
b
b
a
HC
PS
PSV
PSR

Figure 7.

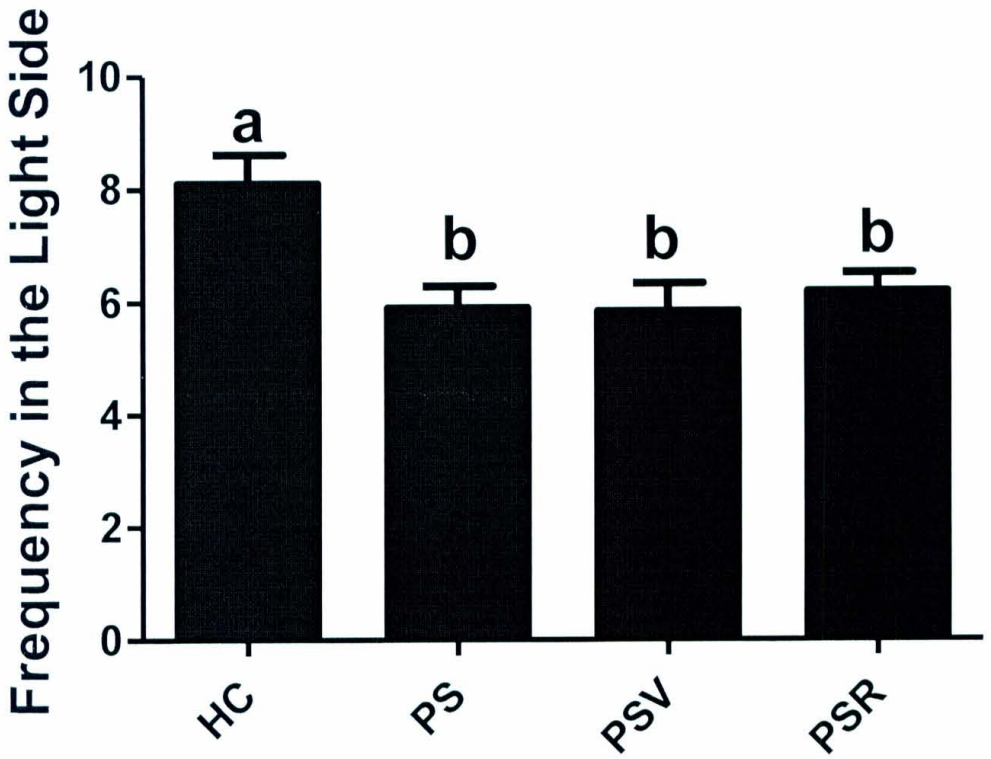


Figure 8.

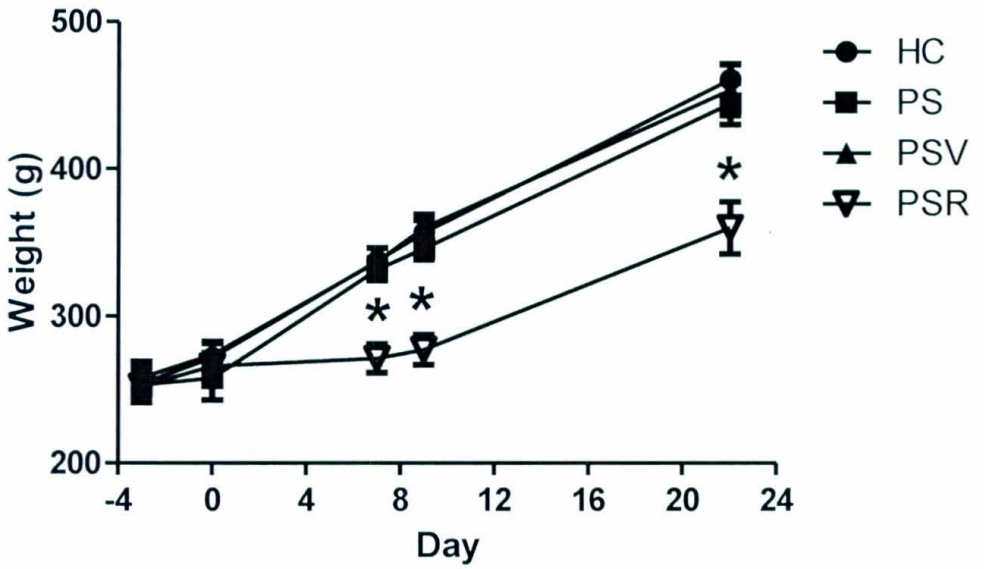
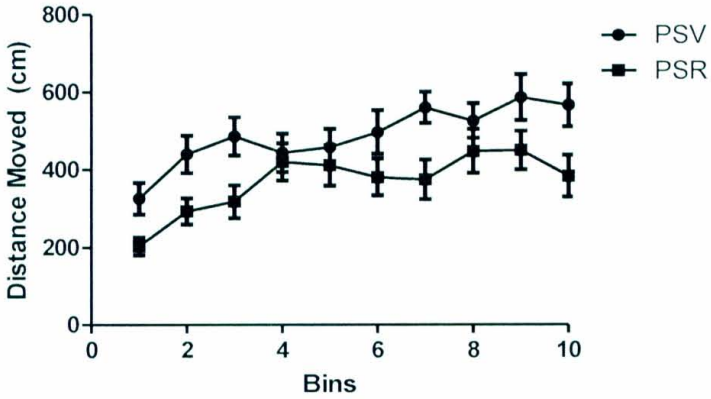
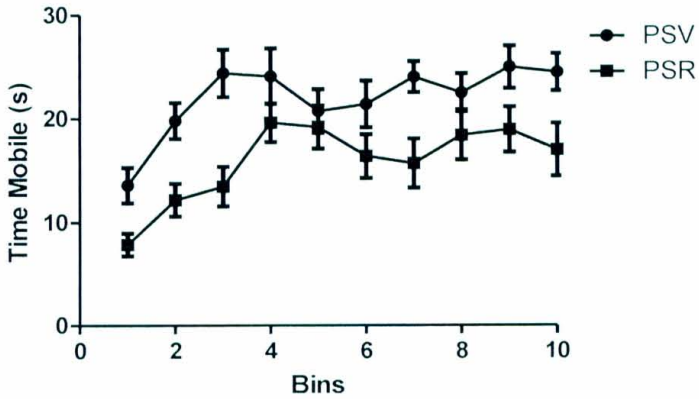


Figure 9.

A.


800
600
400
200
0
Distance Moved (cm)
0
2
4
6
8
10
Bins
PSV
PSR

B.


30
20
10
0
Time Mobile (s)
0
2
4
6
8
10
Bins
PSV
PSR

C.

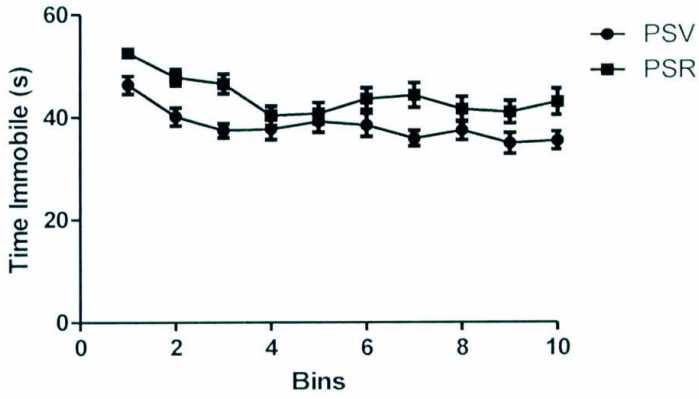

60
40
20
0
Time Immobile (s)
0
2
4
6
8
10
Bins
PSV
PSR

Figure 10.

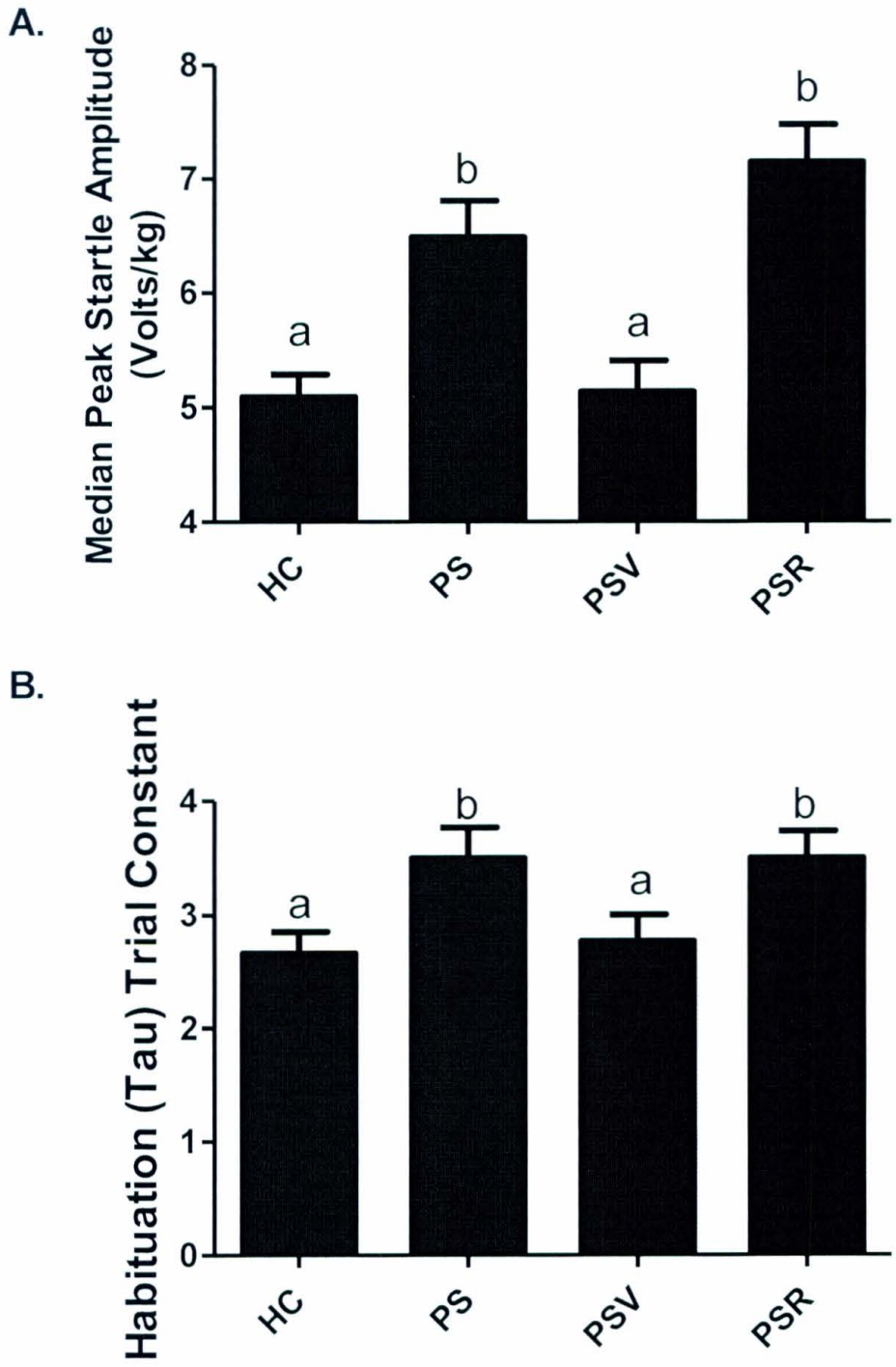

A.
Median Peak Startle Amplitude (Volts/kg)
8
7
6
5
4
a
b
a
b
HC
PS
PSV
PSR
B.
Habituation (Tau) Trial Constant
4
3
2
1
0
a
b
a
b
HC
PS
PSV
PSR

Figure 11.

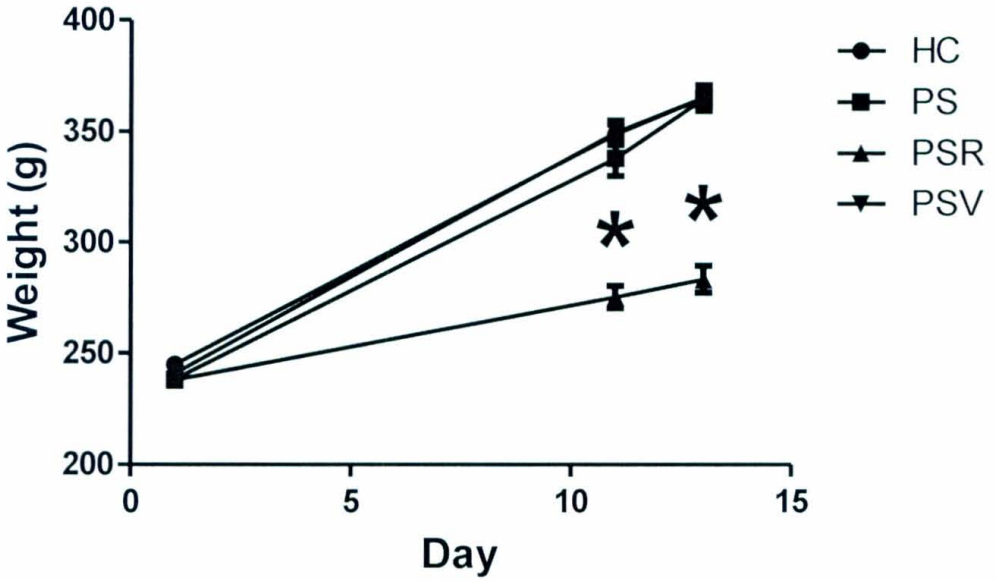
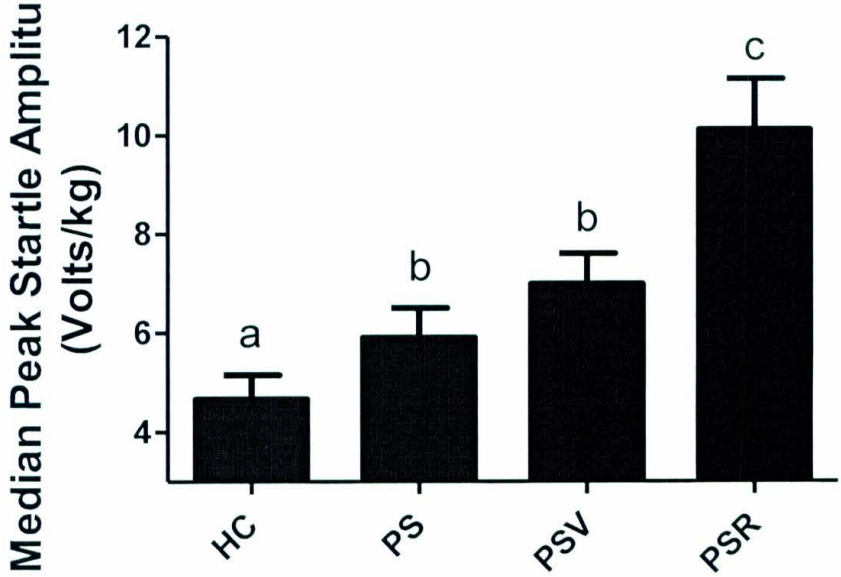


Figure 12.

A.


Median Peak Startle Amplitude (Volts/kg)
12
10
8
6
4
a
b
b
c
HC
PS
PSV
PSR

B.

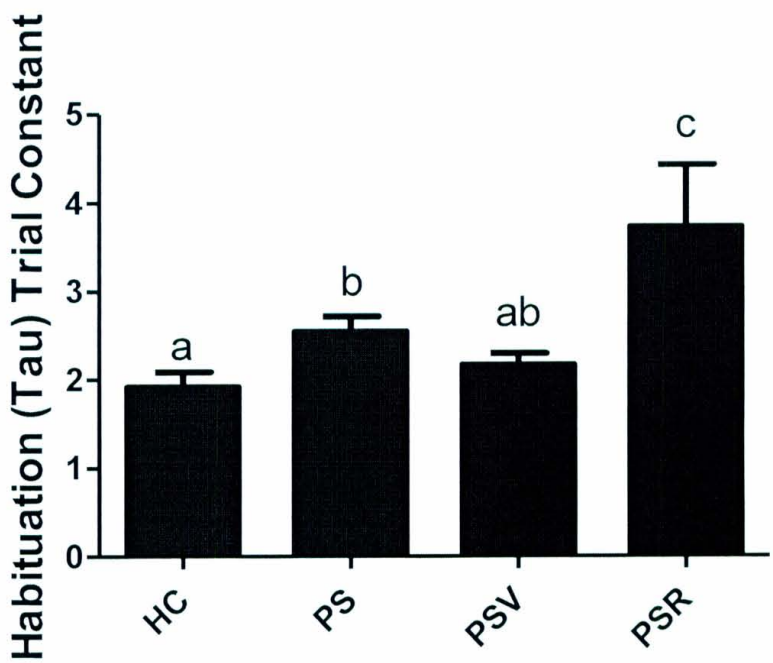

Habituation (Tau) Trial Constant
5
4
3
2
1
0
a
b
ab
c
HC
PS
PSV
PSR

Figure 13.

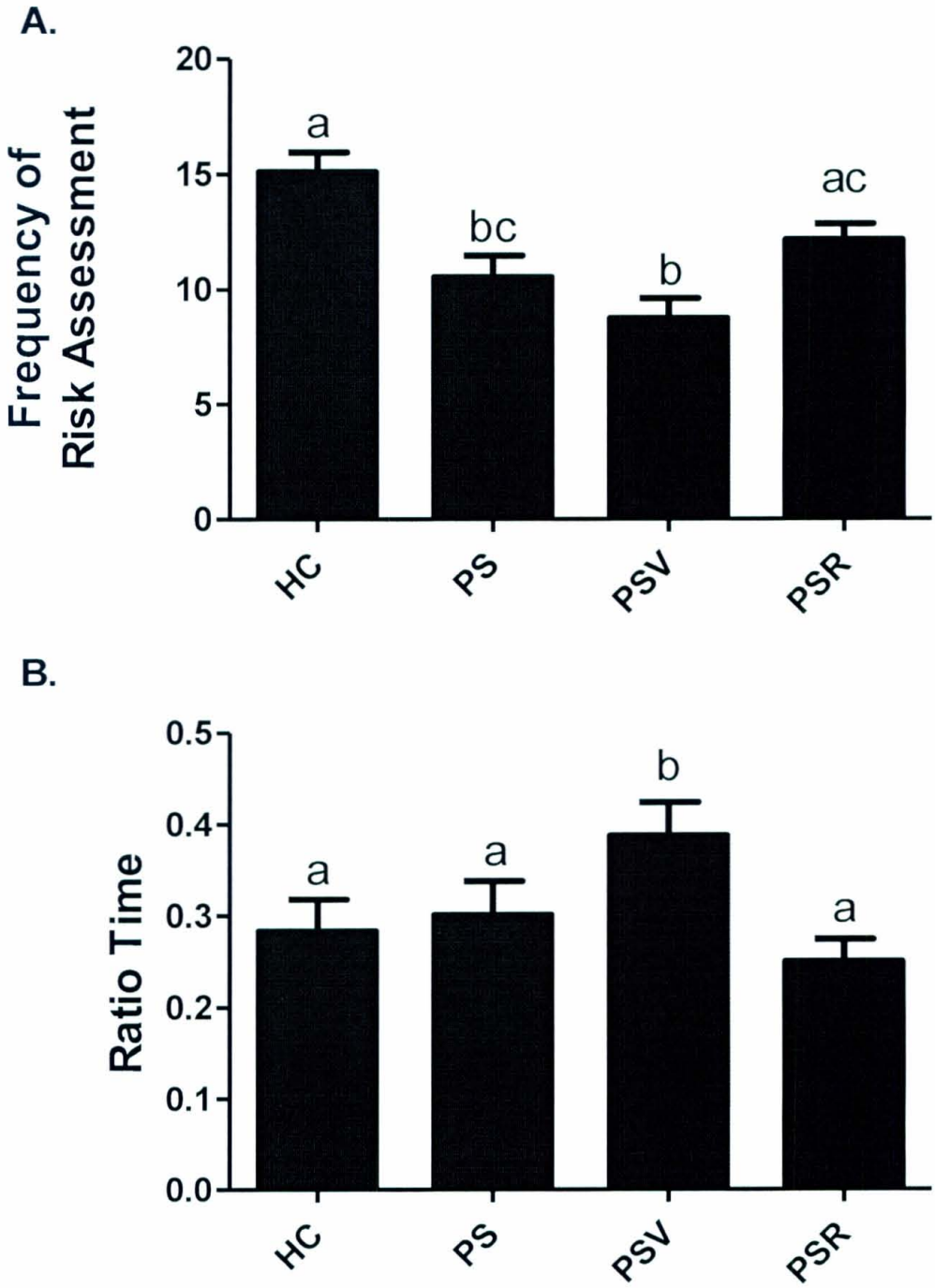

A.
Frequency of Risk Assessment
20
15
10
5
0
a
bc
b
ac
HC
PS
PSV
PSR
B.
Ratio Time
0.5
0.4
0.3
0.2
0.1
0.0
a
a
b
a
HC
PS
PSV
PSR

Figure 14.