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Development of Ethologically-Based Inhibitory Avoidance Models of Fear Memory

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by

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Abstract

Translational research provides a unique opportunity to investigate innate and conditioned fear to develop an integrated understanding of anxiety disorders, ultimately improving treatment for those afflicted. Many fear conditioning paradigms use physically aversive stimuli to induce fear but ethological stimuli may better represent psychological disorders from a translational standpoint. Natural predators and immobilization have been successful in inducing both innate and contextually conditioned fear in rodents but an inhibitory avoidance paradigm that uses ethologically relevant stimuli has yet to be developed. To expand the use of these stimuli into inhibitory avoidance conditioning, an inhibitory avoidance paradigm was developed to include a range of ethologically relevant psychologically (predator exposure, physical restraint) and physically aversive stimuli (electric shock). Adult male Sprague-Dawley rats were conditioned using a step-through inhibitory avoidance model to associate crossing between two compartments with the presentation of an aversive stimulus. Subjects were assessed for conditioned fear measured by crossing latency, freezing behavior and defecation during conditioning and a contextual memory test. Freezing behavior within the conditioning chamber remained constant throughout conditioning regardless of stimulus but all groups conditioned with an aversive stimulus showed significant increases in crossing latency both overtime and during the retention test compared to subjects that received no aversive stimulus after crossing, indicating that inhibitory avoidance conditioning was achieved. Significant increases in defecation were also observed for footshock and predator exposed animals and this effect was intensified by predator exposure, but only after repeated exposures. With this, both predator-based and restraint-based

variations of the inhibitory avoidance model (PBIA and RBIA, respectively) have been successfully established and have been shown to induce evidence of emotionality similar to those seen in traditional shock-based inhibitory avoidance (SBIA) models. Successful development of PBIA and RBIA expands the range of stimuli that can be used with conventional inhibitory avoidance models, allowing for investigation into topics that have yet to be addressed in inhibitory avoidance conditioning.

Chapter One:

Introduction

Disorders characterized by excessive feelings of fear and anxiety that interfere with day-to-day living are among the most prevalent of all psychological disorders, afflicting 18.1% of the adult population of the United States (National Institute of Mental Health, 2016). Post-traumatic Stress Disorder, a sub-category of trauma- and stressor-related disorders, is often triggered by the experience of a stressful event in which an individual is exposed to "actual or threatened death, serious injury or sexual violation", either through direct means, witnessing the event or learning about an event experienced by someone that individual is close to (American Psychiatric Association, 2013). The prevalence of PTSD is steadily climbing and estimates have projected that as many as 8 in every 100 people in the U. S. will be diagnosed with PTSD at some point in their lifetime, with 8 million people experiencing symptoms of PTSD during a given year (Gradus, 2016). Therapies, such as cognitive behavioral therapy or antidepressant medications, may be used to help cope with or alleviate some of the more debilitating symptoms of these disorders but a wholly effective treatment has yet to be established (National Institute of Mental Health, 2016). A thorough understanding of the causes and manifestations of anxiety disorders must be developed in order to generate and improve treatment for those in need.

Paradigms Used To Study Fear and Anxiety

Translational research that uses animal models to simulate human experiences allows for the investigation of innate and conditioned fear during memory consolidation and retrieval to develop an integrated understanding of anxiety disorders. The use of animal models that resemble

the human experience provides an opportunity to investigate these processes with more detail than can be obtained using human research alone as it allows for more invasive measurements of these systems. Research on the physiological effects of fear can be assessed alongside behaviors observed in response to an aversive stimulus or situation to allow for a clearer understanding of fear and anxiety as a whole. Rodent models of learning and memory often use conditioning paradigms that incorporate elements of fear, termed 'fear conditioning', to study the development of anxiety-like behaviors.

Fear conditioning models can be used to assess a subject's memory of an experience or specific stimulus, often in the form of an aversive stimulus. Contextual fear conditioning utilizes classical conditioning procedures to associate a context with the presentation of an aversive stimulus, frequently in the form of a mild electric shock to the animal's footpads, e.g., a footshock, although other stimuli are possible (see Figure 1). Through repeated pairings of an aversive stimulus and a conditioning context, the conditioning context acquires some of the affective qualities of the aversive stimulus, resulting in increased species-typical fear behaviors, such as freezing or risk assessment, in anticipation of the presentation of the aversive stimulus.

Fear conditioning models assess both fear and anxiety. An important distinction between the two is that fear requires a danger on which the emotion is focused that is both known and external whereas anxiety is induced by the attempt to internally cope with a stimulus, suggesting that this state is characterized more by internal rather than external reactivity (Sapolsky, 2004; Steimer, 2002). Even though the divergence may seem clear, it is often difficult to separate fear and anxiety as they share similar overt features and can occur simultaneously (Gross & Canteras, 2012). As a whole, fear conditioning models assess both innate and conditioned, or learned, fear. Innate fear is directed towards a specific, intrinsically threatening stimulus, such as a footshock or

a live predator. Learned fear, which may present with similar overt behaviors, is directed towards a context or a stimulus that has become associated with the presentation of the aversive stimulus (Gross & Canteras, 2012; Ledoux, 2000).

Inhibitory Avoidance Conditioning

Inhibitory avoidance conditioning is a complex form of contextual fear conditioning that requires learning through both classical and operant conditioning. In this task, a subject learns to associate a specific context with the presentation of an aversive stimulus and also that this experience is contingent on the subject's choice to move into that context (Cammarota, Bevilaqua, Kerr, Medina, & Izquierdo, 2003; Liang, 2009; Ogren & Stiedl, 2015). Step-through inhibitory avoidance conditioning occurs within a rectangular chamber that is divided into two compartments, one brightly lit and the other devoid of light (Fig. 1).

Figure 1. Contextual fear conditioning paradigms. Schematic demonstrating the setup of common Contextual Fear Conditioning (A) and Inhibitory Avoidance Conditioning (B) tasks.

A unique factor of this model is that it relies on the subject's natural tendency to move away from bright, open spaces and seek shelter in dark, enclosed spaces due to the innate fear of exposure to threatening stimuli. During conditioning, fear of the bright compartment promotes movement into the dark but, when the subject moves into the new compartment, an aversive stimulus is administered in the form of a footshock. With repeated pairings, the task becomes more cognitive in nature as the subject learns to inhibit its natural tendency to move toward the dark in favor of remaining inside the bright compartment. When conditioning has successfully occurred,

subjects will display conditioned behaviors of fear and anxiety, such as increased crossing latency and freezing behavior, in anticipation of the aversive stimulus when they are returned to the chamber at a later time (Ogren & Stiedl, 2015; Quillfeldt, 2015). Without interference, memory for shock-based inhibitory avoidance conditioning is long lasting and can be retrieved as far as a year from the initial conditioning session (Zoladz, Woodson, Haynes, & Diamond, 2010).

Behavioral Assessments in Fear Conditioning Paradigms

Fear conditioning models rely on species-typical behaviors to assess learning and memory for an aversive event or stimulus. These species-typical responses are innately determined and are produced in response to a multitude of threatening stimuli, such as footshock or a predator (Bolles, 1970; Fanselow, 1994; Gross & Canteras, 2012).

Freezing, or immobilization of the animal in response to a threat, is a behavior frequently observed in response to an aversive stimulus and involves the complete behavioral arrest of any movements other than breathing. Freezing behavior is useful in fear research as it is an innate behavioral response shared across animal species and is produced in response to a multitude of threatening stimuli, allowing for comparisons both across species and aversive stimuli (Bolles, 1970; Gross & Canteras, 2012). Generally, freezing is considered a sign of intense fear and the amount or degree of this behavior can be used to assess the intensity of fear that an animal is experiencing (Kalin, Shelton, Rickman, & Davidson, 1998). Conditioned freezing can also be expressed when an animal is returned to the context in which the original conditioning occurred (Maren, 1999; Wallace & Rosen, 2001; Wilensky, Schafe, Ledoux, & Keck, 2000; Yang & Liang, 2014; Zoladz, Fleshner, & Diamond, 2012). This behavior is commonly regarded as a physical representation of fear or emotionality in the subject and is used across a variety of fear conditioning models, including contextual fear conditioning. In inhibitory avoidance, this behavioral measure

is assessed during both conditioning and the retention test as it is produced to both unconditioned and conditioned stimuli.

Inhibitory avoidance models also use crossing latency as a measure of memory for the aversive stimulus. Crossing latency, or the time it takes for the subject to move into the enclosed compartment, is used to measure 'inhibitory avoidance' of the dark compartment. This measure, unique to inhibitory avoidance models, assesses the degree to which the fear generated by the aversive stimulus is greater than the global anxiety induced by exposure to potential threats within the bright compartment. Step-through inhibitory avoidance tasks rely on the subjects' innate preference for the dark compartment. As the bright compartment contains little cover, leaving the subject exposed and vulnerable to potential threats, relocation into the dark compartment where the subject can be concealed is preferable (Ogren & Stiedl, 2015). Through the process of operant conditioning, in which movement into the dark compartment becomes associated with an aversive experience, the subject must 'inhibit' its natural tendency and instead 'avoid' the dark compartment. Crossing latency is an assessment of the inhibition of this propensity and directly measures the subject's avoidance of the dark compartment. Crossing latency can be used to measure memory strength for the aversive stimulus as the longer the subject remains in the bright compartment, the longer they avoid the presentation of the aversive stimulus. In general, longer crossing latencies indicate a powerful memory of the aversive experience (Ogren & Stiedl, 2015).

Brain Structures Involved in Inhibitory Avoidance Conditioning

Unconditioned and conditioned fear responses in shock-based inhibitory avoidance models have been shown to rely on a few key brain structures, namely the hippocampus and amygdala (Ledoux, 2000). Similarly, in humans, retrieval of emotionally charged information, in the form of a fearful memory or an association developed in a laboratory setting, has been shown to increase

activity within the hippocampus and amygdala more so than emotionally neutral information (Cahill et al., 1996; Dolcos, LaBar, & Cabeza, 2005; LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998).

The Hippocampus

The hippocampus, a large structure located within the temporal lobe of the brain, is implicated in the consolidation and retrieval of episodic memory for both neutral and emotionally charged information. It has been suggested that the hippocampus influences memory by establishing and storing a conjunctive representation of an emotional experience, which includes contextual, sensory, temporal and spatial information (Halonen, Zoladz, Park, & Diamond, 2016; Matus-Amat, Higgins, Barrientos, & Rudy, 2004; Pasquini et al., 2002; Pentkowski et al., 2006; Sanders, Wiltgen, & Fanselow, 2003; Yang & Liang, 2014). Studies have shown that lesions of the ventral hippocampus decrease crossing latency in shock-based inhibitory avoidance tasks and similar measures of anxiety in the elevated plus maze (Bannerman et al., 2002; Kjelstrup et al., 2002; McHugh, Deacon, Rawlins, & Bannerman, 2004).

The Amygdala

Associations developed during fear conditioning are stored within the hippocampus and are relayed through direct and indirect connections to other emotional processing centers, such as the amygdala and the medial hypothalamus, to aid in memory consolidation and retrieval (Risold & Swanson, 1997). Several of the twelve regions of the amygdala, particularly its lateral (LA), basolateral (BLA), medial (MeA) and central (CEA) nuclei, have been shown to contribute in unique ways to the development of fear conditioning but their functional specificity is still being identified (Canteras & Swanson, 1992; Gross & Canteras, 2012; Ledoux, 2000; Petrovich, Canteras, & Swanson, 2001). The LA, BLA and CEA of the amygdala are believed to modulate

acquisition and memory consolidation in inhibitory avoidance as inactivation of these areas cause dose-dependent impairments in conditioning to shock-based inhibitory avoidance (Coleman-Mesches & McGaugh, 1995; Daher & Mattioli, 2015; Izquierdo et al., 1997; Roozendaal & McGaugh, 1996; Wilensky et al., 2000). Indicators of recent neuronal activity are also seen within the LA, BLA, and CEA after training (Pasquini et al., 2002). Huang et al. (2013) observed an increase in immediate early gene immunoreactivity, indicating recent neuronal activity, within the rodent amygdala during acquisition of shock-based inhibitory avoidance conditioning.

The Effect of Various Stimuli on Brain Activity in Other Fear Conditioning Models

Footshock is an aversive stimulus commonly used in fear conditioning models for the robust conditioned freezing it produces and the ease in which this stimulus can be applied. Often, standard inhibitory avoidance conditioning chambers do not allow for the presentation of alternative aversive stimuli and variations of the inhibitory avoidance task may be limited, for example, to shock strength and presentation timing (Canto-de-Souza & Mattioli, 2016; Izquierdo et al., 1997; Lovitz & Thompson, 2015; Parfitt, Campos, Barbosa, Koth, & Barros, 2012).

The choice of stimulus used in any type of fear conditioning is crucial for its ability to represent the psychological and physiological development of anxiety disorders from a translational standpoint. While the use of an electric shock allows for investigation into how physically aversive stimuli influence learning, the use of ethologically relevant stimuli could shed light on the complex nature of anxiety disorders (Canteras, Mota-Ortiz, & Motta, 2012; Goswami, Rodríguez-Sierra, Cascardi, & Paré, 2013; Hegab, Kong, Yang, Mohamaden, & Wei, 2014). Other fear conditioning paradigms have begun to address this issue and permit the use of aversive stimuli that may be encountered in an animal's natural environment. Aggressive conspecifics, natural predators, and physical restraint have been successful in inducing both innate and long-lasting

conditioned fear in rodents but, as it stands today, an inhibitory avoidance paradigm that uses ethologically relevant stimuli has yet to be developed (Gross & Canteras, 2012; Pentkowski, Blanchard, Lever, Litvin, & Blanchard, 2006; Silva et al., 2013; Zoladz, Fleshner, & Diamond, 2012).

Research addressing the effect of various stimuli across similar fear conditioning paradigms suggests that different stimuli activate distinct areas of the brain that ultimately converge on downstream pathways to produce similar overt fear responses. Although it has not been tested using inhibitory avoidance models, studies using alternative fear conditioning methods suggest that the areas of the brain accessed during conditioning to aversive stimuli may not be universal, but rather the extent of each area's individual involvement is dictated by the type of stimulus used during conditioning.

The Hippocampus

Further division of the hippocampus into its dorsal and ventral segments suggests that the dorsal and ventral hippocampus contribute differently to emotional memory consolidation and retrieval (Fanselow & Dong, 2010).

The dorsal hippocampus (DH) has been shown to contribute to the consolidation and retrieval of contextual information about an emotional experience while the ventral hippocampus (VH) contributes to the regulation of anxiety-like behavior (Bannerman et al., 2004). Lesions to the entire hippocampus reduce conditioned defensive responses to contextual stimuli but spare unconditioned responses to threats (Kim, Rison, & Fanselow, 1993; Phillips & LeDoux, 1992). With functional or irreversible lesions that spare passing axons, DH lesions show no effects in tasks of both innate and conditioned emotional responses to stimuli (Bannerman et al., 2003; Degroot & Treit, 2002, 2004; Kjelstrup et al., 2002; Pentkowski et al., 2006).

It has been suggested that the VH is preferentially involved in regulating anxiety to anticipatory threat rather than fear. Pentkowski et al. (2006) observed that excitotoxic lesions of this region do not affect innate, unconditioned behavioral responses to threats of immediate nature, such as footshock or the presence of a predator. Pentkowski et al. (2006) also found that, although these lesions did not affect responses to immediate threat, VH lesions impaired responses to exposure to a predator odor as well as to the context in which the threat was experienced, two stimuli that suggest the potential or future occurrence of a stressor. Additionally, post-training lesions of the VH but not DH impair measures of emotionality, such as freezing, to both auditory and contextually conditioned stimuli (Ballesteros et al., 2014; Trivedi & Coover, 2004). Consistent with this perspective, McHugh, Deacon, Rawlins, & Bannerman (2004) observed that excitotoxic lesions of the VH decreased latency to cross from the bright to the dark compartment in a variant of the inhibitory avoidance task. Similarly, electrolytic lesions of the VH have also been shown to decrease similar measures of anxiety in the elevated plus maze (Bannerman et al., 2002; Kjelstrup et al., 2002).

Connections between the hippocampus and other brain structures provide avenues for the transmission of information about fearful events. The hippocampus maintains direct projections to the amygdalar complex, among many others. The ventral CA1 region of the hippocampus maintains direct reciprocal connections with the LA, BLA, MeA and Basomedial (BMA) nuclei of the amygdala, which contribute to information processing in response to aversive stimuli (Canteras & Swanson, 1992; Petrovich, Canteras, & Swanson, 2001).

Consolidation and retrieval of contextually conditioned stimuli to a variety of stressors is known to rely on the hippocampus (Pasquini et al., 2002; Pentkowski et al., 2006; Yang & Liang, 2014). It is well established that the hippocampus is important for the consolidation of fear

conditioning; however, involvement of the DH and VH may again be split. Application of corticosterone, a glucorticoid whose release is significantly elevated in response to stress, to the DH and VH produces inverse effects, impairing and enhancing neural plasticity, respectively (Krugers, Zhou, Joëls, & Kindt, 2011; Maggio & Segal, 2009). Further evidence suggests that the DH be limited to memory consolidation and may not be necessary for retrieval as protein synthesis in this area is required for consolidation. In spite of this, re-exposure to a footshock conditioned context has been shown to increase c-fos expression, a protein associated with recent neuronal activation, within the DH (Canto-de-Souza & Mattioli, 2016; Phillips & LeDoux, 1992; Strekalova et al., 2003).

The Amygdala

The amygdala contains approximately twelve different regions that influence the response to fear (Gross & Canteras, 2012; Ledoux, 2000). The LA, BLA, MeA, and CEA have been shown to contribute to fear conditioning but recent evidence has come to suggest that nuclei within the region respond specifically to different stimuli.

Lesions to the LA and the BLA have been shown to impair conditioning in shock-based fear conditioning paradigms (LeDoux, 2012; Ledoux, 2000; Maren, 2001; Nader, Majidishad, Amorapanth, & LeDoux, 2001; Wilensky et al., 2000). Additionally, lesions to these areas have been shown to impair conditioned fear to predator fur and odors (Takahashi, Hubbard, Lee, Dar, & Sipes, 2007; Vazdarjanova, Cahill, & McGaugh, 2001). However, Martinez et al. (2011) observed an attenuation of impairments in conditioned and unconditioned responses to a live predator and its context when the LA and BLA were individually lesioned but extensive impairments were observed when lesions were given to the MeA, suggesting that amydalar nuclei may have similar but not overlapping functions in regards to the type of stimulus in question.

Additionally, the MeA has been shown to respond to predator rather than footshock related cues (Blanchard, Canteras, Markham, Pentkowski, & Blanchard, 2005; Carvalho et al., 2015; Dielenberg & McGregor, 2001; Nader et al., 2001; Takahashi et al., 2007). Pérez-Gómez et al. (2015) report that portions of the MeA show evidence of neuronal activation in response to cat fur odor but that TMT, a derivative of the urine of a rat's natural predator, the fox, does not elicit the same activation within the MeA despite producing similar fear behaviors. Interestingly, evidence of recent neuronal activation within the MeA is also observed with prolonged periods of physical restraint as well as to other natural predators of the rodent, such as the ferret (Roseboom et al., 2007; Trnečková, Armario, Hynie, Šída, & Klenerová, 2006).

The contribution of the CEA to fear conditioning have been less clear as some studies report activation in the CEA in response predator and footshock whereas others do not (Day, Masini, & Campeau, 2004; Dielenberg, Hunt, & McGregor, 2001; Roseboom et al., 2007). It is also notable that lesions to the CEA impair acquisition of conditioned fear but have no effect on unconditioned behaviors to a predator or conditioned behaviors to its associated context (Martinez et al., 2011; Wilensky, Schafe, Kristensen, & LeDoux, 2006). Evidence of activation within the CEA in response to restraint stress has also been observed (Hsu, Chen, Takahashi, & Kalin, 1998).

The Hypothalamus

Recently, the existence of independent fear circuits within the hypothalamus, a region involved in feeding, sex, and aggression, has been proposed. Much like the nuclei of the amygdala, areas within the medial hypothalamus have been functionally divided into circuits that respond to different types of fear, such as fear of pain, predators, or social threats, as independent areas of the medial hypothalamus are activated in response to each of these types of stimuli (Gross & Canteras, 2012; Kunwar et al., 2015; Martinez, Carvalho-Netto, Amaral, Nunes-de-Souza, & Canteras,

2008; Motta et al., 2009; Pagani & Rosen, 2009; Silva et al., 2013, 2016; for a review, see Zha & Xu, 2015). The hypothalamus contains several highly interconnected structures that make up the Medial Hypothalamic Defense Circuit (MHDC) and, within this circuit, the ventromedial hypothalamic nucleus (VMH) responds robustly to predatory threats (Canteras, Chiavegatto, Ribeiro do Valle, & Swanson, 1997; Dielenberg, Hunt, & McGregor, 2001; Wang, Chen, & Li, 2015).

The dorsomedial and ventrolateral segments of the VMH have recently been implicated in the response to predatory and social threats, respectively (Silva et al., 2013). Lesions to the dorsomedial portion of the VMH (VMHdm) decrease defensive behaviors to live predators but not to threats of a social or physical nature in both rats and mice (Martinez, Carvalho-Netto, Amaral, Nunes-de-Souza, & Canteras, 2008; Silva et al., 2013; see Zha & Xu, 2015 for a review). When the VMHdm is genetically inhibited or ablated, mice show reduced unconditioned behavioral responses to the presence of a live predator but this inhibition has no effect on unconditioned responses to a footshock (Kunwar et al., 2015; Pagani & Rosen, 2009b; Silva et al., 2013). This area also receives connections from both the hippocampus and the amygdalar complex via the lateral septal nucleus (LSN) through the anterior hypothalamic nucleus (AHN) and the bed nucleus of the stria terminalis (BST), respectively (Canteras, 2002; Gross & Canteras, 2012; Janitzky et al., 2015; Risold & Swanson, 1997; Walker & Davis, 1997). As inhibitory c-aminobutyric acid (GABA)ergic neurons participate in the connections between the LSN and the AHN, connections between the hippocampus and LSN result in inhibitory effects on the AHN and, ultimately, the MHDC and the VMHdm (Canteras, 2002).

While the VMHdm responds robustly to innate predatory threats, this structure also shows further specificity to different types of predatory odors. It has been suggested that this area

integrates information for predator perception as it is also activated in response to the presence of predatory kairomones, such as odors produced from cat fur, and non-olfactory predatory information via its reciprocal connections with the MeA and BMA (Canteras, Simerly, & Swanson, 1995; Dielenberg et al., 2001; Pérez-Gómez et al., 2015; Petrovich, Risold, & Swanson, 1996). Specificity to certain types of predatory odors is observed within the VMHdm as TMT does not elicit activation in this area though behavioral responses to cat fur odors and TMT are similar (Janitzky et al., 2015; Pagani & Rosen, 2009b; Pérez-Gómez et al., 2015). In behavioral tests of anxiety, lesions to the VMHdm result in reductions in avoidance tasks and antagonism of this area with the application of inhibitory GABAa agonists decrease measures of anxiety in open field tests. Currently, its contribution to inhibitory avoidance conditioning to a predator has yet to be tested (Bueno, Zangrossi, & Viana, 2007; Colpaert & Wiepkema, 1976).

Whether or not the MHDC is involved in contextual fear conditioning is unclear. Neural markers of activity, such as c-fos expression, can be observed in the ANH and the dorsal premammillary nucleus (PMd), an area responsible for freezing behavior, following re-exposure to a context associated with a predator, suggesting that unconditioned and conditioned predatory stimuli follow a similar processing circuit (Baisley, Cloninger, & Bakshi, 2011; Canteras, Kroon, Do-Monte, Pavesi, & Carobrez, 2008; Cezario, Ribeiro-Barbosa, Baldo, & Canteras, 2008; Staples, Hunt, Cornish, & McGregor, 2005). Yet, Silva et al. (2016) observed reduced expression of conditioned fear responses when animals with lesions of the VMH were returned to the context in which they had been exposed to a predatory threat but markers of recent neuronal activity were not observed in VMH-intact animals after re-exposure to the context.

Processing Converges on Downstream Areas to Generate Behavior

Although the structures discussed previously show specificity to different threat stimuli, information from these areas converges on downstream nuclei to produce common, species-typical behaviors in response to threat (Adamec, Walling, & Burton, 2004; Brandão, Zanoveli, Ruiz-Martinez, Oliveira, & Landeira-Fernandez, 2008; Canteras, 2002; Janitzky et al., 2015; Johansen, Tarpley, LeDoux, & Blair, 2010; Risold & Swanson, 1997; Wang et al., 2015). The VMHdm, AHN, BST and the LSN send heavy projections the ventrolateral portion of the PMd (Canteras et al., 1997; Canteras, Ribeiro-Barbosa, & Comoli, 2001; Canteras, 2002). This area, included in the MHDC, regulates defensive behaviors to psychological threat as lesions to the PMd that impair predatory and social fear do not effect learned fear of footshock (Blanchard et al., 2005; Cezario et al., 2008; Motta et al., 2009).

Information from predatory and pain threats is sent to the dorsolateral and ventrolateral periaqueductal grey (PAG) from its connections with the PMd and the CEA, respectfully (Fanselow, 1980; Vianna & Brandão, 2003). The PAG is important for the expression of freezing behavior as lesions to this area disrupt freezing to predator, social and pain threats (Cezario et al., 2008; Fanselow, Decola, De Oca, & Landeira-Fernandez, 1995; Silva et al., 2013, 2016). It has been suggested that the PAG mediates innate and conditioned defensive responses, such as freezing and avoidance behaviors, to pain and predatory threats as activation in this area is observed when the subject is exposed to a stimulus or its conditioning context (Brandão et al., 2008; Comoli, Ribeiro-Barbosa, & Canteras, 2003; De Oca, DeCola, Maren, & Fanselow, 1998; Ledoux, 2000; Vianna & Brandão, 2003; Wang et al., 2015). Patterns of neural activity within the brain vary with different stimuli but, ultimately, converge on descending nuclei that control the behavioral expression of fear.

Conditioning to Various Stimuli and the Inhibitory Avoidance Task

In many cases, experimenters must generalize across a multitude of experimental paradigms (chronic or acute, Pavlovian or Inhibitory Avoidance, for example) and different aversive stimuli (psychological or physical) as some designs do not allow for the use of a range of aversive stimuli. Overt fear behaviors, such as increased freezing and reduced exploratory behavior, are similar for both conditioned and unconditioned responses to a variety of aversive stimuli; however research suggests that individual stimuli may be processed in unique regions of the brain that ultimately converge on descending nuclei that control the expression of innately determined, species-typical behaviors (Bolles, 1970; Fanselow, 1994; Gross & Canteras, 2012). This may cloud interpretation of fear conditioning studies as similar behavioral responses are produced by activation in different areas of the brain. It is also possible that these variations in brain activity exist similarly in human populations and may contribute to differences in symptomology and development of anxiety disorder subtypes.

Representation of anxiety disorders from a translational standpoint relies on a model's ability to recreate the psychological and physiological development of disorders in a way that reflects the human experience (Canteras et al., 2012; Daskalakis & Yehuda, 2014; Daskalakis, Yehuda, & Diamond, 2013; Goswami et al., 2013). The use of stimuli that are ethologically relevant, such physical restraint or a natural predator, could help uncover the complex nature of these disorders but an inhibitory avoidance model that allows for the use of different classes of stimuli has yet to be developed. To expand the use of ethologically relevant stimuli into inhibitory avoidance conditioning, a step-through inhibitory avoidance model was developed to include a range of psychologically and physically aversive stimuli.

Chapter Two:

Design and Methods

Hypotheses

It was hypothesized that subjects conditioned using ethologically relevant (exposure to a natural predator, physical restraint) or an aversive stimulus traditionally used during fear conditioning (footshock) would show greater measures of emotionality, manifested as increased crossing latency, freezing behavior, and defecation, when returned to the conditioning context compared to subjects that received no aversive stimulus during conditioning, indicating that inhibitory avoidance conditioning had successfully occurred.

In addition, it was expected that subjects conditioned using ethologically relevant stimuli would show increased crossing latencies, freezing behavior, and defecation relative to those that did not receive an aversive stimulus during conditioning, but that exposure to a natural predator would intensify these differences relative to subjects that received physical restraint during conditioning. Also, similarly elevated levels of emotionality were expected for groups receiving footshock or predator exposure as they are both highly aversive stimuli.

Methods

Subjects

Thirty adult male Sprague-Dawley rats weighing between 250 and 275g at the time of acceptance were obtained from Charles River Laboratories (Wilmington, MA) and given one week to acclimate to laboratory housing before experimentation began. Subjects were housed in pairs in standard Plexiglass^{TM} cages and maintained on a 12-hour light/dark schedule in a temperature and

humidity controlled vivarium. Ad libitum access to food and water was given while subjects were in their cage. All procedures were approved by the Institutional Animal Care and Use Committee at the University of South Florida and were conducted in accordance with the principles of laboratory animal care and the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Apparatus

Figure 2. Photograph of the Shuttle Box. All subjects were exposed to a metal shuttle box with both light (left) and dark (right) compartments separated by a metal door.

Subjects were conditioned to associate the presentation of an aversive stimulus with crossing between two compartments of a Shuttle Box. The apparatus consisted of a 25.5 × 30 × 29cm metal chamber with a metal roof, clear plastic walls and a metal door dividing the larger chamber into two smaller compartments (Fig. 2). The two compartments, although identical in size and physical features, differed by a single factor: brightness. The first compartment was illuminated by a bright 120w lamp while the second was darkened by placing black plastic over any area that would let in light. A small section of the second compartment was left uncovered to allow enough light into the chamber so that its features were visible. Stainless-steel rods 5.5mm in diameter and spaced 5cm apart, through which electric footshocks were administered, created the

floor of the chamber. Corn cob bedding was placed underneath to catch any excretions produced during each session. Conditioning sessions and the retention test were conducted inside the Shuttle Box and each chamber was cleaned thoroughly with an ethanol solution before and after each animal.

Procedure

Conditioning. Subjects were trained on a step-though inhibitory avoidance conditioning model in which they learned to associate passing from one compartment to the other with the presentation of an aversive stimulus (Fig. 3, located on pg. 20). At the start of each conditioning session, subjects were placed inside the bright compartment with the door connecting the two compartments closed. After 30 seconds, the connecting door was opened, allowing the animal to cross freely between the two compartments. The time it took for the animal to cross from the bright to the dark compartment after the door opened was recorded as crossing latency. The subject was considered to have crossed when all four paws were inside the dark compartment. Once this criterion had been met and the subject had fully crossed into the dark compartment, the door between the compartments was closed and one of several aversive stimuli were administered. Conditioning sessions were repeated once daily for four days with one trial per session. Each subject received a single aversive stimulus after crossing.

Aversive Stimuli

After crossing into the dark compartment during conditioning sessions, subjects received either an electric shock administered to their footpads, were exposed to an adult female cat or were physically restrained immediately after crossing. Subjects in the Footshock condition received a single 1 sec, 0.3mA footshock generated by a scrambler and administered through the stainless-

steel rods of the compartment floor (Coulbourn Instruments, Allentown, PA) to produce a carefully controlled and consistent electric shock. The level of footshock used was determined

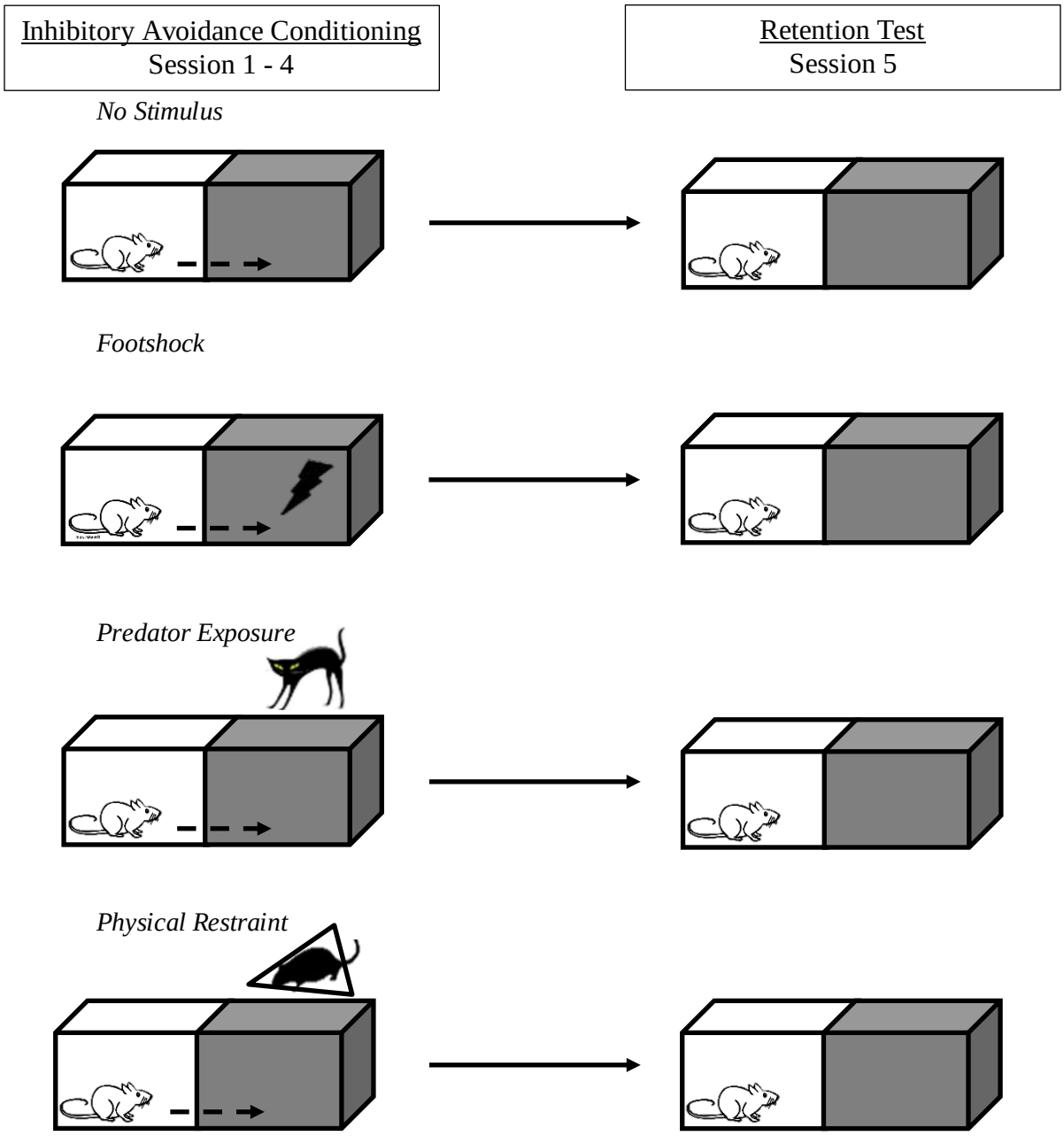

Figure 3. Inhibitory Avoidance Procedure. Schematic demonstrating conditioning procedure and memory assessment. Illustrations represent 0.3mA footshock, exposure to an adult female cat, and physical restraint. Footshock was administered within the conditioning chamber. Both predator exposure and physical restraint were administered outside of the conditioning chamber but in close temporal proximity to crossing.

based on pilot testing and prior reports of ceiling effects produced by higher shock levels in previous adaptations of the inhibitory avoidance task (Quillfeldt, 2015). Fifteen seconds after the termination of the shock, subjects were returned to their cage (Fanselow, Landeira-Fernandez, Decola, & Kim, 1994; Landeira-Fernandez, DeCola, Kim, & Fanselow, 2006).

Subjects in the Predator condition were removed after crossing, restrained using a DecapiCone restraint bag (Braintree Scientific, Braintree, MA) and placed inside a round Plexiglas™ 'rat pie' in the presence of a an adult female cat, a natural predator of the rat, for 10 minutes (Fig. 4). Restraints were used to facilitate conditioning to predator exposure: as subjects were transported across a short hallway to the predator room immediately after crossing, restraint bags served as an intermediary to connect crossing into the compartment with predator exposure. Previous studies have found that this procedure is an effective way to successfully establish and enhance trace conditioning, especially to cat exposure (Conrad, Magariños, LeDoux, & McEwen, 1999; Halonen et al., 2016). Subjects were restrained in the presence of a predator for a total of 10 minutes, a method that has been shown to produce strong conditioned responses and physiological changes in other fear conditioning models (Halonen et al., 2016).

Figure 4**.** Exposure to a predator. Subjects were exposed to cat while being restrained in a rat pie.

Although prior fear conditioning studies have shown that a repeated, brief restraint is minimally aversive, it is possible that physical restraint could contribute to the intensity of the predator stimulus (Gameiro et al., 2006; Melia, Ryabinin, Schroeder, Bloom, & Wilson, 1994; Zhang et al., 2014). In order to isolate the effects of predator exposure, another group of subjects, Physical Restraint, were similarly restrained in DecapiCones and placed in an identical rat pie within a predator-free room for 10 minutes. Additionally, physical restraint is an aversive stimulus that has not previously been used to induce inhibitory avoidance conditioning in past adaptations of the task, allowing the opportunity to test its efficacy as an aversive stimulus.

Subjects in the No Stimulus condition were allowed to cross from one compartment to the other in the manner previously described. However, when a subject in this condition moved into the compartment, no aversive stimulus was administered and the subject was returned to their cage.

Conditioning sessions within the chamber were terminated after the subject crossed into the dark compartment or after 10 minutes, whichever occurred first. If a subject did not cross within 10 minutes of the door opening (i.e., remaining inside the bright compartment for the full 10 minutes), an aversive stimulus was not administered and they were returned to their cage. All subjects were returned to their cages after the termination of their respective stimulus.

Retention Test

Conditioning sessions were repeated once daily for four days with a single aversive stimulus administered immediately after crossing. On the fifth and final day, subjects were returned to the bright compartment of the chamber and were allowed to cross just as they had during conditioning sessions. However, if the subject crossed, no aversive stimulus was administered and the subject was, instead, returned to their cage. Much like during conditioning sessions, subjects that did not cross within 10 minutes were returned to their cage. Expressed

emotionality was measured during conditioning sessions and the retention test using the following assessments: crossing latency, freezing behavior, and defecation.

Chapter Three:

Results

Crossing Latency

Crossing Latency was operationalized as the time it took for the subject to cross from one compartment to the other after the door was opened. Crossing latency was measured on a 0-600 second scale, with larger crossing latencies representing more time spent in the bright compartment before crossing.

In general, crossing latency is used to measure 'inhibitory avoidance' of the dark compartment as the longer the subject remains in the bright compartment, the longer they avoid the presentation of the aversive stimulus. Through the process of operant conditioning, the subject learns to 'inhibit' its natural tendency to search for shelter in enclosed spaces and, instead, 'avoid' the dark compartment. Crossing latency is an assessment of the inhibition of this natural tendency and directly measures the subject's avoidance of the dark compartment. Crossing latency can be used to measure memory strength for the aversive stimulus and longer crossing latencies indicate a powerful memory (Ogren & Stiedl, 2015).

For this behavioral measure, transformation of the data before assessing between-group differences with statistics was required. As testing was terminated after 600 seconds and heavy individual variation is often observed with this task, outliers, ceiling effects and heterogeneity of variance frequently occur. Transformation of the data insured that any statistical analyses were more resistant to these features while also maintaining the integrity of the relationships between data points.

Data were initially assessed for normality and homogeneity of variance and it was found that both normality and homogeneity of variances were violated, as assessed by the Shapiro-Wilk test and Levene's test of homogeneity of variance. Significant positive skewness and kurtosis values for each condition at multiple time-points (see Table A1 within the appendix) suggested that the analysis would benefit from a square-root transformation to normalize the data (Ghasemi & Zahediasl, 2012; Laerd Statistics, 2015; Manikandan, 2010). All further statistical analyses were conducted using the transformed data and the means and standard errors for both untransformed and transformed datasets can be found in Table A2.

A 4 (stimulus) x 5 (session) mixed factor Analysis of Variance was conducted to examine changes in crossing latency. Normality was observed for all combinations of stimulus and session, as assessed by Shapiro-Wilk's test ($p > 0.05$). Homogeneity of covariance, as assessed by Box's test of equality of covariance matrices, was not achieved ($p < 0.01$). Mauchly's test of sphericity indicated that the assumption of sphericity had been violated for the two-way interaction, $\chi^2(9) = 39.43$, $p < 0.01$, and, therefore, degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.60$). A significant effect of stimulus on crossing latency was observed, $F(3, 26) = 12.09$, partial $\eta^2 = 0.58$, $p < 0.01$. Similarly, a significant effect of session was observed, $F(2.40, 62.32) = 49.34$, partial $\eta^2 = 0.66$, $p < 0.01$.

A significant effect of a stimulus x session interaction was also observed, $F(7.19, 62.32) = 9.01$, $p < 0.01$, partial $\eta^2 = 0.51$, suggesting that the main effects for stimulus and session may be misleading. To evaluate the interaction, the simple effects of stimulus and session were individually examined.

A simple effects analysis of stimulus during the first conditioning session suggested that crossing latency was not significantly different between Footshock, Predator, Physical Restraint,

and No Stimulus groups, indicating that there were no observable differences in crossing latency between groups on the first day of training as each group spent similar amounts of time within the bright compartment before moving into the dark compartment (F[3, 26] = 1.73, partial η^2 = 0.17, *ns*).

However, crossing latency significantly increased during the subsequent conditioning sessions. A significant effect of stimulus on crossing latency was observed for the second conditioning session, $F(3, 26) = 3.30$, partial $\eta^2 = 0.28$, $p < 0.05$, but follow-up Bonferroni pairwise comparisons failed to meet the dedicated significance level. Additionally, a significant effect of stimulus on crossing latency was observed for the third conditioning session, $F(3, 26) = 5.10$, partial $\eta^2 = 0.37$, $p < 0.01$. Follow-up pairwise comparisons indicated that subjects in the Footshock group had significantly longer crossing latencies relative to their No Stimulus counterparts during the third conditioning session (Fig. 5). A significant effect of stimulus on crossing latency was also observed for the fourth conditioning session, $F(3, 26) = 9.30$, partial $\eta^2 = 0.52$, $p < 0.01$. Follow-up pairwise comparisons indicated that Footshock, Predator, and Physical Restraint groups had significantly longer crossing latencies than their No Stimulus counterparts during the fourth conditioning session. A significant difference in crossing latency was also observed for the retention test, $F(3, 26) = 28.63$, partial $\eta^2 = 0.77$, $p < 0.01$. Follow-up pairwise comparisons indicated that subjects in the Footshock, Predator, and Physical Restraint groups had significantly longer crossing latencies than their No Stimulus counterparts during the retention test. Differences in crossing latency between the Footshock and Physical Restraint groups also approached significance during the retention test, $p = 0.056$. Overall, this suggests that exposure to an aversive stimulus during conditioning induces longer crossing latencies than being exposed

to no stimulus and that this increase in crossing latency does not depend on the type of aversive stimulus administered during conditioning.

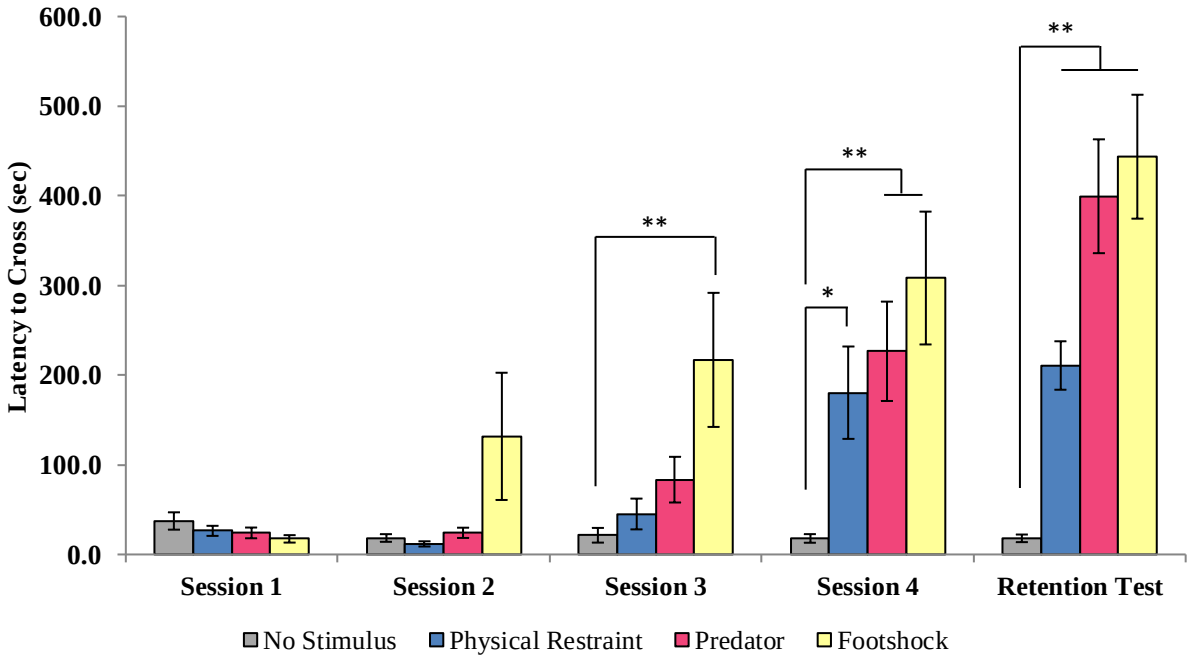

Figure 5. Crossing latency across conditioning sessions and retention test. Results of the simple effect of stimulus on crossing latency are included. Bars indicate standard errors, (*) $p < 0.05$, and (**) $p < 0.01$. Physical Restraint: $N = 6$; No Stimulus, Footshock, or Predator: $N = 8$.

Additionally, the simple effect of session was examined. A simple effects analysis suggested there was a significant effect of session on crossing latency for all groups, indicating that crossing latency changed over time. A significant effect of session was observed for the No Stimulus group and follow-up Bonferroni pairwise comparisons indicated that crossing latency significantly decreased in the fourth conditioning session in comparison to the first session, $F(4, 28) = 3.28$, partial $\eta^2 = 0.32$, $p < 0.05$.

A significant effect of session on crossing latency was observed for Footshock, $F(2.01, 14.04) = 11.94$, partial $\eta^2 = 0.63$, $p < 0.01$, $\varepsilon = 0.50$. Follow-up pairwise comparisons indicated that crossing latency significantly increased during the fourth conditioning session and the retention test individually compared to the first and second conditioning sessions.

A significant effect of session on crossing latency was also observed for Predator exposure, $F(4, 28) = 43.93$, partial $\eta^2 = 0.83$, $p < 0.01$. Follow-up pairwise comparisons indicated that crossing latency significantly increased during the fourth conditioning session and the retention test individually compared to the first, second, and third conditioning sessions. A significant increase in crossing latency was also observed between the fourth conditioning session and the retention test.

Additionally, a significant effect of session on crossing latency was observed for subjects in the Physical Restraint group, $F(1.80, 9.00) = 30.39$, partial $\eta^2 = 0.86$, $p < 0.01$, $\varepsilon = 0.50$. Follow-up pairwise comparisons indicated that crossing latency significantly increased during the retention test compared to the first conditioning session. Also, crossing latency significantly increased during the fourth conditioning session and the retention test individually compared to the second and third sessions.

Overall, the significant effect of stimulus on crossing latency during the condition session and the retention test suggests that inhibitory avoidance conditioning was successfully achieved using both ethologically relevant and traditional fear conditioning stimuli. Additionally, significant increases in crossing latency over time for all three groups exposed to an aversive stimulus suggest that the association between the dark compartment of the conditioning chamber and the presentation of the aversive stimulus became stronger over time, regardless of the type of aversive stimulus.

Freezing Behavior

Additional measurements of species-typical fear behaviors were also measured. Behavioral freezing is an example of a fear response that is stable across animal species. Freezing, or immobilization of the animal in response to a threat, involves the complete behavioral arrest of

any movements other than breathing. Generally, freezing is considered a sign of intense fear and the amount or degree of this behavior can be used to assess the intensity of fear that an animal is experiencing (Kalin et al., 1998).

In this task, freezing behavior was defined by the percent of time the subject was immobile after the door connecting the two compartments was opened. Movement was measured using infrared tracking devices near the top of the conditioning chamber. Each period of arrest was assessed for length and any period greater than three seconds was classified as freezing (Lee & Kim, 1998). The length of each freezing period was combined to generate the total amount of time the animal spent freezing within the bright compartment. As the inhibitory avoidance task terminates when the subject crosses into the dark compartment, the amount of time spent in the bright compartment is dictated by each subject's crossing latency. As crossing latencies can vary, freezing behavior was standardized by dividing a subject's total time freezing by their individual crossing latency. With this, the percent of time spent freezing was obtained and was compared across subjects and groups.

A 4 (stimulus) x 5 (session) mixed factor Analysis of Variance was conducted to examine changes in freezing behavior. Homogeneity of covariance was observed, as assessed by Box's test of equality of covariance matrices, *ns*. Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated for the two-way interaction, $x^2(9) = 27.3$, $p < 0.01$, and, therefore, degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.691$). The main effect of stimulus was not significant, $F(3, 26) = 1.44$, partial $\eta^2 = 0.14$, *ns* (Fig. 6). The main effect of session was also not significant, $F(2.76, 71.83) = 2.09$, partial $\eta^2 = 0.07$, *ns*. Additionally, a stimulus x session interaction was not observed, $F(8.29, 71.83) = 1.06$, partial $\eta^2 = 0.11$, *ns*. This suggests that subjects spent similar amounts of time exhibiting freezing behavior

during each conditioning session and the retention test, regardless of the type of stimulus used. As neither significant main effects nor an interaction effect were observed, follow-up tests were not conducted.

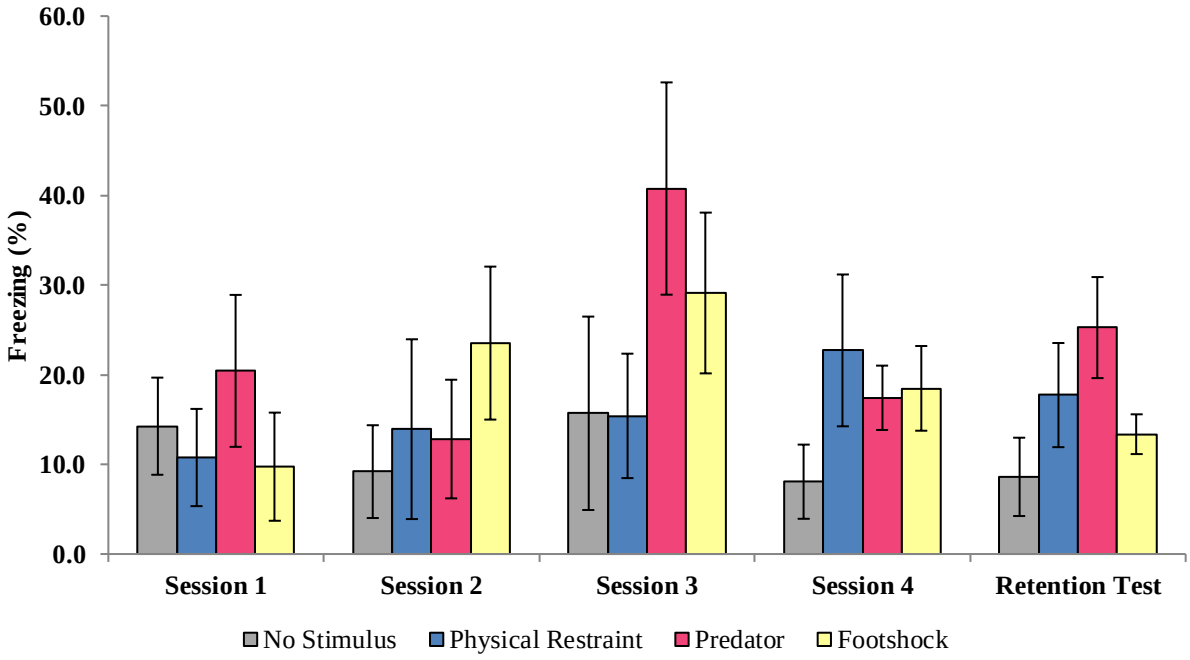

Figure 6. Freezing behavior across conditioning sessions and retention test. Bars indicate standard errors. Physical Restraint: $N = 6$; No Stimulus, Footshock or Predator: $N = 8$.

Defecation

Many animals exhibit freezing behavior in response to an aversive stimulus; however, alternative responses such as increased defecation are possible. Changes in this natural phenomenon can be used as a biomarker of the animal's emotionality (Goldstein, Rasmusson, Bunney, & Roth, 1996; Kjelstrup et al., 2002; O'Mahony et al., 2009). Diamond, Fleshner, & Rose (1994) observed a correlation between the number of fecal boli produced and levels of corticosterone, a hormone released during stress. Similarly, conditioned and unconditioned aversive stimuli have been shown to increase defecation and its use as a measure of emotionality is slowly gaining popularity (Bannerman et al., 2003; Diamond, Park, Heman, & Rose, 1999;

Goldstein et al., 1996; Mönnikes, Schmidt, & Taché, 1993; Nikaido, Miyata, & Nakashima, 2011; Seetharaman, Fleshner, Park, & Diamond, 2016; Zhang et al., 2014; Zoladz, Fleshner, & Diamond, 2013).

As it has not yet been addressed in the context of inhibitory avoidance, changes in defecation were measured as an additional assessment of emotionality. Though defecation can vary with several factors, it serves as a complementary measurement of autonomic responses in addition to other behavioral measures of emotionality. Changes in defecation were assessed by a manual count of the number of fecal units, or boli, present in the conditioning chamber at the end of each session.

A 4 (stimulus) x 5 (session) mixed factor Analysis of Variance was conducted to examine changes in defecation. Homogeneity of covariance was observed, as assessed by Box's test of equality of covariance matrices, however, homogeneity of variances was violated, as assessed by Levene's test ($p < 0.05$). An analysis of the normality of the data produced skewness and kurtosis values, as well as significant Shapiro-Wilk scores, suggestive of a positive skew of the data (see Table A3 within the appendix). Similarly to crossing latency data, a square-root transformation was applied. Mauchly's Test of Sphericity of the transformed data indicated that the assumption of sphericity had not been violated, $x^2(9) = 16.28$, *ns*. Means and standard errors for both untransformed and transformed data can be found in Table A4. A significant effect of stimulus on defecation was observed, $F(3, 26) = 3.49$, partial $\eta^2 = 0.29$, $p < 0.05$. Similarly, a significant effect of session was also observed, $F(4, 104) = 2.69$, partial $\eta^2 = 0.09$, $p < 0.05$.

Additionally, a significant stimulus x session interaction was observed, $F(12, 104) = 2.91$, $p < 0.01$, partial $\eta^2 = 0.25$, suggesting that the main effects for stimulus and conditioning session may be misleading**.** To evaluate the interaction, the simple effects of stimulus and session were

examined. A simple effects analysis of stimulus suggested that defecation did not significantly differ for any of the stimulus types during the first conditioning session, $F(3, 26) = 0.93$, partial $\eta^2 = 0.10$, *ns*, the second conditioning session, $F(3, 26) = 2.05$, partial $\eta^2 = 0.19$, *ns*, or the third conditioning session, $F(3, 26) = 0.84$, partial $\eta^2 = 0.09$, *ns*. However, defecation significantly increased during the fourth and fifth conditioning sessions, $F(3, 26) = 5.28$, partial $\eta^2 = 0.38$, $p < 0.01$ and $F(3, 26) = 5.53$, partial $\eta^2 = 0.39$, $p < 0.01$, respectively. Follow-up pairwise comparisons at both time points indicated that Footshock and Predator groups showed significant increases in defecation relative to their No Stimulus counterparts during the fourth conditioning session (Fig. 7). Similarly, during the retention test, Predator exposed subjects showed a significant increase in defecation relative to their No Stimulus and Physical Restraint counterparts.

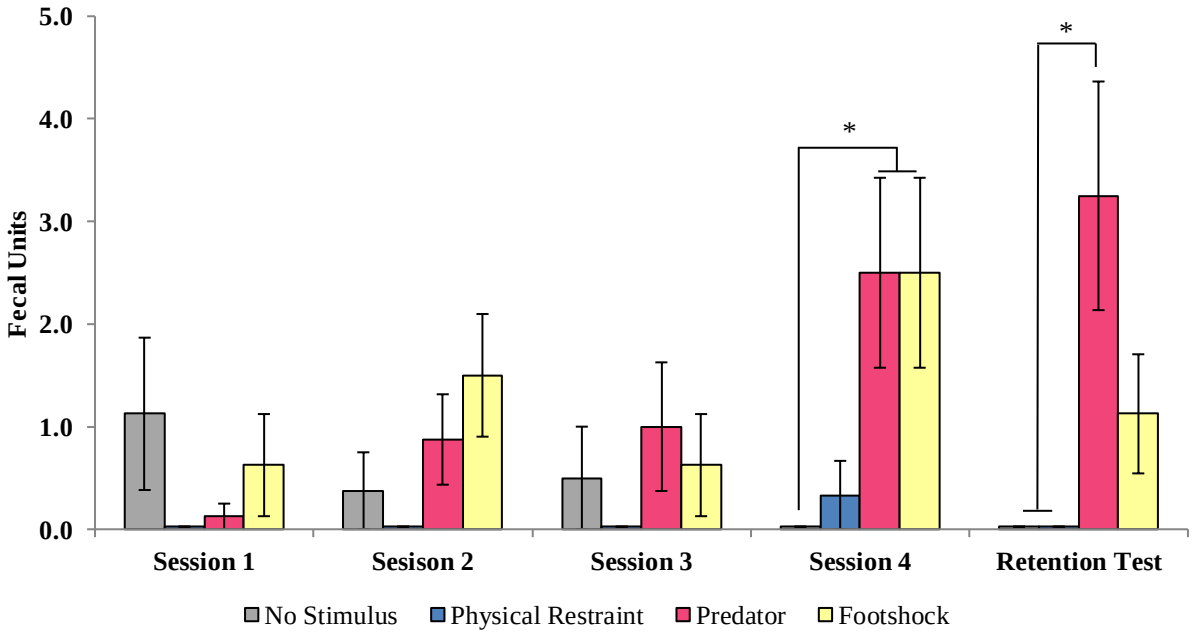

Figure 7. Average number of fecal boli produced within the conditioning chamber across conditioning sessions and retention test. Results of the simple effect of stimulus are included. Bars indicate standard errors and (*) $p < 0.05$. Physical Restraint: $N = 6$; No Stimulus, Footshock or Predator: $N = 8$.

Additionally, the simple effect of session was examined. A simple effects analysis suggested there was a significant effect of session on defecation for Predator exposed subjects,

$F(4, 28) = 3.28$, partial $\eta^2 = 0.319$, $p < 0.05$. Follow-up Bonferroni pairwise comparisons indicated that defecation trended towards a significant increase between the third and fourth conditioning sessions, $p = 0.09$.

Overall, this suggests that various aversive stimuli increase defecation during inhibitory avoidance conditioning and that this effect is intensified for stimuli related to natural predators, but only after repeated presentations (i.e., greater than four pairings of the aversive stimulus and inhibitory avoidance task).

Chapter Four:

Discussion and Future Directions

An inhibitory avoidance model of fear memory was developed to include a range of psychologically and physically aversive stimuli. Subjects were trained in a step-through inhibitory avoidance procedure in which one of several aversive stimuli was administered after the subject crossed between compartments. Subjects either received a footshock, were exposed to an adult female cat while being restrained, or were physically restrained as an aversive stimulus. Crossing latency, freezing behavior and defecation were measured across conditioning sessions and the retention test to assess memory for the aversive stimulus.

Overall, groups exposed to an aversive stimulus during conditioning showed significant increases in crossing latency during the retention test, suggesting a strong association between the conditioning chamber and the presentation of the aversive stimulus. Additionally, the strength of this association grew over time, as evidenced by significant increases in crossing latency across conditioning sessions. Both the significant increase in crossing latency on the retention test as well as over time for Footshock, Predator and Physical Restraint groups indicates that inhibitory avoidance conditioning was successfully achieved using ethologically relevant and traditional fear conditioning stimuli within the same procedure. Similar increases in crossing latency during the retention test for Footshock, Predator and Physical Restraint groups suggest that ethologically relevant aversive stimuli are as effective as physically aversive stimuli in inhibitory avoidance conditioning.

Additionally, all groups exhibited similar amounts of freezing behavior during conditioning and the retention test, suggesting there was no effect of stimulus on freezing behavior. Successful inhibitory avoidance conditioning is often evidenced by both increased crossing latency

and freezing behavior during the retention test for all conditions exposed to an aversive stimulus; however, in this inhibitory avoidance model, it is possible that the type of aversive stimulus influenced specific behaviors exhibited during conditioning. Research suggests that the strategy used to cope with the stress of the aversive stimulus during fear conditioning is associated with the specific type of stimulus used during conditioning (Gross & Canteras, 2012; Hegab et al., 2014; Killcross, Robbins, & Everitt, 1997; Nader, Amorapanth, & LeDoux, 2000; Papes, Logan, & Stowers, 2010; Takahashi, 2014). For example, whereas freezing behavior occurs to a similar extent during both conditioning to footshock and re-exposure to the conditioning context, exposure to a live predator has been shown to induce robust freezing behavior during conditioning but re-exposure to its associated context often results in both freezing and risk assessment behaviors (Ledoux, 2000; Maren, 2001; Pentkowski et al., 2006; Pérez-Gómez et al., 2015). In addition, the low levels of freezing behavior exhibited during conditioning and the retention test by the Footshock group may be due, in part, to the moderate level of footshock used during conditioning (Han et al., 2008; Luyten, Vansteenwegen, Van Kuyck, Deckers, & Nuttin, 2011; Santos, Gárgaro, Oliveira, Masson, & Brandão, 2005).

In comparison to freezing behavior, crossing latency is a more reliable measure of fear in the inhibitory avoidance task because it is less susceptible to variations in behavior. For example, as long as the dark compartment is avoided, crossing inhibition can also be expressed as movement within the bright compartment (Netto & Izquierdo, 1985). Increased grooming behavior is another possible behavior that is commonly expressed when an animal attempts to cope with anxiety induced by fear conditioning tasks (Martinez et al., 2011; Ribeiro-Barbosa, Canteras, Cezário, Blanchard, & Blanchard, 2005). Alternatively, risk assessment behaviors could be produced in response to a previously experienced predatory stimulus. Any of these instances would result in a

decrease in the observed freezing behavior without altering a subject's crossing latency, making it a more reliable measure of fear. Despite the low levels of freezing behavior, increased crossing latency both over time and during the retention test for groups conditioned with an aversive stimulus suggests that inhibitory avoidance conditioning successfully occurred using three different aversive stimuli. An advantage of inhibitory avoidance conditioning is that it takes measurements from multiple forms of expressed of emotionality, making it less sensitive to differences in coping strategies. This provides an advantage for the use of ethologically relevant and traditional fear conditioning stimuli in inhibitory avoidance conditioning as they can confidently be used in future experiments without fear of stimulus-specific coping strategies influencing results.

Significant increases in defecation were observed during inhibitory avoidance conditioning for Footshock and Predator exposed groups and this effect was intensified by a natural predator, but only after repeated exposures. Increased defecation in response to footshock, predators, and their associated contexts is commonly expressed (Moscarello & LeDoux, 2013; Seetharaman et al., 2016; Staples et al., 2008). However, the sustained increase in defecation in predator exposed animals may be due, in part, to the systems that this stimulus engages, namely its engagement of the hypothalamus and its associated structures. Increases in defecation have been observed with stimulation of the hypothalamus and lesions to the VMH have been shown to decrease fecal output in contextual fear conditioning (Colpaert & Wiepkema, 1976; Monnikes, Schmidt, & Tachi, 1993). This is also reinforced by the significant increase in defecation during the retention test between Predator and Physical Restraint groups. Although both groups involved the psychological stress of immobilization, only predator exposed subjects showed increased defecation inside the conditioning chamber.

As prior studies indicate that brief repeated restraint is a mildly aversive stimulus when used in alternative fear conditioning paradigms, it was hypothesized that predator exposure would increase differences in crossing latency and freezing behavior relative to physically restrained subjects (Melia et al., 1994; Sanger, Yoshida, Yahyah, & Kitazumi, 2000; Zhang et al., 2014). However, aside from a significant increase in defecation on the retention test with predator exposure, no significant differences were observed between Predator and Physical Restraint groups on the examined measures. Additional analysis of crossing latency at the retention test suggested that differences in crossing latency between the Footshock and Physical Restraint groups approached significance. One limitation of this study is its small sample size. As noted previously, behavioral responses in inhibitory avoidance tasks are prone to individual variation, which can have noticeable effects when sample sizes are small. That being said, it is possible that any potential differences between groups in this experiment are masked by the effects of variability. Future studies should be conducted with larger sample sizes in order to better address potential differences between groups when conditioning with a variety of aversive stimuli.

It was also hypothesized that similarly elevated levels of emotionality would be expected for Footshock and Predator conditions, as they are both highly aversive stimuli. Although both groups showed significant increases in emotionality compared to subjects that received no stimulus during conditioning, no significant differences in crossing latency, freezing behavior, or defecation were observed between Footshock and Predator groups during the retention test or at any time during conditioning. This further suggests that ethologically relevant aversive stimuli are as effective as a common physically aversive stimulus in inhibitory avoidance conditioning. Although similar behavioral outcomes were produced in response to both stimuli, the sustained increase in defecation with predator exposure suggests that these two aversive stimuli have

different effects on the autonomic nervous system and future research should focus on addressing this issue at a neuroanatomical level (Bannerman et al., 2003; Daviu, Delgado-Morales, Nadal, & Armario, 2012; Gross & Canteras, 2012; Moscarello & LeDoux, 2013; Pagani & Rosen, 2009; Pentkowski et al., 2006; Silva et al., 2013).

With this, both predator-based and restraint-based variants of the inhibitory avoidance task (PBIA and RBIA, respectively) have been successfully developed and have been shown to induce evidence of increased emotionality similar to those seen in traditional shock-based inhibitory avoidance (SBIA). Successful development of these variants expands the range of stimuli that can be used with conventional inhibitory avoidance tasks to include ethologically relevant stimuli in addition to physically aversive stimuli.

Understanding the causes and manifestations of disorders through a translational standpoint will ultimately aid in the improvement of treatments for anxiety disorders. The use of translational models that address potential differences in the effects of various aversive stimuli or conditioning procedures could contribute to a better understanding of differences in symptomology and development of PTSD as well as other disorders related to fear and anxiety. Development of inhibitory avoidance variants that provide a range of aversive stimuli within a single task allows for investigation into topics that have not yet been explored in inhibitory avoidance conditioning. This procedure facilitates the use of pharmacological manipulations to address drug or lesion effects on memory consolidation and retrieval to a multitude of stimuli, opening additional avenues to investigate the complex learning procedures involved in inhibitory avoidance conditioning. Additionally, much like alternative fear conditioning models have already addressed, expanding the range of stimuli used in inhibitory avoidance tasks allows for effects caused by the type of stimulus to be explored. Future studies should investigate the potential differences in neurological

processing and memory retrieval based on the class of aversive stimulus using pharmacological and histochemical methodologies.

Another issue that could be addressed using PBIA and RBIA is differences between types of conditioning paradigms. Studies addressing differences in memory processing and retrieval for contextual fear conditioning conducted within a single chamber in comparison to dual-chamber inhibitory avoidance tasks suggest that, while these tasks are both motivated by fear and result in similar behavioral outcomes during memory assessment, fundamental differences exist between the two tasks that are not often acknowledged (Kim & Jung, 2006; Maren, 2003; Tinsley, Quinn, & Fanselow, 2004; Wilensky et al., 2000). An important aspect of contextual fear conditioning is that, during both conditioning and the retention test, subjects are often confined to a single chamber and the stimulus is presented independent of the subject's behavior. In this conditioning paradigm, a subject may only react to a stimulus and its conditioned context, not control or cope with the presentation of the stimulus. In contrast, inhibitory avoidance tasks assess contextual conditioning to the stimulus but allow the subject to have an active response to fear as the presentation of the stimulus is contingent on the choice to move into the conditioned context, resulting in the recruitment of additional higher-order processing centers (Liang, Yen, Chang, & Chen, 2008; Yang & Liang, 2014). For this reason, differences have been observed in the neural mechanisms underlying Pavlovian and inhibitory avoidance conditioning (Wilensky et al., 2000; Yang & Liang, 2014). Wilensky et al. (2000) reported dose dependent impairments in conditioned responses when the BLA was inactivated with muscimol before contextual fear conditioning, suggesting that the BLA is required for acquisition of this task. Inactivation of the BLA after, but not before, inhibitory avoidance conditioning using similar levels of footshock impaired crossing latency,

suggesting that the amygdala may serve to modulate the strength of the aversive memory elsewhere in inhibitory avoidance conditioning.

The potential differences in the brain regions responsible for conditioning in these tasks make it difficult to compare across paradigms. Until now, the restricted range of aversive stimuli that could be used in the inhibitory avoidance task has limited the ability to address these issues and has required experimenters to compare across different stimuli and paradigms, unintentionally allowing for variations in processing, consolidation and retrieval dictated by the requirements of the task. Widening the stimulus range for the inhibitory avoidance task is the first step in establishing clearer comparisons of stimuli within the inhibitory avoidance task as well as in comparison to their effects on other forms of contextual conditioning.

Finally, future studies should look to integrate PBIA and RBIA into animal models of PTSD and anxiety disorders. Current animal models of PTSD utilize a combination of physiological, psychological, and social stressors, such as electric shock, predator exposure, or chronic stress with a variety of stimuli, to induce the formation and retrieval of emotional memories. In some cases, translational models also attempt to replicate the human experience post-trauma by simulating inadequate or unstable social support, a feature commonly seen in patients who develop PTSD, and have successfully induced long-term behavioral and physiological changes similar to those with PTSD (Brewin, Andrews, & Valentine, 2000; Daskalakis et al., 2013; Ozbay et al., 2007; Sippel, Pietrzak, Charney, Mayes, & Southwick, 2015; Zoladz, Conrad, Fleshner, & Diamond, 2008; Zoladz, Park, Fleshner, & Diamond, 2015; Zoladz & Diamond, 2016). While PBIA and RBIA, taken alone, do not constitute a model of PTSD or other anxiety disorders, future development of the PBIA and RBIA variants should strive to incorporate these

social components to provide a more holistic representation of PTSD and anxiety disorders from a translational standpoint.

Chapter Five

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Chapter Six:

Appendices

Appendix I: Tables

Table A1
Skew, Kurtosis and Shapiro-Wilk results for Crossing Latency

	Conditioning 1		Conditioning 2		Conditioning 3		Conditioning 4		Conditioning 5	
Condition	*Skew*	*Kurt*	*Skew*	*Kurt*	*Skew*	*Kurt*	*Skew*	*Kurt*	*Skew*	*Kurt*
No Stim.	2.06	4.27	1.10	1.40	2.25	5.50	0.94	-0.33	0.32	-1.16
Footshock	1.81	3.52	2.26	5.42	0.92	-0.33	0.33	-0.66	-1.04	0.36
Predator	-0.06	-2.26	0.76	-0.01	1.16	-0.17	0.70	-1.04	0.13	-2.46
Restraint	0.43	-0.81	1.50	2.25	1.55	3.03	1.76	3.29	0.27	-2.23
	S-W	*p*	*S-W*	*p*	*S-W*	*p*	*S-W*	*p*	*S-W*	*p*
No Stim.	0.73	*< 0.01*	0.93	*ns*	0.72	*< 0.01*	0.89	*ns*	0.93	*ns*
Footshock	0.77	*< 0.05*	0.67	*< 0.01*	0.88	*ns*	0.93	*ns*	0.83	*ns*
Predator	0.84	*ns*	0.91	*ns*	0.82	*< 0.05*	0.90	*ns*	0.80	*< 0.05*
Restraint	0.96	*ns*	0.87	*ns*	0.83	*ns*	0.82	*ns*	0.90	*ns*

Note: For skewness, No Stimulus, Footshock, and Predator: SE = 0.75; Restraint: SE = 0.85. For kurtosis, No Stimulus, Footshock, and Predator: SE = 1.48; Restraint: SE = 1.74. No Stimulus, Footshock, or Predator: $N = 8$; Physical Restraint: $N = 6$.

Table A2
Untransformed Mean and Standard Errors for Crossing Latency

Untransformed Data										
	Conditioning 1		Conditioning 2		Conditioning 3		Conditioning 4		Conditioning 5	
Condition	M	SE	M	SE	M	SE	M	SE	M	SE
No Stim.	37.33	9.67	18.35	4.27	21.40	8.19	17.85	4.79	17.96	4.24
Footshock	17.38	4.16	131.71	70.96	217.00	74.78	308.30	74.07	443.64	69.12
Predator	24.01	5.98	24.11	5.70	83.40	25.48	226.58	55.41	399.53	63.49
Restraint	26.32	5.63	11.75	2.89	45.10	17.17	180.42	51.51	210.75	27.02

Transformed Data										
	Conditioning 1		Conditioning 2		Conditioning 3		Conditioning 4		Conditioning 5	
Condition	M	SE	M	SE	M	SE	M	SE	M	SE
No Stim.	5.84	0.68	4.08	0.49	4.19	0.74	3.96	0.74	3.99	0.54
Footshock	4.00	0.44	9.18	2.60	12.95	2.66	16.24	2.66	20.39	2.00
Predator	4.56	0.68	4.66	0.59	8.44	1.32	14.23	1.32	19.53	1.61
Restraint	4.97	0.56	3.31	0.39	6.11	1.25	12.86	1.25	14.37	0.93

Note: Means represent average crossing latency, measured in seconds. No Stimulus, Footshock, or Predator: $N = 8$; Physical Restraint: $N = 6$.

Table A3
Skew, Kurtosis and Shapiro-Wilk Results for Defecation

	Conditioning 1		Conditioning 2		Conditioning 3		Conditioning 4		Conditioning 5	
Condition	*Skew*	*Kurt*	*Skew*	*Kurt*	*Skew*	*Kurt*	*Skew*	*Kurt*	*Skew*	*Kurt*
No Stim.	1.51	0.45	2.83	8.00	2.83	8.00	0.00	0.00	0.00	0.00
Footshock	2.53	6.50	0.36	-1.98	2.53	6.50	0.41	-1.78	1.03	-0.69
Predator	2.83	8.00	0.90	-1.13	2.05	4.19	1.43	2.42	0.57	0.10
Restraint	0.00	0.00	0.00	0.00	0.00	0.00	2.45	6.00	0.00	0.00
	S-W	*p*	*S-W*	*p*	*S-W*	*p*	*S-W*	*p*	*S-W*	*p*
No Stim.	0.60	*< 0.01*	0.42	*< 0.01*	0.42	*< 0.01*				
Footshock	0.54	*< 0.01*	0.80	*< 0.05*	0.54	*< 0.01*	0.84	*. ns*	0.73	*< 0.01*
Predator	0.42	*< 0.01*	0.72	*< 0.01*	0.67	*< 0.01*	0.87	*. ns*	0.86	*ns*
Restraint							0.50	.000		

Note: For skewness, No Stimulus, Footshock, and Predator: SE = 0.75; Restraint: SE = 0.85. For kurtosis, No Stimulus, Footshock, and Predator: SE = 1.48; Restraint: SE = 1.74. No Stimulus, Footshock, or Predator: $N = 8$; Physical Restraint: $N = 6$.

Table A4
Untransformed Mean and Standard Errors for Defecation

	Untransformed Data									
	Conditioning 1		Conditioning 2		Conditioning 3		Conditioning 4		Conditioning 5	
Condition	M	SE	M	SE	M	SE	M	SE	M	SE
No Stim.	1.13	0.74	0.38	0.38	0.50	0.50	0.00	0.00	0.00	0.00
Footshock	0.63	0.50	1.50	0.60	0.63	0.50	2.50	0.93	1.13	0.58
Predator	0.13	0.13	0.88	0.44	1.00	0.63	2.50	0.93	3.25	1.11
Restraint	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.33	0.00	0.00

	Transformed Data									
	Conditioning 1		Conditioning 2		Conditioning 3		Conditioning 4		Conditioning 5	
Condition	M	SE	M	SE	M	SE	M	SE	M	SE
No Stim.	0.53	0.35	0.22	0.22	0.25	0.25	0.00	0.00	0.00	0.00
Footshock	0.38	0.26	0.86	0.33	0.38	0.26	1.20	0.39	0.64	0.32
Predator	0.13	0.12	0.57	0.28	0.58	0.31	1.30	0.34	1.40	0.43
Restraint	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.24	0.00	0.00

Note: Means represent average number of fecal units. No Stimulus, Footshock, or Predator: $N = 8$; Physical Restraint: $N = 6$.

Appendix II: IACUC Approval for Rats

USF UNIVERSITY OF SOUTH FLORIDA

DIVISION OF RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE_USE COMMITTEE

MEMORANDUM

TO:	David Diamond, Ph.D. Dept. of Psychology PCD4118-G
FROM:	Jay B. Dean, Ph.D, Chairperson Institutional Animal Care & Use Committee Division of Research Integrity and Compliance
DATE:	2/6/2013
PROJECT TITLE:	Neuroendocrine Mechanisms and Therapeutics in an Animal Model of PTSD – Rat
AGENCY/SOURCE OF SUPPORT:	Dept. of Veterans Affairs
IACUC PROTOCOL#:	**V 4385**
PROTOCOL STATUS:	**APPROVED**

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC requested modifications/further information in response to that review and has received the required information. The IACUC **APPROVED** your request to use the following animals in your protocol for a one-year period **beginning 2/5/2013 :**

- 2592 Rats, Male

Please reference the above IACUC protocol number in all correspondence regarding this project with the IACUC, Comparative Medicine, or the Division of Research Integrity and Compliance. In addition, please take note of the following:

- **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol.** After three years all continuing studies must be completely re-described in a new application and submitted to IACUC for review.
- **All Comparative Medicine pre-performance safety and logistic meetings must occur prior to implementation of this protocol** [IACUC policy V.10]. Please contact the program coordinator at compmed@research.usf.edu to schedule a pre-performance meeting.
- **All changes to the IACUC-Approved Protocol must be pre-approved by the IACUC [IACUC policyIII.11].** Minor changes can be submitted to the IACUC for review and approval as an amendment or procedural change, whereas major changes to the protocol require submission of a new IACUC application. Minor changes are changes considered to be within the scope of the original research hypothesis or involve the original species and are submitted to the IACUC as an Amendment or Procedural change. Any change in the IACUC–approved protocol that does not meet the latter definition is considered a major protocol change and requires th submission of a new application. More information on what constitutes a minor versus major protocol change and procedural steps necessary for IACUC review and approval are available on the Comparative Medicine web site at http://www.research.usf.edu/cm/amendments.htm
- **All costs invoiced to a grant account must be allocable to the purpose of the grant [IACUC policies IV.5 and V.10].** Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons of convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.

For more information on IACUC policies and procedures, please visit the Comparative Medicine web site at http://www.research.usf.edu/cm/default.htm.

cc: Comparative Medicine
Division of Research Grants

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Appendix III: IACUC Approval for Cats

USF UNIVERSITY OF SOUTH FLORIDA

DIVISION OF RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE USE COMMITTEE

MEMORANDUM

TO:	David Diamond, Ph.D. Dept. of Psychology PCD4118-G
FROM:	Jay B. Dean, Ph.D, Chairperson Institutional Animal Care & Use Committee Division of Research Integrity and Compliance
DATE:	2/5/2013
PROJECT TITLE:	Neuroendocrine Mechanisms and Therapeutics in an Animal Model of PTSD - Cat
AGENCY/SOURCE OF SUPPORT:	Dept. of Veterans Affairs
IACUC PROTOCOL#:	**V 4384**
PROTOCOL STATUS:	**APPROVED**

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC requested modifications/further information in response to that review and has received the required information. The IACUC **APPROVED** your request to use the following animals in your protocol for a one-year period **beginning 2/5/2013 :**

- 4 Cat-Adult, Female

Please reference the above IACUC protocol number in all correspondence regarding this project with the IACUC, Comparative Medicine, or the Division of Research Integrity and Compliance. In addition, please take note of the following:

- **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol.** After three years all continuing studies must be completely re-described in a new application and submitted to IACUC for review.
- **All Comparative Medicine pre-performance safety and logistic meetings must occur prior to implementation of this protocol** [IACUC policy V.10]. Please contact the program coordinator at compmed@research.usf.edu to schedule a pre-performance meeting.
- **All changes to the IACUC-Approved Protocol must be pre-approved by the IACUC [IACUC policyIII.11].** Minor changes can be submitted to the IACUC for review and approval as an amendment or procedural change, whereas major changes to the protocol require submission of a new IACUC application. Minor changes are changes considered to be within the scope of the original research hypothesis or involve the original species and are submitted to the IACUC as an Amendment or Procedural change. Any change in the IACUC–approved protocol that does not meet the latter definition is considered a major protocol change and requires th submission of a new application. More information on what constitutes a minor versus major protocol change and procedural steps necessary for IACUC review and approval are available on the Comparative Medicine web site at http://www.research.usf.edu/cm/amendments.htm
- **All costs invoiced to a grant account must be allocable to the purpose of the grant [IACUC policies IV.5 and V.10].** Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons of convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.

For more information on IACUC policies and procedures, please visit the Comparative Medicine web site at http://www.research.usf.edu/cm/default.htm.

cc: Comparative Medicine
Division of Research Grants

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