Ethopharmacological approaches to the study of fear learning and memory: focus on the mTOR Kinase pathway and predator stress models

By

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A thesis submitted to the

School of Graduate Studies

In partial fulfillment of the requirements for the degree of

Master of Science in Experimental Psychology (Behavioural Neuroscience)

Department of Psychology

Memorial University of Newfoundland

May 2017

St. John's Newfoundland and Labrador

Acknowledgements

I heartily thank my supervisor Dr. Jacqueline Blundell for her exceptional support, mentoring, and encouragement throughout my thesis, and for her great passion for neuroscience research. I thank Dr. Mark Hébert for his instruction and guidance in behavioural and pharmacological lab techniques. The assistance of Therese E. Kenny, Travis Jacobs, Shruti Raheja, Sarah-Lynn Boyle, Katelyn Fallon, and Phillip MacCallum in running the experiments is gratefully acknowledged. Thanks and gratitude are also offered to the late Dr. Robert E Adamec and Dr. Robert J. Blanchard, who pioneered many of the ideas explored here. Finally, I thank NSERC for funding Dr. Blundell's research, as well as constituting a portion of my funding through an Alexander Graham Bell CGS-M scholarship.

Abstract

Traumatic experiences (rape, assault, combat) can cause Post-Traumatic Stress Disorder (PTSD), a source of substantial psychological suffering in those so affected. PTSD is defined by symptoms of traumatic re-experiencing, avoidance, and increased startle response (hyperarousal), along with disruptions in mood and cognition. The substantial social and individual burdens of the disorder strongly motivate research into its neural basis. Predator Stress (PS) models have been introduced to the literature over the last 30 years in order to facilitate this. However, the cat exposure test (Adamec & Shallow, 1993) has proven variable in its effects on rodent subjects. The experiments described here were performed with the aim of developing a more reliable and robust predator stress-based animal model of PTSD. Experiment 1 tested whether predator vocal sounds (cat calls) produced a PTSD-like phenotype in rats, and did not produce any significant effects. Experiment 2 modified the Rat Exposure Test (RET; Yang et al., 2004) and demonstrated predator stress effects on measures of contextual fear memory, anxiety-like behaviour, and hyperarousal, suggesting the RET is a useful model of PTSD. Experiment 3 tested whether inhibition of the mTOR kinase pathway with Rapamycin (RAP) would attenuate the consolidation of these memories. RAP blocked contextual fear memories and attenuated anxiety, but the effects of the RET were not as consistent as in Experiment 2. Reasons for the continued variability in predator stress models (and the neuroscience of learning in general) are discussed.

Keywords: *Predator Stress, learning, memory, mTOR, Rapamycin, Consolidation, Reconsolidation, ethology, psychopharmacology*

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1.0 Introduction

1.1 Post-Traumatic Stress Disorder

Individuals exposed to highly traumatic experiences (physical assault, rape, natural disaster, kidnapping, combat, etc.) can develop Post-Traumatic Stress Disorder (PTSD). The DSM-V classifies PTSD as a stress and trauma-related disorder, defined by a set of symptom clusters that appear for at least 30 days following severe trauma. Symptoms include *re-experiencing* of the trauma (unwanted intrusion of the memory in nightmares and flashbacks, intense upset evoked by cues or conditioned stimuli), *avoidance* of cues related to the traumatic event (situations, places, activities), and *hyperarousal* (increased startle response, irritability, sleep problems). Disturbances in mood and cognition are also core symptoms (American Psychiatric Association, 2013). Epidemiological studies have found that between 37 and 92% of people (76% percent of Canadians) report past exposure to at least one traumatic event (Van Ameringen, Mancini, Patterson, & Boyle, 2008; Kessler & Wang, 2008). Women are twice as likely to develop PTSD as men, and the disorder is often comorbid with other anxiety disorders, as well as depression and substance abuse (Kessler et al., 1995).

Between 25% and 35% of trauma survivors go on to develop PTSD (Yehuda, 2001; Kessler et al., 2005). These figures contribute to the lifetime prevalence of the disorder (percentage of the population that will experience the disorder at some point during their lives), which is currently estimated at 6.1% in the United States and 9.2% in Canada (Goldstein et al., 2016; Van Ameringen et al., 2008). PTSD is therefore one of the most common psychiatric disorders- for comparison, Obsessive Compulsive Disorder (OCD) has a lifetime prevalence of 1-2% and Generalised Anxiety Disorder (GAD) 3-5%

(Statistics Canada, 2015). Among "highly exposed" groups (e.g. low-income, urban populations like inner-city Detroit), lifetime rates of PTSD soar to 40% (Breslau et al., 1998). Rate and prevalence in particular locales are often affected by disastrous world events like the 9/11/2001 terror attacks, which increased PTSD rates in the New York City area (Galea et al., 2002), and Hurricane Katrina, which did the same in the Mississippi Delta (Galea, Tracy, Norris, & Coffey, 2008). In addition, a dose-response relationship exists between symptom severity and frequency of trauma experience: the more traumatic events a person experiences, the greater the intensity of their PTSD symptoms (Binder et al., 2008).

Current thinking in psychology, psychiatry, and neuroscience classifies PTSD as a condition of disturbed emotional learning and memory processes, where in particular the *consolidation* of traumatic fear memories is enhanced, fear *cues* are generalized, and the *extinction* of fear memories is impaired (Murray, Keifer, Ressler, Norrholm, & Jovanovic, 2014; Mahan & Ressler, 2012). The disorder is succinctly described by Bailey and Balsam (2013), who note PTSD is a syndrome where "old memories evoke responses ill-suited to current circumstances"(p. 245). These few words capture the plight of a traumatized combat veteran induced to panic, terror, or rage by the gunshot-like sounds of a holiday firecracker or otherwise innocuous car backfire.

This vividness ensures that understanding trauma is not just another research topic in the behavioural sciences. Indeed, the considerable suffering caused by PTSD, and its high prevalence, contribute a real and potent urgency to research on neural mechanisms underlying the disorder. Clarification of these mechanisms will help clinicians and other scientists understand the development of PTSD and identify candidate drug treatments

(Steckler & Risbrough, 2012; Hauger et al., 2012; Reul & Nutt, 2008). However, in order to understand the logic of this body of research, it is necessary to review the structures of "normal" learning, memory, and emotional systems and their neural substrates. This will allow a clearer view of how these processes are distorted in PTSD.

1.2 The Behavioural Neuroscience of Learning and Memory

Research linking cell/molecular neurobiology to behavioural measures of learning and memory has focused on "simple system" types of learning in invertebrates and rodents (for comprehensive reviews, see: Kandel, Dudai, & Mayford, 2014; Mayford, Siegelbaum, &Kandel, 2012; Sweatt, 2010; Squire & Kandel, 2008). This approach distinguishes between associative and non-associative categories of learning. Associative learning is typified by Pavlovian (classical) fear conditioning, a laboratory paradigm where the pairing of a neutral (conditioned) stimulus or context with an aversive stimulus allows the animal to learn to respond to the neutral stimulus or context with fear the next time it is encountered. Fear is usually defined as the visible performance of species-typical defence behaviours such as freezing (Bolles, 1975; Maren & Fanselow, 1996; Panksepp, 1998). In broad terms associative processes allow an animal to learn about relations between events in its environment, and how to respond to them appropriately. They are algorithms providing a primary means by which the animal represents contingency in its external world, solving complex problems in multivariate non-stationary time series analysis (Rescorla, 1988; Spear, Miller, & Jagielo, 1990, Timberlake, 1994; Gallistel, 2003).

Non-associative learning includes habituation and sensitization. These occur with relatively hard-wired, reflexive processes (such as the "jumping" startle response to a

loud noise) whose expression can be attenuated (habituation) or increased (sensitization) with repeated exposure to the triggering stimulus. Behaviourally, habituation is a decreased response to a stimulus (i.e. "learning what not to do"[Razran, 1971]), and sensitization is an increased response to a stimulus (i.e. the sea snail *Aplysia's* gill withdrawal reflex can be sensitized by applying a mild electric current stimulus to its tail [Byrne, 2013]). While much of what we know about the physiological basis of non-associative learning comes from studies of invertebrates like *Aplysia* (Byrne, 2013; Kandel, 2001; Carew & Kandel, 1973) and the roundworm *C. elegans* (Lau, Timbers, Mahmoud, & Rankin, 2013), habituation and sensitization are readily studied in rodents and humans as well (Lissek & Van Meurs, 2015; Orr et al., 2002; Piltz & Schniltzer, 1996; Leaton & Supple, 1986; Davis, 1970, 1972).

The study of the neural substrates of associative and non-associative learning processes is crucial to understanding the 'pathophysiology' of PTSD. The clarification of these two different types of learning is a useful theoretical distinction, because the symptoms of the disorder can be split naturally into those shaped by associative learning (i.e. the re-experiencing and cue-avoidance symptoms) and those shaped by non-associative learning (the hyperarousal symptoms and comorbid anxiety behaviour). Any animal model intended to allow us to study the neural basis of the disorder should produce as many symptoms of the human disorder as possible, and do so by eliciting species-specific responses to species-relevant threats (Goswami et al., 2013; Adamec, 1997; Skolnick & Paul, 1983). Both associative and non-associative fear memories follow the standard stages of processing for any memory trace: they are subject to acquisition and consolidation, and can be altered by intrinsic updating processes like reconsolidation

and extinction. As the symptoms of PTSD suggest that these memory processes are altered to extreme in people with the disorder, they are now discussed in detail.

1.2.1 Fixing to Learn: Consolidation

Researchers in psychology and neuroscience operationally define learning as a relatively permanent change in behaviour as a result of experience (Gluck, Mercado, & Myers, 2016; Bouton, 2007; Smock, 1999) or as the acquisition of information as a result of experience (Squire, 1987; Tulving, 2000). A memory is therefore an experience-dependent internal representation (Dudai, 2004) or simply the capacity to retain learnt information (Alberini, 2009). This computational terminology often goes undefined in neuroscience, but can be made explicit (Churchland & Sejnowski, 1993). *Information* can be rigorously defined as the reduction in uncertainty about some state of the world that a receiver gains from a message (Shannon & Weaver, 1949); this information-theoretic definition is amenable to mathematical studies of neural activity (Reike, Warland, Van Steveninck, & Bialek, 1997; Dayan & Abbott, 2001; Gallistel, 2003). A *representation* is a functioning isomorphism, where a pattern in one system stands for an entity in another system. Thus $y=mx+b$ is an algebraic (representing system) representation of a straight line in geometry (represented system). When cognitive scientists use the term to describe a memory, they are defining the memory as a pattern in neural activity (representing system) carrying information about an aspect of the experienced world (represented system). However, a memory in the brain is not a static entity. Five decades of studies on the pharmacological manipulation of learning and memory in animals have led to two broadly accepted stages of memory formation, acquisition and consolidation (Nader & Hardt, 2009; Ledoux & Alberini, 2013; McGaugh & Itzquierdo, 2000; Squire, 1987).

The learning experience is naturally the crux of memory acquisition. In fact, when defined using pharmacological methods, the acquisition phase of memory envelops the learning experience or (in a laboratory setting like fear conditioning) the *training*. Once information is learned and a memory is acquired (e.g., a memory trace or "engram" is generated), however, it remains in a labile state where its strength and even its existence are sensitive to pharmacological manipulation. This has been shown through a variety of CNS-targeted drug interventions, most comprehensively with protein synthesis inhibitors (e.g., *Anisomycin* -McGaugh, 1966; McGaugh & Herz, 1972; Davis & Squire, 1984; McGaugh, 2000; Klann & Sweatt, 2008). Protein synthesis inhibitors given following acquisition (training) will block formation of "long-term memory" (LTM is memory evident when tested hours to years following training) but not "short term memory" (STM is memory evident minutes to hours following training). This suggests there is a time-limited neurophysiological process where the initial memory trace must be 'laid down' in the brain in order to be transferred to a long-term/permanent storage format, a process termed consolidation (Muller & Pilzecker, 1900; Dudai, 1996; 2004; McGaugh, 2000; Squire & Bayley, 2007; Kandel, Dudai, & Mayford, 2014).

The term *consolidation* is used with two different meanings in neuroscience (Polster, Schacter, & Nadel, 1991; Dudai, 2004; Eichenbaum, 2011; Eichenbaum & Mackenzie, 2011). The first use refers to a process working on a timescale of minutes to hours that requires new protein synthesis. This is cellular (or synaptic) consolidation, which is dependent on translation-driven molecular changes to synaptic efficacy (Nader et al., 2002; Dudai, 2004; Kandel, 2001). Cellular consolidation is sometimes referred to as *fixation* to distinguish it from systems consolidation, a much lengthier process (weeks

in rodents and years in humans) where the memory trace becomes largely independent of the hippocampus, the site of most initial encoding, and is thereafter represented in more distributed cortical and cortical-hippocampal networks (Eichenbaum, 2011; Squire & Alvarez, 1995; Scoville & Milner, 1957). Most behavioural neuroscience research focuses on cellular consolidation, which can be formally defined as a 'time-dependent stabilization process leading eventually to permanent storage of a new memory' (Nader & Hardt, 2009). It is therefore the process that transfers the trace from STM to LTM, as these stages are defined above.

1.2.2 Thanks for the Update: Reconsolidation and Extinction

Five decades of research have provided rich support for the pharmacological consolidation theory (Rudy, 2014; McGaugh, 2000). However, it is explicit in this theory's original form that once it is consolidated a memory trace is more-or-less permanent, and not subject to change (an idea implied in the early term *fixation* for cellular consolidation). Troublesome for the theory is that such a memory system, where particular snapshot representations of the world are fixed as if in amber, would not be all that useful to organisms. This fixation model is unlikely to be how memory actually functions, using the rigorous criteria for *function* from evolutionary biology. As Klein, Cosmides, Tooby, and Chance (2002) note: "Memory evolved to supply useful, timely information to the organism's decision-making systems"(p. 306), and Tulving (2000) similarly remarked: "Owners of biological memory systems are capable of behaving more appropriately at a later time because of their experiences at an earlier time (p.727)". That is, in order to help the organism solve problems which must be solved for it to survive and reproduce (the biological definition of function), well-adapted memory systems must

have computational mechanisms to update the information about the world that is represented in memories, an idea consistent with the definition of learning as an *adaptive* change in behaviour (Bouton, 2007; Timberlake, 1994; Gallistel, 1990).

Psychologists and neuroscientists have studied two such representational updating processes in detail. One, *extinction,* was discovered by Pavlov and is familiar from decades of research on animal learning (Pavlov, 1927; Humphreys, 1939; Bullock & Smith, 1954; Bouton & Bolles, 1979; Morgan, Romanski, & Ledoux, 1993). The second process is *reconsolidation*, a concept also first discovered several decades ago (Misanin, Miller, & Lewis, 1968; Lewis, 1979) but that has attracted far more interest since its revival by Nader, Schafe, and Ledoux of New York University (2000). Reconsolidation challenges a strict interpretation of traditional consolidation theory by allowing the dynamic updating and bidirectional modulation of a stored memory; that is, not only can memory traces be updated to account for changed states of the world, they can be both degraded and enhanced by this mechanism (Alberini & Ledoux, 2013; Hardt & Nader, 2010; Lee, 2009). Because these processes have important implications for the consolidation concept, they are now briefly discussed.

1.2.3 Extinction

Extinction is defined as the reduction in a conditioned response that occurs when the conditioned stimulus is repeatedly presented without the unconditioned stimulus (Todd, Verbic, & Bouton, 2014; Quirk & Miller, 2008, Myers & Davis, 2007). In formal Pavlovian jargon, extinction training involves repeatedly exposing a previously trained animal to a conditioned stimulus (CS) *without* its previously paired unconditional stimulus (US). The animal soon learns that the CS no longer predicts the US, and the CR

is then said to be *extinguished* (Rescorla, 1996; Delamater, 2004). Importantly, the original CS-US memory trace is not forgotten, unremembered, or otherwise deleted by the new learning (despite contrary claims built into some formal learning models, e.g., Rescorla & Wagner, 1972, McLaren & Mackintosh, 2000). Instead, a new and competing "CS-noUS" trace is said to be formed during extinction learning (Bouton, 1994, 2002, 2004; however, just how exactly the brain would represent CS-noUS is a real conceptual and computational problem-see Gallistel, 1995 & Gallistel, 2012).

In computational terms, extinction learning allows an animal to deal with "stochastic parameters" in the world (following extinction, the animal remembers that there was previously *mutual information* [very roughly, correlation] between CS and US, but now there is not- so this aspect of the world is *non-stationary*- from the brain's point of view it can take on many values). Thus for the brain extinction is a Bayesian updating process (Gallistel, 2012; Courville, Daw, & Touretzky, 2006).

A great deal of research has examined how extinction learning and memory can be enhanced pharmacologically, producing a pharmacopeia of candidate substances including *D*-cycloserine, Propranolol, and exogenous hydrocortisone, among others (Kroes et al., 2016; Sartori et al., 2015; Vupic, Gold, & Bouton, 2011; Graham, Langton, & Richardson, 2011; Clay et al., 2011; Davis, Ressler, Rothbaum, & Richardson, 2006; Cai, Blundell, Han, Greene, & Powell, 2006; Ressler et al., 2004; Ledgerwood, Richardson, & Cranney, 2003). In the clinic, extinction is widely used as a treatment for PTSD, but this application is limited by the fact that the original traumatic memory is not deleted but can return (phenomena also seen in the laboratory such as spontaneous recovery, reinstatement, etc. [Rescorla, 1996; 2004]). These limitations to the clinical use

of extinction have resulted in translational research directed at the reconsolidation concept, and how it may be modulated with drug treatments.

1.2.4 Reconsolidation

Reconsolidation has been a fairly controversial subject since its revival by Nader, Schafe, and Ledoux at the turn of the millennium. Some researchers continue to deny its existence or its independence as a distinct phenomenon from extinction and other forms of new learning, or insist it is an experimental artefact (see the various perspectives reviewed in: Dudai, 2012; Besnard, Cabard, & Laroche, 2012; McKenzie & Eichenbaum, 2011; Lee, 2009; Nader & Hardt, 2009, Alberini, 2005; McGaugh, 2004; Biedenkapp & Rudy, 2004). Some of these reservations are the products of confusion and are semantic in nature- although the two processes share many properties and physiological components, memory reconsolidation is not a literal recapitulation of memory consolidation, making the name somewhat misleading (Dudai, 2006). The phenomenon so influentially demonstrated by Nader et al. (2000) showed that a reactivated fear memory trace (i.e. a previously learned association between tone and shock) could be blocked by protein synthesis inhibition following cue-induced retrieval.

Thus when a memory trace is reactivated by a reminder cue of some sort, it becomes labile yet again, and (in the laboratory) is subject to attenuation by protein synthesis inhibitors. Reconsolidation is therefore protein synthesis-dependent like consolidation, although the molecular players are somewhat (but not totally) different (Li, Meloni, Carlezon, Milad, Pitman, Nader, & Bolshakov, 2013; von Hertzen & Giese, 2005; Debiec & Nader, 2004; Lee, Everett, & Thomas, 2004). The boundaries between extinction and reconsolidation have also been disputed on methodological and molecular

grounds, as the retrieval trial(s) technically constitutes an extinction trial (Dudai, 2012; Monfils, Cowansage, Klann, & Ledoux, 2009). However, as with consolidation and reconsolidation, many studies have shown that reconsolidation and extinction have differing cell and molecular signatures (Lin et al., 2003; Suzuki et al., 2004; Power, Berlau, McGaugh, & Steward, 2006, Mamaya et al., 2009), and different temporal parameters (Pedreira & Maldonado, 2003; Pedreira, Perez-Cuesta, & Maldonado, 2004).

Additionally, researchers specify "boundary conditions" that are necessary for reconsolidation and distinguish it from extinction. One derives from the fact that a reminder cue will elicit several associations, and the associative trace that dominates behaviour will be the one to actually reconsolidate (Eisenberg, Kobilo, Berman, & Dudai, 2003). This is simply a statement that the trace that most powerfully controls behaviour is the one vulnerable to protein synthesis blockers at the time of retrieval. More critical to defining the reconsolidation concept in its own right is a requirement that novel information be present during the reactivation session- as Pedreira and colleagues (2004) showed, blocking reactivated LTM with Anisomycin only works if there is a mismatch between what the animal expected and what actually occurred. Dudai (2012) points out that this sort of mismatch or *prediction error* is what drives learning in most computationally explicit theories (Rescorla & Wagner, 1972; Schulz, Dayan, & Montague, 1997; Zhang, Berridge, Tindell, Smith & Aldridge, 2009) and is consistent with the idea of reconsolidation as a representational updating mechanism.

If we consider how the brain must actually instantiate memory, the nature of consolidation and reconsolidation as mechanistically overlapping but temporally distinct processes makes sense, and offers another line of reasoning against traditional

consolidation theory. Indeed, far from being the mechanism for permanently embedding a particular trace into the brain, cellular consolidation mechanisms can be understood computationally as subroutines that continually form, modify, and update experience-dependent representations (memories). In this sense, "consolidations never end" (Dudai, 2012). However we can certainly retain the conceptual categories for consolidation (the initial laying down of a trace), reconsolidation (cue-dependent updating of a trace that embeds new information in it) and extinction (event pairing-sensitive updating mechanism that uses competing THIS MEANS THAT and THIS NO LONGER MEANS THAT traces to navigate relationships between events in the world that change over time, i.e., the multivariate time series algorithms mentioned above.

1.2.5 Getting Emotional: What is Fear, Anyway?

While a significant literature exists on the behavioural and neurobiological properties of instrumental and incentive-based learning (Dolan & Dayan, 2013; Berridge & Kelley, 2002; Stellar & Rice, 1989; Yeomans, 1988), there is little doubt that the vast majority of research into the neural basis of learning and memory has used one particular protocol: Pavlovian fear conditioning. This has been in large part because the paradigm is simple and reliable. While bringing 'fear' into the mix means that researchers are not studying a putative 'domain-general' or content-independent learning system (which is unlikely to exist-Gallistel, 1990, 2000; Gallistel & Balsam, 2014; Cosmides & Tooby, 1994, 2000; Pinker, 1997; Barrett, 2012), its evolutionary basis is intuitive, and thus gives systems for 'fear learning' some claim to being a central feature of brain design, conserved as a major avenue for navigating environmental threats (predators, cliffs, deep

water, etc.) keeping animals alive to reproduce (Ohman & Mineka 2001; Cosmides & Tooby, 2000; Marks, 1987).

While uses of the terms "fear conditioning" or "conditioned emotional response" to describe what goes on in Pavlovian protocols have been uncontroversial over the years, the recent growth in research on subjective human emotional experience (e.g., Davidson & Begley, 2013; Armony & Vuilleumier, 2013; Lindqvist & Barrett, 2012; Mechias, Etkin, & Kalisch, 2010) has prompted concern from at least one prominent researcher that it is misleading to refer to what is studied in the Pavlovian paradigms discussed above as "fear". Ledoux notes that the (unconscious) activity of the "fear circuits" activated by conditioning does not directly create the conscious *feeling* humans label as fear. By this reasoning, we risk conflating the effects of neural circuits operating below conscious awareness (what researchers like Ledoux actually study using fear conditioning paradigms) with the still poorly understood neural substrate of our conscious feeling of being afraid (Ledoux, 2012; 2014; 2015).

Ledoux has argued for the reframing of emotional circuits in the brain as survival circuits, circumventing any implications about their relation to conscious awareness (Ledoux, 2012). This dovetails fairly well with the computational definition of emotion from evolutionary psychology as a "superordinate program" that adjusts physiological and behavioural parameters into an optimal configuration for dealing with a particular evolutionarily recurrent threat such as predators, thirst, or mate selection (Cosmides & Tooby, 2000; Nesse, 2005, 2007), although his emphasis on individual survival is naïve compared to true evolutionary research; selection sculpts neural circuits that propagate the genes that build said circuits, not necessarily ones devoted to *an individual's* well-

being or survival (Tooby & Cosmides, 2008). In any case, in Ledoux's approach, fear conditioning is simply renamed threat conditioning, a term meant to be agnostic on the role of conscious feelings in Pavlovian learning (and reciprocally, the role of circuits driving this learning in conscious feelings) that retains the image of a defensive behavioural response being entrained. Whether one accepts this argument or not, the need for terminological precision is quite real. The superordinate program definition advanced by Cosmides &Tooby is helpful, as it provides a definition of emotion where no one facet (conscious feeling, facial expression, neural circuit activation, peripheral physiological changes, etc.) need be dominant or taken to singularly define emotion- they are a constellation of factors that the emotion program adjusts into an optimal configuration for dealing with an ancestral threat. Thus the responses studied in fear conditioning protocols *can* be viewed as fear behaviour, as long as it is clear that their neural underpinnings are not the totality of the brain basis of the emotion, or that their existence implies conscious feeling of a sort identical to human awareness. The same need for precision extends beyond the conditioning laboratory to the more ethologically-oriented models of fear described below, especially as these are often explicitly aimed at recapitulating evolutionarily thematic dangers.

1.3 From Molecule to Memory: Brain Mechanisms of Fear Learning

Semantic issues aside, neuroscience research into the workings of memory has profited greatly from the associative and non-associative learning paradigms described above. Much of what we presently know about how brain circuits, cells, and molecules create, maintain, and modify memories comes from invertebrate models of non-associative learning and rodent models of associative learning, especially Pavlovian fear

conditioning. Beginning with the insights of D.O. Hebb (1949), behavioural neuroscientists have searched for a physiological bonding process in neural connections that parallels the nature of behavioural associative learning processes. That is, they look for some sort of *coincidence-detecting* mechanism, such as where a synapse linking two neurons is strengthened when both of these neurons are active at the same time (Bliss & Collingridge, 1993).

1.3.1 Synaptic Plasticity

Lomo and Bliss (1973) discovered just such a process, which they termed Long-Term Potentiation or LTP. Briefly, LTP refers to the enhancement of neurotransmission at a given synaptic junction (electrophysiologically, the strengthening of synaptic conductance) by repeated stimulation of a presynaptic neuron- thus a 'weak' synapse on this cell that is active at the same time that another 'strong' synapse is active is *potentiated,* and this neuron consequently responds to the same input with greater depolarization. (Nicoll & Roche, 2013; Dudek & Bear, 1992). When a population of cells is being studied, they are first given weak stimulation, producing a weak electrophysiological response. Strong stimulation (Tetanus) is then applied, and the response to the weak stimulus is recorded. A glance at the oscilloscope then shows it has been strengthened, or potentiated (Rudy, 2014; Sweatt, 2009).

The molecular mechanisms underlying LTP have been worked out in considerable detail (Frankland & Josslyn, 2016; Malenka & Bear, 2004). Consistent with the hypothesis of LTP processes as a mechanism for memory consolidation, long-lasting LTP (L-LTP) requires protein synthesis, specifically of various synaptic elements (e.g., adhesion and scaffolding related structures such as PSD95 and HOMER, as well as new

glutamate receptors-Rudy, 2014). Further, researchers have found the NMDA glutamate receptor to have precisely the sort of coincidence-detecting properties needed to underlie a synaptic potentiation process; NMDA receptor-mediated LTP remains the most studied variety of the process (Collingridge & Bliss, 2013). With many of the synaptic elements participating in LTP identified (Panja & Branham, 2014; Mayford, Seigelbaum, & Kandel, 2012), research is now focused on the intracellular signalling cascades that mediate synaptic changes by driving protein synthesis. Brain-Derived Neurotrophic Factor (BDNF) has emerged as a key molecule in synaptic plasticity and LTP as related to learning and memory (Panja & Branham, 2014). This molecule provides a mechanistic link between learning and consolidation of fear memory (Monfils, Cowansage, & Ledoux, 2007).

1.3.2 BDNF, TOPs, & mTOR

Given that a long tradition of research points to the amygdala as a key hub for plasticity in fear learning-related processes (Rogan, Staubli, & Ledoux, 1997, Blair, Schafe, Bauer, Rodrigues, & Ledoux, 2001), experimenters have focused in on the role of amygdalar BDNF activity in fear memory consolidation, with much evidence of BDNF transcription during fear memory consolidation (Rattiner, Davis, French, & Ressler, 2004a; Rattiner, Davis, French, & Ressler, 2004b; Ou & Gean, 2006; Ou & Gean, 2007). BDNF has been show to initiate protein synthesis through downstream activation of the mechanistic Target Of Rapamycin (mTOR) kinase pathway (Takei et al. 2004) and this activation appears to regulate expression of the GluR1 glutamate receptor, a component necessary for memory formation (Slipczuk et al., 2009). mTOR has previously been shown to drive LTP (Tang et al., 2002). The link between BDNF and mTOR is part of a

complex feedback loop bridging synaptic function and translation enhancement. Local (dendritic) translation activates terminal oligopyrimadine tracts (TOPs), a subset of mRNAs coding for the translation machinery needed to synthesize synapse-specific elements (Tsokas et al., 2005; Tsokas, Ma, Iyengar, Landau, & Blitzer, 2007).

mTOR regulates synthesis of TOP mRNAs (Thoreen, Chantranupong, Keys, Wang, Gray, & Sabatini, 2012). LTP-generating synaptic activity increases BDNF concentrations, and blocking BNDF activity prevents translation of TOP mRNAs (Braham & Massoudi, 2005). BDNF binds TrkB receptors, which are co-localized with glutamate receptors. BDNF activation of TrkB activates mTOR, and iniates a positive feedback loop of increased BDNF levels. This is thought to represent the mechanistic contribution of BDNF to consolidation, where it recruits intracellular calcium to restore depleted amounts of the cation in synapses undergoing plasticity. Further, blocking TrkB receptors (and thus BDNF activity) has been shown to block both TOP translation and LTP. It is currently thought that the BDNF-TrkB cascade is needed to activate the mTOR-TOP system in order to increase translation activity in the region undergoing plasticity (Rudy, 2014; Braham & Messaoudie, 2005).

Based on these and other findings, there is now a large and growing research literature on the role of the mTOR kinase pathway in memory processes, specifically addressing how upstream signals from the synapse (not limited to TrkB) activate mTOR, and how it in turn drives translation of products needed for the ongoing synaptic plasticity underlying fear memory acquisition, consolidation, and updating, mostly through the biochemical cascades outlined above. Before turning to a review of this work in the context of

Pavlovian and Predator Stress models of fear learning, the molecular and pharmacological details of mTOR and its inhibitor Rapamycin are discussed.

1.4 mTOR and Rapamycin (RAP)

mTOR is a serine-threonine kinase (an enzyme that phosphorylates the -*OH* group of these amino acids) at the centre of a complex signalling pathway that is strongly conserved across phyla (Li, Kim, & Blenis, 2014; Hay & Sonenberg, 2004). It contributes to synaptic plasticity by controlling a subset of protein synthesis through its downstream effectors, and responds to signal transduction pathways mediated by postsynaptic receptors such as NMDA and TrkB that are crucial to synaptic plasticity (Graber, McCamphill, & Sossin, 2013; Hoeffer & Klann, 2010). mTOR also plays a crucial role in cellular processes of growth, proliferation, and metabolism, and responsive to a variety of extracellular signals including nutrient levels, stress, and energy in addition to memory and plasticity related cascades (Hartford & Ratain, 2007). Abundant nutrients or energy levels promote mTOR signalling, while energy depletion and stress down-regulate the pathway's activity (Wullschleger, Loeweth, & Hall, 2006). The 2,549 amino acid, 250-289kD mTOR molecule is found in eukaryotic cells as a component of two different molecular complexes, complex 1 (mTORC1) and complex 2 (mTORC2) (Hay & Sonenberg, 2004; Hoeffer & Klann, 2010).

mTORC1's structure and function are well-characterized. It is bound to the proteins RAPTOR (Regulatory Associated Protein of TOR) and mLST8, and is inhibited by the bacterium-derived drug Rapamycin (RAP). mTORC2 structure and function remains less well-understood. It is bound to the proteins RICTOR (Rapamycin Insensitive Companion of TOR), mSIN1, and mLST8 (Howell & Manning, 2011). As the RICTOR

name implies, mTORC2 activity is not usually inhibited by Rapamycin (Sarbassov et al., 2006), although evidence is accumulating that it is affected by repeated doses of RAP (Howell & Manning, 2011; Costa-Mattioli & Monteggia, 2013). mTOR's phosphotransferase activity is promoted by the G Protein RHEB when RHEB is bound to GTP. RHEB is in turn regulated by the Tuberous Sclerosis 1&2 (TSC1& TSC2) heterodimer. TSC2 acts as a GTPase-activitating protein on RHEB and converts it into an inactive, GDP-bound form. The result of these biochemical interactions is that the TSC1/TSC2 heterodimer negatively regulates mTORC1 activity (Hay & Sonenberg, 2004). In addition, some hormones and other upstream signals (e.g. growth factors) can activate Tyrosine kinases and G protein receptors, which then activate signal transduction pathways (e.g., PI3K-AKT and Ras-ERK) that exert a stimulatory effect on mTORC1 activity by inhibiting TSC1/TSC2. This inhibition involves the phosphorylation of TSC2 by kinases that include AKT, ERK, and ribosomal S6K (Ma & Blenis, 2009). A summary of this pathway can be found in Figure 1.

Less is known about the function of mTORC2 than mTORC1, largely because it is less amenable to manipulation with Rapamycin (Laplante & Sabatini, 2013). However along with contributions to energy homeostasis, neural mTORC2 is apparently involved in learning and memory like mTORC1, with a specific role in actin dynamics-mediated LTP and LTM; in fact, its key role is regulation of actin polymerization (Huang et al., 2013; Jacinto et al., 2004). Also relevant to synaptic plasticity is mTORC2's control of neural spine structure and shape, where it works along with mTORC1 to control dendritic arbour morphology (Urbanska, Gozdz, Schwiech, & Jaworski, 2012), and may also be involved in long-term depression, or LTD (Costa-Mattioli & Monteggia, 2013).

In response to upstream signals (e.g., NMDAR, TrkB-R) mTORC1 acts downstream on two substrates: S6 kinase 1 (S6K1) and eIF4E-binding protein 1 (4EBP1), which interact with mRNAs to control ribosomal biogenesis and the initiation and progression of translation; that is they help control neural protein synthesis (Ma & Blenis, 2009; Hay & Sonenberg, 2004). Protein synthesis is divided into three stages: initiation, elongation, and termination (Alberts et al., 2008). Initiation is the rate-limiting step, and is where mTOR effectors act (Hay & Sonenberg, 2004). 4EBP1 is in fact an inhibitor of mRNA translation, but when it is phosphorylated by mTORC1 it dissociates from eIF4E and shuttles the translation-initiating factor eIF4G to a subset of mRNAs (Hara, Yonezawa, & Kozlowski, 1997). The term "subset" is noteworthy, as mTOR is ubiquitous in cells but its role in protein synthesis promotes expression of a relatively small amount of products (Parsons, Gafford, & Helmstetter, 2006). Specifically, the eIF4E molecule helps recruit the 40*S* ribosomal subunit to the 5'-end of mRNAs, the rate-limiting step in cap-dependent translation (Ma & Blenis, 2009). mTORC1's phosphorylation of 4EBP1 therefore enables translation by blocking a substrate that inhibits translation.

Phosphorylation of S6K1 by mTORC1 leads to the phosphorylation and binding of various proteins (e.g., eEF2K, eIF4B), which promote the initiation of translation (Zoncu et al., 2011). S6K1 also increases transcriptional activity of ribosomal RNA polymerase 1, making mTORC1 a positive regulator of ribosomal RNA synthesis (Mayer et al., 2004). Structurally, mTOR's *C*- terminal end contains a kinase catalytic domain (KIN) with several phosphorylation sites associated with higher levels of mTOR activity,

most notably serine 2448, whose phosphorylation state is the immunohistochemical index of mTOR activation (Hoeffer & Klann, 2010; Reynolds, Bodine, & Lawrence, 2002).

1.4.1 Rapamycin

Nearly all research aimed at understanding the mTOR pathway has made some use of Rapamycin (RAP) to inhibit activity of the mTORC1 pathway. This antibiotic peptide was originally discovered on Easter Island (*Rapa Nui* in Polynesian) and is derived from the soil bacterium *Streptomyces hygroscopicus* that was sampled from that location (Vezina, Kudelski, & Sehgal, 1975). First studied in yeast, it was later found to work by similar processes in mammals, giving mTOR its initial name (*Mammalian* Target Of Rapamycin, which has recently been supplanted by *Mechanisti*c Target).

1.4.2 RAP Pharmacodynamics and Pharmacokinetics

RAP exerts its inhibitory effect on mTOR signalling at a site called the FKBP12-Rapamycin binding domain (FRB), which is located next to mTOR's catalytic KIN domain (Hoeffer & Klann 2010; Hay & Sonenberg, 2004). RAP first binds to FK506-binding protein 12 (FKBP12) and forms a larger protein complex. The RAP-FKBP12 complex then binds to the mTOR molecule's FRB domain and acts as an allosteric inhibitor, proceeding to disrupt protein-protein interactions that are essential to normal mTOR function. Specifically, the binding of the RAP- FKBP12 complex to the FRB disrupts the mTOR-RAPTOR protein-protein association, uncoupling mTORC1 from its substrates and disrupting normal signalling (Bove, Martinez-Vincente, & Vila, 2011). Therefore RAP does not directly inhibit mTOR catalytic activity (by inactivating the catalytic domain), but instead disrupts its formation of protein complexes, and thereby effectively blocks downstream signalling (Kim et al., 2002; Beretta et al., 1996). Acute

RAP treatment selectively inhibits mTORC1, but long-term RAP exposure may inhibit mTORC2 in certain cells by sequestration of freshly made mTOR molecules (Laplante & Sabatini, 2013). Indeed, it is thought that long-term treatment with high doses of RAP can inhibit mTORC2 activity by inhibiting binding and assembly of RICTOR and mSIN1, protein components known to be specific to mTORC2 (Sarbassov et al., 2006).

RAP pharmacokinetics are well-characterized, if metabolically complex. The drug has a bioavailability of 5% and a tmax (time to maximal concentration) of three hours in the rat (Napoli et al., 1997; Napoli & Taylor, 2001). Radioligand binding studies have demonstrated that first-pass metabolism of the drug is inversely dose-dependent. For example, 40% of a 0.5 mg/kg dose and 3% of a 5-mg/kg dose are metabolised by the liver in this fashion (Crowe et al., 1999). Specifically, RAP is metabolised in the liver by cytochrome P450 3A-class enzymes, and rat studies have demonstrated as many as 16 demethylated or hydroxylated metabolites (Trepanier, Gallant, Legatt, & Yatscoff, 1998).

The drug crosses the blood brain barrier following systemic administration, with brain tissue concentrations being an exponential function of blood concentrations (Banarkee, 2011). The half-life of RAP is fairly long, having been measured at longer than five hours in rats, primates, and humans (Trepanier et al., 1998).

1.5 Animal Models of PTSD

There is a large literature on the human psychobiology of PTSD, encompassing neuroendocrine, psychophysiological, and neuroimaging approaches (e.g., Acheson et al., 2014; Pole, 2007; Etkin & Wager, 2007; Bryant et al., 2005; Yehuda, 2009; Rasmusson et al., 2003). However these approaches are largely non-invasive for practical and ethical reasons and therefore provide only correlational data. Direct manipulation of the brain in

order to clarify causal mechanisms requires the use of animal models. A vast amount of work has aimed to model symptoms of PTSD in rodents in order to discover underlying cellular and molecular mechanisms of the disorder (especially consolidation, extinction, and reconsolidation of traumatic memories), an approach that can identify targets for potential pharmacological treatments.

While there is no one ideal animal model of PTSD that recapitulates all symptoms of the disorder, Pavlovian fear conditioning paradigms and predator stress paradigms are the major approaches used by researchers and are discussed in detail here. Pavlovian fear conditioning effectively models the re-experiencing and cue symptoms of PTSD, while predator stress captures these symptoms as well as producing hyperarousal and anxiety-like behaviour.

1.5.1 Fear Conditioning

The fear memories produced by Pavlovian paradigms involve the organism learning that a previously innocuous or neutral cue (a conditioned stimulus, or CS) such as a light or buzzer predicts the onset of a naturally fear-producing stimulus (unconditioned stimulus, US, such as a painful footshock) to which an animal has an innate and quite reflexive behavioural fear response. This is the unconditioned response, (UR) such as tonic immobility (*freezing)* seen in both rodents and humans (Maren, 2001; Ledoux, 2003). Unsurprisingly, little experience is required for animal to "associate" these stimuli in memory, and very quickly the CS comes to elicit the fear response, now referred to as the conditioned response or CR (Gluck, Mercado, & Myers, 2016).

As noted above, conditioning is about the animal learning about relations between events in its world, and fear has been powerfully shaped as survival mechanism

over evolutionary time (Ledoux, 2012; Ohman & Mineka, 2001, Cosmides & Tooby, 2000). Thus little experience is needed for objects and contexts predictive of danger or pain to prime the animal to respond with fear physiologically and behaviourally to these cues when encountered again. Pavlovian paradigms have been successful in modeling one set of PTSD symptoms because the fear learning mechanisms activated in these protocols are dramatically recalibrated in the disorder. As noted above, extinction allows an animal to update its awareness of predictive relationships. This process appears to fail in PTSD (Morgan et al., 2014; Mahan & Ressler, 2012), so cues and contexts related to the original trauma continue to generate powerful fear responses long after they have had any predictive value (Bailey & Balsam, 2013).

1.5.2 Limitations of Fear Conditioning as a PTSD model

By generating strong fear memories for contexts and cues, fear conditioning superbly captures the associative aspects of PTSD symptomology. The disadvantages of Pavlovian fear conditioning are that it does not involve exposure to a truly life-threatening event, nor does it mimic other PTSD symptoms such as persistent generalized hyperarousal (Pitman, 1997), or increased anxiety-like behaviour (Pitman, Orr, & Shalev, 1993). Exposure to a predator (or predator odour), however, produces strong associative fear memories, as well as hyperarousal and anxiety-like behavior (Fifield et al., 2013).

1.5.3 Predator Stress

Predator Stress (PS) paradigms typically involve acute exposure of a prey species (typically a mouse or rat) to a predator (typically a cat, rat, or ferret). Predator scent stress (PSS) involves exposing the prey species to a chemical given off by the predator in fur or urine (Wallace & Rosen, 2000; Dielenberg, Carrive, & McGregor, 2001; Blanchard,

Yang, Li, Gervacio, & Blanchard, 2001; Hebb et al., 2003; Vyas, Kim, Giacomini, Boothroyd, & Sapolsky, 2007; Rosen, Pagani, Rolla, & Davis, 2008). A considerable literature exists documenting the effects of acute (5-10 minute) unprotected cat exposure as a rodent model of PTSD, as it can generate high levels of both associative fear, non-associative fear, and anxiety behaviour (Adamec & Shallow, 1993; Adamec, Shallow, & Budgell, 1997; Adamec, 1998; Adamec, Burton, Shallow, & Budgell, 1999; Adamec, 2001; Adamec, Bartoszyk, & Burton, 2004; Amadec, Walling, & Burton, 2004; Adamec, Blundell, & Burton, 2005; Blundell, Adamec, & Burton, 2005; Adamec, Head, Soreq, & Blundell, 2008; Fifield, Hebert, Adamec, & Blundell, 2013; Fifield et al., 2015; Lau, Whiteman, & Blundell, 2016).

1.5.4 Predator Vocalization paradigms

In addition to direct exposure and scent exposure, ecologically minded researchers have examined the responses of prey species to other predator stimuli that they would be likely to encounter in the wild. Predator sounds have been especially amenable to this approach and have generated a growing literature of field and lab studies on prey responses to predator vocalizations (Hettena, Munoz, & Blumstein, 2014; Clinchy, Zanette, Sheriff, McGowan, & Boonstra, 2011; Hendrie, Weiss, & Eilam, 1996). This approach has been used with a large number of species, including marmots (Blumstein, Cooley, Winternitz, & Daniel, 2008), voles (Eilam, Datan Ben-Eliyahu, Schulman, Shefer, & Hendrie, 1999), and non-human primates (Seyfarth & Cheney, 1990; Hauser & Wrangham, 1990; Bshary & Noe, 1997; Friant, Campbell, & Snowdon, 2008), in addition to rats and mice (Abramsky, Strauss, Subach, Kotler, & Riechman, 1996; Hendrie, Weiss, & Eilam, 1998, Edut & Eilam, 2003).

Such studies have found a number of effects of predator sounds on prey. For example, Zanette, White, Allen, & Clinchy (2011) found that predator vocal sounds decreased the reproductive success of female songbirds- perceived predation threat caused these females to generate few offspring, lay fewer eggs, produce more eggs that failed to hatch, and have more offspring die before first feeding, compared to songbirds exposed to a control (non-predator) sound. In combination with work suggesting this sort of stressor increases corticosterone levels, evidence suggests that exposure to predator sounds can be a long-lasting, physiologically powerful stressor (Clinchy, Sheriff, & Zanette, 2013).

While most predators typically don't vocalise when hunting, prey species do appear to respond to predator calls with various sorts of defensive (fear) behaviour (Blumstein et al., 2008), including risk assessment, hiding, rearing, and decreased foraging (Blanchard & Blanchard, 1988). However, many of these studies have been field experiments, and thus don't necessarily offer the controlled environment of the laboratory in addition to their ecological realism (a combination that is an advantage of live predator exposure paradigms). Thus, it is not known if exposure to predator vocalizations (i.e. cats) produce similar changes in behaviour as exposure to a live predator.

1.6 Predator stress, consolidation, and protein synthesis.

Ample evidence demonstrates that protein synthesis is necessary for consolidation of predator stress-induced non-associative fear memories such as hyperarousal and anxiety-like behaviour, paralleling associative fear memories from the fear conditioning literature (Adamec et al., 2006; Cohen et al., 2006; Blundell et al., 2005; Kozlovsky et al., 2008). Adamec et al. (2006) found that systemic injection of Anisomycin following

predator exposure blocked the increase in anxiety-like behavior and response to acoustic startle when measured 7- 8 days later. Similarly, Cohen and colleagues (2006) found that intracerebroventricular microinfusion of Anisomycin either before or after Predator Scent Stress reduced anxiety and startle responses. While the identity of the target proteins is unknown, these data confirm that the synthesis of novel proteins is necessary for consolidation of non-associative fear memories.

Work by the Adamec and Cohen groups suggests that predator stress-induced fear memories are susceptible to protein synthesis inhibitors and thus require translation in order to be consolidated. While this seems like a normal property for any memory trace, it is notable given that predator stress paradigms produce non-associative memories, which must differ at some level (molecular, cellular, computational,circuit, etc.) from the better-understood associative memories familiar from fear conditioning work. The studies discussed above used Anisomycin, which reduces protein synthesis by as much as 60-80% and is thus a 'global' protein synthesis inhibitor. In contrast RAP only reduces protein synthesis by about 10%, and given its effects in conditioning paradigms, the transcripts it blocks appear quite specific to learning and memory processes (Helmstetter et al., 2008).

More recent work from the Blundell lab has demonstrated a role for the mTOR pathway in predator stress-induced associative and non-associative fear memories. Rats exposed to a cat and then injected with systemic RAP showed decreased freezing when re-exposed to the cat room context and lower anxiety in the elevated plus maze (EPM), along with lower hyperarousal in the acoustic startle test, compared with vehicle-injected controls. In all cases, RAP brought the behaviour of predator-exposed rats in-line with

that of handled control animals (Fifield, Hebert, Angel, Adamec, & Blundell, 2013). Thus RAP had blocked consolidation of the predator stress-induced fear memories. Consistent with this, immunohistochemical work shows elevated mTOR phosphorylation in the hippocampus and PAG of predator Stressed rats one hour following cat exposure (Whiteman, Smith, Ralph, Kenny, Walling, & Blundell, 2016; in preparation).

1.7 Goals and aims

While the research described above demonstrates a role for mTOR in consolidation of predator stress-induced fear memories, results from the classic Predator Stress paradigms (Adamec & Shallow, 1993) have proven to be quite variable (see Adamec, Walling, & Burton, 2004; Clay et al., 2011; Fifield et al., 2015;Apfelbach et al., 2005; McGregor et al., 2002). Moreover, our lab has not been able to generate a robust, consistent PTSD-like phenotype using predator odour (see Smith, 2009). Thus, the goals of these experiments were to 1) develop a robust and comprehensive animal model of PTSD and 2) use this model to study the effects of RAP on predator-induced associative and non-associative fear memories. Experiment 1 was designed to be a laboratory version of predator vocalization experiments, with controlled exposure of subjects to either the sound of a predator or a computer-generated control sound. This experiment was unsuccessful in generating fear memories, with no evidence of predator sound inducing contextual fear, non-associative fear, or hyperarousal.

The failure of this experiment led our laboratory to search for a more reliable and efficacious predator stress paradigm, one that would consistently generate PTSD-like symptoms in rodents and allow for pharmacological manipulation and study of the neural basis of the consolidation of the fear memories underlying these symptoms. The Rat

Exposure Test (RET) introduced by Yang and colleagues (2004), is a predator-prey model originally designed for the study of mouse defensive behaviour in the presence of a rat. Given our laboratory's interest in the neural basis of fear memory consolidation, we shifted the focus of the test from its original basis in mouse behaviour *during* interaction with a rat to examining whether this interaction would produce fear memories in mice, as evident in post-exposure testing of context re-exposure, anxiety-like behaviour, and hyperarousal. Thus, experiment 2 tested the effects of the RET on lasting associative (contextual fear) and non-associative (anxiety-like behaviour and hyperarousal) fear memories. Despite methodological issues with the contextual memory test, we showed that exposure to a rat produced lasting changes in anxiety-like behaviour and hyperarousal. Experiment 3 was designed to test the role of mTOR in consolidation of RET-induced associative and non-associative fear memories. Consistent with previous findings using a different predator stress model (Fifield et al., 2013), we show that aspects of RET-induced fear memories are mTOR-dependent.

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.

2.0 Methods

All procedures and protocols for experiments 1-3 and animal housing followed the guidelines of the Canadian Counsel on Animal Care and Memorial University of Newfoundland's Animal Care Committee.

2.1 Experiment 1 Assessing predator vocalizations as a stressor in rats

2.1.1 Subjects

Eighty Long-Evans rats (male, 6 weeks) from Charles River Canada (St. Constant, QC) were used in experiment 1. Subjects were housed individually in standard clear polycarbonate cages with metal covers. Food and water were available *ad libitum* and each cage contained bedding, cardboard nesting and enrichment objects. Rats were adapted to the colony room on a reverse 12-hour light/dark cycle, with lights off at 7 AM for two weeks prior to experimentation, with handling taking place during the second week. Handling involved picking each rat up for 1 minute each day for 5 days. Rats were held on the experimenter's forearm and gently petted. Reverse scheduling and handling procedures followed standard lab procedure for Predator Stress experiments (Adamec & Shallow, 1993; Fifield et al., 2013).

2.1.2 Groups

Rats were randomly assigned to one of four groups according to exposure condition: predator sounds for 10 minutes (PS10), control sounds for 10 minutes (CX10), predator sounds for 60 minutes (PS60), or control sounds for 60 minutes (CX60). All rats were returned to their home cages immediately following testing.

2.1.3 Sounds

Catcalls were recorded from a lab cat (Xavier) housed at the Memorial University Vivarium. Recordings were made using the sound recorder application on a standard Samsung *SII Galaxy* mobile phone. Six distinct calls were identified and analyzed using the program *Sound Analysis Pro 2011.04* to give a readout list of peak frequencies (1 peak per ms). A Python script was used to generate a sine-wave pure tone of the same

length (1 ms) for each of the peak frequencies. Combining the matched pure tones from each list of peak frequencies resulted in six control sounds. The open-source recording and editing software program *Audacity 2.0* was then used to match the amplitude of each control to its exemplar, and a low pass filter was applied to remove any high-end frequency (defined as any signal with a frequency above 3 kHz).

The predator recordings were created by randomly spacing catcalls at a rate of two per minute, preceded by a one-minute habituation period. Therefore rats heard the calls for the last nine minutes of the ten-minute condition, and the last 59 minutes of the 60-minute condition. Control recordings were generated by locking matched control sounds to the identical timestamp from the predator condition during the ten and 60-minute conditions- that is, both predator and control sound rats heard the sounds at the same intervals during the training period. Before training began (and with no animals present), the sounds were played through the boxes, and amplitude was measured, ensuring that the sound intensity emitted from the boxes for both conditions was exactly 80 dB, a value below that shown to engage the neural circuit for the acoustic startle reflex, which could have confounded the rats' responses to the sounds.

2.1.4 Procedure

All animals were habituated to the fear conditioning boxes for 10 minutes a day for three days prior to exposures. No sounds were played through the boxes during habituation. On exposure day, all animals were brought to the testing room and allowed to acclimate in a dark room for 1 hour before exposures began. All testing was conducted between 9:00 am and 12:30 pm. There were four fear-conditioning boxes (labeled 1-4) and all four rats being trained at a given time were in the same exposure condition. As

such, the order of the conditions was alternated for cohorts. Animals were weighed before they were placed in the chambers. Boxes were cleaned with 40% ethanol between training sessions and faecal boli were recorded. All animals were returned to their home cages in the colony room following exposures.

The computer program *FreezeFrame3* was used to record the animals' behaviour while inside the boxes. A threshold was established to determine what was considered immobility (or freezing behavior) and the program applied this to all animals and automatically scored the amount of time that the animal spent not moving. This freezing measure was recorded as a proportion of the total time spent in the box (to allow for comparisons between 10 and 60 minute conditions, as the latter would almost certainly show larger raw numbers, being 6 times longer than the former).

Forty-eight hours after exposure, rats were returned to the exposure chamber for 10 minutes, without any sounds played. Freezing proportion was again measured using FreezeFrame3 software, with the same threshold as used for the initial exposure measurement. On each successive day, rats were tested for Anxiety-Like Behaviour (ALB) on the Elevated Plus Maze, Open Field, and Light Dark Box and hyperarousal in the acoustic startle test. A description of the ALB behavioral tests and startle can be found in section 2.5.

2.2 Experiment 2: The Rat Exposure Test (RET) as a predator stress paradigm for the study of fear memory processes

2.2.1 Subjects & Groups

Thirty-two C57BL/6 mice (male, 6 weeks) were obtained from Charles River Canada (St. Constant, QC) and randomly assigned to one of two experimental conditions: Predator Stress (PS) or Stuffed Control (SC). Animals were pair-housed in fully enriched environments and had *ad libitum* access to food and water. Animals were kept on a normal lighting schedule (lights on at 7am), following the initial RET experimental protocol performed by Yang et al. (2004). Following exposure day, experimental and control animals were kept on separate shelves on opposite sides of the colony room to minimize likelihood of rat scent on experimental mice providing an olfactory fear cue to the control mice and thus confounding the results. All mice were acclimated to the upstairs lab environment (antechamber outside of the exposure room) for 30 minutes before testing. Prior to and during testing, all animals were handled daily for identification marking with non-toxic markers and routine husbandry duties during the light-phase. All testing was done between 8 a.m. and 2 p.m.

Following the original RET design, Long-Evans rats were used as predators. Four of these rats (male, 8 weeks) were acquired from Charles River Canada (St. Constant, QC). Each rat served as predator to four consecutive mice (a ratio of 4 rats: 16 PS mice). Prior to exposure day, all four rats were food deprived for 24 hours (i.e., food removed 24 hours before beginning of exposures) in order to maintain a constant high activity level throughout the experiment (without the pharmacological manipulation [D-amphetamine] used for this purpose in Yang et al.).

2.2.2 Procedure

2.2.2.1 RET Habituation

For three consecutive days prior to exposure, animals were placed in the exposure chamber for five minutes. The exposure chamber was a standard Plexiglas rat cage (47 cm x 26 cm x 20 cm) containing a clear Plexiglas divider with small holes (not large enough to allow the mouse to pass through to the rat side or vice versa, but intended to allow free olfactory flow). Animals were placed in the same cage that would later contain either the rat or the control "stuffed toy" rat. During habituation the "rat side" contained only clean bedding. Animals were immediately returned to the colony room following habituation. A picture of the rat exposure chamber can be seen in Figure 2.

2.2.2.2 Exposure

Similar to habituation trials, the mouse was placed in the left side of the exposure chamber. The right side of the chamber contained either a live rat (predator exposed mice) or a control "stuffed toy" rat created to match the live rat in size, colour, and shape (after Yang et al.). All control animals were exposed first to reduce/prevent rat scent exposure. Once the mouse was in the exposure chamber, the cover was replaced and animals were exposed to the rat or stuffed toy for five minutes. Following exposures, all animals were returned to their home cages in the colony room. All exposures were video recorded and hand-scored at a later time. Freezing time and freezing frequency were recorded. As with all other experiments described, freezing was defined as any point where the animal was immobile except for respiration. All cages were wiped down with 40% ethanol between exposures.

2.2.2.3 Re-Exposure

Forty-eight hours following exposure, all mice were placed back into the left side of the exposure chamber. The five-minute re-exposure was video-recorded and hand-scored at a later time. Items scored included time and frequency of freezing. Animals were returned to their home cages in the colony room immediately following re-exposure, and exposure cages were wiped down with 40% ethanol between animals.

2.2.2.4 ALB and Hyperarousal

As with Experiment 1, animals were tested for ALB and startle on successive days following re-exposure. The order of these tests (EPM, OF LD, startle) was kept constant across all experiments. Details of these tests can be found in section 2.5.

2.3 Experiment 3: The effects of Rapamycin on consolidation of predator stress-induced fear memories

2.3.1 Subjects & Groups

Sixty-four C57Bl/6 mice (male, six weeks) from Charles River (St. Constant, QC) were randomly assigned to four groups of 16: Predator Stress + Rapamycin (PSR), Predator Stress + Vehicle (PSV), Stuffed Control + Rapamycin (SCR), and Stuffed Control + Vehicle (SCV). RAP groups received an *i.p*. injection of Rapamycin (40 mg/kg) immediately following predator or control stimulus exposure. VEH groups received an injection of vehicle containing 5% EtOH, 5% PEG400 and 5% Tween80 dissolved in dH_20. As with experiment 2, exposures took place in the exposure chamber (see figure 2).

In order to ensure activity and predatory behaviour from the rats, they were food-restricted to 80% of free-feeding weight prior to exposure day (extended from the 24 deprivation in experiment 2).

Mice were housed 4 per cage with *ad libitum* access to food and water on a 12 h light–dark cycle (lights on at 7 a.m.). Prior to and during experiments, all animals were handled daily for identification marking with non-toxic markers and routine husbandry duties during the light-phase. All testing was done between 8 a.m. and 2 p.m.

2.3.2 Habituation and RET Testing

As with experiment 2, mice were habituated to the exposure context for five minutes a day for five days prior to actual exposures. However, unlike experiment 2, rats were also habituated to the chamber before exposure day. On each day of habituation and testing mice and rats were habituated to the laboratory anteroom for 30 minutes before habituation to the exposure chamber or testing. Rats were always brought into the laboratory after mice (habituation period) or after control mice were returned to their colony room (exposure day) in order to ensure control mice did not have even trace olfactory exposure to the rats, to the greatest extent possible. Exposures were recorded with a standard digital video camera for later analysis. Mouse behaviour was again scored for frequency and duration of freezing during predator/stuffed rat exposures. After the five-minute exposure period, each mouse was immediately injected with either RAP or VEH and then returned to his home cage.

48 hours after exposure and injections, all mice were re-exposed to the chamber for five minutes and their contextual fear memory assessed by measurement of freezing frequency and duration. On each following day ALB was measured in the EPM, OF, and

LD box, followed by startle testing. Another control procedure was added for this experiment as well. Because both associative and non-associative testing were done in the same room, the context was made to differ- salient features of the room were covered with white sheeting during habituation, exposures, and re-exposures. The sheeting was removed for non-associative testing. All devices were thoroughly cleaned with 40% ethanol between trials.

2.4 Drug Administration

For experiment 3, mice received an *i.p*. injection of Rapamycin (40 mg/kg dose, injection volumes of 10 ml/kg, volume dependent on mouse weight) or vehicle (5% ethanol, 4% PEG400, and 4% Tween 80 in sterile water, volume dependent on mouse weight).

2.5 Behavioral Testing

2.5.1 Elevated Plus Maze

The elevated plus maze (EPM) consisted of four arms arranged in the shape of a plus sign, with two opposite arms uncovered and two covered. For the rat-sized apparatus, each arm was 10 cm wide, 50 cm long and elevated 50 cm above the ground. The four arms were joined at the 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 were open at the top. For the mouse-sized EPM, each arm was 5.1 cm wide, 29.2 cm long and the maze was elevated 45.7 cm above the ground. The four arms were joined at the center by a 6.4-cm square platform.

The animal was placed in the center of the EPM and behaviour was recorded for 5 minutes. Rodents were then returned to their home cages. Behavioural measures

included time spent in the open arms, time spent in the closed arms, frequency in the open, frequency in the closed arms, and *ratio* measurements of these variables. Ratio time is defined as time in open arms/(time in open)+(time in closed). Ratio frequency follows the same formula.

2.5.2 Open Field

The open field (OF) is a square Plexiglas box (rat-sized apparatus:60 cm long x 60 cm wide x 35 cm high; mouse-sized apparatus: 48 cm x 48 cm x 48 cm) painted with grey enamel. Rodents were placed in the center of the floor at the beginning of each trial. The rodents were then videotaped for 5 minutes trials. Behaviours measured included time in the centre of the box and number of rears. Rears were defined as any instance where the mouse or rat raised itself up on its hind legs, with its forepaws leaving the ground (with the exception of obvious grooming behaviour). Rodents were considered in the center when the full body was within the center area defined by white masking tape, and near the wall when all four feet were between the masking tape and the wall.

2.5.3 Light/Dark Box

The light/dark box was a single alley apparatus constructed of Plexiglas, divided into two chambers of equal size. For the rat LD box, each chamber was 31.75 cm long, 10.48 cm wide and 14.6 cm high. Both chambers were covered by a transparent Plexiglas top, hinged so it could not be opened. Both tops had center pieces cut out to provide ventilation. One chamber had a solid wooden floor and was painted white. The other chamber had a metal mesh floor and its walls were painted black. The chamber painted black had its Plexiglas top rendered opaque with a black plastic covering. In addition, a 100-Watt LED light was positioned 66 cm above the white chamber. Testing took place

in a darkened room illuminated only by the lamp over the white chamber. This produced a light intensity at the center of the floor of the white chamber of 55 foot candles (fc), and an intensity of 2 fc at the center of the floor of the dark chamber.

The mouse light dark box was a 50 cm long, 15 cm high structure with two square-shaped boxes (20 x20 cm) connected by a short (10cm) tunnel. The dark side was covered by a removable lid, while the light side had a hinged Plexiglas lid with air holes to provide proper ventilation. Illumination and light intensity were the same as for the rat apparatus. Behaviour in the testing apparatus was videotaped for later analysis with a video camera mounted directly over the apparatus. Rodents were placed in the light chamber at the start of the test and their activity was videotaped for 5 minutes. Rodents were then returned to their home cages. Behavioural measures included time spent in each chamber, number of entries into each chamber (defined as having all four paws in the chamber) and number of faecal boli in each chamber.

2.5.4 Acoustic Startle Testing

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

2.6 Scoring and analysis

Across experiments, scoring for EPM and OF was done using the software program EthoVision (Noldus Technologies), while LD box data was hand-scored by an experimenter blind to the treatment group of the animals. RET exposures and re-exposures for experiments 2 and 3 were also hand scored by blind experimenters. Data for acoustic startle testing is generated by San Diego Instruments SR-Tech software and converted into Excel format. All data analysis for experiments 1,2, and 3 was performed using SPSS version 21 (IBM), with data imported into SPSS from Excel spreadsheets. For experiments where a direct comparison of two independent means was appropriate, Student's *t*-tests were used to compare groups (e.g., PS vs. SC). For experiments where multiple independent variables were used (such as exposure time in experiment 1 or drug in experiment 3), 2-way Analysis of Variance (*ANOVA*) tests were used instead to detect any overall (omnibus) effects, while Tukey LSD post-hoc tests were used to determine where the mean differences lay. These tests were used for all dependent variables with the exception of startle habituation, for which a repeated-measures *ANOVA* (group x trial) was used.

3.0 Results

3.1 Cat vocalizations do not produce an anxiety phenotype in rats

A two-way (stress x time) Analysis of Variance (*ANOVA*) tests was conducted on control sound and cat-sound exposed groups for each variable of interest (exposure freezing, re-exposure freezing, EPM ratio time, OF centre time, OF rears, LD box time, LD box entries, peak startle amplitude).

For exposure freezing, PS10 groups (M=5.57, SD=4.49) did not differ significantly from CX10 groups (M=8.23, SD=10.19), Tukey LSD *p*=.517. Surprisingly, CX60 (M=36.22, SD=12.99) controls froze more than the PS60 catcall animals (M=21.48, SD=19.32), LSD *p*=.001. This effect is in the opposite direction of what was anticipated. It is likely spurious since neither group appeared to generate a contextual fear memory. While PS10 rats (M=5.98, SD=20.48) had higher mean freezing on re-exposure than CX10 rats (M=2.18, SD=3.06), and PS60 rats (M=1.82, SD=2.44) had higher mean freezing than CX60 rats (M=.312, SD=.50), neither effect was significant, with omnibus $F(3, 76)=1.06$, $p=.37$.

In addition to the lack of a context effect, vocalizations also did not produce ALB in the EPM. The omnibus *ANOVA* detected no group differences in ratio time (time in open arms/[time in open arms + time in closed arms]), $F(3, 66) =.564$, $p= .64$. Means and standard deviations are in table X.X. Similarly, no effect was seen in the Open Field test, with no significant differences between groups in time in centre, $F(3, 74)= .66$, $p=.57$. Groups also did not differ in number of rears in the Open Field, $F(3, 74)=.551$, $p=.65$. Means and standard deviations for both OF measures are in table 1. The LD box was the final test of ALB performed, and no group differences were detected in either time, $F(3, 76)= 1.77$, $p=.16$, or entries; $F(3, 76) =.620$, $p=.60$. Means and standard deviations for both LD box variables are in table 1.

A final *ANOVA* was conducted to examine whether groups differed on average peak startle amplitude in the acoustic startle test. No effect was observed, $F(3, 76)= .620$, $p=.60$. A repeated-measures ANOVA was used to examine habituation to startle (group

by trial interaction term). As with peak startle, no effect was observed, $F(1, 87)=1.066$, $p= .32$. Means and standard deviations for peak startle amplitude can be found in table 1.

3.2 The RET produces a robust ALB phenotype

A series of *t*-tests for independent means were used to determine whether predator stressed mice (PS) differed from mice exposed to a stuffed control (SC) rat. As with experiment 1, dependent variables of interest were exposure freezing, re-exposure freezing, EPM ratio time, Open Field centre time, OF rears, LD box time and entries, peak startle amplitude, and startle habituation.

With regard to freezing during initial exposure to the real or stuffed rat, predator stress mice (M=17.82, SD=.4.37) froze more than controls (M=5.20, SD=3.42), $t(30)= 9.17$, $p<.0001$. Despite this considerable mean difference in exposure freezing, there was no significant difference between PS (M=28.35, SD=18.84) and SC (M=25.80, SD=9.23) groups in freezing at re-exposure, t(30)=.485, p=.63. Frequency of freezing was also analyzed to determine whether it provided a complementary measure. No significant difference was detected between PS (M=25.20, SD=9.6) and SC (M=20.47, SD=5.28), however, $t(30)= 1.73$, $p=.09$. Note the considerable variability for both measures-methodological/scoring reasons for the lack of a contextual fear effect are discussed below.

PS mice (M=.078, SD=.042) did show greater ALB on the EPM in terms of ratio time, with lower mean ratio time than controls (M=.17, SD=.058), $t(30)=3.58$, $p<.001$. A similar effect was seen in the OF, with controls (M= SD=) spending more time in the centre and rearing more frequently than PS mice (), t(30)=3.342, $p=.001$ and t(30)=4.22, $p<.001$, respectively. PS mice also demonstrated greater ALB in the DL box compared to

controls. Controls spent more time in the light (M=75.25, SD=41.70) than PS mice (M=46.62, SD=24.47), $t(29)=2.311$, $p=.028$, and also entered the light side more often (M=7.25, SD=3.20) than PS mice (M=4.50, SD=2.16) did, $t(30)=2.85$, $p=.008$.

In addition to the full range of ALB, PS mice also displayed greater hyperarousal than their control counterparts. For peak startle amplitude, PS mice (M=1066.59, SD=252.16) showed higher average values than SC mice (M=876.15, SD=260.44), $t(30)=2.101$, $p=.04$. RET-exposed mice were also slower to habituate to startle compared to their Stuffed Control-exposed counterparts, with a significant Group by Trial interaction term emerging from a repeated-measures *ANOVA*, $F(1, 29)=4.10$, $p<.001$ (see figure 19).

3.3 Selective Effects of RAP on Consolidation of RET-Induced Fear Memories

Methodological issues regarding scoring of freezing behaviour in experiment 2 were resolved for experiment 3. As expected, both PS groups showed elevated freezing (both duration and frequency) compared to controls, but did not differ from each other (thus they were equally "stressed" before RAP or VEH injection) during initial exposure. This led to a robust context effect; an omnibus *ANOVA* followed by LSD post-hoc tests revealed that PSV mice froze more than both control groups (as well as PSR), omnibus $F(3, 53)=35.12$, $p=.001$, all LSD multiple comparisons from PSV = $p<.001$. Thus RAP significantly attenuated contextual fear in PS mice, but did not reduce it entirely to control levels. The same pattern was observed with frequency of freezing.

Ratio time in the EPM also demonstrated a significant main effect of predator stress, omnibus $F(3, 53)=2.75$, $p=.05$. Here PSV mice displayed lower values (and thus greater ALB) compared to controls (LSD p-values=.024 and .022, respectively) but RAP

didn't significantly attenuate this effect- PSR mice were not significantly different from PSV mice (p=.45). In contrast to the robust findings in experiment 2, no effects of either PS or RAP were visible in the OF, with no differences among the four groups in centre time, omnibus $F(3, 58)$=.37, p=.77, or in rear frequency, Omnibus $F(3, 58)$=1.725, p=.17. Means and standard deviations for the OF are found in table 2. Similarly, there were no significant differences in the LD box. No group differences were detected in entries, omnibus $F(3, 58)$=1.646, p=.19. The same held true for light side time; means and standard deviations for the LD box are in table 2.

Further, no main effects of stress or drug were apparent for acoustic startle. No group differences were found for either average peak startle amplitude, or for startle habituation, with Group x Trial term of repeated-measures ANOVA not significant, $F(3, 58)$=1.007, p=.46. Examination of the habituation curves (fig. 29) shows that the expected pattern (slower and less dramatic habituation in stressed animals compared to controls) is essentially reversed, with RAP-injected controls atop the other groups in a stochastic, vaguely descending saw-tooth pattern.

4.0 Discussion

The set of experiments described were conducted in order to create a predator stress model of PTSD that was both reliable in producing this phenotype in rodents and allowed for manipulation of the fear memory trace-e.g., allowed for manipulation of memory consolidation, reconsolidation, and extinction. The overall results for experiments 1-3 were however mixed; experiment 1 failed to generate any effects of note and led to the use of the RET model in experiment 2. This experiment was successful, and in turn led to the examination of mTOR's role in consolidation of RET-induced fear

memories in experiment 3. This final experiment was only partially successful- exposure and re-exposure freezing rates indicate a main effect of Predator Stress, and of RAP for attenuating the predator stress-induced contextual memory, but less consistent results with respect to ALB.

Experiment 1 was conducted with the aim of generating a predator stress model with greater reliability than the direct cat exposure, which had produced increasingly variable results over years of experiments (Adamec, Walling & Burton, 2004; Fifield et al., 2015). Furthermore, previous work in the Blundell lab had been unsuccessful in producing fear memories with Predator Scent Stress (e.g. Smith, 2009 [unpublished]). The present experiment was designed to be in line as much as possible with previous exposure studies, making use of the same experimental subjects (male Long-Evans rats, 6-8 weeks old) tested on the same set of behavioural measures of associative and non-associative fear. Great care was taken to produce a "control sound" that matched the psychophysical parameters of the catcall used for the experimental groups. However, as with the PSS work, the predator vocalizations did not produce an anxiety phenotype. As noted in the results above, the cat sounds did not produce so much as elevated freezing during the initial exposure, much less any other forms of ALB in the exposed rats, as compared with their controls. In fact absolute freezing levels for both groups was very low, suggesting the issue wasn't that the control sound (which, while matched to the catcall sound wave physically, was itself subjectively heard as a 'screech' quite unpleasant to human ears) somehow engendered increased fear and anxiety in control rats, and thus washed out an effect for the catcalls on the experimental group. In reality, both 10-minute groups froze less than 10% of the time upon re-exposure, whereas a

successful cat exposure trial could induce rats to immobility for as much as three-quarters of the 10-minute trial (for an example, see Fifield et al., 2013; Fig. 4C). With regard to non-associative fear, controls and catcall-exposed rats did not differ across several measures in the EPM (risk assement, ratio time, ratio frequency), open field (time in centre, rears, boli), and LD box (light side entries).

There are several possible reasons why the experiment did not work. Perhaps the simplest stems from the nature of how predators vocalize when hunting prey. Often, they are silent, but cats can produce various "chattering" or "chirp" noises specific to prey observation, some of which mimic the noises produced by birds and rats (Scholtz, 2013). However, the cat sounds recorded and used in present study were more of a loud, wailing meow that may be an example of the feline isolation cry (plausible, given the cat was a singly housed lab cat; feline isolation cries are discussed at length in Buchwald et al., 1988). If rats have evolved to innately fear any vocalizations from cats, they are surely more likely to be the hunting-related chattering/chirp sounds than an isolation cry, given that the former is a clear danger signal while latter at most indicates vulnerability on the cat's part.

Even this explanation is confounded by two factors related to human domestication of both species. Cats raised in human homes tend to vocalize across the lifespan, whereas in the wild this is generally restricted to kittenhood- thus cat sounds are an unlikely selection pressure for the evolution of a fear circuit in rats that promotes memory of the predatory experience. In addition, lab rats have been domesticated by humans and bred in lab environments for several decades- it is possible that inadvertent selection (e.g., for amicability) has produced rats with such well-primed fear circuitry

literally bred out of them- their innate response to predators has been indirectly attenuated by generations of domestication. Even beyond the evolutionary sculpting of these circuits, lab-raised rats also do not have the learning environment that wild-type rats do; if a "fear of predator" system has to be primed by some experience, these animals lack that relevant experience. This would be a specific example of a wider confound in rodent research, hinted at by the finding that even rats raised in enriched laboratory environments have much less developed cortical wiring than wild-type counterparts (McEwen & Davidson, 2012).

Further, while cat exposure paradigms and the present study are intended to have a high degree of ecological validity, the exposure environment may not resemble a "wild" situation enough for its cues and contexts to completely mimic the predatory scenario the prey species is primed to learn about, and this may dilute the effect of the predator exposure. That is, without visual or odour cues and a "wild" environment, the sound of a cat being piped into a fear conditioning chamber may not 'make sense' to the animal in a way that is salient enough to produce a fear memory. A final issue with experiment 1 is methodological- there was no control condition in which animals were simply exposed to silence, in addition to the "control sound", which may have allowed a more complete comparison of the effects of the predator sound and control sound on behaviour. However, the comparison with freezing levels in actual cat exposure studies mentioned above suggest that the null results are not a product of flaws in experimental design, but that the vocalizations simply didn't frighten the rats to any visible extent.

The failure of catcalls to produce fear memory and a PTSD-like phenotype led our laboratory to modify the Rat Exposure Test (RET), originally developed by Robert and

Caroline Blanchard's lab at the University of Hawaii (Yang et al., 2004; Wall, Blanchard, Yang, & Blanchard, 2004). As the Blanchard lab studies mouse defensive behaviour, they used the RET to measure within-exposure behaviour by the prey species only. We were interested in whether this exposure paradigm (of a mouse to a rat) would produce associative and non-associative fear memories on the part of the mouse. As with cats and rats, rats prey upon mice, and will kill and eat them if given the opportunity (Adamec & Himes, 1978; Drew, DeRossett, & Gotsick, 1981). This model has been shown by other laboratories to induce a corticosterone response (Amaral, Gomes, & Nunes-de-Souza, 2010) and have its effects on mice be pharmacologically malleable (Campos et al., 2013).

The results of experiment 2 show the RET producing a fairly robust anxiety and hyperarousal phenotype. Consistent effects were observed in the EPM, OF, LD Box and on both components of the startle test. Exposure freezing was dramatically higher in predator-stressed mice than controls, an important "manipulation check", that indicates that the mice do indeed find the rat frightening and the stuffed toy comparatively neutral, a prerequisite for acquisition and consolidation of fear memory in the PS group, and experimental differentiation of stressed mice from controls. Surprisingly in light of the exposure results is that only test that did not show a significant group difference in experiment 2 was freezing upon re-exposure, where the effects for both freezing time and frequency were "in the expected direction" (e.g. PS group showed higher mean freezing than SC group) yet not at criterion for statistical significance.

The lack of a significant context effect may have been more a product of methodology than a true lack of salience for the contextual fear memory on the part of the animals. As with previous studies, the re-exposures were videotaped from above using a

ceiling mounted camera and hand-scored later. However, the camera lenses were not at full 'zoom', giving a somewhat distant view of the cage. Given that discriminating immobility from mobility in a small animal like a mouse is a task that requires great attention and care even with an ideal viewing angle, it may not have been possible to accurately discriminate freezing, and the effect may have been underestimated or otherwise rendered highly variable. The issue with camera placement (along with other minor parametric 'bugs' in the new RET model) was resolved for subsequent RET work, as suggested by certain experiment 3 results.

With results from experiment 2 suggesting the RET is a reasonably robust model of PTSD-like fear memories (at least non-associative and hyperarousal types), we asked the question of whether the (associative and) non-associative fear memories elicited by it were mTOR dependent. As results above indicate, the answer to this question appears to be a qualified or partial yes. The effects on non-associative fear (ALB) observed in experiment 2 were largely not replicated, with no differences in the OF, LD Box, or startle across the drug and stress conditions. A strong context effect was demonstrated however, and the memory consolidation to be at least partially mTOR dependent, as PSV mice froze more on re-exposure, and PSR freezing nearly being rescued to control levels. In the EPM, a similar pattern was seen for ratio time, as RAP increased the proportion of time PS animals spent on the open arms as compared to VEH mice, but again not entirely to control levels. Previous research in the Blundell laboratory has tended to find LD and OF results somewhat variable, and the 'core' of a predator stress animal model of PTSD can be reduced to contextual/cued fear, ALB, and hyperarousal in predator context, EPM, and startle, respectively (e.g., Cohen et al., 2006).

The results from experiment 3 captured the first two components of this model, but no semblance of a startle effect was seen- indeed, the curves over the course of the 30 trials are nearly reversed from the expected pattern (fig. 29). Further research is needed to examine whether this means that the hyperarousal memory is not under control of the mTOR pathway or if hyperarousal induced by the RET is simply more variable than the context or EPM effects. Given that consolidation of hyperarousal memory appears to be under mTOR control in the cat exposure test (Fifield et al., 2013), and that effects in other non-associative tests (LD, OF) are somewhat variable, the latter is more likely the case. It is also possible that the contributions of mTOR to consolidation of hyperarousal are more nuanced than currently understood, as previous research using cat exposure indicated time-dependent effects of RAP on this variable, for example (Fifield et al., 2015).

Indeed, mTOR's ubiquity in neurons (and all other cells) does introduce complexity; research into its role in ingestive behaviour has come to the consensus that the effects of the pathway on eating and body weight are highly dependent on signaling stimulus, cell population, and behvioural context (Haissaguerre, Saucisse, & Cota, 2014). It would not be surprising of the effects of mTOR on learning and memory were similarly complex, with amygdalar and hippocampal activity promoting fear memory acquisition and consolidation, but prefrontal activity of the pathway promoting extinction or modulation of such memories. Such a scenario would explain the variable nature of results with systemic RAP injected reported here and in Fifield et al. (2013, 2015). It will be critical for future research using the RET to cannulate RAP into specific sites and measure the effects on contextual and non-associative fear memories. This will allow the

dissociation of effects of the pathway in different regions, in parallel to work showing site-specific effects in fear conditioning (Helmstetter et al., 2008).

At any rate, the variable effects seen across these experiments speak in part to the difficulty of balancing ecological validity on one hand with experimental control and replicability on the other. For example, epidemiological research finds only a subset of trauma-exposed individuals develop PTSD. For animal researchers, this means that we should expect a significant number of 'stressed' rodents to be resilient, and that our models are perhaps more ecologically relevant when this is the case. Some models explicitly take this into account (e.g., with use of cut-off behavioral criteria, Cohen & Zohar, 2004), but this requires not only very large sample sizes (given the loss of statistical power engendered when dividing groups up) but a consistent and clear dissociation between strongly and weakly-responding rodents in the PS paradigm. This is best done using a validated test such as the EPM, but this unfortunately removes the EPM from the core of ALB measures used to examine fear memory.

4.1 Theoretical Considerations

While there are well-known general factors that contribute to the perpetuation of null results in the behavioural sciences (publication bias, limited power, etc.) that may well have affected the experiments presented here in tandem with parametric and chance factors, the contradictory nature of the results may actually have a deeper theoretical basis. The theory of learning and memory dominant in behavioural neuroscience since its inception has been built on combining neurobiological techniques with research methods designed by behaviourists to study animal learning, namely Pavlovian (classical) conditioning and Skinnerian operant conditioning. The view of learning in this paradigm

is intellectually descended from 18th century British Associationists like Locke, Berkeley, and Hume. Under this theory, an *association* between phenomena in memory is formed when stimuli or events are repeatedly paired together, very close in time (contiguity).

Contiguity is especially critical in this case, and provides much of the basis for LTP as a candidate memory mechanism given its parallel properties to the putative association (both are thought to be based on temporal contiguity). This is non-trivial, as the understanding of learning and memory in animal neuroscience has been based on the division of learning types described above- that is, into associative and non-associative categories. But if the nature of the processes we are attempting to study is different than this, our measurements and models may not quite capture how they are working, and experimental subjects may behave as if they are sticking a square peg into a round hole- a metaphor that also holds for the neural firing patterns and signalling cascades (like mTOR) that certainly underlie learning and memory in some manner but whose effects can appear variable under present associative theory, as in experiment 3.

Indeed, the understanding of how conditioning works has changed dramatically over the last 30 years (Rescorla, 1988, Timberlake, 1994, Ward, Gallistel, & Balsam, 2013; Gallistel, & Balsam, 2014) and this much more cognitive or computational view (CS provides information[reduction in uncertainty] about US onset, rate and timing) has not extended far into neuroscience, in part because the elements required for its neural basis do not fit easily into an associationist framework for the neurobiology of learning and memory. The psychologist Randy Gallistel of Rutgers University (1990, 1995, 2000, 2001, 2003, 2012) has spent a great deal of time pointing out that the computational complexity of conditioning phenomena mean that the brain cannot simply form

associations to instantiate these memories, and that most forms of animal learning cannot be reduced to association formation, even in very simple brains (e.g., desert ants foraging for food and navigating back to the nest use a form of dead reckoning- which requires symbols [elements standing for aspects of the world] to compute vector integration; similarly, many bird species learn to navigate by the constellations; bees learn the solar ephemeris function and compute the variance of flower patches, etc.).

The original properties of associative theory made synaptic plasticity via long-term potentiation (LTP) an extremely attractive model for the physiological basis of learning and memory. The strengthening of synaptic connections in LTP requires contiguity, and thus appears to represent a cellular bond or coincidence detector (from the Hebbian postulate that neurons that fire together, wire together; Hebb, 1949) that corresponds to forming an associative link. To this day the standard view of learning in neuroscience derives from associative models, usually Pavlovian conditioning (especially the fear conditioning paradigm discussed at length above, but non-associative learning is also viewed as changes in synaptic efficacy). In this view, the temporal pairing of CS and US creates new excitatory conductive links (associations), which are modified synapses between neurons, that is, Hebbian synapses.

Conductance at these synapses is changed by temporal pairing of pre and postsynaptic activity (where CS causes presynaptic activity and US the postsynaptic activity). Thus association formation is gradual, with successive pairings of CS and US close in time strengthening the same association (Gallistel & Gibbon, 2001). An implication of this is that current strength of a given association reflects several aspects of the animal's conditioning experience and therefore does not represent any objective

aspect of the experience (for example, CS-US interval). Associations are not symbols (they don't represent objective facts about the world), and the associative bond doesn't participate in any sort of computation per se (Gallistel, 2003).

However, doubt is cast on this by the cognitive updates of learning theory noted above, which show that temporal contiguity is neither necessary nor sufficient for association formation, and stress the informativeness of stimuli about contingency as the central value of this type of learning (Rescorla, 1988; Spear et al., 1990; Timberlake, 1994, Gallistel & King, 2009; Gallistel & Matzel, 2013). Further, the old equipotentiality assumption- that any stimulus can be associated with any other stimulus with equal ease- has undergone complete dismantling beginning with the work of Garcia and colleagues on taste aversion learning in the late 1960s, providing increasing evidence that even in rodents there exist several types of learning which do not necessarily share the same neural properties (Garcia, 1990; Gallistel, 1990; Gallistel, 2000).

Additionally there is a common intuition that classical and operant conditioning are general-purpose forms of learning, a sort of neural "stickiness", and this is incorrect. When what the brain must compute is closely analyzed, conditioning processes are revealed to be multivariate non-stationary time series analyses- complex algorithms for dealing with a changing world (see discussion above). As with the workings of the visual system, our intuition that a neuropsychological faculty appears simple can mask deep computational complexity (Cosmides & Tooby, 1994).

On the information-processing view, learning is the process of computing objective facts about the experienced world from raw sensory input, and storing the results in a memory. Memories are therefore not associative conducting links but

repositories of information, like patterns of bits in computer memory or the genes on a chromosome (Gallistel & Gibbon 2001). Careful studies have shown conditioning effects to be time-scale invariant, which casts major doubts on associative theories that hang on temporal contiguity, especially LTP as the neural learning mechanism (Gallistel, 1990, 2003; Balsam & Gallistel, 2009; Balsam, Fairhurst, & Gallistel, 2006). A major asset of PS paradigms as PTSD models is that they transcend conditioning theories and encompass non-associative learning and memory.

However, because a component of PS models is still based on association (context) and the model itself neatly but intrinsically divides into memory effects defines as associative and non-associative, it remains vulnerable to the limitations of this conception of learning. There does remain a significant drawback to the information processing view of learning and memory, that its neural basis is presently not known (although the role of signalling cascades like mTOR and changing mRNA transcription profiles in neurons would be at least as critical as they are from the associative point of view, given the information-carrying abilities of these molecules; for example a transcription factor is analogous to a pointer in computer memory, functioning to control or channel access to information at other locations like promoter regions [Gallistel & King, 2009]). In any sense, this perspective offers a theoretical reason for variable results in learning experiments, in addition to parametric limitations (power, chance factors) and endemic issues in psychology and neurobiology literature (other research labs may only publish PS experiments that work, giving a false impression of the stability and power of the paradigm). This last issue is one of increasing visibility and concern in the

behavioural sciences, and more researchers are perhaps sympathetic to the publication of null results like those visible in parts of the present set of experiments.

4.2 General Conclusions

The series of experiments discussed here speak in equal measure to the fragile nature of ethologically-inspired fear memory models and to the fact that these models nonetheless give a view of real phenomena, if one somewhat distorted by a persistent variability. A possible theoretical explanation is offered, in addition to typical experimental and parametric limitations. While predator sounds on their own clearly did not generate fear memories in experiment 1, the robust effects of the RET in experiment 2 suggest that the model does indeed generate fear memories. The more modest findings from experiment 3 do (positively) resolve any ambiguity over whether the RET generates contextual fear, and speak to a role for mTOR in consolidation of this memory. Findings for the EPM do suggest non-associative fear memory/ALB generated by the RET is also at least partially mTOR-dependent. This is in agreement with work using cat exposure (Fifield et al., 2013), but unlike that work, experiment 3 did not find show the RET produced a startle effect, or that such an effect was subject to modification by mTOR blockade with RAP. More work with the RET is needed, especially brain-region specific cannulation of RAP in order to tease out the likely very complex contributions of mTOR to memory modulation.

While the results discussed above are somewhat qualified, they do produce information relevant to PTSD: first, the RET emerges as a useful model for studying the modulation of predator-stress induced fear memories, and thus is a helpful tool in translational research aimed at modeling and developing cures for PTSD symptoms.

Indeed, the results of experiment 3 add to evidence that blockade of mTOR with RAP may be a useful pharmacological treatment, given its attenuation of contextual fear memory and some anxiety behaviour- however, inconsistent results with respect to hyperarousal symptoms mean that future research will be required to fully tease apart the complex contribution of mTOR to fear memory formation and modulation, and thus clarify the best uses of RAP as a PTSD treatment.

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Tables

TABLE 1: Non-associative measure results for experiment 1.

GROUP	TEST	MEASURE	MEAN	SD
PS10	**EPM**	**Ratio Time**	129.92	34.20
PS60	**EPM**	**Ratio Time**	147.13	36.99
CX10	**EPM**	**Ratio Time**	142.76	30.58
CX60	**EPM**	**Ratio Time**	136.26	56.72
PS10	**OF**	**Centre Time**	15.10	13.14
PS60	**OF**	**Centre Time**	16.11	10.84
CX10	**OF**	**Centre Time**	13.81	9.30
CX60	**OF**	**Centre Time**	11.69	8.00
PS10	**OF**	**Rears**	36.88	9.07
PS60	**OF**	**Rears**	36.55	11.2
CX10	**OF**	**Rears**	33.15	9.16
CX60	**OF**	**Rears**	35.80	10.70
PS10	**LD**	**Light Side Time**	150.07	18.11761
PS60	**LD**	**Light Side Time**	150.02	23.41786
CX10	**LD**	**Light Side Time**	161.11	22.95
CX60	**LD**	**Light Side Time**	162.44	26.07
PS10	**LD**	**Light Side Entries**	9.75	1.77
PS60	**LD**	**Light Side**	10.65	2.62

		Entries		
CX10	**LD**	**Light Side Entries**	10.25	1.74
CX60	**LD**	**Light Side Entries**	10.40	2.35
PS10	**Startle**	**Peak startle amplitude**	1576.15	382.66
PS60	**Startle**	**Peak startle amplitude**	1964.96	889.21
CX10	**Startle**	**Peak startle amplitude**	2318.86	915.54
CX60	**Startle**	**Peak startle amplitude**	2357.89	674.99

Table 2: OF and LD results for experiment 3.

GROUP	TEST	MEASURE	MEAN	SD
SCV	**OF**	**Centre Time**	99.34	13.64
SCR	**OF**	**Centre Time**	108.76	22.54
PSV	**OF**	**Centre Time**	87.54	19.40
PSR	**OF**	**Centre Time**	92.33	33.58
SCV	**OF**	**Rears**	18.70	4.23
SCR	**OF**	**Rears**	16.90	2.65
PSV	**OF**	**Rears**	13.15	4.86
PSR	**OF**	**Rears**	14.58	3.79
SCV	**LD**	**Light Side Time**	39.86	12.84
SCR	**LD**	**Light Side Time**	40.34	10.92
PSV	**LD**	**Light Side Time**	43.04	14.77
PSR	**LD**	**Light Side Time**	46.21	19.32
SCV	**LD**	**Light Side Entries**	7.75	1.83
SCR	**LD**	**Light Side Entries**	8.18	2.26
PSV	**LD**	**Light Side Entries**	5.65	1.59
PSR	**LD**	**Light Side Entries**	6.10	2.29

Figure Captions

Figure 1: Schematic of the mTOR kinase pathway.

Figure 2: The RET chamber used in experiments 2-3.

Figure 3: Mean exposure freezing, experiment 1.

Figure 4: Mean re-exposure freezing, experiment 1.

Figure 5: Mean EPM ratio time, experiment 1.

Figure 6: Mean OF centre time, experiment 1.

Figure 7: Mean OF rears, experiment 1.

Figure 8: Mean LD light time, experiment 1.

Figure 9: Mean LD light-side entries, experiment 1.

Figure 10: Mean peak startle amplitude, experiment 1.

Figure 11: Startle habituation curves, experiment 1.

Figure 12: Mean exposure freezing time, experiment 2.

Figure 13: Mean re-exposure freezing time, experiment 2.

Figure 14: Mean re-exposure freeze frequency, experiment 2.

Figure 15: Mean EPM ratio time, experiment 2.

Figure 16: Mean OF centre time, experiment 2.

Figure 17: Mean OF rears, experiment 2.

Figure 18: Mean LD light time, experiment 2.

Figure 19: Mean LD light-side entries, experiment 2.

Figure 20: Mean peak startle amplitude, experiment 2.

Figure 21: Startle habituation curves, experiment 2.

Figure 22: Mean exposure freezing time, experiment 3.

Figure 23: Mean re-exposure freezing time, experiment 3.

Figure 24: Mean re-exposure freezing frequency, experiment 3.

Figure 25: Mean EPM ratio time, experiment 3.

Figure 26: Mean OF centre time, experiment 3.

Figure 27: Mean rear frequency, experiment 3.

Figure 28: Mean LD light time, experiment 3.

Figure 29: Mean LD light-side entries, experiment 3.

Figure 30: Mean peak startle amplitude, experiment 3.

Figure 31: Startle habituation curves, experiment 3.

FIGURES

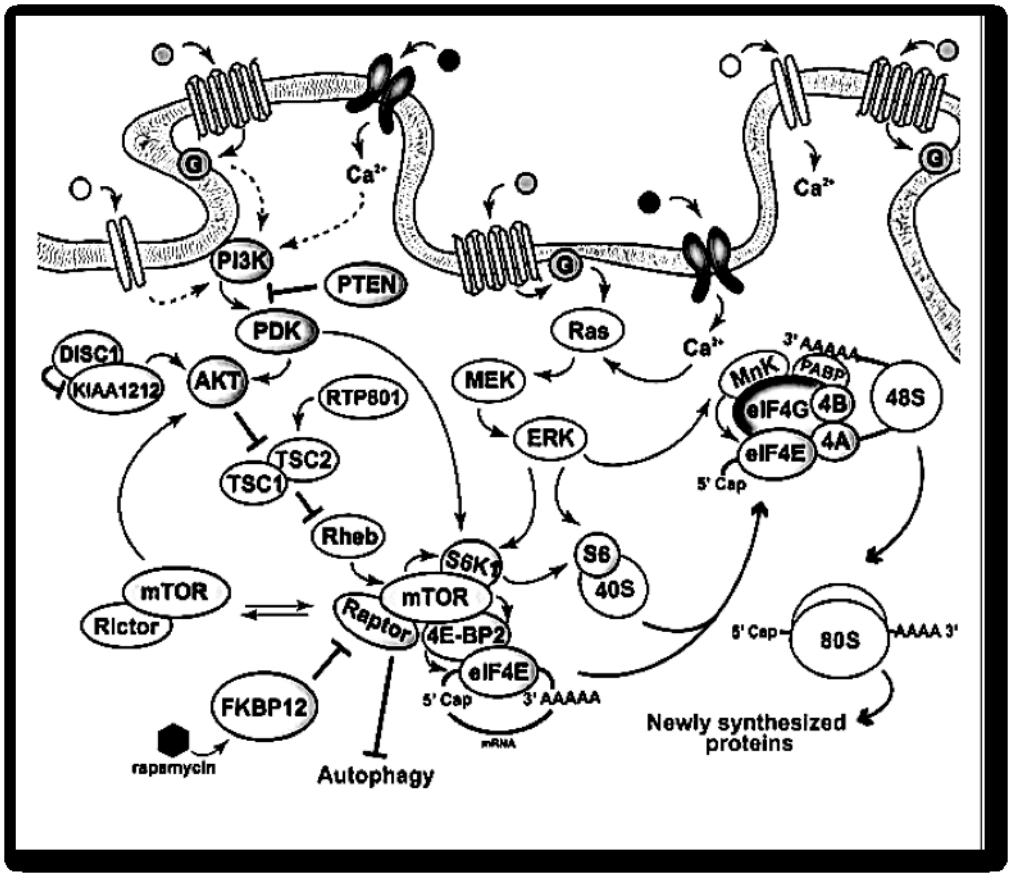

Figure 1: The mTOR pathway. Modified from Santini & Klann (2011).

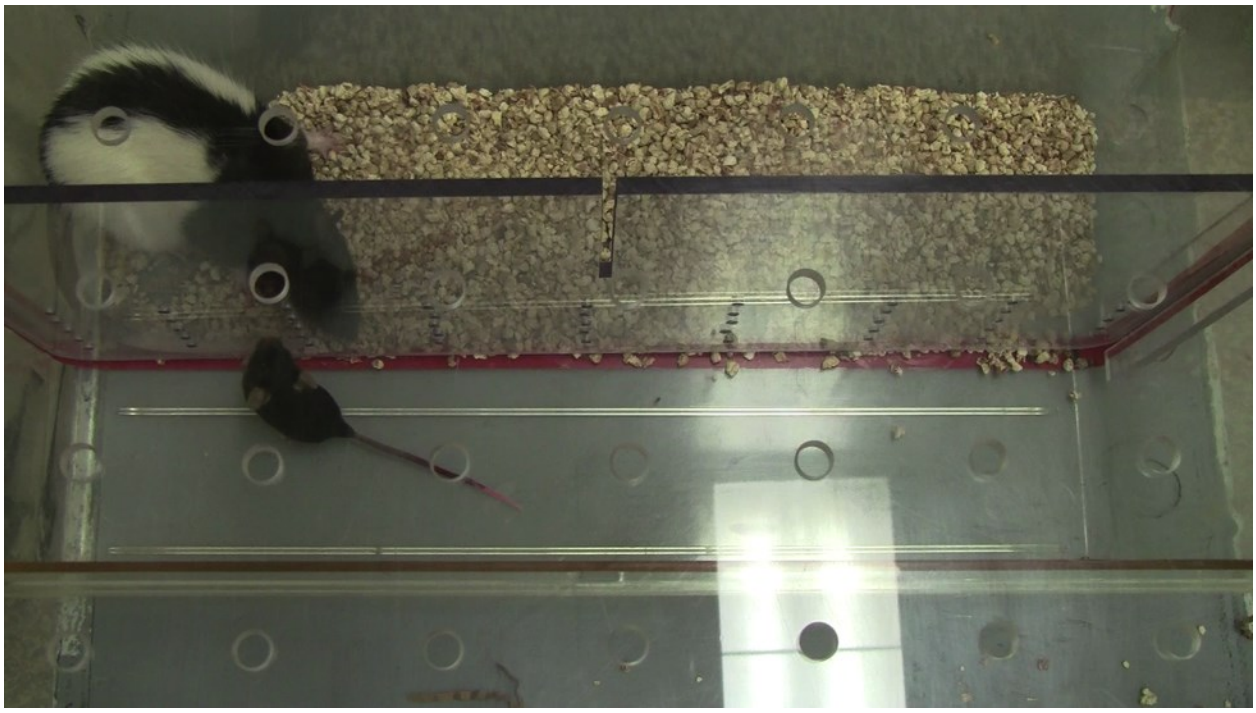

Figure 2: The Exposure chamber for the Rat Exposure Test (RET) as used in experiments 2 and 3.

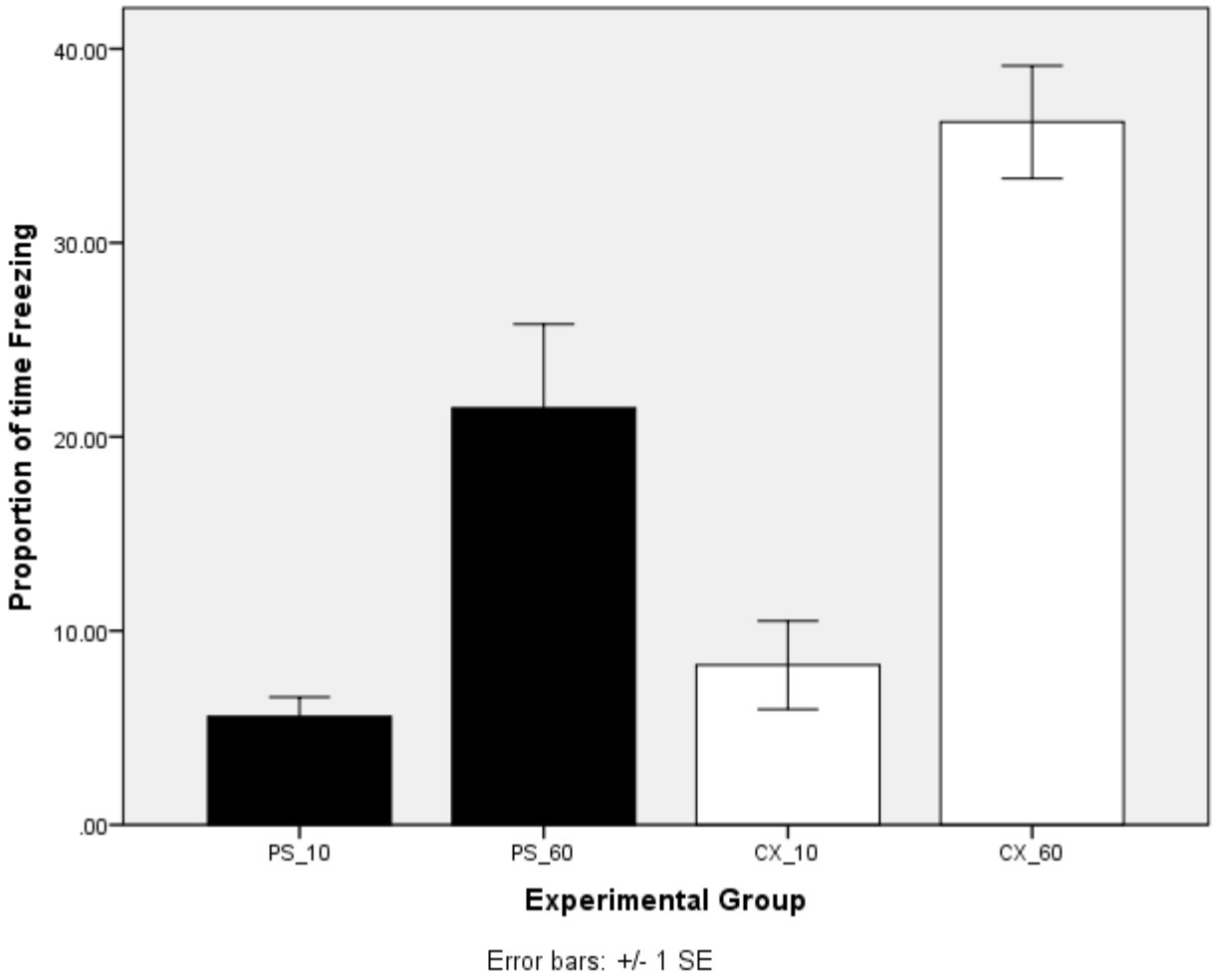

Figure 3: Proportion of Exposure Trial spent freezing, Experiment 1. PS10=Predator Sound 10 Minutes, PS60=Predator Sound 60 Minutes. CX10=Control Sound 10 Minutes, CX60=Control Sound 60 Minutes. Note that CX60 group shows highest mean freezing-an unexpected finding.

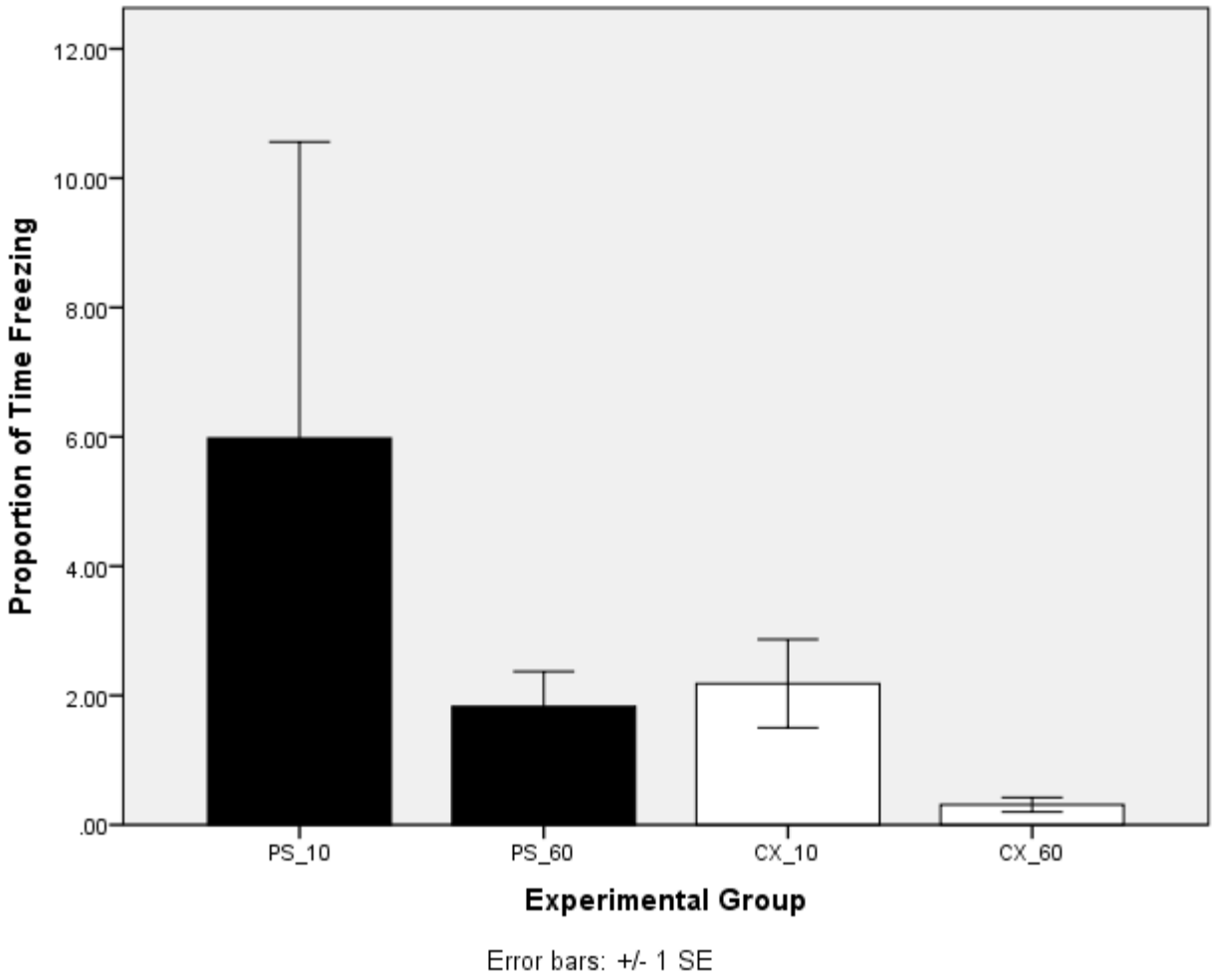

Figure 4: Proportion of Re-exposure Trial spent freezing, Experiment 1.

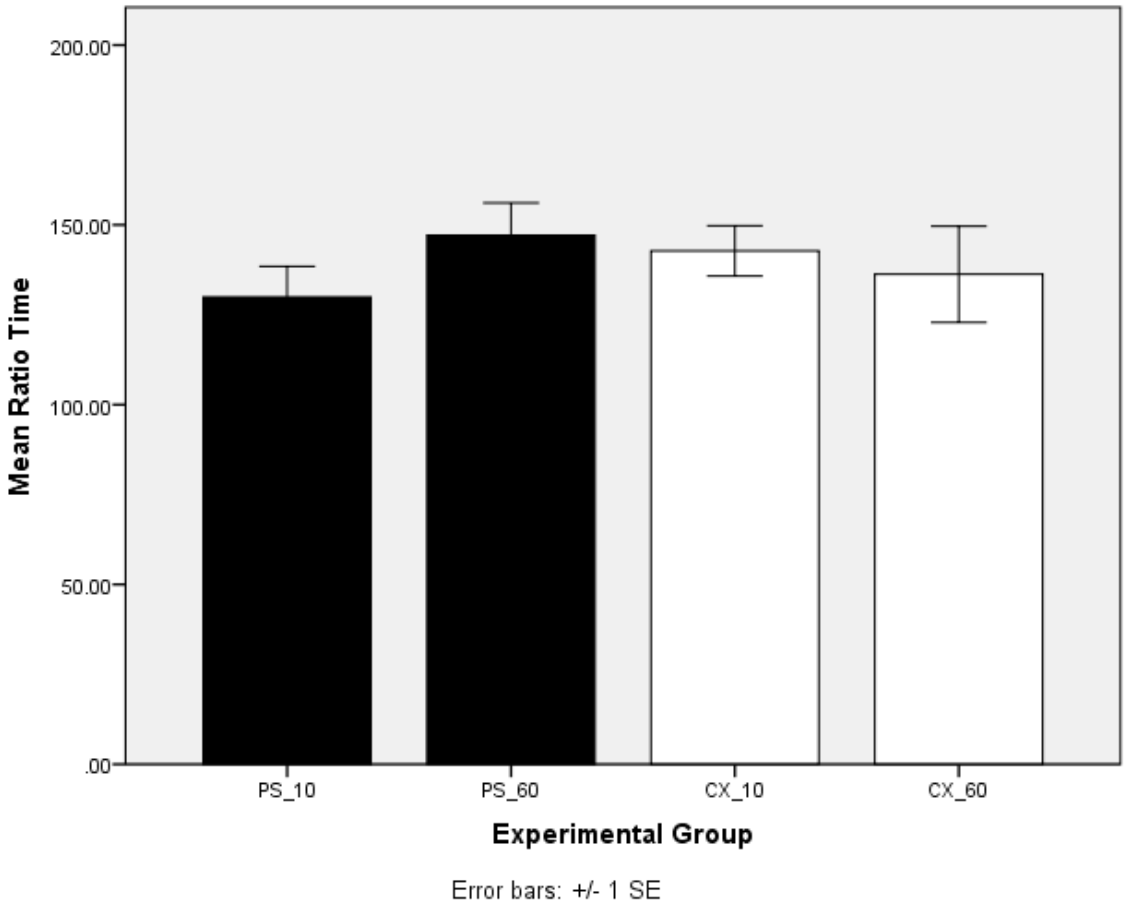

Figure 5: Ratio Time (Open Time/Open Time + Closed Time) in the Elevated Plus Maze (EPM), Experiment 1.

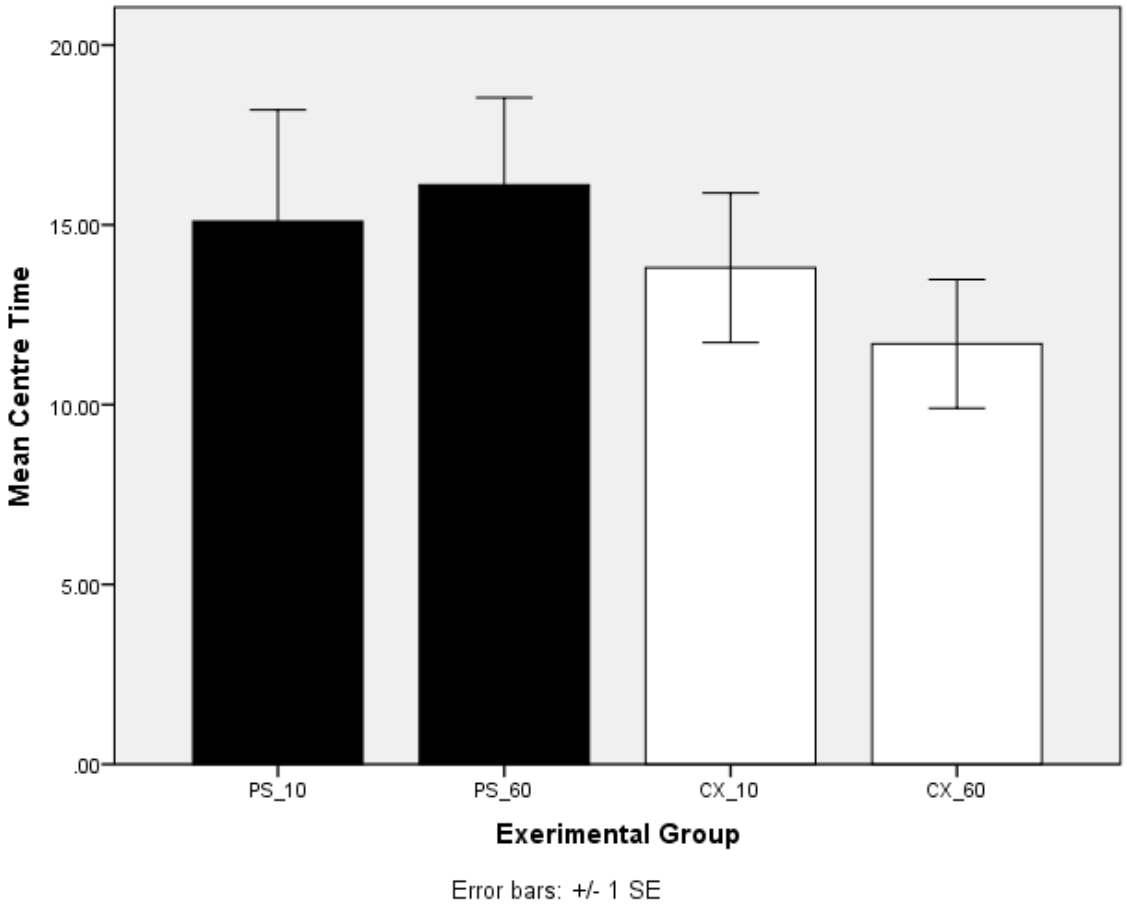

Figure 6: Mean time in Centre of Open Field (OF), Experiment 1.

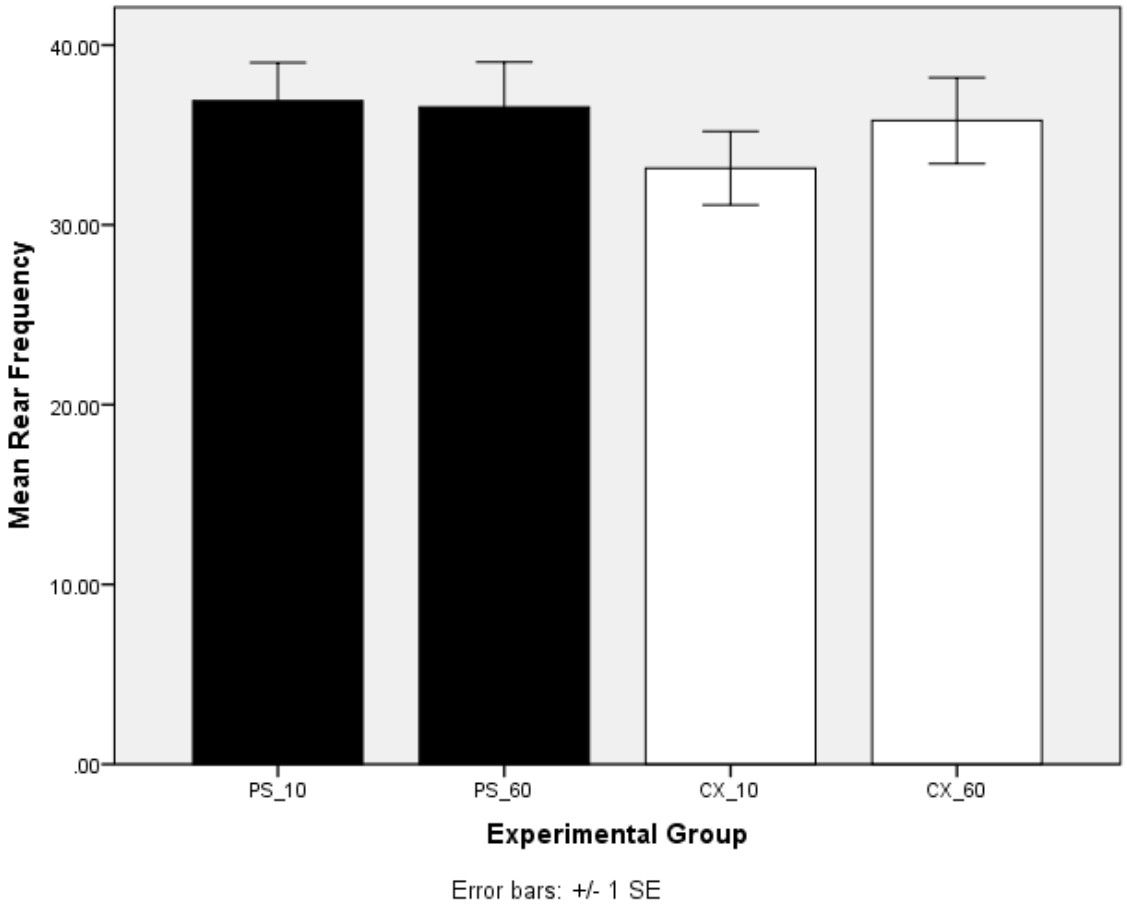

Figure 7: Mean Number of Rears in the Open Field (OF), Experiment 1.

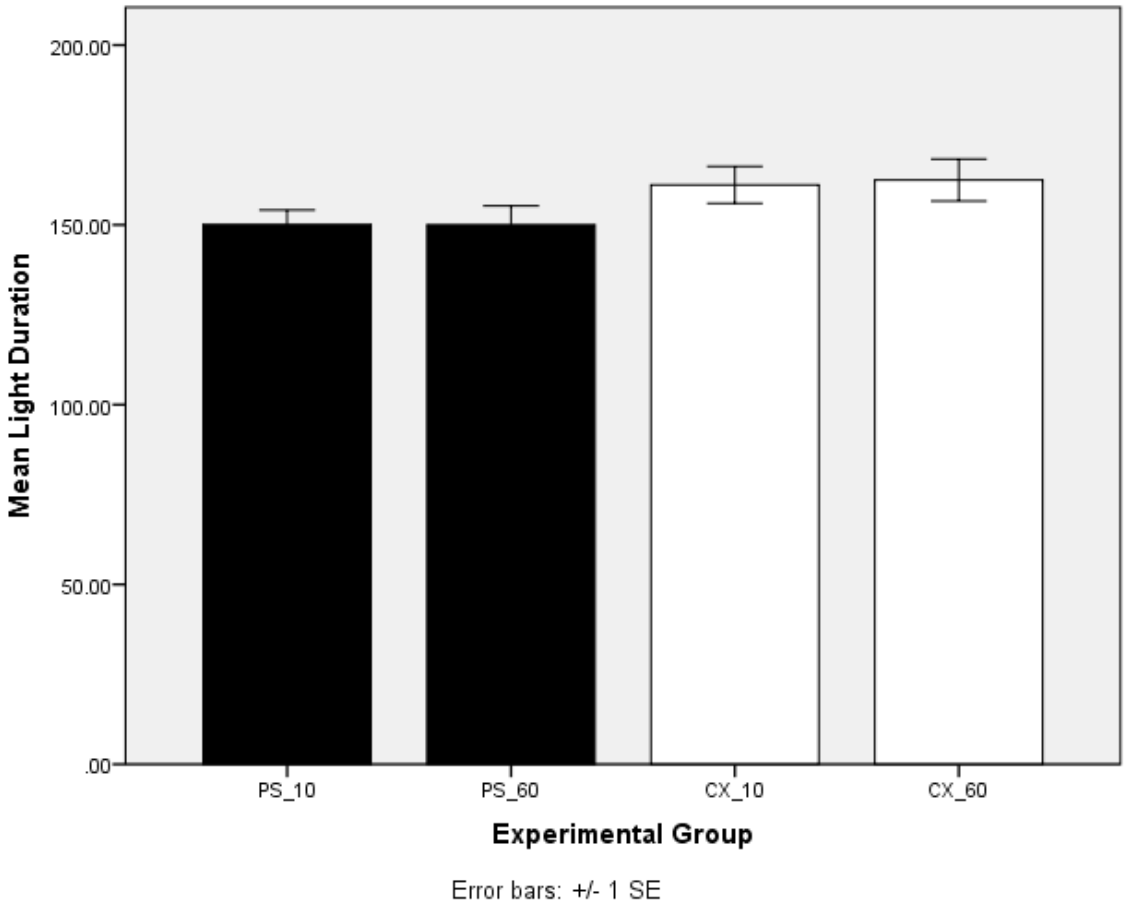

Figure 8: Mean time (seconds) spent in Light Side of Light Dark Box (LD), Experiment 1.

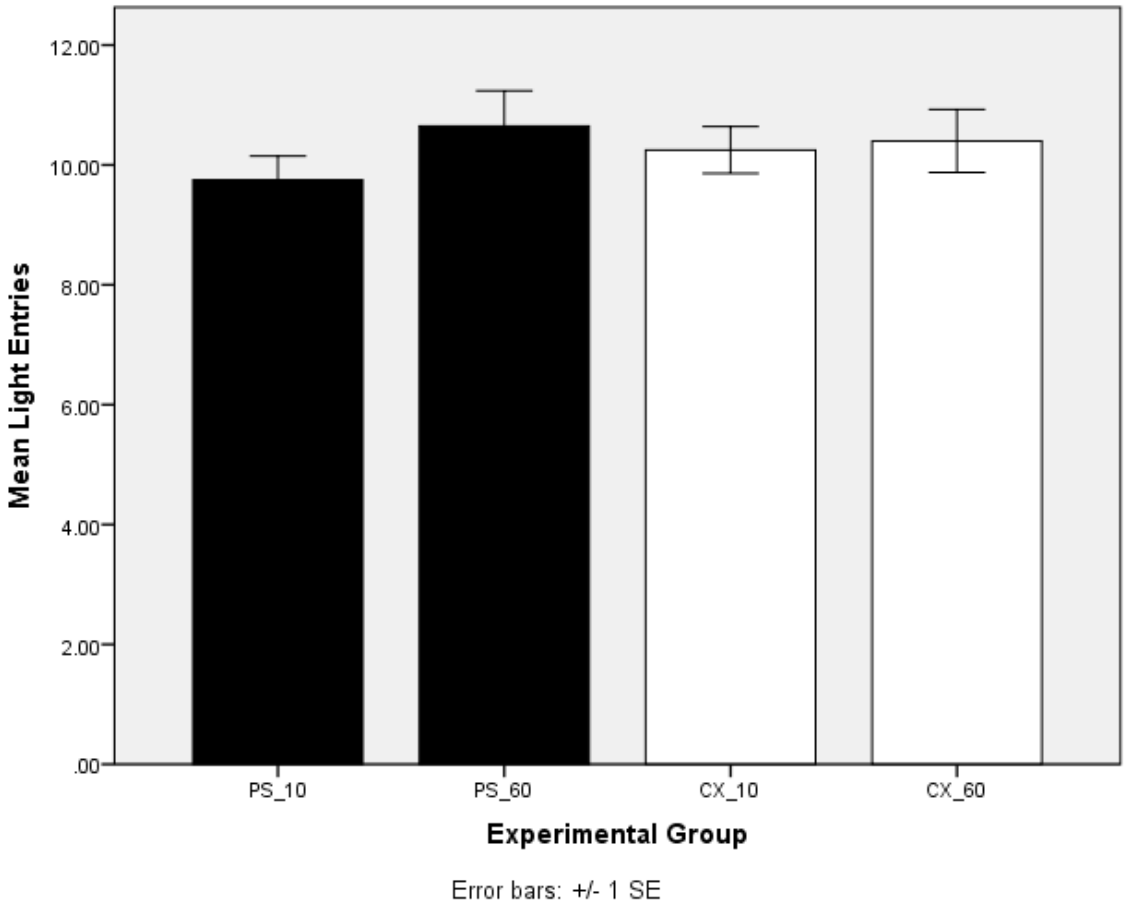

Figure 9: Mean Number of Entries into the Light Side of the Light Dark Box (LD), Experiment 1.

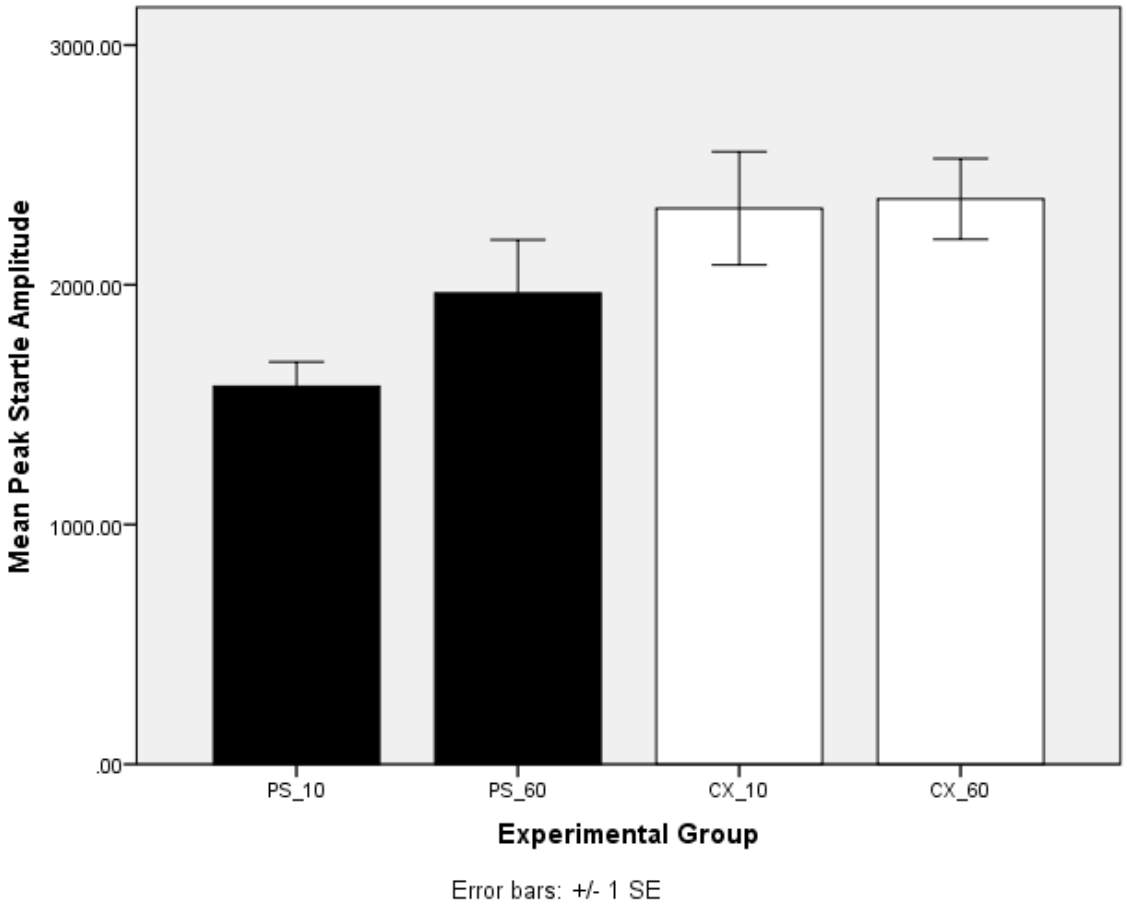

Figure 10: Average Peak Startle Amplitude (PSA) in Volts per Gram (V/g), Experiment 1.

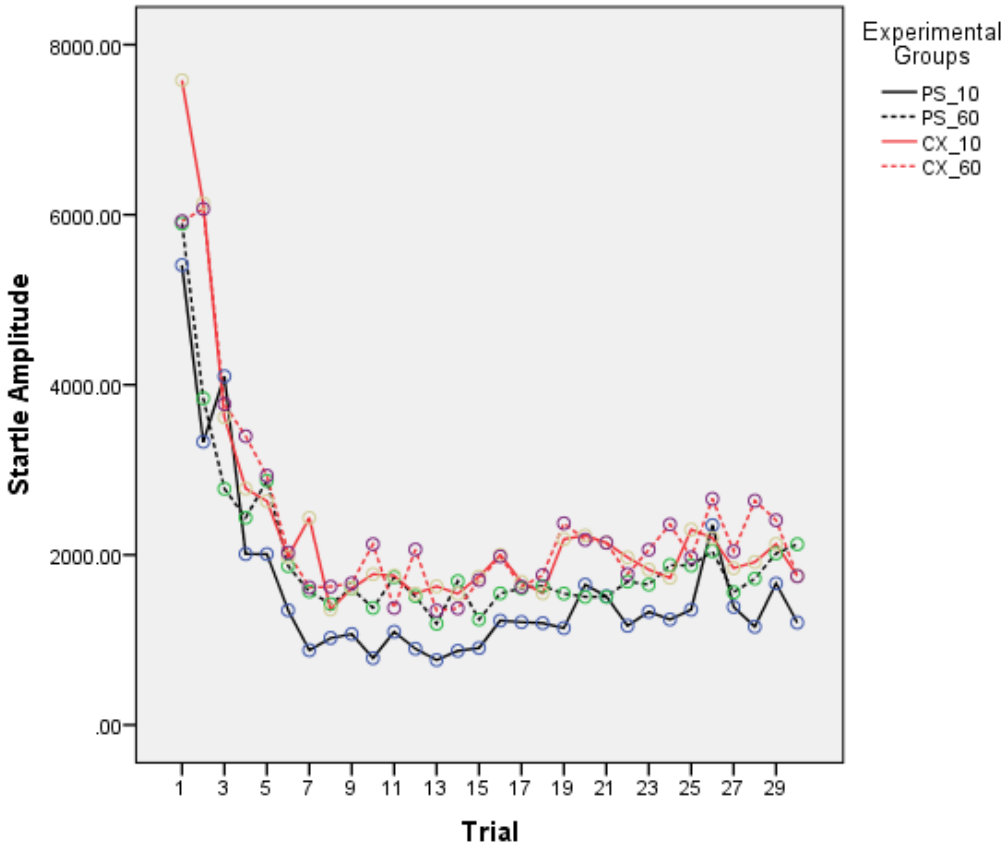

Figure 11: Startle Amplitude Habituation Curves for Experiment 1. Amplitude (V/g) values are plotted over the 30 noise Trials.

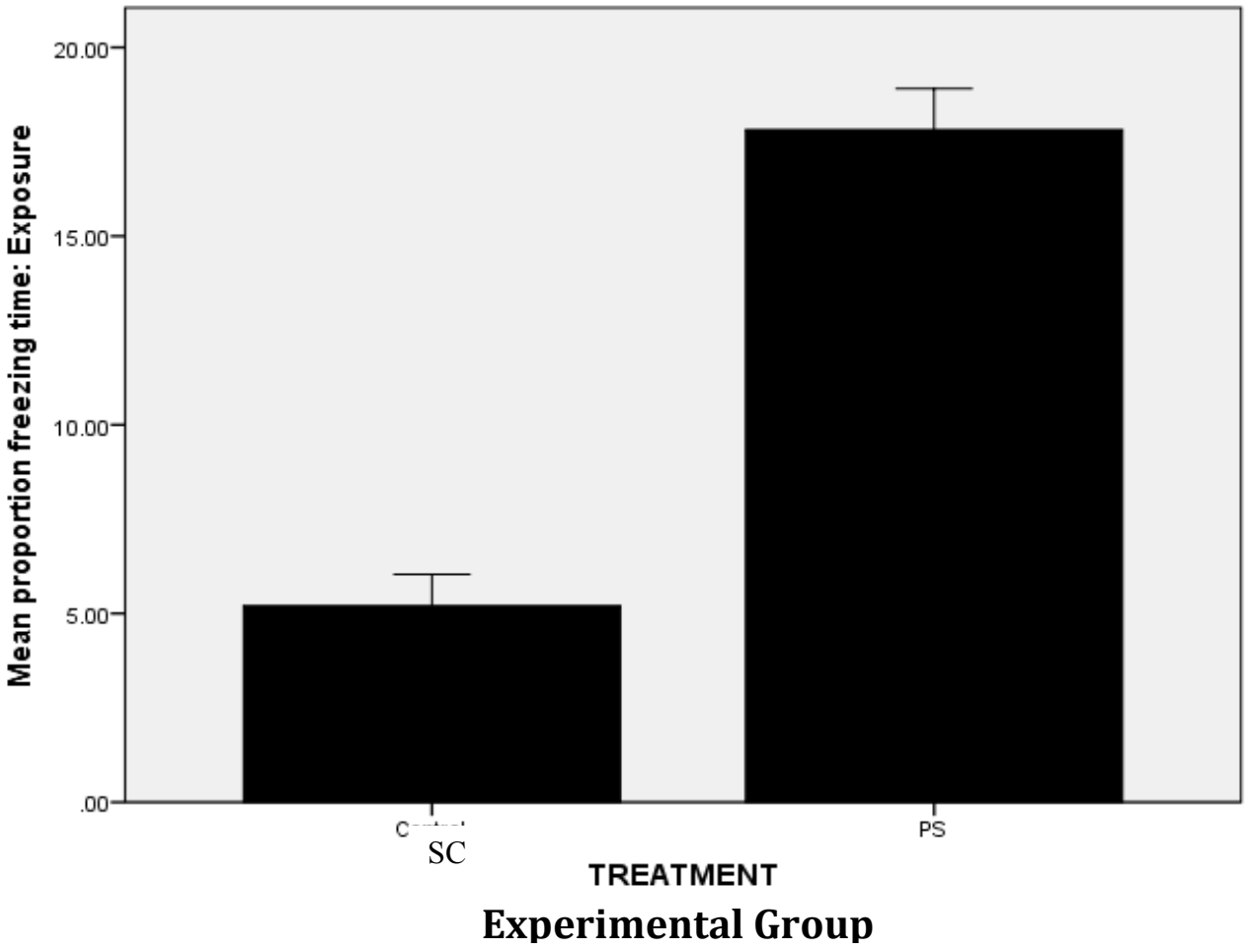

Figure 12: Mean proportion of Exposure Trial spent freezing, Experiment 2. SC= Stuffed Control. PS= Predator Stress (RET exposure)

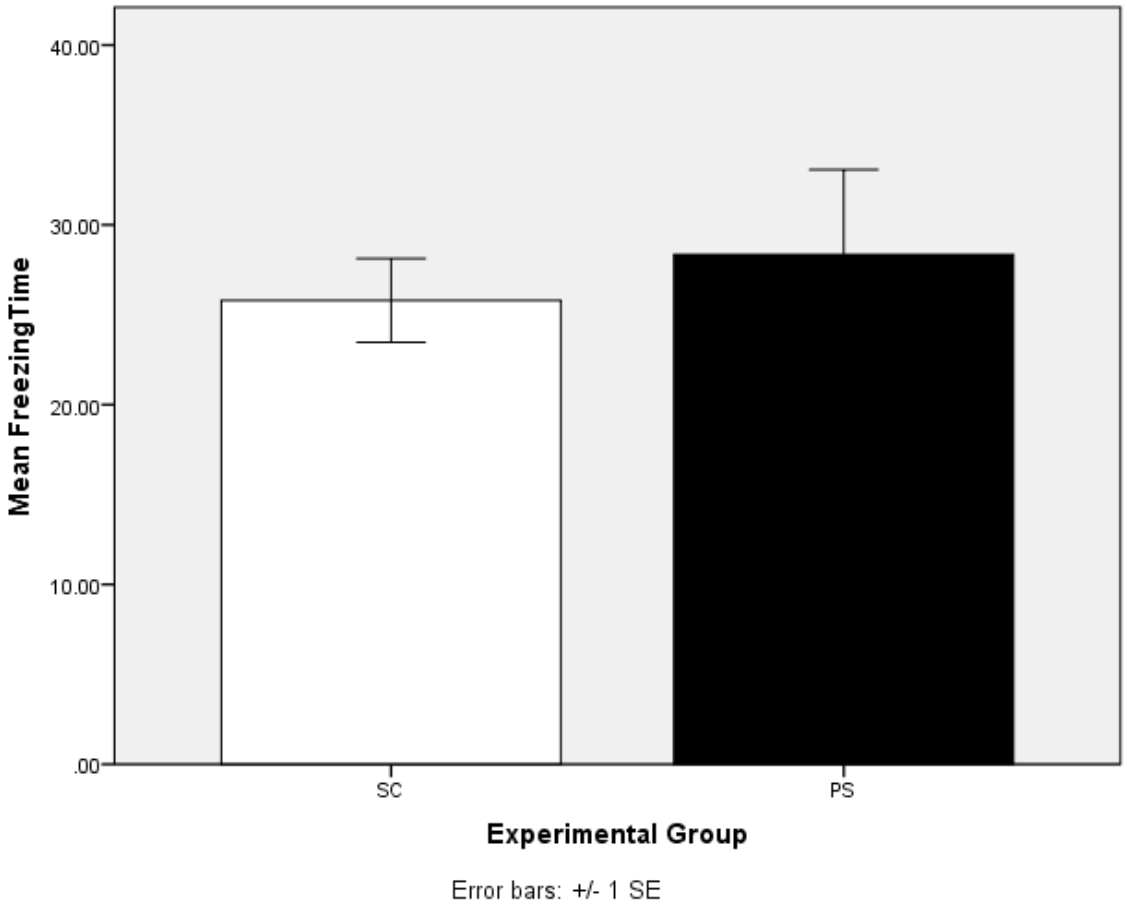

Figure 13: Mean Re-exposure Time spent freezing, Experiment 2.

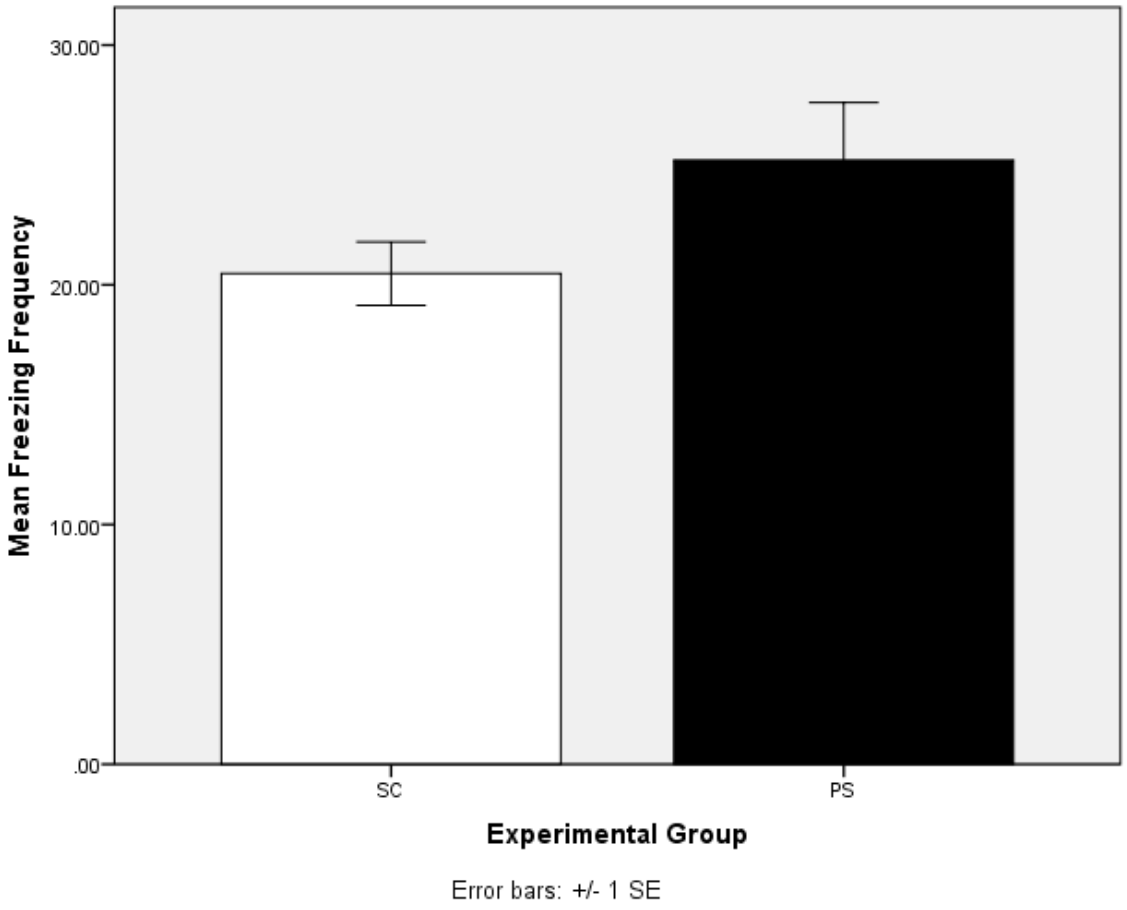

Figure 14: Mean Re-exposure Frequency of freezing, Experiment 2.

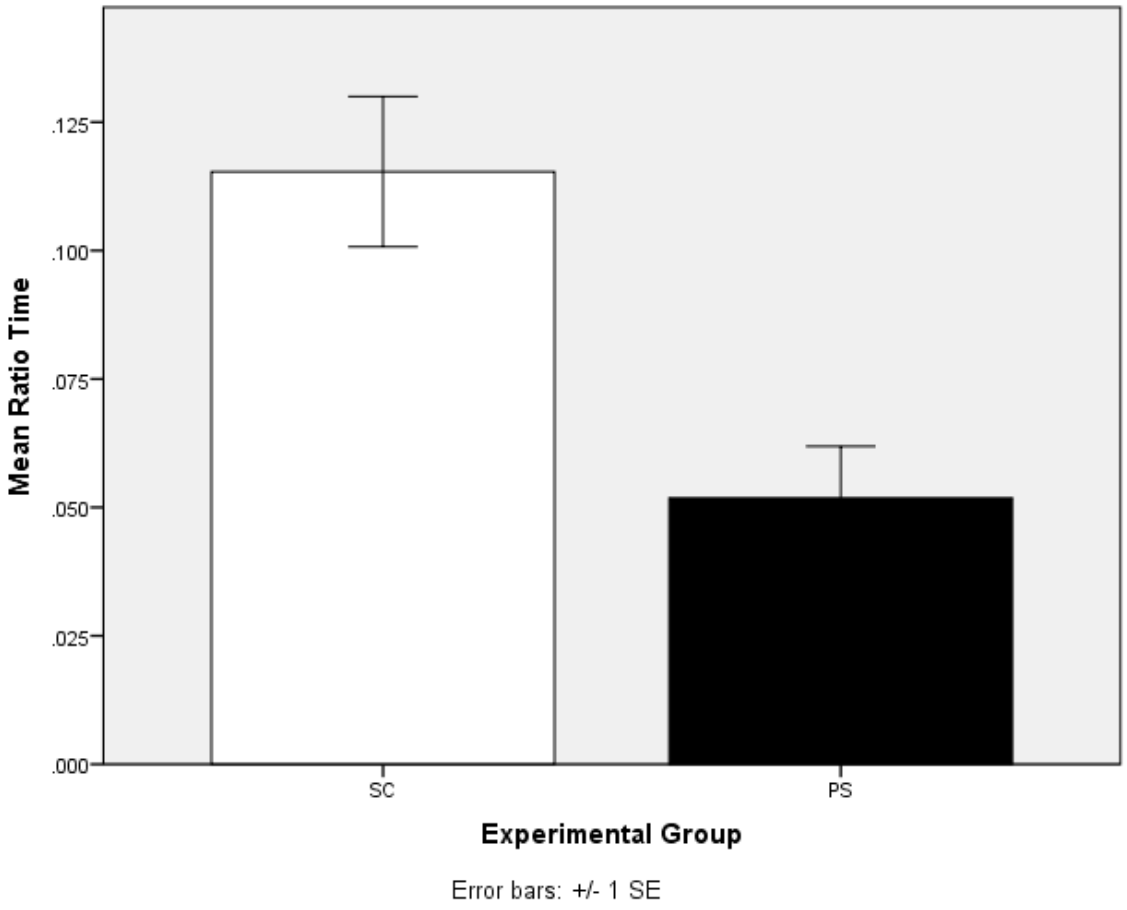

Figure 15: Mean Ratio Time (Open Time/Open Time + Closed Time) in the Elevated Plus Maze (EPM), Experiment 2.

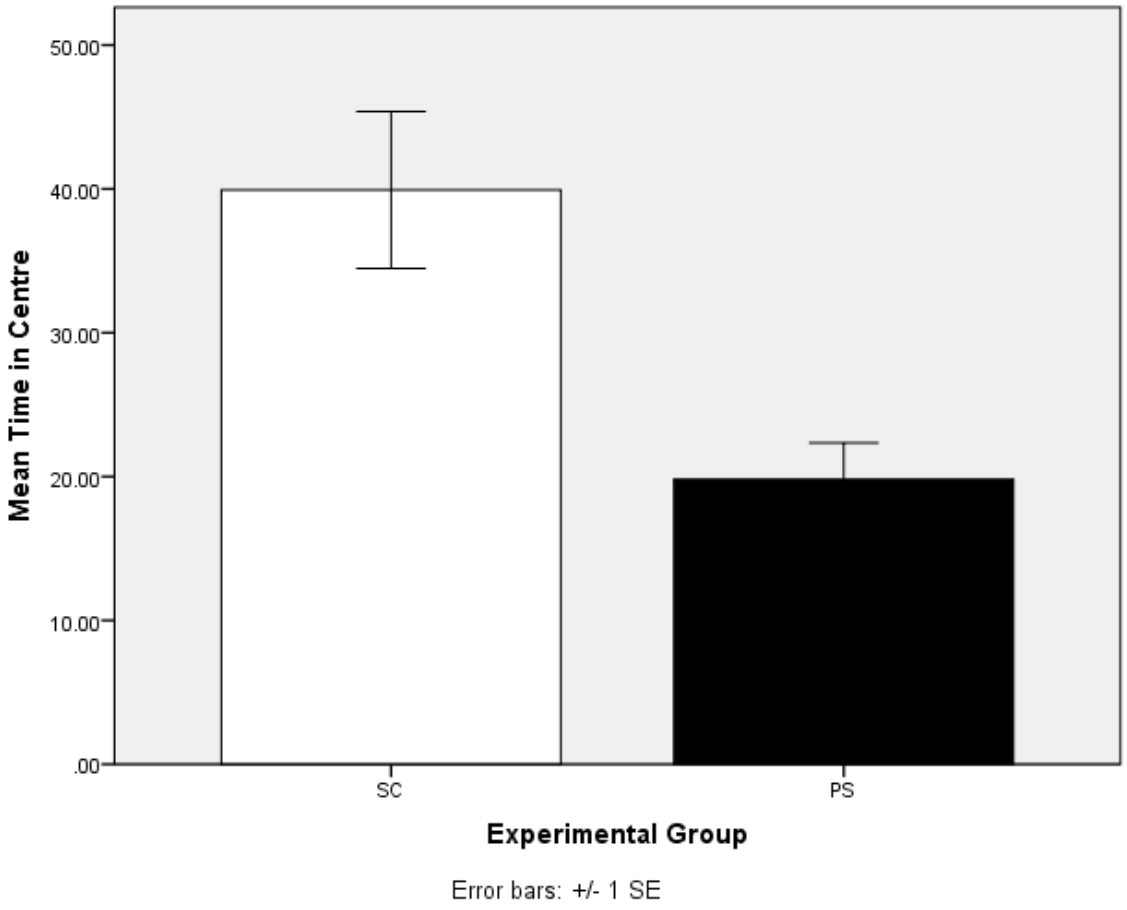

Figure 16: Mean Centre Time in the Open Field (OF), Experiment 2.

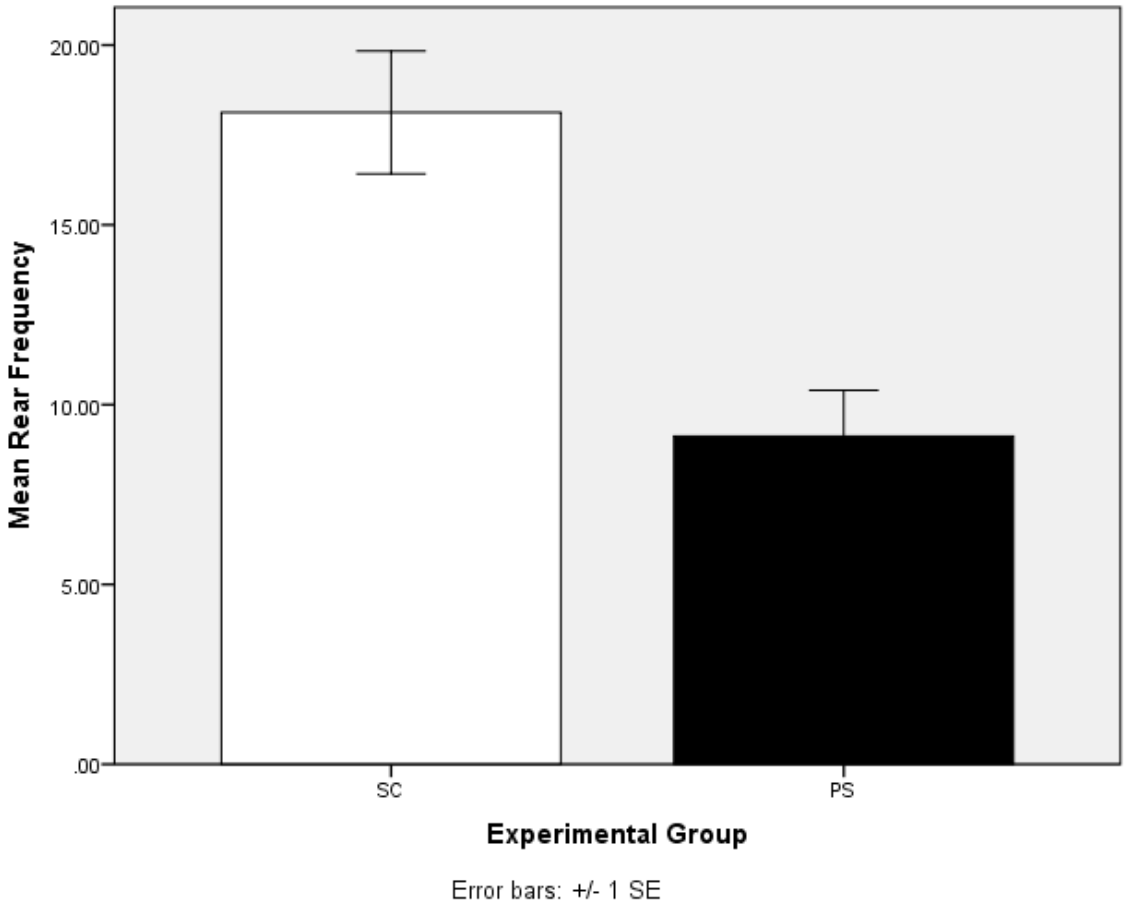

Figure 17: Mean Number of Rears in the Open Field (OF), Experiment 2.

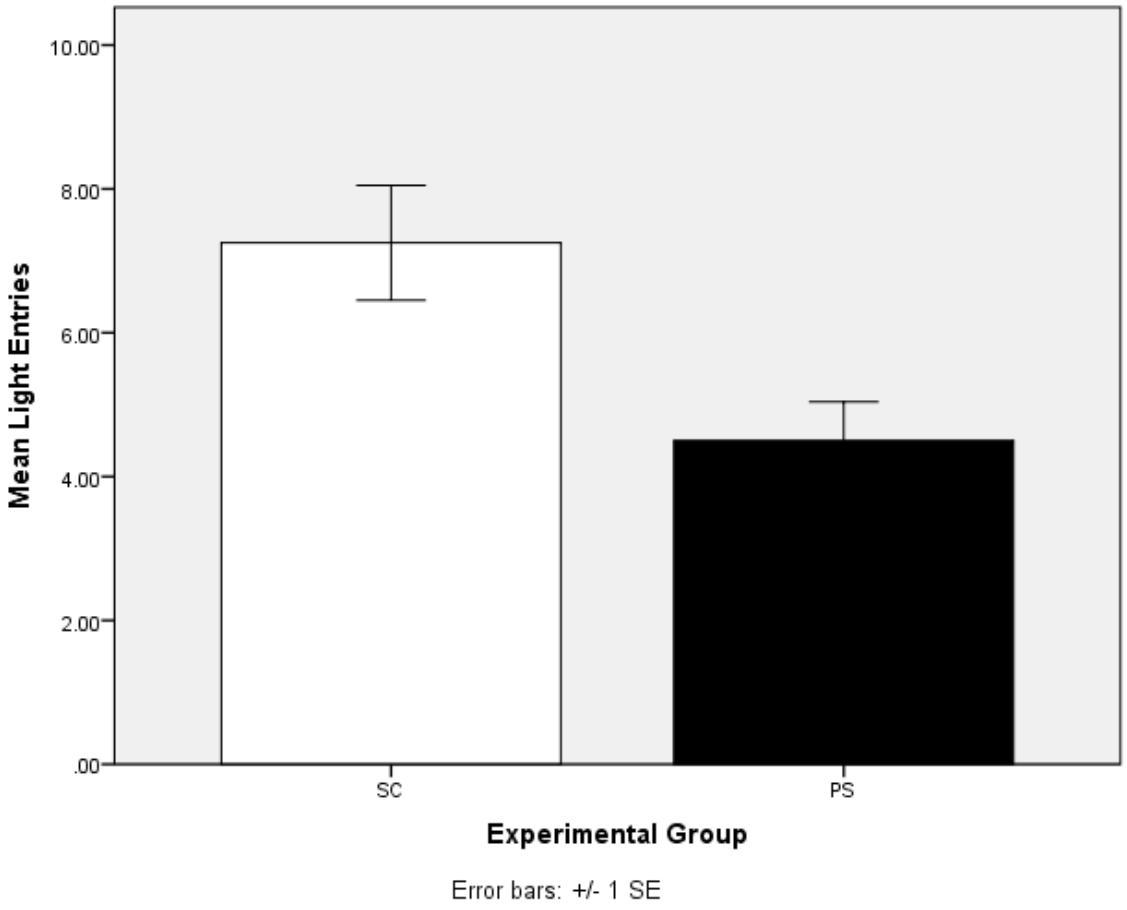

Figure 18: Mean Light-Side Entries in Light Dark Box (LD), Experiment 2.

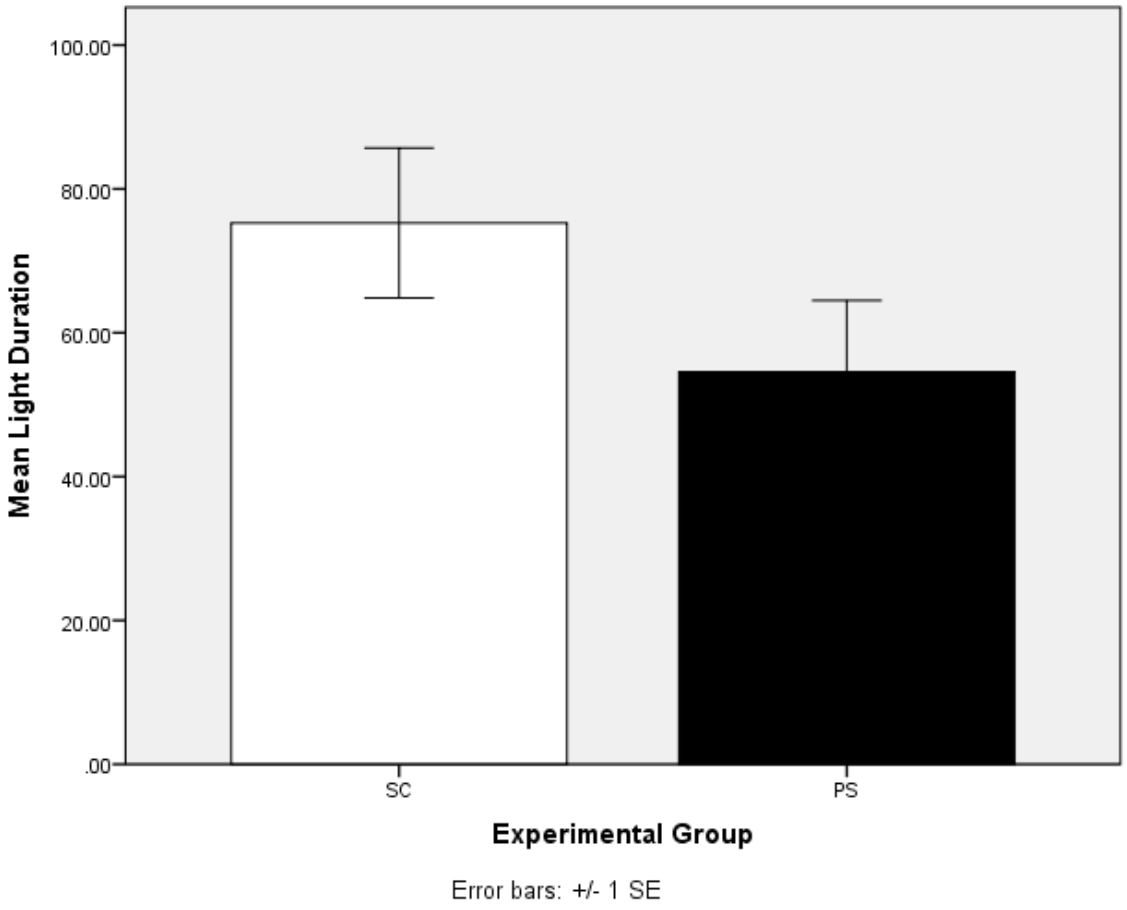

Figure 19: Mean Time spent on Light Side in Light Dark Box (LD), Experiment 2.

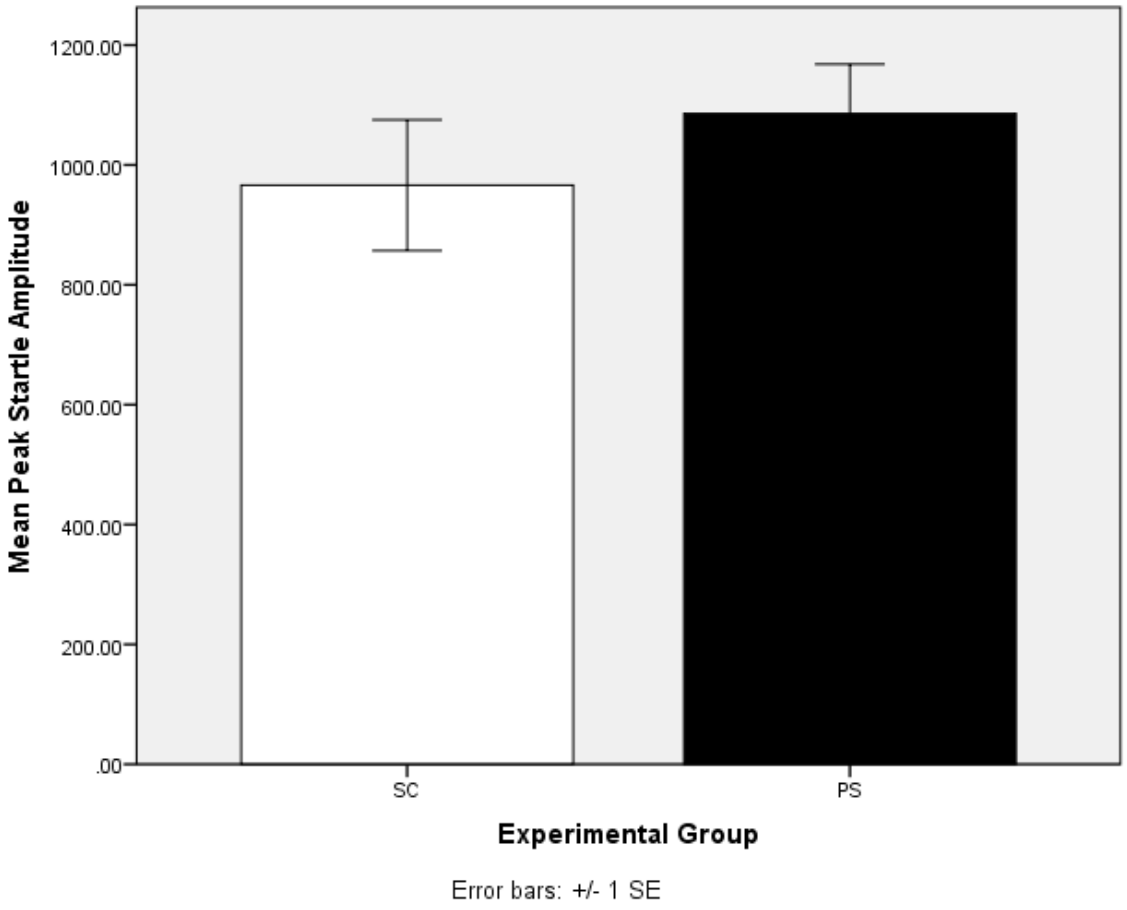

Figure 20: Average Peak Startle Amplitude (PSA), Experiment 2.

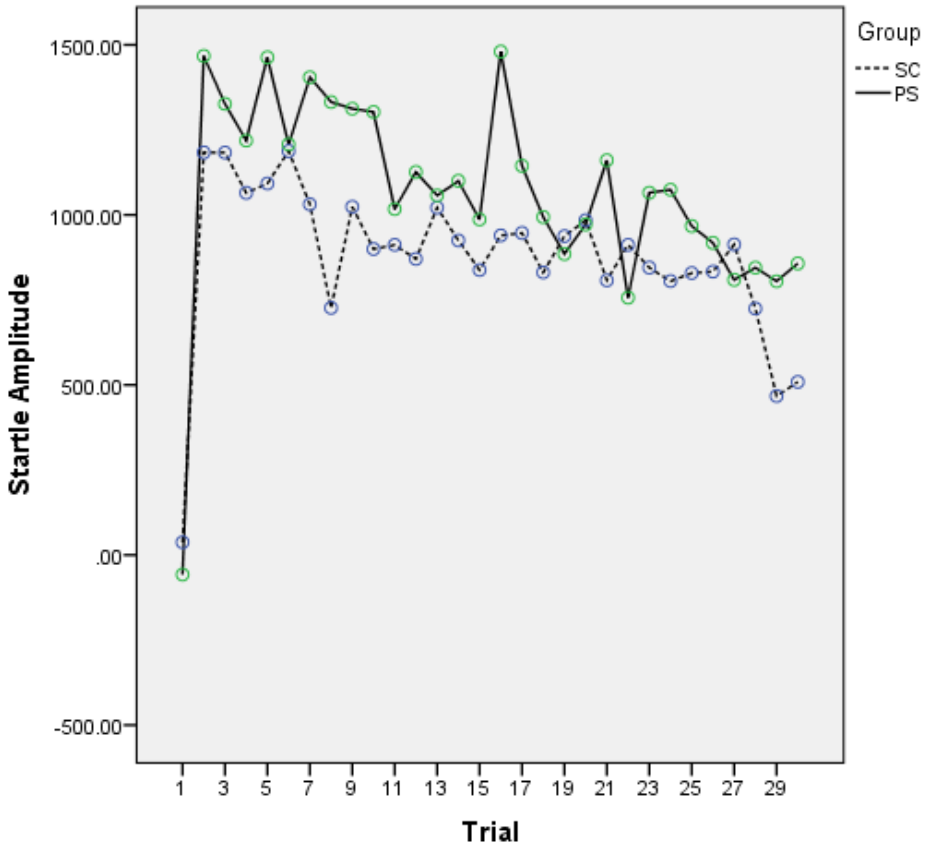

Figure 21: Startle Habituation Curves, Experiment 2.

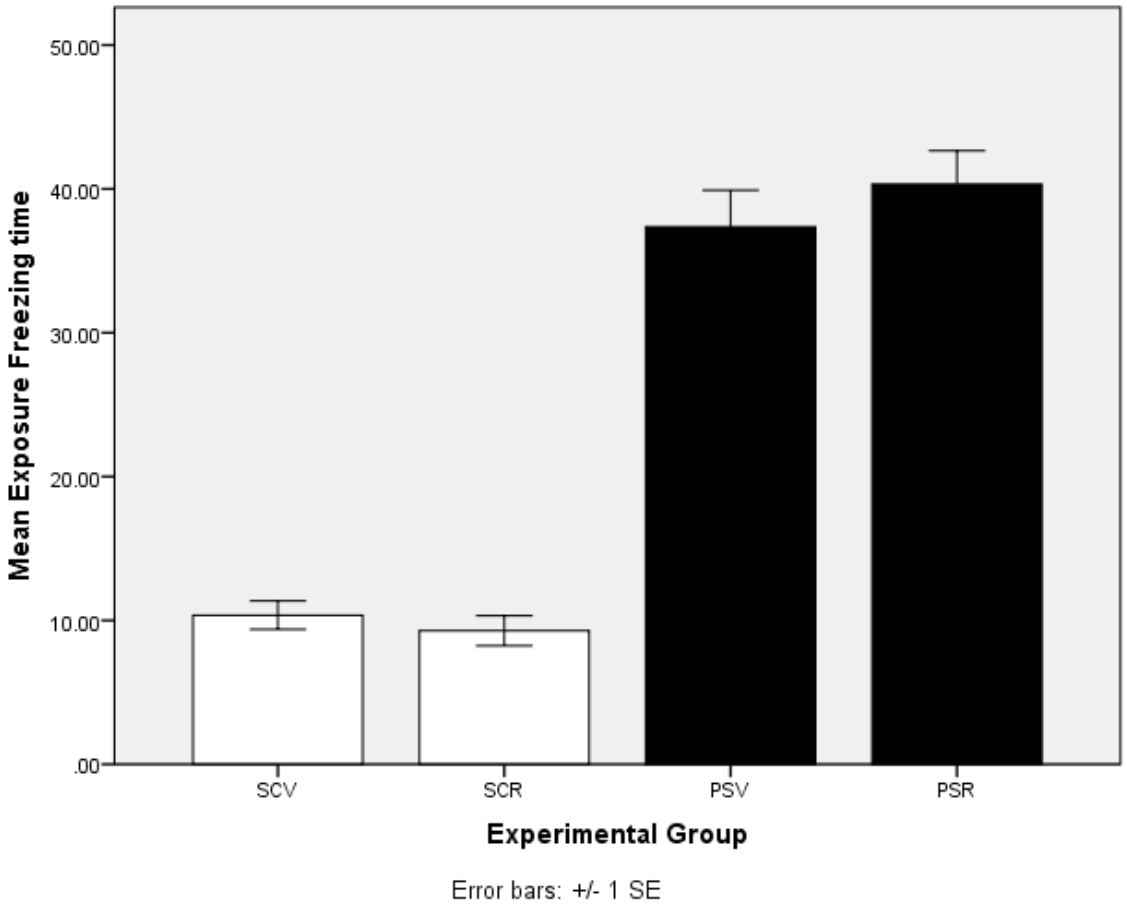

Figure 22: Mean Proportion of Exposure Trial freezing time, Experiment 3. SCV= Stuffed Control+Vehicle; SCR= Stuffed Control+Rapamycin; PSV=Predator Stress+Vehicle; PSR=Predator Stress+Rapamycin.

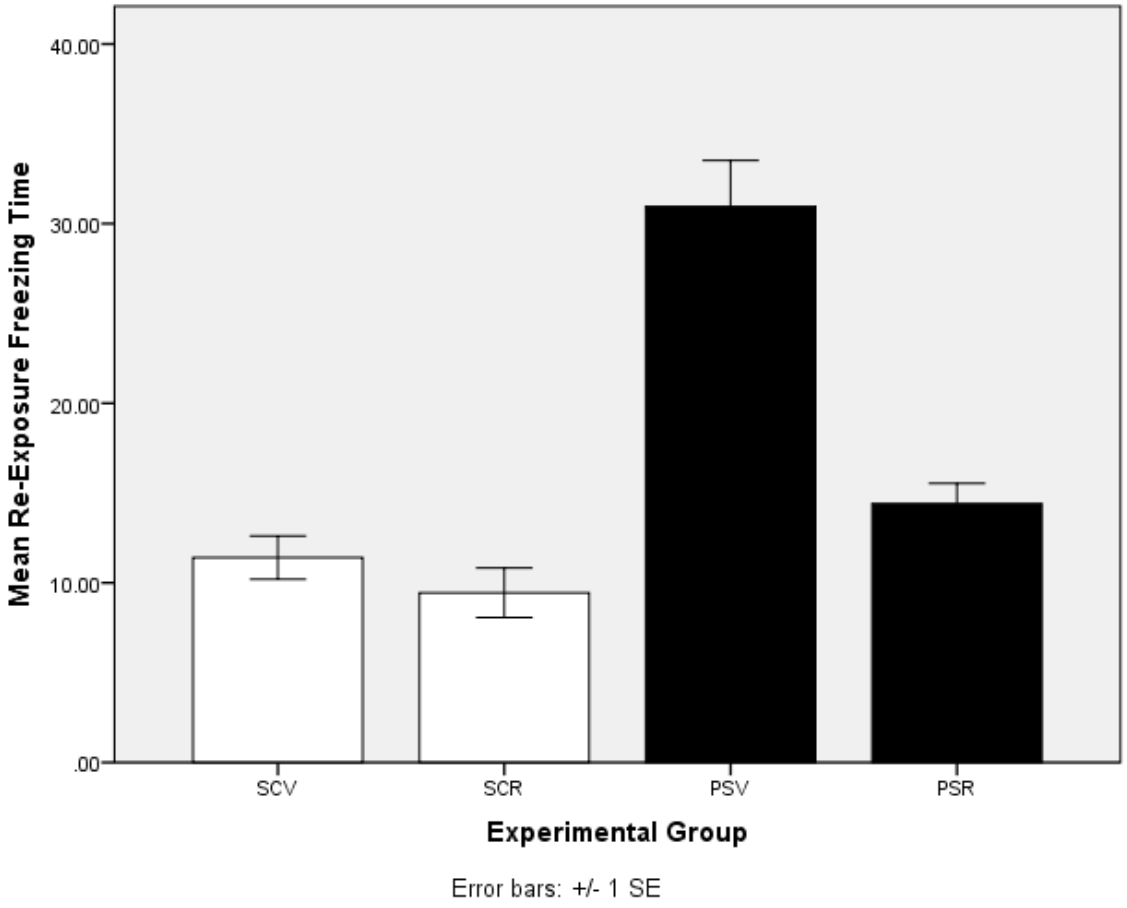

Figure 23: Mean Proportion of Re-exposure Trial spent freezing, Experiment 3.

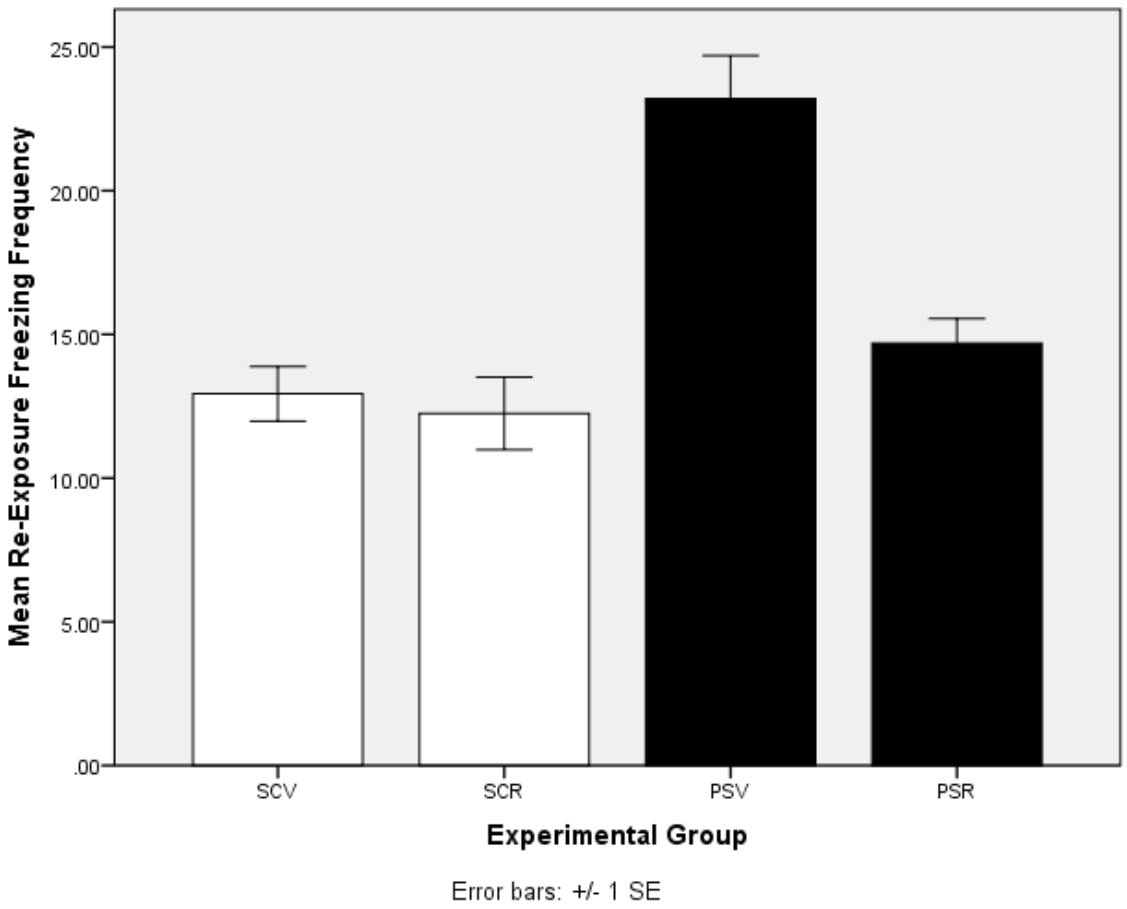

Figure 24: Mean Frequency of Re-exposure Trial freezing, Experiment 3.

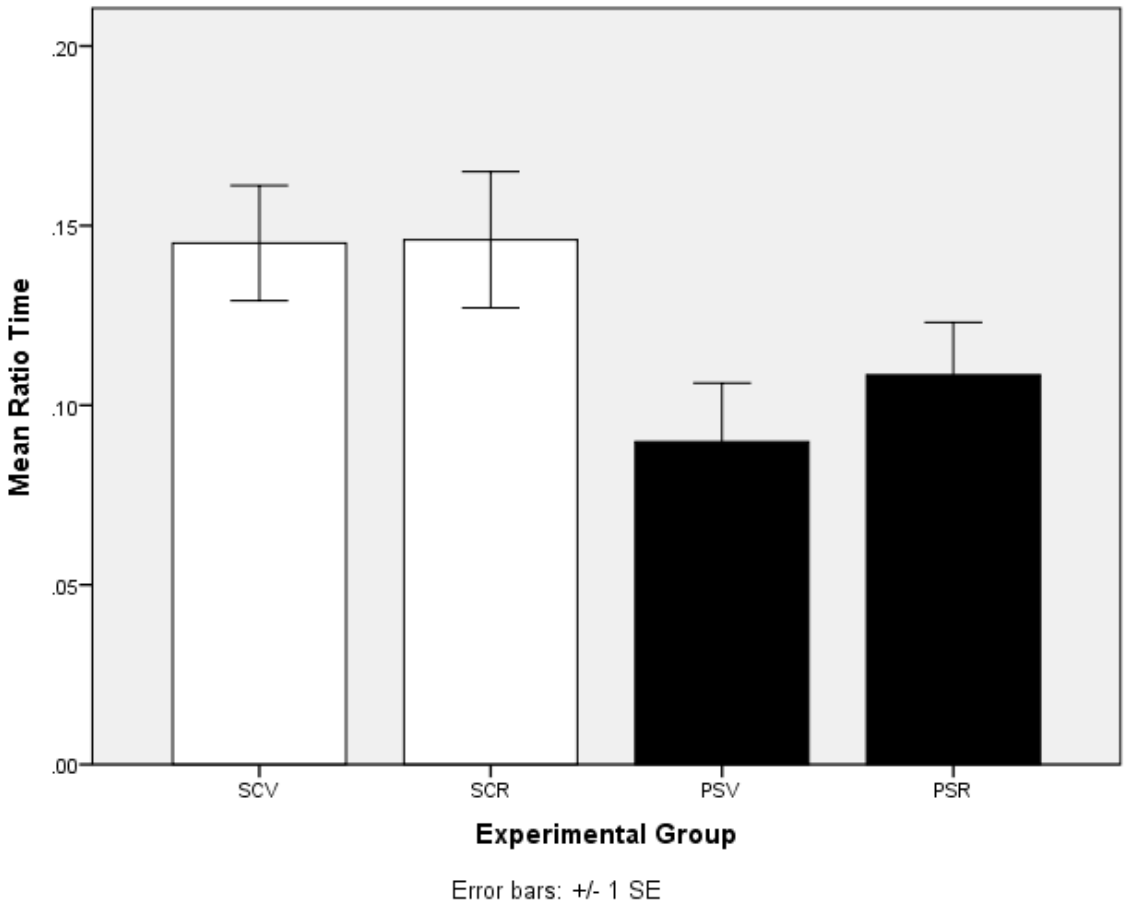

Figure 25: Mean Ratio Time in Elevated Plus Maze (EPM), Experiment 3.

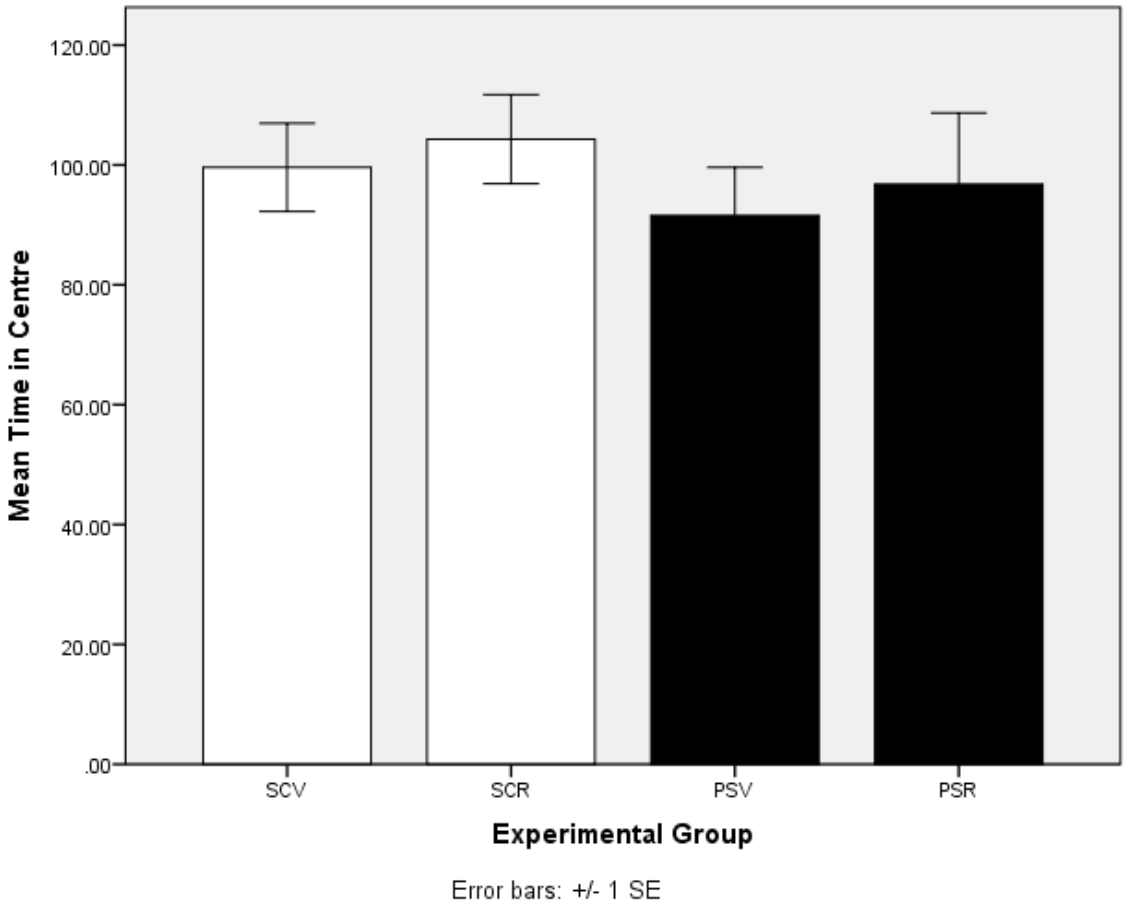

Figure 26: Mean Time in Centre of Open Field (OF), Experiment 3.

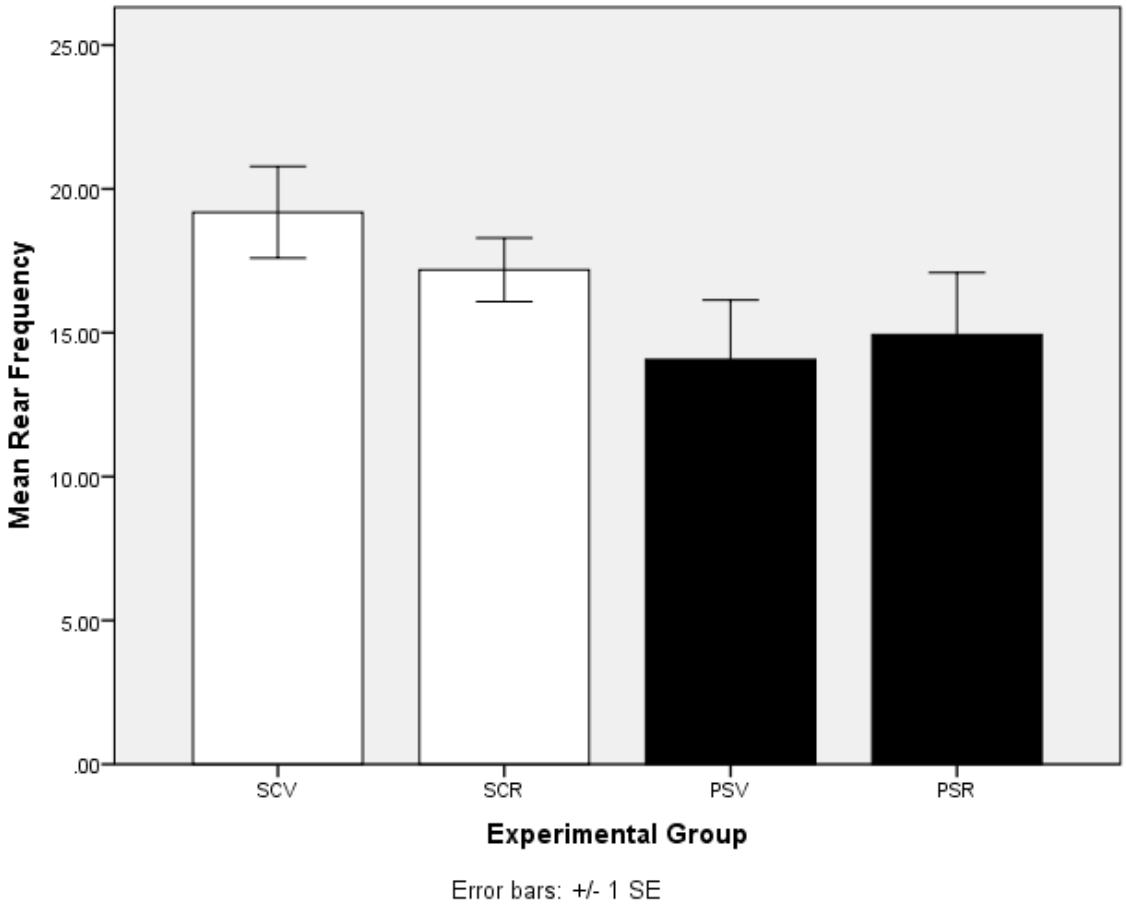

Figure 27: Mean Number of Rears in Open Field (OF), Experiment 3.

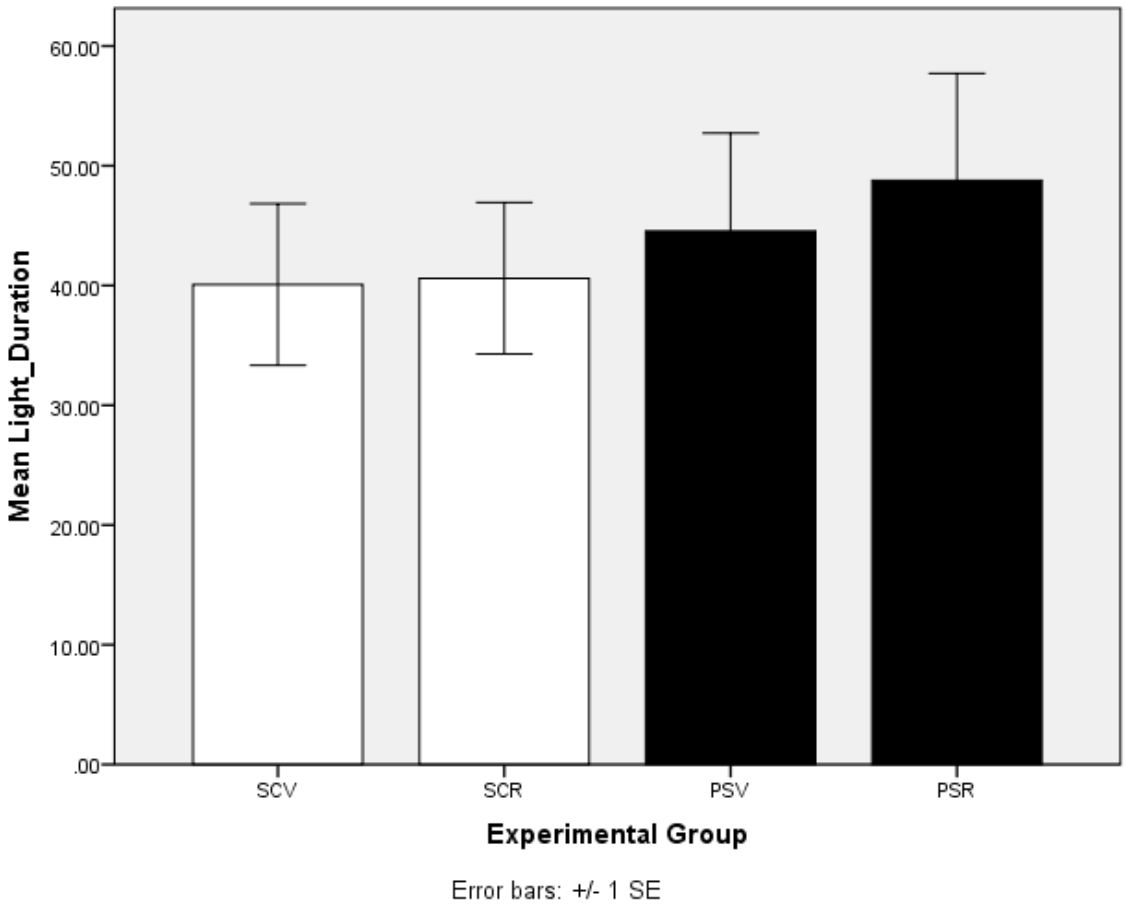

Figure 28: Mean Time spent on Light Side in Light Dark Box (LD), Experiment 3.

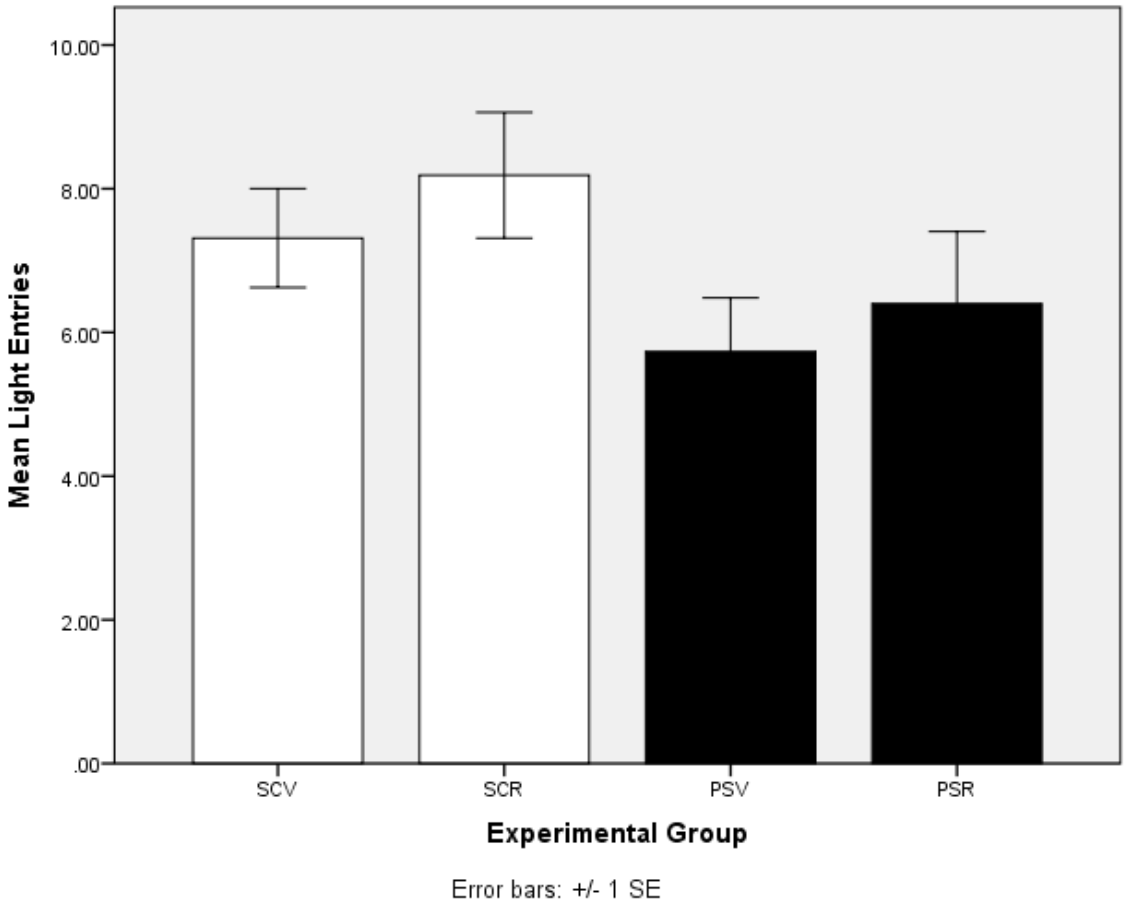

Figure 29: Mean Number of Entries to Light Side in Light Dark Box (LD), Experiment 3.

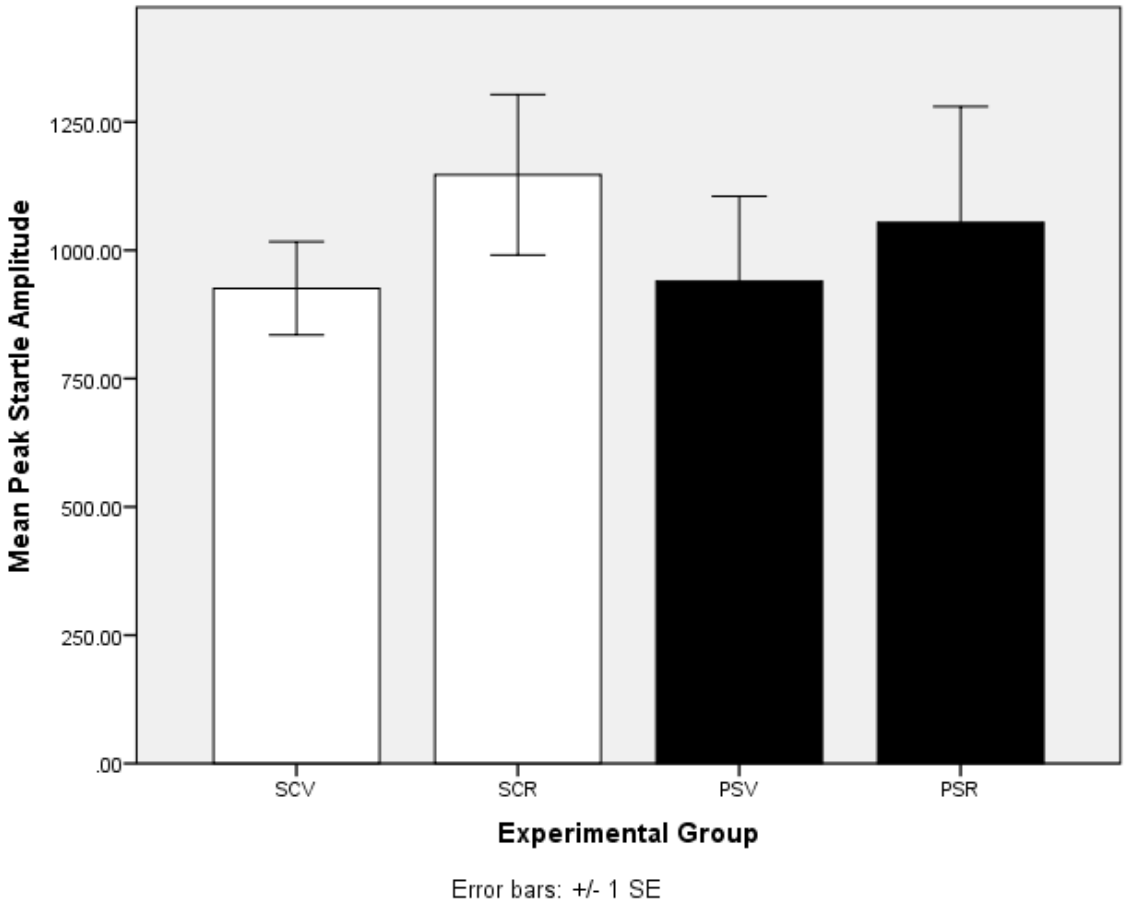

Figure 30: Average Peak Startle Amplitude (PSA), Experiment 3.

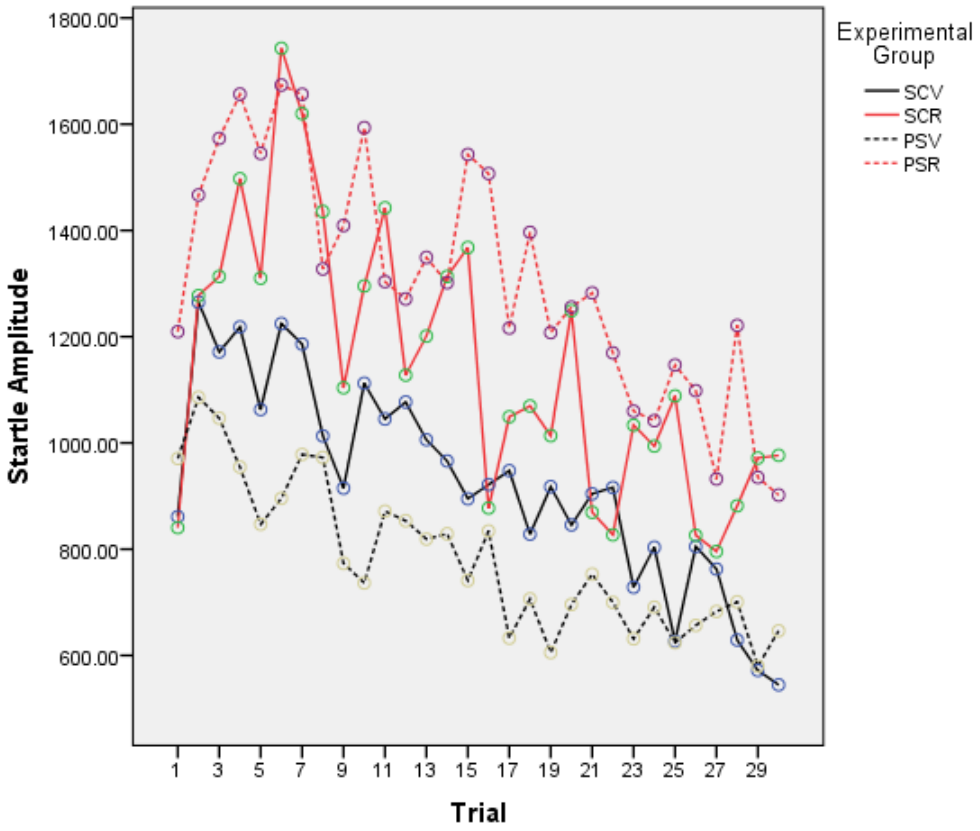

Figure 31: Startle Amplitude Habituation Curves, Experiment 3. Note much more stochastic pattern compared to previous experiments.