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Predator-Based Fear Conditioning: A Novel Approach to the Study of the Neurobiology of Memory

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Predator-Based Fear Conditioning: A Novel Approach to the Study of the Neurobiology of Memory

by

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Abstract

This series of experiments developed novel paradigms involving the integration of conventional and ethologically relevant forms of reinforcement in the study of fear conditioning in rats. Experiment 1 compared the effects of foot shock, immobilization and predator exposure, alone and in combination, on the expression of conditioned fear memory and extinction. The combination of all 3 reinforcers produced a significantly stronger fear memory and greater resistance to extinction, compared to when each reinforcer was administered alone. Furthermore, whereas conditioning with foot shock, alone, resulted in rapid extinction of the fear memory, the combination of immobilization and cat exposure, or all 3 reinforcers together, produced a robust extinction resistant fear memory. Experiment 2 explored the effects of giving extinction trials every two versus every seven days. This experiment demonstrated extinction when the trials were given every 2 days, with no evidence of extinction when trials were given every 7 days. Experiment 3 focused on extending predator-based conditioning to enhance the development of cue-based fear conditioning. Rats were administered multiple predator-based conditioning trials in one session to enhance the formation of both contextual and cue-based fear memories. Experiment 4 tested the hypothesis that hippocampal involvement during learning is necessary for predator-based contextual, but not cued, fear memory. This work provided support for this hypothesis with the finding of impaired contextual memory, with no effect on cued memory, in rats that had a pharmacological suppression of hippocampal activity during fear conditioning. Experiment 5 developed an

entirely novel form of inhibitory avoidance conditioning. This work demonstrated that rats learned to avoid entering a place which was paired with immobilization and predator exposure. Experiment 6 investigated the effects of sleep deprivation occurring prior to fear conditioning on the expression of fear memory. This experiment showed that pre-training sleep deprivation blocked the development of contextual (hippocampal-dependent), but not cue (hippocampal-independent), fear memory. Overall, this series of experiments established the groundwork to use ethologically relevant stimuli, including predator exposure, in conjunction with conventional reinforcers, such as foot shock and immobilization, to advance our understanding of the neurobiology of emotional memory.

Chapter 1: Development of Predator Based Fear Conditioning

1.1 A Brief History of Fear Conditioning

In *The Expression of Emotions in Man and Animals* (1872), Darwin discussed his observations of similar behaviors in different species. Darwin speculated the similarity of behaviors was evidence of evolutionary precursors to human reactions. For example Darwin stated, "With mankind some expressions, such as the bristling of the hair under the influence of extreme terror, … can hardly be understood, except on the belief that man once existed in a much lower and animal-like condition" (pg 14). Darwin's statement of the widespread similarity of behaviors across species is the basis for comparative research.

Whereas Darwin observed the similarity of a broad range of responses, including fear, across species, it was Pavlov who developed a systematic approach to study associative learning. In his book *Conditioned Reflexes* (1927), Pavlov studied learning as an association between a neutral stimulus (the conditioned stimulus or CS) and biologically relevant events (the unconditioned stimulus or US). By pairing a CS, such as a bell, with food (US), Pavlov demonstrated how to form associations between the CS and US. The bell (CS) becomes an anticipatory signal associated with the US, which reliably elicits a reaction. Using food as the US evokes the physiological response of salivation. Salivation, in this example, is an unconditioned response (UR). The term "unconditioned" refers to the fact that no learning is required for the stimulus to elicit a

response. The final component of Pavlovian conditioning is the conditioned response (CR), defined as when the bell (CS), by itself, reliably evokes salivation (now the CR). In this case the animal has learned the association between the bell and food, indicated by a behavioral response (salivation) elicited by the food, now being elicited by the bell *before* food is presented. Pavlovian conditioning provides researchers a systematic way to study behavior using appetitive and aversive associations made among environmental stimuli.

Pavlov's systematic approach to study associations is the foundation for behavioral conditioning research. Although Pavlov is known for pairing a bell with food and measuring salivation in dogs, he also studied associative learning using aversive stimuli in dogs. Fear conditioning is conceptually based on an animal's ability to learn associations between previously neutral stimuli or behaviors (CS) and aversive stimuli (US). For example, pain can be used as an aversive stimulus and is quickly associated with environmental stimuli. An animal's ability to form fear associations is part of their defensive behavior system that serves to protect the animal from danger (Fanselow, 1994; Maren, 2001).

The hypothesis underlying fear conditioning research is that aversive events are associated with environmental stimuli (Holahan & White, 2002). Researchers utilize observable behaviors to measure fear, for instance, research on humans use skin conductance and cardiac responses (Alvarez, Biggs, Chen, Pine, & Grillon, 2008; Cook, Hodes, & Lang, 1986; Hodes, Cook, & Lang, 1985; Labar, Spencer, & Phelps, 1995; Milad et al., 2007). There are also numerous ways to measure fear in rodents. Operant avoidance behavioral paradigms involve training a rat to press a lever to terminate a shock being administered (Brennan, Beck, & Sevatius, 2002). Rats are able to quickly

associate pressing the lever with the termination of the shock. In another paradigm, known as inhibitory avoidance, rats are placed in a brightly lit side of a two-chambered conditioning apparatus (LaLumiere, 2004; Liu, Zheng, & Li, 2009; Roozendaal & McGaugh, 1996; Wilensky, Schafe, & LeDoux, 2000). Rats prefer the darker compartment of the conditioning box and readily cross from the light to the dark side. Upon crossing into the dark box, the rat is shocked. Subsequently, conditioned animals inhibit crossing into a preferred chamber in order to avoid the foot shock. Thus, the animal's crossing behavior into the darker box is associated with shock.

Both the operant and inhibitory avoidance behavioral paradigms allow the rat to control the exposure to an aversive stimulus. Another behavioral paradigm that does not allow the animal to control the occurrence of the aversive stimulus, is classical fear conditioning. In this paradigm the behavioral measure of fear is called "freezing" and has no effect on the occurrence of the aversive stimulus in experimental designs. Freezing is a behavior expressed in rodents defined as the absence of movement except that required for respiration. Freezing could be viewed as an adaptive behavior because predators often use movement to track their prey. Thus, suppressing movement, under aversive circumstances, is hypothetically advantageous. The percentage of time an animal spends motionless is used as a measure of fear. Rodents freeze when shocked or when in the presence of predators and related cues. Freezing is also expressed to places and specific cues that have been paired with aversive stimuli.

Fear associated with the place an aversive stimulus is encountered is one component of Pavlovian fear conditioning. The place where conditioning occurs is known as the context. A rigorous definition of context, as provided by Nadel (2008), is it

entails the cognitive representation of environmental stimuli into a coherent spatial arrangement. Contextual fear conditioning is a basic procedure involving placing an animal in an environment and administering an aversive stimulus. The animal expresses fear when it is returned to the same place. The fear response indicates the animal learned an association between the place and the aversive stimulus.

In addition to investigating associations between the context and aversive stimuli, researchers are characterizing the way specific modalities of sensory stimuli are associated to aversive stimuli using cue fear conditioning. Whereas context fear conditioning associates the place to an aversive stimulus, cue fear conditioning involves pairing a salient cue within the context to an aversive stimulus. Cue-based fear conditioning experiments typically pair a tone or light CS with an electric shock US. Tested later, in a different context, the subject expresses fear upon delivery of the CS. The fear of a specific cue is transportable across contexts, that is to say, cued fear can be expressed in a new place. Therefore, much of the focus of research has been on distinguishing between mechanisms underlying context and cue fear conditioning.

Fear conditioning, in general, has been demonstrated in many species (Kim & Jung, 2006). Humans, rats and snails are among the many species shown to form associations between places or other sensory cues and fear provoking events (Walters, Carew, & Kandel, 1979; Walters & Kandel, 1981). Fear conditioning allows humans and other animals to detect threats and initiate survival behaviors (Sehlmeyer et al., 2009). However, fear conditioning as an adaptive process, can go awry and render safe stimuli threatening and elicit inappropriate fear and anxiety. Human anxiety disorders, such as posttraumatic stress disorder (PTSD) and phobias are linked with persistent fear. Peri et

al. (2000) found that PTSD patients had higher autonomic nervous system responses (skin conductance and heart rate) at rest and that aversive conditioning augmented responses to conditioned stimuli compared to healthy control subjects. The enhanced fear conditioning in PTSD patients was also significantly more difficult to extinguish.

Various fear conditioning paradigms are based on evolutionary foundations that provide means for animals to pass on their genes by avoiding potentially fatal, aversive situations. Fear can be associated with places and discrete environmental cues. Researchers use experimentally generated fear associations to understand physiological changes in the nervous system. The next section outlines a subset of research that is aimed at elucidating the neurobiological aspects of fear memory, including brain structures and modulatory hormones.

1.2 Neurobiology of Fear Conditioning

1.2.1 Neural Structures and Plasticity

Neural Structures. Fear conditioning is a powerful tool used to investigate the underlying neural mechanisms of associative learning (Curzon, Rustay, & Browman, 2009). There are dissociable aspects of fear, such as the fear of an overall context and fear of specific, discrete sensory stimuli. It is not surprising, then, that different neural structures are involved in the different aspects of fear conditioning. The most critical neural structure in fear conditioning is the amygdala. Lesions of the amygdala block Pavlovian fear conditioning (Fanselow & Ledoux, 1999). Amygdala lesions block the freezing expressed to contexts and cues associated with foot shock (Blanchard & Blanchard, 1972; Maren, 1998, 1999; Maren & Quirk, 2004; Martinez, Carvalho-Netto, Ribeiro-Barbosa, Baldo, & Canteras, 2011; Phillips & LeDoux, 1992). Lesions of the

amygdala of rodents made one week before or up to one month after training block freezing (Maren, 1998; Maren, Aharonov, & Stote, 1996). Furthermore, lesions of the amygdala do not result in hyperactive rats, indicating that reduced freezing is not due to a change in general motor activity (Maren, 1998). Similarly, pharmacological inactivation of the amygdala before fear conditioning impairs subsequent expression of the emotional memory. Inactivation of the amygdala approximately one hour before fear conditioning impairs acquisition of conditioned fear to the context (Helmstetter & Bellgowan, 1994) and auditory cues (Muller, Corodimas, Fridel, & LeDoux, 1997; Wilensky, Schafe, & LeDoux, 1999) when tested 24 hours after training. The midbrain central gray in rats is a recipient of amygdalar projections (LeDoux, Iwata, Cicchetti, & Reis, 1988) and lesions of the midbrain central gray block freezing responses globally. Lesions of the medial geniculate nucleus of the thalamus (MGN), which relays auditory information to the amygdala, impair auditory, but not visual, fear conditioning (LeDoux, Iwata, Pearl, & Reis, 1986). These and other studies implicate the amygdala as a crucial hub of processing fear related associations between the US and the CS.

The amygdala is necessary for fear conditioning in general, with the hippocampus playing a crucial role in a subset of fear conditioning. One of the first studies demonstrating that the contextual component of fear conditioning is dependent on the hippocampus and that certain cue fear conditioning paradigms are not hippocampal dependent was Phillips & LeDoux (1992). Investigations of the role the hippocampus plays in Pavlovian contextual fear conditioning show that lesions of the dorsal hippocampus made prior to (Kim, Rison, & Fanselow, 1993; Phillips & LeDoux, 1992, 1994; Selden, Everitt, Jarrard, & Robbins, 1991; Young, Bohenek, & Fanselow, 1994), or

soon after (Kim & Fanselow, 1992), conditioning block freezing upon re-exposure of the subject to the conditioning context. These were some of the preliminary, modern studies into the neurobiological underpinnings of fear conditioning have supported and extended this work (Bannerman et al., 2001; Gewirtz, McNish, & Davis, 2000; Maren & Fanselow, 1997; Maren, Aharonov, & Fanselow, 1997; Maren & Holt, 2004; Mei et al., 2005; Misane et al., 2005; Parsons & Otto, 2008; Quinn, Loya, Ma, & Fanselow, 2005; Rudy & O'Reilly, 2001; Rudy & Matus-Amat, 2005; Sanders, 2003; Yoon & Otto, 2007).

In hippocampal lesioned animals associations formed to discrete cues presented in conjunction with the aversive stimulus remains intact, indicated by freezing to discrete stimuli, such as auditory cues. Parsons & Otto (2008) used the GABA receptor agonist muscimol to temporarily inactivate the dorsal hippocampus to investigate the effects on context, auditory, and olfactory cue fear conditioning. Muscimol infusions into the dorsal hippocampus prior to training, testing, or both produced anterograde and retrograde deficits in contextual conditioning. Freezing was expressed to both auditory and olfactory conditioned stimuli regardless of muscimol or saline infusions. Therefore, the hippocampus is critical for the formation of fear associations to the context, but not discrete cues, in rodents. Additionally, electro-physiological rhythms in the lateral nucleus of the amygdala became synchronized in a theta frequency with the dorsal CA1 area of the hippocampus in fear conditioned mice expressing freezing behavior when confronted with the conditioned context (Seidenbecher, Laxmi, Stork, & Pape, 2003). Thus, the amygdala and hippocampus, together, are integral to processing components of Pavlovian conditioned fear associations.

Mei et al. (2005) used high-density microarrays to investigate fear conditioning induced gene expression profiles in the hippocampus and amygdala of mice after fear conditioning. Of the 11,000 genes and expression sequence tag (ESTs) profiles investigated, in the amygdala 222 genes were influenced by conditioning. Twenty-two percent of the amygdalar genes changed by conditioning coded for structural and cell adhesion proteins, including genes regulating synaptic, dendritic and axonal structures (e.g., actin, brain Spectrin, tubulin, & microtubule associated proteins). Half an hour after conditioning these researchers found up-regulated proteins that interact with NMDA and AMPA glutamatergic receptors, such as β-III spectrin, a vesicle-related protein. Additionally, microtubule associated protein (MAP4) and cytosolic chaperonin (CCT) were upregulated. In the hippocampus, the same amounts of gene-related molecules were analyzed and 38 signaling molecules were affected. For example, protein kinase regulator, a learning related gene (Skoulakis & Davis, 1996), was down-regulated 6 hours after conditioning. This kinase regulator interacts with GABA receptors in neuronal culture (Couve et al., 2001), and Mei et al. (2005) reported that the alpha-1 subunit of the GABA receptor was decreased at the 6 hour time point. Synaptotagmin, pantophysin, and vesicle-associated membrane protein (VAMP) also were down-regulated at the same time-point. These results support the hypothesis that fear conditioning changes the physiology of neurons in the amygdala and hippocampus at the genetic level.

Plasticity. The neuronal processes of the amygdala and hippocampus related to memory are a target of many investigations. The fundamental neuronal process studied related to memory is plasticity. Konorski (1948) described neuronal plasticity as the persistent, activity-driven changes in synaptic efficiency as the mechanism underlying the

storage of information in the brain. Hebb (1949, p.62) formally advanced the theory with his classic postulate that "When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased." Hebbian theory postulates that networks of cells (cell assemblies) act upon each other, such that when one cell's firing is repeatedly facilitated by another cell, an association between the cells' activity is formed. The changes in the strength of connections between neurons is widely theorized to be the basis for memory (Martin, Grimwood, & Morris, 2000).

Experimental evidence for augmented synaptic connectivity from electrophysiological experiments reported that a high frequency train of electrical impulses enhanced the long-term excitability of synaptic connections within the hippocampus of intact rodents (Bliss & Lømo, 1973). The long-term enhancement of synaptic efficiency was termed long-term potentiation (LTP). The groundbreaking finding of enhanced synaptic efficiency by Bliss and Lømo (1973) is an attractive experimental model for memory. The properties of LTP that make it attractive as a model for memory include the persistent increase in synaptic strength, the associative nature of stimulation required to induce LTP and the input-specificity demonstrated in LTP experiments (Bliss & Collingridge, 1993; Howland & Wang, 2008; Martin, Grimwood, & Morris, 2000; Sigurdsson, Doyère, Cain, & LeDoux, 2007). The similarities between the properties of LTP and memory support the hypothesis that endogenous LTP-like neural plasticity underlies memory formation (Kim, Song, & Kosten 2006).

The hypothesis that LTP-like changes in the amygdala are involved in fear conditioning is supported by work done both in vitro and in vivo. Rogan & Ledoux (1995) showed that LTP induction at auditory inputs of the amygdala enhanced auditory-evoked responses in a similar manner to the enhanced response to the conditioned stimulus in fear conditioned animals (Rogan, Stäubli, & Ledoux, 1997). Schimanski & Nguyen (2005) showed mutant mouse strains that had poor induction of LTP in the amygdala also had impaired cued fear conditioning. In vitro work also supports the hypothesis that plasticity in the amygdala is involved in fear conditioning memory. For example, McKernan & Shinnick-Gallagher (1997) demonstrated enhanced electrical transmission between cells of the MGN and lateral amygdala in tissue from auditory fear conditioned animals compared to control animals. A recent investigation demonstrated that the fear conditioning-induced enhanced potentiation of amygdala whole-cell recordings was reduced during extinction and subsequently reinstated by re-conditioning (Hong et al., 2011). These studies indicate amygdalar plasticity is associated with the formation of fear memories to auditory cues.

As mentioned earlier, the amygdala is not the only brain region involved in fear conditioning. The hippocampus plays a role in the contextual component of fear conditioning. That is, the hippocampus aids in generating a representation of the context in which the learning event occurs. One example supporting the hypothesis that hippocampal plasticity underlies contextual fear conditioning is the finding that mutant mice, deficient in hippocampal LTP induction, also show contextual fear deficits (but no deficits in auditory cue delay fear conditioning)(Abeliovich, Chen, et al., 1993; Abeliovich, Paylor, et al., 1993; Bourtchuladze et al., 1994; Huerta, Sun, Wilson, &

Tonegawa, 2000). Endogenous LTP-like plasticity in the hippocampus, initiated by fear conditioning, could be responsible for the formation of the contextual memory (Diamond, Park, & Woodson, 2004).

Sacchetti et al. (2001) provided support for the hypothesis that contextual fear conditioning involves hippocampal processing. By demonstrating that rats conditioned to associate fear to a context, as measured by freezing, showed increases in extracellular electrical responses in hippocampal in vitro preparations using a single low-intensity electrical stimulus. The increases in evoked responses were found in tissues from rats immediately, 1, and 7 (but not at 28) days after conditioning, compared to control groups. As stated by the authors, the lack of an increase at 28 days is congruent with the hypothesis that hippocampal plasticity is necessary for storage of relatively short-term information (as indicated by the immediate, 1, & 7 day results), but not the recall of long-term information. The control group that was allowed to explore the conditioning context also showed an increase in extracellular electrical response comparable to the fear conditioned group, but only immediately after exploring the novel context. These results support the hypothesis that hippocampal plasticity is involved in the exploration of novel environments (Fushimi, Matsubuchi, & Sekine, 2005; Straube, 2003). Though, the changes in tissue of fear conditioned animals at 1 and 7 days in Sacchetti et al. (2001) suggest that emotional learning has a more prolonged impact on hippocampal plasticity than novelty.

Diamond and colleagues (Diamond, Campbell, Park, Halonen, & Zoladz, 2007; Diamond et al., 2004; Diamond, Park, Campbell, & Woodson, 2005) hypothesize that stress, such as that induced by fear conditioning, results in an endogenous hippocampal

LTP-like phenomenon. The hypothesis that stressful situations are better remembered than non-stressful suggests the result would be the formation of a durable episodic memory. Support for this hypothesis is found in research suggesting that the amygdala activates the hippocampus during emotional learning, such as fear conditioning. Investigators have found that electrical stimulation of the amygdala mimics the emotion-driven enhancement of hippocampal LTP (Akirav & Richter-Levin, 1999a, 1999b; Akirav & Richter-levin, 2002; Frey, Bergado-Rosado, Seidenbecher, Pape, & Frey, 2001; Ikegaya, Saito, & Abe, 1995; Nakao, Matsuyama, Matsuki, & Ikegaya, 2004). The evidence of the involvement of the amygdala and the hippocampus in fear conditioning is convincing. To understand how fear conditioning influences memory, an awareness of what modulates the processing of the hippocampus and amygdala is needed. Thus, the next section will briefly describe some of the neuromodulators, released in the peripheral and central nervous systems that are hypothesized to influence neural plasticity and memory.

1.2.2 Neuromodulatory Hormones

Fear conditioning to discrete cues and the overall context is dependent on plasticity in the amygdala and hippocampus. Neural plasticity in these brain regions is significantly influenced by peripheral and central nervous system hormones released in response to stressful events, such as fear conditioning. Two classes of neuromodulator receptors receiving an extensive amount of attention are adrenergic (epinephrine and norepinephrine) and glucocorticoid systems (cortisol in humans and corticosterone in rodents). Post-training treatments with drugs that affect catecholamines and glucocorticoids influence memory (for review, Roozendaal & McGaugh, 2011).

Additionally, in vitro work demonstrates modulation of LTP by catecholamines and glucocorticoids in the hippocampus and amygdala. Together these findings suggest that endogenous processes that activate adrenergic and glucocorticoid systems facilitate memory consolidation processes.

Glucocorticoid-Adrenergic Interactions. Epinephrine and glucocorticoids are released during stressful experiences and there is extensive evidence that these hormones influence memory consolidation (Oitzl, Reichardt, Joëls, & de Kloet, 2001; McGaugh & Roozendaal, 2002; de Kloet, Oitzl, & Joëls, 1999). Specifically, catecholamine and glucocorticoid interactions influence neural plasticity and memory consolidation (Joëls, Fernandez, & Roozendaal, 2011; Pu, Krugers, & Joëls, 2009; Roozendaal, Okuda, de Quervain, & McGaugh, 2006). The corticosterone synthesis inhibitor metyrapone reduces the elevation of circulating corticosterone induced by aversive stimuli and reduces memory augmentation of norepinephrine in an inhibitory avoidance fear conditioning paradigm (Roozendaal, Carmi, & McGaugh, 1996). Furthermore, hippocampal LTP is impaired after foot- and tail-shock, restraint stress and other forms of stress (Artola et al., 2006; Foy, Stanton, Levine, & Thompson, 1987; Shors & Dryver, 1994; Shors, Gallegos, & Breindl, 1997; Xiong et al., 2004) and is correlated with circulating corticosterone levels (Diamond, Bennett, Fleshner, & Rose, 1992). Glucocorticoid and adrenergic receptors in the amygdala have also been shown to modulate hippocampal plasticity (Vouimba, Yaniv, & Richter-Levin, 2007). Therefore, within the neural structures involved in fear conditioning, the interactions among neurohormones influence the consolidation of memory.

The level of arousal interacts with experimental behavioral manipulations, resulting in measurable differences in memory. For example, rats placed in a novel apparatus exhibit arousal, as indicated by elevated circulating plasma levels of norepinephrine and corticosterone, and repeated exposures to the same apparatus reduce the expression of these neurohormones (De Boer, Koopmans, Slangen, & Van der Gugten, 1990). Nonhabituated rats given corticosterone immediately post-training on a novel object recognition task enhanced 24 hour memory of the objects, in contrast, there was no memory enhancement when corticosterone was administered to habituated rats (Okuda, Roozendaal, & McGaugh, 2004). These findings suggest that arousal induced by novelty enables exogenous glucocorticoids to enhance memory consolidation. Furthermore, a β-adrenoreceptor antagonist or α_2-adrenoreceptor antagonist coadministered with corticosterone after novel object recognition, either blocked the corticosterone-induced enhancement of memory, or induced a dose-dependent memory augmentation (Roozendaal, Okuda, de Quervain, & McGaugh, 2006). These findings support the hypothesis that enhanced memory consolidation is a consequence of an interaction between adrenergic and glucocorticoid activity. Therefore, there is an arousal component that interacts with glucocorticoid hormone treatment, resulting in effects on memory.

Glucocorticoid and other endocrine system dysfunctions are reported in patients with PTSD (Krystal & Neumeister, 2009; Pervanidou & Chrousos, 2010; Vidović et al., 2011). Baseline levels of the glucocorticoid, cortisol, are reported to be abnormally low in people with PTSD (for reviews, Yehuda, 2009; Yehuda et al., 2005). People with PTSD have increases in the number and sensitivity of glucocorticoid receptors (Rohleder,

Joksimovic, Wolf, & Kirschbaum, 2004; Stein, Koverola, Hanna, Torchia, & McClarty, 1997; Yehuda, Boisoneau, Mason, & Giller, 1993; Yehuda, Giller, Southwick, Lowy, & Mason, 1991; Yehuda, Boisoneau, Lowy, & Giller, 1995). Increased suppression of cortisol release and adrenocorticotropic hormone (ACTH) following dexamethasone administration has also been reported in clinical populations suffering from anxiety disorders, such as PTSD (Duval et al., 2004; Goenjian, Yehuda, Pynoos, Steinberg, & et al, 1996; Grossman et al., 2003; McFarlane, Barton, Yehuda, & Wittert, 2011; Newport, 2004; Yehuda, 2002; Yehuda, Golier, Halligan, Meaney, & Bierer, 2004). In conjunction with investigations using the dexamethasone-corticotropin releasing hormone (CRH) challenge (Rinne et al., 2002; Ströhle, Scheel, Modell, & Holsboer, 2008; de Kloet et al., 2006) that report PTSD patients display reduced ACTH levels, these lines of research strongly suggest that trauma enhances the negative-feedback of the hypothalamic-pituitary-adrenal axis in people that develop PTSD. However, not all investigations support this hypothesis, likely reflecting the heterogeneity of trauma and measurements used to investigate PTSD populations (Begić & Jokić-begić, 2007; Bonne, 2003; Klaassens, Giltay, Cuijpers, van Veen, & Zitman, 2012; Marshall & Garakani, 2002; Metzger et al., 2008; Pitman & Orr, 1990; Radant, Tsuang, Peskind, Mcfall, & Raskind, 2001; Shalev et al., 2008). Therefore, the development and use of animal models provides means to understand the mechanisms modulating memory for traumatic experiences.

1.3 Development of Predator Based Fear Conditioning

Pavlovian fear conditioning research has provided valuable insight into the neurobiology of memory. A related line of research is concerned with innate behavioral reactions of rodents to predators. Decades ago, investigators described evidence of innate fear reactions by rats to cats and predator related stimuli (Curti, 1935, 1942; Griffith, 1920). These observers reported that rats spent the majority of time freezing in the presence of a cat. Decades later, the Blanchard's (1972) extended this work with some of the first neurobiological investigations into fear conditioning and predator exposure. They showed that lesions of the amygdala dramatically changed the behavior of rats around cats. The difference in freezing behavior was significant, with lesions of the amygdala producing an almost complete lack of freezing during cat exposure or to an electric shock. Thus, the amygdala has been shown to be critical in Pavlovian fear conditioning and innate fear to predators.

The Blanchards and colleagues continued to characterize the relationships among predator-based stimuli and the brain and behavior of rodents. Blanchard, Yang, Li, Gervacio, & Blanchard (2001) described conditioned defensive behaviors to cat odor paired with a context, and the subsequent extinction after unreinforced re-exposures. Furthermore, using Fos immunoreactivity as an indication of neural activation, these researchers have shown activation of the locus coeruleus (one of the major adrenergic outputs of the brain) during exposure to a predator and subsequent re-exposure to a paired context (Ribeiro-Barbosa, Canteras, Cezário, Blanchard, & Blanchard, 2005). Based on data from Fos activity, electrolitic and excitotoxic lesions, the dorsal premammillary nucleus of the thalamus appears to be specifically involved in the control of antipredator

defense behaviors (Blanchard, Canteras, Markham, Pentkowski, & Blanchard, 2005). Recently, colleagues of the Blanchards (Corley, Caruso, & Takahashi, 2011) demonstrated resistance to extinction of freezing and other defensive behaviors in fear conditioned rats, when exposed to cat odor during training.

Other laboratories have investigated predator based reactions in rodents. Ademac and colleagues have demonstrated long lasting anxiogenic effects of predator exposure on rodents in hole-board (Adamec & Shallow, 1993; Adamec, Blundell, & Collins, 2001; Adamec, Kent, Anisman, Shallow, & Merali, 1998), inhibitory avoidance (Adamec, 2001) and elevated plus maze tasks. Adamec and colleagues have also characterized some of the neural mechanisms that are associated with increases in these anxiety-like behaviors (Adamec & Shallow, 1993; Adamec, Blundell, & Collins, 2001; Adamec, Strasser, Blundell, Burton, & McKay, 2006; Adamec, Blundell, & Burton, 2005, 2006; Rosen, Adamec, & Thompson, 2005).

Cohen and colleagues have investigated the effects of predator odor exposure on behavior and physiology of rodents. For example, the anxiogenic agent cholecystokinin was found to additively enhance the cat-induced anxiety on the elevated plus maze (Cohen, Friedberg, Michael, Kotler, & Zeev, 1996). Cohen & Zohar (2004) showed that rats that had significantly more anxiety-like behavior on the elevated plus maze and acoustic startle responses induced by predator odor, also showed higher plasma corticosterone and adrenocorticotropin hormone concentrations than rats that showed less reactivity to the odor. Cohen, Matar, Richter-Levin, & Zohar (2006) investigated the role early-life predator odor exposure had on anxiety-like behaviors later in life using specific rat strains bred to have different hypothalmic pituitary adrenal axis activity.

Diamond and colleagues have demonstrated the effects of predator exposure on neural plasticity and memory in rats. Predator exposure impaired spatial memory on difficult, but not easy, hippocampal dependent mazes (Diamond, Park, Heman, & Rose, 1999). The acute stress-induced impairment of spatial memory found in predator exposed rats was associated with decreased expression of neural cell adhesion molecule in the hippocampus and prefrontal cortex (Sandi et al., 2005). Vouimba, Muñoz, & Diamond (2006) found that acute predator exposure blocked potentiation in the hippocampus and enhanced LTP in the amygdala. Additionally, the predator stress-induced spatial memory impairment is associated with a differential expression of molecular markers hypothesized to regulate neural plasticity (Vanelzakker et al., 2011; Zoladz et al., 2011). The Diamond lab has also developed an animal model of PTSD using predator exposures and social instability that results in heightened anxiety-like behavior, exaggerated startle, increased cardiovascular reactivity and augmented response to yohimbine administration (Zoladz, Conrad, Fleshner, & Diamond, 2008), all of which are reported in people with PTSD (Brewin, Andrews, Valentine, & Link, 2000; Elzinga & Bremner, 2002; Nemeroff et al., 2006; Newport & Nemeroff, 2000; Stam, 2007). Recently, this model has demonstrated hippocampus-specific augmentation of DNA methylation (Roth, Zoladz, Sweatt, & Diamond, 2011); this change in DNA might be a basis for understanding the robust, extinction resistant traumatic memories in PTSD patients (Yehuda & Bierer, 2009). The use of predator-based fear as an unconditioned stimulus in behavioral research is attractive due to the innate qualities of predator generated fear on behavior, memory, and physiology.

In summary, predator and predator-related stimuli increase anxiety-like behaviors and memory in rodents. Research indicates that these predator effects on behavior and memory involve an interaction between the hippocampus and amygdala. Furthermore, associations formed between aversive stimuli and environmental contexts and cues appear to involve the results of interactions of glucocorticoid and adrenergic receptors. One hypothesis generated from the previously discussed lines of research is that predators and their related stimuli activate an endogenous interaction of neuromodulatory hormones that affect the amygdala and hippocampus. The resultant effects of predator-stimuli on memory and behavior are presumed to be evolutionarily advantageous and, therefore, are relevant to comparative research dealing with emotional enhancement of memory in humans.

1.4 The Experimental Rationale for Predator Based Fear Conditioning

Great strides have been made toward understanding the nature and neurobiology of associative learning using fear conditioning. The discipline of fear conditioning has developed from Darwin's observations of similar behaviors across species and Pavlov's systematic approach to understanding behavior. Fear conditioning research has provided the basic understanding of the neural mechanisms involved in memory of aversive associations, with much of the research focused on the hippocampus and the amygdala. The rationale for the following series of experiments is based on fear conditioning and the work done investigating predator effects on behavior, physiology and neural plasticity. This series of experiments set out to behaviorally characterize the effects of predator-based fear on associative memory in rats. This series of experiments is partially based on the hypothesis that as a situation becomes increasingly adverse and more

stressful, hippocampal and amygdala mediated memory can be augmented. What sets this work apart from previous work is it aimed to integrate predator-based fear into the rules of established fear conditioning methodologies. This new line of research will extend our understanding of predator-fear, as tested by utilizing standard conditioning methodologies.

Chapter 2: Experimental Testing Predator Based Fear Conditioning

2.1 Experiment 1

2.1.1 Comparison of different unconditioned stimuli on contextual and cued fear conditioning and rate of extinction

Experiment 1 assessed the effects of different aversive stimuli on context and cue fear conditioning and extinction. This experiment addressed the hypothesis that shock, immobilization, and predator-exposure, alone or in combination, results in a synergistic effect on fear memory. Standard foot shock conditioning was used as a control group and provided a means to compare the effects of other aversive stimuli to established conditioning paradigms. Immobilization was utilized because restraint stress enhances fear conditioning and resistance to extinction (Conrad, LeDoux, Magariños, & McEwen, 1999; Miracle, Brace, Huyck, Singler, & Wellman, 2006; Sandi, Merino, Cordero, Touyarot, & Venero, 2001). Immobilization produces alterations in neurotrophic factors such as glutathione (Ghizoni et al., 2006), brain-derived neurotrophic factor (Marmigère, Givalois, Rage, Arancibia, & Tapia-Arancibia, 2003; Murakami, Imbe, Morikawa, Kubo, & Senba, 2005; Rage, Givalois, Marmigère, Tapia-Arancibia, & Arancibia, 2002), c-fos (Trnecková, Armario, Hynie, Sída, & Klenerová, 2006), and CRH (Givalois, Arancibia, & Tapia-Arancibia, 2000). Furthermore, immobilization stress influences HPA axis and adrenergic systems modulated by glucocorticoid receptors in the brainstem, hypothalamus and locus coeruleus (Makino, Smith, & Gold, 2002). Thus,

immobilization, alone, was assessed as an aversive stimulus by pairing it to contexts or cues. Predator exposure was used as a novel US, alone and in conjunction with foot shock and immobilization.

Recent work using predator odor as the US paired with the learning context and other cues has demonstrated fear conditioning in rodents (for review see Takahashi, Chan, & Pilar, 2008). For example, Corley et al. (2011) demonstrated augmented foot shock to an auditory cue in a trace conditioning paradigm. The design of their experiments involved shocking the rats in one context (acute stress) then placing them in a standard housing cage and exposing them to an auditory cue (clicking noises). While in the standard housing cage, a predator-odor laden cloth was placed on top of the cage for 30 seconds midway through the auditory conditioning, this procedure was repeated for a total of 5 training trials. To test extinction of conditioned fear, the rats were placed in a "runway with hide box" and re-exposed to the auditory cues for 5 consecutive days. The results of their experiments demonstrated that stress-induced fear conditioning exhibited persistent freezing to the cue over the 5 days of testing in the runway-hide box. However, these investigators did not test contextual fear conditioning in their paradigm and the auditory conditioning and testing conducted is not directly comparable to standard foot shock paradigms. Therefore, experiment 1 was designed to fear condition rats using immobilization and predator exposure without using a shock and to investigate differences in the magnitude of the fear expressed to conditioning with foot shock. In addition, this experiment explored the durability of the association of the context and cues to the various aversive stimuli.

2.1.2 Method

Animals. A total of 48 male Sprague-Dawley rats (Charles River) weighing 225-250g on arrival were acclimated to the vivarium and cage cleanings for at least 7 days before any experimental manipulations were conducted. Rats were housed 2 per cage (standard Plexiglas – 46 x 25 x 21 cm). Tap water and rat chow were available *ad libitum*. The animal housing room was maintained at 20 ± 1° C with a humidity range of 60 ± 3%, and a 12hr light cycle (on at 0700 hr). All procedures were approved by the Institutional Animal Care and Use Committee at the University of South Florida.

Conditioning Apparatus and General Procedure. All animals received the same general treatment outlined as follows. Rats were transported in their homecages to the laboratory approximately 30 minutes before conditioning. The rats were removed from their homecage and placed inside 1 of 2 identical standard fear conditioning boxes (25.5 x 30 x 29 cm; Coulbourn Instruments; Allentown, PA) which were inside separate larger sound attenuation chambers. The conditioning boxes consisted of aluminum sides, an aluminum ceiling, and Plexiglas front and back covered with black plastic. The floors consisted of 18 stainless steel rods, spaced 1.25 cm apart. These boxes were also the context test apparatus, the cue test apparatus is outlined in the fear association section. After the rats were in the box for 2 minutes they were presented a 10-second 74 dB 2500 Hz tone, followed by a 40-second interstimulus interval, followed by another tone. The tones served as the auditory cues and were each paired with a 2-second 0.4 mA shock that terminated with the tone. Shocks were administered to only 2 of the 6 total groups. Total exposure of the rats to the box lasted for 3 minutes. After 3 minutes, all of the rats were removed from the conditioning box and received one of the following treatments.

The rats were randomly assigned to each treatment and as described in the following sections.

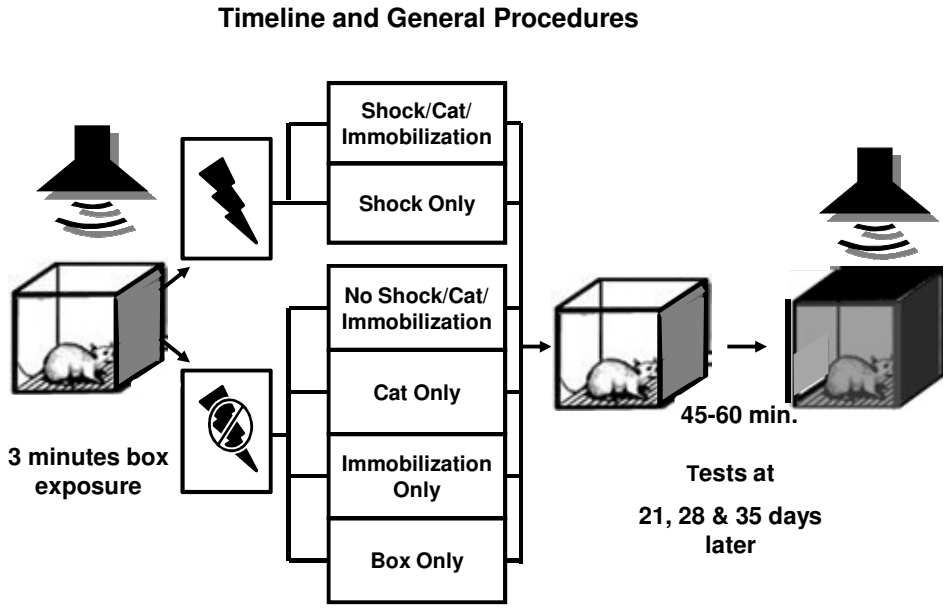

Figure 1: All of the groups were given 3 minutes of exposure to the conditioning chamber with 2 tones presented to the rats. Two of the groups received shocks paired with the tones (as illustrated by the lightning bolt symbol). The other 4 groups were not shocked (indicated by the "no" symbol through the lightning bolt). All of the groups received memory tests consisting of unreinforced re-exposure to the conditioning context and, in a different apparatus, the tone 21, 28 & 35 days after conditioning.

Shock/Cat/Immobilization. This group received shocks as described in the previous section. After the termination of the second shock/tone pairing the rats were immediately immobilized using a plastic DecapiCone (Braintree Scientific; Braintree, MA). Within 2 minutes of being immobilized, the rats were placed in a triangle-shaped wedge (20 x 20 x 10 cm) of a pie-shaped Plexiglas enclosure (Braintree Scientific; 46 cm diameter x 8 cm in height) in the cat housing room for 30 minutes.

Shock Only. This group received shocks as described in the conditioning apparatus and procedure section. After the termination of the second shock-tone pairing the rats were immediately returned to their homecage.

No Shock/Cat/Immobilization. This group received no shocks. After the termination of the second tone the rats were immediately immobilized using a plastic DecapiCone. Within 2 minutes of being immobilized, the rats were placed in a wedge of a pie-shaped Plexiglas enclosure (as described in the Shock/Cat/Immobilization group section) in the cat housing room for 30 minutes.

Immobilization Only. This group received no shocks. After the termination of the second tone the rats were immediately immobilized using a plastic DecapiCone. Within 2 minutes of being immobilized the rats were placed in a wedge of the pie-shaped Plexiglas enclosure located in another room for 30 minutes, not in the cat housing room.

Cat Only. This group received no shocks. After the termination of the second tone the rats in this group were immediately placed in a small unrestrictive novel Plexiglas box (28 x 9 x 14 cm). Within 2 minutes of being put in the box, the rats were placed in the cat housing room for 30 minutes.

Box Only. This group received no shocks. After the termination of the second tone the rats in this group were immediately returned to their homecage. This group received no immobilization or cat exposure.

Fear Memory and Extinction Testing. Unreinforced context and cue fear memory tests occurred on days 21, 28 and 35 after conditioning. On testing days the rats were returned to the laboratory and tested 30 minutes after arriving in the laboratory. Rats were placed in the same fear conditioning box as the one in which they were placed

during conditioning. The immobility of each rat was monitored by computer for five minutes. Immobility data were analyzed using the time window after the first 30-seconds until the beginning of the last minute of the 5 minute chamber exposure. Thus, a total of 3.5 minutes were analyzed for immobility in the context. This served as a measure for contextual fear memory. Approximately 45-60 minutes after the contextual memory test, rats were individually placed in a novel illuminated conditioning box (25 x 22.5 x 33 cm, Coulbourn Instruments; Allentown, PA) that consisted of two aluminum sides, an aluminum ceiling, and a Plexiglas front and back and a square metal floor (21.5 x 21.5 cm). The use of this second box reduced the similarities between the original conditioning chamber and the auditory cue testing box. The tone (74 dB; 2500 Hz) used during training was presented for the last 3 minutes of the 6 minute test. Immobility was measured by a 24-cell infrared activity monitor (Coulbourn Instruments; Allentown, PA), mounted on the top of the boxes. Freezing was defined as continuous periods of immobility lasting at least 3 seconds. A Microsoft Excel macro designed to analyze the percent time freezing calculated the total number of seconds spent freezing by each animal in 30-second epochs.

Statistical Analyses. Repeated measures AVOVA were used to detect significant differences between groups in freezing to the context and cue tests. Post hoc LSDs tested group and weekly test differences. Alpha was set at 0.05. Data points were considered outliers if they were more than 3 standard deviations from the exclusive mean. The analyses for all groups of the context and cue tests had 7-8 rats per group.

2.1.3 Results

Context Memory and Extinction. Repeated measures ANOVA analyses of the context tests indicated a significant within-subject effect of Group on freezing with $F(2,84) = 5.24$, $p < 0.01$. The within-subject Group x Test interaction indicated a trend, but was not statistically significant ($F(10,84) = 1.82$, $p = 0.07$). Between-subjects analyses showed a significant effect of Group ($F(5,42) = 13.18$, $p < 0.01$). Post hoc tests of the first contextual memory test revealed the Shock/Cat/Immobilization group froze significantly more than all other groups. The Shock Only group and No Shock/Cat/Immobilization group froze significantly more than the Cat, Immobilization and Box Only groups. Post hoc LSD test showed during the second context tests the Shock/Cat/Immobilization and No Shock/Cat/Immobilization groups froze significantly more than the Shock, Cat, Immobilization and Box Only groups. The third contextual fear memory test showed that the Shock/Cat/Immobilization and No Shock/Cat/Immobilization groups froze significantly more than the Shock, Cat, Immobilization and Box Only groups.

Cue Memory and Extinction. Statistical analysis of the freezing during the tone indicated a significant within-subjects Test x Group interaction ($F(2,81) = 2.36$, $p < 0.05$). Between-subjects analysis also revealed significant differences in freezing ($F(5,39) = 12.84$, $p < 0.01$). Post hoc tests showed that on the first cue test the Shock/Cat/Immobilization and Shock Only groups froze during the tone significantly more than the No Shock/Cat/Immobilization, Cat, Immobilization and Box Only groups. During the second cue test the Shock/Cat/Immobilization group froze during the tone significantly more than the Shock Only, No Shock/Cat/Immobilization, Cat,

Immobilization and Box Only groups. The third cue test analysis showed that the Shock/Cat/Immobilization group again froze during the tone significantly more than any other group. The Box Only group spent significantly less time freezing during the tone than the No Shock/Cat/Immobilization group, which was statistically equivalent to the Cat Only and Immobilization Only groups.

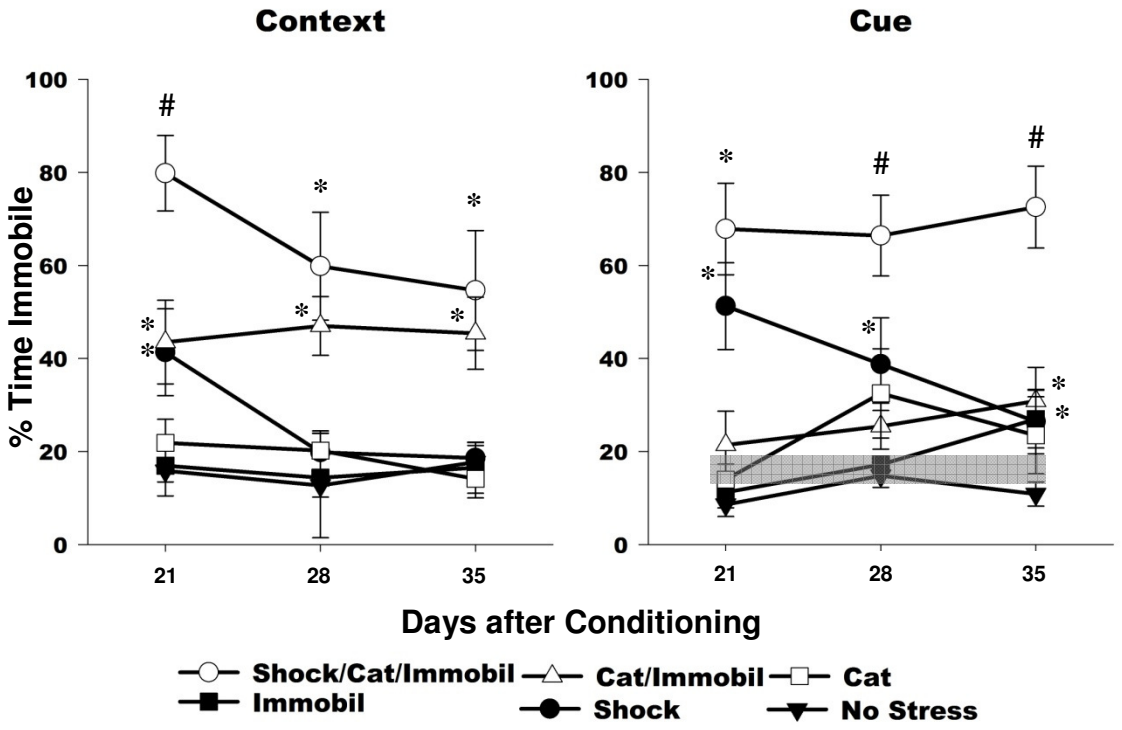

Figure 2. The left graph shows that the combination of shock, cat and immobilization resulted in freezing more in the context than any other group 21 days after conditioning. The cat and immobilization group also exhibited freezing to the context equivalent to the shock alone group. Both the predator exposure/immobilization and shock alone groups spent significantly more time freezing in the context compared to cat, immobilization and box only groups 21 days after conditioning. The shock alone group extinguished their freezing to the context, as indicated by reduced freezing on tests at 28 and 35 days. Both the shock and no shock cat exposure and immobilization groups maintained statistically equivalent freezing percentages on tests at 28 and 35 days relative to their initial memory test.
The right graph shows that the groups that received shocks spent more time freezing to the tone than groups that were not shocked, but only the group that was shocked, cat exposed and immobilized

expressed extinction resistant freezing to the tone. The grey box illustrates the baseline-mean freezing, plus and minus the SEM, for all groups combined in the cue test box prior to the delivery of the tone across test sessions.
# indicates $p < 0.05$ vs. all groups, * indicates $p < 0.05$ vs. No Stress group.

2.1.4 Discussion

This experiment studied how three aversive stimuli, used alone or in various combinations, affected fear conditioning memory and extinction. The standard method of electric foot shock paired with a context and auditory cue resulted in conditioned fear in rodents, expressed as freezing, three weeks after conditioning. The effects produced by foot shock alone extinguished after one unreinforced trial. The effect of foot shock was augmented by immobilization and exposure to a cat in two ways. First, the combination of the three aversive stimuli produced the greatest amount of freezing to both the context and cue. Second, the effect on freezing was extinction resistant when tested four and five weeks after conditioning. This experiment demonstrated the memory enhancing effect of predator exposure and immobilization on a standard foot shock fear conditioning paradigm.

There are three novel findings to come out of this experiment. The first finding is immobilization and predator exposure augmented standard foot shock context and cue fear conditioning. The second novel finding is that the enhanced contextual and auditory cues associated with foot shock predator-based fear conditioning were resistant to extinction. The third finding is that, to our knowledge, this is the only experiment to demonstrate fear conditioning to a context paired with immobilization and predator exposure using a single Pavlovian trace conditioning session. It is important to point out that this experiment utilized a combination of delay and trace conditioning.

Hypothetically, foot shock alone, the delay US, was associated with the context and auditory cue because of the co-occurrences of the stimuli. The immobilization and predator-exposure, as utilized in this experiment, were trace conditioning stimuli. The use of each of immobilization or predator exposure, alone, behaved in a similar pattern to the ultimate no stress control group. Using almost immediate immobilization and predator exposure within 2 minutes initially resulted in contextual fear comparable to the no stress condition. Therefore, the context and cues conditioned, in this experiment are comparable to other standard paradigms. What sets this work apart from standard foot shock and predator-related conditioning paradigms is that, a considerable trace interval (approximately 2 minutes) between context exposure and the predator exposure, robust and extinction resistant fear was produced by using predator-exposure in conjunction with immobilization. Together, the results of this experiment support the hypothesis that immobilization combined with predator exposure enhance fear conditioning and are a sufficient unconditioned stimulus.

The effects of immobilization and predator exposure together, without shock, resulted in extinction resistant freezing in the context memory tests. Predator exposure and predator-related cues (e.g. odor) are an effective US in this and other research. The use of a live cat or the use of cat odor, produces strong reactions in rats and is hypothesized to be based on the salience of the stimulus, namely the presence of a live predator (Blanchard et al., 2005; Blanchard & Blanchard, 1988). Experiment 1 demonstrated that cat exposure or immobilization, used alone is ineffectual at producing long-term fear memories. The relatively long trace period between the time the rat was removed from the conditioning box and placed in the presence of the cat is a possible

explanation for the lack of conditioned freezing at testing. Long trace intervals (more than 30 seconds) reduce the associative qualities in Pavlovian conditioning (Marlin, 1982; Mcechron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998). Immobilization occurred immediately after the cessation of box exposure and the trace interval was only a few seconds. Thus, immobilization or cat exposure as an aversive stimulus might be more suited to delay conditioning paradigms.

The combination of immobilization and predator exposure was sufficient to form long-lasting aversive contextual associations in rats. One explanation for these findings is that the resulting expression of fear is dependent on the intensity of the aversive stimulus and is analogous to the intensity of shock in conventional fear conditioning (Weiss, Krieckhaus, & Conte, 1968). The predator or immobilization manipulations, alone, were not intense enough to reach the threshold necessary to form the fear association, just as very low shock intensities do not produce fear conditioning (Yerkes & Dodson, 1908). The combination of immobilization and predator exposure was intense enough, to reach a conditioning threshold, with the trace methodology used. The intensity of the stimulus interpretation also accounts for the augmented memory in the group that received all three aversive stimuli.

Another interpretation of these findings is, instead of a qualitative difference of intensity, the quantity of aversive stimuli was responsible for the effects on memory. Analogous to standard shock fear conditioning paradigms showing that the intensity of shocks influence memory (Cordero, Merino, & Sandi, 1998), the increased number of aversive stimuli could account for the robust memory. Previous work has also demonstrated that the use of a single (2 hour) restraint stress session 2 days prior to fear

conditioning enhanced contextual freezing, but not freezing to an auditory cue (Cordero, Venero, Kruyt, & Sandi, 2003). Thus, it is possible that immobilization, alone, would enhance contextual foot shock fear conditioning. However, this experiment did not address this possibility. Regardless of whether the quality or quantity of aversive stimuli used drove the effects on memory, the combination of all of the stimuli produced the greatest levels of fear memory.

Similar to results from other labs using predators, or predator-related related stimuli, that suggest the resulting fear associations are amygdala mediated (Blanchard et al., 2005; Corley et al., 2011; Martinez, Carvalho-Netto, Ribeiro-Barbosa, Baldo, & Canteras, 2011), the present experiment hypothetically involves amygdala and hippocampal processing. The amygdala and hippocampus are involved in foot shock, immobilization, and predator-related conditioning. All of these factors were used in the current experiment and the effects on memory hypothetically are mediated by the hippocampus and amygdala.

The findings of experiment 1 are applicable to the study of post traumatic stress disorder (PTSD). A subset of individuals, who experience or witness life-threatening events, go on to develop PTSD. Some of the hallmark symptoms of PTSD are hypervigilance, enhanced startle to cues similar to those experienced around the traumatic event and the avoidance of the place or places similar to where the traumatic event occurred. This experiment demonstrated that rats expressed significantly more fear to the place and cues experienced before being immobilized and exposed to a predator, after being shocked. Similar to humans with PTSD, these animals continued to express extinction resistant fear. Therefore, these findings support the hypothesis that the

combination of shock, immobilization, and predator exposure model PTSD-like memory phenomenon. This model can be used to investigate behavioral, drug, and neural interventions that could be used to alleviate extinction resistant memories.

2.2 Experiment 2

2.2.1 Influence of multiple conditioning trials on contextual fear conditioning and rate of extinction training

Experiment 2 investigated the effects of immobilization and predator exposure conditioning trials on of the expression and extinction of contextual fear. Experiment 1 indicated that immobilization and predator exposure, without the use of shock, resulted in extinction resistant contextual fear. Extinction of fear conditioning is indicated by reduced expression of fear, as a result of presenting the CS in the absence of the aversive US (Myers, Ressler, & Davis, 2006). The study of the fear system, because of the known neural mechanisms, has provided an effective approach toward understanding extinction (Quirk & Mueller, 2008). Extinction is the basis for many effective therapies for the treatment of anxiety disorders (Delgado, Nearing, Ledoux, & Phelps, 2008).

Extinction is a form of new learning that results in reduced behavioral expression of fear conditioning. This hypothesis is supported by the renewal effect, which occurs when a previously extinguished conditioned response to a conditioned stimulus returns in a different context (Bouton & King, 1983). Additionally, an emphasis is placed on the role of the context in gating the expression of extinction (Bouton, 1993). Subsequently, Bouton and colleagues, among others, have gone on to investigate the role that an NMDA receptor partial agonist, D-cycloserine (DCS), has on context specific extinction (Bouton, Vurbic, & Woods, 2008; Ledgerwood, Richardson, & Cranney, 2003, 2004; Woods &

Bouton, 2006). The premise of a drug that facilitates extinction is appealing for the treatment of anxiety disorders. An NMDA receptor agonist hypothetically enhances the new extinction learning. However, DCS appears to only facilitate extinction to a CS in the context that unreinforced exposures occurred (Bouton, Vurbic, & Woods, 2008; Woods & Bouton, 2006). Expression of fear is reinstated when the previously extinguished CS is experienced in the original conditioning context. Bouton and colleagues (2006 & 2008) have shown that the administration of DCS only facilitates the extinction to a CS in the context that the drug and extinction training are performed. In their experiments extinction training to an auditory cue, with DCS administration, in a context other than the original conditioning context, does not eliminate renewal of fear to the auditory cue in the original conditioning context when tested after extinction training.

Although drug therapies are not always effective, behavioral techniques used in conjunction with medication can be optimized for extinction (Bouton, Westbrook, Corcoran, & Maren, 2006). Research indicates that a greater delay between extinction trials and tests of memory reduces extinction of fear (Quirk, 2002). Based on the established work on extinction and the results of experiment 1, the effects of rate of extinction training on predator-based expression of contextual fear were investigated. The predator-based fear conditioning developed in experiment 1 was modified, giving animals multiple trials over days to the context alone. Part of the aim of this experiment was to optimize extinction of contextual fear associations. Extinction of predator-based context fear conditioning was conducted using unreinforced memory tests separated by two or seven days. This experiment tested the hypothesis that extinction training given

more often would reduce fear expression compared to the same number of extinction trials given less often.

2.2.2 Method

Animals. A total of 32 male Sprague-Dawley rats (Charles River) weighing 225-250g on arrival were acclimated to the vivarium and cage cleanings for at least 7 days before any experimental manipulations were conducted. Rats were housed 2 per cage (standard Plexiglas – 46 x 25 x 21 cm). Tap water and rat chow were available *ad libitum*. The animal housing room was maintained at 20 ± 1° C with a humidity range of 60 ± 3%, and a 12hr light cycle (on at 0700 hr). All procedures were approved by the Institutional Animal Care and Use Committee at the University of South Florida.

Procedure & Fear Conditioning. The same conditioning apparatus described in Experiment 1 was used. Rats were randomly assigned to receive immobilization and predator exposure (Cat) or homecage (No Cat). Conditioning sessions took place on Days 1, 3, and 5. Sessions consisted of individual cages being transported to the lab one at a time. Rats were immediately placed in the conditioning chambers and given 3 minutes exposure time. No tones or lights were presented during the conditioning. After 3 minutes in the conditioning chamber, Cat group rats were immediately immobilized and placed in the presence of an adult female cat as described in the previous experiment. A cat exposure lasted 10 minutes. Rats in the No Cat groups were returned back in their homecages and taken to another room for 10 minutes. After completion of the final conditioning session freezing data were used to construct statistically equivalent groups of Cat and No Cat animals, which received extinction tests separated by 48 hours (2-Day)

or 7 days (Weekly) after training. Testing consisted of placing the rats in the same box, for 5 minutes, experienced on the training day.

Statistical Analyses. Repeated measure ANOVA was used on the context freezing tests with post-hoc LSD tests. All groups consisted of 7-8 animals. Alpha was set at 0.05. Data points were considered outliers if they were more than 3 standard deviations from the exclusive mean.

2.2.3 Results

Between-subject tests revealed significant effects of Cat ($F(1,21) = 33.20$, $p < 0.01$), Extinction ($F(1,21) = 5.63$, $p < 0.03$), and a significant Cat x Extinction interaction ($F(1,21) = 7.88$, $p < 0.02$). Within-subjects tests of freezing in the context were significant across exposures ($F(3,63) = 6.59$, $p < 0.01$) and there was a significant Exposure x Cat x Extinction interaction ($F(3,63) = 7.68$, $p < 0.01$). The Exposure x Cat and Exposure x Extinction interactions both were not significant ($F(3,63) = 2.25$, $p = 0.09$ and $F(3,63) = 2.03$, $p = 0.12$). Post-hoc analysis revealed the No Cat groups were statistically similar to each other across each exposure regardless of extinction training. The Cat groups had been treated identically at the time of the initial test session and were statistically equivalent to each other on day 12. The second context exposure test revealed a statistically significant difference in freezing among the Weekly exposed Cat group and all other groups. The 2-Day exposed Cat group spent significantly more time freezing in the context than the 2-Day No Cat group on the second exposure test. During the third and fourth testing sessions the 2-Day Cat group was statistically equivalent to the No Cat 2-Day group. The Weekly exposed Cat group froze significantly more than all other groups during each of the last two testing sessions. (See Figure 3)

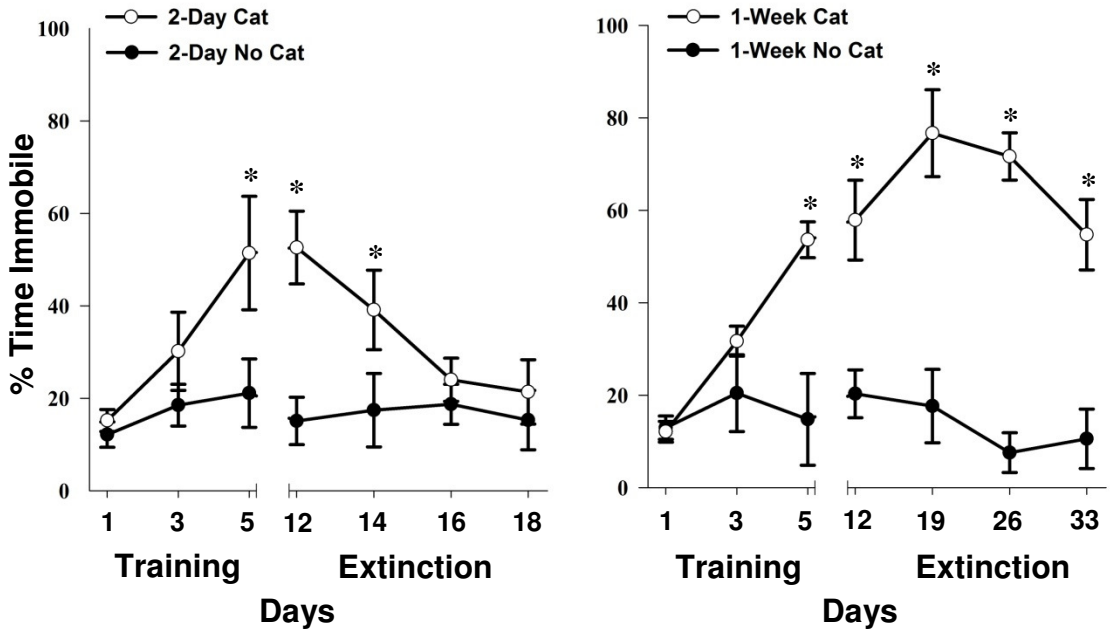

Figure 3. Each of the cat and no cat groups were treated the same until after the first context test on day 12. The left graph shows that the cat group re-exposed to the context every 2 days extinguished freezing to the context. The right graph shows that the cat group exposed to the context every 7 days displayed extinction resistant freezing behavior.
* indicates $p < 0.05$ Cat group vs. No Cat group.

2.2.4 Discussion

This experiment investigated the effects of multiple training sessions on fear memory and what effect the rate of extinction training had on memory. This study showed that multiple fear conditioning trials pairing the context to the US increased freezing by the third session. This also work replicated the finding of the first experiment that immobilization and predator exposure generates extinction resistant memory when tested at weekly intervals. This experiment extended our understanding of how extinction of predator-based conditioning is impacted by the rate of unreinforced exposures to the conditioning context. That is, when the rats were re-exposed every two days, instead of

every seven days, fear to the context was reduced. This experiment is consistent with previous findings using foot shock conditioning (Quirk, 2002). Despite all of the animals in the aversive condition expressing the same level of fear at the first test, fear was extinguished by unreinforced exposures in shorter time intervals. This experiment supports the hypothesis that more frequent re-exposure to a context associated with intense aversive psychological stimuli increases the rate of extinction.

Interest in the extinction of fear conditioning is based on the recognition that neural systems involved in the suppression of fear also are involved in anxiety disorders (Quirk & Gehlert, 2003). In particular, deficits in extinction might play a role in PTSD (Bremner & Vermetten, 2004; Milad, Wright, Orr, Pitman, Quirk, & Rauch, 2007; Orr et al., 2006; Rauch, Shin, & Phelps, 2006; Rothbaum & Davis, 2003). The hypothesis that extinction of fear is not a result of an erasure of the original memory is supported by evidence that fear responses can last months and even years, in the absence of additional fear conditioning (Gale et al., 2004). The general consensus is that extinction involves new learning that results in the inhibition of fear (Bouton, Vurbic, et al., 2008). As discussed previously, the hippocampus plays an important role in the contextual aspect of fear conditioning and is, therefore, likely involved in fear extinction.

Evidence for the hypothesis that the hippocampus is involved in extinction of fear conditioning comes from both and rodent human research. Corcoran & Maren (2001) inactivated the dorsal hippocampus during extinction training to an auditory CS in the same and different contexts and found a selective impairment to context-specific extinction training. Dorsal hippocampal impairment causes extinguished responses to perseverate outside of the extinction training context (Corcoran & Maren 2001, 2002; Ji

& Maren, 2005). Permanent lesions of hippocampus, as well as the fimbria/fornix, eliminate the reinstatement of conditioned responding after extinction as well (Frohardt, Guarraci, & Bouton, 2000; Wilson, Brooks, & Bouton, 1995).

Milad et al. (2006) demonstrated that psychiatrically healthy adults, who underwent fear conditioning, exhibited significant activation of the hippocampus and significant activation of the ventral-medial area of the prefrontal cortex (vmPFC) when undergoing extinction training. The finding that the vmPFC is involved in the extinction of fear conditioning is replicated in rodent research (Barrett, Shumake, Jones, & Gonzalez-Lima, 2003; Herry & Garcia, 2002; Milad & Quirk, 2002). Morgan, Schulkin, & LeDoux, (2003) suggested that lesions of the vmPFC of rats prohibited the processing of contextual cues that influence extinction acquisition. Additionally, the amygdala receives a large amount of fibers from the vmPFC in rodent (McDonald, Mascagni, & Guo, 1996) and primates (Chiba, Kayahara, & Nakano, 2001; Ghashghaei & Barbas, 2002). Clinical studies of PTSD patients indicate a tonically elevated concentration of norepinephrine in the central nervous system (Pervanidou & Chrousos, 2010; Strawn & Geracioti, 2007). Prazosin, an α1-adrenergic antagonist, has been used to treat PTSD and other anxiety disorders (Boehnlein & Kinzie, 2007; Dierks, Jordan, & Sheehan, 2007; Miller, 2008; Raskind et al., 2007; Taylor, Freeman, & Cates, 2008). This drug, when infused in to the vmPFC of rodents, enhances extinction of conditioned contextual fear (Do-Monte, Allensworth, & Carobrez, 2010). Enhancing norepinephrine signaling in the lateral amygdala with the β-adrenergic receptor agonist isoproterenol after extinction training impairs extinction (Debiec, Bush, & LeDoux, 2011).

The results of this experiment indicate that extinction of predator-based fear conditioning can be enhanced with more frequent re-exposures. Neural mechanisms that have been implicated in extinction include the hippocampus, PFC, and amygdala (Akirav & Maroun, 2007; Akirav, Raizel, & Maroun, 2006; Berlau & McGaugh, 2006; Boccia, Blake, Baratti, & McGaugh, 2009; Bruchey, Shumake, & Gonzalez-Lima, 2007; Delgado et al., 2008; Maren, 1998a, 1999b; Markram, Lopez Fernandez, Abrous, & Sandi, 2007; Phelps, Delgado, Nearing, & LeDoux, 2004; Schimanski & Nguyen, 2005; Sotres-Bayon, Bush, & LeDoux, 2004; Yang, Chao, Ro, Wo, & Lu, 2007; Do-Monte, Allensworth, & Carobrez, 2010; Herry & Garcia, 2002; Herry & Mons, 2004; Milad, Vidal-Gonzalez, & Quirk, 2004; Milad et al., 2005, 2007; Miracle, Brace, Huyck, Singler, & Wellman, 2006a, 2006b; Morgan & LeDoux, 1999; Morgan, Romanski, & LeDoux, 1993; Morgan, 2003; Phelps et al., 2004; Quirk, Russo, Barron, & Lebron, 2000; Quirk, Likhtik, Pelletier, & Paré, 2003; Rhodes & Killcross, 2007; Rodriguez-Romaguera, Sotres-Bayon, Mueller, & Quirk, 2009; Santini, Ge, Ren, Peña de Ortiz, & Quirk, 2004; Sotres-Bayon et al., 2004; Sotres-Bayon, Cain, & LeDoux, 2006). Infusions of NMDA receptor antagonists and kinase inhibitors into the BLA of the amygdala blocked extinction (Falls, Miserendino, & Davis, 1992; Lin, Yeh, Lu, & Gean, 2003; Lu, Walker, & Davis, 2001; Quirk & Mueller, 2008). Muscimol infusions into the BLA reduce fear expression during extinction without affecting retrieval 24 hours later (Akirav et al., 2006). Berlau & McGaugh (2006) enhanced BLA activity using the GABA antagonist bicuculline and found a norepinephrine-dependent enhancement of extinction. Lesions that do not include lateral nuclei (LA) of the basolateral amygdala (BLA) have no effect on extinction learning (Anglada-Figuero & Quirk, 2005; Sortes-Bayon et al., 2004). During extinction

a subset of LA neurons continue to produce conditioned firing-responses, in lieu of reduced behavioral expression of fear (Repa et al., 2001). Based on their review, Quirk and Mueller (2008) hypothesize that extinction depends on the function of the amygdala, hippocampus and the PFC. The amygdala is inhibited by the PFC in the extinction context, this contextual information is supplied by the hippocampus. However, outside of the extinction context the PFC inhibition of the amygdala does not occur, due to the hippocampal modulation of the circuit.

An investigation by Adamec's group (Clay et al., 2011) demonstrated that extinction of associative contextual memory for predator exposure was independent of manipulating glucocorticoid levels during extinction using metyrapone and exogenous corticosterone. In this study, mice were exposed to a cat in an experimental context and demonstrated less mobility when re-exposed to the predator-paired context, as well as increases in anxiety-like and hyperarousal behaviors. After five daily unreinforced exposures to the associative context, mice moved significantly more in the context than on the initial re-exposure. Furthermore, in their series of experiments these authors showed that exogenous manipulation of glucocorticoids with metyrapone administered prior to extinction conditioning had no effect on contextual fear conditioning and giving exogenous corticosterone after extinction training also had no effect on memory. The authors contrasted their findings with shock-induced fear conditioning research that has demonstrated glucocorticoid-dependent extinction (Abrari, Rashidy-Pour, Semnanian, & Fathollahi, 2008; Blundell, Blaiss, Lagace, Eisch, & Powell, 2011; Cai, Blundell, Han, Greene, & Powell, 2006; Yang, Chao, Ro, Wo, & Lu, 2007). Clay et al. (2011) suggested

that, based on the discrepancy of their results from shock-induced conditioning, predator-based fear memory extinction is dependent on different physiological mechanisms.

2.3 Experiment 3

2.3.1 Multiple predator-based conditioning trials in one day result in fear 3 days later.

The previous experiments have demonstrated the effectiveness of immobilization and predator exposure as aversive conditioning stimuli. In experiment 1, only the groups that were shocked conditioned to the cue. In experiment 2, multiple conditioning sessions were used over a period of days allowing consolidation of each pairing of the predator-based stimulus and the context. Experiment 3 aimed to facilitate fear conditioning to both contextual and cues stimuli by increasing the number of pairings. Research demonstrates that shock intensity correlates with corticosterone secretion and the degree that conditioned fear is expressed (Cordero et al., 1998). Multiple CS-US pairings in a single day to the context and an auditory cue aimed to construct a conditioning paradigm to facilitate fear to the auditory cue. Therefore, the number of pairings of the aversive stimuli was increased to three times in one day to strengthen the associations formed. Hypothetically, the more pairings with the tone and the aversive stimulus will increase the associative memory. This experiment assessed memory at shorter intervals than the previous experiments. Repeated CS-US pairings were hypothesized to strengthen fear conditioning tested at a shorter interval than the previous two experiments.

The addition of cued conditioning will extend the usefulness of the model by allowing assessment of contextual and auditory memory. As discussed in the introduction, fear conditioning is dissociable into hippocampal-mediated contextual and

amygdala-mediated cue memories. In order to address differences in neurobiological underpinnings of memory for conditioned fear, this experiment aimed to develop predator-based conditioning to an auditory cue and context. A paradigm that produced conditioning to the context and an auditory cue would be useful to investigate how drugs and behavioral manipulations affect memory dependent on the amygdala and hippocampus.

2.3.2 Method

Animals. A total of 44 male Sprague-Dawley rats (Charles River) weighing 225-250g on arrival were acclimated to the vivarium and cage cleanings for at least 7 days before any experimental manipulations were conducted. Rats were housed 2 per cage (standard Plexiglas – 46 x 25 x 21 cm). Tap water and rat chow were available *ad libitum*. The animal housing room was maintained at 20 ± 1° C with a humidity range of 60 ± 3%, and a 12hr light cycle (on at 0700 hr). All procedures were approved by the Institutional Animal Care and Use Committee at the University of South Florida.

Fear Conditioning. Rats were randomly assigned to receive immobilization and predator exposure (Cat) or homecage (No Cat). Rats were placed in the conditioning chamber and given 3 minutes exposure time. During the last 30 seconds of conditioning a 70 dB, 2500 Hz auditory tone was presented. Immediately after the cessation of the tone, the rats were immobilized using a plastic DecapiCone. Within 2 minutes of being immobilized the rats were placed in the pie-shaped Plexiglas enclosure (as described earlier) in the presence of an adult female cat for 10 minutes. After the 10 minutes in the presence of the cat, the rats were placed in their homecage for 25 min before another identical conditioning trial occurred. This was repeated for a total of 3 conditioning trials.

Testing for contextual and cued fear memory, as described in section 2.1.2, was performed 72 hrs after conditioning.

Statistical Analyses. Separate independent sample t-tests were used to detect significant differences between Cat (context test n = 10, cue test n = 9) No Cat (n = 8) groups' freezing behavior to the context and cue. Alpha was set at 0.05.

2.3.3 Results

There was a significant difference in freezing between groups in the context with the Cat group spending significantly more time immobile than the No Cat group ($p < 0.05$). Analysis of the freezing to the cue indicated a borderline significant effect of the Cat group to have spent more time immobile during the cue than the No Cat group ($p = 0.07$).

2.3.4 Discussion

In this experiment a conditioning paradigm consisting of multiple CS-US pairings in a single day to produce context and cue memory. In rats multiple conditioning trials within one day produced significantly more freezing in the context three days after conditioning in the immobilized and cat exposed group compared to the control group. The association of an auditory cue with the predator based stimulus was borderline significant. While only a trend was found for cue conditioning, this experiment does provide a paradigm that is suited toward exploring how other behavioral manipulations can affect fear conditioning. This experiment provides means to understand context and cue predator-based fear conditioning in a comparable manner to standard foot shock conditioning.

Recent work has shown that exposure to a predator and the predator-related context activates the amygdala (Martinez, Carvalho-Netto, Ribeiro-Barbosa, Baldo, & Canteras, 2011). In this study Fos activity was significantly increased in the medial, posterior basomedial and lateral nuclei of the amygdala after cat exposure. The cat-associated context induced significant increases in Fos levels in the lateral area of the central amygdalar nucleus. However, Staples, Hunt, Cornish, & McGregor (2005) showed that re-exposure to a cat-odor associated context failed to significantly increase Fos in the amygdala and the authors note that an outlier could account for a lack of statistical difference. Staples and colleagues have consistently shown cat-odor induced up-regulation in hypothalamic nuclei, nucleus accumbens, caudate putamen, olfactory nuclei, and periaquiductal grey (Staples, Hunt, van Nieuwenhuijzen, & McGregor, 2008; Staples, McGregor, & Hunt, 2009). All of these neural structures are associated with assessing environmental stimuli and reacting behaviorally specifically to cat odor and not trimethylthiazoline, a synthetic predator odor derived from fox feces (Staples, McGregor, Apfelbach, & Hunt, 2008; Staples & McGregor, 2006). Context fear conditioning using foot shock results in the up-regulation of immediate early genes zif268 and CREB in the amygdala (Hall, Thomas, & Everitt, 2001). Methodological differences between foot shock and predator-based conditioning studies could account for the inconsistencies in immediate early gene expression. Using methodologies more similar to foot shock research, such as the methods of this experiment, can address discrepancies between foot shock and predator-related conditioning.

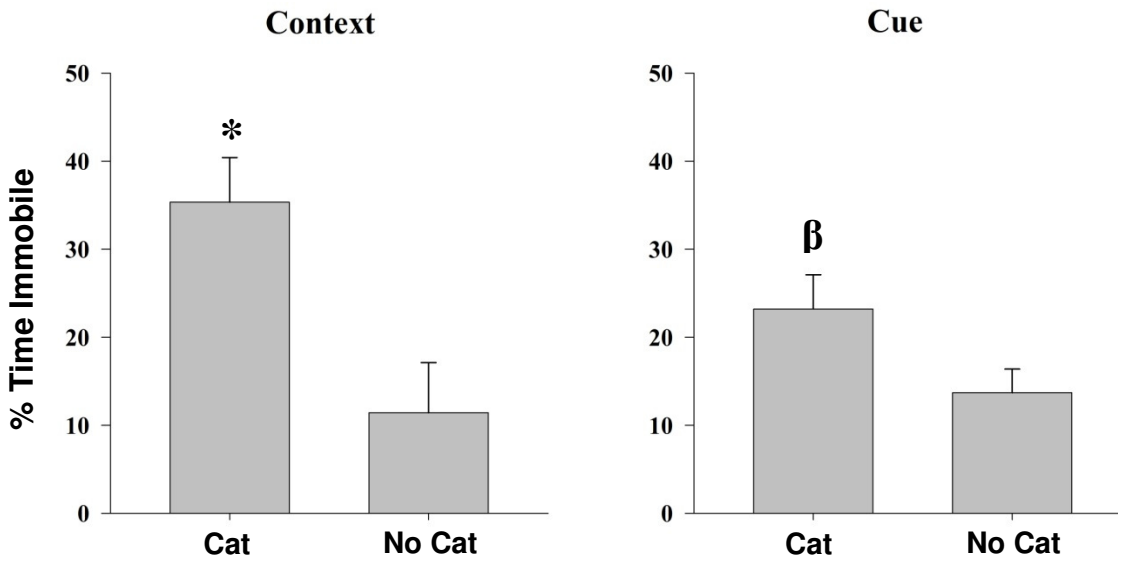

Figure 4. Multiple cat exposure and immobilization conditioning trials in 1 day produced significantly more freezing to the context than the no cat group (left graph). The graph on the right illustrates borderline differences in freezing to the tone induced by the immobilization and cat exposure treatment compared to the no cat group. * = $p < 0.05$, β = $p = 0.07$.

2.4 Experiment 4

2.4.1 Inactivation of CA1 area of hippocampus impairs contextual but not cued fear conditioning.

The importance of the amygdala and the hippocampus in fear conditioning has been established. Amygdala lesions impair Pavlovian fear associations to contexts and cues, and hippocampal lesions impair context, but not cue, Pavlovian fear associations. In experiment 3 predator-based fear conditioning to a context and an auditory cue was successful. Experiment 4 aimed to investigate hippocampal involvement in predator-based conditioning.

The target of this experiment is the dorsal CA1 region of the hippocampus. Hippocampal divisions are based primarily on the cellular organization and

neuroanatomical features of each region conserved across mammals. The perforant pathway is fibers from the entorhinal cortex that terminate in the dentate gyrus and CA3 regions. Schaffer collaterals, which are axons from the CA3 pyramidal cells, project to CA1 pyramidal cells. Neurons in the CA1 project to entorhinal cells, which relay to the cortex. This Neuroanatomical arrangement makes the CA1 region of the hippocampus integral in memory because it receives input from various modalities and outputs to the cortex (Akirav, Sandi, & Richter-Levin, 2001; Artola et al., 2006; Cao, Chen, Xu, & Xu, 2004; Kim, Foy, & Thompson, 1996).

Shapiro & Eichenbaum (1999) hypothesized that the capacity of the hippocampus to receive and integrate information from different senses allows the hippocampus to generate a coherent representation of the context through the associations made between the information. Thus, the hippocampus is important for acquiring new declarative memories (Bunsey & Eichenbaum, 1996; Eichenbaum, 2004) which can be either emotional or neutral in nature. In laboratory animals, damage to the hippocampus seven days before contextual learning (Selden et al., 1991) or muscarinic cholinergic receptor antagonism of the hippocampus fifteen minutes prior to the learning (Anagnostaras, Maren, & Fanselow, 1999) impair performance on contextual fear conditioning.

The dorsal hippocampus is implicated in fear conditioning. Wanisch, Tang, Mederer, & Wotjak (2005) manipulated NMDA receptors with the antagonist AP5 or disrupted protein synthesis with anisomycin in the dorsal hippocampus of mice. Blocking NMDA receptors or protein synthesis prior to trace but not delay auditory conditioning reduced freezing tested 24 hours later. Another group using inhibitory avoidance found that AP5 infused into the CA1, pre-training but not pre-testing, impaired retention of the

avoidance memory (Roesler, Vianna, Schröder, Ferreira, & Quevedo, 2006). Contextual fear conditioning is impaired at 24 hour testing by post-conditioning infusions of propranolol into the CA1 5 minutes but not 6 hours after training, supporting the hypothesis that adrenergic modulation within the hippocampus has time-dependent effects on memory (Ji, Wang, & Li, 2003). Rogers, Hunsaker, & Kesner (2006) demonstrated that chemical lesions of the dorsal CA1 area of the hippocampus produced significantly less freezing to the conditioned context, yet these did not significantly affect trace conditioned auditory cue conditioning. Based on these results, this experiment tested the hypothesis that inactivation of the hippocampus would result in impaired context, but not cue, predator-based fear expression.

2.4.2 Methods

Design. A 2x2 factorial design with artificial cerebral spinal fluid (aCSF) used as Vehicle or Muscimol, and immobilization with cat exposure (Cat) or homecage (No Cat) as the levels.

Animals. A total of 38 male Sprague-Dawley rats (Charles River) weighing 225-250g on arrival were acclimated to the vivarium and cage changes for at least 7 days before any experimental manipulations were conducted. Rats were housed 2 per cage (standard Plexiglas – 46 x 25 x 21 cm) until surgery, after which they were singly housed. Tap water and rat chow was available *ad libitum*. The animal housing room was maintained at 20 ± 1° C with a humidity range of 60 ± 3%, and a 12hr light cycle (on at 0700 hr). All procedures were approved by the Institutional Animal Care and Use Committee at the University of South Florida.

Surgery. On the day of surgery, the rats were brought to the laboratory, where all surgical procedures were performed under aseptic conditions. Rats were deeply anesthetized using isoflurane. Their heads were shaved and placed level on a stereotaxic device. After the skull was exposed, the topographical coordinates for the landmarks of bregma and lambda were recorded for targeting purposes. All targets were in reference to the skull surface of bregma in millimeters and insertions were made with 26-gauge, stainless steel, guide cannula (Plastics One Inc., Roanoke, VA).

The target was the dorsal CA1 region of the hippocampus (coordinates: -3.8 AP, ±3.0 L, -2.8 DV). Guide cannula were held in place by dental cement and anchored to the skull with four skull-screws. Removable stylets projecting 1mm from the tip of the guide cannula were inserted and held in place with a screw-on dust cap (Plastics One Inc., Roanoke, VA) to keep the cannula patent.

Intracerebral Infusions. All animals were given one week to recuperate from surgery before data collection. All infusion and behavioral procedures were performed between 0900-1500 hours. For three consecutive days animals were brought into the laboratory and approximately 30 minutes later underwent the following series of manipulations. On the first day, the dust cap was removed and a mock injection tube placed on the cannula pedestal. The second and third day consisted of the removal the dust cap and stylet, and gently placing the injectors (Plastics One) in the guide cannula. A Harvard Apparatus pump (Holliston, MA), connected to 25µl syringe injectors (Hamilton) by plastic tubing (Plastics One), infused aCSF at a rate of 0.1µl/min for 3 minutes. After the infusion, the pump was turned off and the fluid given 1 minute to

diffuse before the dummy cannula was replaced and dust cap screwed back on the top of the pedestal. On the third day, aCSF or muscimol was administered.

Histology. A total of 36 rats completed testing. Upon completing the behavioral tasks all animals were euthanized with an overdose of Ketamine and Xylaxine, cresyl violet was infused into the cannula at a rate of 0.1µl/min for 5 minutes to give allow visual inspection of cannula placement. The brains were extracted and flash frozen in methylbutane and the tissue was stored at -80°C until it was sliced in coronal sections in 40µm increments on a Cryostat held at -16°C and mounted on microscope slides. There were 2 animals excluded from analysis for cannula placement outside of the target area.

Cat Exposure Procedure. Approximately 15 minutes after the rats were infused with aCSF or muscimol, they were placed in a fear conditioning chamber (as described in section 2.1.2). Exposure to the chamber for 3 minutes terminated with the presentation of a single 30-second, 74 dB 2500 Hz tone, which served as the auditory cue. Animals in the Cat groups were immediately immobilized and then placed in close proximity to a cat as previously described, except that they remained with the cat for 1 hour. Animals in No Cat groups were placed back in their home cages.

Statistical Analyses. Data were analyzed with 2x2 ANOVAs. A priori planned comparisons were tested with two-tailed Student's t-tests, between Cat- and No Cat-aCSF and -Muscimol treated groups in each behavioral test of the experiment. Alpha was set at 0.05 for all analyses. Freezing analysis was conducted with 6-9 rats per group.

2.4.3 Results

Context Memory. Analysis of variance for the CA1 targeted groups' contextual fear revealed an overall significant effect with $F(3,28) = 4.11$, $p < 0.05$. There was a

significant main effect of both Cat ($F(1,28) = 4.46$, $p < 0.05$) and Inactivation ($F(1,28) = 5.95$, $p < 0.05$). The Cat x Inactivation interaction was not significant ($F(1,28) = 1.34$, $p = 0.26$). Planned comparison tests showed Muscimol infused prior to the Cat procedure significantly reduced ($p < 0.03$) freezing compared to aCSF.

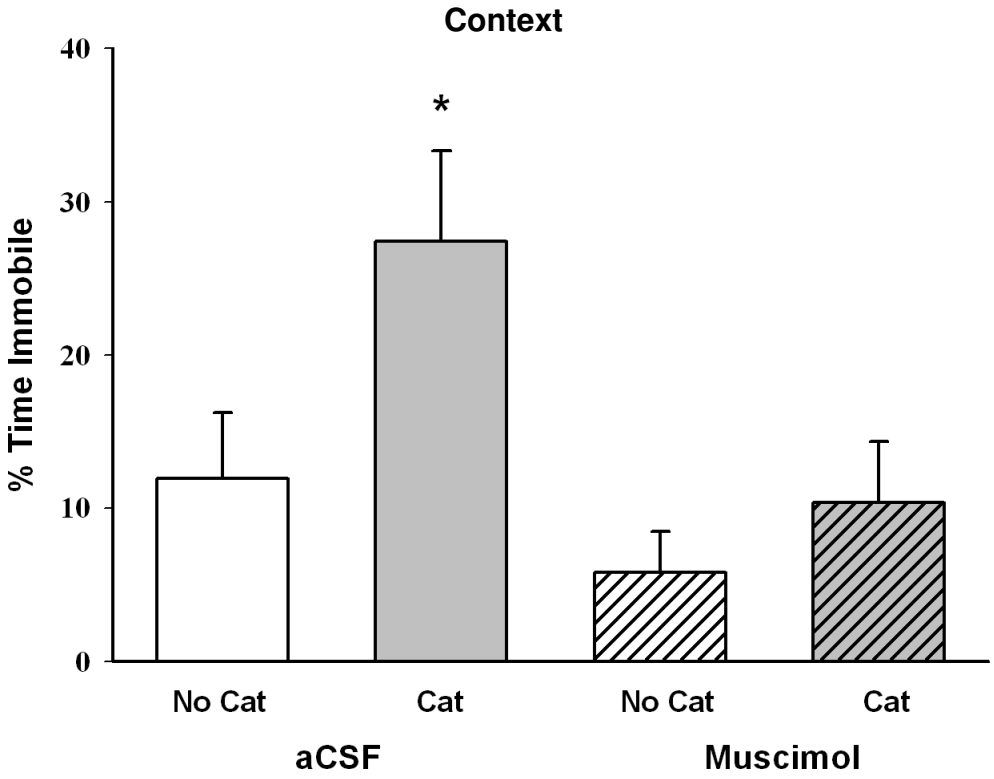

Figure 5. The aCSF-Cat group froze significantly more than the Muscimol-Cat group. Muscimol application to the dorsal CA1 area of the hippocampus blocked contextual memory in the cat exposed group compared to the cat group administered aCSF. * indicates $p < 0.05$ all groups

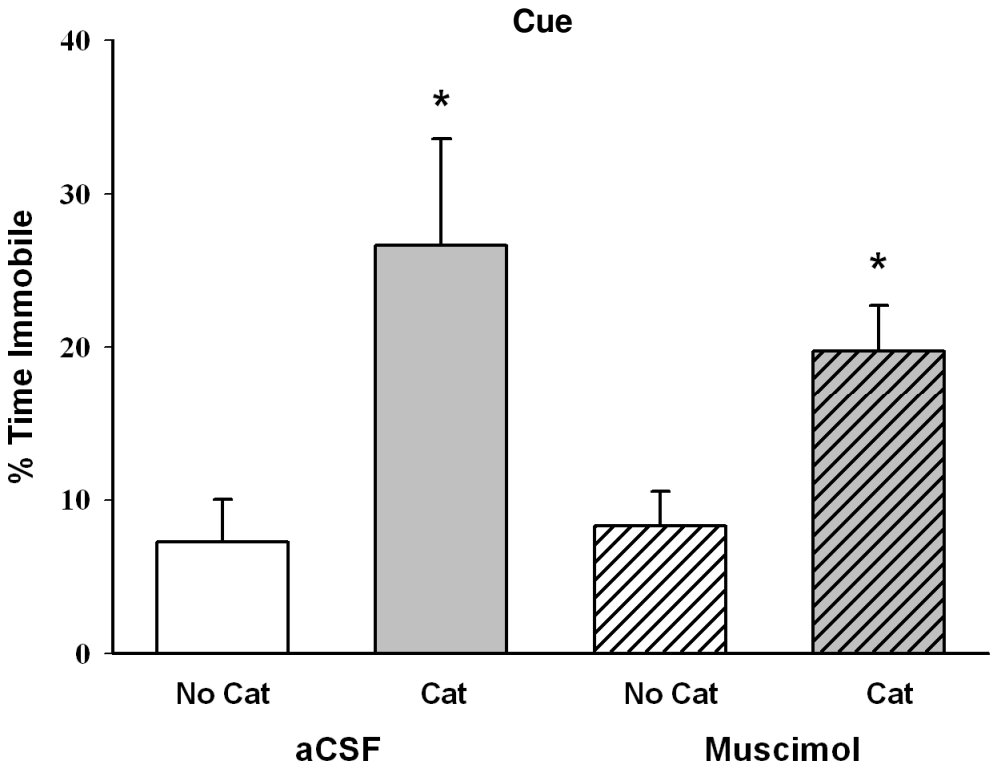

Figure 6. The predator exposure and immobilization treatment resulted in significantly more freezing to the tone than the no cat groups. Muscimol administration to the dorsal CA1 area of the hippocampus did not block the cued freezing in cat groups. * indicates $p < 0.05$ Cat group vs. No Cat group.

Cue Memory. Analysis of the cued fear response in CA1 targeted animals showed significant overall differences ($F(3,29) = 3.83$, $p < 0.05$); with no significant main effect of Inactivation ($F(1,29) = 0.35$, n.s.) or the Cat x Inactivation interaction ($F(1,29) = 0.64$, n.s.). A significant main effect was observed in the Cat manipulation with $F(1,29) = 9.69$, $p < 0.01$; where the Cat procedure resulted in animals freezing more to the cue than No Cat animals. Planned comparison t-tests revealed the Cat-aCSF and –Muscimol animals froze significantly more than the No Cat-aCSF and –Muscimol groups ($p < 0.05$).

2.4.4 Discussion

In this experiment the role of the dorsal CA1 region of the hippocampus in Pavlovian predator-based contextual and cue fear conditioning was investigated. This experiment extended the predator-based fear conditioning paradigm by demonstrating a cue association to the US. Lesions or inactivation of the dorsal hippocampus made prior to foot shock conditioning block expression of fear when the subject is re-exposed to the conditioning context; however, there is intact cue-dependent memory (Kim et al., 1993, Phillips & LeDoux, 1992, 1994; Selden et al., 1991; Young et al., 1994; Kim & Fanselow, 1992). This work applied what has been learned about the hippocampus using shock-based conditioning to predator-based conditioning. This experiment demonstrates that the dorsal hippocampus is necessary for predator-based trace context, but not cue, fear memory. The findings of hippocampal involvement in only context fear conditioning is consistent across this predator-based paradigm and paradigms utilizing foot shock.

Pentkowski, Blanchard, Lever, Litvin, & Blanchard (2006) presented results that implicated that the ventral, not dorsal, hippocampus in unconditioned and conditioned defensive responses. The results of their experiment suggest that the inactivation of the dorsal CA1 in experiment 4 would not significantly affect behavior. However, the lesions in Pentkowski et al. (2006) were made one week before behavioral testing and previous studies indicate that other brain structures can compensate for memory affected by dorsal hippocampal damage (Fanselow, 2000; Sanders et al., 2003; Matus-Amat et al., 2004). Considering the results of experiment 4, support is found for the hypothesis that the dorsal CA1 area of the hippocampus plays a vital role in the conditioned spatial, but not auditory cue-based, associations formed in predator-based trace conditioning.

2.5 Experiment 5

2.5.1 Predator-based inhibitory avoidance.

In experiment 5, an alternative behavioral approach to the Pavlovian conditioning method was investigated using the predator-based aversive stimulus used in the previous experiments. Avoidance conditioning, involves pairing aversive stimuli with a volitional response (Gold, 1986; Roozendaal & McGaugh, 1996; Wilensky et al., 2000). Avoidance conditioning associates a behavior with a consequence. Consequences resulting in increases of the frequency of a behavior are reinforcers and those resulting in decreases in a behavior are punishers. Single trial avoidance training consists of placing a rat in the illuminated side of a two-chambered box, separated by a door. When the door is opened the rats naturally approach and cross into the dark side of the chamber. When the rat crosses into the dark chamber it is shocked. In this paradigm the association between the act of crossing into the dark chamber and the shock is formed, indicated by the rat taking longer to cross into the dark side, from the light side of the conditioning chamber at testing.

Avoidance paradigms differ from Pavlovian conditioning paradigms in that an animal's behavior dictates whether or not it receives punishment. Pavlovian fear conditioning paradigms do not allow the animal's behavior to influence whether or not the aversive stimulus is presented. Therefore, avoidance paradigms explore the inhibition of natural responses based on the previous aversive associations made with a behavior. This experiment tested the hypothesis that repeated pairings of immobilization and predator exposure with crossing by the rats from the light- to the dark-side would result in an avoidance response.

2.5.2 Method

Animals. A total of 16 male Sprague-Dawley rats (Charles River) weighing 225-250g on arrival were acclimated to the vivarium and cage cleanings for at least 7 days before any experimental manipulations were conducted. Rats were housed 2 per cage (standard Plexiglas – 46 x 25 x 21 cm). Tap water and rat chow were available *ad libitum*. The animal housing room was maintained at 20 ± 1° C with a humidity range of 60 ± 3%, and a 12hr light cycle (on at 0700 hr). All procedures were approved by the Institutional Animal Care and Use Committee at the University of South Florida.

Inhibitory Avoidance Conditioning. Prior to conditioning all rats were brought to the laboratory for 3 consecutive days for handling. Conditioning took place in a standard shuttle box (Coulbourn Instruments; Allentown, PA; 25 x 22.5 x 33 cm) divided into an illuminated chamber and a darker chamber by a remote guillotine door. Conditioning occurred daily for 5 consecutive days and consisted of placing a rat into the illuminated side of the apparatus. Thirty seconds after the rat was placed into the apparatus, the door was lifted and the rat could access the dark compartment. Each rat was allowed a maximum of 10 minutes to cross to the dark chamber. Rats in the No Cat group were removed from the apparatus and returned to their home cage immediately after crossing. Rats in the Cat group were immobilized immediately after crossing into the dark chamber and within 2 minutes, placed in the presence of a cat for 30 minutes.

Statistical Analysis. Repeated measures ANOVA were used to analyze the latency to cross into the dark chamber during conditioning. An independent samples t-test was used to analyze the memory test data. All analysis consisted of 7-8 rats per group.

2.5.3 Results

Acquisition. The repeated measure ANOVA indicated significant within subject effects of Training Day ($F(4, 52) = 13.65$, $p < 0.05$) and Training Day x Group Interaction ($F(4, 52) = 6.04$, $p < 0.05$). Post hoc LSD showed that Cat group was significantly different than the No Cat group on Training Days 3-5.

Avoidance Test. The independent samples t-test showed that the Cat group had significantly longer crossing latencies on testing than the No Cat group.

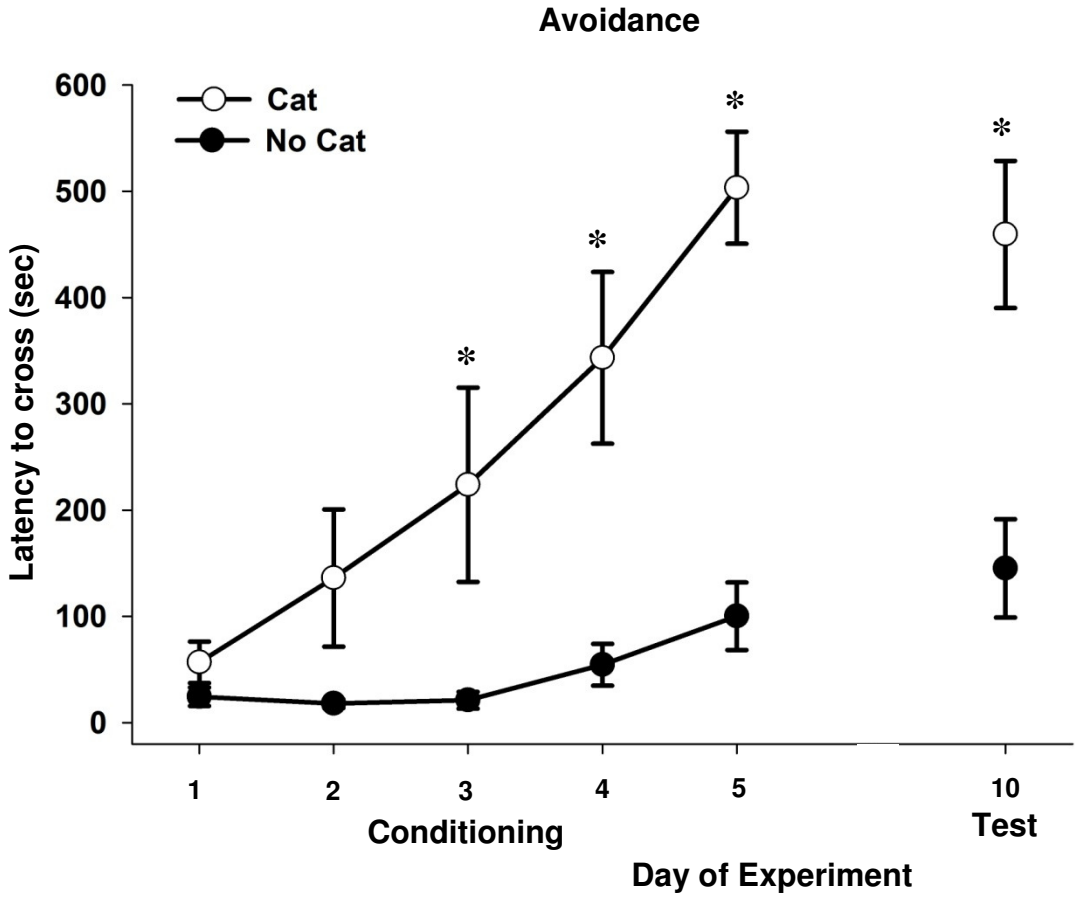

Figure 7. Latency to cross in the cat group significantly increased across training days compared to the no cat group. When tested on day 10, the Cat group took significantly longer to cross than the No Cat group. * indicates $p < 0.05$ Cat group vs. No Cat group.

2.5.4 Discussion

This experiment demonstrated that the predator-based US used in the previous Pavlovian conditioning experiments can be implemented in an inhibitory avoidance conditioning paradigm. Avoidance conditioning using foot shock has been thoroughly characterized. The interactions between noradrenergic receptor function and glucocorticoids in amygdala have been shown to modulate memory for inhibitory avoidance conditioning. Initial work from Gold & Van Buskirk (1975) demonstrated that post-training systemic epinephrine administration enhanced inhibitory avoidance memory in a dose- and time-dependent manner. Furthermore, administration of general and specific β_1- and β_2-adrenergic receptor antagonists into the basolateral nucleus of the amygdala (BLA) blocked the post-training, systemic administration of the synthetic glucocorticoid (dexamethasone), enhancement of inhibitory avoidance memory (Quirarte, Roozendaal, & McGaugh, 1997). The same adrenergic receptor antagonists infused into the central nucleus of the amygdala failed to block the glucocorticoid memory enhancement. Thus, the interaction effects of glucocorticoids and adrenergic receptors on inhibitory avoidance memory are partially mediated within the amygdala.

Similar to amygdala-mediated memory, hippocampus-dependent memory is influenced by glucocorticoid-adrenergic interactions. Also, memory that is mediated by the hippocampus is influenced by the amygdala. Inhibitory avoidance and Pavlovian fear conditioning paradigms both are partially modulated by adrenergic interactions with glucocorticoids within the amygdala and hippocampus. The effect of predator-based fear conditioning on increasing the latency to cross in this experiment is hypothesized to be based on the endogenous release of adrenergic and glucocorticoid sequelae.

2.6 Experiment 6

2.6.1 Sleep Deprivation and Fear Conditioning

Sleep loss is associated with negative impacts on mood, motor function and cognitive performance (Goel, Rao, Durmer, & Dinges, 2009). The effects of sleep deprivation on human neural systems that control circadian and homeostatic mechanisms have focused on the function of the hypothalamus (Hastings, 2002; Mignot, Taheri, & Nishino, 2002; Saper, Chou, & Scammell, 2001; Thomas et al., 2000). The suprachiasmatic nucleus of the hypothalamus modulates both waking- and sleeping-rhythms, making it the "biological clock" to what is considered daily cycles. The functions of this biological clock include modulating more than just sleepiness in waking behavior and has been suggested to be involved in attention and cognitive performance (Van Dongen, & Dinges, 2000; Van Dongen & Dinges, 2003).

Extensive research has shown that sleep deprivation impairs cognitive functioning (Harrison & Horne, 1998, 2000; Kleitman, 1987; Kribbs & Dinges, 1994; Patrick & Gilbert, 1896; Pilcher & Huffcutt, 1996). In humans chronic mild sleep restrictions, of 2-6 hours of sleep a night, and complete acute sleep deprivation (SD) impair cognitive performance compared to non-sleep deprived individuals (Van Dongen, Maislin, Mullington, & Dinges, 2003). The cognitive impairments associated with SD are found in episodic and declarative memory, two forms of memory that involve hippocampal function. Cognitive deficits in episodic memory resulting from SD are positively correlated with reduced hippocampal blood flow in functional magnetic resonance imaging scans in humans (Yoo, Hu, Gujar, Jolesz, & Walker, 2007). Research in rodents indicates that SD interferes with learning and memory associated with the hippocampus.

Recent research indicates that context (hippocampal dependent) fear conditioning is impaired, but cued (hippocampal independent) fear conditioning is not affected in SD rats (Graves, Heller, Pack, & Abel, 2003; Hagewoud et al., 2010; Ruskin & Lahoste, 2008; Ruskin, Liu, Dunn, Bazan, & Lahoste, 2004). These findings suggest the effects of SD are on hippocampal-dependent processing.

Sleep deprivation not only impairs cognition associated with the hippocampus, it impairs hippocampal plasticity as well. Previous research has shown that long-term potentiation (LTP), is impaired in the hippocampus of SD rats (Kim, Mahmoud, & Grover, 2005). LTP involves a calcium dependent cascade, including activation of calcium-calmodulin dependent kinase II (CAMKII). The activation of CAMKII is a regulator of short-term memory and LTP (Malenka, 1999). Twenty-four hours of SD before training impaired hippocampal dependent spatial- and short-term memory in rats (Alhaider, Aleisa, Tran, Alzoubi, & Alkadhi, 2010). The effects of SD on hippocampal-dependent tasks are likely due to the effects SD has on hippocampal LTP.

The hypothesis that sleep deprivation would affect hippocampal-dependent contextual fear, but not amygdala mediated cue fear was tested. The paradigms used in the experiments resulted in hippocampal dependent fear conditioning. Based on the previous work demonstrating that SD in rodents impairs hippocampus-based memory, 24 hour sleep deprivation prior to predator-based fear conditioning is hypothesized to decrease contextual freezing, while unaffecting or enhancing auditory cue conditioning.

2.6.2 Effects of Sleep Deprivation on Fear Conditioning

2.6.3 Method

Animals. A total of 44 male Sprague-Dawley rats (Charles River) weighing 225-250g on arrival were acclimated to the vivarium and cage cleanings for at least 7 days before any experimental manipulations are conducted. Rats were housed 2 per cage (standard Plexiglas – 46 x 25 x 21 cm). Tap water and rat chow were available *ad libitum*. The animal housing room was maintained at 20 ± 1° C with a humidity range of 60 ± 3%, and a 12hr light cycle (on at 0700 hr). All procedures were approved by the Institutional Animal Care and Use Committee at the University of South Florida.

Procedure

Sleep Deprivation. Rats were randomly assigned to receive Sleep Deprivation (SD) or No Sleep Deprivation (NSD). Modified home cages setup to accommodate the flower pot technique and allow the availability of food and water throughout the 24 hrs before conditioning were used. The SD cages were standard clear plexiglass "shoe box" rodent cages modified with a vertical extension such that when 4 platforms (polypropylene jars PCG Scientific, 05-8333-30, 9 cm high x 6 cm diameter) are placed in the cage rats can move freely and access food and water from the standard wire lid. The cages were filled with room temperature tap water raised to within 1 cm of the top of the platforms. The NSD rats were housed in homecages for 24 hrs prior to conditioning in same room that the SD procedure took place. All rats were continually housed with their regular cagemates throughout the 24 hr prior to conditioning.

Fear Conditioning. The same Colbourn Instruments conditioning apparatus used in experiments 1-4 were utilized. Rats were randomly assigned to receive immobilization

and predator exposure (Stress) or homecage (No Stress). Rats were placed in the conditioning chambers and allowed 3 minutes to explore the context. During the last 30 seconds of conditioning a 70 dB, 2500 Hz auditory tone was presented. Immediately after the cessation of the tone rats in the Stress groups were immobilized and placed in the presence of a cat (a described earlier) for 10 min. After the 10 min in the presence of the cat, rats were placed either in their homecage or in the SD apparatus for 25 min before another identical conditioning trial occurred. This was repeated for a total of 3 conditioning trials. Testing for contextual and cued fear, as previously described, was performed 72 hrs after conditioning.

Analyses. Separate 2x2 ANOVAs were used to detect significant differences between SD-Stress (n = 14), SD-No Stress (n = 10), NSD-Stress (context test n = 10, cue test n = 9) and NSD-No Stress (n = 10) groups' freezing behavior to the context, novel environment and cue. Alpha was set at 0.05.

2.6.4 Results

There was a significant differences in freezing between groups in the context as indicated by an omnibus effect ($F(3, 40) = 11.08$, $p < 0.01$), as well as significant main effects of SD ($F(1,40) = 15.23$, $p < 0.01$) and Stress ($F(1,40) = 9.25$, $p < 0.01$). The Stress x SD interaction also reached significance with $F(1,40) = 9.85$, $p = 0.01$. Post hoc analysis showed that the NSD-Stress group (M = 39.03%, SEM = 9.43%) spent significantly more time immobile in the conditioning context than the SD-Stress (M = 6.06%, SEM = 1.32%), SD-No Stress (M = 6.50%, SEM = 1.17%), and NSD-No Stress (M = 9.90%, SEM = 1.42%) groups.

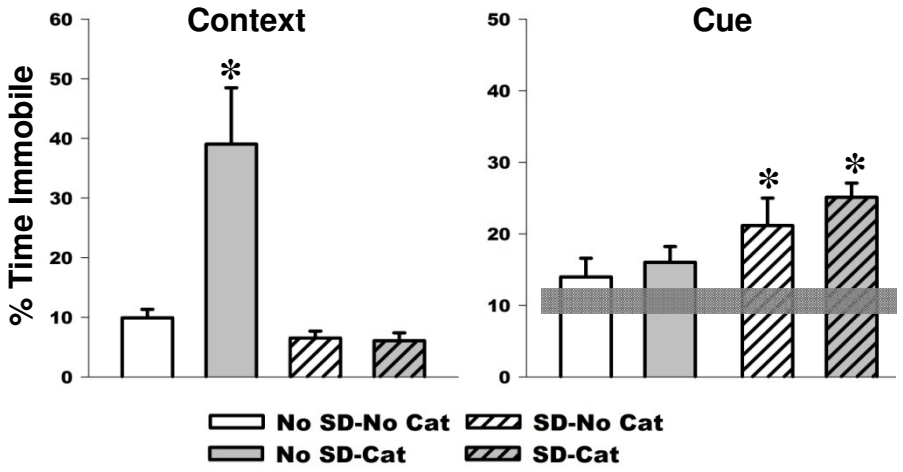

Figure 8. Sleep deprivation significantly impaired freezing to the hippocampal-dependent contextual aspect of predator based memory tested 72 hours after conditioning, shown on the graph on the left. However, predator based freezing to the amygdala-dependent auditory cue was not significantly affected by sleep deprivation, shown on the graph on the right. The grey box illustrates the baseline mean, plus and minus the SEM, freezing for all groups in cue test box prior to the delivery of the tone.* indicates $p < 0.05$, using LSD tests between Cat and No Cat groups.

There were no significant differences in freezing between groups in the novel environment. Data for freezing to the cue did yield a significant overall ANOVA ($F(3,39) = 2.87$, $p = 0.05$). A significant main effect of Stress was indicated with $F(1,39) = 5.05$, $p = 0.03$. The Stress groups ($M = 24.02\%$, $SEM = 2.12\%$) spent significantly more time immobile during the cue than the No Stress groups ($M = 14.99\%$, $SEM = 1.68\%$). Both the main effect of Sleep Deprivation ($F(1,39) = 2.69$, $p = 0.11$) and the Stress x SD interaction ($F(1,39) = 0.60$, $p = 0.81$) were not significant.

2.6.5 Discussion

This experiment replicated and extended the predator-based Pavlovian fear conditioning paradigm developed in the previous experiments. Sleep deprivation before predator-based fear conditioning impaired contextual fear conditioning as indicated by

the significant sleep deprivation and predator-stress interaction. Notably, sleep deprivation had no effect on predator-based fear conditioning to an auditory cue. Therefore, these findings support the hypothesis that sleep deprivation is detrimental to memory associated with hippocampal function without affecting more amygdalar-mediated memory.

A recent large-scale, multi-site sleep disturbance study reported that sleep disturbances immediately prior to a physically traumatic event increased the risk of a range of psychiatric disorders (Bryant, Creamer, O'Donnell, Silove, & McFarlane, 2010). In their meta-analysis, Bryant et al. (2010) pointed out that sleep disturbance predicted clinical disorders, such as PTSD and major depression, better than age, gender, severity of trauma and previous psychiatric disturbances. The authors acknowledge that there are likely common underpinnings for the relationship among the disorders; however, they do recognize that there could be disorder-specific mechanisms posed by sleep disturbances prior to trauma. One explanation proposed for their finding is that sleep impairment reduces emotional, cognitive, and physical resources that would, otherwise, mitigate the aftermath of trauma exposure. Fatigue, a commonality with impaired sleep (Shapiro et al., 2002), produces cognitive impairment, such as reduced attention and concentration (Moul et al., 2002). Thus, individuals who are deprived of sleep and have depleted abilities to deal with trauma could develop fragmented memories of stimuli associated with the trauma. Intelligence and the ability to realistically appraise events could protect against the development of clinical disorders brought on by trauma and these cognitive abilities may be limited by sleep deprivation, and render individuals prone to the development of disorders.

The clinical disorders implicated in these studies have been theorize to involve the amygdala (Bracha, 2006). This line of research would suggest that an interaction between sleep deprivation and trauma would enhance amygdala processing and, by extension, auditory fear conditioning. Evidence from fMRI studies of increased amygdala activation and reduced amygdala-PFC functional connectivity as a result of sleep deprivation suggest that individuals are less able to adapt to trauma after sleep deprivation than non-sleep deprived individuals (Yoo et al., 2007). One hypothesis attempting to explain insomnia posits that hyperarousal is at the core of the inability to sleep (Bonnet & Arand, 2002). Therefore, sleep deprivation before trauma could add to the development of robust fear conditioning. Models of PTSD suggest that hyper-sympathetic arousal (release of glucocorticoids, norepinephrine and epinephrine) at the time of trauma result in over-consolidation of traumatic memories (Pitman, 1989). However, the findings of the current study do not support this hypothesis. Fear conditioning to an auditory cue occurred as a result of the predator-based stimulus, as indicated by the significant main effect of cat exposure, but was no greater in sleep deprived than non-sleep deprived animals.

Other investigators have presented a model of cognitive dysfunction as a result of sleep disorder based on the function of the prefrontal cortex (PFC) (Beebe & Gozal, 2002). As discussed previously, the PFC is pivotal to extinction learning and the link between sleep dysfunction and fear extinction should be investigated further. Thus, the PFC, amygdala, and hippocampus work in concert to form memories and dysfunction of these same brain regions can lead to pathologies, such as PTSD.

Summary

The first experiment developed and characterized the effects of three different reinforcers: electric shock, immobilization, and predator exposure, alone or in combination on the expression of fear memory and extinction. The results of experiment 1 indicated that the combination of immobilization and cat exposure, in conjunction with foot shock produced the most powerful fear memory. Additionally, experiment 1 provided the basis for immobilization and cat exposure, without the use of foot shock, to be used as an unconditioned stimulus.

Experiment 2 expanded on the findings of extinction resistant immobilization and predator exposure associated contextual memory. This experiment exposed rats to three of the predator-based CS-US pairings within six days and tested memory seven days after the final pairing. The second experiment also explored how the frequency of extinction trials given to rats influenced the expression of contextual fear memory. Contextual fear memory was extinguished by giving the rats extinction trials every two days, but not every seven days.

In the first two experiments, rats that were immobilized and exposed to a cat expressed long-term context memory. In experiment 1 cue memory was not significantly different from controls and experiment 2 did not include cue conditioning. Therefore, rats in experiment 3 were given multiple training trials in one day to both the context and a cue, to increase expression of fear memory. This procedure resulted in a significant

increase in context memory and a greater, but not quite significant ($p = 0.07$), expression of fear to the cue. This experiment provides the basis for a valuable paradigm that allows the dissociation between hippocampal-dependent context and hippocampal-independent cue memories.

Experiment 3 provided the framework to measure both context and cue fear memory, therefore, experiment 4 tested the hypothesis that predator-based context memory formation requires a functioning hippocampus. A transient inactivation of the dorsal CA1 area of the hippocampus of rats during context and the predator-based stimulus association blocked contextual memory. The results of experiment 4 also indicated that cue-based memory formation was hippocampal-independent. This experiment demonstrated that, just as in foot shock conditioning paradigms, the predator-based, context, but not cue fear memory is dependent on a functioning hippocampus.

The effects of immobilizing rats and exposing them to a cat on freezing and avoidance behaviors have begun to be established in the previous four experiments. Experiment 5 tested the novel hypothesis that rats can associate immobilization and predator exposure with a volitional behavior and form an inhibitory avoidance response. Rats learned to avoid a preferred context that was paired with the predator-based stimulus. Therefore, the predator-based US can also be associated with the consequence of volitional behaviors, expanding the scope of how effective the US is on rodent memory.

Finally, experiment 6 aimed to impair hippocampal function using sleep deprivation. Impaired sleep is associated with clinical disorders and deficits in memory associated with impaired hippocampal function. Experiment 6 showed that sleep

deprivation impaired the predator-based context, but not cue fear memory. This finding supports the hypothesis that sleep deprivation impairs hippocampal-mediated memory, but spares other memory systems, such as amygdala-mediated cue conditioning.

Conclusion

The development of this predator-based fear conditioning paradigm provides a model for studying the neurobiology of fear memory with an ethologically relevant reinforcer. The findings indicate that predator-based fear conditioning and extinction appear to involve the same neural structures (hippocampus, amygdala, and prefrontal cortex) as conventional foot shock-based fear conditioning, but were produced using more ethologically relevant stress (predator exposure). In summary, this series of experiments has provided the groundwork for integrating the classical fear conditioning paradigm with ethologically relevant reinforcement to extend our understanding of the neurobiology of human traumatic memory.

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