Effects of Predator Odor-Induced Stress on the Rodent Prefrontal Cortex and Cocaine Self-Administration

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ABSTRACT

Post-traumatic stress disorder (PTSD) and substance use disorder (SUD) affect a combined 30 million Americans as of 2014 (Hedden et. al., 2015). An individual who is diagnosed with one has a greatly increased risk of being diagnosed with the other later in life. This suggests some type of biological link between the two. Unfortunately, it is difficult to study the molecular underpinnings of either disease in humans because of ethical concerns. Therefore it would be prudent to develop an animal model that allows for a standardized examination of both disorders. This investigation was designed as an attempt to create an animal model that encompasses both the mammalian stress response as well as substance abuse. Specifically, in the first Aim we investigated the effects of the predator odor 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) on the mRNA levels of several genes in the rodent medial prefrontal cortex (mPFC). In the second Aim we investigated TMT exposure’s ability to affect rodent cocaine self-administration (SA). In the former Aim, we exposed rats to TMT for 15 minutes a day for 5 consecutive days and then euthanized them for analysis of the mPFC by real-time PCR. In the latter Aim we exposed rats to TMT in a similar manner and then trained them on a cocaine SA paradigm. Following the SA the animals underwent extinction training and then a series of reinstatement tests in order to measure drug-seeking. Although the stressful nature of TMT exposure was validated in both Aims, the stress exposure had no effect on the transcription levels of genes of interest or on drug-seeking behavior.
CHAPTER 1: INTRODUCTION

1.1 Background

Stress is a phenomenon experienced worldwide. Humans across all socio-economic strata experience stress everyday. However, some individuals experience stress in excess. When this occurs to a level that impedes the individual’s ability to perform routine daily tasks it is generally classified as an anxiety disorder (US Department of Health and Human Services, 2015). One type of anxiety disorder is post-traumatic stress disorder (PTSD) which is characterized by intrusive thoughts, avoidance of trauma related stimuli, and alterations in arousal and reactivity (Bolduc et. al., 2015). About 8% of the general population suffers from PTSD (Andreski et. al., 1998; Chilcoat et. al. 1998). To further complicate the lives of these individuals, over 75% of PTSD patients receive a secondary lifetime diagnosis (Kessler et. al., 1995). One of the most common co-occurring diagnoses is substance use disorder (SUD). According to one study that analyzed surveys from 34,653 people, the rate of SUD in individuals suffering from lifetime PTSD was 46.4% (Pietrzak et. al., 2011). These data would suggest that there is some intrinsic relationship between PTSD and SUD. In order to identify exactly what this relationship is, it would be beneficial to have an animal model for both PTSD and SUD.

1.2 Literature Review

Animal models for SUD have been under investigation for nearly a half century and have involved a multitude of paradigms (Robinson et. al. 2003; Hyman et. al. 2006; Belin et. al. 2012; Wise et. al. 2014). Generally, animal models consist of exposing animals to an addictive drug and monitoring how much effort they are willing to exhibit, or
punishment they are willing to overcome, in order to be exposed to more of that drug or cues that have been associated with the drug’s availability. However, there is no one animal model for PTSD that has both face and construct validity. Currently researchers are only able to promote the expression of specific symptoms, such as avoidance of trauma-related stimuli and levels of arousal, and then examine the neurobiological mechanisms that might be responsible. Considering that approximately 30 million people in the US alone suffer from either a SUD or PTSD (Hedden et. al., 2015) this is an area of research that deserves investigation.

In order to model a stress disorder in rats, we had to first identify the similarities between the human and rat stress response. It is safe to say that humans and rats experience stress in vastly different ways. However, there are certain hallmarks of the mammalian stress response that can certainly be observed in both species. Stress, in the context of this paper, is defined broadly as “anything which causes an alteration of psychological homeostatic processes” (Burchfield, 1979). The destabilization of an animal’s homeostasis induces the activation of many different physiological processes that are regulated, in large part, by the hypothalamic-pituitary-adrenal axis (HPA) (Koob, 2015). These processes occur in both rats and humans. One hallmark of HPA activation is the release of glucocorticoids, cortisol in humans and corticosterone in rats, by the cortex of the adrenal gland (Rodrigues et. al., 2009). Release of glucocorticoids is preceded by an environmental stimulus being classified as “threatening” by the amygdala, which signals the paraventricular nucleus to secrete corticotropin-releasing factor (de Kloet, 2004). It is this secretion of corticotropin-releasing factor that causes the adrenal gland to release glucocorticoids and prepares the organism for a “fight or flight” behavior. Plasma
glucocorticoid levels can be measured quantitatively in both species. Another hallmark of rodent stress is avoidance of the stressor-associated context even after the stressor has been removed (Whitaker et. al., 2016). This avoidance behavior would support the idea that not only was the stimulus itself stressful, but that the rat has formed a memory of the stimulus and now avoids anything related to it. There are dozens of methods that elicit increases in corticosterone and avoidance responses in rats that can be found in the literature. However, one specific method has proven successful in our lab: predator odor exposure.

Predator odor exposure is a popular method for inducing stress in rodents, although there are numerous competing methods. Other animal models of stress can include injection of a noxious substance, physical pain (i.e. footshock), forced-swim, social-defeat, etc. These various stress paradigms are efficacious depending on the objective of the particular study in which they are used. Two of the most common methods are electrical footshock (FS) and the force-swim test (FST). Footshock paradigms involve the delivery of electrical current through the metal rods on which the rat is placed. Although FS does not typically cause tissue damage, its value as a stressor is heavily tied to the pain it inflicts in the animal. On the other hand, the FST involves placing a rat in a cylinder containing enough water such that the rat cannot stand, rest, or escape and must continuously swim. While both of these methods produce robust stress responses in rats (Sutanto and de Kloet, 1994), they do not accurately model the specific type of psychological stress associated with PTSD that we are trying to recreate in our animal model. The fear-inducing aspects of FS and FST are inherently tied to an unpleasant physical sensation (i.e. the electrical shock or simulation of drowning). With predator
odor, the rat is being exposed to a stimulus that predicts danger is approaching (i.e. a predator is in the area) and this causes the stress response (Fendt et. al., 2008). We believe this non-physical stress more accurately models the psychological trauma of a life-threatening event. Furthermore, the stress response produced by predator odors can be reproduced for multiple days without habituation (Takahashi et. al, 2005; Staples et. al., 2010) and can cause neuronal and behavioral alterations in the animal weeks after exposure has ceased (Wang et. al., 2012; Ojo et. al., 2014). We believe predator odor exposure can be used as a model of repeated exposure to traumatic memories often seen in PTSD (Zoladz et. al., 2008). For these reasons, we chose predator odor as our stressor.

The benefits of predator odor exposure versus other models of stress include being ecologically relevant to the rat and being devoid of any physically painful stimuli. Almost every rat will exhibit heightened corticosterone levels and avoidance behaviors in response to a predator odor exposure (Rosen et. al., 2015). In addition to these acute responses, predator odor exposure has been shown to increase ethanol consumption in a two-bottle choice task (Manjoch et. al., 2016), alter appetitive behavior (Wernecke et. al., 2016), and shift rat behavior towards striatal response-learning as compared to hippocampal spatial-learning in a dual-solution plus-maze task (Leong et. al., 2014). In light of these interesting findings, predator odor exposure seems to be an effective method of inducing stress and altering rat behavioral homeostasis. We chose the single molecule 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) because it is synthetic and therefore does not suffer from batch discrepancy, and also because TMT exposure’s ability to induce a reliable and robust unconditioned fear response in rodents. However, there are other types of predator odor that are commonly used in research including ferret
urine, cat urine, and items that have been associated with predators, like a cat’s collar (Takahashi et. al., 2005; Fendt et. al., 2008; Rosen et. al., 2015). While these odors also produce a fear response akin to TMT, it is difficult to ensure a constant level of exposure with these methods due to their inherently variable odor potencies. Because TMT is a synthetic single molecule odor it is much easier to maintain a constant level of exposure across all experiments.

Our lab has developed a model of rodent stress involving TMT. TMT serves as a stressor to rodents and, after five days of consecutive exposure, produces both an acute stress response as well as long-lasting behavioral alterations in methamphetamine-seeking (Ferland et. al., 2016). TMT has been shown by other labs to be an intense ethologically relevant stressor (Venton et. al., 2006; Fendt et. al., 2008; Janitzky et. al., 2014; Rosen et. al., 2015). The rats in Ferland et. al. (2016) that were exposed to TMT showed significantly higher levels of corticosterone in their blood and avoided the center of the chamber where the TMT was presented. Stress-exposure in Ferland et. al. (2016) was then followed by a self-administration paradigm that involves two measures of drug seeking: a cue-test and a stress-reinstatement test. We have shown that rats that have a history of TMT exposure will show increased levels of drug seeking (i.e. pressing the active lever significantly more during reinstatement tests) compared to their non-TMT exposed cohorts. We believe these behaviors model some aspects of comorbid PTSD/SUD and suggest this is a robust animal model of stress and substance use.

Our lab continues to investigate the effects of TMT exposure on methamphetamine self-administration and reinstatement of drug-seeking. This research also prompted a further investigation into how TMT exposure might affect other drugs of abuse. Sareen
et. al. (2006) found that a person who suffers from PTSD is nearly three times as likely to report using cocaine compared to a non-PTSD patient. A separate study found that a person diagnosed with cocaine use disorder was nearly twice as likely to later be diagnosed with PTSD (Saunders et. al., 2015). Although PTSD has been correlated with many drugs of abuse, our lab specializes in investigating the neurobiological underpinnings of cocaine abuse so this was chosen as our main focus for this investigation.

Another area of interest in our lab is the medial prefrontal cortex (mPFC). Many studies have shown the mPFC to be heavily impacted by both stress and addiction (Arnsten et. al., 2009; Goldstein et. al., 2011; Brenhouse et. al., 2013). Although the prefrontal cortex is a very complex region of the brain that has a seemingly infinite number of inputs and outputs, one of its main roles appears to be that of a cognitive regulator. Our lab has shown that one protein, namely brain-derived neurotrophic factor (BDNF), can play a pivotal role in the mPFC’s ability to regulate rodent drug reinstatement. We showed that an intra-cranial infusion of BDNF into the mPFC immediately following the final day of cocaine self-administration significantly reduces cocaine-seeking on the first day of extinction following six days of abstinence as well as during both a cue-test and cocaine-challenge (Berglind et. al., 2007). Therefore we chose to investigate whether or not TMT exposure can alter levels of BDNF in the mPFC and if these alterations might influence cocaine self-administration in rats.

1.3 Aims and Hypotheses

The following investigation is primarily designed to add to the knowledge of animal models for both stress and addiction. Our main goal was to merge the primary line of
research in our lab that deals with psychostimulant addiction, the mPFC, and BDNF with the growing field of PTSD-like animal models in the hopes of discovering a new path forward that might benefit both our lab and the scientific community at large. We decided to split our project into two Aims: 1) looking at the molecular differences between rats who have been exposed to either TMT or saline, and 2) investigating whether TMT exposure has the same effect on rodent cocaine-seeking as it does with methamphetamine-seeking. With the first Aim we hypothesized that TMT exposure would increase levels of Bdnf transcript in the mPFC. With the latter, we hypothesized that increased cocaine-seeking would be observed in rodents who had a history of TMT exposure. We believed these findings would help elucidate the link between BDNF in mPFC and drug-seeking as well as further the goal of creating a PTSD model in rodents to better understand the role between that disease and SUDs.
CHAPTER 2: STRESS AND THE PREFRONTAL CORTEX

Investigate molecular differences between TMT- and SAL- exposed rats, specifically in the mPFC.

2.1 Rationale

It is a well-established phenomenon that high levels of stress in rats can cause molecular changes in various regions of the brain. For example, just one exposure to TMT has been shown to alter c-fos and egr-1 levels in the amygdala and hippocampus (Asok et. al., 2013) and also alter the subunit composition of voltage-gated calcium channels in the amygdaloid complex (Nasca et. al., 2013). Brain-derived neurotrophic growth factor (BDNF) is a protein shown to be affected by, and regulate, stress and drug-seeking behavior. For instance, our lab has shown that a single infusion of BDNF (0.75 ug/0.5ul/side) into the mPFC immediately following the final day of cocaine self-administration suppresses cocaine-seeking weeks later (Berglind et. al., 2007).

Furthermore, our lab has preliminary results showing that 5 days of TMT exposure followed by methamphetamine self-administration (SA) increases levels of Bdnf exon IV, and decreases levels of a key regulator, Hdac5, in the mPFC (Ferland, unpublished). Rats that were not pre-exposed to TMT did not show these increases after methamphetamine SA. However, a study elucidating the effects of TMT on the mPFC without a methamphetamine SA paradigm is missing. In this Aim, we examined mRNA levels of Bdnf exon IV, Hdac5, as well as BDNF’s primary receptor, TrkB, in the mPFC, a region involved in both stress and addiction in rats who have been exposed to either 5 days of TMT or saline.
2.2 Materials and Methods

Animals

Twelve male Sprague-Dawley rats (Charles River, MA) weighing between 275g and 325g were housed individually on a reverse 12h-light/dark cycle in a humidity and temperature-controlled vivarium. Rats had free access to both water and standard rat chow. All protocols were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996).

Predator Odor Exposure and Locomotor Behavioral Testing

Animals were allowed to acclimate to the vivarium for 3 days before beginning the predator odor paradigm. After acclimation, rats were brought down from the vivarium to the exposure room and allowed to habituate (15 minutes/day) to the Digiscan Animal Activity Monitor (21x19.5x12 inches; Accuscan Instruments) for 3 days before predator odor exposure. On days 4 through 8 of predator odor exposure, rats were exposed to either 10ul of 1% 2,5-dihydro-2,4,5-trimethylthiazoline (TMT; Phero Tech Inc.) or 10ul of saline for 15 minutes. This created two experimental groups: TMT pre-treatment (TMT-PE) and saline pre-treatment (SAL-PE). The TMT or SAL was placed in the center of the Digiscan box on a piece of filter paper. SAL exposure occurred in the same room but before TMT exposure for that day. SAL and TMT exposure occurred 4-5 hours after the beginning of the dark cycle. At the end of the day all boxes were cleaned with 70% ethanol and allowed to air-dry overnight. Immediately following the final day of predator
odor exposure, rats were decapitated for trunk blood acquisition and brain extraction. Also, the adrenal glands were dissected and weighed.

**Tissue Collection**

Brains were removed from the skull as quickly as possible and dropped into Millipore ultrapure RNAse/DNase-free water kept cold on wet ice. Brains were then sectioned using a Rodent Brain Matrix (ASI Instruments) before 3 mm punches were taken from the mPFC (AP= +3.0; ML= +0.6; DV= -1.6, relative to bregma, Paxinos and Watson, 1998). Punches were stored in RNAlater (Thermo Fisher Scientific, Inc.) at 4°C for less than 1 month.

**Extraction and rtPCR Analysis**

For analysis, total RNA was processed using RNeasy Mini Kit (Qiagen Inc.). RNA was reverse-transcribed using iScript cDNA Synthesis Kit (Biorad). Samples were checked for purity using Epoch Microplate Spectrophotometer (Biotek Instruments, Inc.) and were only used if the 260/280 values were less than 0.1 away from 2.0. Samples were quantified using rtPCR with iTaq Universal SYBR Green Master Mix (Biorad). Each reaction was done in triplicate with gene efficiencies between 80-90%. Assays were only quantified if a clear peak was seen in melt curves for each gene. Results were analyzed using rtPCR Miner Software (Zhao and Fernald, 2005). The primers used for each gene were as follows: Actin, forward 5’-AGCCATGTACGTAAGCATCC-3’; reverse 5’-GCTGTGGTGGTGAAGCTGTA-3’; Bdnf exon IV, forward 5’-TGCGAGTATTACCTCCGCCC-3’; reverse 5’-TCACGTGCTCAAAAAGTGTCAG-3’; Hdac5, forward 5’-AGGAGGAAGAGGAGGACTGC-3’; reverse 5’-
GTACACCTGGAGGGGCTGTA-3’; TrkB, forward 5’-AGCCTTCTCCAGGCATCGT-3’; reverse 5’-CGGGTCAACGCTGTTAGGTT-3’

Radioimmunoassay

Blood samples were collected in centrifuge tubes containing 100ul of sterilized heparin and spun down for 20 minutes at 2000g before being stored at -80°C. Following behavioral testing, corticosterone levels were analyzed using Corticosterone Double Antibody RIA (MP Biomedicals).

Statistical Analysis

Unpaired two-tailed T test was used for all analyses.

2.3 Results

Weight Gain and Corticosterone Levels

The weight of the animals was recorded daily and the weight gain between the first and final day of stress was analyzed. It was found that predator odor exposure caused a significant reduction in overall weight gained ($t_{10}=2.783$, $P<0.05$; Figure 1.1). Additionally, a radioimmunoassay showed that corticosterone levels were significantly higher in rats exposed to TMT compared to rats exposed to SAL ($t_{10}=2.445$, $P<0.05$; Figure 1.2). These findings are consistent with evidence that TMT exposure is a stressful experience.
Figure 1.1 Weight gain differed significantly between rats exposed to saline (SAL-PE) or TMT (TMT-PE) for 5d. TMT-PE rats gained less weight than their SAL-PE peers. (* indicates $P<0.05$; $n=6$ per group)

Figure 1.2 Corticosterone levels from trunk blood showed a significant difference between SAL-PE and TMT-PE rats. TMT-PE rats had higher levels of corticosterone than their SAL-PE peers. (* indicates $P<0.05$; $n=6$ per group)
mRNA Quantitation

Using rtPCR, we analyzed the levels of several genes of interest following exposure to either TMT or SAL with the idea that TMT exposure might have an effect on the expression of genes known to play a role in stress and cocaine reinstatement. All genes of interest were normalized to the housekeeping gene Actin, which did not significantly differ between the TMT-PE and SAL-PE groups ($t_{10}=0.4877, P>0.05$; Figure 1.3). We found no difference in any of the following genes of interest when comparing the TMT-PE and SAL-PE groups. Predator odor exposure had no significant effect on Bdnf exon IV ($t_{10}=0.7419, P>0.05$; Figure 1.4). Predator odor exposure had no significant effect on Hdac5 ($t_{10}=0.2952, P>0.05$; Figure 1.5), a gene demonstrated to regulate Bdnf exon IV (Bredy et. al., 2007). Furthermore, predator odor exposure had no significant effect on TrkB ($t_{10}=0.4877, P>0.05$; Figure 1.6), the tyrosine kinase receptor for mature BDNF.

![Figure 1.3 Typical RO levels observed in samples taken from rats in this Aim. No significant difference in Actin mRNA levels was detected between groups for any plate analyzed in this investigation. (n=6 per group)](image-url)
Figure 1.4 No significant difference was detected in *Bdnf* exon IV mRNA levels between SAL-PE and TMT-PE groups. (n=6 per group)

Figure 1.5 No significant difference was detected in *Hdac5* mRNA levels between SAL-PE and TMT-PE groups. (n=6 per group)
Figure 1.6 No significant difference was detected in *TrkB* mRNA levels between SAL-PE and TMT-PE groups. (n=6 per group)

2.4 Discussion

Although the stressful nature of TMT was supported by a marked reduction in weight gain and significantly increased levels of corticosterone seen in the TMT-PE group when compared to the SAL-PE group, no difference in mRNA levels of *Bdnf* exon IV, *Hdac5*, or *TrkB* was observed. In designing this experiment, our line of reasoning was based on our observation that after TMT pre-exposure and a history of methamphetamine self-administration, rats who pressed higher on a TMT-reinstatement test also had higher mRNA levels of *Bdnf* exon IV, and lower mRNA levels of the regulator *Hdac5*, in the mPFC than their counterparts who had never been exposed to TMT previously but also had a history of methamphetamine self-administration (Ferland, *unpublished*). With the data above, it seems that increased levels of *Bdnf* exon IV mRNA are only observed with the combination of both TMT exposure and methamphetamine SA; increased levels of *Bdnf* exon IV mRNA in the mPFC are not observed after TMT exposure alone.
Out of the nine different mRNA forms of *Bdnf*, the one containing exon IV is thought to be the most activity-dependent (Zheng et. al., 2011). Neuronal Ca$^{2+}$ influx, through either L-type voltage gated Ca$^{2+}$ channels or the NMDA receptor, has been shown to induce *Bdnf* exon IV upregulation through a CREB-dependent pathway (Tao et. al., 1998). For this reason, levels of *Bdnf* exon IV have been referred to as a measure of neuronal activity. By examining rats that have only experienced TMT exposure and no self-administration protocol, we were looking to identify the root cause of these increased levels of *Bdnf* exon IV in the rats that had a history of both TMT exposure and methamphetamine self-administration. One possible explanation is an increase of excitatory input within the mPFC.

The mPFC, specifically the prelimbic and infralimbic cortices, has been heavily implicated in the extinction and reinstatement processes (Peters et. al., 2008; Kalivas, 2008). It is possible that with pre-exposure to TMT, the mPFC is primed for activation by methamphetamine SA, possibly via corticotropin releasing factor (CRF) activation of CRF receptors. CRF receptors are, for the most part, G-αs coupled protein receptors (Hauger et. al., 2006). G-αs couple protein receptors are classically thought to stimulate adenylyl cyclase and therefore prime the neuron for activation. Both CRF and CRF receptors are prominently found throughout the PFC (Hupalo et. al, 2016). It is well known that methamphetamine drastically increases the levels of dopamine, as well as glutamate, in the prefrontal regions of the rodent brain (Baldwin et. al., 1993; Shoblock et. al., 2003). Perhaps there is a synergistic effect of this continued assault on the mPFC that results in not only the molecular differences seen in rats with a history of both TMT exposure and methamphetamine SA, but also the behavioral effects observed in Ferland
et. al. 2016 which will be discussed in Aim 2. It possible that this proposed synergistic
effect is the reason we did not see any changes with TMT exposure alone. It is also
possible that if we were to look in regions other than the mPFC, we would see altered
levels of *Bdnf* exon IV with our TMT-only paradigm as many studies have shown that
stressful conditions alter *Bdnf* mRNA in regions like the hippocampus and amygdala
(Vaidya et. al., 1997; Kosizek et. al., 2008). In the future investigating TMT exposure’s
effects on regions outside of the mPFC could prove interesting.

In light of the lack of difference in *Bdnf* exon IV levels between the TMT-exposed
and SAL-exposed group, it is not entirely surprising that there was also no difference in
*Hdac5* or *TrkB* transcript levels. Histone acetylation has been shown to be one of the
main factors influencing BDNF exon IV transcript levels, specifically the actions of
Hdac5 (Bredy et. al., 2007). Since we saw no difference in the *Bdnf* mRNA, it is
reasonable that we saw no changes in *Hdac5*. Furthermore, it is not entirely surprising
that we saw no difference in *TrkB* mRNA levels, as there is little data in the literature
supporting a rapid transient response of *TrkB* transcription to any environmental stimuli.
It has been shown that multiple weeks of anti-depressant treatment may alter TrkB
transcription (Nibuya et. al., 1995); however, TrkB seems to be primarily regulated post-
transcriptionally via phosphorylation and intracellular trafficking (Kozisek et. al., 2008).

Overall, although there were no molecular differences seen between the TMT-
exposed and SAL-exposed groups, we had a better idea about what to investigate next
after the completion of this Aim. It appears that TMT exposure alone is not able to alter
transcription of *Bdnf* exon IV, *Hdac5*, or *TrkB* in the mPFC. It is possible that
transcriptional alteration is occurring outside of the mPFC and could be responsible for
the lasting behavioral effects of TMT exposure seen in rats that undergo methamphetamine SA.
CHAPTER 3: COCAINE SELF-ADMINISTRATION

Investigate whether TMT significantly increases both cue- and predator odor-induced reinstatement in rats that have been trained to lever-press for cocaine infusions.

3.1 Rationale

Previous studies in our lab have demonstrated that chronic exposure (5d) to TMT can induce short and long-term behavioral changes in rodents. Rats that are exposed to 5 days of TMT display acute elevations in peripheral corticosterone and increased avoidance behavior, both established markers of stress in rodents (Ferland et. al., 2016). Furthermore, TMT-exposed rats respond at a significantly higher rate, compared to saline exposed controls, on both cue- and TMT stress-induced reinstatement tests after being trained to press a lever for methamphetamine infusions (Ferland et. al., 2016). Our lab, and many others that investigate SUDs, use these reinstatement tests as a measure of drug seeking. Another study by Venton et. al. (2006) used microdialysis to show that TMT exposure can alter glutamate and GABA levels in the nucleus accumbens, a region heavily involved in cocaine self-administration and reinstatement. To date, no published study has looked at TMT-exposed rats and cocaine self-administration (SA) so this aim was designed to investigate whether or not the increase in reinstated lever-pressing observed in Ferland et. al. 2016 would also be observed following cocaine self-administration.
3.2 Materials and Methods

Timeline

Refer to Figure 2.1 for an overview of the timeline for this study.

Figure 2.1. Rats proceeded from acclimation through to the end of behavioral testing to euthanasia. No rats were excluded. (n=8 per group;)

Animals

Sixteen male Sprague-Dawley rats (Charles River, MA) weighing between 275g and 325g were housed individually in a humidity and temperature-controlled vivarium with a reverse 12h-light/dark cycle. At the beginning of the study, up until self-administration, rats had free access to both water and standard rat chow. During cocaine self-administration rats were limited to 20g a day to promote lever-pressing. All protocols were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996).
Surgery

Animals were given 3 days to acclimate to the vivarium. After acclimating, rats were anesthetized using a mixture of ketamine hydrochloride (66mg/kg, i.p.) and xylazine (1.33mg/kg, i.p.), followed by Equithesin (0.5ml/kg, i.p.) and ketorolac (2mg/kg, i.p.). Chronic intravenous catheters were then inserted into the right jugular vein. Catheters consisted of silastic tubing that was tunneled from the neck to the back, below the shoulder blades, where it connected to a plastic pedestal (Plastics One) that allowed it to be connected to an infusion pump. Post-operative care consisted of daily monitoring with administration of 0.1 ml of the antibiotic cefazolin (10mg/ml) as well as 0.05 ml of Taurolidine-Citrate Catheter Lock Solution (TCS; Access Technologies) to maintain catheter patency. Following surgery, rats were allowed to recover for 5 days before beginning the predator odor paradigm.

Predator Odor Exposure and Locomotor Behavioral Testing

For the first two days of predator odor exposure, rats were brought down from the vivarium to the exposure room and allowed to habituate (15 minutes/per day) to the (21x19.5x12 inches; Accuscan Instruments) for 2 days before any data was acquired. On day 3 of habituation, baseline motor activity was taken in the dark. Following locomotor assessment, 0.2ml of blood was taken from the catheter and frozen at -80°C to be used as a baseline level of corticosterone. On days 4 through 8 of predator odor exposure, rats were exposed to either 10ul of 1% 2,5-dihydro-2,4,5-trimethylthiazoline (TMT; Phero-Tech Inc.) or 10ul of saline for 15 minutes. These exposures created two experimental groups: TMT-pretreatment (TMT-PE) and saline-pretreatment (SAL-PE). The TMT or SAL was placed in the center of the Digiscan box on a piece of filter paper. On days 1
and 5, the TMT or SAL was removed from the box and locomotor activity was assessed for a 15-minute period following predator odor or saline exposure. Also, 0.2ml of blood was taken from the catheters and frozen at -80°C. Blood samples were taken immediately following predator odor or saline exposure and before locomotor activity was acquired. SAL exposure occurred in the same room but before TMT exposure for that day. SAL and TMT exposure occurred 4-5 hours after the beginning of the dark cycle. At the end of the day all boxes were cleaned with 70% ethanol and allowed to air-dry overnight.

**Locomotor Activity**

Time spent in the center of the box was divided by total time in the box (15 minutes) and multiplied by 100 to yield a percentage of time spent in the center. Versamax software and Digiscan Animal Activity Monitors were used to capture locomotor behavior.

**Cocaine Self-Administration**

Following the final day of predator odor or saline exposure rats immediately began the cocaine self-administration paradigm (SA). SA sessions occurred 4-5 hours after the beginning of dark cycle. SA consisted of 2-hour sessions in modular chambers (30x20x20 cm; Med Associates Inc.) Chambers consisted of left and right levers that extended at the beginning of the 2-hour session and retracted at the end. The left lever was programmed to produce no response when pressed and deemed the “inactive lever”. The right lever was programmed to elicit a 2-second infusion of cocaine hydrochloride (0.2mg/50ul/infusion; National Institute of Drug Abuse). Infusions were under a fixed-ratio 1 (FR1) schedule of reinforcement. Infusions were delivered via Tygon tubing that connected the rat’s catheter to a 10ml syringe infusion pump. In addition to the cocaine
infusion, the active lever caused the illumination of a white light directly above the lever and a 2kHz/15dB tone. Each infusion was followed by a 20-second timeout period during which active lever presses resulted in no infusion/light/tone. SA continued until each rat had met criteria (≥ 10 infusions per day) for at least 14 days. Following 14 days of meeting criteria, rats began a 6-day abstinence period where they were left alone in the vivarium with free access to food and water.

Extinction and Reinstatement

Extinction training consisted of placing rats into modular chambers for 2h sessions during which presses on either lever elicited neither a cocaine infusion nor a light/tone. The first day of extinction following the 6 days of abstinence is deemed the Post-Abstinence test. Extinction training continued for at least 6 days and then until the rats had at least 3 consecutive days of ≤10 active lever presses per day. Following extinction training rats underwent a Cue test. The Cue test consisted of placing rats into modular chamber for a 2-hour session during which active levers elicited only the light/tone. Following the Cue test rats underwent extinction training once more for at least 6 days and then until extinction criteria (i.e. 3 consecutive days of ≤10 active lever presses per day) were met after which they were put through a Cocaine Prime test. The Cocaine Prime test consisted of administering an injection of cocaine (10mg/kg, i.p.) immediately before putting the rat into a 2-hour extinction session. Following the Cocaine Prime test rats underwent a final round of extinction training. After criteria were met, rats underwent a TMT test that consisted of a 2-hour extinction session during which 10ul of TMT was dropped onto a piece of filter paper in the center of chamber. TMT was present for only the first 15 minutes of the session after which it was removed. Immediately following completions of
the TMT test rats were decapitated without anesthesia and trunk blood was collected and stored at -80°C. Also, adrenal glands were dissected and weighed at time of euthanasia.

**Radioimmunoassay**

Blood samples were collected in centrifuge tubes containing 100ul of sterilized heparin and spun down for 20 minutes at 2000g before being stored at -80°C. Following behavioral testing, corticosterone levels were analyzed using Corticosterone Double Antibody RIA (MP Biomedicals).

**Statistical Analysis**

For analysis of locomotor activity, corticosterone levels, and lever presses for the last three days of SA Two-Way Analysis of Variance (ANOVA) and Sidak’s multiple comparison tests were used. For all other analyses, an unpaired two-tailed T test was used.

**3.3 Results**

**TMT Exposure Decreases Time Spent in Center and Increases Corticosterone Levels**

Data gathered before and after TMT exposure suggested that predator odor exposure is a stressful experience for the rats. TMT exposure significantly decreased time spent in the center compared to SAL-PE ($F_{(1,14)}=14.73$, $P<0.01$; Figure 2.2). Rats who were exposed to TMT for 5 days showed a significantly lower mean time spent in the center on Day 1 (11.39%) and Day 5 (16.6%) of predator odor exposure compared to rats exposed to saline (Day 1 = 29.3%; Day 5 = 29.6%)($t_{42}=4.271$, $P<0.05$;$t_{42}=3.105$, $P<0.05$). This finding is supported partially by the results of the radioimmunoassay that quantified levels of corticosterone found in the plasma from rats before and after predator odor
exposure (Figure 2.3). TMT exposure significantly increased corticosterone levels on Day 5, but not Day 1, of exposure ($F_{2,24}=7.830, P<0.05$). TMT-PE rats had significantly higher mean levels of corticosterone (177.36ng/ml) on Day 5 of exposure than SAL-PE rats (67.25ng/ml) ($t_{24}=3.422, P>0.05$). Although mean corticosterone levels in TMT-PE rats were increased on Day 1 (131.06ng/ml) compared to SAL-PE (81.42ng/ml), the difference was not significant ($t_{24}=1.543, P>0.05$).

![Graph](image)

**Figure 2.2** There was no difference between the times spent in center before stress began. Rats in TMT-PE group spent significantly less time in the center of the box than SAL-PE rats on Day 1 and Day 5. (* indicates $P<0.05$; n=8 per group)
TMT Exposure Does Not Affect Cocaine Self-Administration or Reinstatement

Previous studies from our lab involving methamphetamine and predator odor exposure demonstrated that rats that were exposed to TMT before SA pressed the active lever significantly more during Cue test than did their saline exposed counterparts. If rats had a history of methamphetamine self-administration, TMT exposure could itself serve as an inducer of reinstatement. Furthermore, our previous studies also demonstrated that rats that had a history of TMT exposure pressed the active lever more than rats without a history of TMT during this TMT stress-induced reinstatement. In this study we saw no effect of TMT exposure on any reinstatement measure. Importantly, TMT exposure was not able to induce reinstatement in rats with a history of cocaine SA regardless of their TMT history. Cocaine self-administration acquisition was not significantly different in TMT-PE compared to SAL-PE on the last three days of SA. Predator odor exposure had
no effect on lever pressing or cocaine infusions ($F_{(1,42)}=0.8549, P=0.3605$; Figure 2.4). Predator odor exposure had no effect on the Post-abstinence test ($t_{14}=0.4670, P>0.05$; Figure 2.5). Predator odor exposure had no effect on the Cue test ($t_{14}=0.4407, P>0.05$; Figure 2.6). Predator odor exposure had no effect on the Cocaine Prime test ($t_{14}=0.7522, P>0.05$; Figure 2.7). Predator odor exposure had no effect on the TMT test ($t_{14}=0.4168, P>0.05$; Figure 2.8).

![Figure 2.4](image.png)

**Figure 2.4** There were no significant differences in inactive or active lever pressing. There was also no difference in number of infusions received. These data are the last three days of self-administration averaged for both groups. (n=8 per group)
Figure 2.5 There was no significant difference detected in active lever presses for the Post-abstinence test between the SAL-PE and TMT-PE group. (n=8 per group)

Figure 2.6 There was no significant difference detected in active lever presses for the Cue test between the SAL-PE and TMT-PE group. (n=8 per group)
Figure 2.7 There was no significant difference detected in active lever presses for the Cocaine Prime test between the SAL-PE and TMT-PE group. (n=8 per group)

Figure 2.8 There was no significant difference detected in active lever presses for the TMT test between the SAL-PE and TMT-PE group. (n=8 per group)
Adrenal Gland Size and Trunk Blood Corticosterone Levels

In our previous studies, higher lever pressing during Cue test and TMT test coincided with higher adrenal weights and elevated levels of corticosterone found the in the trunk blood of rats with a history of TMT exposure. This is based on the theory that higher adrenal weights and corticosterone levels are associated with higher levels of stress (Ferland et. al., 2016). We found no significant difference in adrenal gland weights ($t_{14}=0.4747, P>0.05$; Figure 2.9) or corticosterone levels ($t_{14}=0.4753; P>0.05$; Figure 2.10).

![Graph showing adrenal glands normalized to body weight for SAL-PE and TMT-PE groups.](image)

Figure 2.9 There was no significant difference detected in adrenal weights between SAL-PE and TMT-PE groups. Adrenal glands were dissected from rats on day of euthanasia and weighed immediately. (n=8 per group)
Figure 2.10 There was no significant difference detected in corticosterone levels between the SAL-PE group and TMT-PE group. Corticosterone was detected in serum of trunk blood taken on day of euthanasia. (n=8 per group)

3.4 Discussion

Once again, TMT exposure’s ability to induce an acute-stress response was supported by both the locomotor activity data and corticosterone levels. Rats who were exposed to TMT avoided spending time in the center of the box where TMT had previously been. Studies by other labs support the idea that predator odors are capable of producing avoidance behaviors (Vernet-Maury et al., 1983; Whitaker et al., 2016).

Although a stress response was observed, there was no long-lasting effect with regard to cocaine SA or reinstatement rates.

As expected, based on the findings of Ferland et al. (2016), no significant difference was seen between the TMT-PE group and the SAL-PE group with regard to lever pressing or cocaine infusions. Although certain types of stress can cause increases in drug-taking, this is not usually evident in an FR1 schedule of reinforcement which is used in our lab. This finding is common in the literature. Eagle et al. (2015) found that single-
prolonged stress (SPS), another attempt at producing PTSD-like symptoms in rodents, increased cocaine sensitization in rats but did not alter responding during FR1 SA. This study also found no difference in extinction or Cue test responses and used a dose per infusion similar to our own (0.2mg/infusion). However, another study found that intermittent social defeat stress increased cocaine consumption in a 24-hour binge after rats had acquired a stable pattern of responses (Boyson et. al., 2014). Boyson and colleagues used a higher dose (0.75mg/infusion) and an FR5 schedule. These variances in techniques and results are common in behavioral literature. It is possible that with a higher schedule of reinforcement or even a progressive ratio we might have seen differences in our own paradigm.

Considering that TMT exposure produced no effect on methamphetamine SA, it was not entirely surprising that TMT exposure also produced no effect on cocaine SA. However, we hypothesized that because of the similar psychostimulant nature of cocaine and methamphetamine that we would observe similar results with regard to the reinstatement tests. This hypothesis was proven false. Whereas the TMT-PE group in Ferland et. al. (2016) had significantly higher levels of active lever responding in both the Cue test and TMT test, the TMT-PE group in our study showed no difference from the SAL-PE group. It should be noted that there were differences in the reinstatement protocols. The animals in the methamphetamine study went directly from SA into extinction training and did not receive six days of abstinence. We chose to use this protocol in the cocaine study as it is our typical cocaine SA protocol and one of the goals of this study was adapting our stress model to our cocaine SA model. Additionally, all of the animals in the cocaine study also experienced a Cocaine Prime test after extinction
criteria were met following the Cue test and before the TMT test. This was, once again, an attempt to adapt our stress model to our cocaine SA model. Considering the stress and actual self-administration aspects were as identical as possible we believe that if there were an effect of TMT exposure on cocaine-seeking, then it would have been clearly observed in one of our reinstatement tests. We believe the major factor underlying the lack of difference between the TMT-PE and SAL-PE groups was the different way in which cocaine and methamphetamine act on the rodent brain.

Cocaine and methamphetamine are similar in that they both cause increased monoamine levels in various regions of the brain by altering the activity of the transporters responsible for reuptake (Mandell and Knapp, 1976). However, of all the monoamines, dopamine (DA) appears to be the major factor behind most of the behavioral effects related to substance abuse. This has been suggested by numerous DA antagonist and ablation studies (Kelly and Iversen, 1976; Scheel-Kruger et. al., 1977; Roberts et. al., 1980). Beyond this similarity, however, the two drugs differ vastly. The amount of DA increase, and the regions in which it occurs the most, vary heavily depending on the drug in question. One study showed that injections of methamphetamine increase dopamine in the nucleus accumbens at levels four times greater than injections of cocaine, at equivalent doses (Zhang et. al., 2001). The difference observed is most likely due to methamphetamine’s ability to reverse DA transporters thereby increasing the amount of DA released from striatal terminals of midbrain dopaminergic neurons to concentrations well above normal physiological levels (Fleckenstein et. al., 2007). Also, there is a drastic difference in the half-life of each drug. Cocaine has a half-life of 90 minutes and methamphetamine has a half-life of 11 hours.
(Mahoney et. al., 2014). Mahoney and colleagues (2014) also found that in human subjects, methamphetamine users were significantly more likely to experience psychotic hallucinations during withdrawal than cocaine users. In the case of the differences observed in this investigation, it could be that the increased disturbance of homeostasis associated with methamphetamine causes a more intense withdrawal. It appears there are inherent differences in withdrawal from methamphetamine, compared to cocaine. These inherent differences could interact in a synergistic way with the stress induced by TMT pre-exposure. It might also be that the variance observed is due to a difference in the stress response rather than a difference in the rewarding properties of the two drugs. It is also possible that the two drugs interact with the mammalian stress response in different ways.

One of the key features of the HPA-axis is the corticotropin-releasing factor (CRF) system. When an animal is stressed, CRF is released from the paraventricular nucleus (PVN) of the hypothalamus, which initiates a cascade of events that eventually causes the release of glucocorticoids, two of which are cortisol and corticosterone (Koob, 2015). This initial action of CRF makes it an integral part of the mammalian stress response. It is possible that the differences observed in our study between methamphetamine and cocaine are due to differences in the responsiveness of the CRF system to each drug. One series of studies by Giardino et. al. (2012) showed that cocaine and methamphetamine sensitization are different in that they are dependent on the CRF1 and CRF2 receptor, respectively. Using knockout mice, they showed that without the CRF1 receptor, cocaine sensitization did not occur, and vice versa. The paper goes on to suggest that because the CRF1 receptor is the primary receptor responsible for HPA activation, it is likely that
methamphetamine engages the HPA axis through a different mechanism, possibly the vasopressin (AVP) system. Ferland et. al. (2016) showed that pre-treatment with oxytocin, a molecule notoriously similar to vasopressin that can bind to vasopressin receptors, ameliorates the increase in lever-pressing seen in the TMT-PE rats. Perhaps this pre-treatment with oxytocin interferes with the possible meth-AVP-engagement of the HPA-axis and prevents the stress-induction of reinstatement lever pressing. The cocaine SA rats, in contrast, do not experience the cumulative effects of TMT exposure and methamphetamine withdrawal. Therefore it is possible that the cocaine SA rats are able to recover sufficiently from the TMT pre-exposure such that there is no difference in drug-seeking as measured by our reinstatement tests. There is also ample evidence suggesting a role for CRF receptors in many extra-hypothalamic regions (Fischman and Moldow, 1982; Miguel et. al., 2014; Farooq et. al., 2013). One study showed that chronic cocaine exposure led to a depression of synaptic transmission from basolateral amygdala (BLA) projections into the mPFC (Orozco-Cabal et. al., 2008). The authors of that study suggested this was due to chronic simultaneous activation of CRF1 receptors and D_{1/5} DA receptors. It could be that cocaine self-administration somehow masked the lasting effects of TMT exposure in a way that methamphetamine did not.

Considering the differences in methamphetamine and cocaine, there are a variety of plausible explanations that could account for the lack of TMT effect in the cocaine SA rats. After exploring the literature, it seems the interaction of methamphetamine with the mammalian stress response vastly differs from the interaction of cocaine with the mammalian stress response. Therefore a lack of TMT effect in our cocaine SA paradigm is not entirely surprising. This does not rule out the effectiveness of TMT exposure as a
stressor to rats or as a possible method for inducing PTSD-like symptoms in rats outside the confines of our specific cocaine SA protocol.
CHAPTER 4: OVERALL DISCUSSION

Although neither of our Aims produced the results that we hypothesized, this set of studies was, in the author’s opinion, a step towards a better model for both post-traumatic stress disorder and substance use disorder. Both disorders are immensely complex and are, like all mental health disorders, inherently difficult to study using animal models given the obvious fact that animals cannot verbally communicate their symptoms nor explain their behaviors. However, by making small steps using the methods available to us, progress continues to be made.

Our goal for this investigation was to attempt to merge the two primary lines of research in our lab. On one hand we have an extensive history investigating the role of BDNF and the mPFC in the mechanisms underlying cocaine abuse and relapse. On the other we have a burgeoning interest in developing an animal model for PTSD-like symptoms and methamphetamine abuse and relapse. In this investigation we wanted to see if we could observe predator-odor induced differences in the mRNA of BDNF and related proteins within the mPFC. We also wanted to investigate whether or not we would observe an effect of predator-odor exposure on cocaine-seeking similar to what we have seen with methamphetamine-seeking. Our investigations did not reveal a difference in either of these paradigms. There was no significant difference in mRNA levels or reinstatement lever pressing, our measure of drug-seeking, in rats who were exposed to TMT.

One major factor underlying TMT’s inability to induce mRNA changes in our paradigm could be due to the brain regions examined. Although the mPFC surely plays a pivotal role in both stress and addiction, it is possible that after only five days, changes in
Bdnf exon IV, Hdac5, and TrkB mRNA levels had simply not occurred yet. However, regions like the hippocampus and amygdala have been shown to change quickly, sometimes in a transient manner, in response to stressful stimuli in rats (Andero et. al., 2011; Bennet et. al., 2014; Revest et. al., 2014). That’s not to say that similar rapid changes do not occur in the mPFC, it just does not appear to be at the transcriptional level for these particular genes. Perhaps, examining post-transcriptional regulation would reveal an effect of TMT that we did not see at the transcript level. For instance, it could be that stress leads to differences in the phosphorylation levels of TrkB or an increase in the conversion of immature pro-BDNF to mature-BDNF. Many possibilities exist that have yet to be investigated.

As for the lack of effect of TMT on cocaine self-administration and reinstatement, it appears that there is some inherent, likely CRF-related, aspect of methamphetamine that interacts with TMT pre-exposure in a way that cocaine does not. It could be that cocaine is not a potent enough stimulant to bring about the behavioral changes seen with methamphetamine or that methamphetamine exposure brings about a stress response that is not found with cocaine exposure. Regardless, it is clear that exposure to TMT is a stressful experience for the rat; perhaps a different schedule of reinforcement or even long-access self-administration (six or more hours a session compared to our two hour sessions) would show an effect on cocaine-seeking that was not observed in this investigation.

If given the chance to expand on these two studies, the author would proceed immediately to examining the molecular effects of TMT exposure on the prefrontal cortex before and after methamphetamine self-administration, as well as possible changes
occurring in the amygdala and hippocampus. Additionally, it might be beneficial to look at protein levels as well as transcript levels. It seems that, at least with our cocaine self-administration protocol, TMT pre-exposure does not induce any changes in drug-seeking. Therefore, unless a drastic change in the protocol is made, it would likely be best to focus primarily on TMT and methamphetamine self-administration in the future.

Even with negative results, this investigation gives us a clearer picture of the possibilities of using TMT to induce PTSD-like symptoms in an animal model of substance abuse. Certainly, if no researcher ever found negative results than the field of science would be a very confusing place.
REFERENCES


stress avoidance is attenuated by corticosterone and associated with brain levels of steroid receptor co-activator-1 in rats. *Stress, 19*, 69-77.


