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Influence of Temporary Inactivation of the Prefrontal Cortex or Hippocampus during Stress on the Subsequent Expression of Anxiety and Memory

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Influence of Temporary Inactivation of the Prefrontal Cortex or Hippocampus during Stress on the Subsequent Expression of Anxiety and Memory

by

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of the requirements for the degree of
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In loving memory of Eugene Halonen

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Inactivation of the Prefrontal Cortex or Hippocampus Differentially Affects Predator-Induced Fear Memories and Blocks Non-Stressful Memory Impairments

Joshua D. Halonen

ABSTRACT

The neural pathways underlying the symptoms of Post Traumatic Stress Disorder (PTSD) have not been fully elucidated. Intrusive memories, persistent anxiety and other cognitive deficits have been attributed to maladaptive or otherwise aberrant processing in specific brain regions, including the hippocampus, amygdala and prefrontal cortex. Our laboratory has developed an animal model of PTSD which results in the enhancement of memory for a place associated with exposure to a predator, anxiety-like behavior, increased startle and impaired memory in a non-aversive memory task. To better understand how the interaction of the hippocampus and prefrontal cortex contribute to the different symptoms of the disorder, we investigated the transient inactivation of each structure during an intense stressor. Our results show that long-term contextual fear associations involve activity in both the hippocampus and the prefrontal cortex, but only the prefrontal cortex is involved in cued fear memories as well.

Chapter One: Background

Multiple Memory Systems

The idea that brain structures network with one another during learning has lead to a better understanding of the multiple systems that mediate the formation of memories (McDonald & White, 1993; McDonald & White, 1995; McDonald, Devan, & Hong, 2004; Sutherland, McDonald, Hill, & Rudy, 1989; Kim, Lee, Han, & Packard, 2001; Packard & Cahill, 2001; Packard & Teather, 1998; Packard, Hirsh, & White, 1989; Poldrack & Packard, 2003). Although there is debate among researchers about the distinctions between which brain structures are involved in particular memory functions, multiple memory theory is accepted as the nature of memory processes (Meeter, Veldkamp, & Jin, 2009; Weber et al., 2005). Neural networks orchestrate distinct types of skill learning, declarative neutral and emotional memories in parallel. Individual structures process information and communicate with other structures to form a memory and influence behavior to later stimuli.

Strong emotional experiences have powerful effects on the formation of memory, often facilitating durable memories. People who experience acute trauma and respond with intense fear, helplessness or horror and then relive the trauma through intrusive flashback memories are prone to be diagnosed with PTSD. Not everyone exposed to trauma develops PTSD, but in those individuals that develop the disorder, it seems the abnormally durable memory of a particular event comes at the cost of concentration and

memory for trauma-neutral information (Moores et al., 2008; Gil, Calev, Greenberg, Kugelmass, & Lerer, 1990). The following sections will briefly outline PTSD and the paradox between emotional enhancement of traumatic memory and impairment of post-trauma working memory, followed by a summary of the brain structures implicated in these phenomena.

Post-Traumatic Stress Disorder

One of the diagnostic criteria of PTSD is experiencing a traumatic event (McNally, 2003; Moores et al., 2008). To be considered "traumatic," the event must pose an actual or at least a perceived threat to the individual's physical well being and cause a sense of loss of control. Individuals diagnosed with PTSD experience trouble concentrating and functioning in their daily lives. These symptoms are exacerbated by reminders of the trauma which trigger intrusive memories (Bryant, 2003; Reynolds & Brewin, 1999). Accordingly, individuals with PTSD make great efforts to avoid stimuli that remind them of their trauma. While the type (rape, combat, natural disaster, etc.) and characteristics (duration, intensity, or stage of life) of the trauma (Heim & Nemeroff, 2001; Stam, 2007) play a role in whether or not an individual will develop PTSD, only about 25% of individuals who are exposed to trauma develop the disorder (Yehuda, 2001). This supports the hypothesis that there is some fundamental difference between individuals that do and individuals that do not develop the disorder.

One criticism of the reports of cognitive impairments in PTSD patients is that often these investigations report high comorbidity with major depressive disorder (MDD) and a history of substance abuse in PTSD groups, making it difficult to attribute the

cognitive deficits to a single cause. Cognition can be negatively affected by both MDD (Veiel, 1997) and substance abuse (Goldman, 1999; Goldman, Brown, Christiansen, & Smith, 1991). In order to control for these factors Neylan et al. (2004) excluded individuals with MDD or substance abuse and found no differences between PTSD patients and controls on measures of cognitive functioning, including assessments of attention. These findings suggest that deficits in cognitive functioning may not be a characteristic of PTSD; rather, they could be a predisposition to mental illness in general.

Enhancement

The sights, sounds, or even smells related to trauma can evoke a powerful memory. In patients with PTSD reminders of the traumatic event can lead to reliving the initial experience, commonly referred to as a "flashback". In order to conceptualize these flashbacks, researchers have put forth the notion that negative emotion is associated with information learned around the time of a trauma (Ehlers & Clark, 2000). Thus, when presented with a sensory cue associated with negative emotions people with PTSD have anxiogenic intrusive memories specific to the trauma (Diamond, Campbell, Doan, & Park, 1999; Diamond, Park, & Woodson, 2004; Ehlers, Hackmann, & Michael, 2004; Ehlers & Steil, 1994). These intrusive memories can lead people with PTSD to avoid reminders of the trauma because of the intense anxiety they cause (Burstein, 1985) and negatively affect their daily life functioning, as mentioned earlier.

Understanding how memory is enhanced by emotion has long been of interest to psychologists. One type of this enhancement has been labeled a "flashbulb memory" (Berntsen & Rubin, 2006; Brown & Kulik, 1977; Conway et al., 1994; Diamond, Campbell, Park, Halonen, & Zoladz, 2007). In this form of memory, strong emotion

enhances the salience of the sensory information of an experience resulting in a powerful form of learning (Christianson, 1992; Richter-Levin & Akirav, 2003; Diamond et al., 1999; Diamond et al., 2004; Ehlers & Clark, 2000). Thus, emotion enhances recall compared to learning the same information under non-emotional circumstances. People with PTSD show notable differences in brain activity in the amygdala, hippocampus and the prefrontal cortex as compared to controls (Bremner, 1999). The amygdala has been heavily implicated in PTSD and a vast literature exists describing the role it plays in emotion regulation and fear conditioning. The hippocampus and prefrontal cortex are critical for learning and memory (Goldman-Rakic, 1987; Morris, Garrud, Rawlins, & O'Keefe, 1982; O'Keefe & Speakman, 1987). The interactions among these structures during an intensely emotional event, such as a trauma, play a role in the enhancement of memory. In summary, the hippocampus and prefrontal cortex in conjunction with the amygdala are likely candidates for the neural processing responsible for forming flashbulb memories.

Impairment

Many investigators have suggested that intrusive memories are detrimental to concentration and working memory in PTSD patients (Bremner, Vermetten, Afzal, & Vythilingam, 2004; Chemtob et al., 1999; Halligan, Clark, & Ehlers, 2002; Jelinek et al., 2006; Kivling-Boden & Sundbom, 2003; Litz et al., 1996; McFarlane, Weber, & Clark, 1993; Schonfeld & Ehlers, 2006; Sutker, Winstead, Galina, & Allain, 1991; Tapia, Clarys, El Hage, Belzung, & Isingrini, 2007; Vasterling, Brailey, Constans, & Sutker, 1998b; Yehuda et al., 1995). Chemtob et al. (1999) examined the ability of Vietnam veterans with PTSD to attend to a primary digit detection paradigm while concurrently

viewing either neutral or Vietnam –related distracters, and found that PTSD patients' performance was worse than other groups of combat exposed or psychopathology patients when trauma related pictures were presented. These and other results (Bremner et al., 1995) indicate that intrusive memories can interfere with daily functioning in PTSD patients by reducing their ability to pay attention to information like names and other pieces of information. Physiological studies using event-related potentials (ERP) suggest that trauma-neutral information is abnormally processed, such that PTSD augments the way people evaluate the significance of a stimulus and the subsequent executive processes associated with working memory (McFarlane et al., 1993; Galletly, Clark, McFarlane, & Weber, 2001). The stimulus is then processed as a danger signal, rather than a neutral environmental stimulus.

Stress can have detrimental effects on hippocampus-dependent learning and memory. In fact numerous studies have reported declarative and working memory impairments, along with deficits in attention, in PTSD patients (Bremner, Krystal, Southwick, & Charney, 1995; Bremner et al., 1993; Gilbertson, Gurvits, Lasko, Orr, & Pitman, 2001; Golier et al., 2002; Jenkins, Langlais, Delis, & Cohen, 1998; Sachinvala et al., 2000; Moradi, Doost, Taghavi, Yule, & Dalgleish, 1999; Uddo, Vasterling, Brailey, & Sutker, 1993; Vasterling, Brailey, Constans, & Sutker, 1998; Barrett, Green, Morris, Giles, & Croft, 1996). However, some researchers have found no differences between individuals with PTSD and healthy controls or subjects when not using trauma related material to influence cognitive functioning (Barrett, Green, Morris, Giles, & Croft, 1996; Crowell, Kieffer, Siders, & Vanderploeg, 2002; Neylan et al., 2004; Zalewski, Thompson, & Gottesman, 1994). This means that not all cognitive capabilities of PTSD

patients are always affected by the disorder and they are able to perform some tasks normally.

Animal Models of PTSD

Although PTSD remains a disorder that is unique to humans, the limitations of human research necessitate a valid animal model of PTSD. Such a model would potentially allow investigations into the factors that contribute to the disorder's development, as well as, the neurobiological progression of the disorder. This would pave the way to study effects of therapeutic agents on the treatment of the disorder. Stressors such as electric shock, immobilization (i.e., restraint stress), underwater trauma, and predator stress have been used to produce behavioral effects in rodents that are comparable to those observed in humans with PTSD. However, the fact that many of these models do not reliably generate the range of symptoms displayed in PTSD patients warrants a set of criteria that all animal models of PTSD should meet before they are accepted by the scientific community. According to Yehuda & Antelman (1993) animal models of PTSD should attempt to incorporate five key aspects: 1) Very brief stressors should be capable of inducing the biological and behavioral sequelae of PTSD. 2) The stressor should be capable of producing the PTSD-like sequelae in a dose-dependent manner because the disorder is produced by a threshold "dose" of stress in humans. 3) The stressor should produce biological alterations that persist over time or become more pronounced with the passage of time. 4) The stressor should induce biological and behavioral alterations that have the potential for enhanced or reduced responsiveness to different aspects of the environment. 5) Variability in response to a stressor should be

present either as a function of experience (e.g., prior history and post-stress adaptations), genetics, or an interaction of the two (Yehuda & Antelman, 1993, p. 480-482). Once an animal model satisfies these criteria, the behavioral and biological consequences of trauma can be further elucidated.

To model PTSD, most investigators expose rodents to some form of stress and then assess the effects of that stress on physiology and behavior. The investigators typically compare the entire stressed groups to control animals. Cohen, Zohar, & Matar (2003) argued that some animals appear to be more vulnerable to the stress than others, which supports the fifth criterion in Yehuda & Antelman's (1993) manuscript. Given this argument, Cohen et al. (2003) examined the differential response of rats to intense stress by exposing 150 rats individually to a cat for a period of 10 minutes and then examined their behavior on the elevated plus maze one week later. As a group, the stressed rats exhibited greater levels of anxiety on the elevated plus maze, relative to controls. Interestingly, within the stressed group of rats, some did not show elevated levels of anxiety and freely explored the open arms of the maze. Therefore, the investigators used cutoff behavioral criteria to divide the stressed rats into well-adapted (WA) or maladapted (MA) rats, based on time spent in the closed arms and entries into the open arms. Physiological analyses indicated that the MA rats exhibited greater levels of adrenal hormones shortly after the stress compared to WA rats. The MA rats also displayed greater sympathetic nervous system tone and lower vagal tone based on lower heart rate variability, showing higher high-frequency and lower low-frequency component of their heart rates. Cohen and colleagues have replicated and extended this work by reporting similar effects on other behavioral measures, such as the acoustic

startle response; as well as the use of a different stressor (underwater trauma) to manifest similar results (Cohen, Zohar, Matar, Kaplan, & Geva, 2005; Cohen et al., 2004). Collectively, these findings support the notion that stress does not affect all rodents the same; rather, some appear to be more vulnerable to the effects of stress. These studies relate to humans in that not every traumatized individual reacts the same way to stress and that trauma can come in different forms.

The study of varying types, intensities, and durations of stress have also provided valuable insight into the physiological and behavioral changes in rodents. Chronic restraint stress (6 hrs/day for 21 days) leads to the remodeling of hippocampal dendrites (Magarinos, McEwen, Flugge, & Fuchs, 1996; Magarinos & McEwen, 1995) and impairments of hippocampus-dependent, spatial memory (Conrad, Galea, Kuroda, & McEwen, 1996; Luine, 1994; Luine, Villegas, Martinez, & McEwen, 1994). Other investigators have studied the effects of a small number of stress sessions or a single stress session with periodic reminders of the "trauma" on long-term behavior in rodents. (Pynoos, Ritzmann, Steinberg, Goenjian, & Prisecaru, 1996) exposed mice to footshock (2 mA for 10 seconds) and then assessed their behavioral response 1, 21, or 42 days later. Some of the stressed mice were reminded weekly throughout the experiment by placing them back in the apparatus where they received the shock. Only mice that were given reminders of the shock exhibited increased anxiety on the elevated plus maze 1, 21, and 42 days after being shocked, but they did not demonstrate an exaggerated startle response until six weeks post-stress. Servatius and colleagues (Servatius, Ottenweller, Bergen, Soldan, & Natelson, 1994; Servatius, Ottenweller, & Natelson, 1995; Adamec & Shallow, 1993) also observed a delayed sensitization of startle following exposure to

repeated restraint and tailshock stress. These findings have been inconsistent, stress may induce a delayed sensitization of rats' startle response, but the timeline for this effect is unclear.

An investigation by Adamec & Shallow (1993) found heightened anxiety-like behavior in rats following a single five minute exposure to a cat, as indicated by a reduction in the ratio of time spent in the open- to closed-arms on the elevated plus maze up to three weeks later. This effect is theoretically based on NMDA-receptor-dependent plasticity in the amygdala, which is responsible for the lasting effects of cat exposure on anxiety (Adamec, Muir, Grimes, & Pearcey, 2007). Further work by Adamec and colleagues (Adamec, Burton, Shallow, & Budgell, 1999a; Adamec, Burton, Shallow, & Budgell, 1999b; Blundell, Adamec, & Burton, 2005) has supported the argument that these effects are mediated, in part, by NMDA-receptor-dependent plasticity. When rats were administered competitive NMDA-receptor antagonists 30 minutes prior to cat exposure, it blocked lasting increases in anxiety-like behaviors. However, these drugs were incapable of blocking the stress-induced increase in anxiety-like behaviors if they were administered 30 minutes after cat exposure, suggesting that they had to be present at the time of the stress to be effective.

Studies have observed NMDA-receptor-dependent synaptic plasticity in the amygdala as a result of fear conditioning (Bauer, Schafe, & LeDoux, 2002; Rogan, Staubli, & LeDoux, 1997), and the administration of NMDA-receptor antagonists within the ventricular system (Fanselow, Kim, Yipp, & De Oca, 1994; Kim, DeCola, Landeira-Fernandez, & Fanselow, 1991; Kim, Fanselow, DeCola, & Landeira-Fernandez, 1992) or amygdala (Maren, Aharonov, Stote, & Fanselow, 1996) prevents the formation of fear

memories in rodents. These findings support the idea that stress induces NMDA-receptor-dependent plasticity, probably within the amygdala, results in heightened anxiety-like and fear behavior.

Our laboratory has recently developed a novel form of modeling PTSD in rats using the combination of predator exposure and restraint, in conjunction with daily social instability (Zoladz, Conrad, Fleshner, & Diamond, 2008). In this model two stress sessions with social instability were sufficient to produce enhanced anxiety-like behavior on the elevated plus maze, increased startle and an impaired ability to recognize a familiar object. Animals that went through the trauma-like experiences also exhibited differences in their hypothalamic-pituitary axis and cardiac functions compared to controls. The following experiments were designed to manipulate rats' information processing in specific brain regions to exacerbate or mitigate the expression of PTSD-like symptoms. These experiments will also extend our model by manipulating the number and type of stressors to investigate the effects on contextual and cued fear memory, and other behavioral consequences, that a single cat and immobilization session have on rats.

Neuroanatomy

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. According to some reports, the CA1 region of the hippocampus plays an integrative role 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).

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 according to Shapiro & Eichenbaum (1999). 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, Everitt, Jarrard, & Robbins, 1991) or muscarinic cholinergic receptor antagonism of the hippocampus fifteen minutes prior to the learning (Anagnostaras, Maren, & Fanselow, 1999; Selden, Everitt, Jarrard, & Robbins, 1991) impair performance on contextual fear conditioning.

Prefrontal Cortex

The prefrontal cortex is particularly important for flexible behavioral reactions (Aston-Jones, Rajkowski, & Cohen, 2000), and has demonstrated involvement in sustained attention in rodents (Granon, Hardouin, Courtier, & Poucet, 1998). The prefrontal cortex monitors incoming environmental information and initiates appropriate behavior based on the circumstances at any given time (Dalley, Cardinal, & Robbins, 2004). Trauma-related memories in abused women result in overactive prefrontal cortex (Bremner et al., 2005). Extinction of fear conditioning in rodents has also been shown to

depend on the prefrontal cortex (Herry & Garcia, 2002; Morgan & LeDoux, 1999; Quirk, Garcia, & Gonzalez-Lima, 2006; Sotres-Bayon, Cain, & LeDoux, 2006). These effects have been attributed to a failure of the prefrontal cortex to suppress attention to trauma-related stimuli subsequently allows over excitability of the amygdala and results in resilient, extinction resistant memories (Gilboa et al., 2004).

The ventromedial area of the prefrontal cortex, including the prelimbic and infralimbic sections have strategic connections involved in the extinction of fear behaviors in rodents (Amat et al., 2005; Dalley, Cardinal, & Robbins, 2004; Ishikawa & Nakamura, 2003; Vertes, 2004). Direct neural pathways with the hippocampus and the amygdala, along with lower brainstem areas that are involved in the regulation of neurotransmitter systems such as the ventral tegmental area, dorsal raphe nucleus and locus coeruleus support the role for the prefrontal cortex in the regulation of behavior (Burette, Jay, & Laroche, 1997; Herman, Prewitt, & Cullinan, 1996; Irle & Markowitsch, 1982; Ishikawa & Nakamura, 2003; Shu, Wu, Bao, & Leonard, 2003). Thus, the prefrontal cortex likely plays an integral role in the formation of memories and behavioral reactions to environmental stimuli.

Hypotheses

In order to gain insight into the multiple components of memory researchers have utilized the GABA agonist muscimol to suppress neural activity in specific structures. Understanding the functional interactions among the prefrontal cortex and hippocampus during a traumatic experience may lead to effective diagnostic and treatment strategies for PTSD and other anxiety disorders.

The present experiments were designed to selectively and transiently suppress activity in the ventromedial portion of the prefrontal cortex or CA1 region of the hippocampus before conditioning rats in a single stress model of flashbulb memories. This model generates a durable fear association to a context and produces high levels of anxiety along with deficits in non-aversive memory of intact rats that receive inescapable intense predator stress. To test the extent of interactions between the hippocampus and prefrontal cortex in the formation of long-term fear memories, anxiety-like behaviors, startle and general non-emotional memories, muscimol was used to inactivate the neural activity of these structures at the time of the inescapable cat exposure.

My first hypothesis was that suppression of the prefrontal cortex at the time of emotional learning would allow more amygdalar activation than during vehicle treatment rendering a more emotional memory. This should have facilitated a more durable and salient fear memory and increased anxiety, rendering a more fearful animal in the presentation of the context and cues associated with a strong stressor and a more anxious animal in general. My second hypothesis was that suppressing the hippocampus before the stress would weaken the formation of contextual fear. However, these animals should have exhibited heightened anxiety like behaviors, but maintained equivalent cognitive capacity as compared to vehicle stress animals. All vehicle treated stressed animals were hypothesized to show heightened fear, anxiety, and poorer object recognition memory as compared to non-stressed controls.

The general hypothesis was that in both humans and rats the hippocampus and prefrontal cortex interact to mediate memory and anxiety. During a traumatic experience

each structure provides unique processing, such that the inactivation of each individual area during trauma would influence different aspects of memory and anxiety.

Chapter Two: Experiments

Methods

Design

Hippocampal and prefrontal manipulations were conducted in 2 experiments based on the targeted brain structure. Each of the experiments utilized a 2x2 factorial design with artificial cerebral spinal fluid (aCSF; Harvard Laboratories) used as vehicle or muscimol (1µg/µl), and immobilization with cat exposure (Cat) or homecage (No Cat) as the levels.

Animals

A total of 78 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 are 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 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.

Surgery

On the day of surgery, 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 are in reference to the skull surface of bregma in millimeters and insertions were made with 26-gauge, stainless steel, guide cannulae (Plastics One Inc., Roanoke, VA).

Target coordinates for the vmPFC were +2.7 anterior-posterior (AP), ±0.5 medial-lateral (ML), and -5.0 dorsal-ventral (DV). The target of the hippocampus was the dorsal CA1 region, and the coordinates used were -3.8 AP, ±3.0 L, -2.8 DV. These target coordinates were based on the Paxinos & Watson rat brain atlas and pilot data. Bilateral guide cannulae 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.

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, in order to acclimate the animals to the infusion procedure, animals were brought into the laboratory and given at least 30 minutes to adjust to the surroundings. On the first day, the dust cap was removed and a mock injection tube placed on the cannulae pedestal to familiarize the rats to the sensation of the tube on their head. The second and third day consisted of the removal the dust cap and stylet, and gently placing the injectors (Plastics One) in the guide cannulae. A Harvard Apparatus pump (Holliston, MA) was connected to 25µl syringe injectors (Hamilton) by plastic tubing (Plastics One) and 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 cannulae were replaced and dust cap screwed back on the top of the pedestal. On the third day, aCSF or muscimol were administered.

Histology

A total of 70 rats completed the battery of tests. Upon completing the behavioral tasks all animals were euthanized with an overdose of ketamine and xylaxine, cresyl violet was infused into the cannulae at a rate of 0.1µl/min for 5 minutes to allow visual inspection of cannulae placement. The brains were extracted and flash frozen in 2-methylbutane and the tissue was stored at -80°C until it was sliced in coronal sections in 40µm sections on a Cryostat held at -16°C and mounted on microscope slides. After histological analysis of 30 representative samples four animals were eliminated for placement outside the target area.

Behavior

Stress Procedure. Approximately 15 minutes after the rats were infused with aCSF or muscimol, they were placed in a dark fear conditioning chamber (25.5 x 30 x 29 cm; Coulbourn Instruments; Allentown, PA) that consists of two aluminum sides, an aluminum ceiling, and a Plexiglas front and back covered with black plastic. The chamber served as the context associated with a cat. The floor consists of 18 stainless steel rods, spaced 1.25 cm apart. Exposure to the chamber for three minutes terminated with 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 using a plastic DecapiCone (Braintree Scientific; Braintree, MA) and then placed in a pie-shaped

Plexiglas enclosure (Braintree Scientific; Braintree, MA; 20 x 20 x 8 cm). This container was placed inside a cage containing a female cat to keep the cat in close proximity to the rats. To direct the cat's activity to the container, a small amount of wet cat food was smeared on top of the container. Rats then remained with the cat for one hour. This procedure has been developed to produce a fear memory in rats to a context that is, otherwise, innocuous. Animals in the no cat groups were placed back in their home cages for one hour.

Fear Association. Three weeks later, each rat was brought back to the laboratory, and allowed 30 minutes to acclimate to the environment. After acclimation rats were placed in the same fear conditioning chamber as the one in which they were placed just before receiving the stress treatment 3 weeks earlier. The freezing behavior of each rat was monitored by computer for five minutes. Approximately 45-60 minutes after the contextual memory test, rats were individually placed in the light side (25 x 22.5 x 33 cm) of a shuttle box (Coulbourn Instruments; Allentown, PA) that consisted of two aluminum sides, an aluminum ceiling, and a Plexiglas front and back, with the shuttle door in the closed position. A house light was turned on, and a metal plate (21.5 x 21.5 cm) placed on the floor to eliminate the sensation of the stainless steel rods beneath their paws. These conditions reduce the similarities between the conditioning chamber context and the auditory cue testing chamber. The rats remained in the light side of the shuttle box for a total of six minutes. Our paradigm consisted of no tone present for the first three minutes, and introducing a tone (74 dB; 2500 Hz) for the last three minutes. This paradigm provides a measure for a novel context and a more direct measure of the cue memory. Therefore, rats' freezing behavior in response to the tone was considered as a

measure of their memory for the tone-cat association, independent of the context. The amount of time freezing and fecal boli were recorded and considered as an index of fear. Freezing was measured by a 24-cell infrared activity monitor (Coulbourn Instruments; Allentown, PA), mounted on the top of the fear conditioning chamber, which uses the emitted infrared body heat image (1300 nm) from the animal to detect relative changes in movement. Freezing was defined as periods of inactivity for more than three seconds, except for movement required for respiration. A Microsoft Excel spreadsheet with a macro designed to analyze freezing behavior was used to calculate the total number of seconds spent freezing by each animal at 30-sec epochs divided by the total amount of time in the chamber, providing a percentage of time freezing for each animal. The use of automated parameters have been employed by laboratories elsewhere (Lee & Kim, 1998) and have been shown to significantly correlate with time sampling observer methods often employed to assess freezing behavior (Kim, DeCola, Landeira-Fernandez, & Fanselow, 1991).

Elevated Plus Maze (EPM; see Figure 1). Twenty-four hours after the context and cue tests, all rats were brought to another room of the laboratory and subjected to the EPM assessment. The EPM (Hamilton-Kinder; San Diego, CA) is an apparatus that has been used extensively to study anxiety-like behavior in rodents (Korte & De Boer, 2003). It consists of 2 open (10.80 x 51.17 cm) and 2 closed arms (10.80 x 51.17 cm) that intersect each other to form the shape of a plus sign. The intersection area is 10.80 by 10.80 cm, and the walls of the closed arms are 40.01 cm high. The more time rats spend in the closed arms is considered to be indicative of anxiety-like behavior. In other words, time spent in the open arms is considered risk-taking behavior, as it theoretically places

the rat in open view to predators and susceptible to danger. Each rat was placed on the EPM for 10 minutes, and its behavior monitored by 48 infrared photobeams connected to a computer program (Motor Monitor) that analyzes the behavior. The program enables the experimenter to assess the rats' total movement, distance traveled in each area of the maze, distance traveled overall, and time spent in each area of the maze. The primary measurement of concern is the percentage of time that each rat spends in the open arms, as compared to the closed arms. The EPM was wiped down with 25% ethanol solution after removal of fecal boli between sessions to reduce odor between testing.

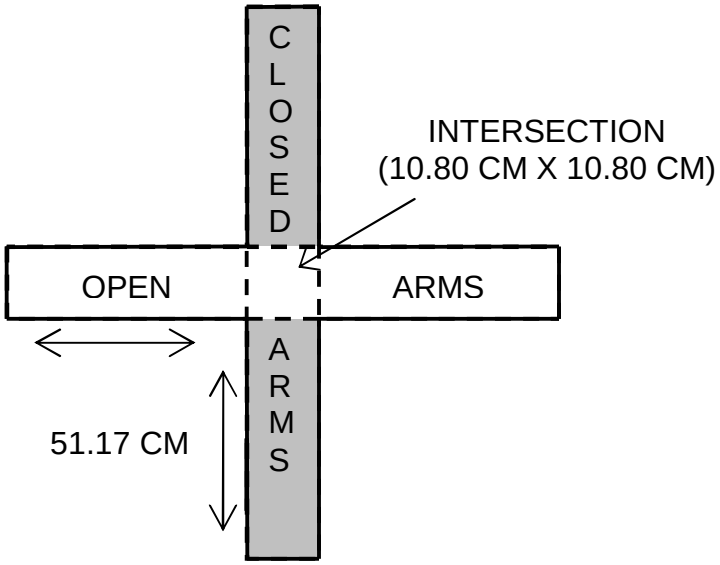

Figure 1. Schematic Diagram of the Elevated Plus Maze

Startle Response. Approximately one hour after the EPM assessment, all rats were subjected to tests of startle reflex. To measure the startle, each rat was placed inside a restraint box that is inside of a larger startle monitor cabinet (Hamilton-Kinder; San

Diego, CA; 35.56 x 27.62 x 49.53 cm). Within the recording chamber, the rat sits on a sensory transducer, which records startle reflexes. The startle trial began with a 5-minute acclimation period, followed by the presentation of 24 noise bursts, eight from each of three auditory intensities (90, 100, and 110 dB). The noise bursts were presented in sequential order (i.e. 8 bursts at 90 dB, followed by 8 bursts at 100 dB, etc.), and the time between each noise burst varied in a pseudorandom fashion between 25 and 55 seconds. Upon the commencement of the first noise burst, the startle apparatus provides an uninterrupted background noise of 57 dB. Each startle reflex was recorded in Newtons, and the complete session lasted 16 minutes.

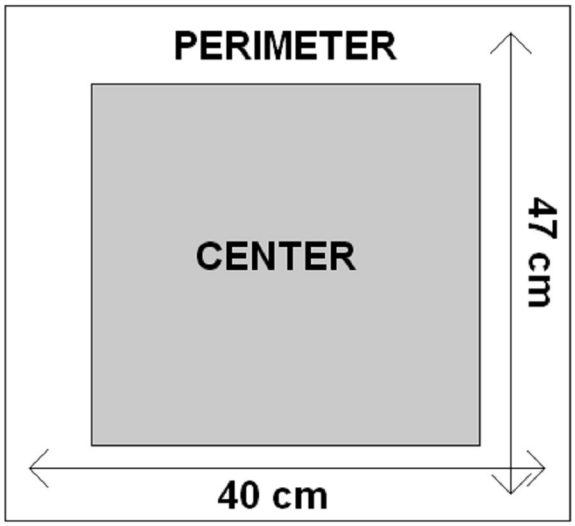

Figure 2. Schematic Diagram of the Open Field Apparatus

Object Recognition. Twenty-four hours after the startle test, animals were returned to the laboratory. After acclimation animals were placed individually into an Open Field (see Figure 2). This field is a large, black walled, plastic box (Hamilton-Kinder, San Diego, CA – 40 x 47 x 70 cm). It has an open top, and is in a light- and

sound-attenuated room for 5 minutes. The rats' behavior was monitored by a video feed to a computer program (Any Maze; Stoelting) and analyzed. The program allows for assessment of the rats' total distance traveled in each area of the open field (center and perimeter), total time spent in each area of the open field, rearing, and entries into each quadrant of the open field. This exposure served as habituation to the apparatus and provided an assessment of general behavior. Another day after the five minute habituation trial, the animals were again brought back to the laboratory and re-exposed to the open field; but this time 2 identical objects were placed diagonally opposite to one another for the rat to explore for a total of 5 minutes (training phase). Three hours later, rats were replaced into the open field, a replica of one of the original objects and a completely novel object were placed in the same orientation as the training procedure for 5 minutes (testing phase). The amount of time spent with the novel object was then recorded by Any Maze monitoring the head of the rat in relation to the objects in the field and served as an index of memory for the original familiar object. Video files were also made for experimenter coding. Rats normally spend more time with the novel object than with the familiar. This paradigm is interpreted as a form of non-stressful memory.

Statistical Analysis

Most of the data were analyzed through use of appropriate types of two-way analyses of variance (ANOVA). 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 experiments. Alpha was set at 0.05 for all analyses.

Results

Fear Conditioning (see figures 3-6). The 3-week fear conditioning retention tests (contextual fear conditioning, cue-based fear conditioning) were analyzed separately. Contextual fear conditioning freezing percentages, excluding the first 30-seconds and last minute of the 5-minute tests, were compared using a two-way ANOVA for each brain target, with Stress (cat or no cat) and Inactivation (aCSF or muscimol) serving as the between-subjects variables. Cued fear conditioning was analyzed using the percent time spent freezing during the 3-minute tone presentation.

Analysis of the vmPFC group's contextual fear response at three weeks revealed a significant overall effect with $F(3,31) = 7.80$, $p < 0.01$. A significant main effect of Stress was found ($F(1,31) = 15.21$, $p < .01$) with the stress procedure producing higher levels of freezing (M = 17.08, SEM = 2.02) compared to animals not receiving the stress procedure (M = 6.58, SEM = 1.18). Inactivation also produced a significant main effect ($F(1,31) = 7.15$, $p < 0.05$), animals having aCSF infused into the vmPFC prior to conditioning expressed higher levels of freezing (M = 15.43, SEM = 1.86) than animals infused with muscimol (M = 8.23, SEM = 1.95). The interaction between Stress and Inactivation was not significant with $F(1,31) = 3.61$, $p = 0.07$. However, planned comparison two-tailed t-tests indicated that Stress rats with the vmPFC inactivated prior to conditioning froze (M = 10.92, SEM = 2.38) significantly ($p < 0.05$) less than Stress – aCSF animals (M = 23.25, SEM = 4.92); in addition, these animals froze significantly ($p < 0.01$) more than Unstressed – aCSF animals (M = 7.62, SEM = 1.60). Analysis of the cued fear response of vmPFC targeted animals indicated no significant differences overall with $F(3,31) = 1.03$, $p = 0.39$.

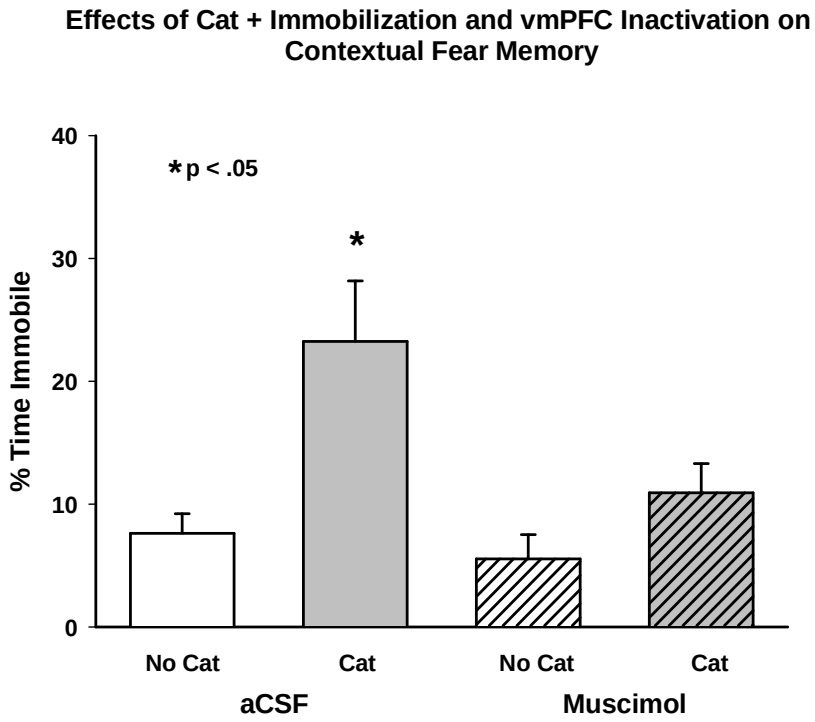

Figure 3. Inactivation of the vmPFC with muscimol before conditioning blocked the stress induced increase in freezing to the context 3-weeks later. (* $p < 0.05$)

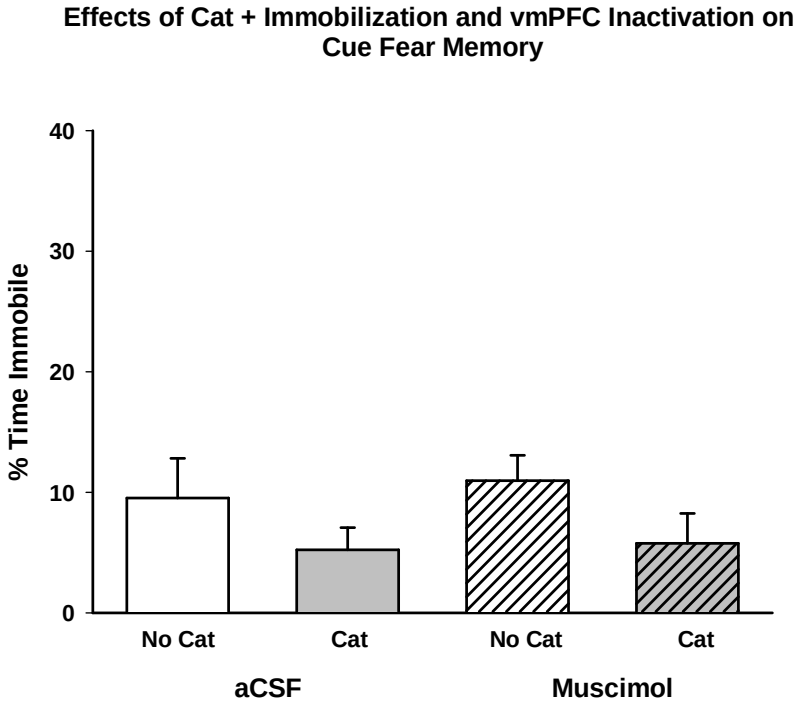

Figure 4. There was no effect of stress or inactivation on the cued freezing.

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 Stress ($F(1,28) = 4.46$, $p < 0.05$) and Inactivation ($F(1,28) = 5.95$, $p < 0.05$). In similar fashion to the vmPFC group, the stressed animals froze more (M = 18.88, SEM = 3.12) than unstressed (M = 8.86, SEM = 3.58), and aCSF treated animals expressed more fear (M = 19.66, SEM = 3.22) than muscimol treated animals (M = 8.08, SEM = 3.49). The Stress by Inactivation interaction was also not significant ($F(1,28) = 1.34$, $p = 0.26$). A similar pattern to the vmPFC group emerged in the CA1 group when analyzed using planned comparison t-tests. Muscimol infused prior to the stress procedure (M = 10.35, SEM = 3.99) significantly reduced ($p < 0.03$) freezing compared to aCSF (M = 27.41, SEM = 5.91), which was also significantly greater than ($p \leq 0.05$) aCSF unstressed animals (M = 11.90, SEM = 4.31). The cued fear response in CA1 targeted animals did show significant overall differences ($F(3,31) = 3.65$, $p < 0.05$); with no significant main effect of Inactivation ($F(1,31) = .93$, $p = 0.34$) or the Stress by Inactivation interaction ($F(1,31) = 1.39$, $p = 0.25$). However, a significant main effect was observed in the Stress manipulation with $F(1,31) = 8.10$, $p < 0.01$; where the Stress procedure resulted in animals freezing more to the cue (M = 21.487, SEM = 3.11) than unstressed animals (M = 7.80, SEM = 3.67). Planned comparison t-tests revealed the Stressed-aCSF animals froze significantly more (M = 29.65, SEM = 7.10) than both Unstressed-aCSF and –muscimol (M = 7.29, SEM = 2.75 and M = 8.31 , SEM = 2.26; $p < 0.05$). However, Stressed-muscimol animals (M = 16.34, SEM = 3.21) were not significantly different from their Stressed-aCSF counterparts in percent time freezing to the tone ($p = 0.096$).

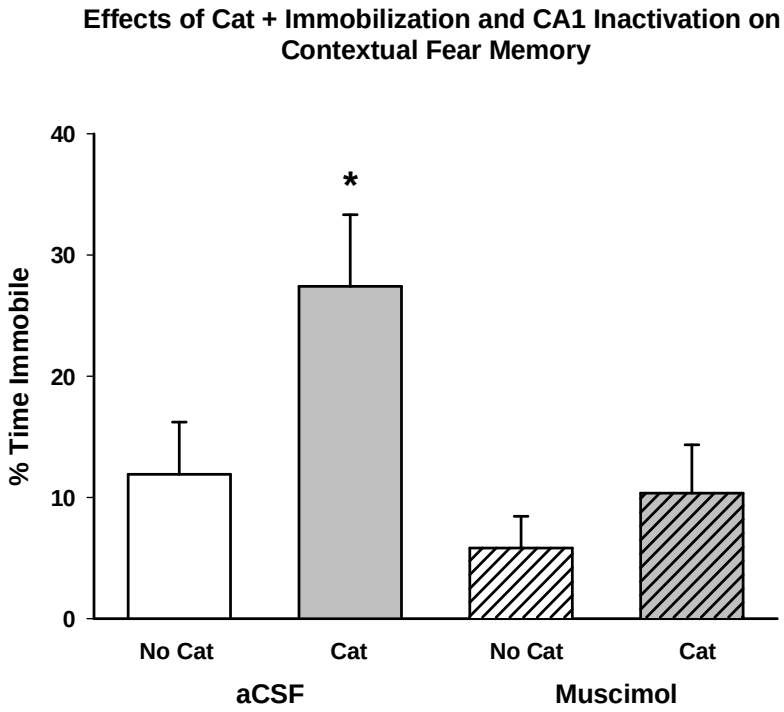

Figure 5. Muscimol inactivation of the hippocampus before conditioning blocked the stress induced increase in freezing to the context 3-weeks later. (* $p < 0.05$)

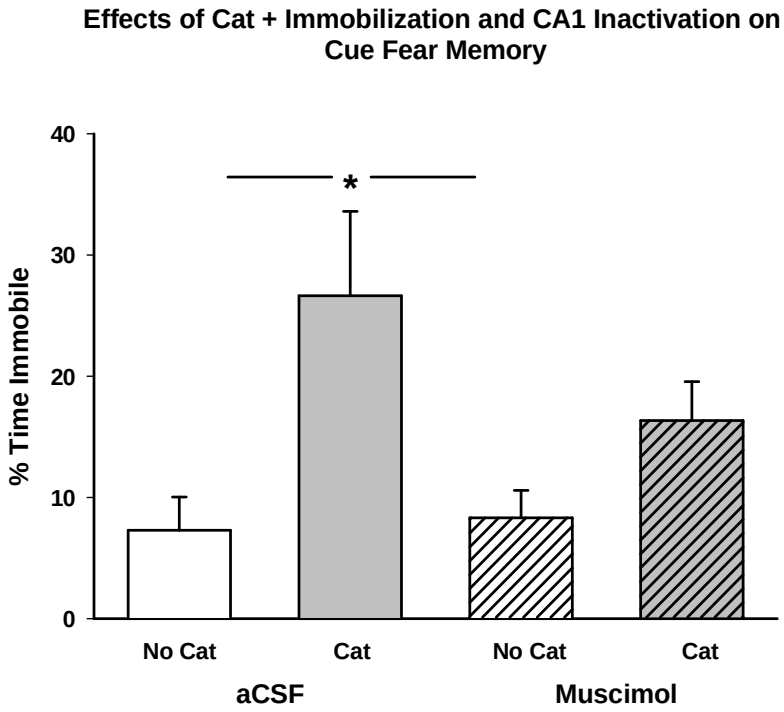

Figure 6. Stressed animals infused with aCSF into the hippocampus exhibited heightened cued fear conditioning after 3-weeks as compared to non-stressed rats. Animals infused with muscimol were not significantly different than any group.

EPM (see figures 8 & 9). The data for the first five minutes of the ten minute test analyzed separately for each brain region using two two-way ANOVAs, comparing the percent time each group spent in the open arms or closed arms. For the vmPFC group a significant overall difference for percent time spent in the open arms was found with $F(3,30) = 5.22$, $p < 0.01$. There was a significant main effect of Stress ($F(1,30) = 10.86$, $p < 0.01$) with the stress procedure resulting in less percent time (M = 10.52, SEM = 3.92) in the open arms as compared to animals not put through the procedure (M = 27.77, SEM = 3.48). However, there was no significant main effect of Inactivation ($F(1,30) = 2.13$, $p = 0.16$) or Stress x Inactivation interaction ($F(1,30) = 2.56$, $p = 0.12$) for the percent time spent in open arms. The analysis of the percent time spent in the closed

arms for the vmPFC group indicated a significant overall effect with $F(3, 30) = 6.23$, $p < 0.01$. A significant main effect for Stress ($F(1,30) = 18.49$, $p < 0.01$) was found; however, no significant main effect of Inactivation ($F(1, 30) = 0.02$, $p = 0.90$) or Stress x Inactivation interaction ($F(1,30) = 0.003$, $p = 0.96$) were found. Both the Stress-aCSF (M = 82.35, SEM = 6.77) and Stress-Muscimol (M = 82.87, SEM = 7.24) treated groups spent significantly greater percent time in the closed arms than the Unstressed-aCSF (M = 53.79, SEM = 5.77) and Unstressed-muscimol (M = 54.98, SEM = 6.38). The CA1 groups showed no significant overall differences in percent time spent in the open ($F(3,30) = 1.60$, $p = 0.21$) or closed arms ($F(3,30) = 0.53$, $p = 0.68$).

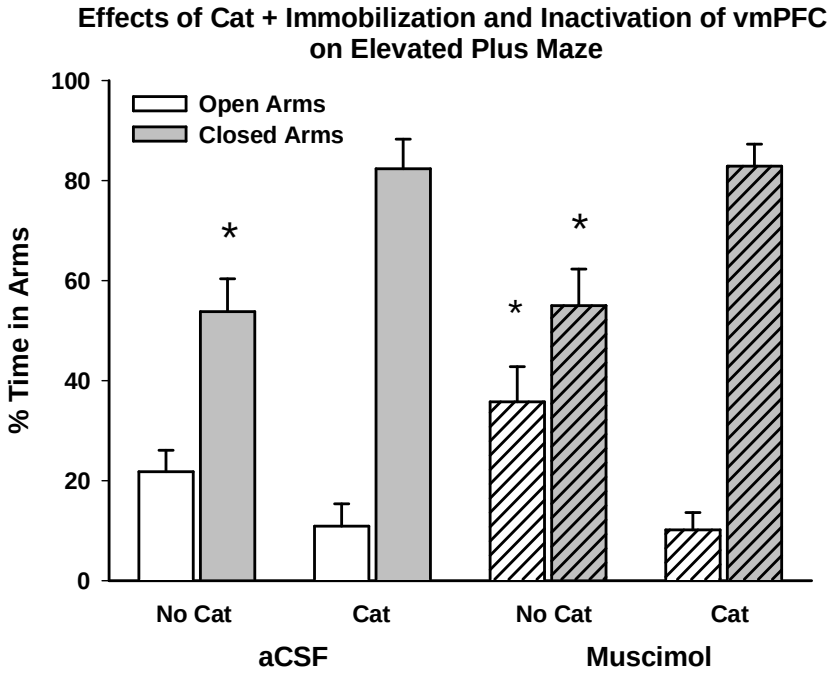

Figure 7. Inactivation of the vmPFC had no effect on stress effects on time spent in open or closed arms of the EPM. (* indicates $p < 0.05$ comparing closed arms or open arms between stressed and unstressed rats)

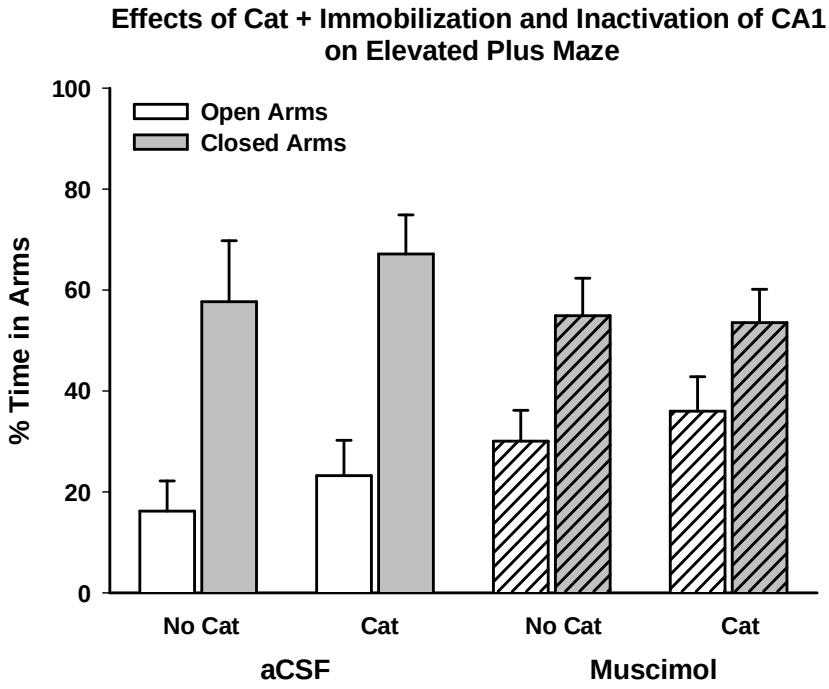

Figure 8. There was no overall effect of stress or inactivation on EPM behavior in the hippocampal manipulation.

Startle Response (not shown). For each rat, eight startle responses at each of three auditory intensities were averaged to create one data point per auditory intensity per rat. The data were analyzed using a mixed-model ANOVA, with Stress and Inactivation serving as the between-subjects variables and Intensity (90, 100, and 110 dB) serving as the within-subjects variable. For each brain structure, only significant differences were found between startle intensities; with the vmPFC ($F(2,66) = 85.30$, $p < 0.01$) and CA1 ($F(2,60) = 86.54$, $p < 0.01$) groups both expressing more startle as the intensities increased.

Object Recognition (see figures 9 & 10). The object recognition data were analyzed using two-way ANOVAs with the same between-subjects variables as before, with time spent with the novel and familiar objects as the within-subjects variables. For

the vmPFC group an overall within-subjects trend was revealed for object preference ($F(1,28) = 2.58$, $p = 0.12$), with the Novel object ($M = 18.79$, $SEM = 2.63$) being investigated more than the Familiar object ($M = 13.68$, $SEM = 1.53$). Planned comparison repeated measure t-tests were also used to investigate the effects of inactivating the brain structures during the stress procedure on this non-stressful memory. These tests indicated that each of the Unstressed-aCSF, Unstressed-muscimol, and Stressed-muscimol groups spent significantly ($p < 0.05$) more time with the Novel object ($M = 19.78$, $SEM = 2.05$; $M = 17.9$, $SEM = 1.93$; $M = 21.3$, $SEM = 5.38$, respectively) than the Familiar object ($M = 13.33$, $SEM = 1.16$; $M = 13.73$, $SEM = 0.83$; $M = 10.26$, $SEM = 1.78$). However, the aCSF-Stress animals showed no difference ($p = 0.43$) between the time spent with the Novel ($M = 13.67$, $SEM = 1.01$) and Familiar ($M = 13.33$, $SEM = 1.09$) objects.

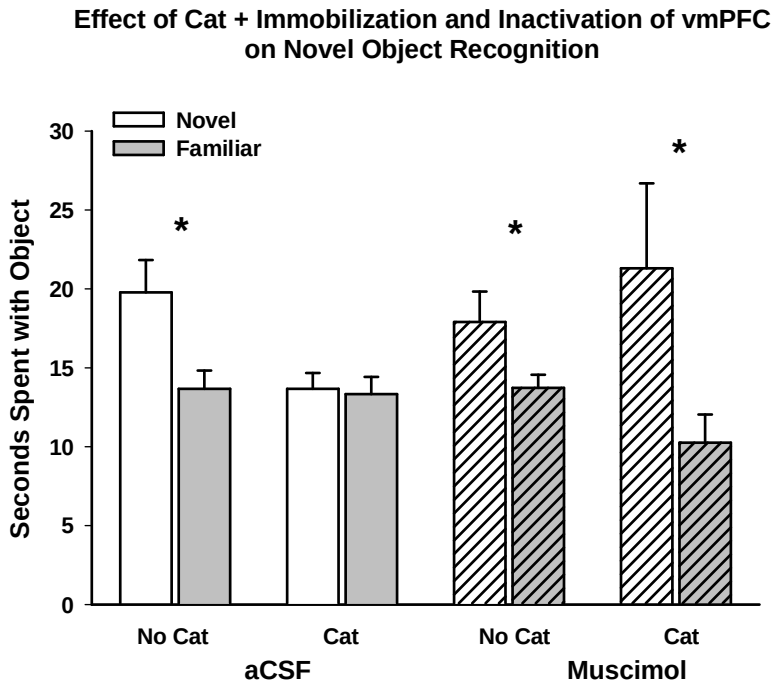

Figure 9. Only the vehicle treated stress animals did not investigate the novel object more than the familiar object. (* $p < 0.05$)

The hippocampal manipulations showed a significant within-subjects difference for object preference as indicated by $F(1,26) = 7.25$, $p < 0.05$, with more time being spent with the Novel object (M = 18.40, SEM = 2.65) than the Familiar object (10.92, SEM = .99). The planned comparisons for these groups showed similar patterns to that of the vmPFC results. The only group not to show a significant preference for the Novel object (M = 17.8, SEM = 4.40) over the Familiar (M = 11.59, SEM = 1.48) was the aCSF-Stress group.

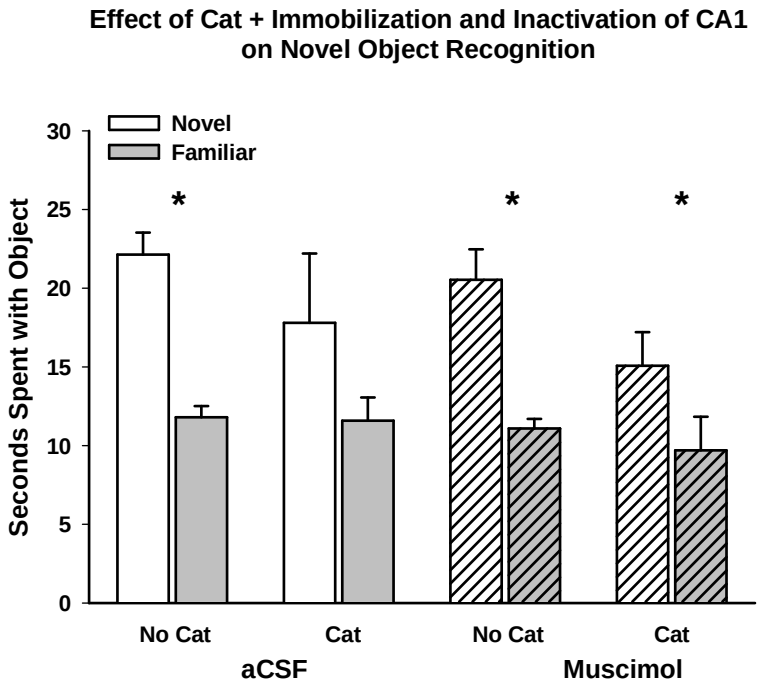

Figure 10. Only the vehicle treated stress animals did not investigate the novel object more than the familiar object. (* $p < 0.05$)

Fecal Boli (see figures 11 & 12). The total number of fecal boli each animal produced for contextual and cued fear conditioning, EPM, Startle, Open Field, Object Training, and Object Recognition behavioral tests was averaged and analyzed using a 2 x 2 ANOVA. For the vmPFC group a significant overall difference was found ($F(3,34) = 4.91$, $p < 0.01$). There was a significant main effect of Stress with $F(1,34) = 11.33$, $p < 0.01$, where the Stress groups (M = 10.75, SEM = 1.30) produced more boli than the No Stress groups (M = 5.02, SEM = 1.13). Planned comparison analysis revealed that the Stress-aCSF group defecated more than both of the No Stress groups; however, the Stress-muscimol group was no different statistically from the Stress-aCSF group.

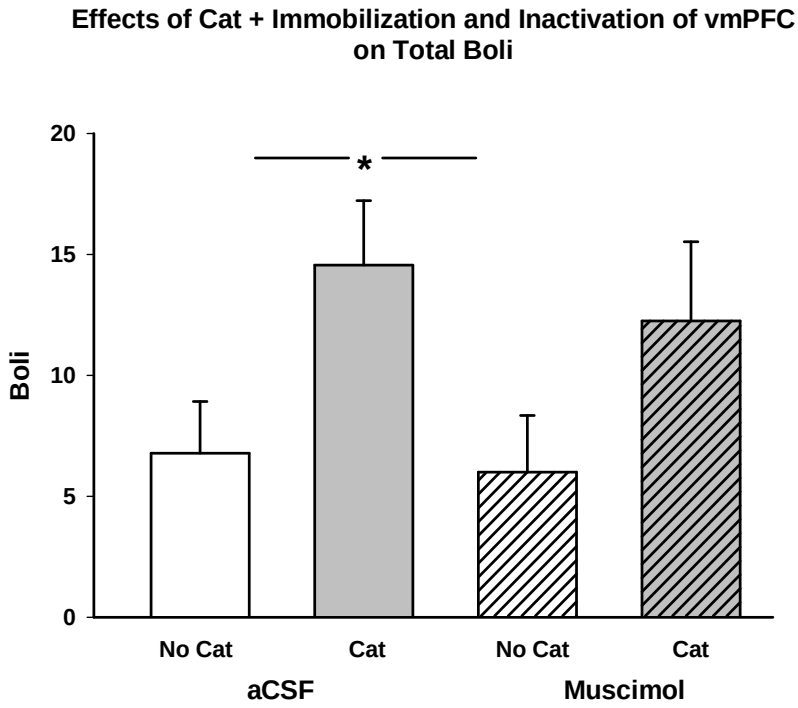

Figure 11. The stressed animals receiving vehicle infusions defecated more than the unstressed groups, but stressed animals receiving muscimol were not significantly different than any other groups. (* $p < .05$)

Analysis of the hippocampal manipulations revealed a main effect of Stress ($F(1,28) = 4.87$, $p < 0.05$), with the Stress groups (M = 13.40, SEM = 1.87) defecating more than the No Stress groups (M = 7.31, SEM = 2.03). Planned comparison t-tests indicated the Stress-aCSF (M = 14.56, SEM = 2.57) produced more boli than either of the No Stress groups (aCSF, M = 6.78, SEM = 2.57; and muscimol, M = 7.83, SEM = 3.15).

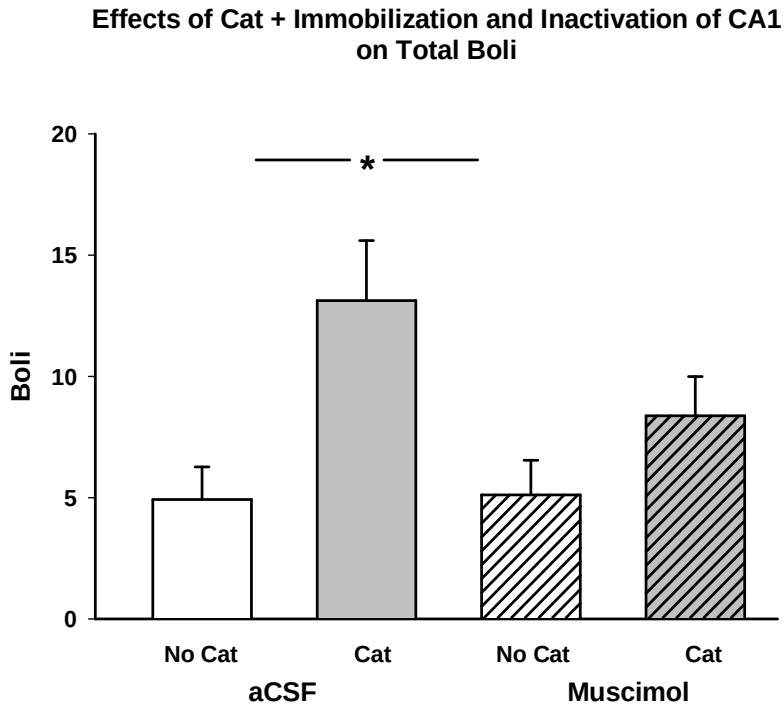

Figure 12. The stressed animals receiving vehicle infusions defecated more than the unstressed groups, but stressed animals receiving muscimol were not significantly different than any other groups. (* $p < 0.05$)

Chapter Three: Discussion

Prefrontal Cortex

The most intriguing finding of these experiments was, counter to the hypothesis, infusion of muscimol into the prefrontal cortex resulted in lower freezing to the contextual fear conditioning tests. While this finding is contrary to the hypothesis derived from the literature that the inactivation of the prefrontal cortex would result in a more active amygdala and produce a more robust fear memory, there is a similar finding in humans. Combat veterans who received brain damage to the prefrontal cortex had significantly less occurrence of PTSD than those with damage to any other brain region, except the amygdala (Koenigs, et al., 2008). The results of the present study are comparable to this finding in humans. The prefrontal circuitry involved has been theorized to rely on the percept of control, such that in rats the infralimbic and prelimbic cortex orchestrate inhibitory influence over the dorsal raphe nucleus (DRN). The DRN provides the majority of 5-hydroxytryptamine signaling to the rest of the brain. It has been demonstrated that the vmPFC is important for controlling the DRN and that the construct of control mitigates these effects in rats (Christianson, Thompson, Watkins, & Maier, 2008; Amat, Paul, Watkins, & Maier, 2008; Baratta, Lucero, Amat, Watkins, & Maier, 2008; Baratta et al., 2007; Maier, Amat, Baratta, Paul, & Watkins, 2006; Amat, Paul, Zarza, Watkins, & Maier, 2006; Amat et al., 2005). This design specifically set out to reduce the perception of control the animal had. Then, why would inhibiting the

prefrontal cortex reduce the memory of the context and cue associated with immobilization and predator exposure? Human imaging studies have provided evidence that the vmPFC is more likely responsible for attention (Geday & Gjedde, 2009). If this is the case in rodents then one possible explanation for the present finding is the animals were unable to attend to the context and the cue when the information would have normally been processed, and were then subsequently unable to recognize them as predictors of an aversive stimulus. The regulation of emotional behavior by the PFC is two-fold. That is, while electrical activation of the prelimbic areas stimulates the parasympathetic nervous system, infralimbic activity is associated with sympathetic nervous system stimulation (Powell, Watson, & Maxwell, 1994). Thus, the obtained results could be due to more consistently ventral placement of the cannula, resulting in a more blunted emotional response at the time of stress.

The stressed animals in the vmPFC group displayed more anxiety-like behaviors on the elevated plus-maze. This demonstrates the single-stress paradigm is sufficient to be anxiogenic, much like our laboratory's model of PTSD. However, the inactivation of the vmPFC did not facilitate any further anxiogenesis. In fact, the reduced amount of time spent in the closed arms in both vehicle and muscimol unstressed rats supports the idea that these animals were less anxious than the stressed animals.

Hippocampus

The hippocampal inactivation in stressed animals resulted in a blockade of the stress induced contextual fear memory, while sparing the cued fear memory. These results supported the hypothesis that hippocampus modulates the contextual, but not

auditory cue information processed in close temporal proximity to fear learning in rats. Thus, this experiment illustrates the importance of the hippocampus in relation to the timing of contextual fear-memory formation. These findings also provide validity of this stress procedure, per Yehuda & Antelman's (1993) criteria, because the single "dose" of stress facilitated fear learning. The lack of an effect on the EPM is possibly due to an anxiolytic effect of the cannulation via cellular remodeling after the surgery. (Dringenberg, Levine, & Menard, 2008) found one second of electrical stimulation of the dorsal but not the ventral hippocampus before behavioral testing reduced the amount of time rats spent in the open arms of an EPM. Furthermore, (McEown & Treit, 2009) showed transient inactivation of the ventral and the dorsal hippocampus during acquisition of a defensive burying task (an anxiogenic type of fear conditioning) reduced anxiety-like responses when retention was tested in the burying apparatus. However, only transient inactivation of the dorsal hippocampus after acquisition resulted in anxiolytic effects on the 24 hr test. These reports indicate the hippocampus is involved in anxiety-like behaviors in rats and that the dorsal hippocampus is particularly important for contextual memory of aversive events.

Limitations

The lack of effects on the startle behavior could have arisen from the fact that these animals underwent stereotaxic surgery, which usually results in the puncture of the tympanic membrane (Kaplan, Allan, & Wolf, 1983). It is also possible that two stress sessions are necessary to generate the hyperarousal behaviors we observe in our PTSD model. There is also indication from research in humans that startle is context dependent.

In a series of experiments Morgan and colleagues (Grillon, Morgan, Southwick, Davis, & Charney, 1996; Morgan, III, Grillon, Southwick, Davis, & Charney, 1995; Morgan, III et al., 1995) examined the startle response in PTSD patients and found PTSD patients exhibited greater startle throughout both baseline and threat conditions. However, (Grillon, Morgan, Southwick, Davis, & Charney, 1996) examined the baseline startle response of Vietnam veterans with PTSD in a familiar environment and found no differences between startle responses of Vietnam veterans with or without PTSD and healthy control subjects. Other studies (Grillon & Morgan, III, 1999; Grillon, Morgan, III, Davis, & Southwick, 1998; Pole, Neylan, Best, Orr, & Marmar, 2003) have found manipulations of the experimental context or the presentation of explicit threat cues consistently leads to enhanced startle responses in PTSD symptoms, indicating the exaggerated startle responses reported in PTSD patients is context dependent, and not necessarily a stable trait of these individuals. Thus, testing startle in the fear-provoking context could have facilitated enhanced startle expression.

There are methodological limitations of this investigation. One limitation is the fact that only one type of GABA agonist was utilized to inactivate the brain regions of interest. It is possible that the single administration of this powerful drug was sufficient to induce a general anxiolytic effect in treated animals. Future investigations could determine whether inactivation of the prefrontal cortex or hippocampus using different pharmacological agents, such as lidocaine or tetrodotoxin result in similar findings. Another limitation arises from the 90 degree angle used in cannulae placement of these investigations. This could account for the lack of cued fear conditioning in the prefrontal cortex manipulations from cortical damage from cannulae placement. The fact that all of

the behavioral tests were done on the same animals; that is separate groups were not used to look at each behavior could have influence the results. However, the most intriguing findings were found in the first and last behavioral tests, indicating that at least the non-aversive memory effects are robust.

General Conclusions

Overall, these studies have shown that the hippocampus and the prefrontal cortex are necessary to form long-term contextual fear memories. Furthermore, these investigations call into question the current theories of how multiple brain regions interact to form traumatic memories. If the prefrontal cortex "goes offline" during a traumatic experience and allows the amygdala to form a more emotional memory than usual, then the stressed rats with muscimol inactivation and the veterans with prefrontal damage (Koenigs et al., 2008) would both have had more robust fear and anxiety symptoms than has been reported. This serendipitous finding calls into question the current theoretical framework that many researchers are using.

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